Biogeography, population genetics and mating systems of natural *Nasonia* populations

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General Introduction

B. K. Grillenberger

Motivation of this thesis

Evolutionary biology aims at understanding the processes leading to the enormous phenotypic diversity we observe in nature and wants to reconstruct the evolutionary history of life on earth (Futuyma 1986b). This includes research on functioning of individual genes and gene-complexes as well as on environmental forces that lead to the adaptive evolution of these gene networks. Adaptation to a local environment can lead to ecological speciation, when the complexity and heterogeneity of the environment leads to reproductive isolation and ultimately the evolution of new species. One approach in evolutionary research is the development of models that describe the essence and most important features of the evolutionary process (Futuyma 1986a). In order to describe the enormous complexity of natural processes, two major types of models have been developed: (1) general models that are limited to a certain range of conditions and rely on many (sometimes optimistic) assumptions; (2) models that are mimicking a specific system and are only applicable to a narrow range of cases. The ultimate goal is to merge these two approaches and develop detailed models that are applicable in a wide range of scenarios. Especially for detailed models a lot of information about the biology of an organism is needed. A common approach to obtain this information is to focus on model organisms. Focusing on the biology of a single organism provides the advantage that many researchers can combine their results and build a more and more complete picture of a specific system. A model organism in evolutionary biology should be amenable to experimental analysis in fields such as ecology and genetics, be open to field studies, easy to be handled in the laboratory and have a short generation time.

The jewel wasp, *Nasonia*, has become a model organism in evolutionary biology, because of its ease of handling and rearing (Barrass 1976), and its

interesting biology. The genus *Nasonia* consists of three closely related species that are found in sympatry as well as in allopatry (see below). *Nasonia* has been used in a large variety of studies in behavioural (e.g. Barrass 1961; van den Assem & Visser 1976; King 1993; Beukeboom & van den Assem 2001; van den Assem & Beukeboom 2004; Leonard & Boake 2006; Lehmann & Heymann 2006; Shuker *et al.* 2006a), physiological (e.g. King 1962; Rivers & Denlinger 1995), developmental (e.g. Lynch *et al.* 2006; Olesnicky & Desplan 2007) and genetic (e.g. Gadau *et al.* 1999; Beukeboom & Werren 2000; Opijnen *et al.* 2005; Velthuis *et al.* 2005) research. A particular fruitful field of research has been sex allocation and sex ratio distorters (e.g. Werren 1980; 1983; 1988; Beukeboom & Werren 1992; Werren & Beukeboom 1993).

In the *Nasonia* system it is possible to compare and cross individuals between closely related species, as well as individuals of one species originating from natural populations that experience different environmental conditions (see below for details). Therefore the *Nasonia* system provides a unique opportunity to test the validity of models on speciation and adaptation.

Given the wide applicability of, and interest in, the *Nasonia* system, the genome of *N. vitripennis* and its sister species, *N. giraulti* and *N. longicornis*, has been sequenced. As a spin off of the sequenced genome many more studies on genetic and genomic level will follow, and application of molecular tools in parasitoid research will be intensified considerably. However, many ecological aspects and basic life history traits of *Nasonia* are still unknown. Factors and assumptions that are included in theoretical models have to be confirmed experimentally, to make these models realistic and to judge the validity of different models. So far, most experimental research on *Nasonia* has been conducted in the laboratory, but laboratory results can often be biased due to the use of a constant set of arbitrarily chosen laboratory conditions, that only resemble a fraction of the range of natural conditions. Hence, the wider applicability and relevance of the models to field populations remains largely to be determined.

To understand the life history of any organism it needs to be considered how events in different stages of the annual cycle interact and influence subsequent events at the level of the individual and, eventually, the population (Webster *et al.* 2002). To accomplish this, one needs to consider the complexity of an organism's natural environment. One consequence of this complexity is that an

organism has to adapt to a large variety of local conditions, to attain optimal fitness. A precondition of the adaptation to local environments, and eventually speciation, is limited gene flow between different populations. If there is gene flow between local populations locally adapted allelic variants and co-adapted gene complexes might be under opposite selection in other habitats. This difference in selection pressure between local populations can eventually inhibit fixation of an adaptive allele in the population. To assess the probability of local adaptation and the potential for speciation, knowledge about migration between and admixture within populations is required.

In this thesis I provide information on the population genetic structure, phylogeography, reproductive strategies and dispersal abilities of field populations of *Nasonia*, in order to judge the validity of previous results on sex allocation behaviour derived from laboratory experiments under natural conditions. I also evaluate the precision of adaptation in sex and resource allocation of *Nasonia* in sympatric and allopatric populations.

Local mate competition theory

A very intensely studied field in evolutionary biology is sex allocation, and as part of that Local Mate Competition (LMC) theory. Due to its haplodiploid sex determination (see below for details) *Nasonia* became the preferred model species in this field. As a large part of the work presented in this thesis is motivated by LMC theory, I will introduce it here.

Fisher (1930) argued that in a large random mating population the only evolutionary stable strategy is an equal investment in the two sexes. The main reason for this is that, as soon as one of the sexes becomes more abundant in the population, it is advantageous to invest more in the rarer sex, because that sex will have a higher fitness on average. As this is true for either sex that is in the minority, the only stable outcome is an equal frequency of both sexes.

LMC theory (Hamilton 1967) gave an explanation for the observed biased sex ratios in nature, relaxing the Fisherian assumption of a large random mating population. This theory is the basis for a large amount of research into adaptive sex ratio adjustment (Taylor & Bulmer 1980; Werren 1983; Herre 1985; Orzack 1986; King & Skinner 1991; Hardy 1994; Godfray & Werren 1996; Antolin 1999; Courteau & Lessard 2000; West *et al.* 2000; Shuker *et al.* 2004b; 2005). The basic assumption of LMC is, that if a female has control over the sex ratio

of her offspring, she can increase her fitness by reducing the competition between her sons. This is an evolutionary stable strategy if females are the only dispersing sex and if mating only takes place at the natal patch (Hamilton 1967). In such a system competition takes place between all local males to mate with females that are available at the patch. If the population is founded by a single female only, all males are brothers and it is beneficial for the founding female to shift the offspring sex ratio strongly towards daughters to reduce local mate competition between her sons, and in order to maximize the offspring yield in the next generation (her grandchildren). With increasing foundress number, competition between unrelated males increases and therefore selection favours females that produce more males to increase the chance that their sons mate with daughters of other females and hence contribute to an increase of the mother's genes in the next generation. This leads to a less female-biased sex ratio. The resulting prediction is that the offspring sex ratio in a patch is a function of the number of females ovipositing in that patch, starting at very low sex ratios (proportion of males) with few foundresses and approaching a balanced sex ratio at very high foundress numbers (Hamilton 1967) (see Figure 1.1).

A central idea of LMC theory is that mating exclusively takes place within the patch, and therefore the population is highly structured, and inbreeding is high. In the case of parasitoids the structuring is thought to be due to the patchy distribution of hosts. With limited dispersal the relatedness among the founding females would increase, and therefore the population inbreeding would be high, leading to an increase of relatedness among competing sons of multiple foundresses in a patch. The consequence would be an increase of inclusive fitness benefits for the individual foundresses via the offspring of their related co-foundresses, which in turn leads to a lower optimal sex ratio (less males). Inbreeding has been considered in several extensions of the basic Hamilton model (Herre 1985; Frank 1985b). Nunney and Luck (1988) modelled the combined effects of male dispersal, inbreeding and asynchronous parasitism on sex allocation, whilst Courteau and Lessard (2000) developed several different scenarios of dispersal, i.e. before or after mating and dispersal probability for haploid, diploid and haplodiploid organisms.

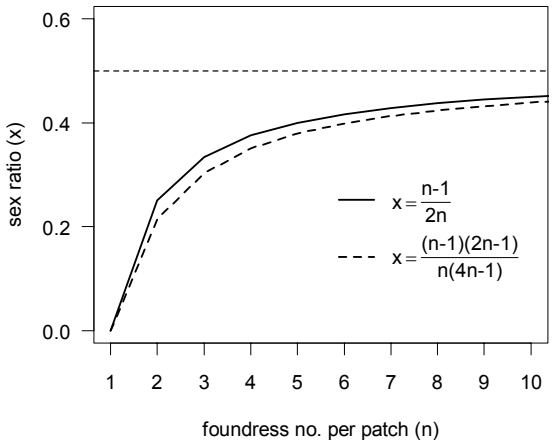

Figure 1.1: Optimal sex ratio (proportion males, x) as a function of foundress number parasitizing a patch (n), as proposed by Hamilton for diploids (1967) (solid line), and corrected for haplodiploids as proposed by Hamilton (1979, as cited in Taylor & Bulmer 1980) (dashed line).

While most authors focused on the actual number of foundresses parasitizing a patch, Werren (1980) considered the relative clutch size of a second parasitizing female compared to the first female to be the crucial factor in sex ratio adaptation. Shuker *et al.* (2005) recently extended Nunney and Luck's (1988) model of asynchronous parasitism by considering two foundresses parasitizing hosts on a patch sequentially but allowing females to use either the same or different hosts. In species such as the parasitic wasp *Nasonia vitripennis* asynchronous parasitism on a single host is thought to have little effect on the timing of emergence, as *N. vitripennis* larvae of later deposited eggs speed up their development to achieve a synchronous emergence of all individuals from a host (Werren 1980). In contrast, asynchronous parasitism of several hosts in a patch leads to asynchronous emergence of the offspring, as each host has a different emergence time. As such, males of an early foundress have a chance to mate with females of a later foundress, whose sons do not have access to the daughters of the early foundress. Such asymmetric LMC leads to a shift of the optimal sex ratio towards more males for the second foundress, compared to previous models (Shuker *et al.* 2005). To what extent asynchronous parasitism is taking place in nature is largely unexplored.

LMC theory has been tested on a number of parasitic wasp species. Among others *Pachycrepoideus vindemiae* (Nadel & Luck 1992), *Muscidifurax raptor* (King & Seidl 1993), and *Spalangia endius* (King 2002) show a clearly female

biased sex ratio, but follow only roughly LMC theory. Since in all three species males can fly and disperse facultatively and therefore do not obey to the primary criteria of LMC, they may experience only partial LMC. Studies on these species show that next to LMC, host quality appears to play an important role in sex allocation (Nadel & Luck 1992; King & Seidl 1993; King 2002). Several field studies on fig wasps support the idea of LMC, but there is a lot of variation and many more factors than just the number of foundresses seem to play a role (Herre 1985; Herre 1987; West & Herre 1998; Moore *et al.* 2002; Greeff 2002; Pereira & do Prado 2005). As a general trend Herre (1987) found that those species that regularly encounter high foundress numbers in a patch produce sex ratios that are closer to the predictions of LMC theory than species that usually parasitize with a single foundress.

N. vitripennis has been widely used for laboratory experiments regarding sex ratio adjustment (Werren 1980; 1983; 1984; Drapeau & Werren 1999; Shuker & West 2004; 2004a; Shuker *et al.* 2004b; 2005). Laboratory experiments and two field studies (Werren 1980; Molbo & Parker 1996) have shown that *N. vitripennis* modulates the sex ratio of its offspring broadly consistent with LMC theory. However, to what extent the experimental laboratory conditions resemble the field situation is little known. One important condition for LMC to occur is that *Nasonia* regularly encounters competitors while parasitizing hosts. The only information on superparasitism rates comes from a small field study in Sweden using allozyme markers (Molbo & Parker 1996). These data are the first in documenting foundress numbers in a natural situation. However, allozymes have limited resolution and may underestimate the actual number of foundresses. Hence there is a need for more field data to evaluate the complementarities of population structure and dynamics of LMC theory. Since the parasitoid wasp *Nasonia* has already proven to be an ideal model to test LMC theory, it is the species of choice in this study. In the following section I will introduce the *Nasonia* system in more detail.

The Nasonia *model system*

Nasonia is an approximately 3 mm large pupal parasitoid of cyclorraphous flies, which are mostly found in bird nests and on carcasses (Whiting 1967). Up to now, three species are known in the genus *Nasonia*: *N. vitripennis* seems to be cosmopolitan (Whiting 1967, and see: http://www.nhm.ac.uk/research-

curation/projects/chalcidoids/), *N. giraulti* is found in the north-eastern and *N. longicornis* in the north-western part of North America (Darling & Werren 1990). Due to the patchy distribution of its host, populations of *Nasonia* are thought to be extremely substructured (Darling & Werren 1990; Molbo & Parker 1996), with the consequences for sex ratio adaptation discussed above. The major morphological difference between the species is the male wing length: *N. vitripennis* has short wings, *N. giraulti* has long wings, and *N. longicornis* intermediate wings (Darling & Werren 1990).

The divergence of the three species is thought to be a relatively recent event. *N. giraulti* and *N. longicornis* are estimated to have separated from *N. vitripennis* around 1 Myr ago. The separation of *N. longicornis* and *N. giraulti* is placed around 0.25 Myr ago (Campbell *et al.* 1993). The species are reproductively isolated due to infection with incompatible *Wolbachia* strains, which causes chromosome destruction in interspecific crosses (Breeuwer & Werren 1990). However, cured strains produce viable and fertile hybrid offspring (Breeuwer & Werren 1995). Behavioural studies have shown that there are clear differences in courtship behaviour between the three species leading to prezygotic isolation (van den Assem & Werren 1994; Beukeboom & van den Assem 2001).

It is assumed that the cytoplasmic incompatibility caused by *Wolbachia* infection played a major role in the initiation of the speciation process (Bordenstein & Werren 1998; Bordenstein *et al.* 2001). However, it remains to be established whether *Wolbachia* was the primary cause or has played a role in the maintenance of genetic separation leading to speciation. It is also not known whether the speciation of *N. longicornis* and *N. giraulti* took place in allopatry or in sympatry with *N. vitripennis* in North America (see below).

The life cycle of *Nasonia* is similar in all three species (Figure 1.2): An adult female (a foundress) searches for host pupae and drills a hole in the puparium with her ovipositor. She stings the host and injects venom into the host pupa, which will eventually kill the host. She lays about 30 eggs, depending on host size and the presence of other female's eggs, directly onto the fly pupa. The eggs hatch inside the host puparium and upon emergence the larvae feed on the body tissue of the host. After two weeks of development at 25ºC, the adult wasps are ready to emerge. Males reach adolescence earlier and emerge shortly before females. They bite their way through the puparial shell and wait at the

exit hole for the females to emerge. Once the females have crawled out of the puparium, the males start courting them. As a consequence, the amount of inbreeding depends on the number, relatedness and produced sex ratios of foundresses that have laid their eggs in a particular host. After mating the females usually disperse and search for a new host patch. In *N. vitripennis,* males have reduced wings and are thought to stay at their place of birth to wait for other females to emerge, until they die (Whiting 1967). It has been found that *N. longicornis* males stay at the mating site as well, while *N. giraulti* males have a tendency to disperse after mating (Leonard & Boake 2006). However, how far *N. giraulti* males disperse, and whether there is a chance for them to find a mate, has not been tested yet.

The mating behaviour after emergence is generally similar for all three species, but especially *N. giraulti* and to some degree *N. longicornis* females mate within the host before emerging (Drapeau & Werren 1999). Within Host Mating (WHM) is rare in parasitoids and the occurrence of this behaviour in natural populations of *Nasonia* would have a large influence on the population structure. In the case of a single parasitized host, the offspring will perform exclusively sib-mating. If most hosts are single parasitized, the degree of inbreeding in the population would therefore be increased if WHM occurs regularly. It has been suggested that WHM is an adaptive behaviour of *N. giraulti* to avoid hybrid mating with *N. vitripennis* (Leonard & Boake 2006). This hypothesis still remains to be tested.

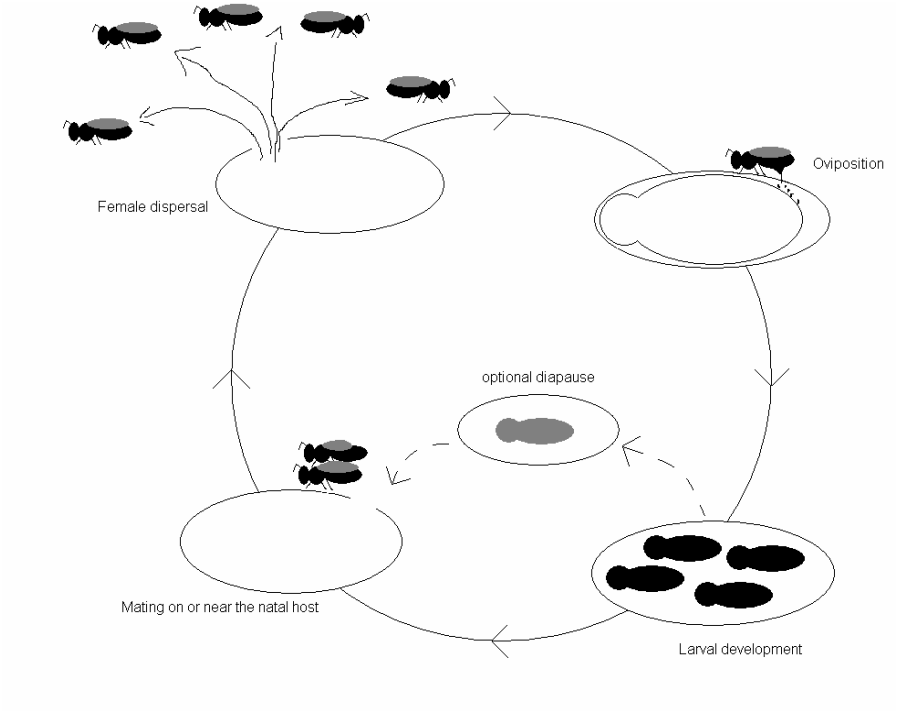

Figure 1.2: Schematic life cycle of *Nasonia vitripennis*

Nasonia has haplodiploid sex determination. Fertilized eggs develop into females and unfertilized eggs into males. After mating sperm is stored in a spermatheca, and due to its anatomy, females are able to facultatively fertilize an egg. This enables females to control the sex ratio of their offspring. The biology of *Nasonia* seems to fit most assumptions of Hamilton's LMC theory and the species has been widely used in LMC research (see above). However, the details of the cues that are influencing sex allocation decisions and the underlying mechanisms are not well understood yet.

As mentioned above, the major morphological difference between the three species in the genus *Nasonia* is the wing size of the males (Darling & Werren 1990). *N. vitripennis* has short wings, *N. giraulti* has long wings that cover the abdomen and *N. longicornis* has intermediate sized wings. The males of *N. vitripennis* are incapable of flight, while those of the other two species can fly, and might therefore disperse (King & Skinner 1991; Leonard & Boake 2006; Lehmann & Heymann 2006). However, there is a report of *N. vitripennis* males

and females mating six meter away from their natal host (Grant pers. comm. in Orzack 1986). The dispersal abilities of *Nasonia* have not been examined in detail yet, but one can imagine that such a small insect might have problems to cover long distances by its own power. If dispersal abilities are limited, the strong population substructuring would lead to very low gene flow between subpopulations and therefore inbreeding should be very high within the subpopulations. If *Nasonia* can disperse by wind drift, as reported for fig wasps (Harrison 2003), migration between the subpopulations would be higher and the level of inbreeding within the subpopulations be reduced. For *N. vitripennis* a high level of inbreeding has already been shown in one natural population using allozyme variation (Molbo & Parker 1996), but it is likely that different habitats induce a different dispersal behaviour in *Nasonia*, affecting the population structure. In addition, allozymes may not have enough resolving power to produce reliable estimates of inbreeding in populations with such a complex life history as *Nasonia*. For the other two species, *N. longicornis* and *N. giraulti*, information on inbreeding and dispersal are totally lacking.

As many insect species (Danks 2007), *Nasonia* larvae have the ability to survive unfavourable environmental conditions in a dormant state, called diapause (Whiting 1967). In most species it is the larva itself that induces and enters diapause upon experiencing unfavourable conditions. In *Nasonia*, however, diapause is induced in the mother but developmental arrest takes place in the fourth larval instar of her offspring. Several cues have been found to trigger the female to produce eggs that will develop into diapausing larvae: low temperature, long dark periods, food shortage and older age (Saunders 1962; 1965a; 1966a; 1966b). The diapausing larvae are able to survive up to two years and then continue normal development into an adult individual. In the laboratory diapause can be broken by exposure to low temperatures (~4ºC) for three months, and subsequent culturing at normal temperature (20-25ºC) (Whiting 1967).

The differences in life history, mating system and dispersal capabilities between the *Nasonia* species, and the various environments in which *N. vitripennis* can be found (sympatric with its sister species in North America, and allopatric in Europe), provide unique opportunities to study the precision of adaptation. In this thesis the main focus will be on the precision of two adaptive traits: sex allocation and diapause.

Population history of Nasonia

The origin and fixation of locally adapted traits in a population usually takes a long time. To estimate the probability of local adaptation it is necessary to obtain information on the population history of a species. In principle two histories of the colonization of the North American continent by *Nasonia* are conceivable (see Figure 1.3): (1) The ancestor of all three *Nasonia* species invaded the New World and the speciation into the three species *N. vitripennis*, *N. longicornis* and *N. giraulti* took place sympatrically. *N. vitripennis* then extended its range from North America to the rest of the globe. If the species complex evolved in this way, high genetic variation in North America and a reduced variation in the rest of the world would be expected, due to a possible population bottleneck during colonization. (2) The ancestor of *N. giraulti* and *N. longicornis* came to North America and the speciation of these two sister species took place in isolation of *N. vitripennis*, which evolved outside the New World. In more recent times *N. vitripennis* was introduced to North America. In this case, the genetic diversity of the North American *N. vitripennis* population would be reduced compared to the European population, due to a possible bottleneck during the colonization event. The time that *N. vitripennis* lives in sympatry with one of its sister species has implications for the time during which behavioural adaptations may have evolved in response to interspecific competition. The population history of *N. vitripennis* in North America has not been investigated yet.

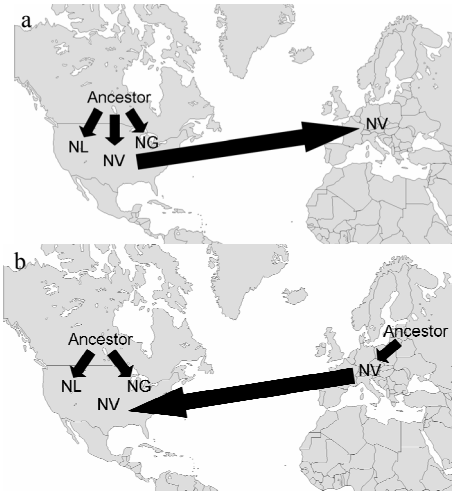

Figure 1.3: Illustration of the two possible population histories in the *Nasonia* system. a) speciation into all three species in North America and subsequent migration of *N. vitripennis* (NV) to Europe; b) speciation of *N. giraulti* (NG) and *N. longicornis* (NL) in North America and of *N. vitripennis* in Europe or Asia from a cosmopolitian ancestor, and subsequent migration of *N. vitripennis* to North America (via the Bering strait bridge).

The goal of this thesis is to evaluate the underlying assumptions of models describing adaptive behaviour (sex allocation and diapause). I aim to acquire information on the precision of adaptation in a natural environment and gain a better understanding of the multiple selective forces that shape life history traits. The results of this study will help to place the many results of theoretical and laboratory studies in an ecological context and can help to identify remaining questions about parasitoid life history evolution.

Methodological approach

When working with small insects, like *Nasonia*, direct observational methods are limited to the laboratory. For field observations the many techniques that are commonly used with larger species (e.g. radio tracking, colour banding, mark-release-recapture over longer time spans) cannot be applied. An alternative to answer many field biological questions is an indirect approach using genetic markers. The choice of the marker is dependent on the application, as different markers have different strengths and weaknesses. In the following I will provide more information and justification for the two types of markers that are used in this thesis: nuclear microsatellites and mitochondrial DNA sequences.

Microsatellites

Microsatellites, or simple sequence repeats (SSRs), are non-coding repetitive DNA sequences consisting of short (1 to 6 bp) repeats which occur in nuclear

and organelle DNA and are widespread across all eukaryotic genomes. Due to their high mutation rate and, in general, selective neutrality these markers have been broadly used in population genetic studies and for paternity analysis. The allelic differences are caused by variation in the number of repeats in the repetitive sequence, which is scorable as the difference in amplified fragment lengths from primer sets flanking the repetitive sequence. These repetitive sequences can be scanned for using a molecular probe, and therefore it is possible to develop primer for microsatellites even without having the genome sequenced. This makes microsatellites a very efficient marker for various applications, especially in non-model organisms.

The statistical tools that can be used to estimate population genetic parameters from microsatellite data are based on different mutation models (see Box 1.1). The most commonly used tool is Wirght's *F*-statistic (Wright 1931), which assumes an infinite number of possible alleles (infinite alleles model, IAM). The so called *R*-statistic (Slatkin 1995) is the counterpart to the *F*-statistic and is based on the stepwise mutation model (SMM). However, from discussions in recent literature it appears that most microsatellites follow a mixture of IAM and SMM (two phase model) and therefore neither the *R*- nor the *F*-statistic would capture the truth. Therefore it is advisable to compare *R*- and *F*-statistical parameters and then draw conclusions (Oliveira *et al.* 2006).

A possible problem that can arise when using microsatellites is their enormous variability. Assuming a SMM and a limited number of possible alleles, the high mutation rate leads to a high chance of homoplasy over longer time spans. This results, over time, in an increasing unreliability of diversity and differentiation estimates (Nauta & Weissing 1996). Another problem of the high variability of the marker is the comparability of results between different studies. However, this problem seems to be largely solved with the invention of variation corrected differentiation estimates (e.g. G'_{ST}) (Hedrick 2005).

Box 1.1: Overview of the proposed mutation models in microsatellites following Oliveira *et al.* (2006):

1. Infinite alleles model (IAM):

   Every mutation alters the length of the microsatellite randomly, irrespective of the magnitude of the change. Therefore repeat units of length 5 and 15 are as closely related as lengths 5 and 6.

2. Step wise mutation model (SMM):

   During mutation, a microsatellite only looses or gains a single repeat unit. Therefore the genetic distance between two alleles is roughly proportional to the length difference between them.

3. Two phase model (TPM):

   A mixture of the IAM and SMM. The length of a microsatellite changes gradually (SMM), but occasionally larger leaps are possible.

4. K-alleles model (KAM):

   The above mentioned models assume an infinite number of possible microsatellite lengths (alleles). In contrast, the KAM assumes a limited number of alleles (k) possible for a microsatellite locus.

On the other hand, the high mutation rate (10^{-2} – 10^{-6}) in microsatellites rapidly creates differences between isolated populations, and when applied in the appropriate time scale, the resolution obtained by these markers can hardly be met with another marker type at the time this project was started. Another property is that every microsatellite locus appears to have its own mutation rate and pattern, depending on the number of repeats and its location in the genome. This leads to variation in the resolution between microsatellite loci, and makes it necessary to test the markers for their suitability to answer a specific question. However, this variation in resolution allows to find microsatellite loci with enough variation to differentiate between close relatives, but also to find loci that are conserved enough to address questions on inter-species level.

Considering the above mentioned properties, microsatellites are the markers of choice for the analysis of population structure and relatedness on a regional scale. With the recent advances in high throughput sequencing more effective methods are becoming available, but at the time this research was started, they were not an option.

Mitochondrial DNA sequences

Mitochondrial DNA (mtDNA) is the circular genome of mitochondria of eukaryotes. Every mitochondrion has one copy of it, and as there are many mitochondria in one cell, there are multiple identical copies of mtDNA per cell. There are 13 genes encoded by the mtDNA that are in close functional connection to nuclear encoded genes. Consequently, there can be a close association between certain mitochondrial types and nuclear genes.

Mitochondria are exclusively inherited via the maternal lineage, as they are transferred through the cytoplasm of the oocyte. There can be interactions between mitochondria and other cytoplasmic symbionts, leading to indirect selection on certain mtDNA haplotypes.

One problem of inferences from mtDNA can be the exclusive maternal transmission. In the case of differences in migration behaviour between males and females, or a different population history of the sexes, mtDNA can only reflect the female side of the story. Another pitfall in reconstructing population histories by mitochondrial information only, is its possible linkage to inherited symbionts, leading to a bias in the information that can be obtained (Hurst & Jiggins 2005). In *Nasonia* the symbiont is *Wolbachia*, which is exclusively maternally inherited as well (Werren 1997).

With 10^{-8} – 10^{-10} substitutions per site and generation, depending on the gene, mtDNA is evolving fast and linear within a time frame of up to 150 Myr (Mueller 2006). It can easily be amplified (no cloning necessary) and, due to highly conserved regions, there are universal primers available, that facilitate the use in non model organisms.

These properties qualify mtDNA sequences to be the marker of choice for intraspecific inferences on a large scale, and for questions regarding the history of closely related species. I will use the information obtained from mtDNA in combination with microsatellites to combine the advantages of both markers, and to circumvent some of the problems when relying on only one type of marker.

Outline of this thesis

As outlined above, in spite of all advances in theoretical and laboratory research there is a profound lack of information on the natural ecology and behaviour of

parasitoids like *Nasonia*. The aim of this thesis is to fill some gaps between theoretical models and the natural situation in *Nasonia* research. In the following I will give an overview of the topics dealt with in the individual chapters of this thesis.

As described above *Nasonia* has been found to follow quite closely the predictions of local mate competition (LMC) theory in the laboratory. However, LMC theory is based on many assumptions about the population genetic structure, and it is unknown to what extent they are validated in nature. In **Chapter 2** (Genetic structure of natural *Nasonia vitripennis* populations: validating assumptions of sex ratio theory) I will evaluate the assumptions made in sex ratio theory on data from two European *N. vitripennis* field populations. In particular I will address the population structure, foundress number per patch, parasitation sequence and clutch sizes. As a follow-up, I will investigate in **Chapter 3** (Facultative sex ratio adjustment in natural populations of wasps: cues of local mate competition and the precision of adaptation) to what extent the predictions made by LMC theory are matching the sex ratios observed in the European populations and where the strengths and weaknesses of present models are.

Whereas in Europe *N. vitripennis* has no closely related competitors, the situation in North America is more complex. Given that *N. vitripennis* and *N. giraulti* are living in close sympatry in parts of North America, the question arises whether adaptations have evolved to avoid hybridization between the species. While it has been found that there are clear differences in courtship behaviour, it is still unknown whether there are adaptations with respect to LMC. In **Chapter 4** (Reproductive strategies under multiparasitism in natural populations of the parasitoid wasp *Nasonia* (Hymenoptera)) I will investigate the reproductive strategies of *Nasonia* in a two species situation, regarding the sex ratio adjustment as well as diapause production, focusing on how well *N. vitripennis* is adapted to the competition with its close relative *N. giraulti*.

In order to evaluate how far adaptation of *N. vitripennis* to the competitive situation in North America might have progressed, information is required on the population history of *Nasonia* in North America. So far, it has been assumed that the cosmopolitan species *N. vitripennis* has its origin in North America, as that seems to be the hot spot of diversity within the genus *Nasonia*. In **Chapter 5** (Population history of *Nasonia vitripennis* (Hymenoptera) in North America)

I test the hypothesis that *N. vitripennis* originates from North America, or from outside the New World. Using a combination of mtDNA, nuclear microsatellites and *Wolbachia* sequences, I am comparing the genetic variability among North American and European samples.

A prerequisite of local adaptation is that there is only limited gene flow between areas with and without selection pressure on the adaptive trait. *N. vitripennis* might have evolved adaptations to the presence of its sister species *N. giraulti* and *N. longicornis* in North America. However, as there are large areas in North America where *N. vitripennis* occurs allopatrically, without selection for competition with a close relative, the question arises whether local adaptation in the sympatric areas is possible. In this context I investigate in **Chapter 6** (Female dispersal and isolation-by-distance of *Nasonia vitripennis* (Walker) in a local mate competition context) the dispersal capabilities of *N. vitripennis* on a local scale with a mark-release- recapture experiment as well as on larger scale with molecular markers.

In the final **Chapter 7** I will merge the results of the previous chapters and sketch the current knowledge of the population structure and history of *N. vitripennis* in Europe and North America.

Acknowledgements

I would like to thank Leo Beukeboom, Louis van de Zande and Jürgen Gadau for all the helpful comments that considerably improved this chapter.

Chapter 2

Genetic structure of natural *Nasonia vitripennis* populations: validating assumptions of sex ratio theory

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Abstract

The parasitic wasp *Nasonia vitripennis* has been used extensively in sex allocation research. Although laboratory experiments have largely confirmed predictions of Local Mate Competition (LMC) theory, the underlying assumptions of LMC models have hardly been explored in nature. We genotyped over 3500 individuals from two distant locations (in the Netherlands and Germany) at four polymorphic microsatellite loci to validate key assumptions of LMC theory, in terms of both the original models and more recent extensions to them. We estimated the number of females contributing eggs to patches of hosts and the clutch sizes as well as sex ratios produced by individual foundresses. In addition we evaluated the level of inbreeding and population differentiation. Foundress numbers ranged from 1 to 7 (average 3.0 ±0.46 SE). Foundresses were randomly distributed across the patches and across hosts within patches, with few parasitizing more than one patch. Of the hosts, 40% were parasitized by more than one foundress. Clutch sizes of individual foundresses (average 9.99 ±0.51 SE) varied considerably between hosts. The time period during which offspring continued to emerge from a patch or host correlated strongly with foundress number, indicating that sequential rather than simultaneous parasitism is the more common. Genetic differentiation at the regional level between Germany and the Netherlands, as estimated by Slatkin's private allele method (0.11) and Hedrick's corrected G'_{LT} (0.23), indicates significant substructuring between regions. The level of population inbreeding for the two localities (F_{IL} = 0.168) fitted the expectation based on the average foundress number per patch.

Introduction

Local mate competition (LMC) theory (Hamilton 1967) is the basis for a large amount of research into adaptive sex ratio adjustment (Werren 1983; Herre 1985; Orzack 1986; King & Skinner 1991; Hardy 1994; Godfray & Werren 1996; Antolin 1999; Courteau & Lessard 2000; West *et al.* 2000; Shuker *et al.* 2004b; 2005). It assumes that a female has control over the sex ratio of her offspring and can maximize her fitness by reducing the competition between her sons. This is an evolutionary stable strategy if males are not the dispersing sex and if mating only takes place at the natal patch (Hamilton 1967). In such a mating system all males are competing to mate with the females that are available at the patch. If the patch population consists of only a single family, the males are brothers and it is beneficial for the foundress female to shift the offspring sex ratio strongly towards daughters to reduce competition among her sons. With increasing foundress number, competition between unrelated males increases and therefore selection favors females that produce more males to increase the chance that their sons mate with daughters of other females as well. This leads to a less female-biased sex ratio. The resulting prediction is that the offspring sex ratio in a patch is a function of the number of females ovipositing on that patch (Hamilton 1967).

A central assumption of LMC theory is that the population is highly subdivided in terms of mating. In the case of parasites this is thought to be due to the patchy distribution of hosts. Hamilton (1967) assumes that clutch sizes are equal and that there is random mating among the offspring of one patch. A patch could be for example all the fly pupae on a carcass or in a bird nest. The resulting population inbreeding F_{IT} follows $F_{IT} = 1 / (4n\text{-}3)$ with n being the mean number of foundresses per patch (Hamilton 1979).

Hamilton's LMC model has been further extended by several authors in various ways. The concept that females can have different clutch sizes and sex ratios has been incorporated by Werren (1980). Inbreeding has also been considered in several ways (Herre 1985; Frank 1985b). Nunney and Luck (1988) modeled the combined effects of male dispersal, inbreeding and asynchronous parasitism on sex allocation, whilst Courteau and Lessard (2000) in turn developed several different scenarios of dispersal, i.e. before or after

mating and dispersal probability for haploid, diploid and haplo-diploid organisms. Shuker *et al.* (2005) recently extended Nunney and Luck's (1988) model of asynchronous parasitism by considering two foundresses parasitizing hosts on a patch sequentially but allowing females to use either the same or different hosts. In species such as the parasitic wasp *Nasonia vitripennis* asynchronous parasitism on a single host is thought to have little effect on the timing of emergence, as *N. vitripennis* larvae speed up their development to achieve a synchronous emergence of all individuals from a host (Werren 1980). In contrast asynchronous parasitism of several hosts in a patch leads to asynchronous emergence of the offspring. As such, males of an early foundress have a chance to mate with females of a later foundress, whose sons do not have access to the daughters of the early foundress. Such asymmetric LMC leads to a shift of the optimal sex ratio towards more males for the second foundress (Shuker *et al.* 2005). Like most models, Shuker *et al.*'s (2005) model has been confirmed under laboratory conditions (see also Shuker *et al.* 2006b), but few field studies have been performed to test these models.

The parasitoid wasp *Nasonia vitripennis* has been widely used for laboratory experiments regarding sex ratio adjustment and behavioral genetics (Werren 1984; Drapeau & Werren 1999; Beukeboom & van den Assem 2001; van den Assem & Beukeboom 2004; Shuker *et al.* 2005; 2006b). Laboratory experiments and two field studies (Werren 1983; Molbo & Parker 1996) have shown that *N. vitripennis* modulates the sex ratio of its offspring largely consistent with LMC theory. As Molbo and Parker (1996) used allozymes, which have a rather low variability, it is possible that they underestimated foundress number. In addition the level of superparasitism might also have been underestimated, as they themselves acknowledged. Werren (1983) on the other hand used the offspring number per patch as an indirect measure of the foundress number and found a strong positive correlation between patch offspring number and sex ratio, leveling off at 50% males.

Other genetic studies on parasitoid Hymenoptera have considered the level of the population rather than the level of individual patches, and have produced varying results on the population substructuring and the level of inbreeding. De Leon and Jones (2005) found for *Gonatocerus ashmeadi* a pronounced genetic structure between samples from the American East- and West coast (G_{ST} =

0.38), while Kankare *et al.* (2005) found differing results for *Cotesia melitaearum* and *Hyposoter horticola*. F_{ST} for *C. melitaearum* was much higher than for *H. horticola* (0.378 vs. 0.063), and both species showed significant isolation by distance. These differences between parasites of the same host species reflect their differences in mobility (Kankare *et al.* 2005). In a study on *Trichogramma pretiosum* (Antolin 1999), a rather high degree of population inbreeding (F_{IT} = 0.246) was found but no significant differentiation between three subpopulations within California. These different findings regarding the population structure of various parasitoid wasps do not allow any generalizations, and do not specifically test assumptions of LMC. In this study we use four polymorphic microsatellites to estimate the level of inbreeding, foundress numbers, timing of parasitism and individual sex allocation in two field populations of *N. vitripennis* in Europe in order to test how well natural populations represent the idealized conditions assumed in models of LMC.

Material and Methods

Sampling

Nasonia vitripennis is a gregarious pupal parasitoid of a wide range of cyclorraphous flies. Like all Hymenoptera, *N. vitripennis* has a haplodiploid reproduction mode: fertilized eggs develop into diploid females, unfertilized eggs into haploid males. In *N. vitripennis* females usually mate at their place of birth and disperse after mating. Males have reduced wings and cannot fly (Whiting 1967).

Fly host pupae were collected from bird nests obtained from 95 nest boxes in a 1.4 km x 2.5 km field site in the Hoge Veluwe National Park (The Netherlands) (referred to as HV) and from baits placed in all HV nest boxes. A second plot consisted of 28 nest boxes along a straight ~600 m long road near Schlüchtern (Hessen, Germany) (referred to as Schl), where only baits were used. The collected host pupae were incubated individually at room temperature (~20˚C) and the emerging wasps, after being identified as *N. vitripennis*, were counted, sexed and stored directly in 90% ethanol for molecular analysis. For the HV-samples we kept record of the first and last day of emergence for every host pupa. Unfortunately we could not record the data per individual wasp. For baiting (in Schl), 25 laboratory hosts (*Calliphora vicina*) were placed in a mesh

bag and left inside the nest box for approximately one week to allow parasitism. As the nest boxes are cleaned out every year and we did not find any host pupae that showed signs of emergence, we assume that our sample represents all offspring that emerged from these nest boxes.

Parentage analysis

DNA isolation followed a standard high salt-chloroform protocol (Maniatis *et al.* 1982). For genotyping we used four polymorphic microsatellites (dinucleotide repeats) (Table 2.1). Nv-22 and Nv-23 have originally been developed by Pietsch *et al.* (2004) but the primers have been redesigned in our laboratory. Primer sets for the other two microsatellites have been developed in our laboratory using the technique described by Rütten *et al.* (2001). The length of the amplified fragments was determined on an ABI Prism 377 DNA sequencer (Perkin-Elmer Applied Biosystems).

The genotypes of the females (here called foundresses) that oviposited on each host were determined from the genotypic data of the offspring following these simple rules: (1) A female can maximally provide two alleles per locus. (2) The father can only provide one allele per locus (being haploid) that is shared by all full sisters. (3) Sons can only have an allele from their mother, as they develop from unfertilized eggs. If several foundress genotypes were possible based on the microsatellite profile, we always preferred the solution with the lowest number of foundresses. We allowed the foundresses to be multiply mated in our paternity analysis. This foundress assignment has been done independently by three of the authors (BKG, TK and MNB-C) to validate the assignment process. It yielded data on the number of foundresses per nest box and per host, as well as on the individual clutch sizes and sex ratio of every foundress.

Table 2.1: Chromosomal location, primer sequences, number of alleles, Nei's overall gene diversity (H_t) (Nei 1987) and annealing temperatures of four microsatellites used.

Primer	Chromo -some*	Sequence	Allele no.	H_t	Ann. temp.	GenBank accession no.
Nv-22	I	5' GAC TGC GTA CCA CTC CAA AAA TA 3' 5' AAG ACC AGC TAG GGA AGA GGA TA 3'	16	0.90	58˚C	AY262041
Nv-23	II	5' ATA CTC AAG CAA GCC ACA GCA TA 3' 5' GCG TAC CAA TCC ACA GAA AAT AG 3'	13	0.39	58˚C	AY262044
Nv-41	IV	5' GTC AGA CGT GGG CTT TGT C 3' 5' TTA TGC GCC ACA CAC ACC 3'	11	0.85	52˚C	EU155141
Nv-46	V	5' TTA CGT CAA GGT ATA GCT GC 3' 5' GAA TAA GTG GCT GAA AGT TTC C 3'	27	0.87	58˚C	EU155142

* chromosome designation according to Rütten *et al.* (2004)

Population structure analysis

Some sex allocation models use the population inbreeding coefficient F_{IT} as a measure of relatedness to estimate the optimal strategy for a foundress (e.g. Frank 1985b and see citations in introduction), assuming that females (and patches) have equal productivity. Furthermore, information about the population genetic structure allows estimations about gene flow and migration rates among populations. For this analysis we divided the samples into the two geographic regions (HV and Schl) which contain several nest boxes, each of which can be considered as a patch (in the LMC terminology) or a subpopulation (in the *F*-statistical sense). As the individuals emerging from one nest are the members of only a few families (Molbo & Parker 1996), the relatedness among these is very high. We therefore decided to use each foundress genotype once, rather than use the genotypes of all the offspring. In this way the sample size was reduced considerably, but we avoided multiple non-independent samples. The most common method for determining population differentiation and inbreeding involves *F*-statistics, which were originally designed for diploid organisms (Wright 1931; Weir & Cockerham 1984; Slatkin 1987; Cockerham & Weir 1993). As we only use the diploid females in our analysis, we can apply F-statistics. However, in their review on population genetics of X-linked genes and haplodiploids, Hedrick and Parker (1997) find that a major effect of

haplodiploid inheritance is a reduced effective population size compared to diploids. Hence, care should be taken in comparing quantitative results with data of diploid organisms.

Hedrick (1999) cautioned against the use of conventional F-statistics on microsatellite data, as the high mutation rate and the high number of alleles of such markers can lead to a severe underestimation of the genetic differentiation. New mutations in separated populations can produce identical alleles that are not identical by descent and therefore mask the differentiation (Nauta & Weissing 1996). He recommended the use of Slatkin's private allele method (Slatkin 1985; Barton & Slatkin 1985). Later Hedrick (2005) developed a standardized measure of F_{ST}, called G'_{ST} which is standardized for the maximal value that G_{ST} (a multi allelic version of F_{ST}) can reach, given a certain genetic diversity in a population. Here we apply all three methods and compare the results.

In the following we will use the F-statistical terminology as used by Hartl and Clark (1997), with the subpopulation (index *S*) being the individual nest box, or patch in the LMC sense, and the sampling areas (Schl or HV) being localities (index *L*). The total population (index *T*) represents the pooled data set of both localities. A classical F-statistical analysis within regions was not possible, as 5 out of the 18 nest boxes were parasitized by one female only (leading to very localised mating prior to female dispersal). As a substitute for F_{ST} we used Rousset's distance *a* (Rousset 2000) between pairs of individuals within a region, within and between patches to test for isolation by distance. The expectation is a linear positive correlation between genetic distance and the logarithmic geographic distance (Rousset 1997).

Population statistics were calculated using FSTAT 2.9.3 (Goudet 2001), GENEPOP (http://genepop.curtin.edu.au/) (Raymond & Rousset 1995), and SPAGeDi 1.2 (Hardy & Vekemans 2002). Statistical tests were performed with SPSS 13.0 or R 2.4.1 (R Development Core Team 2006). All mean values are given as arithmetic mean ± SE unless indicated differently.

Table 2.2**:** Number of foundresses estimated, total number of hosts, and total number of parasitized hosts at 2 field sites. HV = Hoge Veluwe National Park (The Netherlands); Schl = Schlüchtern (Hessen, Germany);

Nest box no.	No. of foundresses	Total hosts	Hosts used
HV 8	1	15	1
HV 13	5	27	27
HV 220	5	NA	9
HV 267	7	16	16
HV 288	1	25	11
HV 306	1	6	1
HV 323	2	8	6
HV 330	5	82	79
HV 344	1	43	4
HV 365	1	35	1
Schl 11	4 (a)	25	15
Schl 13	2(b)	25	3
Schl 16	2	25	4
Schl 20	2(c)	25	25
Schl 21	7(a, d)	25	9
Schl 22	4(b, c, d)	25	14
Schl 23	2	25	1
Schl 28	3	25	15
Total	49 (6 double visits)	466	241

a = 3 foundresses found in nest box Schl 11 that also parasitized nest box Schl 21
b = foundress that parasitized nest box Schl 13 and Schl 22
c = foundress that parasitized nest box Schl 20 and Schl 22
d = foundress that parasitized nest box Schl 21 and Schl 22
NA = the number of total hosts in these nest boxes was not recorded; for the total number of hosts the number of hosts parasitized (9) was assumed

Results

Foundress numbers and pattern of parasitism

From the 95 nests that were inspected at the Hoge Veluwe, 15 (16%) contained fly pupae of which 9 (9.5% of total) yielded *Nasonia vitripennis* emerging from at least one host. The baits in the HV nest boxes only yielded *Nasonia* in one case (HV 288). From the 28 baited nest boxes in Schlüchtern, 8 (29%) yielded *N. vitripennis*. The total number of natural hosts found per nest box ranged from 6 to 82. The number of parasitized hosts per nest box ranged from 1 to 79 (Table 2.2).

We genotyped a total of 3550 individuals emerging from 9 natural nests (HV) and 9 baits (8 Schl and 1 HV) (the complete data can be found as Supplementary Data online). We could identify a total of 49 foundresses (arithmetic mean per patch: overall 3.0 ±0.46, in HV 2.9 ±0.74, in Schl 3.1 ±0.55; harmonic means: overall 1.9, HV 1.6, Schl. 2.4, Figure 2.1A). Assuming that the allele frequencies measured in our sample represent the genetic makeup of the whole population (HV and Schl combined), the chance that two unrelated individuals share the same allele is equal to the frequency of the particular allele in the population. Due to the high allelic variation of our markers, the chance of encountering two or respectively three females that have identical genotype in all four markers is < 0.001. The number of offspring per foundress per host varied between 1 and 39 (mean = 9.99 ±0.41), while the number of hosts being parasitized by a single foundress varied between 1 and 27 (mean = 7.17±1.02). The total number of offspring per foundress across all hosts varied between 1 and 346 (mean = 72.5± 9.46). The total number of offspring per host varied between 1 and 55 (mean = 14.77±0.64). The observed level of superparasitism is high. In 39.5% (N = 241) of all hosts we found evidence for more than one foundress and in 5.5% more than two foundresses (Figure 2.1). In Schlüchtern we found six foundresses parasitizing hosts in two nest boxes each (three on S11 and S21, one on S13 and S22, and one on S20 and S22 Table 2.1). We found no significant difference in the distribution of foundresses across patches or hosts between the natural nests (HV samples) and baits (SCHL samples) (Kolmogorov-Smirnov test: patch-level D = 0.29 n.s, host-level D = 0.5 n.s.).

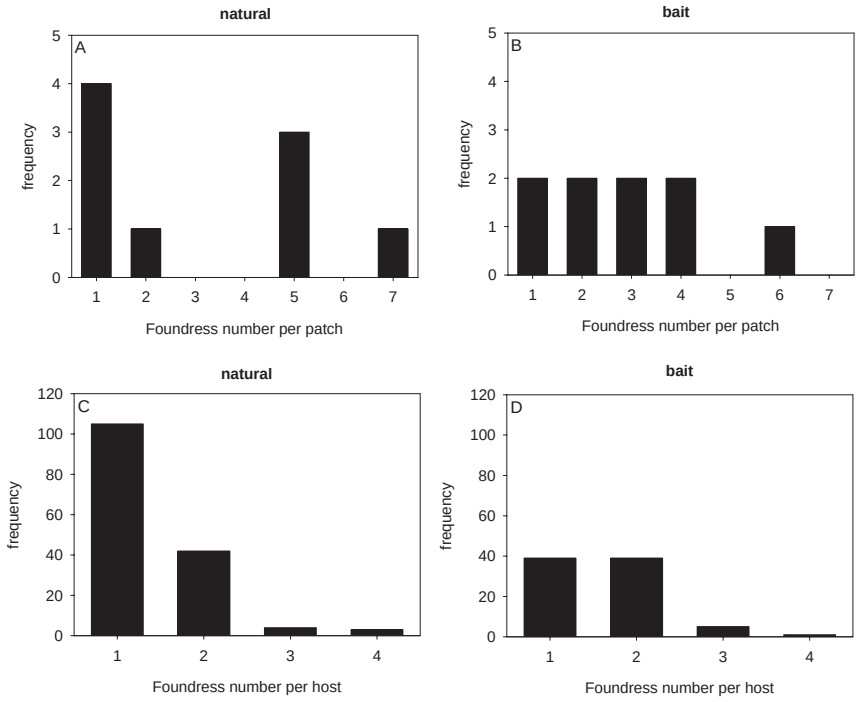

Figure 2.1: Frequency distribution of the number of foundresses per patch (A, B) and host (C, D) under natural conditions (A, C) and baits (B, D).

There was no evidence for female preference for or against patches or hosts used by other females. We found no significant deviation from a random distribution of the foundresses across used patches (dispersion test for a Poisson distribution following Grafen and Hails (2002), $\chi^2 = 69.03$, df = 237, p = 1) or across the hosts within a patch (χ^2-test against Binomial distribution using pooled data of all patches and combining the low represented classes, $\chi^2 = 12$, df = 9, p = 0.213). The total number of hosts present in a patch and the number of foundresses parasitizing also showed no significant correlation (adj. $R^2 = -0.024$, $F_{1,17} = 0.582$, p = 0.456). Although there is a large variation in the clutch

sizes per foundress, there was no significant correlation between foundress number per host and the clutch size per foundress (adj. R^2 = -0.003, $F_{1,342}$ = 0.07, p = 0.798). The mean coefficient of variation within clutch sizes of a particular host is 0.69 ±0.04, and therefore not negligible.

Although the data were not specifically collected to test for synchrony of parasitism, we can obtain some information from our data. The time window in which wasps emerged from a single host ranges from 1 to 10 days and for all hosts of a patch from 1 to 19 days (Figure 2.2). There is a strong positive relationship between foundress number and emergence window of a patch (adj. R^2 = 0.7161, $F_{1,7}$ = 21.18, β = 2.71 ±0.59, p = 0.0024; Figure 2.2A) and of a host (adj. R^2 = 0.093, $F_{1,139}$ = 15.4, β = 0.95 ±0.24, p < 0.001; Figure 2.2B).

Our data strongly suggest multiple mating in two cases (4%, N = 49). Among the HV-foundresses we found one female that was doubly mated (HV 267, foundress #14) and one that was mated three times (HV 330, foundress #26). An alternative explanation would be a genotyping error, but the decision on additional mates is supported by more than one marker, and doubtful individuals have been genotyped twice, which makes genotyping errors unlikely. The high level of inbreeding in the population (see data below) however, increases the relatedness of individuals in the populations and therefore the chance of highly related individuals parasitizing the same patch, which could then lead to the impression of multiple mating.

One nest produced only male offspring (HV 8), which can most easily be explained by a single unmated foundress. We excluded this progeny from further sex ratio analysis. Without this nest the sex ratio (proportion male) of the emerged offspring varied between 0.05 and 0.56 across the nests. The nest and host sex ratios as a function of foundress number roughly fit the theoretical predictions of basic LMC models (Figure 2.3).

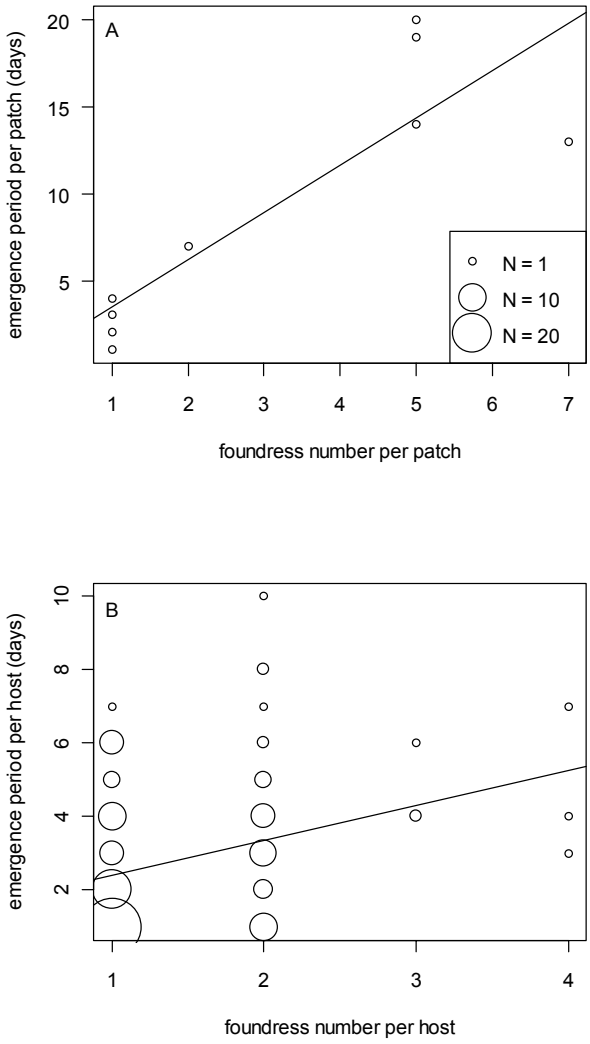

Figure 2.2: Emergence window per patch (A) and per host (B) in days as a function of the foundress number per patch (A) and per host (B). The circle surface is proportional to the sample size. The regression lines are highly significant (see text for details).

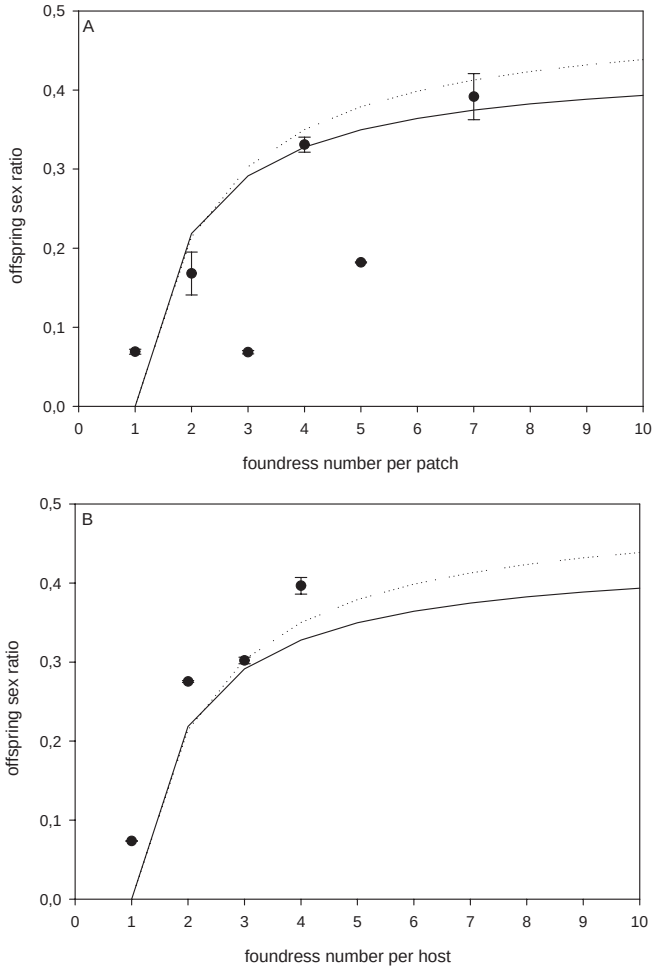

Figure 2.3: Sex ratio (proportion of males) ± SE as a function of foundress number per nestbox (all hosts pooled) (A) and per host (B), compared to the expectation of Hamilton (1979) (dotted line) and Frank (1985) assuming $F_{IL} = 0.168$ (solid line).

Population genetic analysis

Due to the fact that *N. vitripennis* is mating in the natal patch, and the small size (~3 mm) of the dispersing females, the population of this parasitoid is expected to be highly subdivided. Random mating within the regions (Schl and HV) and the total population can be tested indirectly by comparing measured and expected heterozygosity among the foundress females. The mean F_{IL}= 0.168 ± 0.016 indicates a heterozygote deficiency and therefore non-random mating (inbreeding) within the regions; F_{IT} = 0.197 ± 0.014 shows more or less the same for the whole population (HV and Schl pooled) (Hartl & Clark 1997). The differentiation index F_{LT} = 0.035 ± 0.011 indicates a low differentiation among the regions in general (averaging over loci). Slatkin's private allele method results in Nm = 2.62 which corresponds to F_{LT} = 0.11 (following F_{RT} = 1/(1+3Nm)), a threefold higher value. Hedrick's standardized G'_{LT} = 0.23 is even higher (Hedrick 2005).

We found no positive correlation between geographic (ln (geographic distance)) and genetic distance (Rousset's a) within a locality (Mantel's test: Schl r^2 = 0.0047, n.s.; HV r^2 = 0.0043, n.s., Figure 2.4). The mean genetic distance between foundress females of one patch was not different from the mean genetic distance of foundress females from different patches within one region (HV: within patches 0.19 ± 0.04, between patches 0.21 ± 0.02, 2-sided t-test: t = -0.3833, df = 49.825, n.s.; Schl: within patches 0.16 ± 0.07, between patches 0.14 ± 0.02, 2-sided t-test: t = 0.1928, df = 17.09, n.s.).

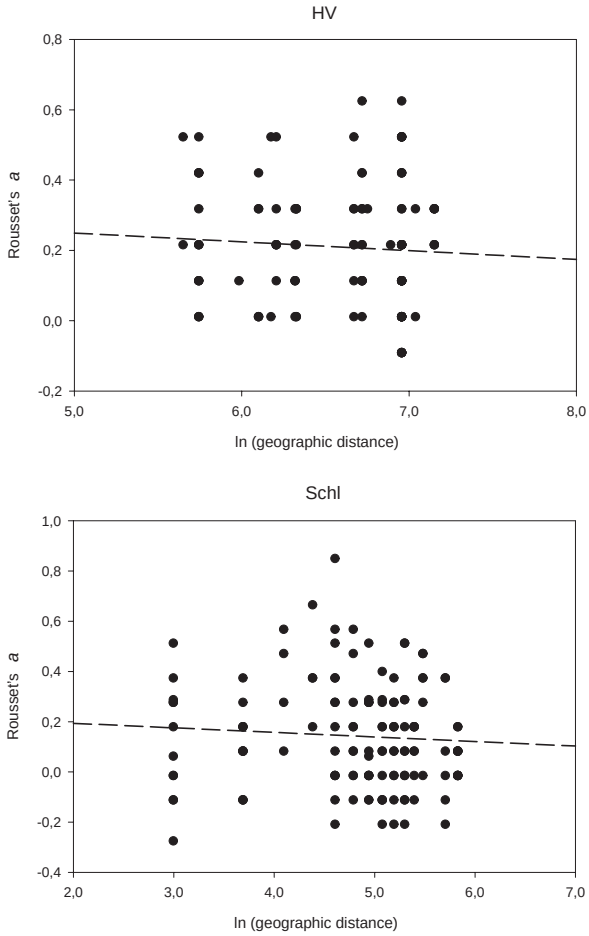

Figure 2.4: Genetic differentiation in *N. vitripennis*. Shown is pairwise genetic differentiation in the form of Rousset's a (Rousset 2000) against logarithmic geographic distance. The upper graph shows the HV data (R2 of regression line 0.0043), the lower graph Schl (R^2 of regression line 0.0047). All pairs of estimated foundresses from different nest boxes are shown, as well as the regression lines.

Discussion

Herre (1985) found that species of fig wasp that are more likely to encounter a conspecific on a patch are more likely to shift sex ratios as predicted by LMC. *N. vitripennis* is known to have a very strong response to LMC in the laboratory. Here we have shown that foundress numbers vary across hosts and patches in the wild, with a high superparasitism rate of 40% at the host level and 63% at the patch level. Therefore we can conclude that conditions favouring facultative sex allocation in *N. vitripennis* are frequent enough for LMC theory to be relevant to our field populations. Given these data, *Nasonia* should have evolved as a result of LMC selection and be an ideal model organism to test assumptions of LMC. Table 2.3 gives an overview of the most important LMC model assumptions and the results of this study.

Table 2.3**:** Overview of assumptions made by several models on local mate competition theory and the results of this study

Reference	Assumption	Found in this study?
General assumptions	(1) Localized mating within patches	Yes
	(2) Random dispersal of mated females	Yes
Hamilton 1967	Equal clutch sizes	No
Hamilton 1967	Random offspring mating within patches	No
Werren 1980, Hamilton 1967, Frank 1985	Synchronous parasitism	No
Nunney and Luck 1988, Shuker *et al.* 2005	Asynchronous parasitism	Yes

Fragmented populations?

A general assumption in LMC theory is that the population is highly subdivided in terms of mating. This is usually thought to be a consequence of the patchy distribution of hosts. Our data confirm that patches are often parasitized by only one female, leading to very localized mating. LMC theory then assumes that mated females disperse randomly from their natal patch. Consistent with this,

the individual based test for isolation by distance did not show an increase of genetic differentiation with geographic distance within localities (Figure 2.4). This lack of differentiation between patches is also shown by the equal level of genetic distance within and between patches of one locality. Using the conventional F-statistic as developed by Weir & Cockerham (1984) to compare the localities, we find a rather low degree of differentiation between the two sampling localities (F_{LT} = 0.035). The private allele method estimates the number of migrants per generation between the populations and can be interpreted as a F_{LT} of 0.11. This resembles more considerable differentiation and is in the same range as Hedrick's G'_{LT} of 0.23. Together, these data indicate that there is high dispersal within the scale of the localities and that the composition of foundresses parasitizing a patch represents a random genetic sample of the local population. Therefore the relatedness among the foundresses of a patch can be expected to be similar to that within a locality. Between the two localities (HV and Schl) however, gene flow seems to be very limited, as expected by the large distance of about 300 km. The low differentiation indicated by the conventional F-statistics can easily be explained by the high variation of the used markers (Hedrick 2005). Therefore, the variation independent measurements G'_{LT} and the private allele method should be more informative. This leads to the conclusion that the relevant scale for LMC is the hierarchical level of localities, and not the total sample.

A common measure of the level of relatedness in a population is the population inbreeding coefficient F_{IT}. Hamilton (1979) predicted that, under the assumptions of random mating within a patch and equal foundress productivity, the population inbreeding under LMC should follow $F_{IT} = 1/(4n-3)$, with n being the harmonic mean number of foundresses per patch. For our study n is 1.9, resulting in an expected population inbreeding coefficient of 0.22 which is very close to the observed value of F_{IL}= 0.168 ± 0.016. We use F_{IL} rather than F_{IT}, as the relevant level for LMC is the local population rather than the total sample as discussed above. However, the assumption of equal productivity is clearly violated and mating within a patch might not be random (as a consequence of asynchronous parasitism; see below). Therefore Hamilton's prediction can only be seen as a rough estimate.

Molbo and Parker (1996) calculated a population inbreeding coefficient F_{IT} of 0.312 for a Swedish population, which is considerably higher than our study. However, Molbo and Parker used all genotyped individuals for a calculation of F_{IT}, in contrast to our study (Molbo, personal communication). A recalculation of F_{IT} in our study using all individuals results in 0.272 ±0.042 which more closely resembles the value of Molbo and Parker (1996). Moreover as Molbo and Parker (1996) used allozymes, the probability of underestimating the real number of foundresses due to limited variation in the marker is much higher than with the microsatellites we used (~10% Molbo and Parker 1996, < 1 % this study). In addition they estimated 1.5 foundresses per patch, while our estimate is 1.9. We also found a higher level of superparasitism (41%) than Molbo and Parker (23%). These differences could be explained by the higher resolution of our microsatellite markers, or by ecological differences between their Swedish population and our Dutch and German populations (such as population densities of parasites and hosts). An overall inbreeding coefficient F_{IL} of 0.168 corresponds to 45% sibmating (using $S = 4F_{IT}/(1+3F_{IT})$, Werren 1987). This is in the same range as the proportion of sibmating that has been found for *Trichogramma pretiosum* (56.6%, Antolin 1999), a gregarious parasitoid of Lepidoptera.

Equal clutch sizes and random mating within patches?

Hamilton (1967) assumed in his original LMC model that there is random mating among all the offspring on a patch and that all females in a patch lay equally sized clutches. Unsurprisingly, females lay varying clutch sizes, and there is a large coefficient of variation in clutch sizes per pupa across the patches (0.69 ± 0.04). This variation could be a consequence of sequential parasitism where the first female usually lays the largest clutch and later females lay reduced clutches (Werren 1980).

Unfortunately we cannot measure deviations from random mating on patch level using our data. One way to do that would be to measure the relatedness between foundresses and their mates. As *Nasonia* males are haploid and we have only information from four microsatellite loci, such measurements would be rather limited in this context, and we therefore did not present such analysis here. However, we can draw some conclusions from our other findings. The data strongly suggest that parasitism of hosts on a patch is asynchronous (see

next section for details), which leads to a bias in the opportunities for individuals from different hosts to mate with each other, as the daughters of early foundresses might have already left when the sons of late foundresses emerge. The sons of early foundresses on the other hand will have the chance to mate with their early sisters as well as with the daughters of late foundresses, as they stay on the patch. This obviously leads to the conclusion that mating among the offspring of a patch cannot be completely random, but only among the offspring that are present at the same time. (Shuker *et al.* 2006a).

Synchronous parasitism?

If all foundresses parasitized hosts at the same time, one would expect no increase in the emergence window with foundress number. As the emergence window on patch and host level is strongly positively correlated with foundress number (Figure 2.2), synchronous parasitism is perhaps the exception rather than the rule (Werren 1980, Hamilton 1967, Frank 1985). However, alternative explanations for the emergence window exist, including delayed developmental time due to crowding in the hosts, or individual foundresses parasitizing the same host several times, which might occur given the large variation in emergence time of the offspring of single foundresses (Figure 2.2B). Multiple parasitism by a single foundress on the same host may change the optimal sex ratio towards more males, if the female parasitized other hosts in between, as found by King (1992). Werren (1980) found that asynchronously laid clutches are synchronized by a speed up of development of the later clutches. Such a behavior would lead to a weaker correlation between foundress number and emergence window, than is evident from our data. However, we only collected data on the emergence window per host. To be able to resolve parasitism strategies of individual foundresses we would need data on the emergence time of individual offspring. Nevertheless, LMC models for species such as *Nasonia vitripennis* should incorporate asynchronous parasitism, as is the case in some more recent models (Nunney & Luck 1988; Shuker *et al.* 2005).

Additional parameters

In addition to the assumptions from existing LMC theory (Table 2.3) that were tested, we also considered some other parameters. Although the total number of hosts may intuitively be considered as a good predictor of patch quality, we did

not find a significant correlation between foundress number and the total number of hosts in a patch. One reason for this might be variation in individual host quality across patches. Also, variation in age of the hosts might play a role in the attractiveness of a patch. Hosts can only be parasitized by *N. vitripennis* if they are at a certain stage of development. If a patch has a large number of hosts suitable for parasitism for a longer period of time due to variation in host age, it might attract more wasps than a patch with an equal number of hosts that are all the same age. This would also explain the inferred patterns of sequential oviposition.

As superparasitism constitutes direct resource competition for a particular host, one may expect that the foundresses have evolved ways to avoid each other when parasitizing the same patch, as has already been shown in several studies (e.g. Shuker *et al.* 2005). Such a behaviour would lead to an underdispersed pattern of parasitism. However, our results do not indicate a significant deviation from a random pattern of parasitism. We should mention though that our sample sizes, especially on patch level, are rather low and that the goodness of fit test that was applicable for our data is not very powerful. Hence, at patch level, we have no strong evidence for preference or avoidance of superparasitism.

The estimated percentage of unmated females (2%) is in the range of what has previously been reported: Beukeboom and Werren (2000) found 2.99% ± 2.32% in a larger field sample from the US. This frequency of so-called constrained females should not have a strong effect on the expected optimal sex ratio at the level of the population (Godfray 1990; Hardy & Godfray 1990). We assumed that the all-male family in a one-foundress patch in our study was due to an unmated female. We also found some all-male families among superparasitized hosts. In these latter cases family sizes were small and the assigned female also produced daughters in other hosts. Hence, such small all-male families can be considered as the outcome of superparasitism as predicted by LMC (Werren 1984).

Although previous studies indicated that single mating appears to be the rule in *Nasonia* (Azab *et al.* 1967; van den Assem & Visser 1976; van den Assem 1977), we found evidence that a small proportion (2 out of 49, ~ 4%) of foundresses are multiply mated. Genotyping errors can almost be ruled out, as

we genotyped doubtful individuals at least twice, but the high level of inbreeding indicates that there is a high chance of highly related foundresses that have similar genotypes. If there would be the tendency that highly related females parasitize the same patch, there should be a correlation between genetic and geographical distance on a local scale. Our isolation by distance analysis however did not show any indication of such a correlation (Figure 2.4).

In general, polyandry reduces relatedness among the female offspring of a particular female. Unlike inbreeding, which would lead to selection for a more female biased sex ratio (Reece *et al.* 2004; Shuker *et al.* 2004a), polyandry does not change the relatedness of a mother to her offspring and should therefore have no influence on sex allocation. It has been shown that multiple mating in *N. vitripennis* increases with time cultured in the lab (van den Assem & Jachmann 1999; Burton-Chellew *et al.* 2007a). Furthermore, van den Assem and Visser (1976) showed that females are willing to mate a second time when they have already laid eggs. Therefore, it is conceivable that a previously mated female encounters a male that was born on the patch where she is ovipositing and mates a second time outside her natal patch. Nevertheless multiple mating seems to be rare in *N. vitripennis* and the effect of this behavior on the population genetic structure is likely to be negligible.

Finally, as predicted, we found a strong positive correlation between sex ratio and number of foundresses per patch, although there were large quantitative deviations from the predictions of Hamilton (1967) and Frank (1985b). We consider sex allocation in more detail elsewhere (Burton-Chellew *et al.* 2008).

To summarize our findings we can state that a suitable model of LMC for species such as *Nasonia vitripennis* should make the following assumptions: (1) large variation in clutch sizes, (2) non-random mating within the offspring of a patch, (3) asynchronous parasitism, (4) regular encountering of competitors, (5) highly structured mating populations (within localities) followed by (6) a random distribution of foundresses across the patches, and across hosts within patches. More recent models of LMC have started to take such factors into account (Nunney & Luck 1988; Shuker *et al.* 2005). Our findings provide empiric values for these factors and this will help to develop more realistic and precise LMC models, and hopefully also stimulate much needed studies of sex allocation in the wild for a wider range of parasitoid species.

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Chapter 3

Facultative sex ratio adjustment in natural populations of wasps: cues of local mate competition and the precision of adaptation

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Abstract

Sex ratio theory offers excellent opportunities for examining the extent to which individuals adaptively adjust their behaviour in response to local conditions. Hamilton's theory of local mate competition (LMC), which predicts female biased sex ratios in structured populations, has been extended in numerous directions to predict individual behaviour in response to factors such as relative fecundity, time of oviposition and relatedness both between foundresses and their mates. These extended models assume that females use different sources of information and have generally been either not tested or only tested in the lab. We use microsatellite markers to describe the oviposition behaviour of individual females in natural populations of the parasitoid wasp *Nasonia vitripennis*, and hence test these various models. The offspring sex ratio produced by a female on a particular host reflected the number of eggs laid on that host, relative to the number of eggs laid on that host by other females. In contrast, the offspring sex ratio was not directly influenced by other potentially important factors, such as the number of females laying eggs on that patch, relative fecundity at the patch level, or relatedness to either a mate or other females on the patch.

Introduction

Sex ratio theory has provided excellent opportunities for examining the precision of adaptation (Charnov 1982; Herre 1987; Hardy 2002; West & Sheldon 2002; Boomsma *et al.* 2003; Shuker & West 2004). One of the most productive areas from this respect has been Hamilton's theory of local mate competition (LMC), which explains why female biased sex ratios are favoured in structured populations, where mating occurs before the females disperse (Hamilton 1967). Specifically, if N diploid females lay eggs on a patch, then the evolutionary stable (ES) sex ratio (r^*; proportion males) is given by $r^* = (N-1)/2N$ (Hamilton 1967), such that decreasing the number of foundresses increases the female bias in the sex ratio. One way of conceptualising this is that a female bias is favoured as it reduces competition between sons (brothers), and increases the number of mates for sons (Taylor 1981). An additional bias is favoured in haplodiploid species because inbreeding makes females relatively more related to their daughters than their sons (Herre 1985; Frank 1985b). There is extensive empirical support for the basic predictions of LMC theory: females of numerous species have been shown to adjust their offspring sex ratios in response to the number of females laying eggs on a patch (N) (West *et al.* 2005).

Extensions of LMC theory have suggested that the pattern of sex ratio adjustment should vary depending upon how much information females are able to process about the environment. Hamilton's original prediction was based on a number of simplifying assumptions, such as females contributing the same number of offspring to each patch, and random mating within the patch (Hamilton 1967). These assumptions implicitly constrain what information females are thought to use. When these assumptions are relaxed, offspring sex ratios are predicted to vary within the patch, between individuals, and over time and space (Werren 1980; Suzuki & Iwasa 1980; Yamaguchi 1985; Frank 1985b; Frank 1987; Stubblefield & Seger 1990; Taylor & Crespi 1994; Abe *et al.* 2003; Reece *et al.* 2004; Shuker *et al.* 2005). For example, Werren (1980) showed that when two females lay eggs sequentially on the same host, the sex ratio produced by the second female to lay eggs on the host should be negatively correlated with the relative size of her clutch (clutch laid by the second female divided by

the clutch size of the first female). This is because, when a female produces a lower proportion of offspring, then her offspring will experience less LMC (i.e. less competition between brothers). It has since been shown that the same qualitative prediction, to produce a less female biased sex ratio, or even a male biased sex ratio, arises in a range of other models with simultaneous oviposition (Yamaguchi 1985; Frank 1987; Stubblefield & Seger 1990). Table 3.1 summarises these models and identifies which variables are predicted to influence sex ratio. Whilst these models have been tested several times in the laboratory (see below), there has been a conspicuous absence of field tests that examine what information females actually use when varying their sex ratio under LMC. This is largely because of the technical difficulties of recording oviposition behaviour in the field.

Here we address this problem by using microsatellite markers to trace the field behaviour of individual females of the parasitic wasp *Nasonia vitripennis*. *Nasonia vitripennis* is an ideal organism for such a study because it is known from both laboratory and field studies that the females adjust their sex ratios in response to the basic tenets of LMC (Werren 1983; Orzack *et al.* 1991; Molbo & Parker 1996; Shuker & West 2004; Grillenberger *et al.* 2008). *Nasonia vitripennis* has also been extremely useful in testing the more complex LMC models, but so far these studies have been restricted to the laboratory (Werren 1980; Orzack & Parker 1986; Orzack & Parker 1990; Flanagan *et al.* 1998; Reece *et al.* 2004; Shuker *et al.* 2004a; Shuker *et al.* 2004b; Shuker *et al.* 2006a; Shuker *et al.* 2007). Here we use the power and resolution of molecular techniques to test these extensions to LMC theory in the wild. Specifically, we (1) test to what extent females adjust their sex ratio in response to predicted environmental parameters (Table 3.1), and (2) test which models of LMC best approximate sex allocation in the wild. By genotyping more than 3500 offspring at four microsatellite loci, we were able to reconstruct the parental genotypes and hence determine the sex ratios produced by 49 females, in 350 broods across 18 natural patches. Our results provide the first detailed analysis of individual sex allocation under LMC in the wild.

Table 3.1: Models of sex allocation under Local Mate Competition, in terms of the information females are predicted to use and the variables associated with the models in our empirical study.

Model	Predicted information use	Empirical variables associated with the model
Hamilton (Hamilton 1967; Hamilton 1979)	Patch foundress number $s^* = (2N-1)(N-1)/N(4N-1)$	Patch foundress number
Stubblefield & Seger model I (S&SI) (Stubblefield & Seger 1990)	Knowledge of own fecundity, no knowledge of co-foundress fecundity ("imperfect knowledge")	Focal female fecundity (defined at the level of the host or patch)[1]
Stubblefield & Seger model II (S&SII) (Herre 1985; Frank 1985b; Stubblefield & Seger 1990)	Knowledge of own fecundity and co-foundress fecundity ("perfect knowledge")	Focal female and co-foundress fecundity (defined at the level of the host or patch) [1]
Werren (host)[2] (Werren 1980; Suzuki & Iwasa 1980)	Relative clutch size (focal female relative to co-foundresses) on a given host	Relative clutch size of focal female on a host (as difference in clutch sizes between focal and co-foundress females)
Werren (patch)[2] (Werren 1980; Suzuki & Iwasa 1980)	Relative clutch size (focal female relative to co-foundresses) across the patch	Relative clutch size of focal female on a patch (as difference in clutch sizes between focal and co-foundress females)
Asymmetrical LMC (Nunney & Luck 1988; Shuker *et al.* 2005)	Knowledge of own and co-foundress fecundities across both individual hosts and the patch as a whole	Focal female and co-foundress fecundities across hosts and patch
Greeff (Greeff 1996; Reece *et al.* 2004)	Relatedness to mating partner and foundress number	Relatedness to mating partner and foundress number
Frank (Frank 1985b; Taylor & Crespi 1994; Frank 1998; Shuker *et al.* 2004b)	Relatedness to co-foundresses and foundress number	Relatedness to co-foundresses and foundress number

1. Originally defined at the level of the patch, but if mating is increasingly non-random within a patch (Shuker et al. 2005), then each host effectively becomes patch.

2. The original Werren model is for sequential oviposition by two females, with the focal female being the second female. The predicted sex ratio is influenced by the primary sex ratio, the population inbreeding coefficient, as well as relative clutch size. We use it here in a general sense to consider sex allocation based on relative clutch size.

Materials and Methods

Study organism

Nasonia vitripennis is a gregarious parasitic wasp, with females laying clutches of eggs on a range of large Diptera pupae such as *Calliphoridae* and *Sarcophagidae* (Whiting 1967). The species is ectoparasitic, with the eggs laid between the pupa and puparium wall, and adults emerging from the host puparium to mate. Males are brachypterous and unable to fly, and are typically the first to emerge. They then mate with the emerging females. When multiple hosts on a patch are parasitised, mating is typically non-random with males and females from the same host more likely to mate with one another (van den Assem *et al.* 1980a; van den Assem *et al.* 1980b; Shuker *et al.* 2005). Females are fully winged and disperse away from the host. The mating system typifies that assumed by LMC, and *N. vitripennis* has long been an outstanding model organism for the study of sex ratios (Shuker & West 2004).

Sampling

We used two field sites, one in Hoge Veluwe (HV) National Park, the Netherlands, and one at a field site near Schlüchtern, Hessen, Germany (Schl). Full details of the sampling and subsequent genetic analysis of wasps are provided by Grillenberger *et al*. (2008). That paper also describes the patterns of oviposition on the patches and the population genetics of the two study populations. Briefly, we collected *Nasonia vitripennis* broods in June 2004 from bird nestboxes ("patches"), either by searching for parasitized host puparia (HV) or by leaving unparasitized host puparia (*Calliphora vicina*) at nestboxes as baits (patch size: 25 hosts, both HV and Schl). The HV samples consisted of ten nestbox samples, 9 of which were 'natural' collections and one that was a successfully baited sample. The Schl samples were eight successfully baited samples. All fly puparia were collected and incubated individually at room temperature.

Each day we brought out the incubated hosts into the daylight for at least 30 minutes before anesthetising any emerged individuals with CO_2 and storing them for molecular analysis. We checked for any un-emerged individuals by opening the fly puparia a month after the last emergence from that host. We recorded the origin of every individual in terms of field site, nestbox, and host.

The full details of the number of parasitised hosts and the individual broods are given in Table S1. Throughout we consider the number of emerged offspring to be the number of eggs laid by females (clutch size), thereby assuming negligible larval mortality. Whilst this has been shown to be the case under laboratory conditions (Werren 1984), we do not know the impact of larval mortality in the wild.

Molecular genetic analysis

We extracted whole genomic DNA from individual wasps by using either a standard high salt-chloroform protocol (Maniatis *et al.* 1982) or Chelex®100 (Bio-Rad California, USA). For genotyping we used four polymorphic, di-nucleotide repeat microsatellites (Nv-22, Nv-23, Nv-41, and Nv-46). Nv-22 and Nv-23 were originally developed by Pietsch et al. (2004) but the primers were redesigned for this study (Table S2). We separated PCR products by fragment length using an AB 3730 DNA analyzer or ABI Prism 377 DNA sequencer (Applied Biosystems, California, USA), and analysed them using either GeneMapper v4.0® or GeneScan 3.1® (Applied Biosystems, California, USA).

We sexed all individuals by external morphology before DNA extraction, checking damaged individuals by their heterozygosity (e.g. heterozygotes have to be female). Parentage was assigned according to Mendelian rules of inheritance under haplodiploidy. The genotypes of the foundresses that oviposited on each host were reconstructed from the genotypic data of the offspring. Each patch was resolved with the minimum number of foundresses required to explain the offspring. For the analysis presented above, two patches were excluded. In the first case, a solitary foundress oviposited on one host in the nestbox, producing only sons. This female may have therefore been a virgin and unable to produce daughters (a “constrained” female). We also excluded a nestbox containing 16 parasitised hosts and up to 7 foundress females. In this case, assigning offspring to foundresses was difficult as some of the foundress females, and their respective mates, appeared to be very closely related. This meant that numerous offspring had multiple possible mothers. Inclusion of these two patches does not qualitatively alter the results presented. The following analysis therefore considers 16 patches, containing 324 clutches from 47 foundress females laid on 222 hosts. These clutches produced 3027 genotyped offspring that were assigned to a foundress.

We calculated the average relatedness between all foundresses on each patch, and between each foundress and her mate(s), following the principles of Queller and Goodnight (1989) We used the Relatedness 5.0.8 program (developed by Goodnight; 2001) to generate relatedness values on a scale from -1.0 to 1.0. We treated the HV and Schl samples as two distinct populations and the estimate of the population allele frequencies was bias-corrected for each foundress by excluding both herself and her mate. We excluded the cases of a single foundress parasitizing a patch from the patch relatedness analysis.

Statistical Analyses

We performed two analyses. First we tested explanatory variables at the host and patch level. For the second analysis we tested specific statistical models appropriate for different models of LMC. For the first analysis the explanatory variables were: patch foundress number; host foundress number; difference in fecundity of focal female versus other foundresses on the host (or on the patch); focal female fecundity; patch size (as total number of hosts); numbers of parasitised hosts (by the focal female, and by all females on patch); proportion of the hosts parasitised; the relatedness between females on a patch (if appropriate – see above); relatedness of a female to her mate. The difference in fecundity between a focal female and the other females on the host (or patch) was calculated by subtracting the number of offspring produced by other foundress females from the number produced by the focal female. This allowed us to consider a form of relative clutch size, a potentially important variable (Werren 1980), usually calculated as (focal female clutch size)/(non-focal female clutch size). However, this latter definition is undefined for females that oviposited by themselves, necessitating the use of difference in fecundity. When we specifically considered just those hosts with more than one foundress (i.e. superparasitism), the more usual relative clutch size of the focal female was used. For one patch the total number of hosts (parasitised plus unparasitized) was not known due to a recording error. Therefore the fixed effects “patch size” and “proportion of parasitised hosts” were tested on the subset of 15 patches with this information. One potentially informative variable that we were unable to measure is laying order (i.e. the sequence in which particular females contributed eggs to a host or patch). Emergence times of wasps do not provide reliable oviposition order data since in *N. vitripennis* superparasitism can lead to

Table 3.2: Analysis of sex ratio variation.

	Fitted together		Fitted alone	
Fixed effect	**t (d.f.)**	**P**	**t (d.f.)**	**P**
Patch foundress no	1.28 (279)	0.20	2.74 (282)	0.007
Host foundress no	1.06 (277)	0.29	6.34 (282)	<0.0001
Relative fecundity (patch)	0.65 (274)	0.52	1.64 (281)	0.10
Quadratic term	1.64 (281)	0.10	0.66 (281)	0.51
Relative fecundity (host)	8.23 (282)	<0.0001	8.09 (281)	<0.0001
Quadratic term	1.18 (278)	0.24	1.57 (281)	0.12
Focal female patch fecundity	0.55 (276)	0.59	2.05 (282)	0.04
No of hosts used by focal female	0.96 (275)	0.34	0.99 (282)	0.32
Total no of hosts used on patch	1.46 (280)	0.15	0.01 (282)	0.99
Patch size	1.43 (34)	0.16	1.43 (34)	0.16
Proportion of hosts used	1.13 (274)	0.26	1.13 (274)	0.26
Patch relatedness	0.92 (268)	0.36	0.59 (269)	0.56
Mate relatedness	0.34 (37)	0.74	0.02 (37)	0.99

Note: Fixed effects were tested either by (1) model simplification, with the all terms fitted together in the full model, with the least significant terms removed in turn, with significance tested after the fitting of any other significant effects, or (2) fitted alone in a model (apart from the relative fecundities which are fitted with their respective quadratic terms). t values are marginal t tests presented with approximate degrees of freedom.

synchronised development of the different broods within a host (Werren 1980). Whilst relative clutch size is a possible proxy of laying order (since in gregarious parasitoids superparasitising females typically produce a relatively smaller clutch size, e.g. Godfray 1994), we could not be sure this would always be the case. This problem with laying order is a necessary constraint of this kind of study (also see the Discussion).

Sex ratios are best modelled within a generalised linear modelling framework assuming binomially distributed errors and with a logit link function (Wilson & Hardy 2002). Since females could contribute multiple clutches, for the first analysis we used a generalised linear mixed modelling approach (GLMM) including female identity as a random effect to take these multiple observations

into account. GLMMs are still an area of active research and current tractable estimation methods do not generate true likelihoods but rather use approximations to complete the integration. We used restricted penalised quasi-likelihood (REPQL) as provided by the glme function in the Correlated Data library in S-Plus 7 (Pinheiro & Chao 2005) methods for binomially distributed data (Laplacian and adaptive Gaussian Quadrature methods) force the dispersion parameter to be 1 (i.e. assume true binomial variance), but our data were slightly over-dispersed (dispersion parameter = 1.555). The fixed effects were tested using marginal t tests with approximate degrees of freedom (Pinheiro & Chao 2005). Models were simplified by removing the least significant terms in turn, to generate the minimum adequate model. For completeness, given that several of the explanatory variables associated with different models of LMC are likely to be correlated with each other, we also tested variables alone in individual models.

For our second analysis, since GLMMs do not yield true likelihoods, we were unable to compare different models using techniques such as likelihood ratio tests or AIC (Akaike Information Criterion). In order to test how well different models of sex allocation predict wild sex ratios we therefore fitted specific models in turn (Table 3.1) to the sex ratio data using a maximum likelihood mixed effects framework. Model fit was examined by way of AIC and the models were then compared. All statistics were performed in S-Plus 7 (Insightful Corporation, Seattle, WA, USA). Means are presented ± standard error (with asymmetric binomial standard errors for sex ratio).

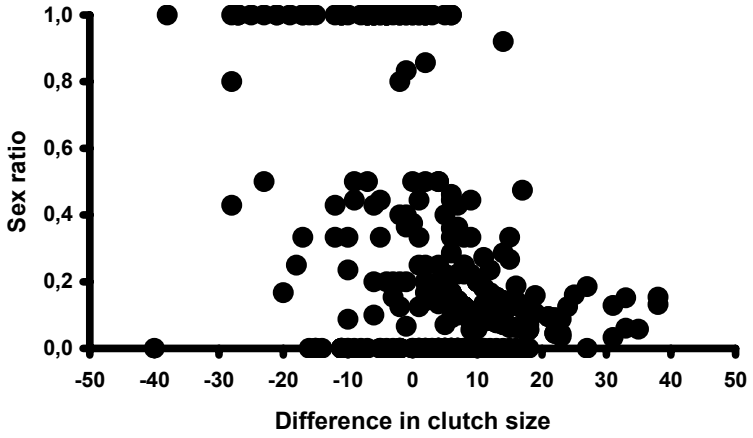

Figure 3.1: Sex ratios are negatively correlated with the difference in clutch size between females ovipositing on a host.

Results

Descriptive statistics

The overall sex ratio across the 16 patches was extremely female biased (0.200 ± 0.007). The number of females laying eggs on patches ranged from 1 to 7, and on individual hosts from 1 to 4. The average clutch size per host per female was 9.34 ± 0.40 wasps (N = 324 clutches). For those hosts where only one female laid eggs it was 11.56 ± 0.64, and in those hosts where multiple females laid eggs it was only 7.74 ± 0.48. Sex ratio did not differ between populations (t_{39} = 0.75, P = 0.46) and so the analysis below considers both populations together. Sex ratios did vary significantly among females (among-female variance component = 1.225, 95% confidence intervals = 0.654 – 2.292). The average relatedness between foundresses on a patch varied from -0.46 to 0.28, with a mean of 0.09 ± 0.04 for HV and -0.05 ± 0.05 for Schl. The average relatedness of a foundress to her mate(s) suggested appreciable levels of sibmating: for HV the mean relatedness was 0.32 ± 0.04 (N = 27); and for Schl it was 0.22 ± 0.02 (N = 19), with values ranging from -0.43 to 0.82.

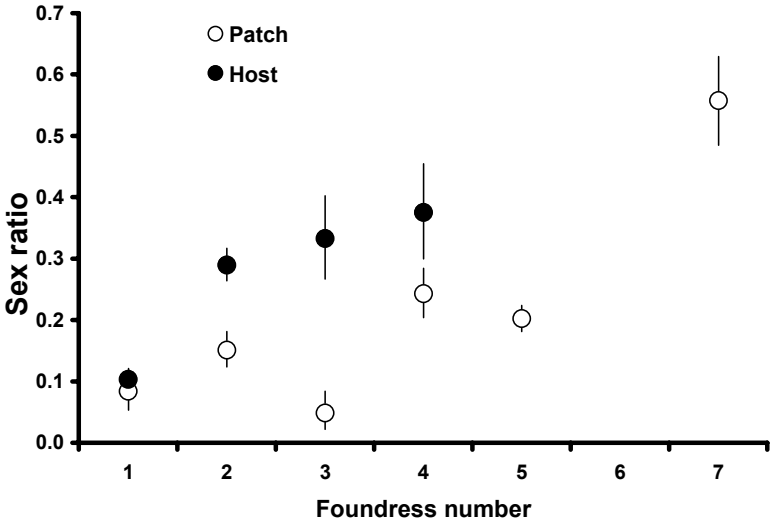

Figure 3.2: Sex ratios vary with the number of foundresses using the patch (open circles) or a particular host (filled circles). Error bars are 95% binomial confidence intervals.

Sex ratios

Sex ratios varied with the difference in clutch sizes that females produced on a host, with females producing more female biased sex ratios when they laid relatively more eggs on a host (t_{282} = 8.23, P < 0.0001; Figure 3.1). The quadratic term was not significant (t_{278} = 1.18, P = 0.24). When difference in clutch size at the level of the host was fitted in the model, no other factors were significant (Table 3.2).

The relative number of offspring that a female produced on a host or a patch was negatively correlated with the number of females laying eggs on that host or patch (host foundress number and difference in fecundity on that host: r_{322} = -0.66; patch foundress number and difference in fecundity on that patch: r_{322} = -0.22; both P < 0.0001). When difference in clutch size was not included in the model, the sex ratio was therefore positively correlated with both the number of females laying eggs on a host (t_{282} = 6.34, P < 0.0001; Figure 3.2) and the number of females laying eggs on a patch (t_{282} = 2.74, P = 0.007; Figure 3.2).

There was also a weak negative correlation between sex ratio and the total number of offspring a female contributes to a patch when fitted alone (t_{282} = 2.05, P = 0.04).

The above data set considers all females and combines different patterns of patch and host use. It is also useful to consider some specific cases. In the simplest case, an individual female was the only foundress on a patch (N = 4). With no cues indicating reduced LMC, sex ratios were highly female biased (sex ratio = 0.084, lower SE = 0.016, upper SE = 0.019) and independent of clutch size (per host: t_{12} = 0.59, P = 0.57; per patch t_2 = 0.12, P = 0.92), although of course the sample is very small. Alternatively, other females used a host individually, but shared the patch as a whole with other females (N = 27). Females did not shift their sex ratios on these hosts in response to the characteristics of the rest of the patch. Their sex ratios were not correlated with patch foundress number (t_{23} = 1.24, P = 0.23), clutch size on the host (t_{91} = 1.05, P = 0.30), total fecundity of the focal female on the patch (t_{23} = 1.01, P = 0.32), or with the difference in fecundity between the focal female and all the other foundresses across the patch (t_{23} = 0.82, P = 0.42). Finally, two or more females shared particular hosts (superparasitism, N = 35 foundresses). Sex ratios were highly significantly correlated with relative clutch size (defined here as [focal female clutch size]/[non-focal female clutch size]; see Methods), with sex ratios declining with increasing relative clutch size as expected by theory (Figure 3.3). Both relative clutch size and its quadratic term were highly significant (t_{151} = 4.47, P < 0.0001, and t_{151} = 3.81, P < 0.0001). The theoretical prediction for sex allocation under superparasitism according to Werren (1980; adjusted for haplodiploidy) includes the sex ratio of eggs already present on a host and the inbreeding coefficient. Using the sex ratio produced by females when ovipositing on a patch alone and F_{IT} = 0.197 (Grillenberger et al. 2008) the Werren model also predicts a highly significant proportion of the variance in sex ratio (t_{152} = 4.04, P < 0.0001; Figure 3.3 and Table 3.3).

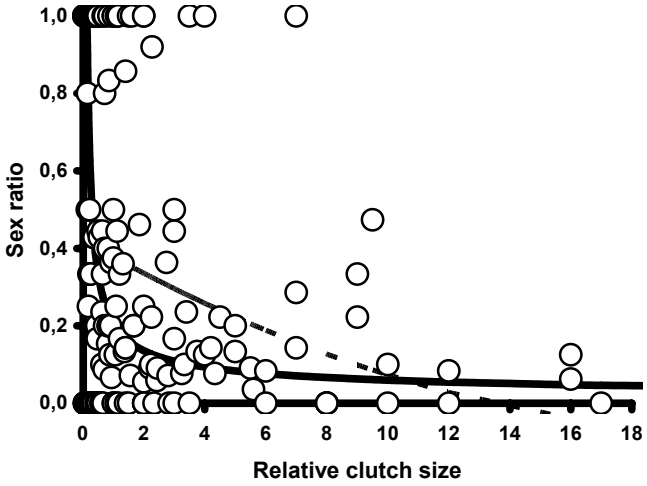

Figure 3.3: Sex ratios vary with relative clutch size when two or more females lay eggs on the same host (superparasitism). The dashed line is the relationship between sex ratio and relative clutch size (RCS) obtained from the analysis (sex ratio ~ 0.4211 – 0.0448*(RCS) + 0.0010*(RCS)2). The solid line is the prediction from Werren (1980) adjusted for haplodiploidy. For clarity, the largest relative clutch size has been omitted from the figure (RCS = 39.0, sex ratio = 0.154).

Testing LMC models

Models of sex allocation under LMC form a hierarchy, with more complicated models assuming that females use increasingly sophisticated information to estimate the level of LMC (Table 3.1). By including the appropriate variables for each model, we can assume that females process increase amounts of information about the patch. Doing so explains increasing amounts of the variation in sex ratios in the field (Table 3.3). The best fitting models suggest that complete knowledge of the clutch sizes of the females on a given host, either in absolute terms or as the difference between them, is crucial for explaining the sex ratio. The best fitting model of all is the "Werren (host)" model (1980). This also corresponds to the empirically derived minimal model from the above analysis, containing the difference in fecundity on a host. For the specific case of superparasitism, the empirically derived model above (relative clutch size and its quadratic term) fits the data marginally better than a fully-parameterised version of the Werren model (1980).

Table 3.3: Testing models of sex allocation that assume different sources of information for estimating the level of LMC experienced by offspring: (a) all females; (b) only those females sharing hosts (superparasitism)

Model	AIC	Log-lik	Residual	% decrease
(a) All females				
Random effect only	221.08	-107.54	0.3111	
Hamilton	217.54	-104.77	0.3100	0.35
S&S I (patch)	216.18	-104.09	0.3099	0.39
S&S II (patch)	211.46	-100.73	0.3094	0.55
S&S I (host)	180.08	-86.04	0.2901	6.75
S&S II (host)	**151.70**	**-70.85**	**0.2800**	**10.00**
Werren (host)	**149.96**	**-70.98**	**0.2804**	**9.87**
Werren (patch)	213.59	-102.80	0.3098	0.42
Asym LMC	**153.27**	**-69.63**	**0.2799**	**10.03**
Greeff	216.53	-103.27	0.3091	0.64
Frank	208.56	-99.28	0.3106	0.16
(b) Superparasitism				
Werren[(1)]	175.14	-83.57	0.3425	4.38[(2)]
Empirical model[(3)]	**171.52**	**-80.76**	**0.3405**	**4.94**

Note: mixed effect models were fitted by maximum likelihood, with Female as a random effect. Each model was fitted in turn. Model fit is described in terms of: AIC = Akaike Information Criterion; Log-lik = log-likelihood of the model; Residual = residual deviance of the model; % decrease = % decrease in residual deviance compared to the model with just the random effect. Models in bold represent the better fitting models. The model "Werren (host)" also represents the minimal model from our empirical analysis. For full details of the models see Table 3.1.

1. The specific version of the Werren (1980) model adjusted for haplodiploidy (Suzuki & Iwasa 1980; Greeff 2002) and parameterised using the single foundress sex ratio, relative clutch sizes, and inbreeding coefficient from this paper and Grillenberger et al. (2008).

2. The residual deviance after fitting the random effect only is 0.3582.

3. Contains the variables Relative Clutch Size and (Relative Clutch Size^2).

Discussion

We used microsatellite markers to determine the sex ratio behaviour in the field of individual *N. vitripennis* females. We found that the only significant variable was the relative clutch size laid on a host: females produced a less female biased sex ratio when they laid relatively fewer eggs on a host (Figure 3.1). When this effect was included in the model, no other factors were significant (Table 3.2). We also tested the extent to which different LMC models could explain variation in sex ratio. We found that whilst models based purely on the number of females laying eggs on a patch (Hamilton 1967), or the relative fecundity on a patch (Stubblefield & Seger 1990), were statistically significant, they did not fit the data as well as models based on relative fecundity at the host level (Werren 1980; Suzuki & Iwasa 1980; Shuker *et al.* 2005; Table 3).

Our results suggest that females are adjusting their offspring sex ratio in response to variation in the extent of LMC, and that the primary cue on which they are basing their behaviour is the relative number of eggs that they are ovipositing on each host. In contrast, they do not appear to be using information about the total number of females on a patch, or the relative fecundity of different females on a patch. This result agrees with a recent laboratory experiment in which females were shown to lay less female biased sex ratios when co-foundress females were present, but that the primary cue was the eggs laid by those other females, and not the presence of the females themselves (Shuker & West 2004). We suggest that the explanation for these results is that females are responding to the cues that are reliable indicators of the extent of LMC that their offspring will experience under natural conditions. Females appear to be able to assess with relative ease whether a host has been previously parasitized (Werren 1984; King *et al.* 1995; Shuker *et al.* 2005; Shuker *et al.* 2006a), and a higher proportion of previously parasitized hosts should correlate with less LMC. In contrast, females may not be able to directly assess the number of females that are laying eggs on that patch, especially if these females visit the patch sequentially. Relative clutch size is also likely to be associated with laying order of females (Godfray 1994), a factor we could not specifically test given the difficulties of accurately resolving the visitation order (see Methods).

Another potentially important factor is that mating will often not be random within the whole patch, as assumed by most LMC models (Shuker *et al.* 2005; Shuker *et al.* 2006a; Shuker *et al.* 2007). Understanding the scale at which LMC occurs is therefore crucial. Laboratory experiments have shown that even when wasps emerge at very similar times, from hosts that are next to each other, they are more likely to mate (albeit not exclusively) with individuals that developed in their own host (van den Assem *et al.* 1980a; van den Assem *et al.* 1980b; Shuker *et al.* 2005). In nature, this effect will be increased because hosts can be spatially separated and emergence times can also be very spread out, as they were for our HV population (emergence times for the Schl population were not recorded), where the mean duration of emergence from the first to the last individual in a patch was 9.00 ± 2.36 days. Sometimes the difference in emergence time between hosts from the same patch was as high as 18 days, which is considerably higher than the mean lifespan of approximately nine days for sexually-active males in the laboratory (Burton-Chellew *et al.* 2007b). This means that the level of LMC actually experienced by wasps may differ from that expected by observers when considering the whole patch, and that wasps from different broods on the same patch may experience different levels of LMC (asymmetrical LMC: Shuker *et al.* 2005). Consequently, whether a host has been previously parasitised, and the relative number of eggs that a female lays on it, may be a more reliable indicator of the level of LMC that the offspring from a host will actually experience. Characteristics of the patch as whole will then not be so important. The importance of this in other species will depend upon natural history details: for example, emergence and mating may be staggered in many parasitoid wasps that attack clumps of hosts (Godfray 1994; West *et al.* 2005), whereas the relatively synchronous oviposition and emergence of fig wasps (Hamilton 1967; Herre 1985; Frank 1985a; Frank 1985b; Herre 1987) should lead to relatively random mating within the patch.

What information do females actually use to produce our observed negative correlation between offspring sex ratio and the relative clutch size that a female lays on a host (Figure 3.1 and Figure 3.3)? Females may respond to their own fecundity, whether or not the host has been previously parasitized, or the number of previously laid eggs on the host (Werren 1980; Werren 1984; Orzack & Parker 1990). Support for the idea that females are responding to previous

parasitism and the number of eggs laid previously is provided by the fact that there is: (1) no correlation between absolute clutch size and sex ratio when females lay eggs on a host alone (whether they share any of the other hosts on the patch or not; Table 3.2); (2) a poorer fit to the data with a focal female's own fecundity when compared to a focal female's fecundity plus other foundress females' fecundity (Table 3.2). In addition, previous experiments have shown that females are less likely to oviposit on, and lay fewer numbers of eggs on, parasitized hosts that have had a greater numbers of eggs previously laid on them (Shuker et al. 2005). As highlighted above, our analyses of the field data will also have underestimated the ability of individuals to assess the number of eggs previously laid on a host because, in superparasitized hosts, we do not know the order in which females laid eggs. Consequently, the first females to visit each host are also included in our analyses, despite the fact that they can have no knowledge of the number of eggs that will be laid later on the host. This limitation of a natural data set may also explain why we did not find support for the experimentally observed pattern that the sex ratio laid on a host is influenced by the extent to which other hosts on the patch have been previously parasitized (Shuker *et al.* 2005). Clearly there is scope for trying to bring experimental approaches to studies in the field, for example by providing hosts on artificial patches with known parasitism histories.

Further complications include that females only superparasitize hosts that have been recently parasitized (i.e. within 48 hours; Shuker *et al.* 2006a) and that, as discussed above, parasitization and emergence can be relatively spread out on natural patches. Females may also be sperm limited, and thus constrained to produce fewer daughters than they would otherwise. One female, excluded from the analysis presented here, did produce only males, which could result from virginity or sperm-depletion. Whilst a single mating in *N. vitripennis* usually provides sufficient sperm to fertilise several hundred eggs, males that have recently mated with 50 or more females do produce smaller ejaculates (or fail to inseminate successfully: Barrass 1961). However, in our dataset only four from the 136 clutches laid singly on hosts had sex ratios in excess 0.4, none of which exceeded 0.5. Sperm limitation therefore seems unlikely to be common.

Our analyses support the results from laboratory studies on *N. vitripennis* and other species that females do not adjust their sex ratio in response to their

relatedness to their mate or the other females on the patch (Frank 1985b; Taylor & Crespi 1994; Greeff 1996; Frank 1998; Reece *et al.* 2004). Females are predicted to lay a more female biased sex ratio when mated to more closely related individuals, because then they will be relatively more related to their daughters than their sons (Herre 1985; Frank 1985b; Greeff 1996; Reece *et al.* 2004). Females are also predicted to lay a more female biased sex ratio when ovipositing with more closely related females, because this will increase the relatedness between the offspring developing on the patch, and hence increase the extent of LMC (Frank 1985b; Frank 1986; Taylor & Crespi 1994). Whilst it could be argued that selection for an effect with relatedness to other females may be weak, because relatives rarely oviposit on the same patch and mating between related offspring emerging from different hosts may also be limited due to their distribution in space or time, there is appreciable variation in relatedness to mates, as mating with both siblings and non-siblings is common. However, such sex ratio adjustment would require reliable cues for kin recognition, and theory suggests that sufficient variability in the cues is unlikely to be maintained (Reece *et al.* 2004). The reason for this is that more common alleles would be recognised more often, indicate a higher relatedness, and hence be under positive selection: less common alleles would thus be eliminated, along with the variability that is required for kin discrimination (Crozier 1986; Rousset & Roze 2007).

Conclusion

Our results show that for species which are shown to fit simple models of LMC (West *et al.* 2005), techniques that allow the testing of more specific models in the wild can tell us a great deal about what limits adaptive behaviour. Our results also emphasise two general points about the extent to which we should expect data to fit theory. First, the ability of individuals to adjust their behaviour in response to environmental conditions depends upon the cues which they can use, and the reliability of those cues (West & Sheldon 2002; Boomsma *et al.* 2003; Shuker & West 2004). Here, we have found that cues concerning whether or not hosts are already parasitized are much more important than social cues, such as the presence of other females or the relatedness between individuals. Second, the pattern of social interactions in natural conditions can be much more complicated than that assumed by theory or laboratory experiments. More

specifically, mating can be structured both temporarily and spatially within patches, leading to a higher likelihood of mating among individuals from the same host, in contrast to the usual assumption of random mating at the patch level (Shuker *et al.* 2005). Studies on sex ratio evolution have been extremely useful for illustrating such general points, because of the relative ease with which the key parameters can be measured and linked to their fitness consequences.

Acknowledgements

We should like to dedicate this paper to the memory of Chris Barnard. Thank you to Christof Pietsch for providing the German samples and two of the primers used. We are extremely grateful to The Royal Society, the Biotechnology and Biological Sciences Research Council, and the Natural Environment Research Council for funding. L.W.B. acknowledges a Pioneer grant of the Netherlands Organisation for Scientific Research.

Supplementary information

Table S3.1: A Summary of the field collection. Wasps were collected at two field sites, either from natural host puparia found in nestboxes, or from baits, containing 25 laboratory host puparia, placed into nestboxes. Not all the host puparia found or baited were parasitized. For various reasons not all offspring could be assigned to a foundress. The sex ratio is that of the assigned individuals within a patch (nestbox).

Nestbox (patch) and Study Site	Parasitised Hosts (total)	Number of Foundresses	Total Offspring (unassigned)	Sex Ratio for analysis
HV 8[1]	1 (15)	1	7 (0)	1.000
HV 13	27 (27)	5	607 (1)	0.211
HV 220	8 (unknown)[2]	5	171 (0)	0.199
HV 267[1]	16 (16)	7	476 (19)	0.222
HV 288[b]	11 (25)	1	141 (2)	0.086
HV 306	1 (6)	1	18 (0)	0.056
HV 323	6 (8)	2	203 (0)	0.094
HV 330	79 (82)	5	593 (3)	0.197
HV 344	4 (43)	1	79 (0)	0.063
HV 365	1 (35)	1	25 (0)	0.160
Schl 11[b]	15 (25)	4	204 (5)	0.317
Schl 13[b]	3 (25)	2	43 (6)	0.108
Schl 16[b]	4 (25)	2	24 (3)	0.333
Schl 20[b]	25 (25)	2	331 (11)	0.178
Schl 21[b]	9 (25)	7	186 (5)	0.558
Schl 22[b]	14 (25)	4	246 (1)	0.188
Schl 23[b]	1 (25)	2	8 (1)	0.125
Schl 28[b]	15 (25)	3	188 (2)	0.048
ALL HV	154 (262)	29	2320 (18)	0.186
ALL Schl	86 (200)	20[3]	1230 (33)	0.241
TOTAL	**240 (462)**	**49**	**3550 (59)**	**0.205**

HV = Sample from Hoge Veluwe (HV) National Park, the Netherlands.

Schl = Sample from Schlüchtern, Hessen, Germany.

b = samples collected from baits.

1These patches were ultimately not included in the analyses, because the foundress in HV 8 was believed to be a constrained or virgin female, and because assigning offspring in HV 267 was problematic due to the foundresses being closely related.

2The number is not known because of a recording error, but it is known to be nine or more, and thus nine is used when compiling the totals.

3The total number of foundresses for Germany does not equal the sum total because six foundresses parasitized puparia in two different nestboxes.

Table S3.2: Information regarding the four microsatellite primer sets used. Name (annealing temperature), location, sequence, size range of PCR products, and fluorescent dye used.

Primer	Chromosome*	Sequence 5'-3'	Size Range	Dye
Nv-22 (58°C)	I	F) GCT ATA ACA CTT TTC CGC TCT CA R) AAG ACC AGC TAG GGA AGA GGA TA	194-222	HEX
Nv-23 (58°C)	II	F) ATA CTC AAG CAA GCC ACA GCA TA R) GCG TAC CAA TCC ACA GAA AAT AG	235-257	FAM
Nv-41 (52°C)	V	F) GTC AGA CGT GGG CTT TGT C R) TTA TGC GCC ACA CAC ACC	326-358	NED
Nv-46 (58°C)	IV	F) TTA CGT CAA GGT ATA GCT GC R) GAA TAA GTG GCT GAA AGT TCC	235-267	FAM

*Chromosome designation according to Rütten et al.(2004).

Chapter 4

Reproductive strategies under multiparasitism in natural populations of the parasitoid wasp *Nasonia* (Hymenoptera)

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Abstract

Parasitoid *Nasonia* wasps adjust their progeny sex ratio to the presence of conspecifics to optimize their fitness. Another trait under female control is the induction of offspring diapause. We analyzed progeny sex ratios and the proportion of diapausing offspring of individual *Nasonia* females in host patches parasitized by two species, *N. vitripennis* and *N. giraulti* in North American field populations using microsatellite fingerprinting. Both *Nasonia* species produced similar sex ratios on hosts that were co-parasitized by their own species as by the other species, indicating that females do not distinguish between con- and heterospecific clutches. The sex ratios of the diapause and adult fractions of mixed broods from single females were not correlated. We found further indications that *N. vitripennis* females take the emergence time of the offspring into account in their sex allocation. The reproductive strategies of *Nasonia* under multiparasitism are largely adaptive, but also partially constrained by information.

Introduction

The evolution of life history traits leads to intricate adaptations to maximize fitness in a local environment (Stearns 1992). Parasitoid wasps have been used extensively for studying adaptation, especially with respect to foraging behaviour and sex allocation (Shuker & West 2004 and citations therein). Parasitoids have only a limited amount of resources to allocate for the development of their offspring. Therefore the quality of the host plays a major role in the optimization of resource allocation (Harvey 2005). If there are enough resources, a parasitoid should prefer to parasitize unparasitized hosts only. However, under natural conditions there is ample competition with con- and heterospecific foundresses. In addition, a founding parasitoid has to deal with variation in host quality and density as well as abiotic factors, such as temperature and seasonality. Facing all these challenges, every parasitoid uses certain reproductive strategies to maximize its fitness, and in the course of time natural selection will lead to adaptation to the local environmental conditions.

The gregarious wasps (females lay more than one egg in a host) of the genus *Nasonia* are pupal parasitoids of cyclorraphous flies found on carcasses and in bird nests (Whiting 1967). In this genus three species are known: *N. longicornis*, that is only found in the west of North America, *N. giraulti* that is restricted to eastern North America, and *N. vitripennis*, that is cosmopolitan and occurs in close sympatry with its sister species in North America (Darling & Werren 1990). The three species are closely related and phylogenetic studies revealed that the lineages split rather recently (between 0.2 and 1 Mya, Campbell *et al.* 1993). The species are reproductively isolated due to *Wolbachia* infection with incompatible strains (Breeuwer & Werren 1990). However, cured strains produce viable and fertile hybrid offspring (Breeuwer & Werren 1995). Behavioural studies have shown that there are clear differences in courtship between the three species (van den Assem & Werren 1994; Beukeboom & van den Assem 2001), which, together with *Wolbachia* infection, makes the occurrence of hybrids in nature very rare.

N. vitripennis has been widely used as a model organism to study sex ratio adjustment in the framework of local mate competition (LMC) theory (Werren 1984; Drapeau & Werren 1999; Shuker *et al.* 2005; 2006b). LMC theory

assumes that a female has control over the sex ratio of her offspring and can maximize her fitness by reducing the competition between her sons. This is an evolutionarily stable strategy if males are not the dispersing sex and if mating only takes place at the natal patch (Hamilton 1967). In such a mating system all males are confined to mate with the females that are available at their natal patch. If the patch population consists of offspring of a single female, males are brothers and it is beneficial for the foundress female to shift the progeny sex ratio strongly towards daughters to reduce competition among her sons. With increasing foundress number, competition between unrelated males increases and therefore selection favours females that produce more males to increase the chance that their sons mate with daughters of other females as well. This leads to a less female-biased sex ratio. The resulting prediction is that the progeny sex ratio in a patch is a function of the number of females ovipositing on that patch (Hamilton 1967). It has been shown in the laboratory (Werren 1984; Drapeau & Werren 1999; Shuker *et al.* 2005; 2006b) and field (Werren 1983; Molbo & Parker 1996; Burton-Chellew *et al*. 2008) that females of all three *Nasonia* species follow the predictions of LMC theory quite closely, when they are the only *Nasonia* species in a patch.

The expectation from LMC theory for a foundress that encounters heterospecific eggs in a host, is that there is no sex ratio response, but only resource competition. The oviposition response of a parasitoid female to the presence of a female of a closely related species has been investigated by several authors in the laboratory: Vet *et al.* (1984) found that *Asobara tabida* and *A. rufescens* mutually avoided parasitism of hosts preparasitized by the other species; Wylie (1973) found that *N. vitripennis* differentiate between hosts pre-parasitized by a conspecific and *Muscidifurax raptor*; Ivens *et al.* (in prep.) found that *N. vitripennis* and *N. longicornis* differentiate between conspecific and heterospecific co-foundresses regarding host acceptance, but not with respect to progeny sex ratios. Here, the focus will be on the sex ratio response of *N. vitripennis* and *N. giraulti* in a sympatric field situation.

In temperate regions most insects can only survive the winter in diapause. An important life history decision is therefore when to enter diapause. The cues that lead to a switch from normal development to diapause appear highly variable among the insects. Photoperiod, light intensity, temperature, thermoperiod,

food, moisture, density, mating status, chemical cues and even the presence of predators have been found to affect diapause (Danks 2007 and citations therein). In most insects the diapause inducing cues are experienced by the individual going into diapause. In *Nasonia*, however, the mother is in control of the developmental mode of her offspring. The main cue influencing the females' decision is the photoperiod, whereas temperature, food shortage and age appear to have a modulating effect (Saunders 1965a; 1965b; 1966a; 1966b).

In an evolutionary context the question arises of how a *Nasonia* foundress uses the limited resources of a host for her sons and daughters, as well as for adult and diapausing offspring, in the presence of a closely related species which is competing for the same resources. We investigated this in natural populations of *N. vitripennis* and *N. giraulti* in North America by fingerprinting offspring of host patches (bird nests) that were parasitized by both species. We ask the following questions: (1) Is the host usage within and between nests random, or do the species actively avoid each other? (2) Do the two species produce different sex ratios when encountering their own species versus the other species on a host? (3) Does a foundress produce a higher proportion of diapausing brood when encountering other conspecific foundresses? (4) Does a foundress produce the same sex ratio among her diapausing and adult offspring? (5) Does a foundress produce a different sex ratio in the presence of diapausing versus adult offspring of another foundress? (6) Does a foundress produce the same brood size when producing diapausing versus adult offspring?

Material and Methods

Sample collection

In July 2005 we collected all fly host pupae out of 108 nest boxes from three regions in New York state in the USA (near Brewerton 32, Ithaca 53, Rochester 23). Most nest boxes were used by tree swallows (101), some by eastern bluebirds (6), and one by chickadees. The pupae were incubated individually at room temperature until either a fly, or parasitoids emerged. If nothing emerged after three weeks, the hosts were opened to check for diapause larvae. Adult parasitoids and diapause larvae were stored in 96% alcohol and empty hosts were discarded. For all adult *Nasonia* the species and sex was determined.. Since females of all *Nasonia* species are phenotypically similar, we determined

the species on the basis of wing length of males; *N. giraulti* has long wings, *N. vitripennis* short wings (Darling & Werren 1990). For a subset of the diapause larvae the species and sex was determined after genetic analysis (see below).

Genetic analysis

For a more detailed analysis of the frequency and offspring sex ratios of the two species within a nest, genetic information was required. Three nest boxes from the study site close to Brewerton (BR12, BR23, and BR29) were chosen which contained males from both species and had a number of progeny that could be logistically processed (up to a total of 2000 individuals). These three nest boxes contained a total of 84 parasitized hosts, which can be seen as independent data points regarding sex allocation as has been shown by Grillenberger *et al.* (2008). DNA was extracted from all individuals using a standard high salt protocol (Maniatis *et al*. 1982).

All individuals were genotyped for seven polymorphic microsatellites using the Qiagen Multiplex kit (see Table 4.1 for primer details and Beukeboom *et al.* in prep.) and fragment lengths were determined on an ABI 3730XL sequencer (Applied Biosystems, CA). All individuals were assigned into fullsib families using three simple rules of haplodiploid inheritance (Grillenberger *et al*. 2008): (1) A female can maximally provide two alleles per locus. (2) The father can only provide one allele per locus (being haploid) that is shared by all full sisters. (3) Sons can only have an allele from their mother, as they develop from unfertilized eggs. For a first assignment the software COLONY 1.2 (Wang 2004) was used. To correct for genotyping errors, the software output was then revised by hand and similar families were combined. Clear differences in allelic composition between the two species allowed species identification based on the microsatellite fingerprint. To ensure correct species identification, one individual per family was sequenced for a 400 bp fragment of the mitochondrial Cytochrome Oxidase I gene (*COI*). Primers were designed on the basis of the *N. vitripennis COI* sequence from GenBank (acc.# LOC100113910) (fwd-primer: 5' GTT ATA CCT KTW ATA ATW GGA GGA TT TGG 3', rev-primer: 5' CTT TGA AA ACC ACG TTA CCC 3', annealing temperature 52ºC, Grillenberger *et al.* in prep.). The resulting sequence was aligned with sequences of known origin. This used *COI* fragment differs in 15% of nucleotides between *N. vitripennis* and *N. giraulti* (Grillenberger *et al.* in prep).

For diapause larvae the sex was determined by the number of loci that were homozygous. Individuals homozygous for all seven loci were scored as males (hemizygous haploids), all other individuals as females (diploids). Using this information, clutch size and sex ratio of individual parental females was determined, as well as the number of co-founding females per host.

Definition of terms

To avoid confusion, we will define some terms that we use in the rest of the paper. A female that is laying eggs is called a foundress. The foundresses are parasitizing fly pupae that are called hosts and these hosts are found in bird nest boxes. In a nest box we find both unparasitized and parasitized hosts. Hosts parasitized by a single foundress are called single foundress hosts. If two or more foundresses of the same species parasitized a host, this is called superparasitism, whereas if a host is parasitized by one or more foundresses of more than one species, the situation is called multiparasitism. A clutch is the eggs of an individual foundress laid in a single host. The foundress' brood is the actual offspring of the foundress in a single host observed in this study. A foundress can parasitize several hosts and can therefore have several broods. A brood that consists of adult offspring only is called an adult brood while a brood that contains only diapausing offspring is called a diapausing brood. Mixed broods contain both diapausing and adult offspring.

Table 4.1**:** Chromosomal location, primer sequences, number of alleles, Nei's overall expected heterozygosity (H_t) (Nei 1987) and annealing temperatures of seven microsatellites used. For further details see Beukeboom et al. (in prep.).

Locus	Chromo-some*	Primer Sequences	Allele no.	H_t	Ann. temp.	Species specific?	GenBank accession no.
Nv-104	IV	5' GAT TTC CCG GAG CTT CCT AC 3' 5' GCC GTT AAT TGA ACC TGT CG 3'	8	0.859	55˚C	Yes	FJ156110
Nv-109	V	5' GCT TAC TCT CGG GAA CTG GA 3' 5' CGA GCA TTA ACC ATC AGC AG 3'	10	0.880	55˚C	Yes	FJ156114
Nv-111	III	5' AGG TCT CAG CCG CAC AAA 3' 5' GCA GCT GCT TTT GGC ACT 3'	16	0.962	55˚C	Yes	FJ156115
Nv-114	IV	5' ATG GGC AAT AAA ACG AAA CG 3' 5' CAT CCT TGC GGA GAC ACT AA 3'	15	0.826	55˚C	No	FJ156231
Nv-300	II	5' ACA TTC CGC AGA GCG ATT AT 3' 5' CGC GAC CGA TGA TTT ACT C 3'	3	0.676	55˚C	Yes	FJ156211
Nv-308	I	5' ATT CGG AAT CCA CGA AAC G 3' 5' TAG GGC GCG TAT AGA TCG AG 3'	10	0.705	55˚C	Yes	FJ555533
Nv-313	V	5' GAA GCT GCG GGT TAA GTG TG 3' 5' CGC TAC TTT ATG CCA GTT ACG G 3'	19	0.902	55˚C	Yes	FJ156221

* chromosome designation according to Rütten *et al.*(2004).

Statistical analysis

Previous research has shown that a *Nasonia* foundress bases her sex ratio decision on a host level rather than nest box level (Grillenberger *et al*. 2008; Burton-Chellew *et al*. 2008). Therefore our statistical analysis is mostly based on foundress numbers on a host level. To answer the questions posed in the introduction, we performed the following statistical analysis on the genetic data obtained from the three fully analyzed nest boxes:

(1) To test whether the host usage of *N. vitripennis* and *N. giraulti* is random we compared the observed foundress frequencies with the expectation under a binomial distribution using Fisher's exact test. To do this, the total number of hosts available for parasitism at one moment in the patch has to be known. In this study it was only observed whether a host was parasitized, or not, but it is unknown for what reason a host is unparasitized. Hence, it was not possible to distinguish between hosts that might have been available for parasitism but were not chosen (the number that is required for estimating the random distribution), and the hosts that were not suitable for parasitism. As host number appears to be the limiting factor for *Nasonia*, we excluded in our analysis hosts that had not been parasitized by at least one species, assuming that they were not available for parasitism.

(2) To compare sex ratios produced under single-, multi- and superparasitism, we distinguished three classes of broods: (i) The single foundress case, where a host contains offspring of one foundress only; (ii) the multiparasitism case, where a single brood of the focal species foundress is accompanied by one or more broods of the other species; (iii) the superparasitism case, where two or more broods of the same species are present in a host without the presence of the other species. For both species we used Kruskal-Wallis H-tests and Nemenyi post-hoc tests for pair wise comparisons between the three brood classes (Zar 1999).

(3) To test whether a foundress produces a higher proportion of diapausing offspring when encountering other foundresses, we first compared the observed frequencies of diapausing broods with the expectation under a binomial distribution with a χ^2-test. Additionally, we investigated whether the proportion of diapausing offspring per host is correlated with the number of foundresses, using a Spearman's rank correlation.

(4) To test for differences between the sex ratio of diapausing and adult broods we investigated whether the sex ratio of adult offspring resembles that of the diapausing offspring produced by the same female using a Spearman's rank correlation. Furthermore, we compared the sex ratio of adult and diapausing broods in single- and superparasitism hosts, using Mann-Whitney U-tests.

(5) To test whether a foundress produces a different sex ratio in the presence of diapausing versus adult offspring of another foundress, we compared the brood sex ratio of the following classes using Kruskal-Wallis H-tests: (i) single foundress diapausing broods; (ii) single foundress adult broods; (iii) hosts with diapausing broods of a single foundress accompanied by one or more adult broods; (iv) hosts that contained multiple diapausing and at least one adult brood. Categories iii and iv were also compared using a Mann-Whitney U-test.

(6) To test whether a foundress produces the same brood size when producing diapausing versus adult offspring, we compared the brood size of diapausing, mixed and adult broods, using Kruskal-Wallis H-tests and Nemenyi post hoc tests.

All statistical tests were performed with R (R Development Core Team 2006). All sex ratios are given as the proportion of males and averages are calculated after arcsin square root transformation and back transformation following Wilson and Hardy (2002). As *N. giraulti* hardly produced any diapause larvae, the diapause analysis was performed on *N. vitripennis* data only.

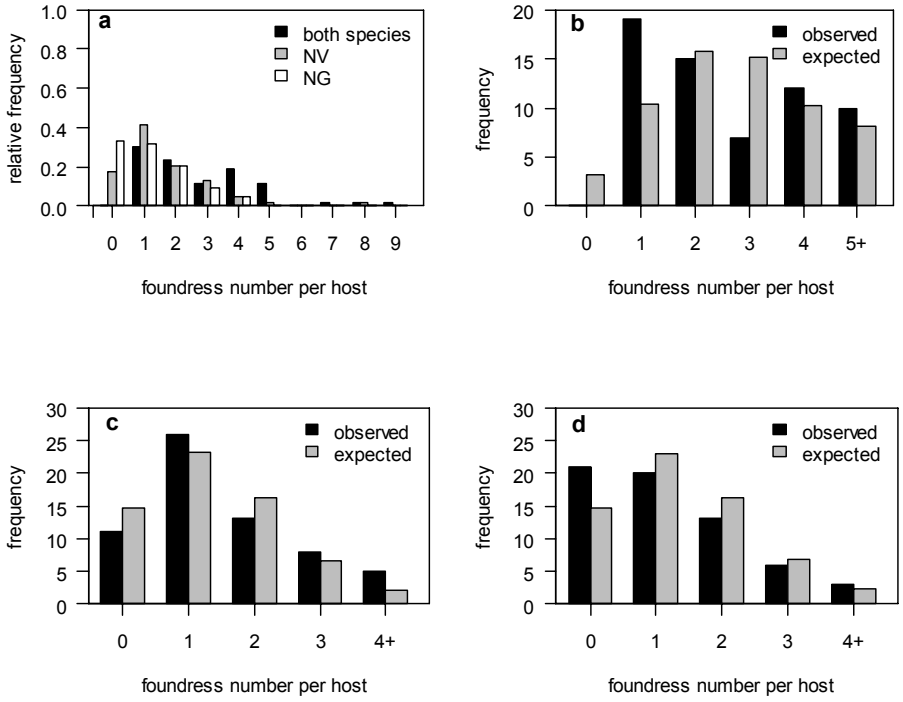

Figure 4.1: Frequency distribution of foundress number per used host in nest BR29. (a) The relative frequencies of hosts used by either both species (black bars), *N. vitripennis* (grey bars, NV) or *N. giraulti* (white bars, NG). (b-d) Comparison of the observed frequency of foundress number per host (black bars) with the expected frequencies assuming a binomial distribution (grey bars, see text for details): (b) both species combined (there are no observed empty hosts in the graph, as the analysis did only include used hosts; see text), (c) only *N. vitripennis*, (d) only *N. giraulti*. Fisher's exact test showed a significant difference between the host usage of the two species (see text for details).

Results

Species abundance and distribution across hosts

Out of the 108 nests collected, 64 contained fly pupae (a total of 2043 pupae), of which 58 (91%) were parasitized by *Nasonia*. 17 nests (29%) contained both *N. vitripennis* and *N. giraulti*, 39 (67%) contained only *N. vitripennis*, and no

nests contained exclusively *N. giraulti* (for two nests the species could not be determined). The number of hosts per infested nest ranged from 1 – 138 (mean 31.9). The proportion of hosts parasitized per nest ranged from 1 – 100% (mean 48%), excluding infested nests that did not yield *Nasonia*.

The three nests that were chosen for the detailed analysis (BR29, BR23, BR12) contained 170 hosts, 84 (49%) of which yielded *Nasonia* offspring (Table 4.2). We genotyped a total of 1906 individuals for seven microsatellites. 51 samples did not amplify and seven could not be assigned to a foundress. Six out of the seven markers showed clear allelic differences between the two species (detailed information upon request from the author), and species assignment based on the microsatellite data was always in concordance with the mitochondrial sequence data (Table 4.1). For the remainder of the analysis we focused on the three genetically analyzed nest boxes.

There were large differences in the composition of the three nests. The largest nest contained 63 parasitized hosts, yielding 19 foundresses. The smallest nest contained seven parasitized hosts and six foundresses. Thirty five out of 84 parasitized hosts yielded both *N. vitripennis* and *N. giraulti* (41.7% multiparasitation rate, all three nests pooled). The number of broods and the average brood size was slightly higher for *N. vitripennis* than for *N. giraulti* (118 vs. 90 broods, 9.62±0.68 versus 7.92±0.75 average brood size, Mann-Whitney U-test: U= 5988.5, p = 0.11) Additionally, there were more *N. vitripennis* than *N. giraulti* foundresses (*N. vitripennis*: 15, *N. giraulti*: 12). These differences result in approximately 60% more *N. vitripennis* than *N. giraulti* offspring (1135 to 713). This reflects a higher productivity of *N. vitripennis* in the study area. The superparasitation rate of *N. vitripennis* was comparable to that of *N. giraulti* (45% and 44%, respectively , Table 4.2).

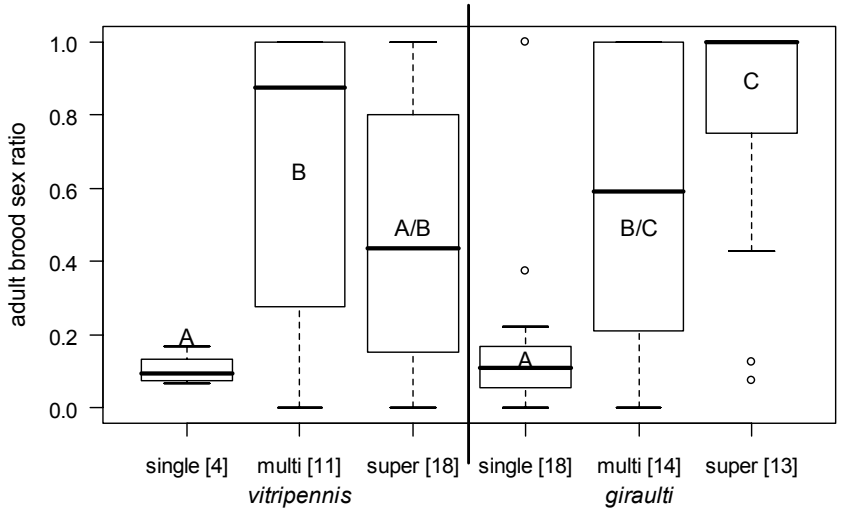

Figure 4.2: Box and Whisker plots showing brood sex ratio of adult offspring for the two species in three different situations: (1) Single foundress broods of the focal species, without the presence of any other broods in that host (single); (2) the sex ratio of the focal brood, when there is one brood of the focal species together with at least one brood of the other species (multi = multiparasitism); (3) multiple broods of one species (super = superparasitism). Numbers in brackets refer to sample size, capital letters indicate significant differences between groups within one species, or within groups between species, using Nemenyi's test.

The foundress distributions across hosts differed between the species [Fisher's exact test with low represented classes combined (≥ 4 foundresses), p = 0.03, Figure 4.1 a]. More hosts parasitized by *N. vitripennis* were unused by *N. girault* than vice versa. There was no evidence for female preference for or against patches or hosts used by other females. We found no significant deviation from a random distribution of the foundresses across used hosts within a patch [Fisher's exact test against binomial distribution using data of the largest patch (BR29) only and combining the low-represented classes (≥ 4 foundresses), for both species: p = 0.20 (excluding unused hosts); for *N. vitripennis* only: p = 0.67; for *N. girault* only: p = 0.79, Figure 4.1b-d].

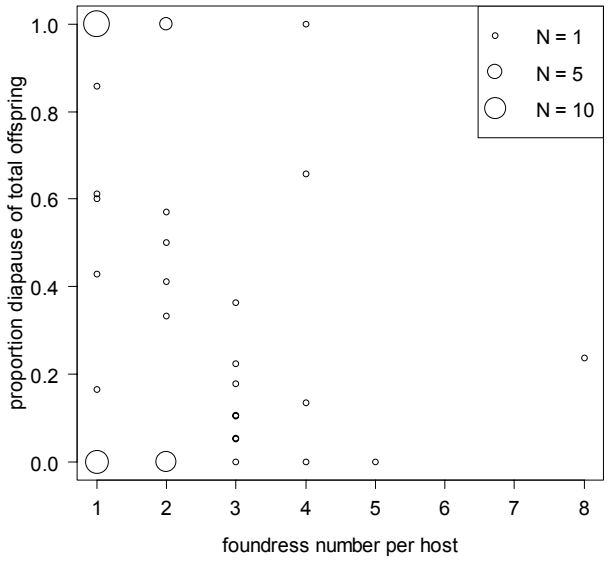

Figure 4.3: Bubble plot of the proportion of diapause offspring per host as a function of the foundress number per host. Spearman rank correlation was not significant. Bubble sizes indicate sample size.

Sex ratios

Overall sex ratios (given as proportion males) of *N. vitripennis* were lower than of *N. giraulti*. Mean sex ratios per host for *N. vitripennis* were 0.34 ±0.003 (N = 63, median = 0.3), and were 0.43 ±0.004 (N = 57, median = 0.33) for *N. giraulti*, however the Mann-Whitney U test was not significant (U = 1666, p = 0.5) (Table 4.2). The sex ratios from single foundress hosts were significantly lower than broods in multi- and superparasitized hosts in *N. giraulti*, as expected from the stronger degree of LMC in the former case. In *N. vitripennis* only the difference between single parasitism and multiparasitism was significant (Kruskal-Wallis H-test within species: *vitripennis*: $H_2 = 7.29$, p = 0.03; *giraulti*: $H_2 = 16.04$, $p < 0.001$; pair wise comparisons with Nemenyi test: *vitripennis*: single – multi: p = 0.03, single – super: p = 0.42, super – multi: p = 0.72; *giraulti*: single – multi: p = 0.008, single – super: $p < 0.001$, super – multi: p = 0.61) (Figure 4.2). This indicates that under multiparasitism both species are producing a sex ratio more similar to the superparasitism case than to the single foundress case, which is in contrast to expectation, as only conspecific competitors play a role in LMC. The variance in the multi- and superparasitism classes is much larger than in the single foundress class. The comparison between the two species within each class showed a significantly higher sex

ratio in the case of superparasitism involving *N. giraulti* (Mann-Whitney U-test: *giraulti* super – *vitripennis* super, U = 166.5, p = 0.043). This means that *N. giraulti* shows a stronger within species LMC response than *N. vitripennis*.

The proportion of offspring in diapause differed greatly between the two species. 39% of all *N. vitripennis* offspring were in diapause compared to only 0.4% in *N. giraulti*. The distribution of *N. vitripennis* diapausing broods across hosts within the largest nest box (BR29), did not differ from a random distribution (χ^2-test against binomial distribution, combining the low-represented classes, $\chi_2^2 = 1.20$, p = 0.55). There was no correlation between the proportion of diapausing offspring per host and the total foundress number (all *N. vitripennis* foundresses) per host (Spearman: S = 49947.8, ρ = -0.199, p = 0.118, Figure 4.3).

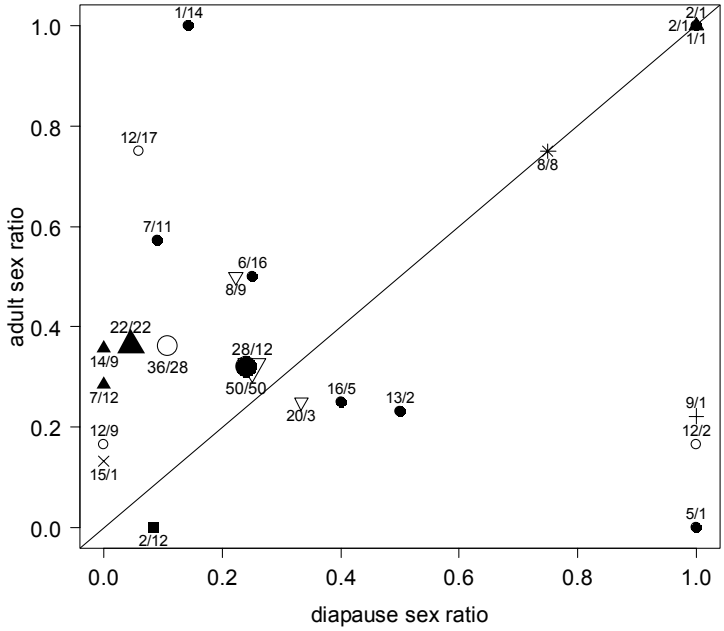

Figure 4.4: Sex ratio of adult offspring versus diapause offspring within clutches of one foundress (N = 20). The solid line represents the theoretical expectation when sex ratio is equal for diapause and adult offspring. Different symbols represent different foundresses (N = 9), the first number near the label the number of adults in that clutch, the second the number of diapause larvae. There is no correlation between the sex ratio of diapause and adult clutches (Spearman rank correlation: S = 1082.399, ρ = 0.186, p = 0.4319). The larger symbols and numbers

Table 4.2: Overview of the three fully analyzed nests. Shown in the left part are the total number of hosts found in a nest, the total number of hosts parasitized per nest, and the fraction of hosts multiparasitized. In the right part are shown per species and nest: the number of hosts parasitized, the fraction of hosts superparasitized, the total number of offspring, the fraction of offspring in diapause, the total and average foundress number per host, the nest-wide sex ratio and brood size, and the average host and brood sex ratio.

Nest	Total host no.	Host no. parasitized per nest	% host. multi-parasitized	UN Ind.	NA ind.	Species	Host no parasitized	% host super-parasitized	Ind. no.	% diap. of total offsp.	foundress number	Avg. foundress no. per host	nest sex ratio	Avg. brood size	Avg. host sex ratio	Avg. brood sex ratio
BR29	85	63	49%	5	23	**NV**	52	50%	926	46%	9	1.94±0.19	0.34	9.26±0.71	0.36±0.003	0.59±0.003
						NG	42	52%	483	0.4%	10	1.81±0.15	0.45	6.36±0.66	0.53±0.006	0.66±0.005
BR23	64	14	7%	0	18	**NV**	4	0%	57	42%	1	1.0±0	0.05	14.25±2.46	0.04±0.004	0.035±0.004
						NG	11	0%	204	0.5%	1	1.0±0	0.12	18.54±2.10	0.12±0.0005	0.12±0.0005
BR12	21	7	43%	2	10	**NV**	7	71%	152	0%	5	2.0±0.33	0.29	10.86±2.57	0.43±0.02	0.46±0.02
						NG	3	0%	26	0%	1	1.0±0	0.23	8.67±3.84	0.39±0.01	0.29±0.01
overall	170	84	42%	7	51	**NV**	63	45%	1135	39%	15	1.81±0.15	0.32	9.62±0.68	0.34±0.003	0.55±0.003
						NG	56	44%	713	0.4%	12	1.68±0.13	0.35	7.92±0.75	0.43±0.004	0.58±0.004

UN Ind.: the number of individuals unassigned; NA Ind.: the number of individuals that gave no PCR amplification; NV = *N. vitripennis*; NG = *N. giraulti.*

The sex ratio of adult and diapausing offspring of an individual foundress were not correlated, and there is no consistent direction in the difference in sex ratio between diapausing and adult offspring (Figure 4.4, Spearman rank correlation: S = 1082.4, p = 0.43, N = 20 broods of 9 foundresses). This indicates that the sex ratio of the diapausing and adult fractions of a brood are independent. The brood sex ratios of single foundresses and foundresses under superparasitism producing diapausing or adult offspring did not differ from each other (Mann-Whitney U-test: single dia – single adult: U = 25.5, p = 0.919; super dia – super adult: U = 21.5, p = 0.796; Figure 4.5). This indicates that superparasitism does not differentially affect the sex ratio of diapausing and adult offspring.

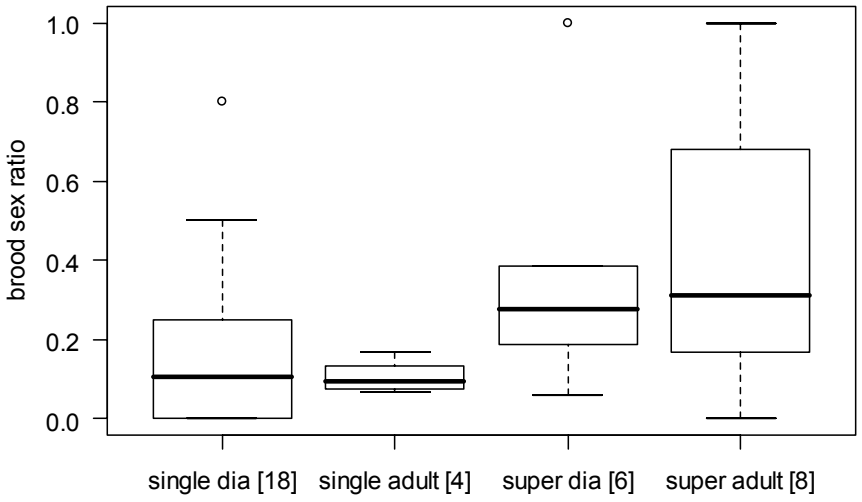

Figure 4.5: Box and Whisker plots showing *N. vitripennis* brood sex ratios of single foundresses producing all-diapause (single dia) or all-adult (single adult) broods, as well as for foundresses under superparasitism (super dia and super adult). Numbers in brackets refer to sample size. Pairwise comparisons between diapause and adult clutches within single and superparasitism classes were not significant (Mann Whitney U-test).

The brood sex ratios of single diapausing broods, single adult broods, and single diapausing broods accompanied by adult broods did not differ

significantly (Kruskal-Wallace H-test: $H_2 = 0.0127$, $p = 0.99$, Figure 4.6). The sex ratios of diapausing broods accompanied by other diapausing and adult broods, however, were significantly higher than the sex ratio of single diapausing broods accompanied by only adult broods (Mann-Whitney U-test: $U = 7$, $p = 0.031$, Figure 4.6). This suggests that females producing diapausing broods increase their sex ratio as a function of the presence of other diapausing broods in a host.

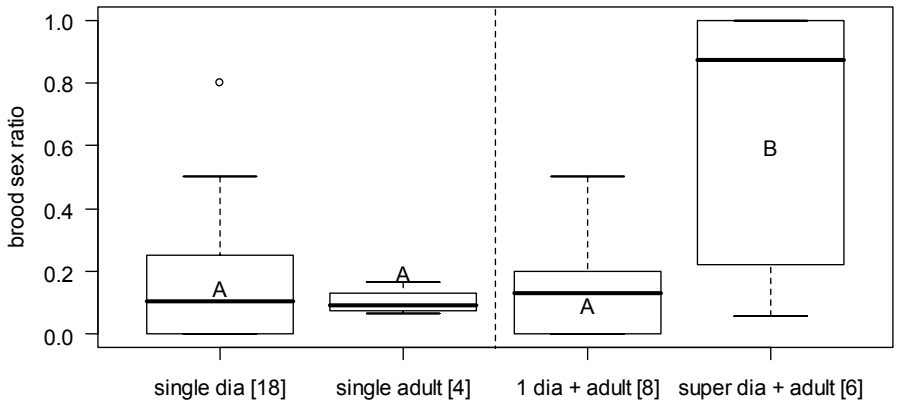

Figure 4.6: Box and Whisker plots showing *N. vitripennis* brood sex ratios of single foundresses producing pure diapause (single dia) and all-adult broods (single adult), as well as of single foundresses producing diapause in the presence of one or more adult broods in the same host (1 dia + adult; the reverse 1 adult + dia, was not found), and of foundresses producing a diapause brood in the presence of at least one other diapause brood and one other adult brood (super dia + adult). Numbers in brackets refer to sample size. (Kruskal-Wallace H-test across the first three groups was not significant; Mann-Whitney U-test between 1 dia + adult and super dia + adult was significantly different; significance indicated by different capital letters in the plot)

Mixed diapause-adult broods were significantly larger than pure adult broods, while the difference between pure diapause and mixed broods was not significant (Kruskal-Wallace H-test: $H_2 = 11.1228$, $p = 0.004$; pair wise comparison with Nemenyi's test: adult – mixed: $p = 0.007$, diapause – mixed: $p = 0.19$, diapause – adult: $p = 0.44$, Figure 4.7). This could indicate that mixed

broods are the product of foundresses that sequentially produced an adult and a diapause brood in the same host (self-superparasitism).

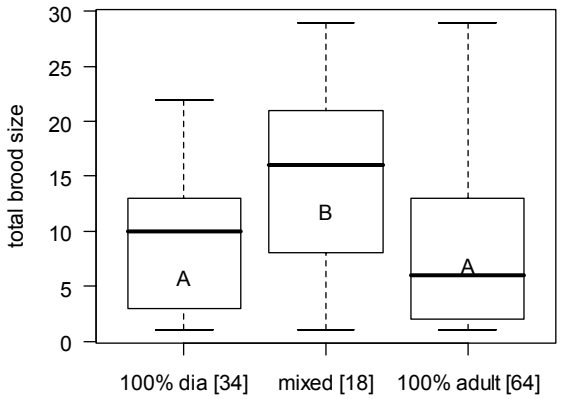

Figure 4.7: Box and Whisker plots showing *N. vitripennis* brood sizes of pure diapause (100% dia), pure adult (100% adult) and mixed broods. Numbers brackets refer to sample sizes. (Kruskal-Wallis H-test: $H = 13.69$, $df = 2$, $p < 0.05$; pair-wise comparison with Nemenyi's test, significance indicated by different capital letters in the plot).

Discussion

Species distribution in the field

Our data clearly show that in the three nests that were chosen for detailed analysis the two species, *Nasonia vitripennis* and *N. giraulti*, encounter each other very frequently, as the multiparasitism rate is very high (42%). The level of superparasitism in *N. vitripennis* (45%) is comparable to that found in a European *N. vitripennis* population (39%, Grillenberger *et al*. 2008), as well as that found in *N. giraulti* (42%). These results are in agreement with the data on foundress numbers, which appear to be comparable for the two species. Hence, the species densities appear to be similar in our research area. For *N. vitripennis,* the density is also more or less equal to the allopatric population in Europe (assuming an equal abundance of hosts), where similar foundress numbers of one to seven per nest box were found (Grillenberger *et al*. 2008).

The distribution of foundresses across hosts between species was significantly different. The main difference is in the number of single-species parasitized hosts (*N. vitripennis* occurs more often alone in a host than *N. giraulti*), which could indicate that *N. giraulti* is more selective regarding the host quality. The across host distribution within species was not different from a random distribution. This gives no indication of clumping of individuals of the same species, or avoidance of the other species in host acceptance. It is conceivable that the random distribution is a product of the lack of choice between available hosts, when a female arrives in a nest box. Note that the composition of a patch (here a nest box) is variable over time. The hosts are only available for parasitism for a few days (depending on temperature), and therefore there are potentially three classes of hosts in a given patch: hosts that are too young to be parasitized, hosts that are too old to be parasitized, and hosts that are in the correct stage. Hence a foundress might have a limited number of hosts to choose from and therefore has limited opportunity to actively avoid or prefer hosts that have already been parasitized by either a con- or heterospecific. Superparasitism is then often her only option. The final outcome of such competition for hosts could be the random distribution that we observed. Ivens *et al.* (in prep.) found in a laboratory study on *N. vitripennis* and *N. longicornis*, that foundresses of both species do not reject hosts that are parasitized by the other species, when parasitizing synchronously, but do reject pre-parasitized hosts of the other species after 24 hours. This indicates that either most multiparasitism in our study happened synchronously, or the species interaction between *N. vitripennis* and *N. giraulti* differs from *N. vitripennis* and *N. longicornis*.

Sex ratios in a two species situation

Surprisingly, the sex ratios produced by *N. giraulti* are higher than those produced by *N. vitripennis*. This is in contrast to the findings of King and Skinner (1991) and Drapeau and Werren (1999), who found lower sex ratios in *N. giraulti* in a laboratory experiment under both single- and superparasitism conditions, and interpreted this as the consequence of frequent within host mating in *N. giraulti*. The difference in sex ratios seems to be largely due to the significantly higher sex ratios of *N. giraulti* under superparasitism (Figure 4.2). This indicates that *N. giraulti* shows a stronger local mate competition towards

conspecifics than heterospecifics, and that its superparasitism response is stronger than in *N. vitripennis*.

Our results show no indication that either *N. vitripennis* or *N. giraulti* is able to distinguish between con- or heterospecific broods in their sex allocation (Figure 4.2). If they distinguished, the multiparasitism sex ratios would be expected to be indifferent from the single foundress sex ratios. An issue with studies using field data is the lack of information on parasitization sequence, as the first foundress on a patch is expected to produce a strongly female biased sex ratio and later foundresses higher proportions of males (e.g. Hamilton 1967; Werren 1980; Shuker *et al.* 2005). To avoid this problem, we compared single foundress broods as one extreme case with superparasitized broods as the counterpart. As the multi-foundress classes contain both first and subsequent foundresses, the variance of sex ratios produced is large.

The multiparasitism sex ratios resemble those of superparasitism more than the single foundress sex ratios. Wylie (1965) found that *N. vitripennis* foundresses cannot detect whether a host has been parasitized previously, until its ovipositor has been inserted into the puparium. In a later study Wylie (1973) showed that *N. vitripennis* females are able to distinguish between eggs of their own species and eggs of the heterospecific competitor *Muscidifurax raptor*, as they showed a stronger sex ratio response when encountering conspecific eggs. Ivens *et al.* (in prep.) found in a laboratory experiment with *N. vitripennis* and *N. longicornis*, that both species show a lower acceptance of hosts that have been pre-parasitized by a heterospecific female compared to a conspecific. However, in agreement with our data, once multiparasitism occurs, there is no influence of multiparasitism on the sex ratio adjustment (Ivens *et al.* in prep.). This means that *N. vitripennis* is able to distinguish the eggs of *M. raptor* from its own species eggs (Wylie 1973), but not the eggs of its sibling species *N. longicornis* (Ivens *et al.* in prep.), or *N. giraulti* as shown by our data. Martel and Boivin (2004) found in a lab experiment that *Trichogramma minutum* produced a different sex ratio when encountering heterospecific competitors, compared to conspecific competitors, but a closely related species (*T. pintoi*) did not show a differential response. They explained this by differences in the distribution range, as *T. minutum* lives sympatricly with other *Trichogramma* species in its natural habitat, while *T. pintoi* does not. Since *N. vitripennis* and

N. giraulti live microsympatrically in our research area, the most likely explanation for our results is that both species have not diverged far enough to discriminate between con- and heterospecific eggs.

Offspring in diapause

We found a difference in the occurrence of diapause between the two species. *N. vitripennis* produced 39% diapause larvae, while *N. giraulti* produced only 0.4% (Table 4.2). This is surprising as both species have been collected at the same time of the year at the same location. Hence, the climatic factors potentially inducing production of diapausing offspring by adult females were identical. Considering that the sampling took place early July, and the active season for *Nasonia* in the area runs until September, the difference is even more puzzling. In a European *N. vitripennis* population (Netherlands) at approximately the same time of year in 2004, few diapause larvae were found (Tosca Koevoets unpublished data). This indicates that there is large intra- and interspecific variation for the response of *Nasonia* to diapause inducing cues, and/or in the actual cues used.

Saunders (1965b) showed that a female's age plays a major role in the proportion of diapause larvae produced. From our data it is not clear whether all eggs of one female were laid at once, or whether sequential parasitization events by the same foundress occurred. It could be that the first clutch contained mostly adult offspring and a later clutch contained mostly diapausing offspring, as the female aged one or more days in the meantime. Self-superparasitism would presumably result in a larger mixed brood. This explanation is consistent with the larger brood size of females in mixed (diapause-adult) broods compared to pure broods of either type (Figure 4.7).

Saunders (e.g. 1962; 1965b; 1966a; 1973) also showed that stressed females (high and low temperatures, host shortage) tend to produce more diapausing offspring. A correlation between the total number of foundresses per host, which could be considered a stress situation in the form of crowding, and the proportion of diapausing offspring was not found in our study. Diapause is usually seen as a state to survive the winter (Danks 2007 and citations therein). However, in the case of diapause larvae in July it is questionable why *N. vitripennis* females do not produce another adult generation. This suggests that

there are other cues triggering the production of diapausing offspring. One possibility could be the presence of its sibling competitor *N. giraulti* in a nest box. Whether there is a difference in the proportion of diapausing individuals among *N. vitripennis* offspring in allopatric and sympatric populations remains to be tested.

Sex ratio and diapause

We did not find a correlation between the sex ratio of adult and diapausing offspring within one brood. Although this could be due to low statistical power, it could be the outcome of different sex allocation between adult and diapause offspring. If foundresses adjust the sex ratio of their offspring no matter whether the eggs are going into diapause or not, and mixed broods are the result of a single clutch, the sex ratio of the diapause and the adult fraction of one clutch should be identical. Therefore, our results suggest a physiological link between diapause induction and sex allocation, or in case of multiple clutches by aging females, a change in sex allocation in diapausing clutches produced later in life.

From an evolutionary point of view sex allocation according to LMC theory applies only to the fraction of offspring that encounters each other. This means that the fraction of a brood that is in diapause is expected to compete only with other diapausing individuals, assuming simultaneous emergence out of diapause after the winter. The prediction would then be that a foundress separates the sex ratio adjustment for adult and diapausing offspring. Indeed, we found that the sex ratio of single diapausing broods in hosts that also contained adult broods did not differ from the sex ratios produced in single foundress diapausing or adult broods (Figure 4.6). In addition, the sex ratios of superparasitized diapausing clutches were significantly higher than those of single foundresses. This observation fits the expectation of a foundress adjusting her progeny sex ratio only to broods that are expected to emerge at the same time (e.g. after the winter), and suggests that a foundress is able to distinguish between eggs of co-foundresses that will develop into adult or diapausing offspring.

Conclusion

We did not find evidence that *Nasonia* foundresses differentiate between hetero- and conspecific co-foundresses in their sex allocation response. As interspecific matings do not yield any hybrid female offspring, *Nasonia* foundresses should

be selected to differentiate between con- and heterospecific co-foundresses to avoid costly interspecific mating among their progeny. There are clear differences in courtship behaviour between the three sister species (van den Assem & Werren 1994) resulting in different degrees of prezygotic isolation. Why species differentiation in sex allocation has not yet evolved can only be speculated upon. One possible explanation is that *N. vitripennis* is a non-native species in the sampled area (Grillenberger *et al.* in prep.), originating from an allopatric population, and adaptation has not yet progressed this far.

We found a large difference in the production of diapausing offspring between *N. vitripennis* and *N. giraulti* in North America as well as between North American and European *N. vitripennis* populations. The data point to variation in the response to environmental cues between as well as within species. We also found evidence for an interaction between diapause induction and sex allocation. We are not aware of any studies evaluating the adaptive potential of various diapause strategies in a life history evolution context. The *Nasonia* system appears promising for further research into the adaptive significance of diapause strategies in nature. Laboratory experiments on sex allocation and different diapause conditions are also needed, since field studies also have their drawbacks (e.g. no information about the sequence of parasitism).

In the history of LMC research a transition took place from simplistic models toward more complex models that capture some of the observed variation. The current model is that sex allocation is largely adaptive, but that there are certain limits due to information constraints (Shuker & West 2004). Our study also shows that *Nasonia* females are not omniscient, but constrained by the information they can use in their sex allocation decision, such as the inability to discriminate against their closely related species. The further transition from evolutionary models that assume perfect adaptation towards models that include information constraints will be a challenge for the future.

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Chapter 5

Population history of *Nasonia vitripennis* (Hymenoptera) in North America

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submitted to Heredity

Abstract

Parasitic wasps of the genus *Nasonia* are thought to have evolved in North America. However, *N. vitripennis*, a cosmopolitan species with populations in North America and Europe showed reduced genetic variation in North America. Therefore it was tested whether the North American *N. vitripennis* populations originated recently from an European ancestor and shows reduced genetic variation due to a founder effect. We analyzed the population history of 89 *N. vitripennis* specimen from Europe and North America using three types of genetic markers: a 399 bp fragment of the mitochondrial cytochrome oxidase I gene, 9 polymorphic nuclear microsatellites and 6 *Wolbachia* genes. The European samples had a 7-times higher genetic variation in their mitochondrial sequence than the American samples. A phylogenetic analysis revealed a close relationship between the American and European samples and only weak structuring among the American samples. The microsatellite data showed an equal variation among the American and European samples. Genetic distances were slightly higher in Europe. The *Wolbachia* genes showed no variation at all. The differences in variation between the markers reflect the differences in mutation rates between the three tested genomes. The data are consistent with two alternative explanations: (a) the North American population of *N. vitripennis* is derived from a founding population from Eurasia (immigration event 30 – 160 thousand years ago based on mitochondrial divergence); (b) a mitochondrial sweep has occurred in North America populations, possibly due to associated *Wolbachia*. The discordance between mitochondrial and microsatellite data does not resolve between these two possibilities.

Introduction

The parasitic wasp *Nasonia vitripennis* is a model organism in developmental and evolutionary biology (Pultz & Leaf 2003; Beukeboom & Desplan 2003; Shuker *et al.* 2003; Werren *et al.* 2004). It has a cosmopolitan distribution and has been found almost everywhere where it has been looked for (Whiting 1967, and see: http://www.nhm.ac.uk/research-curation/projects/chalcidoids/). Rather recently two sister species of *N. vitripennis* have been discovered in North America (Darling & Werren 1990): *N. giraulti*, is found in eastern North America and *N. longicornis* is found in western North America. Both species occur microsympatrically with *N. vitripennis*.

The divergence of the *Nasonia* genus has been placed in the Pleistocene between 0.2 and 1 Mya (Campbell *et al.* 1993), and *Wolbachia* infections are thought to have played a prominent role in this process (Bordenstein & Werren 1998). The *Wolbachia* infections in *Nasonia* cause complete interspecific reproductive incompatibility between the species (Breeuwer & Werren 1990; 1993; 1995; Bordenstein *et al.* 2001). However, elimination of *Wolbachia* by antibiotic treatment leads to the production of viable hybrid offspring (Breeuwer & Werren 1990). Breeuwer and Werren (1995) and Bordenstein et al. (2001) have shown with antibiotically cured strains of each species that *N. vitripennis* has diverged from the other two species genetically to the point where some F2-hybrid breakdown occurs, whereas the two North American species show little or no hybrid breakdown. Given this close relationship within the genus *Nasonia*, and the presence of all three species in North America, one might expect that all three species evolved from an ancestral species in this region. In this scenario *N. giraulti* and *N. longicornis* would have been confined to North America, while *N. vitripennis* has spread throughout the rest of the world, probably due to its association with synanthropic flies (Whiting 1967). However, this hypothesis about the origin of the genus *Nasonia* is based on the current species distribution, and other scenarios are conceivable. In this study we use different genetic markers to determine whether *N. vitripennis* should be considered native to North America, or has been (re)introduced.

One approach to determine the direction of migration is a comparison of genetic diversity between North American *N. vitripennis* populations with

populations of different origin. If the population in one region is the result of a founder event, its genetic diversity is expected to be reduced compared to a native population (Sakai *et al.* 2001).

Mitochondrial DNA (mtDNA) has been the marker of choice in a wide range of phylogeographical studies because the mutation rate is rapid enough to create variability after rather short periods of time, while the reconstruction of genetic distance remains linear due to its strictly maternal inheritance (Avise 2000). The mitochondrial genome only reflects the maternal history, and can also be associated with inherited endosymbionts like *Wolbachia* and thus selective forces working on the symbiont can influence the evolution of mtDNA (Turelli & Hoffmann 1991; Werren 1997; Hurst & Jiggins 2005). A comparison of these maternally inherited elements to nuclear markers can add valuable information about the phylogenetic history of a species. Another advantage of independently segregating markers is the reduction of the bias generated through inferring population history from a single gene (Rosenberg & Nordberg 2002). Nuclear microsatellite loci are highly polymorphic and used in a wide variety of population genetical studies. These markers have the potential to be very informative over short time periods, but lose their information content as divergence times increase. The reason for this is that due to the high mutation rate and a limited number of alleles, homoplasy can be a serious problem (Nauta & Weissing 1996).

Opijnen *et al.* (2005) compared the genetic diversity of all three *Nasonia* species regarding their nuclear, mitochondrial and *Wolbachia* genome. They found a remarkably reduced mitochondrial variation in *N. vitripennis* compared to its two sister species, while the nuclear and *Wolbachia* genomes did not show such pattern. Opijnen *et al.* (2005) explained this result by a recent selective sweep of a well adapted mitochondrial haplotype. However, their *N. vitripennis* samples were largely restricted to North America and only contained two specimens from Europe. Hence, they could not exclude that the reduced variation was due to a recent founder event.

Here we present a comparison of the genetic diversity among North American (N= 52) and European (N= 27) *Nasonia vitripennis* specimens inferred from a fragment of the mitochondrial cytochrome oxidase I gene, 9 polymorphic nuclear microsatellites, and 6 *Wolbachia* genes. Our results show much greater

levels of mitochondrial variation in Europe, but similar levels of mitochondrial diversity in both regions. The data are consistent with either an origin of *N. vitripennis* in Eurasia, or a mitochondrial sweep in North American populations.

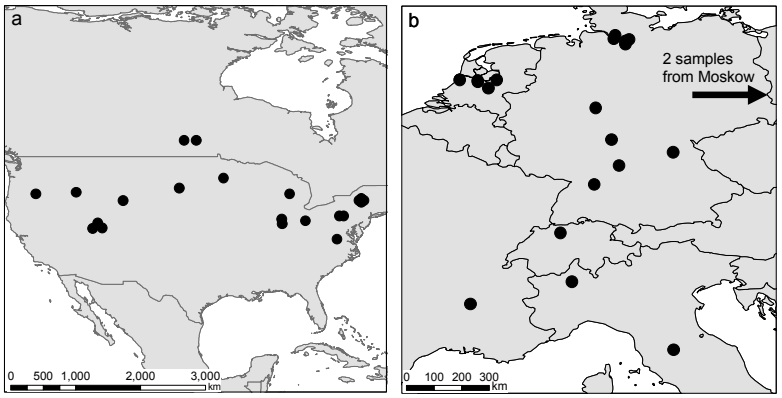

Figure 5.1: Location of the sampling sites in (a) North America and (b) Europe.

Material and Methods

Nasonia sampling and DNA extraction

We acquired 52 specimen from North America and 27 from Europe from bird nests and artificial baits (Figure 5.1, see the supplementary online material for a complete sample list). The North American specimens were obtained from previous samplings by members of the Werren laboratory (University of Rochester) and from field collections by the senior author in 2005. The European specimens were obtained from wild derived stocks in the laboratory in Groningen, and from field collections in summer 2006. All field material was stored at −80 ºC or in 95% ethanol. DNA isolation followed a high salt isolation protocol (Maniatis *et al.* 1982). As some of the laboratory lines had been in culture for a long time and may have lost some genetic variation we only used one individual per line. This precluded any population genetic estimates that are based on the measured heterozygosity in the sampled population (e.g. F_{IS}). For the field samples we also only used one individual per sampling point (mostly a bird nest box) to avoid sampling of highly related individuals.

mt-DNA analysis

We designed primers on the basis of the *Nasonia vitripennis* Cytochrome Oxidase I (*COI*) gene sequence obtained from GENBANK (LOC100113910). The combination of the primers NL COI Fwd (5' GTT ATA CCT KTW ATA ATW GGA GGA TT TGG 3') and NV COI Rev (5' CTT TGA AA ACC ACG TTA CCC 3') amplified a ~400 bp fragment for all three *Nasonia* species using a standard PCR protocol with 52ºC annealing temperature.

The amplification of the fragment was checked on agarose gels (1%) and the PCR product was purified with the Nucleospin II kit (Machery Nagel). The sequencing reaction was performed on both strands using the same primers and the Big Dye Termination sequencing kit (Applied Biosystems, CA). Purified sequencing product was analyzed with an ABI 377 automatic sequencer (Applied Biosystems, CA). The software DNASP4 (Rozas et al., 2003) was used to calculate haplotype diversity and nucleotide diversity indices. For the phylogenetic analysis we used the ALIGNX program included in the VECTOR NTI software package (Invitrogen, CA) to align a 399bp fragment without gaps. To find the ancestral haplotype we used a median joining haplotype network (Bandelt *et al.* 1999) constructed with NETWORK 4.5 (available at: http://www.fluxus-technology.com/sharenet.htm). We used the PHYLIP software package (available at: http://evolution.gs.washington.edu/phylip.html) to construct a neighbour joining tree rooted in an outgroup formed by two *Trichogramma* species obtained from GENBANK (*T. ostriniae* DQ177914, and *T. achaeae* DQ177918). Two *N. longicornis* (GENBANK acc.# EU935415 and EU935416) and two *N. giraulti* (GENBANK acc.# EU935417 and EU935418) sequences obtained in this study were added as comparison.

Table 5.1: Microsatellite primer pairs used in this study. For each primer the chromosome, sequence, number of observed alleles, annealing temperatures and GENBANK accession numbers are given.

Locus	Chrom osome	Primer sequences	Allele#	Ann. Temp.	GENBANK accesion No.
Nv 22	I	5' GAC TGC GTA CCA CTC CAA AAA TA 3' 5' AAG ACC AGC TAG GGA AGA GGA TA 3'	14	58 ºC	AY262041
Nv 23	II	5' ATA CTC AAG CAA GCC ACA GCA TA 3' 5' GCG TAC CAA TCC ACA GAA AAT AG 3	17	58 ºC	AY262044
Nv 41	IV	5' GTC AGA CGT GGG CTT TGT C 3' 5' TTA TGC GCC ACA CAC ACC 3'	15	52 ºC	EU155141
Nv 46	V	5' TTA CGT CAA GGT ATA GCT GC 3' 5' GAA TAA GTG GCT GAA AGT TTC C 3'	12	58 ºC	EU155142
Nv-44	I	5' CCA CTC GAT CGA TTA TTC CT 3' 5' GTG GCC AAT AGT TCA CAT CAA 3'	6	58 ºC	FJ156233
Nv 300	II	5' ACA TTC CGC AGA GCG ATT AT 3' 5' CGC GAC CGA TGA TTT ACT C 3'	6	57 ºC	FJ156211
Nv 303	III	5' GAC AAT AGC CGC TAC GGA AA 3' 5' CGT CGT TCT GCT GCT TCT C 3'	6	57 ºC	FJ156214
Nv 313	V	5' GAA GCT GCG GGT TAA GTG TG 3' 5' CGC TAC TTT ATG CCA GTT ACG G 3'	16	57 ºC	FJ156221
Nv 316	III	5' ACC AGA GAG GGG GAT TTC G 3' 5' CGC AGG ACA ACA TCA AAT A 3'	11	57 ºC	FJ156228

Nuclear DNA analysis

We used 9 polymorphic microsatellites (dinucleotide repeats) spread over all 5 chromosomes to estimate the genetic diversity of the nuclear genome of the European and the North American populations (Table 5.1). The microsatellites Nv 22 and Nv 23 had originally been developed by Pietsch *et al.* (2004), but the primers were redesigned in our laboratory. All other loci were developed in the Groningen laboratory (Beukeboom *et al.* in prep.). PCR followed a standard protocol and the length of the amplified fragments was determined on an ABI Prism 377 DNA sequencer (Applied Biosystems, CA).

As the samples were not distributed evenly over the areas, we grouped samples of neighbouring origin into arbitrary subpopulations to be able to calculate reliable genetic distance estimates. We grouped the samples from the

following locations into corresponding subpopulations: Germany = GER; Netherlands = NL; Switzerland, Italy and France = CH-IT-FR; Canada = CN; Idaho = ID; New York = NY; Utah = UT; Montana, Oregon, Wyoming, Nevada and South Dakota= MT-OR-WY-NV-SD; Pennsylvania and Virginia = PE-VI; Indiana, Michigan, Ohio = IN-MI-OH. The genetic diversity indices were calculated using the F-STAT software package (Goudet, 2001) or by hand. Nei's standard genetic distance D_S (Nei, 1987) and Goldstein's $\delta\mu^2$ (Goldstein and Pollock, 1994) were used to construct Neighbour Joining trees between subpopulations within Europe and North America, using the POPULATIONS 1.2.30 software (available at: http://bioinformatics.org/~tryphon/populations/) and 1000 bootstraps over loci.

Wolbachia analysis

To test the field isolates for *Wolbachia* infection we employed the multi locus sequence typing (MLST) system that has recently been developed (Baldo et al., 2006). Essentially, information from five genes (gatB, coxA, hcpA, ftsZ and fbpA) is used to characterize the *Wolbachia* infections by comparison with other sequences on the PubMLST website (http://pubmlst.org/wolbachia/) which archives the sequences. The combination of the five haplotypes forms a unique allelic profile for each *Wolbachia* infection. Primers have been developed for both super group A (wNvitA) and B (wNvitB) specific *Wolbachia* for the five MLST genes and the sequences and conditions can be found on the website. We screened 21 different field isolates, selected to cover the various mitochondrial DNA haplotypes, for the presence and characterization of *Wolbachia* with primers from both super groups for the MLST genes as well as *wsp*. The primers and their conditions for the A and B super group *wsp* were taken from Zhou et al. (1998). Regular Taq (Invitrogen, CA) was used for PCR reactions and the amplified products were treated with shrimp alkaline phosphatase and exonuclease I (Amersham, CA) to clean the reactions before sequencing. Sequencing was performed at the Functional Genomics Center, University of Rochester, using BigDye v2.0 or v3.0 terminator sequencing kit (Applied Biosystem, CA) and an ABI 3700 or 3730xl automated sequencer (Applied Biosystem, CA). The chromatograms were

manually inspected and cleaned with SEQUECHER 4.7 software (Gene Codes Corp., MI) and the sequences were aligned with BIOEDIT 7.0.1 (Hall, 1999).

Table 5.2: Haplotype frequencies in European (EU), North American (NA) and pooled (total) samples.

haplotype	#NA	#EU	#total	freq NA	freq total	freq EU
1	0	3	3	0	0.0337	0.0938
2	43	4	47	0.7544	0.5281	0.125
3	0	1	1	0	0.0112	0.0313
4	0	1	1	0	0.0112	0.0313
5	6	0	6	0.1053	0.0674	0
6	0	1	1	0	0.0112	0.0313
7	0	4	4	0	0.0449	0.125
8	0	6	6	0	0.0674	0.1875
9	0	4	4	0	0.0449	0.125
10	0	1	1	0	0.0112	0.0312
11	2	0	2	0.0351	0.0225	0
12	1	0	1	0.0175	0.0112	0
13	1	0	1	0.0175	0.0112	0
14	1	0	1	0.0175	0.0112	0
15	0	1	1	0	0.0112	0.0313
16	0	1	1	0	0.0112	0.0313
17	0	1	1	0	0.0112	0.0313
18	0	1	1	0	0.0112	0.0313
19	1	0	1	0.0175	0.0112	0
20[1]	1	0	1	0.0175	0.0112	0
21[1]	1	0	1	0.0175	0.0112	0
22	0	1	1	0	0.0112	0.0313
23[1]	0	1	1	0	0.0112	0.0313
24	0	1	1	0	0.0112	0.0313
Sample size	57	32	89			

1 haplotypes containing one non-synonymous mutation.

Results

mt-DNA sequence data

All *N. vitripennis COI* sequences can be found on GENBANK (acc.# EU935326 – EU 935414; see supplementary online material for sample names and corresponding accession numbers). We identified 24 unique haplotypes containing 34 synonymous and three non-synonymous mutations (Table 5.2). The non-synonymous mutations lead to three haplotypes that were represented by one individual each. The only haplotype that was shared between Europe and North America was the most common haplotype in North America but it was not the most frequent in Europe (haplotype #2, Table 5.2, Figure 5.2). A nested clade analysis (Templeton *et al.* 1992) did not yield any significant geographic clustering of haplotypes within continents. The phylogenetic analysis (Figure 5.3) showed that all North American samples clustered together with a few interspersed European samples. The general topology of the tree resembled previous data (Campbell *et al.* 1993; Opijnen *et al.* 2005).

Table 5.3: Comparison of the mitochondrial DNA results with Opijnen et al. (2005).

	this study	**Opijnen *et al.* 2005**
sequence	399bp COX I	349bp COX I
nucleotide diversity π	NV-EU: 0.0236 NV-NA: 0.0031 NV-World: 0.0149	NV: 0.0046 NG: 0.0127 NL: 0.0114
sample size	32 from EU 57 from NA	NV 2 from EU 18 from NA
Tajima's *D*	NV-EU: 0.53 n.s. NV-NA: −0.88 n.s. NV-World: −0.66 n.s.	NV: −1.81 $p < 0.05$ NG: 0.22 n.s. NL: 0.01 n.s.

NV-EU = *N. vitripennis* Europe, NV-NA = *N. vitripennis* North America, NV-World = all *N. vitripennis* samples pooled, NL = *N. longicornis*, NG = *N. giraulti*.

We found a seven-fold higher nucleotide diversity among European than North American samples (Table 5.3). The level of variation in the North American *N. vitripennis* samples is in the same range as found by Opijnen *et al.* (2005). It is striking that the diversity in the European *N. vitripennis* samples found in this study is in the same range as that of *N. longicornis* and *N. giraulti*

found by Opijnen *et al.* (2005) in North America. Tajima's *D* (Tajima 1989) is −0.88 but not significantly different from 0 for the North American samples and 0.53 (n.s.) for the European samples (Table 5.3). The North American samples differ in their nucleotide sequence (using Nei's D_{XY}) from the European samples by on average 2.3% (compared to 0.3% and 2.4% within NA and EU respectively), while all *N. vitripennis* samples pooled differ from the two sister species by on average 14.2% (*N. vitripennis – N. longicornis* = 13.6%, *N. vitripennis – N. giraulti* = 14.8%) (Table 5.4).

Table 5.4: Average number of nucleotide substitutions per site between populations using Jukes Cantor correction ±(D_{XY} SD, Nei, 1987) based on mt-DNA. The numbers in brackets following the sample abbreviation indicate the sample size.

	NV-EU(32)	NL(2)	NG(2)
NL(2)	0.136±0.021		
NG(2)	0.147±0.022	0.099±0.050	
NV-NA(57)	0.023±0.003	0.137±0.016	0.148±0.017
NV-World(89)		0.136±0.013	0.148±0.014

NV-EU = *N. vitripennis* Europe, NV-NA = *N. vitripennis* North America, NV-World = all *N. vitripennis* samples pooled, NL = *N. longicornis*, NG = *N. giraulti*.

Microsatellite data

Nei's gene diversity $\hat{H}$ (Nei, 1987) and allelic richness *R* are equal in the North American and the European samples. This pattern is consistent over loci with various repeat lengths and motifs (Table 5.5). There is low, but highly significant ($p < 0.001$) differentiation between the two continents ($F_{ST} = 0.037 \pm 0.013$SE). As this measure is highly dependent on the variability of the marker used, we also calculated Hedrick's $G'_{ST} = 0.23$ (Hedrick, 2005) in order to compare the differentiation to other studies. This corrected differentiation index shows the same level of differentiation between the two continents as did a previous study between two populations within Europe (0.23, Grillenberger et al., 2008). Both Neighbour Joining trees, based on the genetic distances (D_S and $\delta\mu^2$) between subpopulations within Europe and North America, indicate no clear split between the two continents. We only present the tree based on Nei's D_S, as the qualitative results for the tree based on $\delta\mu^2$ are identical (Figure 5.4). The mean genetic distance estimated with both parameters indicate no clear

difference between the continents. The variation within Europe is slightly higher than within North America (D_S ±SE: Europe 0.36±0.10, North America 0.35±0.02, between Europe and North America 0.43±0.04; $\delta\mu^2$ ±SE: Europe 22.05±8.06, North America 13.92±2.30, between Europe and North America 17.18±2.54).

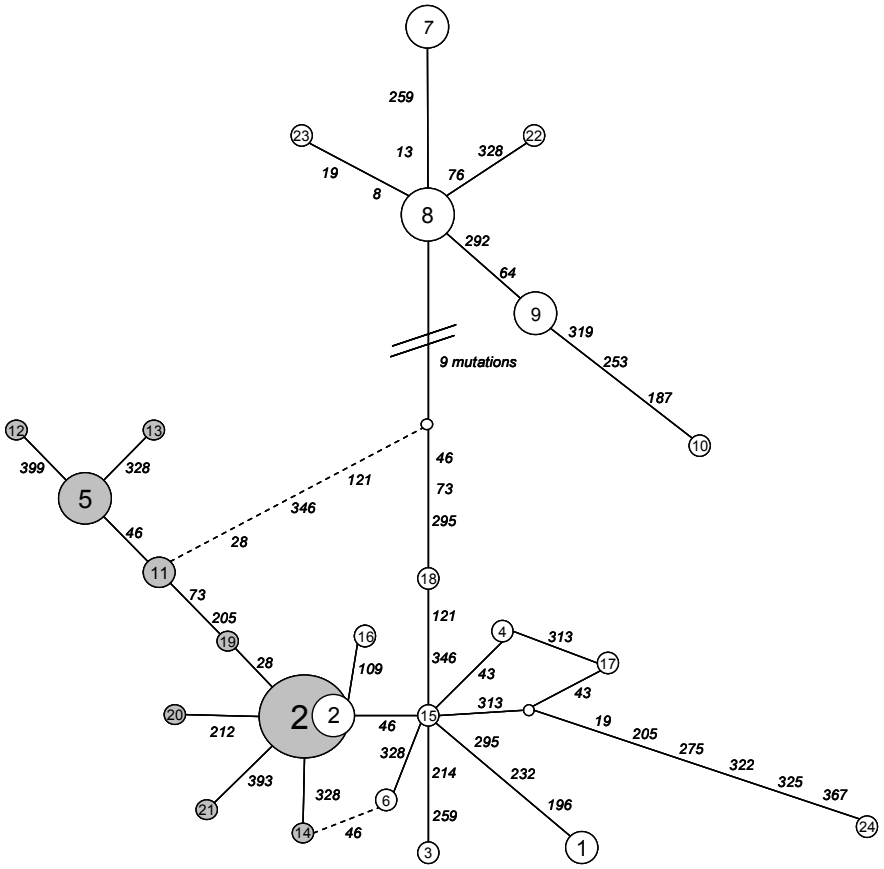

Figure 5.2: Haplotype network based on the 399bp mitochondrial *COI* sequence. Numbers in circles represent particular haplotypes, numbers along lines the nucleotide positions in the sequence that changed. The size of the circles reflect the haplotype frequency. Nodes without a label represent intermediate haplotypes that were not sampled. Dashed lines indicate alternative mutation routes. The grey haplotypes have been found in North America, the white ones in Europe. Haplotye #2 has been found on both continents (see Table 5.2 and text).

Table 5.5: Number of repeats based on sequenced genome, gene diversity ($\hat{H}$), allele number (#), allelic richness (R) per locus and population (NA = North America, EU = Europe) as well as the total sample, and Weir and Cockerham's F_{ST} (Weir and Cockerham, 1984) of the microsatellite data. Means over all loci are given ± s.d. Mean F_{ST} is given ± s.e after Jackknifing over loci.

locus	# repeats	$\hat{H}$ NA	$\hat{H}$ EU	#NA	#EU	#total	R_S NA	R_S EU	R_T total	F_{ST}
Nv-22	26	0.88	0.85	12	11	14	10.47	9.77	10.76	0.04
Nv-23	28	0.79	0.57	14	8	17	12.35	7.63	11.40	0.07
Nv-41	15	0.74	0.89	12	11	15	11.13	12.73	11.94	0.14
Nv-46	21	0.78	0.86	7	12	12	6.89	11.46	8.79	0.07
N1-10	14	0.40	0.40	5	3	6	5.06	5.00	5.09	0.08
Nv-300[1]	6	0.51	0.47	4	4	6	3.36	3.68	3.56	0.58
Nv-303	7	0.76	0.73	6	6	6	5.73	5.90	5.73	0.09
Nv-313	14	0.89	0.81	13	8	16	11.96	8.90	11.41	0.16
Nv-316	16	0.76	0.75	10	6	11	8.61	6.77	8.39	0.14
mean		0.72±0.16	0.70±0.18	9.22±3.55	7.67±3.02	11.44±4.48	8.39±3.28	7.98±3.00	8.56±3.12	0.15±0.054

[1] Nv 300 is a trinucleotide marker, all other markers are dinucleotide repeats.

Wolbachia variation

Out of the 21 individuals analyzed for *Wolbachia*, we found three to be uninfected, three to be infected with type wNvitA only, and 15 to be doubly infected with wNvitA and wNvitB. One sample, HV-736, could not be sequenced completely because of poor DNA quality. In all sequenced individuals we did not find any variation within *Wolbachia* strains across North America and Europe (supplementary online material).

Discussion

We found that the genetic diversity of *N. vitripennis* at the mitochondrial level is much higher in Europe than in North America, even though the latter specimens were spread over a larger geographical area (nucleotide diversity π: NA = 0.0031, EU = 0.0236). As a consequence, the phylogenetic analysis shows a much weaker structuring of the North American cluster (Figure 5.2). There is one predominant haplotype (#2) in North America that is present but infrequent in Europe. The negative Tajima's *D* value suggests that there has been a rapid expansion of *N. vitripennis* in North America, while there is no evidence for such an event in the European data.

The observed pattern can be explained by two scenarios. In scenario (1) a selective sweep in favour of haplotype #2 occurred in the North American population. Although haplotype #2 involves only synonymous substitutions compared to most of the other haplotypes, it may still be possible that a beneficial mutation is located outside the sequenced *COI* fragment and that haplotype #2 swept through the population by hitchhiking. Alternatively, the selective sweep could be associated with an advantageous *Wolbachia* strain, since a selective sweep of a *Wolbachia* type would result in hitchhiking of the associated mitochondrial haplotype due to their maternal co-inheritance (Turelli and Hoffmann, 1991). The alternative scenario (2) is that the North American population went through a severe bottleneck that caused the reduction in mitochondrial diversity. This bottleneck could have happened in the course of a founder event of individuals carrying haplotype #2 coming from Europe. This would be consistent with the rapid range expansion suggested by the negative Tajima's *D* value (and a mismatch distribution analysis, results shown in the supplementary online material Table 2 and Figure 1), as well as with the

stronger substructure of the European population. In this scenario the situation in Europe is the product of a long history resulting in a diverged population, while the North American population is the product of a rather recent migration event. However, this interpretation assumes that the similar levels of microsatellite variation observed in European and North American populations, are due to high mutation rates of microsatellites that restored an equilibrium variation since the bottleneck (see also below).

Although the nested clade analysis did not show significant correlations between genetic and geographic structure, the structure of the mitochondrial haplotype network (Figure 5.2) allows some conclusions. The star like pattern of the American part of the haplotype network can be interpreted as the result of a recently expanding population (Avise, 2000) or as mitochondrial sweep. Such a historical pattern is consistent with the findings of Grapputo et al. (2005) who analyzed the genetic diversity of potato beetles (*Leptinotarsa decemlineata*) in Europe and North America. *L. decemlineata* has been introduced to Europe in 1920 and showed no mitochondrial variation at all in Europe, but considerable variation in its native population (Grapputo et al., 2005). The same pattern has been found by Scheffer and Grissel (2003), who found no genetic variation at all in South American seed feeding wasp (*Megastigmus transvaalensis*) populations and a high variability in the African source population. Aoki et al. (2008) investigated the consequences of a glacial bottleneck on the genetic variation of a seed parasitic weevil (*Cuculio hilgendorfi*) in Japan and found a similar pattern. The structure of the haplotype network of our North American population resembles the bottlenecked population in Japan, and our European populations show a similar pattern as the Japanese populations from a refugial area.

The differences in genetic diversity at the nuclear level are not pronounced. The microsatellite data shows no difference in the allelic richness (Europe 7.98±3.00, North America 8.39±3.28, Table 5.5). The mean genetic distance among the European samples is slightly higher than among the North American samples (D_S ±SE: EU 0.36±0.10, NA 0.35±0.02; $\delta\mu^2$ ±SE: EU 22.05±8.06, NA 13.92±2.30), despite the larger geographical range of the latter. This indicates that there is stronger differentiation between the local populations in Europe than in North America. In the case of a founder effect during the colonization of

North America, or a selective sweep, one would expect a drastic reduction in genetic diversity. However, the differences detected in the microsatellite variation are only subtle, and therefore do not indicate a recent bottleneck. It is obvious that there is no direct gene flow between the two continents. However, the microsatellite data do not reveal a clear split between the European and North American populations (Figure 5.4). The missing phylogenetic differentiation between the two continents together with the equal level of nuclear variation, suggests that the differences between the continents are masked by homoplasy. This could be explained by the high mutation rate of the microsatellite markers, which leads to the relatively fast recovery of genetic variation but a high risk of homoplasy over time (Nauta and Weissing, 1996).

Using Campbell *et al.*'s (1993) estimate of species divergence as a calibration, the genetic distance between the North American and the European *N. vitripennis* population (NV-NL = 13.6%, NV-NG = 14.8%,, NV-NA – NV-EU = 2.3% Table 5.4) corresponds to a separation between the populations between 30 and 160 thousand years ago. Assuming five generations per year in *Nasonia* this corresponds to 0.15 - 0.8 $*10^6$ generations. Nauta and Weissing (1996) found that a divergence time of 20 $*10^3$ generations already drastically reduces reliability of microsatellites. The much longer divergence time could therefore explain the missing signs of a bottleneck, or selective sweep, as well as the evidence for homoplasy (see above) in the microsatellite data.

One of the key features about the natural history of mitochondria is their association with maternally inherited endosymbionts like *Wolbachia* (Johnstone and Hurst, 1996; Ballard and Whitlock, 2004). Since they are co-inherited, *Wolbachia* can have profound effects on the evolutionary history of the host mitochondria. *Wolbachia* induced cytoplasmic incompatibility can drive a particular combination of mitochondria and bacterial types to very high frequencies in a population resulting in a *Wolbachia*-mitochondrial sweep (Turelli and Hoffmann, 1991) in the host population. Such sweeps are indicative of new *Wolbachia* invasions. Therefore, one explanation for the presence of multiple mitochondrial haplotypes could be the presence of different *Wolbachia* infections in the wasps.

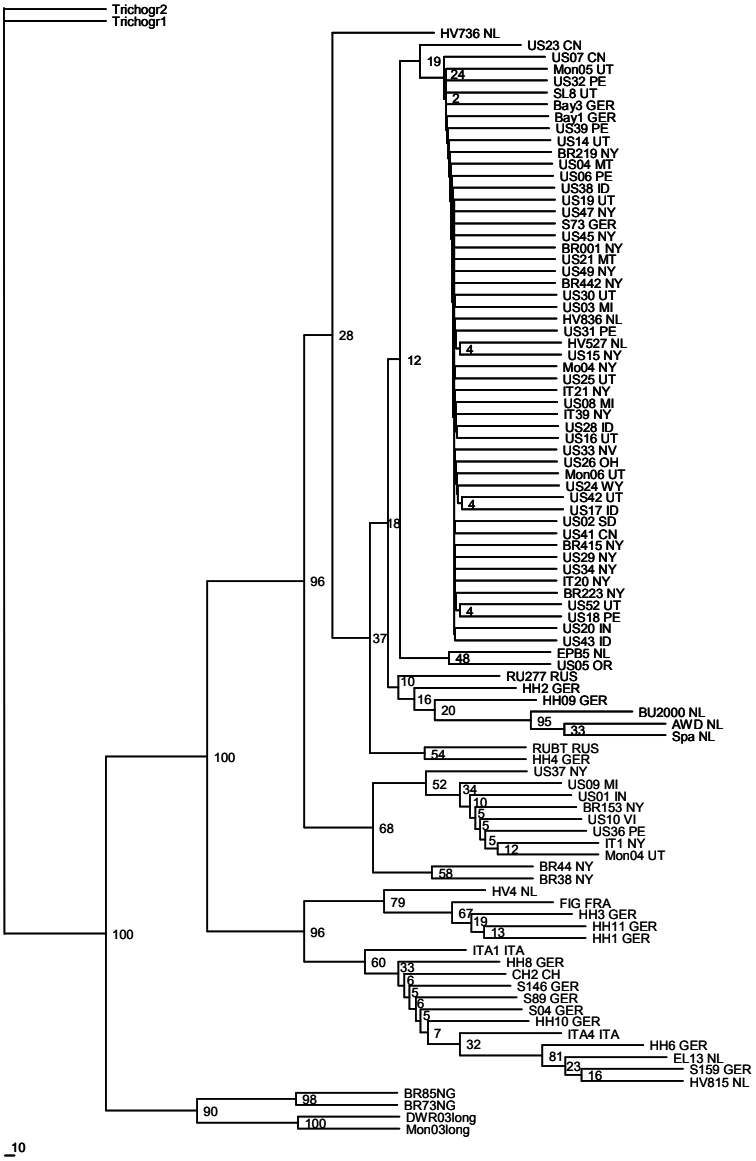

Figure 5.3: Neighbour joining tree based on the 399 bp mitochondrial *COI* sequence. Genetic distances were calculated using the F84 method in DNADIST (Phylip). Branch lengths and internal values indicate bootstrap values out of 100 bootstraps. The tree is rooted in an outgroup formed by Trichogr1 and Trichogr2 (*Trichogramma*). Sequences of the two sister species Mon03long, DWR03long (*N. longicornis*), BR85NG, BR73NG (*N. giraulti*) were added for comparison.

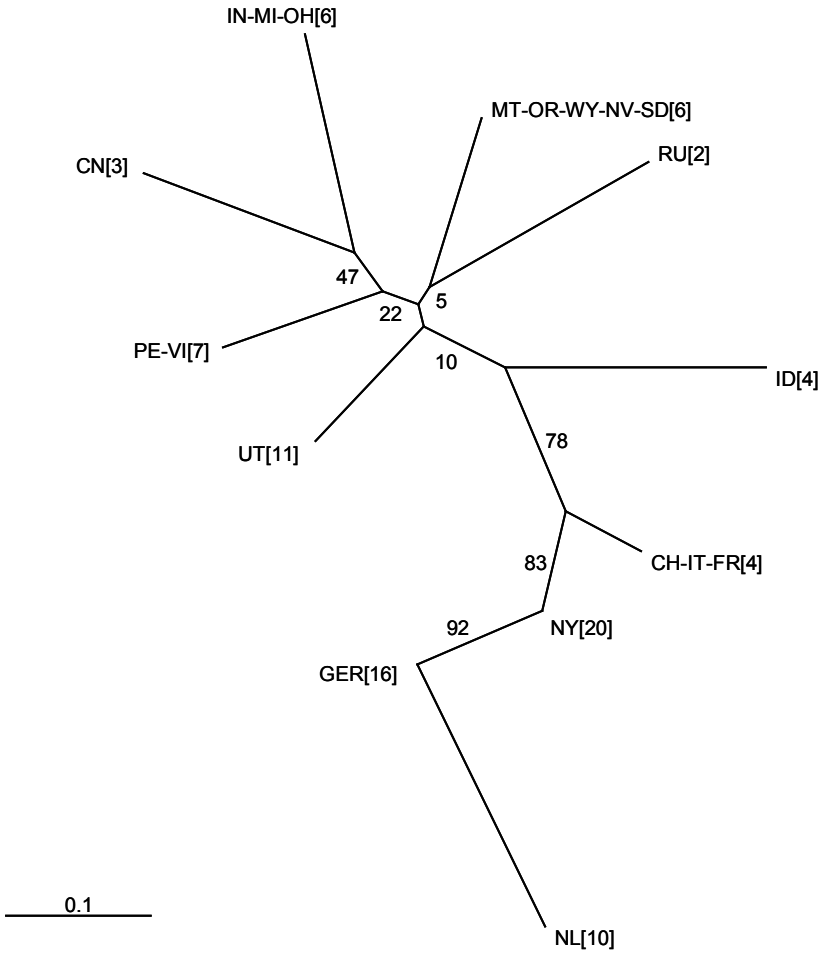

Figure 5.4: Unrooted Neighbour joining tree based on Nei's genetic distance D_S (Nei 1987) between subpopulations calculated on the basis of nine polymorphic microsatellites. Branch lengths indicate genetic distance, numbers at branches the bootstrap value after 1000 permutations. Numbers in brackets indicate the number of individuals combined in the subpopulation. Germany = GER; Netherlands = NL; Switzerland, Italy and France = CH-IT-FR; Canada = CN; Idaho = ID; New York = NY; Utah = UT; Montana, Oregon, Wyoming, Nevada and South Dakota = MT-OR-WY-NV-SD; Pennsylvania and Virginia = PE-VI; Indiana, Michigan, Ohio = IN-MI-OH.

Previous studies have shown that *N. vitripennis* is infected with two *Wolbachia* strains, each belonging to one of the super groups A and B (Werren *et al.*, 1995; Opijnen *et al.*, 2005, Raychoudhury *et al*., submitted), with each

infection causing bi-directional cytoplasmic incompatibility with each other (Perrot-Minnot *et al*., 1996). We found three individuals to be uninfected, and three to be singly infected with type wNvitA. The loss of an infection only occurred in laboratory lines and can be attributed to laboratory condition, where prolonged diapause can result in the loss of infections (Perrot-Minnot et al., 1996). We did not find any variation in the six genes of the *Wolbachia* among the 15 doubly infected individuals. We do not expect that the missing variation is due to a reduced sample size for the *Wolbachia* screen, as we selected the specimens in such a way that we covered all variation based on the mitochondrial data. This warranted detecting a correlation between mitochondrial and *Wolbachia* variation, if present. Nevertheless, the hypothesis that the variation in mitochondria in *N. vitripennis* is a reflection of the variation in *Wolbachia* infections cannot be fully rejected. A key aspect of *Wolbachia* biology in *Nasonia* that has recently emerged is the relative mutation rate of the bacteria and host nuclear and mitochondrial genes (Oliveira *et al*., submitted). *Nasonia* has an extremely high rate of synonymous mutation in its mitochondrial genes which are estimated to evolve at a rate nearly 40 times faster than the nuclear genes (Oliveira *et al*., submitted). On the other hand, the *Wolbachia* genome evolves at a rate that is approximately 1/3 that of the nuclear genes (Raychoudhury *et al.*, submitted). Extrapolating, there are roughly 120 mutations in the *Nasonia* mitochondria for every mutation in *Wolbachia*. This can explain the mitochondrial diversity with respect to the lack of diversity in the *Wolbachia* infections of *N. vitripennis*. Furthermore, given the high mutation rate observed in *Nasonia* mitochondria (~ 40 greater than the nuclear point mutation rate), the high similarity between the common haplotype in North America and an uncommon variant in Europe implies that the selective sweep in North America was very recent. Otherwise, variation would have accumulated in the North American haplotype since the founder event. In contrast, the pattern of microsatellite variation would suggest that the founder event was not recent. Therefore, the severely reduced mitochondrial variation in North America coupled with its high mutation rate would argue for a North American mitochondrial sweep rather than a founder event. Resolution of the issue, however, will require calibrating microsatellite to mitochondrial and nuclear (SNP) mutation rates.

Taken together, our data can be explained by the two scenarios introduced in the beginning of the discussion: either a selective sweep in favour of haplotype #2 (possibly due to associated Wolbachia variants) occurred in North America, or *N. vitripennis* was introduced into North America. To decide conclusively whether there has been a bottleneck or a selective sweep, more nuclear markers with a lower mutation rate (e.g. SNPs) need to be screened, and the advent of high throughput techniques will make this affordable in the near future.

Acknowledgements

We would like to thank H. Beran (Landesbund für Vogelschutz Bayern) and R. Peters (University of Hamburg) for sending us samples, and David Winkler (Cornell University) and John Rogers for giving us access to their nest boxes. This work was supported by a Pioneer grant (ALW833.02.003) to L.W.B., a field grant of the Schure-Beijerinck-Popping Fonds of the Royal Netherlands Academy of Sciences to B.K.G., and US NSF EF-0328363 to J.H.W.

Appendix

Supplementary material**:** List of samples, assigned haplotype, origin, type of sample (field collection or laboratory stock) and GENBANK accession number. Results of *Wolbachia* analysis: U = uninfected, A = only strain wNvitA , AB = doubly infected for strains wNvitA and wNvitB.

Sample ID	haplo type	*Wolbachia* analysis	state and country	location	sample type	GENBANK Accession No.
CH-2	8	AB	Switzerland	Langenthal	field	EU935412
Fig-1-FRA	9	AB	France	INRA-Gotheron Valence	laboratory	EU935344
Bay-1-GER	2	AB	Germany	Bayreuth	field	EU935408
Bay-3-GER	2		Germany	Bayreuth	field	EU935409
S-04-GER	8		Germany	Schlüchtern	field	EU935341
S-73-GER	2		Germany	Schlüchtern	field	EU935370
S-89-GER	8		Germany	Schlüchtern	field	EU935343
S-146-GER	8		Germany	Schlüchtern	field	EU935342
S-159-GER	7		Germany	Schlüchtern	field	EU935338
HH-1-GER	9		Germany	Hamburg	field	EU935339
HH-2-GER	3	AB	Germany	Hamburg	field	EU935346
HH-3-GER	9		Germany	Hamburg	field	EU935331
HH-4-GER	7	AB	Germany	Hamburg	laboratory	EU935347
HH-6-GER	7		Germany	Haseldorf	field	EU935332
HH-8-GER	8		Germany	Bad Arolsen	field	EU935336
HH-9-GER	15	AB	Germany	Eberdingen	field	EU935340
HH-10-GER	8		Germany	Bad Mergentheim	field	EU935361
HH-11-GER	9		Germany	Elmshorn	field	EU935345
ITA-1	22	AB	Italy	Toscany	field	EU935406
ITA-4	23	AB	Italy	Piemont	field	EU935407
AWD-1-NL	1	A	Netherlands	Amsterdam	laboratory	EU935326
BU-2000-NL	1		Netherlands	Bussum	laboratory	EU935411
SPA-1-NL	1		Netherlands	Bussum	laboratory	EU935327
EP-B5-NL	6	U	Netherlands	Elspeet	laboratory	EU935334
EL-13-NL	7	AB	Netherlands	Elspeet	laboratory	EU935335
HV-4-NL	10		Netherlands	Hoge Veluwe	field	EU935348
HV-527-NL	16	AB	Netherlands	Hoge Veluwe	field	EU935362
HV-736-NL	24	AB	Netherlands	Hoge Veluwe	field	EU935410

Sample ID	haplo type	*Wolbachia* analysis	state and country	location	sample type	GENBANK Accession No.
HV-815-NL	7		Netherlands	Hoge Veluwe	field	EU935337
HV-836-NL	2		Netherlands	Hoge Veluwe	field	EU935363
RU-BT-RUS	17	AB	Russia	Moscow	laboratory	EU935369
RU-277-RUS	18	AB	Russia	Moscow	laboratory	EU935371
US-7-CN	2		Ontario- CN		laboratory	EU935388
US-23-CN	19		Ontario- CN		laboratory	EU935391
US-41-CN	2		Ontario- CN		laboratory	EU935400
US-17-ID	2		Idaho USA		laboratory	EU935376
US-28-ID	2		Idaho USA		laboratory	EU935384
US-38-ID	2		Idaho USA		laboratory	EU935398
US-43-ID	2		Idaho USA		laboratory	EU935402
US-1-IN	5		Indiana USA		laboratory	EU935353
US-20-IN	2		Indiana USA		laboratory	EU935379
US-3-MI	2		Michigan USA		laboratory	EU935385
US-8-MI	2		Michigan USA		laboratory	EU935389
US-9-MI	5	A	Michigan USA		laboratory	EU935355
US-21-MT	2		Montana USA		laboratory	EU935380
US-4-MT	2		Montana USA		laboratory	EU935386
US-33-NV	2		Nevada USA		laboratory	EU935396
US-15-NY	2		New York USA		laboratory	EU935374
US-29-NY	2	U	New York USA		laboratory	EU935392
US-34-NY	2		New York USA		laboratory	EU935397
US-45-NY	2		New York USA		laboratory	EU935413
US-47-NY	2		New York USA		laboratory	EU935403
US-49-NY	2		New York USA		laboratory	EU935404
BR-001-NY	2		New York USA	Brewerton	field	EU935328
BR-38-NY	11	AB	New York USA	Brewerton	field	EU935349

Sample ID	haplo type	*Wolbachia* analysis	state and country	location	sample type	GENBANK Accession No.
BR-44-NY	11		New York USA	Brewerton	field	EU935360
BR-153-NY	5		New York USA	Brewerton	field	EU935333
BR-219-NY	2		New York USA	Brewerton	field	EU935329
BR-223-NY	2		New York USA	Brewerton	field	EU935330
BR-415-NY	2		New York USA	Brewerton	field	EU935359
BR-442-NY	2		New York USA	Brewerton	field	EU935350
IT-1-NY	12		New York USA	Ithaca	field	EU935351
IT-20-NY	2		New York USA	Ithaca	field	EU935414
IT-21-NY	2		New York USA	Ithaca	field	EU935364
IT-39-NY	2		New York USA	Ithaca	field	EU935365
M-04-NY	2		New York USA	Mumford	field	EU935366
US-37 NY	13	U	New York USA		laboratory	EU935357
US-26-OH	2		Ohio USA		laboratory	EU935383
US-5-OR	14		Oregon USA		laboratory	EU935358
US-6-PE	2		Pennsylvania USA		laboratory	EU935387
US-18-PE	2		Pennsylvania USA		laboratory	EU935377
US-31-PE	2		Pennsylvania USA		laboratory	EU935394
US-32-PE	2		Pennsylvania USA		laboratory	EU935395
US-36-PE	5		Pennsylvania USA		laboratory	EU935356
US-39-PE	2		Pennsylvania USA		laboratory	EU935399

Sample ID	haplo type	*Wolbachia* analysis	state and country	location	sample type	GENBANK Accession No.
US-2-SD	2		South Dakota USA		laboratory	EU935390
Mon-04-UT	5		Utah USA	Huntsville	field	EU935352
Mon-05-UT	2		Utah USA	Huntsville	field	EU935367
Mon-06-UT	2		Utah USA	Huntsville	field	EU935368
SL-08-UT	2		Utah USA	Strawberry Lake	field	EU935372
US-14-UT	2		Utah USA		laboratory	EU935373
US-16-UT	2		Utah USA		laboratory	EU935375
US-19-UT	2		Utah USA		laboratory	EU935378
US-25-UT	2		Utah USA		laboratory	EU935382
US-30-UT	2		Utah USA		laboratory	EU935393
US-42-UT	20	A	Utah USA		laboratory	EU935401
US-52-UT	21		Utah USA		laboratory	EU935405
US-10-VI	5		Virginia USA		laboratory	EU935354
US-24-WY	2		Wyoming USA		laboratory	EU935381

Chapter 6

Female dispersal and isolation-by-distance of *Nasonia vitripennis* (Walker) populations in a local mate competition context

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submitted to Entomologia Experimentalis et Applicata

Abstract

Dispersal behaviour directly influences the level of inbreeding, but the effect of inbreeding avoidance on dispersal is less well studied. The parasitoid wasp *Nasonia vitripennis* (Walker) (Hymenoptera: Chalcidoidea) is known to mate exclusively on the natal patch, and females are the only dispersing sex. A previous study has shown that foundresses on a patch are typically unrelated, implying that females disperse for a considerable distance from their natal patch after mating. We investigated the dispersal of *N. vitripennis* on two scales. On a local scale we use a mark-release-recapture experiment and for the larger scale we investigated isolation by distance using a population genetic approach. We found that *N. vitripennis* females are long distance dispersers capable of covering at least two kilometres in 48 hours. Whereas populations within a range of 100 km showed no substructure, larger distances or major geographical barriers restricted gene flow and led to significant population structure. The results provide a basis for future research on the dispersal of parasitoids and are discussed in the context of dispersal abilities and inbreeding avoidance in *Nasonia*.

Introduction

The essence of local mate competition (LMC) theory is that a female should adjust the sex ratio of her offspring in such a way that the competition between relatives is minimized (Hamilton 1967). This is an evolutionary stable strategy if females are the only dispersing sex, and if mating takes place exclusively at the natal patch. The most extreme case is that a patch population is founded by a single female. In this scenario all males are brothers and the best strategy for the ovipositing female (here called foundress) would be to shift the sex ratio towards more daughters to reduce the competition among her sons. In a scenario with multiple foundresses, the outcome strongly depends on the relatedness among them. If the foundresses are unrelated, the sons of the different families compete with each other to mate with the available daughters. Therefore, for an individual foundress it is beneficial to produce a higher proportion of sons. In this scenario the patch sex ratio will approach 0.5 when the number of foundresses per patch becomes very large (Fisher 1930). In contrast, related foundresses produce related offspring, which results in the maintenance of the high level of LMC for a female's sons and an expected weaker LMC response (Herre 1985; Frank 1985b). The jewel wasp *Nasonia vitripennis* is a gregarious parasitoid of cyclorraphous flies that is mainly found in bird nests. Since its life history closly resembles the assumptions of LMC theory, it has been used extensively in LMC research (Werren 1984; Drapeau & Werren 1999; Shuker *et al.* 2004b; 2006a; 2006b). Laboratory experiments and three field studies (Werren 1984; Molbo & Parker 1996; Burton-Chellew *et al.* 2008) showed that *N. vitripennis* modulates its progeny sex ratio largely according to LMC theory.

The strict local mating of *N. vitripennis* strongly enhances inbreeding. Inbreeding combined with genetic drift leads to a loss of genetic variation and increased homozygosity, collectively termed as genetic erosion. In diploid organisms this process can lead to the expression of homozygous recessive deleterious alleles. As Hymenoptera are haplodiploid, the effect of inbreeding is thought to be limited due to purging of such deleterious alleles in haploid males (Werren 1993). However, one study on *N. vitripennis* (Luna & Hawkins 2004) and one on *Uscana semifumipennis* (Henter 2003) found evidence that there is some inbreeding depression in parasitoids, as outcrossed strains performed better than the original inbred field strains. Another effect of the loss of genetic

variation is the reduced ability to react to a changing environment (Bijlsma & Loeschcke 2005). Therefore, purging of deleterious alleles in males is not sufficient to avoid the consequences of the loss of variation, as the adaptive potential is dependent on the amount of available genetic variation. These theoretical considerations lead us to seek for mechanisms of inbreeding avoidance in *Nasonia*. A field study on *N. vitripennis* found slightly lower inbreeding than expected under random mating among the offspring of unrelated females in a patch (population inbreeding coefficient F_{IT} = 0.17, compared to an expected value of 0.22 under the assumption of unrelated females parasitizing a patch; Grillenberger *et al.* 2008) The assumption that all foundresses are unrelated requires a large well mixed population. The usual behaviour of a *Nasonia* female is to disperse right after mating and search for new hosts (Whiting 1967, BKG, personal observation). While the primary purpose of female dispersal is the colonization of new host patches, the composition of patches, and the associated level of relatedness among the foundresses, depends as well on the dispersal strategy of the mated females. Low dispersal distances could lead to a high level of relatedness and high dispersal distances to a low level of relatedness between foundresses within a patch, respectively. In *N. vitripennis* it has been shown that the foundress population of a patch is a random sample of the wasp population of the area (Grillenberger *et al.* 2008). This suggests that *N. vitripennis* females do leave their natal patch after mating and disperse a rather long distance. However, this study was confined to *N. vitripennis* in two, rather small areas, and the actual dispersal distances have not been studied, yet. In the present study we use a mark-release-recapture experiment to estimate the dispersal capabilities of *Nasonia vitripennis* and its sister species *N. giraulti* (Darling) on a local scale. To estimate the dispersal capabilities on a larger scale, we as well used a population genetic analysis on *N. vitripennis* samples.

Material and Methods

Mark release recapture experiment

To avoid effects of changes in behaviour due to long laboratory culturing we used recently collected strains for the mark-release-recapture experiment. For *Nasonia giraulti* we used the strain NGVA collected in summer 2006 in

Virginia (USA). For *N. vitripennis* we used either emerging individuals from freshly collected bird nests (species identity was checked by determining the species of the male offspring emerging from a single host), or we used the strain ITH4c which was collected in summer 2006 in Ithaca (NY, USA). The 2006 strains have been in laboratory culture for about 20 generations. All strains were cultured on *Sarcophaga bullata* hosts at room temperature, until one day after emergence, and then kept at 4ºC until release.

Cornell University (Ithaca, NY) allowed us to use their large array of bird nest boxes to perform a mark-release-recapture experiment (Figure 6.1). At this field site nest boxes are mounted on approximately one meter high poles around either small ponds (western part) or in a large shallow pond (east side). For our experiment, we used all nest boxes along a transect from north to south (max. dist = 445m) and along a second transect from east to west (max. dist. = 415m). At the intersection of these transects we released the marked wasps (~95% females) inside an empty nest box. For recapture, we placed mesh bait bags with 20 host pupae (*Sarcophaga bullata*) inside all (34) nest boxes along the transects. Most nest boxes were empty but, if not, we placed the bait under the existing nest material as that is where natural hosts are usually found (BKG, personal observations).

All wasps were counted while transferring them to a new culture vial (100 per vial) and stained with fluorescent dust the evening before the release, by adding a small amount of dust to the vial and rolling the vial until all wasps were covered. The wasps were then kept at room temperature until their release the next morning to give them the opportunity to clean off the excess dust. A previous test in the laboratory had shown that the wasps are able to clean off most of the dust, except at the base of the wings, where the fluorescent dust could be easily detected after one week of maintenance at 25ºC. On the release day we first placed the baits for recapture in the appropriate nest boxes. Around 10:00 AM the culture vials with the stained wasps were placed inside the release nest box and opened. Every 24h we collected all baits and replaced them with fresh ones, until the end of each release experiment. All collected baits were checked under UV light for traces of fluorescent dust, as well as for any *Nasonia* present on the hosts. The baits were kept at room temperature until either flies or wasps emerged to check whether hosts had been parasitized

without a wasp being detected on the bait. For consecutive releases the dust colours were changed, to be able to assign recaptured individuals to a certain release date, because multiple releases were done in the area during the season. Before a consecutive release, all remaining wasps (mostly males) were removed from the release nest box.

Molecular analysis

To evaluate the population genetic consequences of dispersal on a larger scale for *Nasonia vitripennis*, we genetically analyzed samples from North America and Europe. For North America we used samples from two locations in Ithaca, one in Brewerton (New York) and three in Utah; for Europe we used samples from two locations on Corsica and nine localities across central Europe. Ten samples per area/population were examined (with central Europe as one area), using one individual per sampling location (mostly a nest box) to avoid artefacts due to relatedness between the samples of one nest box (for a complete sample list see Appendix).

We used eight polymorphic microsatellites to estimate the genetic differentiation between the study populations (Beukeboom *et al.*, in prep., Table 6.2). PCR was performed using the Qiagen Multiplex kit (Qiagen, CA) and fragment lengths were determined on an ABI 3730XL sequencer (Perkin-elmer Applied Biosystems, CA).

The software package F-stat (Goudet 2001) was used to calculate Nei's G_{ST} values as well as Weir and Cockerham's F_{ST} on different hierarchical levels within North America and Europe as well as between North America and Europe. Hedrick's G'_{ST} (Hedrick 2005) which is adjusted for marker variation, was calculated by hand on the basis of the F-stat output. As G_{ST} values tend to be slightly negative due to rounding errors, negative G_{ST} values were set to zero for the isolation by distance analysis, and the calculation of G'_{ST} (Meirmans 2006). An isolation by distance analysis was performed by correlating the logarithm of geographical distance with G'_{ST}, using linear regression analysis in R (R Development Core Team 2006). We compared these data to that of a previous study (Grillenberger *et al.* 2008) that calculated F_{ST} and G'_{ST} values in *N. vitripennis* as well.

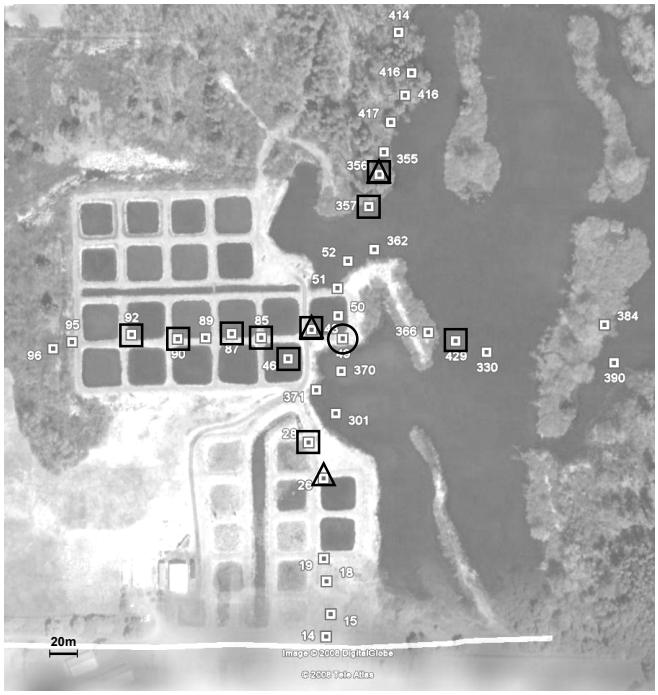

Figure 6.1: Approximate position of the nest boxes used in the mark-release-recapture experiment. Nest box 49 was used as release site (circle). In boxes labelled with a black square we found unmarked wasps, in boxes labelled with a black triangle we recaptured marked wasps. Map drawn with Google Earth.

Results

Mark release recapture experiment

A total of five releases were performed (three times only *N. vitripennis*, once only *N. giraulti*, once both species). The time between release and last collection of the recapture baits was between one and three days. The time between subsequent releases was between two and six days. After 24 hours we exclusively recaptured marked males in the release nest box; all marked females had dispersed. Out of 3150 released *N. vitripennis* specimen only three marked females were recaptured within the study area: 20 m East, 120 m North and 100 m South of the release point. In the same period and area we caught 31 unmarked females in the baited nest boxes (Figure 6.1). Assuming an equal probability of catching marked and unmarked individuals, this leads by

extrapolation to a population in the study area of around 27000 individuals. The low recapture rate does not allow any conclusions about dispersal patterns within the area. However, we recaptured two marked *N. vitripennis* females in a nearby study area in which we conducted a different experiment. This area is about two kilometres east of the release site. This indicates that individual *N. vitripennis* females can cover a distance of at least two kilometres within two days. An onsite weather station recorded primarily westerly winds during the study period, suggesting that the wasps have been carried by wind currents. Most baits on which female wasps were found at collection also yielded wasp offspring, while the baits without signs of wasp presence did not. None of the 1650 released *N. giraulti* were recaptured (Table 6.1).

Table 6.1: Numbers and timing of the mark-release-recapture studies in Ithaca. There were no *N. giraulti* recaptured.

release	#NV	#NG	colors used	release date	last collection date	unmarked NV	marked NV
1	300	0	yellow	27-6-2007	28-6-2007	0	0
2	500	0	blue	2-7-2007	4-7-2007	2	2
3	0	800	green	5-7-2007	7-7-2007	5	0
4	850	850	blue / yellow	7-7-2007	10-7-2007	23	1
5	1500	0	green	12-7-2007	14-7-2007	1	0*
total	**3150**	**1650**				**31**	**3**

NV = *N. vitripennis*; NG = *N. giraulti*

* Two *N. vitripennis* have been recaptured at 2 km distance

Molecular analysis

In *N. vitripennis* the observed amount of genetic variation of the eight microsatellite markers was equal for the European and North American continent (Table 6.2). Between the sampling locations in New York, that were 2 and 100 km apart respectively no significant level of genetic differentiation was found (Mean F_{ST} = -0.01). However, we did find a considerable amount of differentiation between the two locations in New York and a population from Utah (~3000 km) with a mean F_{ST} = 0.10. Between the European mainland and Corsica we found a level of differentiation equal to that between Utah and New

York. The differentiation between the two continents is only slightly higher (see Table 6.3 for all F_{ST} values and geographic distances).

Table 6.2: Markers used and the number of alleles (No. alleles) and expected heterozygosity (H_T, Nei 1987), found in the North American (NA) and the European (EU) samples.

Marker	No. alleles NA	H_T NA	No. alleles EU	H_T EU	No. alleles total	GenBank accession #
NV104	11	0.872	9	0.891	12	FJ156110
NV109	14	0.779	14	0.920	20	FJ156114
NV111	16	0.909	15	0.947	25	FJ156115
NV114	15	0.724	11	0.913	20	FJ156231
NV300	4	0.552	3	0.464	4	FJ156211
NV308	9	0.643	5	0.800	11	FJ555533
NV313	13	0.862	9	0.711	13	FJ156221
NV316	5	0.680	6	0.730	7	FJ156228
average	10.88	0.753	9	0.797	14	

The isolation by distance analysis showed a significant correlation between genetic and geographic distance (linear regression, adjusted $R^2 = 0.679$, $F = 11.58$ on 1 and 4 df, $p = 0.027$). A comparison with the data of Grillenberger *et al.* (2008) on genetic differentiation of European populations shows good consistency between both studies (Table 6.3).

Table 6.3:Pairwise F_{ST} (after Cockerham & Weir 1993), p-value following *G*-statistics over all loci (as implemented in F-stat), Hedrick's G'_{ST} (Hedrick 2005) and approximate geographic distance between the populations within North America and Europe.

sampling groups	F_{ST}	p-value	G'_{ST}	Approximate distance in km
IthacaU1 – IthacaU2	-0.025	0.675	0	2
IthacaU1 – Brewerton	0.002	0.267	0.006	100
IthacaU2 – Brewerton	-0.013	0.233	0	100
IthacaU2 – Utah	0.089	**0.008** *	0.302	3000
IthacaU1 - Utah	0.083	0.016	0.317	3000
Brewerton – Utah	0.111	**0.008***	0.406	3000
Total NY - Utah	0.103	**0.05***	0.357	3000
EU mainland – Corsica	0.185	**0.05***	0.613	500
Total EU – North America	0.133	**0.05***	0.552	5000
Within EU **	0.035		0.23	300

* significant at nominal level of 0.05 after Bonferroni-correction for multiple comparisons; ** data from Grillenberger et al. (2008)

Discussion

The main goal of this paper was to find out how dispersal might influence population-level amounts of inbreeding, using both a local experimental approach and a more global population genetic approach.

Although the data from the recapture experiment are limited, they demonstrate that *N. vitripennis* females are able to disperse at least over a distance of two kilometres. As the wasps were released in high densities, the effect of the release method on dispersal has to be considered. The usual number of wasps emerging from a single host is around 20, and there are up to several tens of hosts parasitized in a single nest (Grillenberger *et al.* 2009, and unpublished data). As hosts are typically available for parasitation during a short period, several hundreds of wasps emerging from a single nest within a

short time span is not uncommon in nature. Therefore we consider it unlikely that the release situation triggered unusual dispersal behaviour. The wind records from an onsite weather station showed mostly westerly winds during the experiment, so the dispersal direction can be explained by wind drift. Small parasitoids are part of the aerial plankton and totally dependent on wind drift for dispersal, but for larger species, very little is known (Godfray 1994; Quicke 1997). Long distance dispersal with the help of wind currents seems to be common among fig wasps and distances of several tens of kilometres, even over open sea, are no major obstacle for dispersal (Harrison 2003; Zavodna *et al.* 2005). The parasitoid *Anagrus delicatus* has also been found to disperse over several kilometres (Antolin & Strong 1987). While these species seem to be mainly transported by the wind, *Leptopilina heterotoma* has been found to preferably fly against the wind (Papaj & Vet 1990). A study on *Cotesia flavipes* (Sallam *et al.* 2001) showed a rather high recapture rate (6.7%) in a 100 x 100 m plot, and a pronounced decline in recapture numbers towards the edges of the plot, indicating a low dispersal distance. Several studies on *Trichogramma spp.* (Kuske *et al.* 2003 and citations therein) found dispersal distances to a maximum of 400m from the release site, which was alsol wind assisted. This indicates that there are clear differences between parasitoid species in their dispersal behaviour. There seem to be two general strategies: (1) short distance dispersal with directed movement and (2) long distance dispersal with the help of wind and presumably followed by directed movement within a close range.

As expected, the *N. vitripennis* males that were released together with the females did not disperse and could still be found at the release site. *N. vitripennis* males have very short wings and are incapable of flight (Darling & Werren 1990). The evidence for long distance dispersal of *N. vitripennis* females is in line with the low genetic differentiation between the New York locations, where we found that gene flow is still possible within a range of 100 km. Also, given the high numbers of offspring emerging from a single host (~20, Grillenberger *et al.* in prep.), a high gene flow over relative long distances is conceivable (Zavodna *et al.* 2005). The concordance of the differentiation measured in European populations with the extrapolation of the North American data, suggests that *N. vitripennis* on both continents have a similar mode of dispersal.

We did not recapture any released *N. giraulti*. This could be attributed to the lower number of individuals released, or the use of laboratory hosts as bait. *N. giraulti* is believed to be specialist of Protocalliphora fly pupae (Darling & Werren 1990), however, the baits were filled with *Sarcophaga* pupa. Hence, it is conceivable that *N. giraulti* was not as attracted to the baits as the generalist *N. vitripennis*. Another reason could be the differences in flight capability. Lehmann and Heymann (2006) showed that *N. vitripennis* females are just able to hover in mid air, but their maximal flight performance does hardly exceed that level. *N. giraulti*, however seems to be able to produce more lift with its wings, and might hence be a better flyer and have left the study area completely.

The intercontinental comparison represents a maximum level for the F_{ST} as there is no gene flow between the continents. The high mutation rate in microsatellites bears the risk of homoplasy and the measured level of differentiation of long diverged lineages could as a result be masked due to saturation in variation (Nauta & Weising 1996). However, the observed high level of differentiation in the intercontinental comparison indicates that homoplasy is not an issue with the makers used in our study. The genetic differentiation between the island population on Corsica and the European mainland, as well as between Utah and New York, indicates that large bodies of water and mountain ranges are impassable barriers for *Nasonia*, as expected.

Taken together our data suggest that *N. vitripennis* is a long distance disperser that uses wind currents, resulting in high levels of gene flow across distances of 100 km. The implications for applying LMC theory to *Nasonia* are that the founding population of a local patch can be considered a random sample originating from a large area.

For a parasitoid such as *Nasonia,* that is exclusively dependent on a patchily distributed host, dispersal is the only way to find new opportunities for reproduction. In the release experiment, a large number of suitable hosts (~8% of the baits have been parasitized by naturally occurring unmarked wasps) were presented within a close range, but most of the released wasps did not use them, and recapture rates were low. Although we cannot exclude that our laboratory cultured wasps were less efficient in finding hosts, a more satisfying explanation is that females initially disperse a large distance to avoid inbreeding. This can be interpreted as an adaptive strategy to minimize the level

of genetic erosion given the characteristics of the life cycle of *N. vitripennis*. As already mentioned in the introduction, a high level of dispersal and the admixture of a large population leads to the maintenance of a large genetic variability that enables the population to react to stresses in a variable environment. Burton-Chellew *et al.* (2008) found that relatedness among the co-foundresses parasitizing a patch has no influence on the produced sex ratio, which is in contrast to LMC theory (Frank 1985b; Taylor & Crespi 1994; Greeff 1996; Frank 1998; Reece *et al.* 2004). However, if *N. vitripennis* generally disperse over long distances, the chance that closely related females meet each other in a patch is likely to be negligible. As such, there would be little selection on the recognition of close relatives among co-foundresses.

To give a more detailed answer to the question how far *Nasonia* females disperse after mating, a more refined study covering distances as large as 1000 km for a population genetic approach and about 2 km for a release experiment would be advisable. We believe that that the insights obtained from the present study will provide valuable background information to conduct such a study.

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Appendix

Supplementary material: List of samples used with location and sampling group for the population structure analysis. Cor = Corsica, EU = European mainland, UT = Utah, Brew = Brewerton, U1 = IthacaU1, U2 = IthacaU2.

Sample ID	location	sampling group
Cor01	Pirio, Corsica	Cor
Cor02	Pirio, Corsica	Cor
Cor03	Pirio, Corsica	Cor
Cor04	Pirio, Corsica	Cor
Cor05	Muro, Corsica	Cor
Cor06	Muro, Corsica	Cor
Cor07	Muro, Corsica	Cor
Cor08	Muro, Corsica	Cor
Cor09	Muro, Corsica	Cor
Cor10	Pirio, Corsica	Cor
Bay1	Bayreuth (Bavaria), Germany	EU
HH01	Hamburg, Germany	EU
HH03	Hamburg, Germany	EU
S004	Schlüchtern (Hessen), Germany	EU
ITA1	Montevarchi (Toscana), Italy	EU
AWD1	Amsterdam, Netherlands	EU
BU2000-2	Bussum, Netherlands	EU
FIG1	Valence, France	EU
SPA1	Spanderswoud, Netherlands	EU
CH02	Oftringen, Switzerland	EU
MON013	Huntsville, Utah	UT
MON014	Huntsville, Utah	UT
MON011	Huntsville, Utah	UT
Mon010	Huntsville, Utah	UT
MON012	Huntsville, Utah	UT
MON015	Huntsville, Utah	UT
PE020	Ogden, Utah	UT
SL010	Provo, Utah	UT
SL012	Provo, Utah	UT
SL011	Provo, Utah	UT

Sample ID	location	sampling group
A252	Brewerton, New York	Brew
A476	Brewerton, New York	Brew
A396	Brewerton, New York	Brew
A342	Brewerton, New York	Brew
A363	Brewerton, New York	Brew
A055	Brewerton, New York	Brew
A091	Brewerton, New York	Brew
A605	Brewerton, New York	Brew
A271	Brewerton, New York	Brew
A652	Brewerton, New York	Brew
A509	Brewerton, New York	Brew
A379	Brewerton, New York	Brew
A255	Brewerton, New York	Brew
A580	Brewerton, New York	Brew
A341	Brewerton, New York	Brew
A235	Ithaca, New York	U1
A238	Ithaca, New York	U1
A318	Ithaca, New York	U1
A336	Ithaca, New York	U1
B105	Ithaca, New York	U1
B110	Ithaca, New York	U1
B115	Ithaca, New York	U1
B128	Ithaca, New York	U1
B142	Ithaca, New York	U1
B155	Ithaca, New York	U1
B300	Ithaca, New York	U1
B311	Ithaca, New York	U1
B317	Ithaca, New York	U1
B731	Ithaca, New York	U1
A083	Ithaca, New York	U2
A086	Ithaca, New York	U2
A197	Ithaca, New York	U2
A227	Ithaca, New York	U2
A247	Ithaca, New York	U2

Sample ID	location	sampling group
B103	Ithaca, New York	U2
B109	Ithaca, New York	U2
B113	Ithaca, New York	U2
B114	Ithaca, New York	U2
B145	Ithaca, New York	U2
B191	Ithaca, New York	U2

Chapter 7

Summarizing Discussion

B.K. Grillenberger

The use of genetical methods in ecological research has offered new possibilities and lead to new insights (Lowe *et al.* 2004). Especially in research with small insects, the molecular approach gives the opportunity to study topics in the field that have so far been reserved to larger organisms (e.g. dispersal), or to the confined possibilities of the laboratory (e.g. resource allocation). While a growing amount of theory is based on primarily laboratory data of a limited number of organisms, tests of underlying assumptions under natural conditions have rarely been performed, yet. The advent of molecular tools for a larger array of organisms offers the possibilities to do so.

A prime example for missing information on ecology and natural behaviour of a tiny model organism is *Nasonia*. The aim of this thesis was to add knowledge about the field biology of *Nasonia*, which has so far mainly been used in laboratory studies and in only very few field studies (see **Chapter 1**). The main focus was on *Nasonia*'s population structure, phylogeography, reproductive strategies and dispersal abilities, to evaluate the underlying assumptions of models of adaptive behaviour. In the following I will try to merge the major findings of this thesis with previous knowledge into a more complete picture of the natural history of the *Nasonia* species complex. I will consider remaining ambiguities and open questions and point out future research topics.

A broader picture of the Nasonia *system*

The theory of reinforcement predicts that postzygotic isolation in the form of unviable hybrids, should favour selection on increased prezygotic isolation (Dobzhansky 1951). In *Nasonia*, postzygotic isolation is rather complete due to *Wolbachia* induced cytoplasmic incompatibility (Breeuwer & Werren 1990), and as there are differences in courtship behaviour, there is as well some

evidence for prezygotic isolation (van den Assem & Werren 1994; Beukeboom & van den Assem 2001). An interesting question is to what extent species divergence has progressed for other life history traits, such as sex allocation, host choice, overwintering strategies (diapause) etc.

In **Chapter 2** we showed with field data that most assumptions that are made in the recent LMC theory are fulfilled by *N. vitripennis*: local mating, random dispersal and asynchronous parasitism. Other assumptions of more basic models, such as equal clutch sizes, random mating among offspring within patches and synchronous parasitism are clearly violated. This shows that the success story of LMC research (Shuker & West 2004) is on the right track, but that there are still some poorly understood factors in this intensively studied system. When fitting the recent LMC models onto the data obtained in **Chapter 2**, we found in **Chapter 3**, that there are some factors included in these models that appear less relevant than previously thought (e.g. the total number of foundresses on a patch). Other factors seem to play a more important role (e.g. the relative clutch size of a parasitizing foundress compared to previous foundresses). The general message of this chapter is that females are limited in the cues they can obtain from their environment and these can differ from our expectations. The limited information poses boundaries to the adaptive response of the individual, as has also been acknowledged in more recent LMC research (Shuker & West 2004). A female might not have the total information on what has happened, and will happen in a patch that she is going to parasitize. This is especially true when a female *N. vitripennis* is confronted with the presence of a close relative, which I investigated in **Chapter 4**.

We found in North American field data that *N. vitripennis* does not adjust its sex ratio to conspecifics only, but reacts similarly when parasitizing hosts that are also parasitized by *N. giraulti*. This indicates that the two species have not diverged far enough yet in so far that a female is able to recognize the eggs of the competitor as different from conspecific eggs. Given that *N. vitripennis* has been found to recognize more diverged species' eggs as different (Wylie 1965; 1970), a foundress appears in principle capable of differentiating between hetero- and conspecific clutches. Our results can clearly be attributed to missing information on species identity of the eggs encountered during oviposition. From an evolutionary point of view the expectation is that an organism has

maximized the precision of adaptation. In the case of *N. vitripennis*, adaptation towards encountering a closely related species is not optimal. In the following I will discuss two hypotheses that might explain this observation.

We showed in **Chapter 6** that *N. vitripennis* is a long distance disperser that can cover at least 2 km, and that populations as far apart as 100 km are still little differentiated. This explains why we did not find differentiation on smaller scale (**Chapter 2**), and helps to demarcate what a population is in *Nasonia*. The high dispersal distance implicates the admixture of a large population that might cover areas of sympatry and allopatry of *N. vitripennis* and *N. giraulti* within North America. This high rate of admixture and therefore potential gene flow from allopatric into sympatric areas could explain the inability of *N. vitripennis* females to recognize eggs of *N. giraulti* as being different. In this scenario selection only acts in the sympatric area, but the population's adaptation is prevented or at least slowed down by gene flow out of the larger allopatric zone (Bridle & Vines 2007). However, this is only the case, when the alleles leading to recognition of the other species are selected against in the allopatric area, otherwise the capability of species recognition would spread through the whole population (Sanderson 1989). Whether the ability to recognize eggs of a heterospecific as being different comes with a fitness disadvantage when there is no closely related competitor present, has not been investigated yet. However, given that selection is only favouring species recognition in the sympatric area, it is conceivable that *N. vitripennis* cannot adapt to the regular encounters with *N. giraulti* and is therefore not able to evolve recognition of the species differences. We could show in **Chapter 4** that there is a high proportion of multiparasitism (*N. vitripennis* and *N. giraulti* parasitizing the same host) among hosts of nests that contain both species, which indicates that the selection pressure on species recognition should be high in these nests. However, to generally evaluate the strength of selection on species recognition in nature, more detailed data on the distribution of both species in North America is required. Especially degrees of sympatry and allopatry of the two species need to be determined in more detail. Another interesting line of further research is to screen for natural variation in species recognition and to artificially select for it.

Another assumption in *Nasonia* research was that all three species originate from North America. However, this has never been investigated thoroughly. In **Chapter 5** we found indication that the North American *N. vitripennis* population is much younger than the European population. This raises the question whether there was sympatric speciation of all three species in North America, or whether both *N. longicornis* and *N. giraulti* evolved in North America while *N. vitripennis* is of Eurasian origin and invaded the New World more recently. So far, the differences in *Wolbachia* infections among the three *Nasonia* species were thought to be the driving force for the speciation process. However, the fact that the distribution ranges of *N. longicornis* and *N. giraulti* do not overlap, and that *N. vitripennis* could have its origin outside of North America, poses doubt to the hypothesis that the differences in *Wolbachia* infection are cause, and not consequence of the speciation.

The immigration scenario would as well fit the results that *N. vitripennis* is not recognizing *N. giraulti* as being different when parasitizing a host, as well as the high levels of diapause of *N. vitripennis* in North America compared to European populations, and to *N. giraulti* in the same location (**Chapter 4**). It is conceivable that there was no selection on recognizing a closely related competitor as being different, in the original (allopatric) habitat (e.g. Europe). Missing adaptations of *N. vitripennis* towards encountering *N. giraulti* when parasitizing a host can be interpreted as evidence for the non-American origin of this cosmopolitan species. As for diapause, one argument accounting for the differences between the two species is that unknown factors play a role in a foundress' decision to produce diapause. Future research has to show which factors are influencing diapause production in *Nasonia*. Seen in the light of a recent immigration of *N. vitripennis* to North America, the diapause production of *N. vitripennis* in North America could be high, because the environmental cues that announce the advent of winter might be different between North America and the region of origin. For both observations, missing species recognition and high diapause production, it is conceivable that due to a rather short time of *N. vitripennis* living in North America, in combination with the high gene flow over large areas, selection might not have had enough time to shape the response of the immigrant species to the conditions in its new habitat. Another possible reason for the missing adaptation of *N. vitripennis* to the new

environment in North America could be the reduced genetic variation after the bottleneck that accompanied the founder event during the colonization of the New World. It is thought that reduced genetic variation decreases the adaptive potential of a population (Baker 1965), but it is still unclear how strong the reduction of adaptive potential after a bottleneck event really is (Dlugosch & Parker 2008). To further evaluate the validity of these hypotheses more data on the factors influencing sex allocation as well as diapause and the underlying genetics are required. Further research also has to reveal how quickly selection can act on these traits and how much variation there is in the cues that are used within and between populations from various latitudes.

Taken together I consider the most probable hypothesis of speciation in the *Nasonia* system as follows: the two sister species *N. giraulti* and *N. longicornis* developed independently from *N. vitripennis* in North America, while the *N. vitripennis* is an Eurasian species that spread to the New World more recently. The consequences of this rather recent event are the low precision of adaptation towards the local climate (diapause production) and towards the presence of a close relative in the habitat (sex ratio adaptation). The high gene flow over large distances and the presumably reduced adaptive potential after the founder event prevented a rapid adaptation.

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Summary

To gain a thorough understanding of the evolutionary processes that lead to the enormous biodiversity in nature, evolutionary biology tries to develop models that describe the essence of the underlying process. These models are getting increasingly complex and are relying on a growing set of assumptions. To be able to gather enough knowledge on a biological system to accommodate and validate a model, evolutionary biologists rely on the study of model organisms. The jewel wasps of the genus *Nasonia* has become a model organism in evolutionary biology for a variety of behavioural, developmental and genetic studies. A particular fruitful field of research has been sex allocation, which is one of the best understood adaptive behaviours.

The genus *Nasonia* consists of three closely related species: *N. vitripennis* which is cosmopolitan, *N. giraulti* which has only been found in north-eastern North America and *N. longicornis* which is endemic to north-western North America. As *N. vitripennis* can be found in sympatry as well as in allopatry with both its sister species and its distribution encompasses multiple environmental conditions, the system offers unique opportunities to study the validity of models on speciation and adaptation. The ability of *Nasonia* to influence its offspring's sex ratio and to induce diapause in its offspring under unfavourable conditions, are two adaptive traits that are in particular being considered in this study. (**Chapter 1**)

The goal of the research described in this thesis was to evaluate the underlying assumptions of models describing adaptive behaviour (sex allocation and diapause). I aimed to acquire information on the adequacy of adaptation in a natural environment and to gain a better understanding of the multiple selective forces that shape life history traits. The results of this study will help to place the many results of theoretical and laboratory studies into the biological reality of a natural environment to identify remaining questions about parasitoid life history evolution.

Nasonia has been found to follow quite closely the predictions of local mate competition (LMC) theory in the laboratory. LMC theory predicts that an

organism should produce a strongly female biased sex ratio, when its offspring is competing for mates among each other. However, LMC theory depends on the validity of many assumptions about the population genetic structure, and it is unknown to what extent these are met in nature. In **Chapter 2** I evaluated some essential assumptions made in sex ratio theory on data from two European *N. vitripennis* field populations. In particular I investigated the genetic population structure, foundress number per patch, parasitation sequence and clutch sizes. I found that most assumptions made in recent LMC theory are fulfilled by *N. vitripennis*: local mating, random dispersal of females and asynchronous parasitism. My research showed that other assumptions of more basic models, such as equal clutch sizes, random mating among offspring within patches and synchronous parasitism are clearly violated and therefore rightfully adjusted in the recent theory.

Subsequently, I investigated in **Chapter 3** to what extent the predictions made by LMC theory are matching the sex ratios observed in the European populations and what the strengths and weaknesses of present models are. I showed that some factors included in these models are less relevant than thought before (e.g. the total number of females parasitizing a patch), whereas other factors play a more important role (e.g. the relative clutch size of a parasitizing female compared to earlier females). The general message of this chapter is that females are limited by the cues they can obtain from their environment, and these can be different from what the researcher expects. A female might not have total information on what has happened and will happen in a patch that she is going to parasitize. The limited information poses boundaries to the adaptive response of the individual.

Whereas in Europe *N. vitripennis* has no closely related competitors, the situation in North America is more complex. Given that *N. vitripennis* and *N. giraulti* are living in close sympatry in parts of North America, the question arises whether traits have evolved to avoid hybridization between the species. While it has been found that there are clear differences in courtship behaviour, it is still unknown whether there are also adaptations with respect to LMC. In **Chapter 4** I investigated the reproductive strategies of *Nasonia* in a two species situation, regarding the sex ratio adjustment as well as diapause production, focusing on how well *N. vitripennis* is adapted to the competition with its close

relative *N. giraulti*. I found that *N. vitripennis* does not adjust its sex ratio to conspecifics only, but responds to host parasitation by *N. giraulti* as if encountering conspecific clutches. This indicates that the two species have not diverged far enough yet in so far that a female is able to recognize the parasitation event of the competitor as different from conspecific competitors. Given that *N. vitripennis* has been found to recognize further diverged species' eggs as different, a foundress may in principle be capable of differentiating between hetero- and conspecific clutches. Furthermore, I found a higher level of diapause production of *N. vitripennis* in North America compared to European populations, and to *N. giraulti* in the same location. This can either indicate that there are species specific differences in the factors, which play a role in a foundress' decision to produce diapause, or it can be interpreted as an imprecise adaptation to the local environment.

In order to evaluate how far adaptation of *N. vitripennis* to the competitive situation in North America might have progressed, information is required on the population history of *Nasonia* in North America. So far, it has been assumed that the cosmopolitan species *N. vitripennis* has its origin in North America, as that seems to be the hot spot of diversity within the genus *Nasonia*. In **Chapter 5** I tested the hypotheses whether *N. vitripennis* originates from North America, or from outside the New World. Using a combination of mtDNA sequences, nuclear microsatellites and *Wolbachia* sequences, I compared the genetic variability among North American and European samples. I found evidence that the North American *N. vitripennis* population is much younger than the European population, which places the species origin into the New World. This raises the question whether there was sympatric speciation of all three species in North America, or whether only *N. longicornis* and *N. giraulti* evolved in North America and *N. vitripennis* is of Eurasian origin and invaded the New World more recently. So far, the differences in *Wolbachia* infections among the three *Nasonia* species were thought to be the driving force for the speciation process. However, as the distribution ranges of *N. longicornis* and *N. giraulti* do not overlap, and given that *N. vitripennis* has its origin outside of North America, it is conceivable that the differences in *Wolbachia* infection are not cause, but consequence of the speciation.

A prerequisite of local adaptation is that there is only limited gene flow between areas with and without selection pressure on the adaptive trait. *N. vitripennis* might have evolved adaptations to the presence of its sister species *N. giraulti* and *N. longicornis* in North America. However, as there are large areas in North America where *N. vitripennis* occurs allopatrically, without selection for competition with a close relative, the question arises whether gene flow can prevent local adaptation in the sympatric areas. In this context I investigated in **Chapter 6** the dispersal capabilities of *N. vitripennis* on a local scale with a mark release recapture experiment as well as on a larger scale with molecular markers. I found that *N. vitripennis* is a long distance disperser that can easily cover more than 2 km, and that populations as far apart as 100 km are still hardly differentiated. This provides an explanation for why previous studies did not find differentiation on a smaller scale, and helps to determine what a population is in *Nasonia*. The high level of admixture of a large population might counteract selection for local adaptation, as selected and unselected sub-populations constantly exchange genetic material.

In the final **Chapter 7** I merged the results of the previous chapters and sketched the current knowledge of the population structure and history of *N. vitripennis* in Europe and North America. The results of the previous chapters made me believe that the most probable hypothesis of speciation in the genus *Nasonia* is the following: the two sister species *N. giraulti* and *N. longicornis* developed independent from *N. vitripennis* in North America, while the latter is an Eurasian species that spread to the New World more recently. The consequence of this rather recent event is the imprecise adaptation towards the local climate (diapause production) and towards the presence of a close relative in the habitat (sex ratio adaptation). The high gene flow over large distances and the presumably reduced adaptive potential after the founder event prevented a rapid adaptation.

Samenvatting

Om een duidelijk inzicht te krijgen in de evolutionaire krachten die tot de enorme biodiversiteit in de natuur leiden, probeert de evolutionaire biologie modellen te ontwikkelen die de essentie van de onderliggende processen beschrijven. Deze modellen worden steeds complexer en zijn op een groeiend aantal aannames gebaseerd. Om voldoende kennis te verzamelen over een biologisch systeem en daarvoor een model op te kunnen zetten en te testen, zijn evolutionair-biologen aangewezen op onderzoek naar model organismen. De juweelwespen uit het genus *Nasonia* zijn in de evolutionaire biologie tot model organismen geworden voor een verscheidenheid aan gedrags-, ontwikkelings- en genetisch onderzoek. Een bijzonder vruchtbaar onderzoeksgebied is seks allocatie - de investering in vrouwelijk of mannelijk nageslacht - één van de best begrepen vormen van adaptief gedrag.

Het genus *Nasonia* bestaat uit drie nauwverwante soorten: *N. vitripennis* die kosmopolitisch is, *N giraulti* die alleen in noordoostelijk Noord-Amerika gevonden is en *N. longicornis* die endemisch is in noordwestelijk Noord-Amerika. Aangezien *N. vitripennis* zowel in sympatrie als in allopatrie met de beide zustersoorten voorkomt en het verspreidingsgebied een grote verscheidenheid aan omgevingsfactoren herbergt, biedt het systeem unieke kansen om de validiteit van soortvormings- en adaptatiemodellen te bestuderen. Het vermogen van *Nasonia* om de geslachtsverhouding van de nakomelingen te beïnvloeden en onder ongunstige omstandigheden diapauze te induceren, zijn twee adaptieve eigenschappen die in in dit proefschrift het bijzonder worden onderzocht (**Hoofdstuk 1**).

Het doel van het onderzoek zoals beschreven in dit proefschrift, was het evalueren van de onderliggende aannames van modellen die adaptief gedrag beschrijven (seks allocatie en diapauze) Ik heb gegevens verzameld over het aanpassingsvermogen in een natuurlijke omgeving om een beter begrip te krijgen van de verschillende selectieve krachten die de levensgeschiedenis (life history) eigenschappen vormgeven. De resultaten van deze studie helpen uitkomsten van theoretisch en laboratorium onderzoek te interpreteren en aan de

natuurlijke situatie te spiegelen. Voorts heeft het geleid tot het identificeren van resterende vragen betreffende de evolutie van de levensgeschiedenis van parasitoïden.

Er is aangetoond dat *Nasonia* de voorspellingen van de lokale partnerconcurrentie (local mate competition, LMC) theorie in het laboratorium vrij precies volgt. De LMC theorie voorspelt dat een organisme een sterk vrouwelijk gerichte geslachtsverhouding (seks ratio) zal produceren wanneer haar nageslacht onderling voor partners concurreert. De LMC hangt echter af van de betrouwbaarheid van veel aannames over de genetische populatiestructuur, en het is niet bekend in hoeverre deze in de natuur van toepassing zijn. In **Hoofdstuk 2** evalueer ik enkele essentiële aannames die gemaakt worden in de seks ratio theorie aan de hand van twee Europese *N. vitripennis* veldpopulaties. In het bijzonder heb ik de genetische populatiestructuur, het aantal stichtsters per voortplantingslocatie, de volgorde van parasitering en de broedselgrootte onderzocht. Ik vond dat *N. vitripennis* voldoet aan de meeste aannames die gemaakt worden in recente LMC theorieën: lokale paringen, willekeurige verspreiding van vrouwtjes en asynchroon parasitisme. Mijn onderzoek liet verder zien dat aan een aantal andere aannames die gemaakt worden in meer basale modellen, zoals gelijke broedselgroottes, willekeurige paringen tussen nageslacht op één plek en synchroon parasitisme, duidelijk niet wordt voldaan en dat deze aannames terecht zijn bijgesteld in recente theorieën.

Vervolgens onderzoek ik in **Hoofdstuk 3** in hoeverre de voorspellingen die de LMC theorie maakt passen bij de geslachtsverhoudingen die gevonden worden in Europese populaties. Ik laat zien dat sommige factoren die de modellen bevatten minder relevant zijn dan voorheen gedacht (zoals het totale aantal vrouwtjes dat op een locatie parasiteert) terwijl andere factoren juist een belangrijkere rol spelen (zoals de relatieve broedselgrootte van een parasiterend vrouwtje vergeleken met die van eerder leggende vrouwtjes). De algemene strekking van dit hoofdstuk is dat vrouwtjes slechts op een beperkt aantal signalen uit de omgeving kunnen reageren, en dat deze signalen kunnen afwijken van wat de onderzoeker verwacht. Een vrouwtje heeft mogelijk geen toegang tot alle informatie over wat er gebeurd is, en wat er gaat gebeuren, op

de plek waar zij gaat parasiteren. Deze beperking van informatie stelt grenzen aan het aanpassingsvermogen van het individu.

Terwijl *N. vitripennis* in Europa geen competitie ondervindt van nauwverwante soorten, omdat deze in Europa niet voorkomen, ligt dat in Noord-Amerika ingewikkelder. Gegeven dat *N. vitripennis* en *N. giraulti* in delen van Noord-Amerika in een hoge mate van sympatrie voorkomen, zou men verwachten dat er eigenschappen zijn geëvolueerd om hybridisatie tussen beide soorten te voorkomen. De vraag is nu of deze evolutie inderdaad is opgetreden. Hoewel er al duidelijke verschillen in baltsgedrag tussen de beide soorten bekend zijn, is het nog niet bekend of de soorten op het gebied van LMC ook aan elkaar zijn aangepast. In **Hoofdstuk 4** onderzoek ik de voortplantingsstrategieën van *Nasonia* in een omgeving waar beide soorten parasiteren. Hierbij kijk ik naar zowel geslachtsverhouding, legselgroote, alsmede de productie van diapauze larven. Hoe goed is *N. vitripennis* aangepast is aan de competitie met haar nauwe verwant *N. giraulti*? Ik heb gevonden dat *N. vitripennis* niet alleen haar geproduceerde geslachtsverhouding aan soortgenoten aanpast, maar ook reageert op het eerder parasiteren van een gastheer door *N. giraulti.* Hierbij gedraagt zij zich alsof de broedsels die ze tegenkomt, broedsels van een soortgenote zijn. Dit duidt erop dat de twee soorten nog niet ver genoeg gedivergeerd zijn, althans in zoverre dat een vrouwtje het parasiteren door een concurrent van de andere soort niet kan onderscheiden van het parasiteren door een concurrent van de eigen soort. Aangezien van *N. vitripennis* bekend is dat zij parasiteringen van andere, minder verwante soorten wel kan herkennen als niet soort-eigen, zou een vrouwtje in principe in staat moeten zijn om parasiteringen van de eigen soort van die van een andere *Nasonia* soort te onderscheiden. Verder vond ik dat *N. vitripennis* in Noord-Amerika een hogere productie van diapauze larven heeft in vergelijking tot *N. vitripennis* in Europese populaties en tot *N. giraulti* op dezelfde locatie. Dit kan er enerzijds op duiden dat er soortspecifieke verschillen zijn in de factoren die een rol spelen bij de beslissing van een vrouwtje om diapauze larven te produceren, of anderzijds dat de aanpassing aan de lokale omstandigheden onnauwkeurig is.

Om te evalueren hoever de aanpassing van *N. vitripennis* aan de competitieve situatie in Noord-Amerika al gevorderd is, is er meer informatie nodig over de

geschiedenis van de populatie van *Nasonia* in Noord-Amerika. Tot dusverre werd aangenomen dat de oorsprong van de wereldwijd verspreide soort *N. vitripennis* in Noord-Amerika ligt, aangezien daar de grootste soortenrijkdom in het geslacht *Nasonia* lijkt te zijn. In **Hoofdstuk 5** test ik de hypothese dat *N. vitripennis* van oorsprong uit Noord-Amerika komt en niet van buiten de Nieuwe Wereld . Met behulp van een combinatie van mtDNA sequenties, microsatellieten voor kern DNA en *Wolbachia* sequenties, heb ik de genetische variatie vergeleken tussen Noord Amerika en Europa. Ik vond bewijs dat de Noord-Amerikaanse *N. vitripennis* populatie veel jonger is dan de Europese populatie. Dit doet de vraag rijzen of er eigenlijk wel sympatrische soortsvorming van alledrie de soorten is opgetreden in Noord-Amerika, of dat alleen *N. giraulti* en *N. longicornis* geëvolueerd zijn in Noord-Amerika, terwijl *N. vitripennis* van oorsprong Euraziatisch is en zich meer recent heeft gevestigd in de Nieuwe Wereld. Tot op heden werd gedacht dat de verschillen in *Wolbachia* infecties tussen de drie *Nasonia* soorten de drijvende kracht voor het proces van soortsvorming zijn. Echter, aangezien de verspreidingsgebieden van *N. longicornis* en *N. giraulti* elkaar niet overlappen, en gegeven dat de oorsprong van *N. vitripennis* buiten Noord Amerika ligt, zou het ook goed kunnen zijn dat de verschillen tussen *Wolbachia* infecties niet de oorzaak, maar juist het gevolg zijn van de soortvorming.

Een vereiste voor lokale aanpassing is dat er slechts beperkte uitwisseling van genen is tussen gebieden met en zonder selectiedruk op een bepaalde eigenschap. In Noord Amerika zouden er in *N. vitripennis* aanpassingen hebben kunnen evolueren als respons op de aanwezigheid van haar zustersoorten *N. giraulti* en *N. longicornis*. Aangezien er grote delen in Noord-Amerika zijn waar *N. vitripennis* allopatrisch, zonder selectie voor competitie met een nauw verwante soort, voorkomt, is het de vraag of die uitwisseling van genetisch materiaal lokale aanpassing in sympatrische gebieden zou kunnen voorkomen. In deze context onderzoek ik in **Hoofdstuk 6** het verspreidingsvermogen van *N. vitripennis* op lokale schaal met een markeer-terugvangst (mark-recapture) experiment en tevens de verspreiding op grotere schaal met behulp van moleculaire markers. Ik vond dat *N. vitripennis* zich verspreidt over grote afstanden, tot wel 2 kilometer en dat populaties, die zelfs 100 kilometer uit elkaar liggen, genetisch nauwelijks van elkaar gedifferentieerd zijn. Dit geeft

een verklaring waarom eerdere studies geen differentiatie vonden op kleine schaal en het helpt te bepalen wat als een populatie moet worden beschouwd in *Nasonia*. De hoge mate van vermenging in een grote populatie zou selectie voor lokale aanpassing kunnen tegengaan, aangezien sub-populaties die wel en subpopulaties die niet onder selectiedruk staan continu genetisch materiaal uitwisselen.

In het laatste **Hoofstuk 7** voeg ik de resultaten uit de voorgaande hoofdstukken samen en aan de hand daarvan schets ik de huidige kennis van de populatiestructuur en -geschiedenis van *N. vitripennis* in Europa en Noord Amerika. De resultaten van de voorgaande hoofdstukken doen me geloven dat de meest aannemelijke hypothese voor soortvorming in het geslacht *Nasonia* de volgende is: de twee zustersoorten *N. giraulti* en *N. longicornis* hebben zich onafhankelijk van *N. vitripennis* in Noord-Amerika ontwikkeld, terwijl *N. vitripennis* een Euraziatische soort is die zich meer recentelijk naar de Nieuwe Wereld heeft verspreid. De consequentie van deze recente gebiedsuitbreiding, is een onnauwkeurige aanpassing aan het lokale klimaat (productie van diapauze larven) en aan de aanwezigheid van nauwverwante soorten in de habitat (seks allocatie) in Noord-Amerika. De hoge mate van uitwisseling van genen over grote afstanden en de verminderde genetische variatie om zich aan te kunnen passen na vestiging, hebben snelle aanpassing tegengehouden.

Zusammenfassung

Um die evolutionären Prozesse, die zu der enormen Artenvielfalt in der Natur geführt haben, eingehend zu verstehen, versucht die Evolutionsbiologie Modelle zu entwickeln, die den Kern des zugrunde liegenden Vorgangs beschreiben. Diese Modelle werden zunehmend komplexer und bauen auf mehr und mehr Annahmen auf. Um so viel Wissen über ein biologisches System sammeln zu können, dass ein Modell angepasst und beurteilt werden kann, konzentrieren sich Evolutionsbiologen auf die Untersuchung von Modellorganismen. Die parasitoiden Wespen der Gattung *Nasonia* wurden zu einem Modellorganismus der Evolutionsbiologie in einer Reihe von verhaltens- und entwicklungsbiologischen, sowie genetischen Studien. Ein besonders erfolgreiches Forschungsgebiet war „sex allocation theory“ (Theorie über die Investition in die jeweiligen Geschlechter), eines der am besten verstandenen adaptiven Verhaltensforschungsgebiete.

Die Gattung *Nasonia* besteht aus drei eng verwandten Arten: die kosmopolitische *N. vitripennis*, *N. giraulti*, die nur im Nordosten Nordamerikas gefunden werden kann und *N. longicornis*, die endemisch für den Nordwesten Nordamerikas ist. Da *N. vitripennis* sowohl sympatrisch als auch allopatrisch mit ihren beiden Schwesterarten vorkommt, und ihre Verbreitung verschiedene Umweltbedingungen umfasst, bietet das System einmalige Möglichkeiten, die Gültigkeit von Modellen zu Artbildung und Anpassung zu untersuchen. Die Fähigkeit von *Nasonia* das Geschlecht ihrer Nachkommen zu beeinflussen, und unter widrigen Bedingungen eine Diapause (= Ruhezustand) in den Nachkommen auszulösen, sind zwei adaptive Fähigkeiten die in der vorliegenden Studie eine wichtige Rolle spielen. (**Kapitel 1**)

Das Ziel der Untersuchungen, die in dieser Arbeit beschrieben werden, war, die zugrunde liegenden Annahmen von Modellen zu beurteilen, die adaptives Verhalten (sex allocation und Diapause) beschreiben. Ziel war es, Informationen über die Zulänglichkeit von Anpassungen in einer natürlichen Umwelt zu sammeln, um ein besseres Verständnis der vielen selektiven Kräfte zu erlangen, die den Lebenszyklus eines Organismus formen. Die Resultate

dieser Studie werden helfen, die vielen Ergebnisse aus theoretischen Studien und Laborstudien in die biologische Realität einer natürlichen Umwelt zu setzen, und die noch ausstehenden Fragen über die Evolution der Lebenszyklen von Parasitoiden aufzuzeigen.

Es wurde gezeigt, dass *Nasonia* im Laborversuch den Vorhersagen der „local mate competition" (LMC) (= lokale Paarungskonkurrenz) ziemlich genau entspricht. Die LMC Theorie besagt, dass ein Organismus ein zu Gunsten der Weibchen verschobenes Geschlechterverhältnis produzieren sollte, wenn seine Nachkommen untereinander in Paarungskonkurrenz stehen. Allerdings ist die LMC Theorie auf der Richtigkeit vieler Annahmen über die Populationsstruktur angewiesen, und es ist noch nicht bekannt, inwieweit diese mit der Realität zu vereinbaren sind. In **Kapitel 2** überprüfte ich einige grundlegende Annahmen aus der LMC Theorie an Hand von Daten von zwei europäischen *N. vitripennis* Feldpopulationen. Um genauer zu sein, untersuchte ich die genetische Populationsstruktur, die Anzahl der Gründerinnen pro Revier, die Reihenfolge der Parasitierung und die Gelegegröße. Ich fand heraus, dass die meisten Annahmen, die in der neueren LMC Theorie gemacht werden, durch *N. vitripennis* erfüllt werden: lokale Paarung, zufällige Ausbreitung der Weibchen und asynchrone Parasitierung, während andere Annahmen von einfacheren Modellen wie gleiche Gelegegröße, zufällige Paarung zwischen den Nachkommen eines Reviers und synchrone Parasitierung eindeutig gebrochen werden, und damit zu Recht in den neueren Theorien angepasst wurden.

Anschließend untersuchte ich in **Kapitel 3** inwieweit die Vorhersagen aus der LMC Theorie mit dem Geschlechterverhältnis übereinstimmen, das in den europäischen Populationen gefunden wurde, und was die Stärken und Schwächen der aktuellen Modelle sind. Ich konnte zeigen, dass einige Parameter, die in diesen Modellen inbegriffen sind, weniger wichtig sind als zuvor angenommen (z.B. die Gesamtzahl der Weibchen die ein Revier parasitieren), während andere Parameter eine wichtigere Rolle spielen (z.B. die relative Gelegegröße eines parasitierenden Weibchens im Vergleich zu früheren Weibchen). Die Hauptaussage dieses Kapitels ist, dass Weibchen durch die Informationen, die sie von ihrer Umwelt bekommen, beschränkt werden, und sich dadurch von den Erwartungen der Wissenschaftler unterscheiden können. Ein Weibchen hat wahrscheinlich nicht die allumfassende Information was, in

einem Revier, das sie parasitieren wird, passiert ist und passieren wird. Diese beschränkte Information setzt Grenzen für die Anpassung eines Individuums.

Während *N. vitripennis* in Europa keine nahverwandten Konkurrenten hat, ist die Situation in Nordamerika komplizierter. In Anbetracht der Tatsache, dass *N. vitripennis* und *N. giraulti* in Teilen Nordamerikas in enger Sympatrie vorkommen, stellt sich die Frage, ob sich Eigenschaften so entwickelt haben, dass eine Hybridisierung der Arten vermieden wird. Während klare Unterschiede im Paarungsverhalten dokumentiert sind, ist noch unbekannt, ob auch Anpassungen in Bezug auf LMC bestehen. In **Kapitel 4** untersuchte ich die Fortpflanzungsstrategien von *Nasonia* in einer Zwei-Arten-Situation, mit Bezug auf die „sex allocation" sowie Diapause Induzierung. Dabei lag der Schwerpunkt auf der Stärke der Anpassung von *N. vitripennis* an die Konkurrenz mit der eng Verwandten Art *N. giraulti.* Ich habe festgestellt, dass *N. vitripennis* das Geschlechterverhältnis nicht nur in Gegenwart von Gleichartigen, sondern auch bei Wirtsparasitierung von *N. giraulti* so reagiert als wäre es die eigene Art. Dies weist darauf hin, dass die Trennung der Arten noch nicht soweit fortgeschritten ist, dass ein Weibchen in der Lage ist, die Parasitierung einer andersartigen Konkurrentin von der einer gleichartigen unterscheiden zu können. Im Angesicht der Beobachtung, dass *N. vitripennis* in der Lage ist, Eier einer entfernter verwandten Art als anders zu erkennen, erscheint klar, dass eine Gründerin im Prinzip in der Lage ist zwischen andersartigen und gleichartigen Gelegen zu unterscheiden. Des weiteren fand ich ein deutlich höheres Niveau an Diapause Produktion in *N. vitripennis* Populationen aus Nordamerika im Vergleich mit *N. giraulti* vom gleichen Standort und europäischen *N. vitripennis* Populationen. Dies kann entweder darauf hindeuten, dass es artspezifische Unterschiede bei den Faktoren gibt, die bei der Entscheidung einer Gründerin Diapause Eier zu legen eine Rolle spielen, oder es kann als eine ungenaue Anpassung an die Umwelt interpretiert werden.

Um zu erörtern, wieweit die Anpassung von *N. vitripennis* an die Konkurrenzsituation in Nordamerika fortgeschritten ist, sind Informationen über die Populationsgeschichte von *Nasonia* in Nordamerika erforderlich. Bislang wurde angenommen, dass die Art *N. vitripennis* ihren Ursprung in Nordamerika hat, da dort der „Hotspot" des Artenreichtums innerhalb der Gattung *Nasonia* liegt. In **Kapitel 5** überprüfte ich die Hypothesen, ob *N. vitripennis* aus

Nordamerika, oder von außerhalb der Neuen Welt stammt. Mit Hilfe einer Kombination aus mitochondrialer DNS Sequenzen und nukleären Mikrosatelliten verglich ich die genetische Variabilität innerhalb nordamerikanischer und europäischer Populationen. Ich fand Hinweise darauf, dass die nordamerikanische Population viel jünger ist als die europäische, was für eine europäische Herkunft der Art spricht. Dieses Ergebnis wirft die Frage auf, ob es eine sympatrische Artenbildung in Nordamerika gab, oder ob *N. vitripennis* eurasischen Ursprungs ist und erst später in die Neue Welt eingewandert ist und *N. longicornis* und *N. giraulti* in Nord Amerika entstanden sind. Bisher wurde angenommen, dass die Unterschiede in der *Wolbachia* Infektion zwischen den drei *Nasonia* Arten die treibende Kraft für den Artbildungsprozess waren. Da sich allerdings die Verbreitungsgebiete von *N. giraulti* und *N. longicornis* nicht überlappen, und unter der Annahme, dass *N. vitripennis* seinen Ursprung außerhalb Nordamerikas hat, ist es denkbar, dass die Unterschiede in der *Wolbachia* Infektion nicht Ursache, sondern die Konsequenz der Artentrennung sind.

Eine Grundvoraussetzung für lokale Anpassung ist, ein eingeschränkter Genfluss zwischen Gebieten mit und ohne Selektionsdruck auf eine bestimmte adaptive Eigenschaft. *N. vitripennis* könnte Anpassungen an die Gegenwart seiner Schwesterarten *N. longicornis* und *N. giraulti* in Nordamerika evolviert haben. Da es allerdings in Nordamerika große Gebiete gibt, in denen *N. vitripennis* allopatrisch vorkommt, also ohne Konkurrenz mit einer nah verwandten Art, stellt sich die Frage ob Genfluss die lokale Anpassung in den sympatrischen Gebieten verhindern konnte. In diesem Zusammenhang untersuchte ich in **Kapitel 6** die Ausbreitungsfähigkeiten von *N. vitripennis* auf lokaler Ebene mit einem „mark-release-recapture“ Versuch und auf globaler Ebene mit molekularen Markern. Es stellte sich heraus, dass *N. vitripennis* eine Langstreckenverbreiterin ist, die ohne Probleme mehr als 2km zurücklegt, und dass selbst Populationen die 100km voneinander entfernt liegen, kaum differenziert sind. Das erklärt auch,warum frühere Studien auf kleinerer Ebene keine Differenzierung gefunden haben, und hilft die Dimension einer *Nasonia* Population zu definieren. Der hohe Austausch innerhalb einer großen Population könnte der lokalen Anpassung entgegenwirken, da selektierte und nicht-selektierte Teilpopulationen ständig genetisches Material austauschen.

Im abschließenden **Kapitel 7** habe ich die Ergebnisse der vorhergehenden Kapitel zusammengefasst und das gegenwärtige Wissen über die populationsgenetische Struktur und Geschichte von *N. vitripennis* in Europa und Nordamerika umrissen.Die Ergebnisse der vorhergehenden Kapitel deuten meiner Meinung nach darauf hin, dass die wahrscheinlichste Hypothese zur Artbildung in der Gattung *Nasonia* die folgende ist: Die zwei Schwesterarten *N. longicornis* und *N. giraulti* entwickelten sich unabhängig von *N. vitripennis* in Nordamerika, während letztere eine eurasische Art ist, welche erst in jüngerer Zeit die Neue Welt eroberte. Die Folge dieses eher jungen Ereignisses ist die ungenaue Anpassung an das lokale Klima (→ Diapauseproduktion) und an die Gegenwart eines nahen Verwandten im Habitat (→ sex allocation). Der hohe Genfluss und die wahrscheinlich geringe genetische Variabilität nach der Kolonisation verhinderten eine schnelle Anpassung.

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Thank you all !!!

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Curriculum Vitae

Bernd Klaus Grillenberger was born on 31st of January 1979 in Nürnberg, Germany. He started his studies of biology at the Friedrich-Alexander University in Erlangen in 1998. In 2000 he spent four months for a field course in Australia to make behavioural observations on Rock Wallabies. After that experience he decided to focus on behavioural- and socio- biology and moved to the Julius-Maximilian University in Würzburg 2001 where he did his Master thesis on "Consequences of intra- and interspecific raiding on the population structure of the honey ant species *Myrmecocystus mimicus* and *M. depilis*" under the supervision of Jürgen Gadau and Bert Hölldobler. During this project he got first contact with Hymenoptera and molecular population genetic techniques. He finished his Master in 2004. The same year he started his Ph.D. project at the University of Groningen under the supervision of Leo W. Beukeboom, Kuke Bijlsma, Jürgen Gadau and Louis van de Zande. The results of this work are presented in this book.