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# Phenotypic and Genetic Correlations Among Mating Traits in Three Species of Nasonia (Hymenoptera: Pteromalidae

Jason E. Leonard
*University of Tennessee - Knoxville*

To the Graduate Council:

I am submitting herewith a dissertation written by Jason E. Leonard entitled "Phenotypic and Genetic Correlations Among Mating Traits in Three Species of Nasonia (Hymenoptera: Pteromalidae." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Ecology and Evolutionary Biology.

Christine R.B. Boake, Major Professor

We have read this dissertation and recommend its acceptance:

Gordon Burghardt, Ben Fitzpatrick, Susan Riechert

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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# PHENOTYPIC AND GENETIC CORRELATIONS AMONG MATING TRAITS IN THREE SPECIES OF *NASONIA* (HYMENOPTERA: PTEROMALIDAE)

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Jason E. Leonard
August 2008

## Dedication

To Kelly, the one person who made me believe in myself and you will always be the love of my life.

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There are many people who have contributed to this body of work that need to be addressed. First, I have to thank my wife Kelly who had to put up with a lot over the eight year stint of graduate school. But, her willingness to stick it out and be there for me when I really needed someone made this accomplishment possible. I also have to thank my parents, John and Cathie, for all the help they provided, both financially and mentally (even though they weren't sure how this would turn out). They were always willing to lend a hand and they gave me a place to stay when it came down to the wire. Oh, and they're the best babysitters too. I need to thank my two children, Julia and Josef, whose two cute faces reminded me of why I was doing this to begin with.

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Thank you all and you will be missed.

## Abstract

Phenotypic correlations between traits are thought to reflect genetic correlations. However, traits related to a central function, like reproduction, need not all be genetically correlated. Identifying genetic correlations between behavioral traits can help identify the evolutionary relationship between different behaviors, as well as provide initial information on the number of genes involved in behavioral variation and the rates and direction of evolution. This body of work describes the phenotypic and genetic relationship between mating traits in the parasitoid wasp genus *Nasonia*. Chapters 2 and 3 describe behavioral differences among the three species of *Nasonia*. Two of the species, *N. vitripennis* and *N. giraulti*, show extreme differences for several behavioral traits. *Nasonia vitripennis* females mate almost exclusively after emergence from the host puparium and show a strong propensity to remate, and males of this species show strong site fidelity after emergence. Conversely, *N. giraulti* females mate almost exclusively inside the host (called within-host mating; WHM), show a low frequency of remating, and males show no site fidelity and disperse after emergence. The third species, *N. longicornis*, is similar to *N. vitripennis* for male site fidelity, but shows between line variation for WHM and female remating. I used the between line variation to create divergent recombinant inbred lines (RILs), 24 of which were screened for ten different behavioral traits (Chapter 4), including WHM, male site fidelity, remating, components of male courtship, and male and female developmental traits. Three behaviors were genetically correlated; WHM was positively correltated with remating frequency and remating frequency was negatively correlated with male courtship duration. The

relationship between WHM and remating frequency could not be explained in terms of timing of female receptivity. However, remating frequency was directly correlated with when females became sexually receptive as early female receptivity was found in lines with high remating frequencies. I also address the evolution of these behaviors in an ecological context (Chapter 5). WHM is thought to have evolved as a byproduct of selection against hybridization in nature, and the ecological distribution of *Nasoni,a* both within nests and host pupae, is consistent with this hypothesis.

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## Chapter 1-Introduction

The study of genetic influences on behavior is a young science and identifying genetic effects on behavior is an essential tool in understanding how behavior evolves (Boake et al. 2002). A central question in behavior genetics is to what extent behaviors are genetically correlated. Phenotypic correlations between behavioral traits, either within or between the sexes, are common, but the underlying genetic effects have not been investigated for most systems (Boake et al. 2002). Additionally, few theoretical models implicitly test the role of behavior in adaptive evolution, and the models where behavior can be applied are mostly related to sexual selection and sexual conflict (Lande 1981, Lande and Kirkpatrick 1988, Iwasa and Pomiankowski 1995, Payne and Krakauer 1997, Gavrilets 2001, Gavrilets 2003). Information on genetic correlations among behavioral traits can provide information on the rates and direction of evolution where the magnitude of the correlation is related to the strength and speed of the response to selection (Falconer and MacKay 1996, Roff 1997). Additionally, highly correlated traits can be indicative of a fairly simple genetic architecture as strong correlations could indicate that traits are affected by few genes or loci of large effect (Falconer and MacKay 1996, Roff 1997).

Adaptive evolution proceeds via selection on traits that improve the fitness of a population or species (Darwin 1859, Fisher 1930). However, not all traits that change over time (like behavior) are under selection and many can change via alternate evolutionary processes (Futuyma 2006). One mechanism of change for traits not under

direct selection is through genetic correlations. In a simple two-trait example, selection will act on one trait and indirectly affect the other trait because of the genetic relationship between them (Falconer and MacKay 1996, Roff 1997). From this perspective, in order to investigate evolution in any population or species, organisms as a whole need to be addressed, and not simply a single trait that is thought to be adaptive. Selection has effects on entire organisms and investigating multiple traits can give us a better understanding of how new traits evolve with respect to the adaptive context of a trait and the genetic relationship between traits.

An alternate mechanism for the evolution of new traits is through transgressive segregation. Transgressive segregation produces hybrid progeny with phenotypes that fall outside the range of the phenotypes of the parents (Reiseberg et al. 1999). In hybrids, alleles from different loci, all of which have an effect on a particular trait, can come together either through random segregation or through selection (Rieseberg et al. 1999, Bell and Travis 2005). Transgressive phenotypes of these hybrids exceed the phenotypic ranges of the parental populations, creating extreme phenotypes on which selection can act. Hybrids could be generated through species, ecotype or population crosses (Riechert and Maynard Smith 1984, Rieseberg et al. 1999). Identifying transgressive phenotypes can generate hypotheses for how new traits have developed in a species, as well as provide a genetic explanation for the evolution of adaptive phenotypes.

Investigating the evolution of behavior is simplified when two populations or two closely related species differ in one or more behavioral traits and the phylogeny is

known. Thus, identifying behavioral differences between closely related species is the first step to developing adaptive and genetic hypotheses for behavioral evolution. In the following chapters, I will discuss multiple behavioral differences between three closely related species of the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae). These three species differ in components of male courtship behavior (van den Assem and Werren 1994) and the location of mating (Drapeau and Werren 1999), so I wanted to identify additional differences in behavior and attempt to explain the evolution of these behaviors from phylogenetic, adaptive and developmental perspectives.

Male behavior outside of courtship is not well studied in the Hymenoptera (Godfray 1994, Quicke 1997). The species differences in the location of mating in *Nasonia* (either inside or outside the host puparium; called Within-host mating or WHM) suggested that there may be an opportunity for species differences in male mating and aggressive behavior. Thus, Chapter 2 addresses the aggressive behavior of males of all three species. I found that *N. vitripennis* males are highly aggressive and females do not mate inside the host, whereas *N. giraulti* males are not aggressive and females mate almost exclusively inside the host puparium. Males of the third species, *N. longicornis*, were aggressive, but the females showed a large range for the frequency of WHM. The behavioral differences discovered among the three species are discussed with reference to the molecular phylogeny in an effort to determine the ancestral and derived conditions of male aggression and site-fidelity, as well as the phenotypic relationship between male host defense and the reproductive status of the females. This chapter was published in Animal Behaviour (Leonard and Boake 2006).

Chapter 3 covers the initial female receptivity to male courtship and a female's willingness to remate. Previous studies had reported a variety of remating frequencies for *N. vitripennis*, ranging between 5% and 50% (Holmes 1975, van den Assem and Visser 1976). This variability surrounding remating frequencies for this species could be due to major differences in methods. My work is the first study of remating frequencies for the other two species, *N. giraulti* and *N. longicornis*. A study of *N. vitripennis* (van den Assem and Visser 1976) had suggested that male performance of a post-copulatory display was a major factor influencing whether or not a female would remate: females receiving post-copulatory displays were unlikely to remate. I tested the remating frequencies of females from all three species and found that *N. vitripennis* females had the highest remating frequencies (~95%), *N. giraulti* females had the lowest remating frequencies (~30%) and *N. longicornis* females were intermediate (~65%). Additionally, for *N. vitripennis* and *N. longicornis*, the duration of first male courtship was associated with the frequency of female remating, but the duration of the post-copulatory display was not. The only species in which the duration of the post-copulatory display was associated with remaing frequencies was *N. giraulti*. This chapter is in press in Animal Behaviour (Leonard and Boake 2008).

The results in the first two chapters showed strong phenotypic correlations within two of the species, *N. vitripennis* and *N. giraulti*, for the majority of behaviors that were investigated. The third species, *N. longicornis*, showed a great deal of population differentiation, which suggested that there was enough variation in behavior to

investigate genetic correlations among the behavioral traits. Two populations from different states differed significantly for six of the ten behavioral traits from the previous two studies. I used these two populations of *N. longicornis* to create 71 recombinant inbred lines (RILs), of which 24 were tested for each of the 10 behavioral traits. Because RILs are a result of random segregation of alleles, and therefore genetically different, determining the behavioral correlations for multiple RILs allowed me to estimate the genetic correlations among all of the behaviors (Chapter 4). I found only three significant correlations among the ten behavioral traits studied. Female remating frequency was positively correlated with WHM, and this relationship was opposite that in the parental lines. Also, the likelihood of female remating was positively correlated with the duration of male courtship, and this correlation was in the expected direction. Additionally, there was a strong positive relationship between male duration of courtship and the number of first head nods he gave, however these traits provide the same information. I was unable to detect significant correlations that matched the phenotypic relationship across species for male aggression and site-fidelity. Interestingly, for three of the behaviors (WHM, copulation duration, and post-copulatory display duration) I found a total of eleven RILs that showed transgressive phenotypes.

Preliminary data analysis of the RILs suggested a role for developmental differences for two of the behaviors, WHM and the frequency of remating (Chapter 4). Behavioral differences found among the RILs appeared to share a relationship to the onset of sexual receptivity in females. In other words, whether the female chose to mate first inside or outside the host puparium and how often she mated were affected by the

age at which she became sexually mature. Therefore, I assessed the developmental times (egg-to-adult and egg-to-emergence times) for six of the RILs, three that showed high levels of WHM, and three that showed low levels of WHM. Additionally, I tested the parental lines and a population from each of the other two species to determine if the patterns seen among the RILs were consistent across species. I also tested the timing of the onset of sexual receptivity by presenting males to females immediately after eclosion (emergence from their own pupal case) until they became receptive. I found that females in RILs with high WHM were sexually receptive earlier than females in RILs with low WHM. However, this trend was the opposite of that in the parental lines. Additionally, the populations of the other two species both had females that were receptive immediately after eclosion. So, the age of the onset of receptivity of the females did not appear to be a good predictor of WHM. However, the age of the onset of receptivity did show a more consistent relationship with remating frequencies: lines with reduced female remating had females that became receptive later.

Chapter 5 provides data to address ecological questions about the distributions of the three species of *Nasonia* and their range overlap in bird nests in the United States. *Nasonia* is a common parasite of *Protocalliphora* blowflies that prey on nestling birds, and two of the species, *N. longicornis* and *N. giraulti*, are found only in bird's nests (Darling and Werren 1990). At least one of the behaviors, WHM, is thought to have arisen as a by-product of selection against hybridization in areas of sympatry. However, the degree of sympatry between the three species had not been reported. To investigate this hypothesis, I used a data set that was provided to me by Dr. Jack Werren at the

University of Rochester, New York. I found that the distribution of the three species was closely associated with that of the host genus *Protocalliphora*. The results indicated that *N. girault*i and *N. longicornis* are highly dependent on this host genus, more so that *N. vitripennis*. Additionally, there were differences in habitat overlap for the three species. There was a higher probablility of finding *N. vitripennis* and *N. girault*i in the same nest than finding *N. longicornis* and *N. vitripennis* in the same nest. I also found that in New York State, *N. vitripennis* and *N. girault*i parasitized the same host pupa, but not at a high frequency. These data were consistent with a hypothesis that suggests species differences for WHM are a result of selection against hybridization in sympatry, but more data are needed to make a substantial conclusion about the mechanism of divergence in this group.

The final chapter suggests research directions that could be profitable.

# Chapter 2 - Site-dependent Aggression and Mating Behaviour in Three Species of *Nasonia* (Hymenoptera: Pteromalidae)

This chapter is a lightly revised version of a paper that was published in Animal Behaviour in 2006 and co-authored with Dr. Christine R.B. Boake.

**Leonard, J.E. and Boake, C.R.B.** 2006. Site-dependent aggression and mating behaviour in three species of *Nasonia* (Hymenoptera: Pteromalidae). *Animal Behaviour*. **71**: 641-647.

## Abstract

Changes in male reproductive tactics are often associated with mate availability, which will lead to a correlation between the male's mating strategy and a female's willingness to mate. However, identifying such a correlation within a population with variable male behaviour may be impossible, so studying variation between populations or species becomes preferable. We tested whether this type of association exists between three species of the parasitoid wasp *Nasonia*, where females show variation in their frequency of mating inside the host puparium (called Within-host mating; WHM) versus outside it. We investigated male aggressive behaviour, site fidelity and dispersal in relation to WHM using six lines of *Nasonia*; 2 from each species. All observations were made during adult emergence from the host puparium. We found that male *Nasonia vitripennis* were highly aggressive and did not leave the area surrounding the host, and 95% of females emerged as virgins. In contrast, *N. giraulti* males showed little to no aggression and a high tendency to leave the area after emerging from the host, and 100% of females tested emerged having already mated. The third species, *N. longicornis*, was variable for

both male aggressive behaviour and WHM and may represent the early stages of behavioural divergence from the ancestral state represented by *N. vitripennis*. The behavioural differences between males of *N. vitripennis* and *N. giraulti* represent the ancestral and derived states respectively, and imply that environmental factors may have had a major influence on the evolution of this phenotypic association between the sexes.

**Introduction**

A male's ability to procure mates is often dictated by either his ability to out-compete other males or by the reproductive state of females (Andersson 1994). Males that are at a competitive disadvantage may adopt alternative tactics or strategies for access to potential mates (Gross 1996, Alcock 1997). These alternate mating tactics result in different fitness optima for weaker males that would otherwise be unable to produce offspring. Many studies have investigated the maintenance of alternate male reproductive behaviours within populations (Andersson 1994, Gross 1996), but few have examined population or species divergence in male tactics (Carroll 1993, Carroll and Corneli 1995). Identifying differences at the species level can help us to distinguish the genetic and environmental changes needed to result in selection for one behavioural phenotype over another.

Changes in male reproductive tactics, for example the location of mating, can be associated with female receptivity or female density (Emlen and Oring 1977). Consequently, a correlation can form between male and female reproductive traits as a result of selection on one trait driving the evolution of the other. Because of the low

repeatability of behavioral traits, it may be difficult to detect a correlation between male and female traits within a species, but such a correlation might be detectable in closely related species. Species differences in male reproductive courtship and other communication behaviours are common (Andersson 1994), and thus it should be possible to detect correlations within a group of closely-related species. In this study, we use the three species of the parasitoid wasp *Nasonia* to test the hypothesis that male aggressive behaviour and area defense are correlated with mate availability.

Many parasitoid wasp species have non-resource based behavioral mating systems: the majority of social interactions occur on or near the host after adult eclosion and just before dispersal (Godfray 1994, Quicke 1997). The vast majority of behavioural studies of parasitoid wasps have focused on female behaviours, including courtship and mating, sex-ratio allocation, and host detection and predation (Godfray 1994, Quicke 1997). Descriptions of male aggressive behaviour in the Hymenoptera are limited to studies of solitary bees and wasps (Alcock 1996, Alcock 1997, Simmons et al. 2000, O'Neill 2001). As in the solitary bees, males of some *Nasonia* species appear to compete aggressively with each other for access to females. The species of *Nasonia* offer a unique opportunity to investigate the factors associated with divergence in male competitive behaviour because a male's aggressive success is of primary importance to his lifetime fitness. Additionally, identifying key differences between species within a known phylogeny may help us to understand the evolutionary associations between male and female behaviour.

The genus *Nasonia* consists of three species: *Nasonia vitripennis*, *N. giraulti*, and *N. longicornis*. The phylogenetic relationship between the three species, based on an internal transcribed spacer and 28s rDNA sequences, indicates that the initial split between *N. vitripennis* and its sister taxa occurred between 0.2 and 1 mya. The other two species, *N. giraulti* and *N. longicornis*, diverged slightly more recently (Campbell et al. 1993). All three species are gregarious ectoparasitoids of the pupae of flesh flies and blowflies of the genera *Protocalliphora*, *Calliphora*, and *Sarcophaga*, and are typically found in bird's nests (Darling and Werren 1990). *Nasonia vitripennis* is a cosmopolitan species and is sympatric with *N. giraulti* and *N. longicornis*, which have only been found in eastern and western North America respectively (Darling and Werren 1990). All three species are gregarious, laying more than one egg on the host; clutch sizes typically range from 20 to 140 offspring depending on the host species and host size (Whiting 1967).

Previous behavioural work with *N. vitripennis* showed that male host defense consists of staying on or near the host surface in an attempt to keep intruding or newly emerging males off the host and away from the emergence hole. Males that successfully defended a host from other males had access to emerging females (King et al. 1969, van den Assem et al. 1980). Therefore, the most aggressive males were able to mate with virgin females as they emerged. This should produce a fitness advantage for aggressive behaviour as long as females are sexually receptive when they emerge.

Comparative analyses became possible with the discovery of *N. giraulti* and *N. longicornis* the early 1990's (Darling and Werren 1990). Drapeau and Werren (1999)

found a significant difference in the tendency of females from the three species to mate inside the host puparium (called Within-host Mating or WHM). *Nasonia giraulti* shows the highest level of WHM, *N. vitripennis* shows the lowest frequency, and *N. longicornis* is intermediate. Drapeau and Werren's results confirm that the majority of *N. vitripennis* females emerge as virgins as was previously suspected. However, because *N. longicornis* and *N. giraulti* show greater variation than *N. vitripennis* in the frequency of emerging females that are virgin, it is possible that male aggressive behaviour may also be variable because the fitness payoff to defending a host might be low when emerging females are not virgins.

We had two main goals of this study. First, we wanted to determine if there were significant differences within and between the three species in male site fidelity, dispersal and aggression. To accomplish this goal we first described the behaviour of male *N. vitripennis* in detail, paying particular attention to aggressive and response behaviours exhibited between two males on the host surface. We then examined the aggressive behavior of males of the other two species, using the same protocols as with *N. vitripennis*. Our second goal was to determine if a correlation exists between male aggression and WHM. Thus, we conducted two sets of tests. The first were observations of male behaviour on the surface of the host, in order to compare levels of aggression between species. The second set of tests was evaluations of the levels of WHM, which we determined by dissecting females to learn whether they were carrying sperm when they emerged from host puparia. Because we assume that females that carry sperm are sexually unreceptive, we predicted that species with high levels of WHM would show

low scores for male aggressive behaviour and species with low levels of WHM would show high scores for male aggression.

## Materials and Methods

### *Stocks*

We tested two strains per species. All strains were collected by J. Werren at the University of Rochester and each was generated from single wild caught female. For *N. vitripennis*, strains NVOH 204 and NVXNVB 401AF (hereafter NV1 and NV2 respectively) were collected from Ohio in 1989 and Nevada in 1991 respectively. The *N. girault*i strain RV2 (NG1) was collected in New York in 1987 and has become a standard laboratory strain that has been in active culture for at least 15 years. The strain NGPA 233F2 (NG2) was collected in Pennsylvania in 1989. For *N. longicornis*, strain NLUT 218 (NL1) was collected from Utah in 1989 and strain NLCA 003270A (NL2) is from California and was collected in 1990. Each strain had been maintained in diapause, having only 1 or 2 reproductive generations per year (with the exception of the *N. girault*i laboratory strain) prior to being sent to us in November 2001. We kept them in continuous laboratory culture from receipt until testing.

*Rearing and experimental conditions*

All stocks were maintained under laboratory conditions at 25 °C, at least 50% relative humidity, on a 16h:8h light:dark cycle. Stocks were maintained by placing 6-8 mated females into each of 5 plastic vials with 5 *Sarcophaga bullata* hosts. Under these conditions, *Nasonia* have an approximate 15-17 day developmental period.

For the experimental trials we allowed three females to parasitize a single host for two days. We chose these hosting methods to decrease the chance of offspring entering diapause. The host was removed and given approximately two weeks for the wasp offspring to develop. This hosting method permits fairly synchronous developmental and emergence times for each host. We conducted our tests during emergence; thus all the wasps were tested approximately 1 day since eclosion. All experimental trials were conducted under the laboratory conditions described above.

*Aggression Trials*

Aggressive interactions consisted of contests between males on the surface of the host puparium. Interactions have the same general sequence of behaviour in all three species (Table 2.1). Beginning with the orienting behaviour, one or both males may then perform the wings up behaviour. An aggressive male may then charge or lunge at the other male, if the males are close to each other, and the interaction may continue with a male

Table 2.1. A partial ethogram of the aggressive and response behaviours of male *Nasonia*. No assumptions are made about behaviours exhibited by winners and losers of bouts. Responses to aggressive behaviours can also be other aggressive behaviours.

| **Aggressive behaviors** | **Description** |
|---|---|
| *Wings up* | - a male places his wings in a 90° vertical position (as described for *N. vitripennis* ; Van den Assem et al. 1980) |
| *Orient* | - a male moves his body so that his head is facing another male. |
| *Charge* | - a male rapidly moves directly towards another male. |
| *Lunge* | - a male raises his head and forelegs while pushing with its back legs toward another male. |
| *Grab* | - after contact, a male partially climbs onto the back of another male and wraps his forelegs around the body or wings, typically the abdomen. |
| *Pursue* | - after an interaction, a male follows another male that is moving away. |
| **Response behaviors** | |
| *Back up* | - a male moves backward or to the side one or two steps. |
| *Flee* | - a male runs in the opposite direction of the other male immediately after an interaction. |
| *Still* | - with another male on its back, the wasp crouches against the host surface and lies motionless. |
| *Kick* | - a male being grabbed from behind by another male raises his back legs toward the grabbing male. |

grabbing or chasing the other male. The second male's responses to his opponent vary from charging or lunging back, to backing away, fleeing or crouching and becoming motionless (Table 2.1). Additionally, either male may retreat from the host surface to the area surrounding the host. It is important to note that a response to an aggressive behaviour can be another aggressive behaviour and in any interaction one or more of the behaviours may be missing. Data collected from aggressive interactions were based on this behavioural description.

When wasps were expected to begin to emerge, the host puparium was placed in a clear Plexiglas observation chamber that measured 28 x 24 x 24 cm. Within the observation chamber, the host was placed in the center of an 11 cm diameter circle (the arena). The host puparium was placed on a non-toxic poster putty to eliminate movement of the puparium while wasps emerged. Observations began when the second male emerged from the host puparium because of the possibility that the first male was disturbed when the host was moved to the observation chamber. A trial was considered to begin when the third male emerged from the host, and continued for 30 minutes. During each trial, males and females were allowed to emerge freely from the host. We measured the following aspects of aggressive behaviour: site fidelity, dispersal and aggressive interactions. Each trial was one unit of sample size.

Site fidelity in this system was defined as a male wasp staying on the host from which he had emerged. We measured this by performing one scan observation every 30 seconds for a period of 30 minutes; thus we made 60 observations per trial. For each time

interval, we recorded the number of males on the host surface as well as the number of males that dispersed from the arena. Males had the opportunity to stay in the arena, but not on the host, which was why we also measured dispersal. A male that left the 11 cm circle was considered to have dispersed from the arena and was removed from the observation chamber. A total of 10 trials were conducted for each strain. We calculated the number of observation periods with at least one male on the host surface, as well as the proportion of males that had emerged that left the arena.

We recorded all aggressive interactions between males that were on the host surface during the same 30 minute time period as above. An aggressive interaction was defined as any interaction that included a 'charge' or 'lunge', followed by a response from the other male. A specific interaction was considered to have ended when none of the listed behaviours (Table 2.1) was being performed or when one of the focal males began an interaction with a different male. We were unable to record the intensity of aggressive interactions due to the speed at which they occurred. Males were also aggressive towards males that had only partially emerged from the host puparium, but these aggressive behaviours were not included in the data because the response of the emerging male was not completely observable. Such unrecorded interactions were approximately 1% of all observed interactions.

Because males were allowed to emerge freely during each trial, the number of males in the arena in any time period varied from 1 to 8 with an average of 3.5 males across all hosts and all strains. To permit comparisons between trials, we developed an

Aggression Index to produce a standardized score. The Aggression Index was the total number of aggressive interactions during the 30 minute trial divided by the average number of possible pairs in the arena per 30 second interval. For example, with three males in the arena, there were three possible pairs, but with four males in the arena there were six possible pairs. We used 30 second intervals because this reflected the rate at which the number of males changed.

*Within host mating (WHM)*

These observations were conducted in a separate experiment from the one described above. When the first wasp emerged from the host, it and all subsequent wasps were collected and isolated. Ten females were examined for WHM by dividing the total number of females that emerged (N) by ten ($i = N/10$). Every $i^{th}$ female was then dissected to locate the spermatheca, a sperm storage organ. For example, if 50 females emerged, every fifth female was dissected. The sperm in the spermatheca have a reflective quality that can easily be seen under a dissecting microscope at 40x magnification. Because the females were collected immediately after emergence, they did not have the opportunity to mate outside the host puparium. Therefore, the presence of sperm in the spermatheca indicated that the female had mated within the host and the absence of sperm indicated that the female was still a virgin upon emergence. Ten collections were made for each strain. Below, we report the average percentage of mated females per host per strain.

Sex ratio might be a nuisance variable in our analyses of WHM because if females were very abundant, they might not encounter a male before emerging from the host. We were able to consider whether sex ratio could contribute to the results because we had collected every wasp that emerged. We define sex ratio as the proportion of males in a clutch.

*Statistical analyses*

Data for both territorial and WHM trials were analyzed using non-parametric Kruskal-Wallis tests with an alpha level of 0.05 by using the statistical software JMP 4.0. Additionally, a two-tailed non-parametric multiple comparison test for equal sample sizes (Siegel and Castellan 1988) was used to identify significantly different strains.

**Results**

*Aggressive behaviour*

In our analysis of site fidelity, the mean number of males on a host differed significantly between the six strains (Kruskal-Wallis test: $H_5$=47.44, P<0.0001) (Figure 2.1). Strain NV1 showed the greatest site fidelity with a male always being observed on the host surface. Strain NV2, and both strains NL1 and NL2, were not significantly different from each other or from NV1. A multiple comparisons test for equal sample sizes was conducted by calculating a critical value Z, which is the same for all tests at an alpha

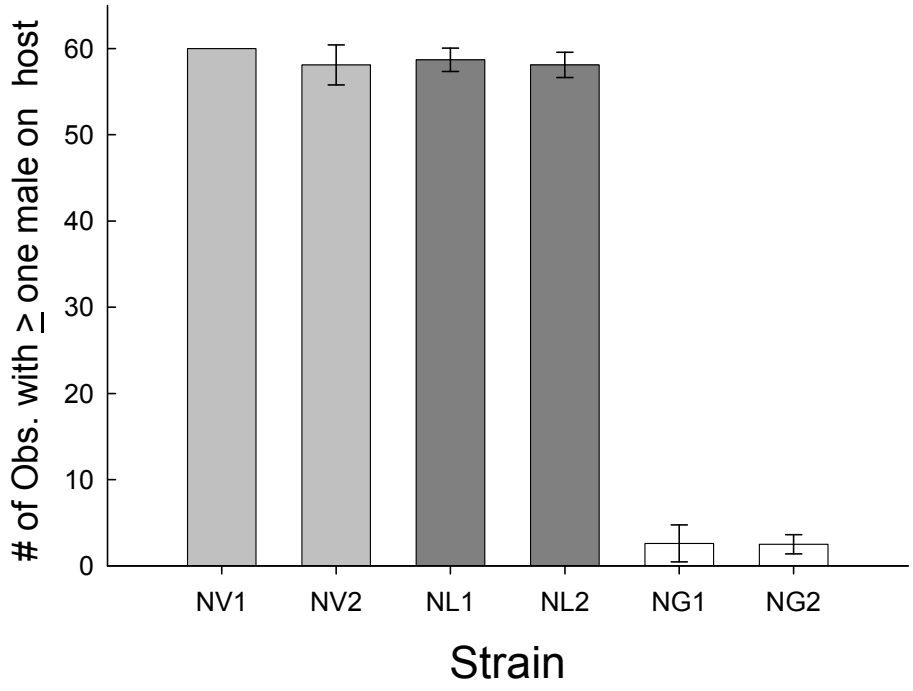


Figure 2.1. Mean number of observations out of 60 (± 2 SE) with at least one male on the host for six strains of *Nasonia*. Different shades indicate different species and different letters indicate significant differences. Both *N. giraulti* strains (NG1 and NG2) differ significantly from all other strains.

level of 0.05 (Z = 22.92). We then calculated independent Z scores for each strain and compared them to the critical value. The significant difference in site fidelity was due to the two *N. giraulti* strains, NG1 and NG2 ($Z > 22.92$ for all comparisons, $P<0.05$), but the two *N. giraulti* strains were not different from each other. This result confirmed our impressions from our observations: The tests of male *N. giraulti* on the host surface rarely provided data on aggression because the males left the host surface after emergence, whereas in almost every observation of *N. vitripennis* and *N. longicornis* there was at least one male on the host (Figure 2.1).

We tested whether *N. giraulti* males stayed near the host or left the arena more frequently than males from the strains of the other two species. The six strains differed significantly in the mean percentage of males to leave the arena (Kruskal-Wallis test: $H5 = 39.75$, $P < 0.0001$) (Figure 2.2). Again the significant difference was due to the two *N. giraulti* strains ($Z >22.92$ for all comparisons, $P<0.05$). Thus we found that the males from *N. giraulti* that left the host surface also left the arena. In contrast, *N. vitripennis* or *N. longicornis* males that left the host surface stayed near the host and rarely left the arena (Figure 2.2).

We expected to find a strong positive correlation between male aggression and site fidelity because we only scored aggressive encounters that took place on the host surface. There was a significant difference in the mean levels of aggression between the six strains tested (Kruskal-Wallis test: $H_5 = 41.70$, $P < 0.0001$) (Figure 2.3). Multiple comparisons tests again show that the significant difference is due to both NG1 and NG2.

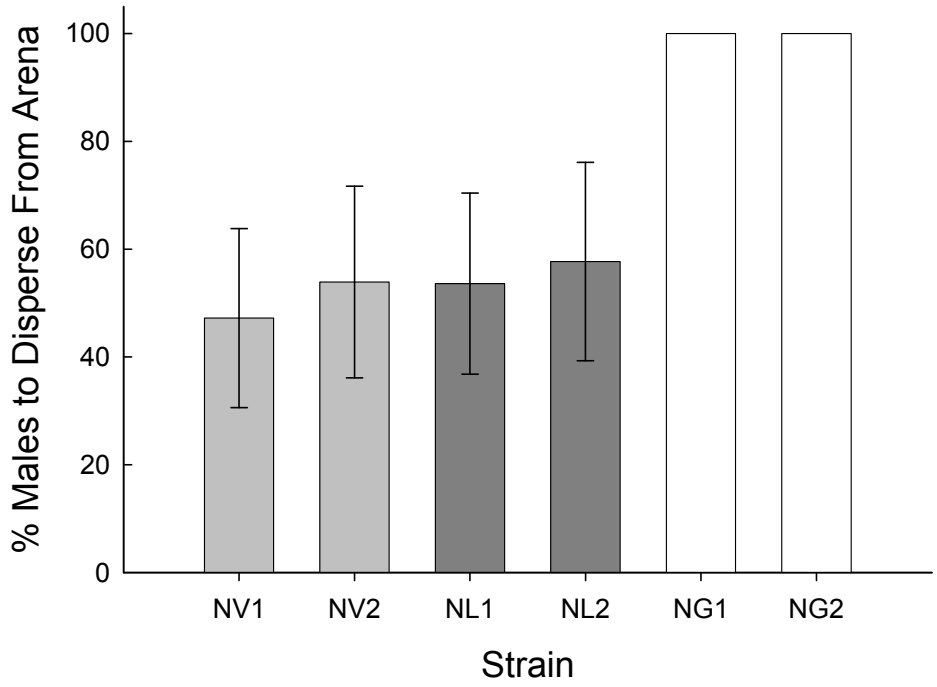


Figure 2.2. Mean percentage of males that emerged during the trial (± 2 SE) to disperse from the arena. Letters indicate significant differences. Every male from both *N. giraulti* strains dispersed during the time interval. Males from *N. vitripennis* and *N. longicornis* did not disperse immediately upon emergence.

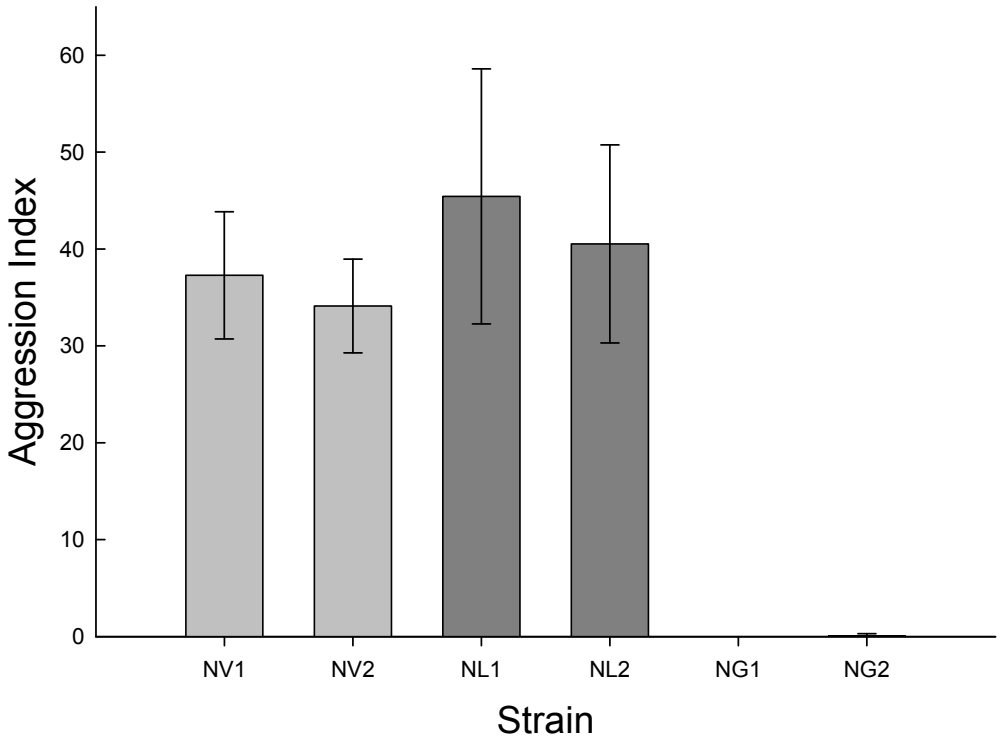


Figure 2.3. Mean level of male aggression (± 2 SE) for each strain of *Nasonia*. Different letters indicate significant differences. Males from both *N. vitripennis* strains and both *N. longicornis* strains are highly aggressive. Only one aggressive interaction for *N. giraulti* (NG2) was observed.

Only one aggressive interaction on the host surface was observed for *N. giraulti* (NG2) (Figure 2.3) and this observation can be attributed to the rapid rate of emergence and the high number of males that emerged during that trial. These results indicate that both *N. vitripennis* strains and both *N. longicornis* strains were highly aggressive, whereas both *N. giraulti* strains showed little or no tendency to be aggressive on or near the host surface. There were no cases of *N. giraulti* males staying near or on the host and being non-aggressive.

*Within-host Mating*

Our results for WHM were similar to results reported previously for these species (Drapeau and Werren 1999). There was a significant difference between strains in the mean frequency of WHM (Kruskal-Wallis test: $H_5 = 47.44$, $P < 0.0001$) (Figure 2.4) and the difference was due to the two *N. giraulti* strains. Both *N. giraulti* strains had WHM values of 100%. In other words, all females tested had mated prior to emerging from the host. *Nasonia longicornis* strain NL2 did not differ significantly from the *N. giraulti* strains. This is probably because NL2 showed the largest variability in the frequency of WHM, with frequencies that ranged from 0% - 60%. Drapeau and Werren (1999) reported that *N. giraulti* showed the highest frequency of WHM, with *N. longicornis* being intermediate, and *N. vitripennis* having the lowest values of WHM. Our data (Figure 2.4) followed the same pattern, but for three of the four strains that were tested in both studies (NG1, NG2 and NV1) our frequencies were higher. We attribute this increase to a difference in methods in that we allowed all wasps to emerge, and therefore

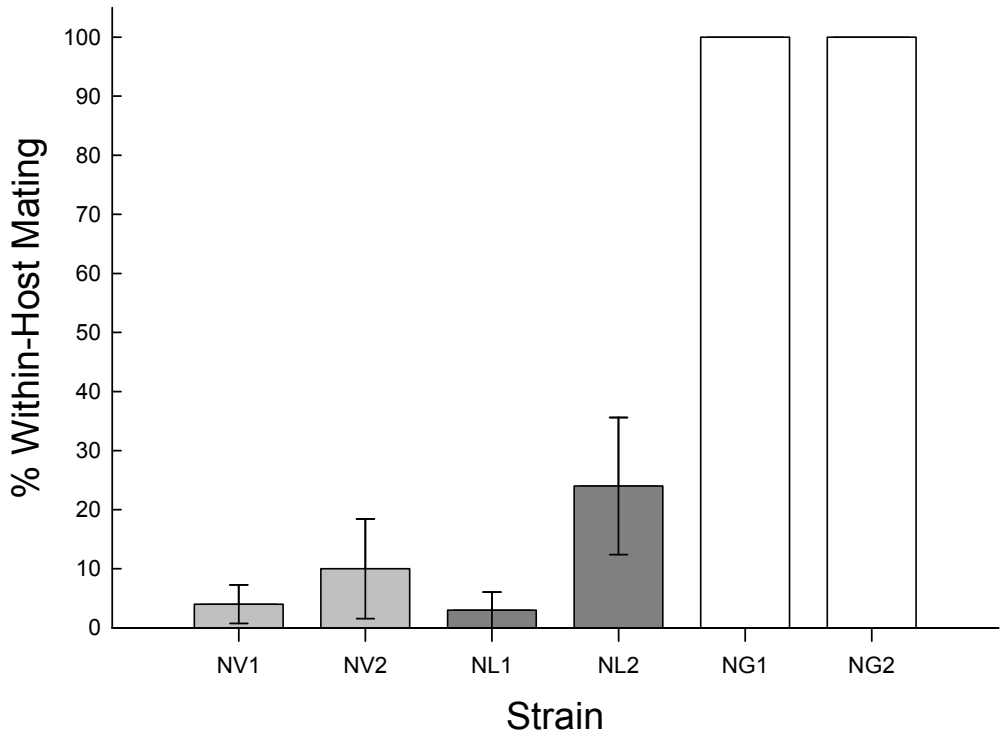


Figure 2.4. Mean level of Within-host mating (WHM; ± 2 SE) for females of 3 species of *Nasonia*. Different letters indicate significant differences. Both *N. giraulti* strains differ significantly from all other strains except NL2.

allowed as much time as possible for them to mate before they came out. In contrast, Drapeau and Werren (1999) opened the host puparium as soon as the first wasp emerged and isolated females immediately, which may have prevented some from mating inside. The fourth strain that was tested in both studies (NL1) had a lower value for WHM than previously reported.

We found no significant relationship between the sex ratio (proportion of males) and the percentage of females that mated inside the host (Table 2.2). These data indicate that WHM is a predominantly female trait. If WHM were a male-regulated behaviour, we would expect to see greater WHM when more males were available to mate, in other words when the sex ratio was more male-biased. Instead we found that the number of males in any given host had no relationship to WHM values.

*The Relationship Between WHM and Site-dependent Aggression*

We found greater WHM in populations with lower male aggressive behaviour (Figure 2.5). However, we did not conduct statistical tests because we tested only two populations per species. Our prediction that there would be a negative linear relationship between these two traits cannot be distinguished from a prediction of a threshold effect because we found no intermediate WHM values. We observed that males from strains with high levels of WHM did not defend the host from other males, whereas males from strains with low levels of WHM were aggressive and defended the host (Figure 2.5). *Nasonia longicornis* showed a wider range of scores between strains than the other two

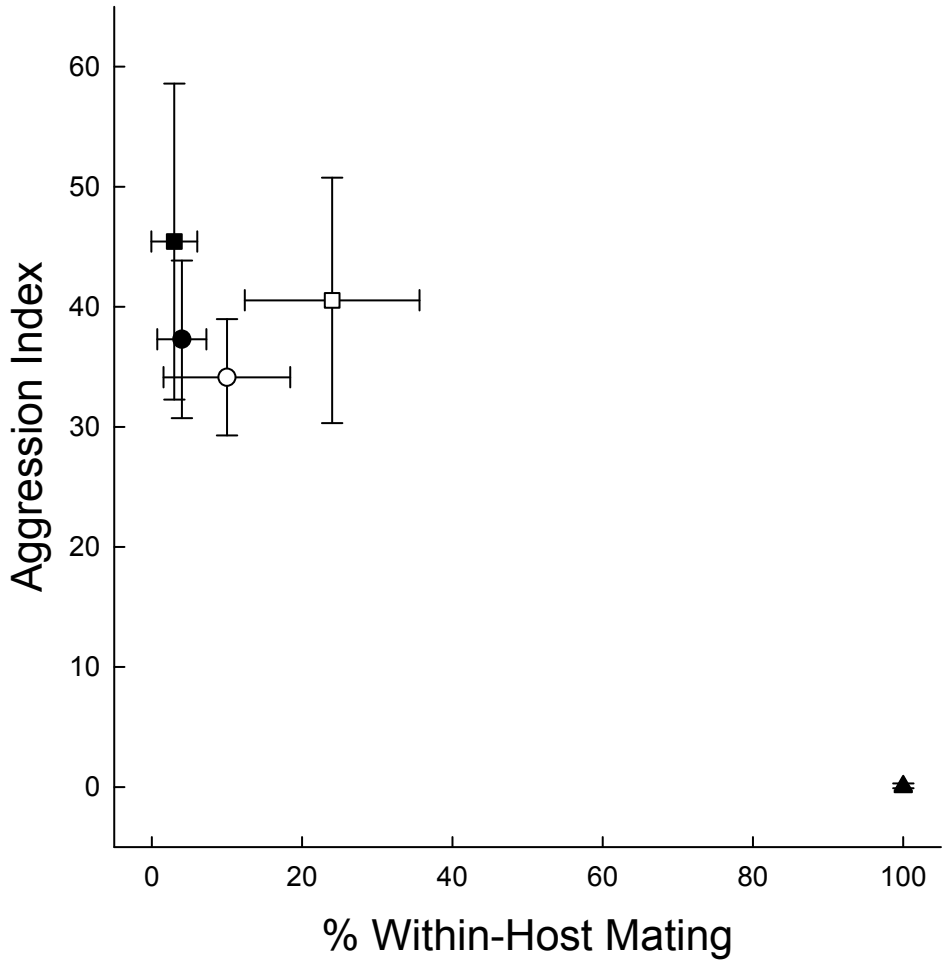


Figure 2.5. Mean level of male aggression (± 2 SE) plotted against mean female WHM (± 2 SE). *N. vitripennis* ( ●, ○); *N. longicornis* ( ■, □); *N. giraulti* ( ▲, 2 points overlap at 100 %). Species with high aggression have low WHM and species with low aggression have high WHM.

Table 2.2. Mean sex ratio (proportion of males; ± SD) for all six strains of *Nasonia*. The correlation coefficients represent the relationship between adult sex ratio and WHM within strains. There is no significant relationship between these two variables across strains. Correlation values are not reported for the *N. giraulti* strains because the WHM values were 100%.

| Strain | N | Adult Sex Ratio | R-squared for correlation between sex ratio and WHM |
|---|---|---|---|
| NV1 | 10 | 0.292 ± 0.085 | 0.089 |
| NV2 | 10 | 0.140 ± 0.051 | 0.017 |
| NL1 | 10 | 0.108 ± 0.042 | 0.083 |
| NL2 | 10 | 0.216 ± 0.155 | 0.055 |
| NG1 | 10 | 0.164 ± 0.037 | --- |
| NG2 | 10 | 0.185 ± 0.117 | --- |

species for both WHM and aggression, suggesting that other populations of this species may exhibit a range of values for WHM.

**Discussion**

*Nasonia vitripennis* and *N. giraulti* may represent the extremes of directional selection on WHM and aggression; *N. vitripennis* shows virtually no WHM and high aggression, and *N. giraulti* has substantial WHM and virtually no aggression (Figure 2.5). Based on the *Nasonia* phylogeny (Campbell et al. 1993), we believe that the high levels of aggression on the host found in *N. vitripennis* and *N. longicornis* represent the ancestral state in this group and that the loss of site-dependent aggression in *N. giraulti* is derived. Additional support for our hypothesis that high aggression is ancestral in *Nasonia* is found in the observations of high levels of male aggression in some other genera in their family (Pteromalidae; Van den Assem et al. 1980, Fitzpatrick and Wellington 1983, Quicke 1997). *Nasonia longicornis* showed the largest ranges within and between strains in both male territoriality and WHM, which suggests that it is in the early stages of behavioural divergence from the ancestral state represented by *N. vitripennis* (Campbell et al. 1993).

*Nasonia giraulti* represents the derived state for aggressive behavior, according to the phylogeny (Campbell et al. 1993). Males showed high levels of dispersal from the arena (Figure 2.2) and very little to no aggression on the host (Figure 2.3). However, *N. giraulti* males have been observed to be aggressive toward conspecifics, away from the host surface (J.L. personal observation). A more appropriate interpretation of the data is

that males of this species show no site fidelity, but may possibly search for alternate hosts elsewhere that they can defend. This is possible for *N. girault*i because males have large wings and can fly. In contrast, male *N. vitripenni*s, which are aggressive on the host surface, have vestigial wings and are unable to disperse via flight from the area surrounding the host (Gadeau et al. 2002). However, there is discordance between the derived and ancestral states of wing size with the behavioural data presented here. *Nasonia vitripennis* represents the derived state for wing size while *N. girault*i represents the ancestral wing type (Gadeau et al. 2002). To date, male dispersal in this genus has not been investigated and only females are known to disperse (Darling and Werren 1990).

If a high level of site-dependent aggression is the ancestral state, then so is a low level of WHM. Thus we hypothesize that *N. girault*i has evolved both high levels of WHM and low levels of male aggression on the host. The evolutionary increase in WHM and decrease in male aggression may be associated with differences in female remating behaviour. The current hypothesis regarding the evolution of WHM is that it evolved as a by-product of selection against mating with individuals that possess different strains of the endosymbiotic bacterium *Wolbachia* (Drapeau and Werren 1999). In *Nasonia*, *Wolbachia* create near complete reproductive isolation between individuals or populations that harbor different strains (Werren 1998, Bordenstein et al. 2001). Because the different species of *Nasonia* can possess different strains of *Wolbachia*, mating with 'unlike' individuals results in strong postzygotic isolation. *Wolbachia* could play a role if it is common for two species to oviposit on the same host individual (called multiple parasitism). Multiple parasitism has been described between *N. vitripennis* and the other

two species (Darling and Werren 1990), although its frequency is unknown. If both *N. vitripennis* and *N. giraulti* parasitize the same host, then in the probable ancestral condition, females that emerged as virgins would risk mating with the wrong species of male on the host surface. *Nasonia giraulti* appears to be the less common species based on its narrower geographic and host ranges (Darling and Werren 1990) and the fact that it was discovered many decades after *N. vitripennis* had become a well-studied laboratory organism (Whiting 1967). A potential consequence of being less common is that there could have been selection on *N. giraulti* females to mate before encountering heterospecific males that carried a different strain of *Wolbachia*, and selection for the females to become unreceptive to remating. If the ancestral male state is to be aggressive, then the lower aggression in *N. giraulti* could have evolved in correlation with the increase in WHM in that species. This hypothesis depends on the frequency of female remating within the time interval from eclosion to emergence. Remating in *Nasonia* has been investigated in *N. vitripennis* and some strains remate at a frequency of almost 50 percent (Barass 1964, van den Assem and Feuth-de Bruin 1977). These results suggest that *N. vitripennis* females are highly sexually receptive upon emergence. However, remating in *N. giraulti* and *N. longicornis* has not been investigated and we would expect, based on the male behaviour pattern of *N. giraulti*, that females of this species would show a lower frequency of remating. We are currently studying remating in *N. giraulti* and *N. longicornis.* Because *N. vitripennis* shows low levels of WHM, rather than a complete absence, the ancestral state may well have been a low level; thus it could be that the reason that *N. giraulti* changed rather than *N. vitripennis* may be arbitrary and dependent on the frequency of encountering populations with different infections.

It appears that in these species the male alternative of being less aggressive is an evolutionary response to changes in female behaviour. This type of correlation can arise as a result of the spatial and temporal distribution of females that are sexually receptive (Orians 1969, Emlen and Oring 1977). Male abilities to exploit female receptivity can be variable both within and between species, and are thus potentially evolvable (Webster and Robinson 1999). For example, different blackbird species have different degrees of polygyny depending on nest density (Robinson 1986, Webster 1992). Male blackbird mating strategies differ in response to female availability where males are able to exploit the number of sexually receptive females in an area. Our data indicate a similar situation in that *Nasonia* males are responding to the number of sexually receptive females inside or outside the host. However, this association exists in species that are ecologically similar where males of all three species have the ability, both spatially and temporally, to mate with multiple females (Darling and Werren 1990). We believe that the loss of male site fidelity in *N. giraulti* could reflect species differences in the temporal component of female receptivity, where *N. giraulti* females become sexually receptive earlier than *N. vitripennis*, resulting in higher levels of WHM.

In these three species the male tactics appear to be fixed at the extremes rather than showing an evolutionarily stable mix within species. Similarly, the female mating behavior seems distinct. This raises the question of whether the two male tactics ever coexisted in one species, and if it was stable for any time. The concept of evolutionarily stable strategies, which is defined within populations, requires an assumption that environmental (i.e. selective) conditions remain constant. If different populations differ in

environmental conditions, within-population behavioural plasticity might allow two populations to diverge due to selection, resulting in fixed differences between species. In species that have evolutionarily stable male behavioural polymorphisms, how much of an environmental change would be sufficient for the polymorphism to disappear? Did *Nasonia* males once show behavioral polymorphism within species, but later lost the polymorphism as a result of environmental changes? Further studies with *N. longicornis* may provide insight into these questions because this species may represent the intermediate stages of divergence from its sister species.

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# Chapter 3 - Associations Between Male Courtship and Female Polyandry in Three Species of *Nasonia* (Hymenoptera: Pteromalidae)

This chapter is a lightly revised version of a paper that is in press in Animal Behaviour. It is scheduled to be published in September 2008 and is co-authored with Dr. Christine R.B. Boake.

**Leonard, J.E. and Boake, C.R.B.** 2008. Associations between male courtship and female polyandry in three species of wasp, *Nasonia* (Hymenoptera: Pteromalidae). *Animal Behaviour*. in press.

## Abstract

Males can affect the number of times that females mate by inducing resistance to remating. We investigated the associations between male courtship behaviour and female remating frequencies using three species of the parasitoid wasp *Nasonia*. We tested females for remating at 10 minutes and 24 hours after the first copulation and found that species differences in female remating frequencies were associated with different first-male behaviours. *Nasonia vitripennis* females showed the highest average frequency of remating, greater than 75% for all tests, and females that did not remate had experienced long first courtships, suggesting that they were generally resistant to courtship. In *N. longicornis*, the two populations tested had a two-fold and significant difference in female remating frequency, from 45% to 90%, but we could not identify any first male behaviour that was significantly associated with this difference. Fewer than 35% of *Nasonia giraulti* females remated 10 minutes after the first copulation but remating increased to about 70% in the 24 hour tests. In *Nasonia giraulti* longer post-copulatory

displays by the first male were associated with a higher chance of female remating. These three species are closely related but male behaviours that appear to manipulate female remating differ between them. The divergence may have evolved as a consequence of sexual conflict over different reproductive optima for each species.

## Introduction

The number of times a female mates can be affected by the first male with which she mates. Remating in females is known to be inhibited by the presence of sperm or male accessory gland substances inside the female (Chapman et al. 1995, Wolfner 2002, Moore et al. 2003). Additionally, male behaviour in the form of post-copulatory courtship has been shown to reduce female receptivity (Alcock & Buchmann 1985, Allen et al. 1994, King & Fischer 2005). These types of male traits can influence the evolution or maintenance of multiple mating either by sexual selection on direct and indirect benefits of multiple mating (Thornhill and Alcock 1983, Arnqvist 1989, Martens and Rehfelt 1989, Wiklund et al. 2001, Arnqvist and Andres 2006) or sexual conflict through costs associated with repeated mating (Parker 1979, Rice 1996, Arnqvist & Rowe 2005). However, the roles of other male behaviours such as pre-copulatory and copulatory courtship have received little attention as alternative mechanisms that can influence female remating frequencies (Eberhard 1996). The goal of this study is to investigate the role of pre- and post-copulatory courtship on female remating using wasp species that show both kinds of courtship.

Post-copulatory courtship reduces female remating in at least three species of Hymenoptera from three genera (Alcock & Buchmann 1985, Allen et al. 1994, King & Fischer 2005). We have examined the role of male courtship in female remating in three other species of Hymenoptera, the three known members of the parasitoid wasp genus *Nasonia*. Males of all three species engage in both pre-and post-copulatory courtship (van den Assem & Werren 1994). Barrass (1960, 1965) suggested that post-copulatory courtship performed by male *N. vitripennis* acts to inhibit further female receptivity. This hypothesis was supported by van den Assem and Visser (1976) who reported that female remating rose from 12.5% to 100% when males were prevented from performing post-copulatory courtship.

Some unique features of the biology of *Nasonia* suggest that female remating frequencies may differ between the species. In particular, *N. giraulti* females mate almost exclusively inside the host puparium, called Within-Host Mating, while *N. vitripennis* females almost always emerge as virgins. The third species, *N. longicornis*, is intermediate for within-host mating and varies depending on the population (Drapeau & Werren 1999, Leonard & Boake 2006). Additionally, *N. giraulti* males disperse from the host immediately after emergence, while both *N. vitripennis* and *N. longicornis* males stay on the host and defend it from other males (Leonard & Boake 2006). The higher rate of dispersal for *N. giraulti* males suggests there is no fitness advantage for them to stay on the host; this may be because the females have already mated and are not receptive to remating. Additionally, the species differ in courtship behaviour (van den Assem &

Werren 1994). These differences led us to hypothesize that there could be species differences in the frequency of female remating among *Nasonia* wasps.

This study had two main goals. First, we wanted to determine if the three species differ in female remating frequency. Second, if female remating frequencies differ between any of the species, we wanted to identify the male behavioural traits that are associated with such differences. We measured the durations of all pre- and post-copulatory male behaviours, and used a multivariate analysis to identify the behaviours that were significantly associated with species differences in female remating frequency.

**Methods**

The parasitoid wasp genus *Nasonia* consists of three closely related species: *Nasonia vitripennis*, *N. longicornis*, and *N. giraulti*. The phylogeny of *Nasonia* shows that *N. vitripennis* initially split from its sister taxa approximately 0.8 million years ago and *N. longicornis* and *N. giraulti* diverged from each other more recently (Campbell et al. 1993). All three are ectoparasitoids of sarcophagid and calliphorid flies, but differ ecologically in that *N. vitripennis* is a cosmopolitan species and a generalist. *Nasonia longicornis* and *N. giraulti* are both specialists, having been found only in bird's nests in western and eastern North America respectively (Darling & Werren 1990). The three species of *Nasonia* also differ in courtship (van den Assem & Werren 1994), female mating behaviour (Drapeau & Werren 1999) and male site fidelity and aggression (Leonard & Boake 2006).

We used van den Assem and Werren's (1994) description of the *Nasonia* courtship sequence. Briefly, courtship begins when a male mounts the female with his head over hers. The male then performs a series of head nods. The first head nod is the most distinct and it is often performed with mouthpart extrusions. It is thought that a pheromone that is released in tandem with the mouthpart extrusions is required to induce receptivity from the female (van den Assem 1980b, Beukeboom & van den Assem 2001). A series of more rapid head nods follows the first nod, after which there is a short period of virtually no movement. If the female does not become receptive, then the male begins a new cycle. The interval from the first head nod of a series to first head nod of the next series is considered one cycle. A courtship bout consists of multiple cycles. The female signals receptivity by lowering her antennae and opening her genital orifice at the same time. Once a female signals receptivity, the male backs up and copulates with her. Female receptivity signals are almost always followed by copulation. After copulation is complete, the male returns to his original courtship position and performs a brief post-copulatory display. This display consists of another cycle of courtship, although there is variation in the number of head nods performed. If a male is unable to induce a receptivity signal during the original courtship attempt, he dismounts from her, and if he encounters her again, he begins a new bout of courtship.

We tested two strains from each species of *Nasonia* (six total). All six strains were provided by J. Werren at the University of Rochester, and had been originally collected from single wild caught females (for full stock information see Leonard and Boake 2006). Briefly, we used two *N. vitripennis* strains, NVOH 204 (NV1) and

NVXNVB 401AF (NV2), two *N. longicornis* strains, NLUT 218 (NL1) and NLCA 003270A (NL2), and two *N. giraulti* strains, RV2 (NG1) and NGPA 233F2 (NG2).

All strains were maintained at 25˚ C and at least 50% relative humidity with a 16:8 h light:dark cycle. Under these conditions, *N. vitripennis*, *N. longicornis* and *N. giraulti* have 15, 16 and 17 day developmental periods respectively. Stocks were maintained by placing 6-8 females into each of five vials with five *Sarcophaga bullata* hosts per vial.

We collected wasps for experimental trials by opening the host puparium approximately one day before the wasps eclosed from their own pupal cases. For each trial day, three hosts were opened and 7-8 male and female pupae were collected from each host, for a total of approximately 40 wasps. Male pupae were mixed to randomize the host of origin and females were treated similarly. Each wasp was isolated in its own vial that contained a grain of sand to facilitate eclosion.

We conducted no-choice mating tests to assess remating frequencies at two different time intervals, 10 minutes and 24 hours after the first copulation. Each trial was performed at 24˚ C. All wasps were approximately 1 day old when used (except some males in the 24h trials, see below). We began each trial by placing a virgin female into a 9 x 30mm glass vial and giving her 5 min to acclimate, after which a male was placed into the vial with the female. If the male did not mount the female in 10 min, the trial was ended and a new male was placed with the same female. No virgin female needed to be

tested with more than two males. Males that mounted a female were allowed three courtship bouts to induce a receptivity signal and if receptivity did not occur the female was given a score of not copulating. Each female that mated was removed from the vial and placed into a clean vial. Ten minutes later, a new inexperienced male was placed with the female to test for remating. So, the latency until remating occurred ranged from 10 min, if remating was immediate, to 19 min in our longest trial.

We also studied remating 24 hours after the first copulation and we followed similar methods to those described above, with two modifications. For these tests, the second male in each trial came from the same group of adults, which resulted in the second male being two days old instead of one day old. Additionally, because we conducted the trials one day later, we used two-day old virgin females as controls to eliminate age as a factor that could influence remating frequencies. We tested 30 females per strain for each retesting time.

It is possible that the strain and species differences in remating were due to reduced time spent courting by the second male. In other words, the second male may not have been willing to court an already mated female. We tested this hypothesis by creating a courtship index, defined as the proportion of time the second male spent courting a female, starting with the first mounting, relative to the total duration of the trial. We compared courtship indices for all strains and species to determine if reduced courtship was responsible for differences in remating.

For both the first and second males, we recorded the latency to mount the female, latency to court, courtship duration, and the durations of copulation and the post-copulatory display. At no time did we prevent any aspect of the courtship sequence. We chose these male behaviours because they represent the entire courtship sequence, and because courtship duration differs between the species (van den Assem and Werren 1994). We recorded whether females produced a receptivity signal and if they copulated with either or both males**.**

Most courtship studies with *Nasonia* use the number of first head nods as a proxy for courtship duration (van den Assem & Visser 1976, Jachmann & van den Assem 1995, van den Assem and Jachmann 1999, Bordenstein et al 2000). Courtship duration was positively correlated with head nods and provided more information than the traditionally used number of first head nods (Appendix).

*Statistical Analyses*

All data were analyzed using the statistical software JMP 6.0 (SAS Institute, Cary, North Carolina, U.S.A.). Chi squared tests were used to identify differences among strains in remating frequencies and we determined which strains differed from each other by partitioning the Chi-squared table (Siegel & Castellan 1988).

Logistic regression was performed at the level of species. We performed two logistic regressions for each species, one each for 10 min and 24 hr after the first

copulation. We used remating as the response variable (Yes or No), and for independent variables we examined the first male courtship components of latency to mount, latency to court, courtship duration, copulation duration and post-copulatory display duration. We also included strain as a factor in the models. We included second order interactions in the two models for *N. girault*i but for the other two species we were unable to include second order interactions due to a lack of power as the sample sizes for the 'No remate' category were very low.

Means comparisons between species were made using non-parametric Kruskal-Wallis tests. Additionally, a two-tailed non-parametric multiple comparison test for unequal sample sizes was used to assess differences between species (Siegel & Castellan 1988). This test calculates a critical value Z that is different for all comparisons. Individual Z scores were computed for each comparison and scores that exceeded the critical value were significant. A Wilcoxon Signed Rank test was used for comparisons between means of different males within a species. Alpha was set at 0.05 for all statistical tests.

## Results

### *Species differences in remating frequencies*

When females were tested for remating 10 min after the first copulation, the six strains differed significantly in remating frequencies ($\chi_5^2$= 54.06; P<0.001: Figure

3.1).We found two groups of stocks that differed from each other but not within the group; the groups were NV1, NV2, and NL1 (remating frequencies were 97%, 77%, and 87% respectively) , which differed significantly from NL2 (47%), NG1(33%) and NG2(27%) ($\chi_1^2$= 10.01; P<0.001: Figure 3.1). *Nasonia vitripennis* females were more than twice as likely to remate as *N. girault*i females. *Nasonia longicornis* was variable with one strain (NL1) resembling *N. vitripennis* and the other strain (NL2) resembling *N. girault*i.

For our tests 24 hr after the first copulation, the strains differed significantly in female remating frequency ($\chi_5^2$= 16.39; P<0.01: Figure 3.1), falling into three groupings. Two strains, NV1 and NL1, differed from all other strains but not each other ($\chi_1^2$= 24.44; P<0.001), having nearly 100% remating. Three of the remaining strains, NV2, NG1 and NG2, formed the second group, with remating frequencies of about 80% ($\chi_1^2$= 5.32; p<0.05 from NV1 and NL1; $\chi_1^2$= 13.75; p<0.001 from NL2; Figure 3.1). About 40% of females of strain NL2 remated. Thus, at 24 hr after the first copulation, remating frequencies no longer showed a consistent level within species, but the variation within *N. longicornis* persisted.

Only two strains showed significant differences in remating frequency between trials at 10 min and 24 hr after the first copulation. These were the two *N. girault*i strains, NG1 and NG2, which doubled in remating frequency when given a 24 hr interval between the introduction of males (NG1: $\chi_1^2$= 12.47; p<0.001, NG2: $\chi_1^2$= 14.62;

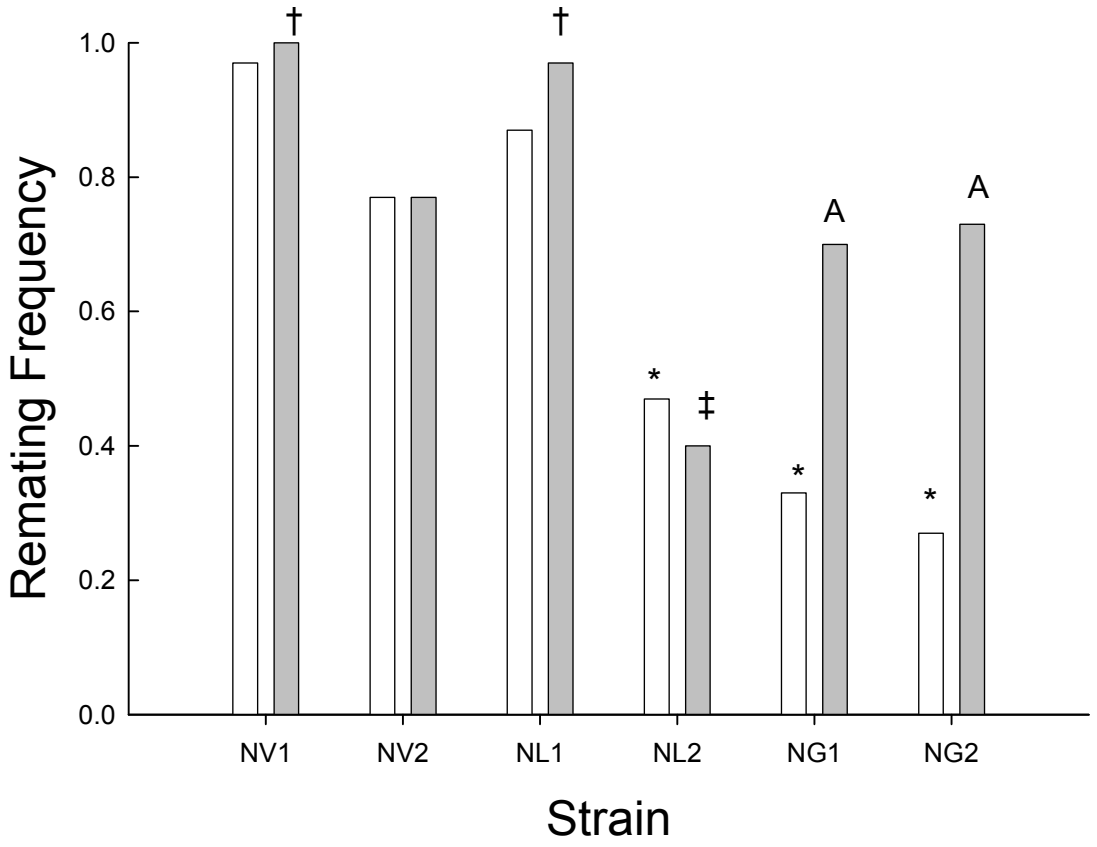


Figure 3.1. Remating frequencies for six strains of *Nasonia*. NV: *N. vitripennis*; NL: *N. longicornis*; NG: *N. girault*i. White bars are for 10 minute tests and shaded bars are for 24 hour tests. N = 30 for all six strains in both retesting times. Bars with different symbols indicate significant differences between strains within each trial type ($\chi_5^2$ test, $P<0.001$). Bars with letters indicate significant differences between trial types ($\chi^2$ test, $P<0.001$) Strains NV1 and NL1 are marginally significantly different from NV2, NG1 and NG2 in the 24 hour trials ($P<0.05$). Only NG1 and NG2 differ between tests 10 min and 24 hr after the first copulation.

p<0.001: Figure 3.1). This change in frequency cannot be attributed to age differences of males (Table 3.1): first males were consistently capable of inducing a receptivity signal from virgin females for the two testing times and the 24h control group ($X_2^2$= 0.13; P > 0.90; Table 3.1).

Males did not appear reduce the time spent courting an already mated female. For those trials in which remating took place, courtship indices were usually 1: successful second males were able to induce a receptivity signal during the first courtship bout for 91% and 85% of tests 10 minutes and 24 hours after the first copulation respectively, so remating generally took place at the end of the first courtship cycle. For cases in which remating did not occur, all strains had average courtship indices greater than 0.65. This drop in courtship index could be interpreted as males being reluctant to court previously-mated females, but the courtship index for the second males did not predict the overall remating frequency across strains for either retesting time (Figure 3.2). Males in the two strains of *N. giraulti* differed substantially in their courtship indices but the two strains had very similar female remating frequencies in both tests. The same pattern is seen in *N. longicornis*. Thus, our results suggest that the behaviour of the first male, rather than the second male, might influence female remating.

*First male behaviours associated with female remating*

For all three species, in trials where remating occurred, we compared the courtship behaviour of first and second males that were paired with the same female. For

Table 3.1. Success of first males at copulation with virgin females for three species of *Nasonia*. Only one test did not result in copulation (*N. longicornis*).

| | | First male mating success | | |
|---|---|---|---|---|
| Retesting time | N | *N. vitripennis* | *N. longicornis* | *N. giraulti* |
| 10 min | 60 | 100% | 100% | 100% |
| 24 h | 60 | 100% | 100% | 100% |
| 24 h control | 60 | 100% | 98.30% | 100% |

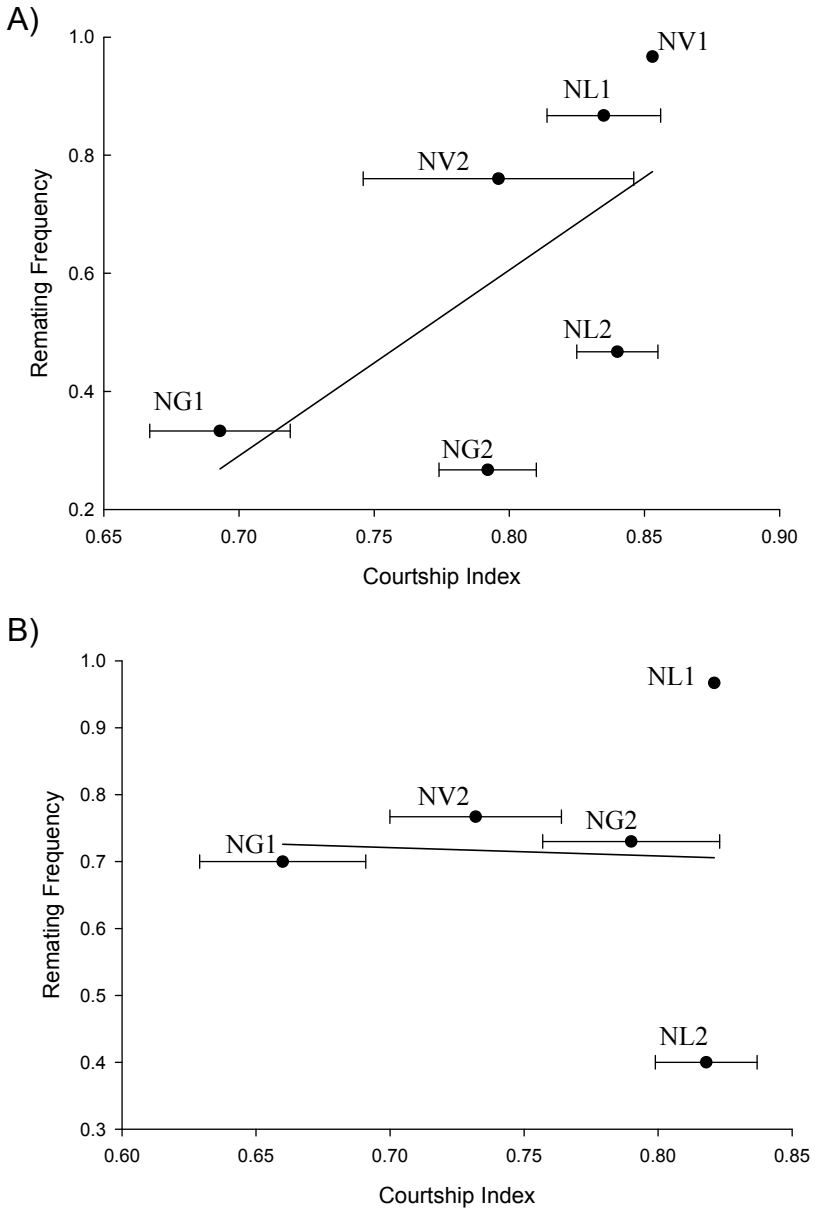


Figure 3.2. The relationship between remating frequency and mean courtship index for second males (±SE) using linear regression for six strains of *Nasonia* in trials where remating did not occur. A) 10 minute tests ($r^2 = 0.39$). B) 24 hour tests ($r^2 = 0.002$). Lines are linear regressions. There is no significant relationship between remating frequency and courtship index (ANOVA, $P>0.1$) for either retesting time. All females remated for strain NV1 in the 24 hour trials and thus courtship index is uninformative.

each retesting time, in all species, the second male always took longer to induce the receptivity signal from the female (Wilcoxon signed-rank test; $P < 0.01$; Figure 3.3 A-C). This result suggests that for those females that were willing to remate, the first male had affected the length of time required for the second male to induce receptivity from the female.

In all remating tests, all components of male behaviour were seen. We examined the durations of the courtship sequence, copulation and post-copulatory displays of first males to find predictors of female failures to remate. The behaviour of first males was significantly associated with the probability of female remating in all three species (Table 3.2, 3.3). However, different variables were significant for the two retesting times and the three species.

In *N. vitripennis*, the only significant predictor of remating in trials 10 min after the first copulation was the first male's courtship duration (Table 3.2A). For tests 24 hr after the first copulation, the overall model was also significant, but none of the first male behaviours was significantly associated with female remating (Table 3.3A). First males in tests of *N. vitripennis* in which the female did not remate had courtship durations that were twice as long as those of first males in tests where the females remated, and this pattern held for both the 10 min and 24 hr tests after the first copulation (Table 3.4). This suggests that females that did not remate needed more courtship from the first male in order to become receptive.

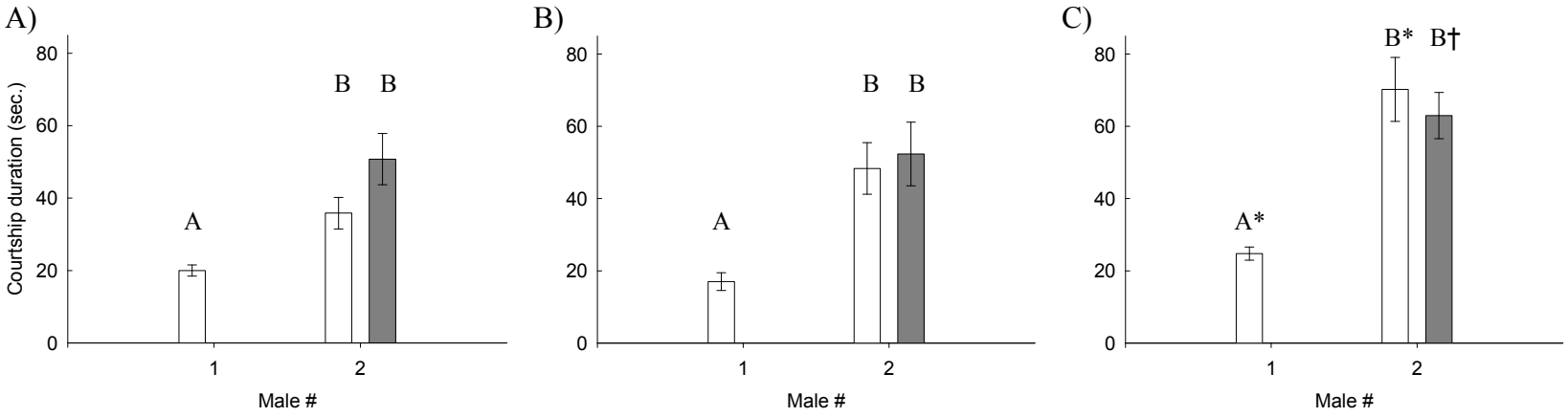


Figure 3.3. Mean durations of courtship in seconds (± SE) between first and second males, and between species, in trials in which remating occurred for three species of *Nasonia*. Data are pooled for first males presented to females. For second males, white bars are for 10 minute tests and dark bars are for 24 hour tests. Letters indicate a significant difference between means within a species. Comparisons were made between males within a retesting time, and between retesting times for each male. Symbols indicate a significant difference between species for each male within each trial type: * - differs from both species; † - differs from *N. vitripennis* only. A) *N. vitripennis*. B) *N. longicornis*. C) *N. girault*i.

Table 3.2. Associations between behaviour of the first male and female remating when females were tested 10 minutes after the first copulation. Results are from logistic regression analysis and present the association between courtship components of the first male and whether the female remated. N = 30 for each of two strains per species.

A) *N. vitripennis*

| *Model* | *d.f.* | *-LLE* | $X^2$ | *P* |
|---|---|---|---|---|
| Difference | 6 | 7.87 | 15.73 | **0.0153** |
| Full | | 15.69 | | |
| Reduced | | 23.56 | | |
| *Parameter* | | *Estimate (SE)* | $X^2$ | *P* |
| Intercept | | 8.328 (4.542) | 3.36 | 0.0667 |
| Latency to mount | | 0.007 (0.011) | 0.44 | 0.5072 |
| Latency to court | | -0.167 (0.145) | 1.33 | 0.2491 |
| Courtship duration | | -0.109 (0.042) | 6.88 | **0.0087** |
| Copulation duration | | -0.201 (0.235) | 0.73 | 0.3931 |
| Post-copulatory display duration | | 0.002 (0.043) | 0.00 | 0.9725 |
| Strain | | 0.204 (0.799) | 0.07 | 0.7984 |

B) *N. longicornis*

| *Model* | *d.f.* | *-LLE* | $X^2$ | *P* |
|---|---|---|---|---|
| Difference | 6 | 7.55 | 15.10 | **0.0195** |
| Full | | 30.64 | | |
| Reduced | | 38.19 | | |
| *Parameter* | | *Estimate (SE)* | $X^2$ | *P* |
| Intercept | | 0.509 (3.023) | 0.03 | 0.8664 |
| Latency to mount | | -0.006 (0.008) | 0.47 | 0.4909 |
| Latency to court | | 0.048 (0.069) | 0.49 | 0.4828 |
| Courtship duration | | -0.028 (0.019) | 2.13 | 0.1442 |
| Copulation duration | | 0.013 (0.183) | 0.01 | 0.9417 |
| Post-copulatory display duration | | 0.038 (0.074) | 0.26 | 0.6091 |
| Strain | | -0.877 (0.412) | 4.53 | **0.0333** |

Table 3.2, cont.

C) *N. giraulti*

| *Model* | *d.f.* | *-LLE* | $X^2$ | *P* |
|---|---|---|---|---|
| Difference | 21 | 20.75 | 41.50 | **0.0049** |
| Full | | 15.90 | | |
| Reduced | | 36.65 | | |
| *Parameter* | | *Estimate (SE)* | $X^2$ | *P* |
| Intercept | | -60.95 | 2.69 | 0.1009 |
| Latency to mount | | 0.10 | 4.13 | **0.0421** |
| Latency to court | | 4.76 | 5.12 | **0.0237** |
| Courtship duration | | -0.26 | 1.18 | 0.2768 |
| Copulation duration | | 0.98 | 0.26 | 0.6122 |
| Post-copulatory display duration | | 0.62 | 5.45 | **0.0195** |
| Strain | | -7.02 | 2.91 | 0.0878 |
| Latency to mount x Latency to court | | 0.02 | 1.63 | 0.2023 |
| Latency to mount x Courtship duration | | 0.00 | 3.27 | 0.0706 |
| Latency to mount x Copulation duration | | 0.03 | 2.28 | 0.1307 |
| Latency to mount x Post-copulatory display duration | | 0.00 | 0.77 | 0.3798 |
| Latency to mount x Strain | | -0.07 | 4.96 | **0.0259** |
| Latency to court x Courtship duration | | 0.00 | 0.00 | 0.9962 |
| Latency to court x Copulation duration | | 0.11 | 0.03 | 0.8707 |
| Latency to court x Post-copulatory display duration | | 0.11 | 2.25 | 0.1333 |
| Latency to court x Strain | | -3.65 | 3.91 | **0.0481** |
| Courtship duration x Copulation duration | | -0.01 | 0.02 | 0.8836 |
| Courtship duration x Post-copulatory display duration | | 0.00 | 0.02 | 0.8952 |
| Courtship duration x Strain | | 0.26 | 3.31 | 0.0691 |
| Copulation duration x Post-copulatory display duration | | 0.03 | 0.17 | 0.6770 |
| Copulation duration x Strain | | 0.35 | 0.36 | 0.5508 |
| Post-copulatory display duration x Strain | | -0.30 | 3.36 | 0.0668 |

LLE – Logliklihood estimate
ns = $P \geq 0.05$

Table 3.3. Associations between behaviour of the first male and female remating when females were tested 24 hours after the first copulation. Results are from logistic regression analysis and present the association between courtship components of the first male and whether the female remated. N = 30 for each of two strains per species.

A) *N. vitripennis*

| *Model* | *d.f.* | *-LLE* | $X^2$ | *P* |
|---|---|---|---|---|
| Difference | 6 | 8.31 | 16.62 | **0.0108** |
| Full | | 13.31 | | |
| Reduced | | 21.61 | | |
| *Parameter* | | *Estimate (SE)* | $X^2$ | *P* |
| Intercept | | 4.676 (64.713) | 0.01 | 0.9424 |
| Latency to mount | | -0.008 (0.007) | 1.35 | 0.2450 |
| Latency to court | | -0.180 (0.289) | 0.39 | 0.5331 |
| Courtship duration | | -0.064 (0.034) | 3.39 | 0.0658 |
| Copulation duration | | 0.373 (0.357) | 1.09 | 0.2963 |
| Post-copulatory display duration | | 0.034 (0.100) | 0.12 | 0.7297 |
| Strain | | -4.705 (64.525) | 0.01 | 0.9419 |

B) *N. longicornis*

| *Model* | *d.f.* | *-LLE* | $X^2$ | *P* |
|---|---|---|---|---|
| Difference | 6 | 13.70 | 27.41 | **0.0001** |
| Full | | 23.76 | | |
| Reduced | | 37.46 | | |
| *Parameter* | | *Estimate (SE)* | $X^2$ | *P* |
| Intercept | | -0.745 (3.619) | 0.04 | 0.8370 |
| Latency to mount | | 0.002 (0.006) | 0.08 | 0.7824 |
| Latency to court | | -0.011 (0.174) | 0.00 | 0.9475 |
| Courtship duration | | 0.008 (0.014) | 0.33 | 0.5674 |
| Copulation duration | | 0.168 (0.206) | 0.66 | 0.4158 |
| Post-copulatory display duration | | -0.026 (0.109) | 0.06 | 0.8115 |
| Strain | | -2.236 (0.638) | 12.27 | **0.0005** |

Table 3.3, cont.

C) N. giraulti

| *Model* | *d.f.* | *-LLE* | *X²* | *P* |
|---|---|---|---|---|
| Difference | 21 | 24.04 | 48.08 | **0.0007** |
| Full | | 11.72 | | |
| Reduced | | 35.76 | | |
| *Parameter* | | *Estimate (SE)* | *X²* | *P* |
| Intercept | | -1.15 | 0.01 | 0.9144 |
| Latency to mount | | -0.02 | 0.90 | 0.3423 |
| Latency to court | | 0.61 | 0.38 | 0.5379 |
| Courtship duration | | 0.02 | 0.05 | 0.8266 |
| Copulation duration | | -0.61 | 0.68 | 0.4101 |
| Post-copulatory display duration | | 0.19 | 2.78 | 0.0956 |
| Strain | | 1.40 | 1.39 | 0.2378 |
| Latency to mount x Latency to court | | -0.02 | 1.46 | 0.2265 |
| Latency to mount x Courtship duration | | 0.00 | 0.32 | 0.5743 |
| Latency to mount x Copulation duration | | -0.03 | 2.96 | 0.0855 |
| Latency to mount x Post-copulatory display duration | | 0.00 | 1.95 | 0.1624 |
| Latency to mount x Strain | | -0.01 | 0.47 | 0.4931 |
| Latency to court x Courtship duration | | 0.25 | 4.77 | **0.0289** |
| Latency to court x Copulation duration | | -0.60 | 0.99 | 0.3193 |
| Latency to court x Post-copulatory display duration | | -0.41 | 4.08 | **0.0433** |
| Latency to court x Strain | | -2.05 | 2.96 | 0.0853 |
| Courtship duration x Copulation duration | | 0.17 | 3.28 | 0.0701 |
| Courtship duration x Post-copulatory display duration | | 0.02 | 2.88 | 0.0897 |
| Courtship duration x Strain | | 0.12 | 1.35 | 0.2446 |
| Copulation duration x Post-copulatory display duration | | -0.10 | 1.26 | 0.2626 |
| Copulation duration x Strain | | -0.79 | 1.77 | 0.1839 |
| Post-copulatory display duration x Strain | | 0.25 | 2.89 | 0.0893 |

LLE – logliklihood estimate
ns = $P \geq 0.05$

Table 3.4. The effects of the first male's courtship duration and post-copulatory display duration (in sec.) on a female's probablility of remating for each species. Mean durations are for the first male presented to the female for tests both 10 minutes and 24 hours after the first copulation.

| Species/ Trial Type | Remate? | (N) | Courtship duration Mean (SE) | Post-copulatory display duration Mean (SE) |
|---|---|---|---|---|
| *N. vitripennis* | | | | |
| 10 min. | Y | 52 | 16.78 (1.63) | 19.75 (1.34) |
| | N | 8 | 40.50 (7.46) | 19.38 (2.77) |
| 24 hr. | Y | 53 | 23.19 (2.57) | 20.04 (0.82) |
| | N | 7 | 54.71 (9.53) | 24.29 (2.63) |
| *N. longicornis* | | | | |
| 10 min. | Y | 40 | 13.75 (2.36) | 18.50 (0.92) |
| | N | 20 | 28.20 (5.01) | 17.40 (0.76) |
| 24 hr. | Y | 41 | 20.22 (4.28) | 17.85 (0.71) |
| | N | 19 | 35.37 (4.33) | 18.26 (1.07) |
| *N. giraulti* | | | | |
| 10 min. | Y | 18 | 23.22 (2.51) | 34.67 (2.13) |
| | N | 42 | 26.05 (2.92) | 44.36 (2.14) |
| 24 hr. | Y | 43 | 25.42 (2.39) | 34.12 (1.61) |
| | N | 17 | 32.94 (4.89) | 41.12 (2.96) |

For *N. longicornis*, which showed the greatest differences in remating between stocks, the only significant effect is of strain for tests both 10 min and 24 hr after the first copulation (Tables 3.2B, 3.3B). This strain effect is probably the result of one strain having nearly complete remating. For the strain with a testable number of remating individuals, longer durations of first courtships were associated with the failure to remate, as we found for *N. vitripennis* (Table 3.4).

In *N. giraulti*, for both retesting times, remating frequency was predicted in part by the post-copulatory display duration (Table 3.2C, 3.3C). The post-copulatory displays received by unreceptive females were approximately 30% longer than those received by females that remated regardless of the interval until the remating test (Table 3.4). Additionally, for tests 10 min after the first copulation, two other factors of the first courtship significantly predicted remating, but these factors did not include the duration of the first courtship. These additional factors were interaction terms, the latency to mount x strain and the latency to court x strain (Table 3.2C). For tests 24 hr after the first copulation, we also found two interactions that predicted remating, the latency to court x courtship duration and the latency to court x post-copulatory display duration (Table 3.3C).

*Species differences in first male behaviour*

We found no discernable species differences in three of the courtship components, latency to mount, latency to court and copulation duration (Appendix Table 1). These

results agree with results previously reported by van den Assem and Werren (1994) for latency to court and copulation duration.

We also compared the durations of first courtships between species in trails in which remating occurred. We pooled the data for the 10 min and 24 hr retesting times for first courtships because all tests with first males had identical methods. The mean durations of first courtships differed between the species (Kruskal-Wallis test: $H_2 = 25.19$, $P < 0.0001$: Figure 3.4 A-C). A multiple comparisons test for unequal sample sizes (Siegel & Castellan 1988), showed that *N. vitripennis* ($N = 105$; $Z > 27.79$) and *N. longicornis* ($N = 81$; $Z > 28.97$) courtships were approximately 21% and 32 % significantly shorter, respectively, than those of *N. girault*i ($N = 61$) but did not differ from each other. These results agree with species differences described by van den Assem & Werren (1994).

For *N. girault*i we showed above that the post-copulatory display appears to affect female remating. The display in *N. girault*i is at least 50% longer than in either of the other two species (Kruskal-Wallis test: $H_2 = 97.90$, $P < 0.0001$: Figure 3.4 A-C). The other two species do not differ from each other. This difference between the three species has not been reported previously.

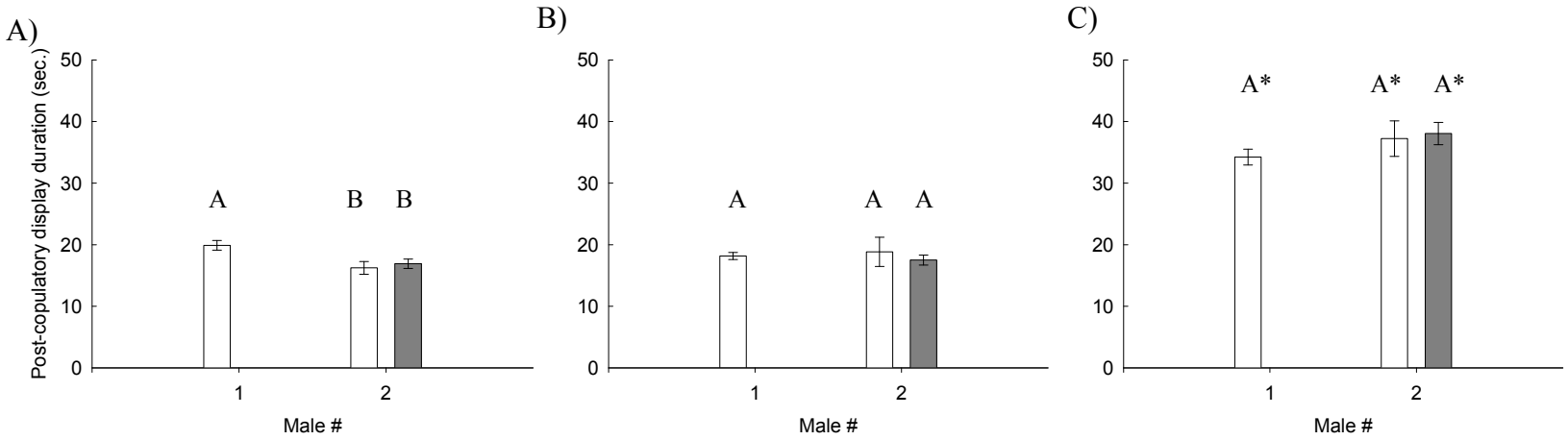


Figure 3.4. Mean durations of the post-copulatory display in seconds (±SE) for first and second males in three species of *Nasonia*. Means are for trials in which remating occurred. Data are pooled for first males presented to females. For second males, white bars are for 10 minute tests and dark bars are for 24 hour tests. Letters indicate a significant difference between means within a species. Symbols indicate a significant difference between species for each male within each retesting time: * - differs from both other species. Comparisons were made between males within a retesting time, and between retesting times for each male. A) *N. vitripennis*. B) *N. longicornis*. C) *N. girault*i.

## Discussion

Monandry appears to be widespread in the Hymenoptera (Thornhill & Alcock 1983, Ridley 1993) and *Nasonia* was thought to be no exception (Barrass 1960, Whiting 1967, van den Assem & Visser 1976). In contrast, we found high probabilities of remating in two species: overall 97% of NV1 females, 75% of NV2 females and 85% of NL1 females remated (Figure 3.1). Our frequencies are higher than any previously reported, which ranged from 7% to 70% (Barrass 1965, Holmes 1975, van den Assem & Visser 1976, Shuker et al. 2007). One possible reason for our high values is that female receptivity and male courtship have been shown to evolve in laboratory populations of *N. vitripennis*, even over short periods of time (van den Assem & Jachmann 1999, Burton-Chellew et al. 2007). However, selection under laboratory conditions may not affect each species equally as the remating frequency of NL2 has not changed over a 3 year period of continuous culture (J.L. unpublished data). Another potential explanation for the differences in remating frequencies is that the majority of studies that have looked at polyandry in *Nasonia* have been with European populations, whereas our populations are from North America. Possibly this genus shows geographic variation in the incidence of polyandry. Nevertheless, polyandry appears to be more common in *Nasonia* than previously thought because in our tests females from all three species remated at least 30% of the time.

We believe an important factor in tests of remating is the latency until a female encounters a second male. This may affect the probablility of remating in *Nasonia,* as has

been found in *Drosophila melanogaster* (Newport & Gromko 1984). Our tests 10 minutes after the first copulation showed high frequencies of remating for *N. vitripennis* and one population of *N. longicornis*, which agree with previous statements about *N. vitripennis* having high remating frequencies when given males in 'rapid succession' (Jachmann & van den Assem 1995). While our study did not show differences in remating frequencies between tests 10 min and 24 hr after the first copulation for *N. vitripennis* and *N. longicornis*, the remating frequencies for *N. giraulti* changed. The only other study of remating for *N. vitripennis* at different time intervals, 4, 24 and 48 hours after the first copulation, reported low remating frequencies (7% - 12.5%; van den Assem & Visser 1976). However, in natural populations female encounters with additional males are most likely to occur immediately after the first mating, and not many hours later. This is because *N. vitripennis* females mate with males on the host surface after emergence. Males that are unable to defend the host surface from other males stay near the host, allowing them to encounter females that leave the host surface before mating (van den Assem et al. 1980a, Leonard & Boake 2006). Once a female has mated, she immediately tries to leave the area (J.L. personal observation). Thus, if a female encounters another male, it seems more likely to be while she is trying to leave the area, and not after she has dispersed from the natal host site.

In *N. giraulti*, remating frequencies increased significantly between tests 10 min and 24 hr after the first copulation. This could be due to changes in sperm storage as well as the effects of accessory gland substances as has been found in other species (Thornhill & Alcock 1983, Wolfner 2002). For example, in *Drosophila* species remating frequencies

increase as the time interval between first and second males rises suggesting that the inhibitory effects of accessory gland substances on female remating diminish over time (recent work shows a causal link between the presence of male-produced sex-peptide in the female storage organs and female receptivity, Singh & Singh 2004). Exhaustion of sperm supply by the female can also influence remating frequencies (Mery & Joly 2002) but our experiment precluded sperm exhaustion because we did not allow oviposition by females. Because remating frequencies of the different strains differed enormously, even within species (Figure 3.1), our results indicate that the proximate control of remating can evolve rapidly, as has been suggested in models of male-female conflict over reproductive decisions (reviewed in Arnqvist & Rowe 2005).

Male manipulation of females during mating or fertilization can result in different fitness optima for the two sexes, which in turn can impose fitness costs, such as a suboptimal mating rate, on females (Gavrilets et al. 2001, Arnqvist & Rowe 2005). This conflict between the sexes can result in the rapid evolution of male traits that manipulate females (reviewed in Arnqvist & Rowe 2005), and ultimately in population divergence and possibly speciation (Gavrilets et al. 2001). The difference between *Nasonia vitripennis* and *N. giraulti* in the male trait associated with reduced female remating could have resulted from divergence related to sexual conflict, as could the difference between populations of *N. longicornis* in the tendency of females to remate. The possibility that the divergence of remating behaviour between closely related *Nasonia* species and populations is due to sexual conflict could be tested by examining the fitness consequences of female multiple mating in these species.

In *N. vitripennis*, females sometimes give a second receptivity signal during the post-copulatory display and this second signal may indicate that the female is no longer receptive to male courtship (van den Assem & Visser 1976). We noted that some females of all three species gave a second and even a third receptivity signal during the post-copulatory display (J.L. personal observation), but we did not measure this variable during our trials. In a later study with a similar design, we found no significant associations between second receptivity signals and the probability of female remating for any of the three species even though remating frequencies were consistent with our first study (Table 3.5).

*Nasonia* is not the only hymenopteran genus in which male post-copulatory behavioural traits inhibit female receptivity. For example, males of the bee species *Centris pallida* perform a post-insemination display consisting of acoustical and tactile signals that suppresses a female's return to receptivity (Alcock & Buchmann 1985). Males of the parasitoid wasp species *Aphytis melinus* and *Spalangia endius* perform post-copulatory courtship which consists of returning to his courtship position on the female after copulation and performing displays that are similar to those seen during pre-copulatory courtship, and that inhibit female receptivity (Allen et al. 1994, King & Fischer 2005).

We found that courtship behaviour is strongly associated with female remating in all three species of *Nasonia*. For *N. giraulti*, the duration of post-copulatory courtship is

Table 3.5. Proportion of post-copulatory displays that resulted in second receptivity signals from females after copulation. No significant differences were found between species for the frequency of the second receptivity signal and the second receptivity signal was not associated with remating frequency.

| Species | *N* | Freq of 2nd signal | Remating frequency | |
|---|---|---|---|---|
| | | | Y | N |
| *N. vitripennis* | 24 | 54.17% | 0.85 | 0.82 |
| *N. longicornis* | 24 | 50.00% | 0.75 | 0.67 |
| *N. giraulti* | 24 | 58.30% | 0.36 | 0.30 |

Y = tests where 2nd signal occurred
N = tests where 2nd signal did not occur

associated with decreased female remating, whereas for *N. vitripennis* and *N. longicornis*, the important display is pre-copulatory courtship (Table 3.4). However, the short durations of the first male's courtship in *N. vitripennis* and *N. longicornis* that were associated with high female remating can be viewed as either increased male vigor or elevated receptivity in females. One way to test this hypothesis would be to use males and females of two species that differ in remating frequency to determine if those differences are under male or female control. However, crosses between the two most divergent species, *N. vitripennis* and *N. giraulti*, cannot be used because of low probabilities of copulations between the species and genetic incomaptibilities (van den Assem & Werren 1994, Bordenstein et al. 2000, Bordenstein et al. 2002). Another kind of test is to use Recombinant Inbred Lines (RILs) from the two *N. longicornis* populations from this study, and if they show a consistent pattern in the remating frequencies, we should be able to determine if the longer courtship durations are due to male or female variability. Additionally, using these RILs for further analysis could help us identify genetic changes associated with behavioural divergence.

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# Chapter 4 - When, where and how often to mate? Genetic correlations among male and female mating behaviors in the wasp *Nasonia*.

## Introduction

In theory, phenotypic correlations can arise through either genetic or environmental influences. Correlations can exist between a suite of behavioral characters, sometimes called a behavioral syndrome, across multiple contexts, for example antipredator, mating, and foraging behaviors, or within a single context, for example all behaviors involved with reproduction (Sih et al. 2004). However, not all behaviors related to a single context need be correlated. A question that arises is, to what extent are multiple behaviors, both male and female, that are related to a central process, correlated both phenotypically and genetically? Cheverud (1988) suggested that any group of traits that is phenotypically correlated should also be genetically correlated. For example, mating behavior encompasses both male and female traits, and theory suggests that genetic correlations should arise between male and female reproductive behaviors either through sexual selection or sexual conflict (Lande 1981, Gavrilets 2001, 2003). Because the male and female behaviors are components of a much broader trait, it is easy to assume that most or all of them would be correlated to some extent within a single species. But, in some cases, each sex-specific trait is capable of evolving independently of another, suggesting that there are no correlations among them (Falconer and MacKay 1996, Roff 1997).

Evolution is a multivariate process in which each trait under selection can be affected indirectly by other traits that are genetically or phenotypically correlated, or, selection may act independently on multiple traits simultaneously (Arnold 1994). The

strength and direction of genetic correlations may indicate potential evolutionary pathways for the evolution of new behaviors. The magnitude and direction of genetic correlations in one species may be able to provide information relevant to evolution in another closely related species (Begin and Roff 2003). However, inferences about evolutionary pathways across species have two assumptions. These are first, that the genetic variance-covariance matrix does not change over time, and second that the covariance structure is the same for all species (Begin and Roff 2003). These assumptions are a point of debate because few studies have shown consistent results, even for different traits within a species like *Drosophila melanogaster* (Phillips et al. 2001, Whitlock et al. 2002). Consequently, identification of genetically correlated behavioral traits related to mating could help explain phenotypic patterns seen in natural populations that show differences in behavior.

Behavioral phenotypic correlations between closely related species can be traced to correlated changes in development (West-Eberhard 2003). For example, the age at which a female first mates may be correlated with the timing of sexual maturity, and females of different species may become sexually mature at different times. Understanding the relationship between development and behavior can provide a mechanistic understanding of species differences in behavior. For example, a change in the timing of sexual maturity may be indicative of delayed ovarian maturation or a change in hormone production (reviewed in Ringo 1996). Selection that shifts developmental timing may have an influence on behavioral divergence and possibly speciation (West-Eberhard 2003). Additionally, identifying the genetic correlations

between development and behavior can allow us to infer behavioral changes from a change in development or potentially identify how selection that alters a behavior pattern could result in a developmental shift.

The parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae) is an ideal organism to study genetic relationships between multiple behaviors and developmental traits. Specifically, most if not all of the male and female behaviors related to mating have been well described (van den Assem and Werren 1994, Drapeau and Werren 1999, Leonard and Boake 2006) and developmental cycles have been documented (Whiting 1967, van den Assem and Werren 1994, Pultz and Leaf 2003). Two of the species, *Nasonia vitripennis* and *N. giraulti*, are highly divergent for most behaviors and show strong phenotypic correlations between many male and female traits (Bordenstein et al. 2000, Leonard and Boake 2006). For example, *N. vitripennis* males defend the host puparium from other males to gain access to emerging virgin females ( King et al. 1969, van den Assem et al. 1980, Leonard and Boake 2006). Also, *N. vitripennis* females will mate more than once (Holmes 1975, van den Assem and Visser 1976). In contrast, *N. giraulti* males do not defend the host puparium, females are not virgin when they emerge (called within-host mating; WHM), and females have greatly reduced remating (Leonard and Boake 2006, 2008). The third species, *N. longicornis*, is intermediate for most traits and can show a high amount of between-line variation (Drapeau and Werren 1999, Bordenstein et al. 2000, Leonard and Boake 2006). At least two *N. longicornis* lines significantly differ for WHM and female remating frequency (Leonard and Boake 2006, 2008). The variability of *N. longicornis*, the rapid generation time, and the recent

sequencing of the *Nasonia* genome (www.hgsc.bcm.tmc.edu/projects/nasonia/NasWhitePaper2004.pdf) make *Nasonia* an excellent organism to investigate the genetic relationship among behavioral traits that are phenotypically correlated between two of the species.

There were two main goals of this study. First, we wanted to use the population variation in *N. longicornis* to determine which of ten different behavioral traits were genetically correlated by using recombinant inbred lines (RILs). We also wanted to determine if correlations were in appropriate directions based on the phenotypic relationship seen among species. We hypothesized for the primary behavioral traits that male site fidelity would be negatively correlated with both WHM and female remating frequencies, but WHM and female remating frequency would be positively correlated. Additionally, we expected that one of the components of the male courtship sequence, duration of courtship, would be positively correlated with female remating frequencies. Second, we wanted to explain the variation seen between RILs from a developmental perspective. Specifically, we tested for relationships between the onset of female sexual receptivity and several behavioral patterns we observed among the RILs to determine if developmental shifts played a role in behavioral variation.

## Materials and Methods

*General stock maintenance*

All species stocks and RILs were maintained at 25˚ C and at least 50% relative humidity with a 16:8 h light:dark cycle. Species and parental stocks were maintained by placing 6-8 females into each of five vials with five *Sarcophaga bullata* hosts. RILs were maintained by placing 6-8 females and one male into a single vial with 5 *S. bullata* hosts.

Hosting methods for experimental trials consisted of placing 3 mated females into each of five vials with one *S. bullata* host. These females were allowed to parasitize the host for two days (except for eclosion and emergence experiments; see below), after which time the hosts were removed, placed into individual vials, and new hosts were put into the vials. The same females were allowed to parasitize a series of five hosts.

*Generation of RILs*

Two *N. longicornis* stocks were used to generate the RILs. The first stock, NLUT 218 (called P1 hereafter), was generated from a single wild caught female in Utah in 1989. The second stock was NLCA 003270A (P2) which was also generated from a single female, caught in California in 1989. These two stocks were hosted for approximately 3 generations a year and primarily kept in diapause in the Werren laboratory, Rochester, N.Y. from collection until 2001. Both stocks have since been maintained at the

University of Tennessee, Knoxville in laboratory culture with approximately 22 generations/year.

Generating RILs is more complex for haplodiploid species than for diploid species. Parental strains were crossed together using 10 pairs of males and females in one direction to produce F1 hybrid females (Figure 4.1). Only one direction was necessary because F1 females from either cross had the same nuclear genes (assuming the parental lines were homozygous). In the F1 generation, males were not hybrids but contained half of their mother's genome. To produce recombinant males, F1 females were hosted individually as virgins to produce only sons. The F2 recombinant males were genetically unique through recombination and provided the source of the lines. F2 males were backcrossed to F1 hybrid females to produce F2 hybrid females. On average, F2 females had 50% of their genes in common with each parental strain, as did their brothers. To begin inbreeding and fixation of alleles, F2 females were collected and hosted as virgins to produce F3 recombinant sons (Figure 4.1). We then mated the F3 sons to their mothers, which had been kept alive for approximately 16 days using a solution of a 3:1 water to honey. We mated the F3 sons to their mothers rather than their sisters because the sons of F2 females shared all their genes with their mother, while they only shared 50% their genes with their sisters on average. We then hosted the F2 mated females to produce F3 females. Each line was then maintained by full-sib mating for ten generations. Under this breeding scheme, < 1% of the genome for each RIL is heterozygous (Caballero 1995, Falconer and Mackay 1996). After ten generations, the lines were maintained using the standard culturing

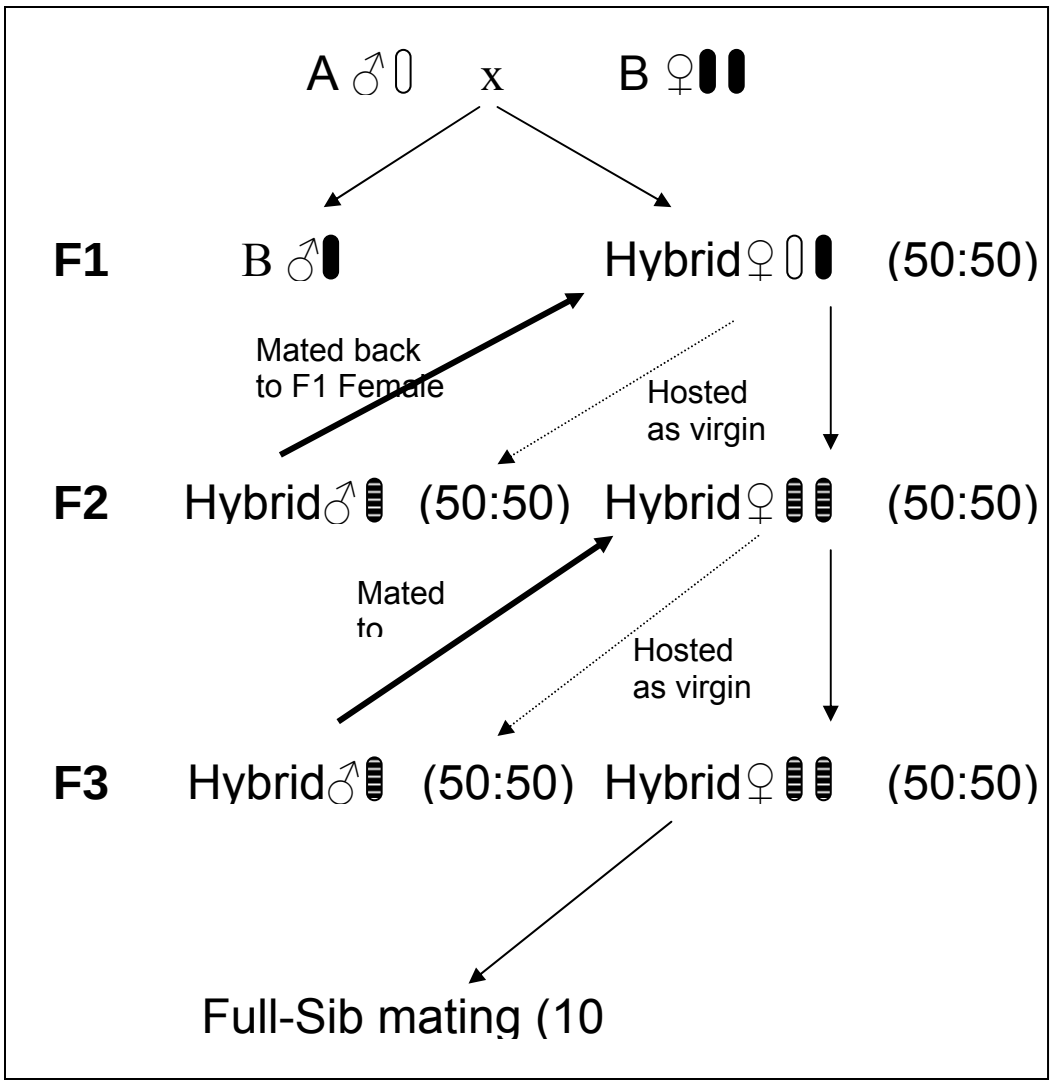


Figure 4.1. Breeding Design for the Generation of Recombinant Inbred Lines in a Haplodiploid System. Generation numbers are listed on the left. Dotted lines indicate the female is hosted as a virgin and solid lines indicate the female is hosted after mating. Bold lines show males are being crossed back to the previous generation. Striped chromosomes show hybrid individuals and numbers in parentheses are the average chromosomal makeup of the individual. Inbreeding begins by mating F2 females to their sons because brothers of the F2 females share only ¼ of their genes with their sisters, whereas sons of the F2 females share ½ of their genes.

procedure described above. We started with approximately 300 F2 recombinant males and after losses to diapause and inbreeding depression, we were able to produce 74 RILs. We tested 24 of the lines.

*Behavioral Experiments*

Within-Host Mating

Each behavior was measured in independent experiments. For tests of WHM, the trial began when the first male emerged from a host puparium. We then placed the host onto a piece of non-toxic poster putty in a small circular observation chamber and collected each wasp and isolated it as it emerged from the host puparium. Wasps were allowed no contact with other wasps outside the host puparium. We collected wasps until the first 20 females had emerged. We collected only the first 20 females because we found that the proportion of females that mate inside the host is accurately represented by the first 20 (see Leonard and Boake 2006). To assess WHM, all 20 females were dissected in saline solution at 40x magnification. We isolated the spermatheca from each female and viewed it under a compound microscope at 100x magnification to determine the presence of sperm, which indicates that the female mated inside the host. We calculated mean WHM frequencies and mean sex ratios for each line using each host as a single datum, and 5 hosts per line.

Male aggression and dispersal

We used the partial ethogram of aggressive and response behaviors developed by Leonard and Boake (2006) to score aggressive interactions on the host surface during emergence. Capitalized words refer to behavioral descriptions. Briefly, an aggressive interaction typically begins when a male Orients toward another male. This behavior is often followed by a Wings Up behavior in which one or both males raise their wings to a 90° vertical position. Then, one male will Charge or Lunge toward the other male resulting in the first contact between males. The aggressive male can then Grab the other male, often around the wings or abdomen with his forelegs, and will then Pursue a Fleeing male across the host surface. Responses by the second male to these aggressive behaviors can be some of the aggressive behaviors above, or can be non-contact behaviors such as Backing Up or Fleeing. Additional response behaviors include Still, when a male crouches against the host surface and remains motionless, or Kick, which is when a male raises his hind legs toward a Grabbing male.

To test for variation in male aggression and dispersal, we monitored hosts for emergence. When the first male emerged, we placed the host in the center of a 28 x 24 x 24 cm Plexiglas observation chamber that had an 11 cm circle marked on the bottom (the Arena). First males were not transferred. Hosts were fixed to the glass using a non-toxic poster putty to make sure the host remained in the center of the circle. Trials began when the second male emerged from the host. All wasps were allowed to emerge freely during trials.

We performed continuous observations during each 10-min trial and recorded the following data using the Observer 2.0 program: the number of males on the host, the number of males and females to emerge from the host, the number of males to disperse from the arena and the number of aggressive interactions between males on the host surface. An aggressive interaction was defined as any male interaction that included a 'charge' or 'lunge' and was followed by a response from the other male. Aggressive interactions were considered to have ended when either none of the behaviors were being performed or the interaction shifted to being between two different males. We observed 5 hosts per RIL.

Because we could not control the number of males that were interacting during trials, we created an aggression index to allow comparison. The index was calculated as the total number of aggressive interactions during the trial divided by the mean number of possible pairs of males in the arena per 20 sec interval. We divided the 10 minute trial time into thirty 20 sec intervals to allow comparison with previous studies.

Female Remating

Wasps used for female remating experiments were collected one day before eclosion. At this stage of development, host puparia can be opened and wasp pupae are easily sorted by sex. Before each trial day, we opened approximately three hosts per RIL and collected 7-8 males and females from each. Males and females were randomized

within each RIL with respect to the host of origin. Each wasp was placed into an individual vial with a grain of sand to aid with eclosion. Wasps were not allowed any contact with other wasps prior to testing. We tested females approximately one day after eclosion (~1 day old females).

We performed no-choice mating and remating tests. A virgin female was placed into a 9 x 30mm glass vial and allowed to acclimate for 5 min. The trial began when we introduced an inexperienced male to the vial. If the male did not court the female in 10 min, the trail was ended and a new male was presented to the female. Every female became receptive with the first or second male. All males were allowed to perform the full courtship sequence including the post-copulatory display (courtship is described in van den Assem and Werren 1994). We recorded the latency to mount, latency to court, number of first head nods and the durations of courtship, copulation and the post-copulatory display. Females were placed into new observation vials following successful copulation. We tested female receptivity to remating 10 min after the first copulation by providing a new inexperienced male to the female, and collected the same data as above. We tested 12 females per RIL.

*The time relationship between adult emergence and female sexual receptivity*

Our preliminary analysis suggested that variation in WHM could be a result of differences in the timing of the developmental onset of female sexual receptivity. To test this hypothesis we used a subset of the RILs (three high WHM lines and three low WHM

lines) to investigate potential differences in the onset of female sexual receptivity. Additionally, we tested both parental *N. longicornis* strains, and one strain from each of the other two species: the *N. vitripennis* strain was NVOH 204 (hereafter NV) and the *N. giraulti* strain was NGPA 233F2 (hereafter NG). Detailed information on the NV and NG stocks can be found in Leonard and Boake (2006). We predicted that females from lines with high levels of WHM would become receptive at younger adult age sooner after ecolsion than females from lines with low WHM.

To identify potential environmental variables associated with sexual receptivity, we measured developmental times from egg to emergence. Hosting methods for this experiment differed slightly from those in the experiments above. We placed three mated females into each of 25 vials. These females were given a single host for approximately 24 hours, after which the hosts were removed and held in individual vials. We repeated this procedure twice more (3 days total) using the same females. Approximately one day before eclosion, we opened four to five of the hosts and collected a sample of up to 5 male and 10 female pupae from each host. Wasp pupae were placed into vials in groups of 5 of the same sex and given a grain of sand to facilitate eclosion. We measured eclosion times by scanning vials and recording the number of wasps in each vial that had fully eclosed. We checked vials every two hours starting at 08:00 h and ending at 22:00 h for 5 days. We estimated the eclosion rate for wasps that eclosed overnight by calculating an average number of males or females to eclose per two hours, within a strain, that came from a single host (~ 6% of wasps eclosed overnight). This study gave us mean times from egg to eclosion for both males and females in each stock.

The hosts that were not used to study eclosion times were used to estimate times from egg to the emergence of the first wasp from the host. The wasps in approximately 20 hosts per trial day were allowed to emerge freely. We checked all hosts every 30 min from 08:00 h to 22:00 h for three days. Fewer than 1% of wasps began to emerge overnight so we made no corrections for overnight hours and overnight data were not used in analyses. This provided us with mean times from egg to emergence for each stock.

We also wanted to determine the age of the onset of female sexual receptivity. We opened host puparia 2-3 days prior to eclosion and separated roughly 20 female and 35 male pupae per stock. Wasp pupae were placed individually into vials. To test for the timing of female receptivity, females were observed during their eclosion process. Testing for sexual receptivity began immediately after eclosion was complete. Each newly-eclosed female was removed from her vial and placed in a 9 x 30 mm observation vial with one male. The pair was allowed 10 min to begin courtship. If a male did not begin courtship in 10 min, he was replaced by a new male. Female receptivity was scored by the success of male courtship, that is, if the female opened her ovipositor for the male, allowing copulation. Each unreceptive female was moved to a new observation vial and given a new male every 30 minutes until she became receptive. If the female did not become receptive during the first day of testing (generally a four to six hour testing period), the trial resumed the following day. Once a female had mated we began a remating trial, which consisted of pairing the female with inexperienced males every 10

min for one hr. We tested 10 females per stock. Generally, only two to three females were tested at a time.

## Results

We asked if there was significant genetic variation for ten different behavioral traits in *N. longicornis* that were related to a central concept, mating. Because the RILs represent different combinations of genes, any significant differences found between RILs represent significant genetic variation for the phenotypes of interest. We found significant differences between the RILs for six of the eleven behavioral traits measured, as described below.

### *Within-host Mating*

First, the two parental lines differed significantly from each other for WHM (Table 4.1). Additionally, WHM varied significantly between the RILs (Table 4.1). The mean percentage of females from the RILs that mated within the host ranged from 0% to 53%. Although the mean WHM values were continuously distributed, the distribution for the RILs was bimodal with each line resembling one of the parental lines. Only one RIL differed significantly from both parental stocks and was transgressive, having an average of twice as many females mate inside the host as parental stock P2.

Table 4.1. Behavioral trait mean values (± 95% CI) for two *N. longicornis* parental stocks and 24 recombinant inbred lines (RILs).

The number of RILs that exhibited phenotypes outside the means of the parental lines is indicated for each trait.

| | | | | | | Components of male courtship | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Line | %WHM | FRM | AI | %DIS | ASR | LM | LC | # FHN | CD | COPD | PCDD |
| P1 | 4.00±2.21 | 0.91 | 20.64±3.06 | 0.26±0.06 | 0.19±0.07 | 69.44±18.87 | 6.75±0.98 | 1.79±0.34 | 9.84±1.84 | 13.28±0.52 | 18.59±0.66 |
| P2 | 24.00±8.25 | 0.42 | 19.91±2.64 | 0.24±0.12 | 0.11±0.05 | 82.61±15.82 | 8.19±1.05 | 3.12±0.54 | 29.81±8.06 | 14.92±1.21 | 17.81±0.52 |
| P1 vs. P2 | ** | * | ns | ns | * | ns | ns | ** | ** | ns | ns |
| RILs (mean) | 8.53±6.01 | 0.81±0.09 | 17.82±1.02 | 0.24±0.04 | 0.14±0.02 | 79.91±9.93 | 6.84±0.54 | 2.31±0.30 | 20.50±4.17 | 14.72±0.60 | 17.82±1.05 |
| RILs (range) | 0-53.00 | 0.33-1.00 | 13.65-23.35 | 0.09-0.38 | 0.09-0.27 | 53.42-142.83 | 5.08-9.25 | 1.33-3.67 | 6.08-37.00 | 11.67-16.67 | 11.42-22.00 |
| # transgressive | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 6 |

Behavioral traits: %WHM (% within-host mating), FRM (proportion of females that remated), AI (aggression index), %DIS (percent males to disperse), ASR (adult sex ratio), LM (latency to mount), LC (latency to court), #FHN (number of first head nods), CD (courtship duration), COPD (copulation duration), PCDD (post-copulatory display duration).

** = $P<0.001$ ;Wilcoxon signed-rank test

* = $P<0.01$

ns = $P>0.05$

*Female Remating*

The two parental lines differed significantly from each other (Table 4.1). In P1, 91% of females tested remated within a 10 min period, while only 42% of females remated in line P2. The range of female remating for the RILs approximated the range of remating in the parental lines. We found significant differences between the RILs indicating that there was significant genetic variation for female remating behavior (Table 4.1).

*Male Site Fidelity, Aggression and Dispersal*

We found no significant differences between the parental lines or the RILs for male aggressive behavior toward conspecific males and none of the RILs differed significantly from the parental stocks (Table 4.1).

*Male courtship behavior*

Four of the six components of the first (naïve) male courtship sequence showed significant genetic variation in the RILs. These were the number of first head nods, the duration of courtship, copulation duration and duration of the post-copulatory display. Both parental lines differed significantly for both the number of first head nods to elicit a receptivity signal from a virgin female and the duration of courtship (Table 4.1). The RILs differed significantly from each other for both the number of first head nods

(Kruskal-Wallis test: $H_{23} = 150.73$; $P<0.001$) and courtship duration (Kruskal-Wallis test: $H_{23} = 155.08$; $P<0.001$).

For the other two components of the male courtship sequence, copulation duration and the duration of the post-copulatory display, the parental lines did not differ significantly from each other but there was significant variation among the RILs (Table 4.1). However, both of these traits had RILs that were transgressive. For copulation duration, 4 of the 24 RILs differed significantly from both parental lines; 3 of the RILs showed longer copulation durations, while 1 RIL had a shorter copulation duration. Additionally, 6 of the 24 RILs differed significantly from both parental lines for the duration of the post-copulatory display; three of the lines had a longer post-copulatory display and three lines had a shorter post-copulatory display. Only one of these lines, R271 was transgressive for both of the above traits. So, in this case, we discovered genetic variation for two traits that did not show differences between the two parental stocks.

*Phenotypic (Genetic) Correlations*

We used the means for each of the eleven behavioral traits for the 24 RILs to determine if the traits were correlated. Phenotypic correlations (non-parametric Spearman's ρ) between RILs provide estimates of genetic correlations, although the correlations are not based on estimates of additive genetic variation. We corrected for multiple comparisons by using a Bonferroni adjustment which lowered the alpha level to

0.0021. We found four significant correlations between the 11 behavioral traits (Table 4.2). There was a strong positive correlation between WHM and female remating frequency where RILs with high WHM also had high female remating frequencies. However, this relationship appears to be a threshold function rather than linear (Figure 4.2). In RILs with remating frequencies ≤ 50%, WHM did not occur. However, WHM was found at varying frequencies for lines with remating frequencies ≥ 50%. In contrast to the parental lines, WHM only occured at appreciable frequencies (> 15%) when the remating frequency was 100%. Line P1 had the higher remating frequency (91%), but had the lower mean frequency of WHM (4%), while line P2 had a low remating frequency (42%) but had the higher mean frequency of WHM (24%; Table 4.1, Figure 4.2).

The three remaining significant correlations were centered around two of the components of courtship. The number of first head nods was strongly positively correlated with the courtship duration for first males (Table 4.2). Males that produced fewer head nods had shorter courtship durations and males that produced more first nods had longer courtship durations. The strong relationship is most probably due to these two traits being essentially the same measure. They were treated as independent here because most other studies assumed that the number of first nods is the more important measure (Jachmann and van den Assem 1993, 1996), until a recent study suggested that courtship duration was the more important measure as the number of head nods does not account for much of the variation in time spent courting a female (Leonard and Boake 2008). Both of the above traits are strongly negatively correlated with female remating

Table 4.2. Phenotypic correlations (Spearman's ρ) for 11 behavioral traits between 24 *N. longicornis* RILs.

| | | | | | Male courtship components | | | |
|---|---|---|---|---|---|---|---|---|
| | WHM | Male dispersal frequency | Remating frequency | Adult sex ratio | # First head nods | Courtship duration | Copulation duration | Post-copulatory display duration |
| WHM | - | | | | | | | |
| Male dispersal frequency | **-0.413*** | - | | | | | | |
| Remating frequency | **0.777**** | -0.232 | - | | | | | |
| Adult sex ratio | **-0.450*** | 0.159 | -0.363 | - | | | | |
| # First head nods | -0.333 | 0.167 | **-0.652**** | **0.450*** | - | | | |
| Courtship duration | -0.337 | 0.163 | **-0.650**** | 0.401 | **0.966**** | - | | |
| Copulation duration | -0.065 | -0.279 | -0.295 | 0.099 | 0.264 | 0.364 | - | |
| Post-copulatory display duration | -0.340 | 0.328 | -0.118 | -0.120 | -0.313 | -0.180 | 0.312 | - |

Bold numbers = P<0.05 (not significant after Bonferroni correction)
* = p<0.001 (significant after Bonferroni correction)

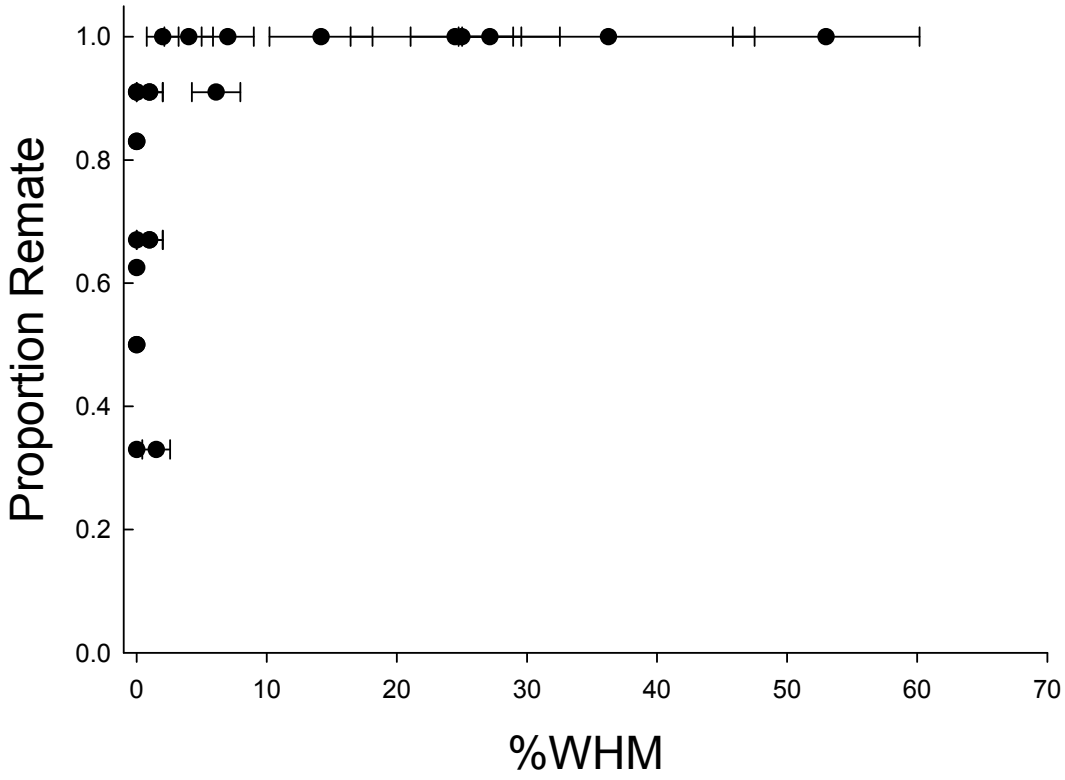


Figure 4.2. A positive correlation between remating frequency and WHM for 24 *N. longicornis* RILs. The proportion of females that remated is plotted against the mean percentage of females that mated inside the host (± 95% CI). Grey dots are the parental lines.

frequency (Table 4.2): RILs with high female remating frequencies had first males that produced fewer first head nods and shorter courtship durations (Figure 4.3A).

*Relationship Between Development and Behavior*

Our preliminary data analysis suggested a role for female sexual receptivity in the observed variation in WHM and female remating frequencies. We found that females from RILs with high remating frequencies, and thus high WHM, became receptive rapidly when courted by a second male, whereas females from RILs with lower remating frequencies took a longer time to become receptive during the second courtship (Figure 4.3B). Thus, early receptivity may afford females the opportunity to mate before emergence, and mate more often than females that do not become receptive early. Based on these data, we hypothesized that females from RILs with high levels of remating and WHM became sexually receptive earlier during development than females from RILs with low levels of remating and WHM.

Initially we needed to determine if there were differences in the developmental time for both males and females, as well as the time from egg to emergence from the host puparia. We tested 6 RILs plus both parental lines and one line each from *N. vitripennis* (NV1) and *N. giraulti* (NG1). We chose three high WHM lines and three low WHM lines from the RILs. We found developmental differences both within *N. longicornis* and between the species. The parental lines showed significant differences in both male and female developmental times and time from egg to male eclosion (Figure 4.4). Males and

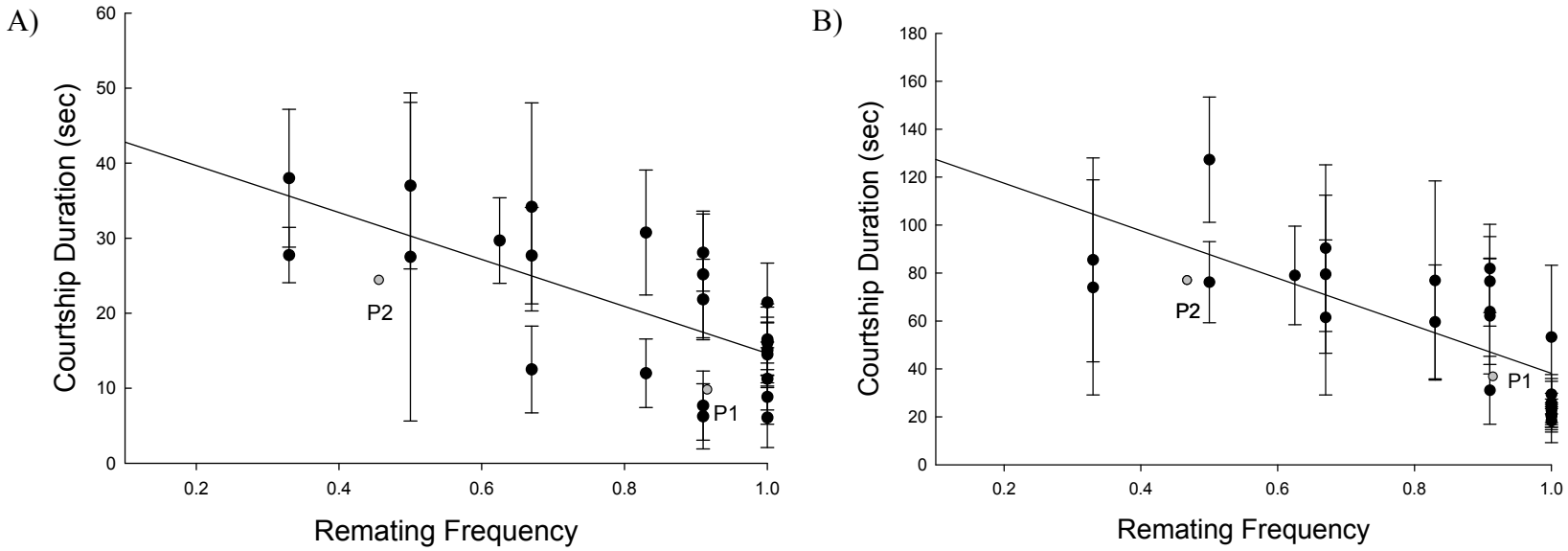


Figure 4.3. Linear regression of mean courtship duration (± 95% CI) on remating frequency for 24 N. longicornis RILs. A) Courtship durations are for first males presented to virgin females (ANOVA; $P<0.001$). B) Courtship durations are for second males presented to already mated females in trials in which remating occurred (ANOVA; $P<0.001$). Grey dots are for parental lines.

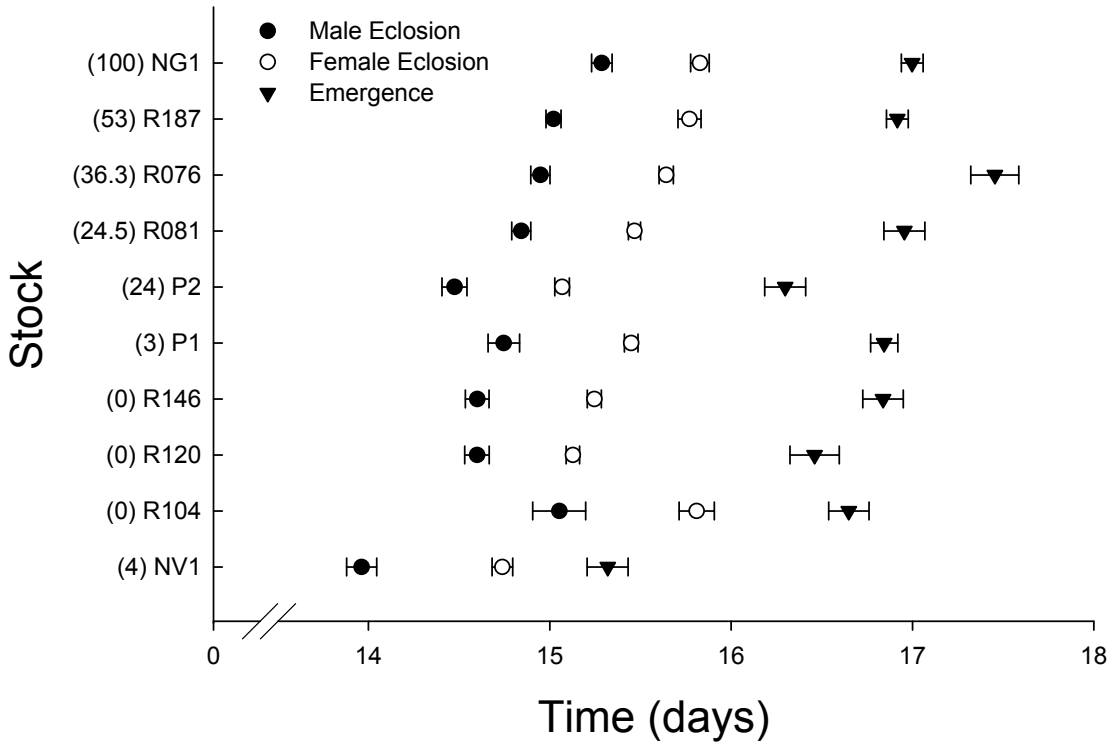


Figure 4.4. Developmental differences for six *N. longicornis* RILs, the parental lines and one stock each of the other two species, *N. vitripennis* and *N. giraulti*. Mean times from egg to adult eclosion (± 95% CI), as well as adult emergence from the host puparia. Numbers in parentheses on the Y-axis are mean WHM percentages for that line. Once a hole has been chewed in the host puparium, adults emerge in a pretty constant stream, and the sex of the first individual to emerge is usually male.

females from line P1 took more time from egg to eclosion and egg to emergence than from line P2 (Figure 4.4).

The RILs differed significantly for all three traits (Figure 4.4). Three of the six RILs tested (R104, R076, and R187) had significantly longer developmental times than either parental line (Figure 4.4). The remaining three lines resembled parental line P1. Egg to emergence times were more variable in the RILs and did not show as consistent a pattern as in the parental lines (Figure 4.4). We found no significant difference in developmental times of lines R076 and R187, but a significant difference in their emergence time: line R076 emerged from the host puparia approximately 0.5 days later than R187 (Figure 4.4).

We also measured these traits in one line each for the other two species. *Nasonia vitripennis* stock NV1 had the shortest male and female developmental times, as well as egg to emergence time (Figure 4.4) and differed significantly from both other species for all three traits. *Nasonia giraulti* stock NG1 had the longest developmental times of the three species and differed significantly from NV1 and both *N. longicornis* parental lines. The *N. longicornis* parental lines were intermediate between the other two species (Figure 4.4).

We tested for a relationship between developmental timing and WHM within *N. longicornis* males and females. Within the RILs, the three lines with high WHM showed consistently longer male and female developmental times than the three lines with low

WHM (Figure 4.4). So, while there appears to be a general relationship within the RILs, the relationship found across the RILs is the opposite of the pattern for the parental lines (Figure 4.4).

The developmental onset of female sexual receptivity could influence WHM and female remating frequencies. We investigated potential differences, both within *N. longicornis* using the RILs and the parental lines, and between the species using one line each of *N. vitripennis* and *N. giraulti*. First, we found that the parental lines differed from each other in when females became sexually receptive after eclosion. Seventy percent of females tested for parental line P1 became receptive within 30 min after eclosion with the remainder being receptive in the tests 24 hr after eclosion (Table 4.3). In contrast, none of the females tested for line P2 were receptive until 24 hr after eclosion.

The RILs showed a more variable pattern for the onset of sexually receptivity in females, with these differences being significant. In some lines females became sexually receptive very soon after eclosion, generally within 30 min and in one case within 2 hr (Table 4.3). Lines R081, R076 and R187 shared the pattern of becoming sexually receptive almost immediately after eclosion (Table 4.3). In the remaining three RILs (R104, R120, and R146) the pattern was bimodal, with some females becoming receptive within 30 min of eclosion (Table 4.3) and the rest not becoming receptive until the 24 hr test.

Table 4.3. Proportion of females that became receptive at five different time intervals following eclosion. Stocks below the dashed line are different species.

| | | Time from ecolsion to receptivity in females | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Stock/RIL | *N* | 30 min | 1hr | 2hr | 3hr | 4hr | 24hr | %WHM |
| R104 | 6 | 0.5 | - | - | - | - | **0.5** | 0 |
| R120 | 7 | 0.29 | - | - | - | - | **0.71** | 0 |
| R146 | 10 | 0.2 | - | - | - | - | **0.8** | 0 |
| P1 | 10 | **0.7** | - | - | - | - | 0.3 | 4 |
| P2 | 6 | - | - | - | - | - | **1.00** | 24 |
| R081 | 10 | **1.00** | - | - | - | - | - | 24.5 |
| R076 | 10 | **1.00** | - | - | - | - | - | 36.3 |
| R187 | 9 | **0.88** | - | 0.12 | - | - | - | 53 |
| NV1 | 10 | **0.9** | 0.1 | - | - | - | - | 3* |
| NG1 | 10 | **1.00** | - | - | - | - | - | 100* |

Bold numbers indicate significant differences between time categories, lines that have bold numbers within a time category do not differ from each other.
* = WHM values are from Leonard and Boake (2006)

The two lines tested from the other two species of *Nasonia* showed almost identical patterns with regards to female receptivity after eclosion. Lines NV1 and NG1 did not differ from each other : > 90% of females from both lines were receptive immediately after eclosion (Table 4.3). Additionally, parental line P1 did not differ from either of the other two species. Parental line P2 appears to be the only species stock that differed from stocks of the other two species (Table 4.3).

Because only line P2 of the pure species stocks tested had females that were not receptive immediately, we needed to determine that the lack of receptivity in this line was not due to age of the males used in the study (that is, recently eclosed males could be less capable of inducing female receptivity than older males). We performed an experiment with a factorial design in which we paired recently-eclosed females with males that had eclosed three days earlier, as well as pairing two-day-old virgin females with recently-eclosed males. In both tests, male age had no effect on female receptivity: all older females were receptive, while all recently-eclosed females were unreceptive (Table 4.4).

Within the RILs, there appeared to be a general relationship between the onset of sexual receptivity and WHM and female remating. RILs with high levels of WHM (and a high probability of remating) were receptive earlier than RILs with low levels of WHM (and a lower probability of remating). However, this relationship between these three traits did not appear between the parental lines. In fact, the majority of females from parental line P1 (low WHM), were receptive immediately and the females from line P2 (high WHM) were not receptive immediately. For these two traits, the parental lines and

Table 4.4. Results of experimental pairing between differently-aged males and females and controls of line P2.

| Pairing Type | *n* | Female receptive? |
|---|---|---|
| Young Male x Young Female | 6 | 0 % |
| Old Male x Young Female | 6 | 0 % |
| Young Male x Old Female | 10 | 100 % |
| Old Male x Old Female | 10 | 100 % |

Young = <30 min. after eclosion
Old = ~1 day after eclosion

the RILs showed opposite patterns. Interestingly, the relationship between sexual receptivity and female remating does appear to hold across RILs, parental stocks and species as all lines with early female receptivity showed high probabilities of female remating (*N. giraulti* was the exception, but see discussion).

**Discussion**

In a behavioral syndrome, not all phenotypically correlated traits need be genetically correlated even though all traits may be related to a central function, like reproduction. Cheverud (1988) suggested that genetic correlations should be detectable in any phenotypically correlated set of traits. However, the extent to which multiple behaviors are a genetically cohesive unit is an issue that needs to be addressed. Some traits, like behaviors, are context dependent and may only be used under certain environmental conditions, or a behavior may have no function in a particular species. For example, in some Hymenoptera, male post-copulatory behaviors are often phenotypically correlated with female remating frequency, but in some cases performance of these types of behaviors can be affected by male density and the receptivity status of the females (Alcock and Buchmann 1985, Allen et al. 1994), or in some cases species lack the behavior (Quicke 1997). *Nasonia longicornis* males perform a post-copulatory display, but this display has no detectable function in this species (Leonard and Boake 2008). These types of phenotypic relationships do not necessarily result in a genetic relationship between the behaviors. So, while we were able to detect significant correlations between three of the behaviors, the remaining seven behaviors appear to be genetically

independent of each other even though they are functionally related to reproduction. Thus, it is important to distinguish the phenotypic relationship and the genetic relationship between any set of behavioral traits.

*Genetic correlations*

The genetic correlation structure within *N. longicornis* weakly mimics that of the phenotypic correlations between the species. We are unable to determine from our data if the genetic correlation structure within *N. longicornis* is an accurate representation of the genetic relationship among behavioral traits between species. Begin and Roff (2003) found that for three species of *Gryllus* crickets the genetic covariance structure for five morphological traits was conserved across species. However, the covariance structure need not be the same for all species because correlations between different traits can result in parallel changes in behavior. For example, Moore et al. (2002) performed artificial selection on social dominance traits in *Nauphoeta cinerea* and showed that two different responses to selection resulted in an increase in social dominance. Pheromone composition was genetically correlated with dominance and in each case, pheromone composition changed, but the proportions of the components were different in the two lines after selection (Moore et al. 2002). The positive genetic correlation between WHM and female remating frequency is relatively consistent across species if we consider that *N. giraulti* males have evolved an apparent ability to reduce female remating using their post-copulatory display (Leonard and Boake 2008). In other words, for all three species there is a positive correlation between WHM and female remating, but *N. giraulti* males are capable of reducing female remating frequencies, which can account for why the

correlation does not appear in this species. Additionally, the lack of a genetic relationship between female remating and the post-copulatory display in *N. longicornis* is most likely due to a lack of function of this behavior in this species (Leonard and Boake 2008). So, it is possible that the genetic correlations presented here are accurate representations of the correlations between species, but more data are needed to determine if selection for either WHM or remating would result in appropriate changes in these behaviors.

The probability of remating was correlated across the RILs with two other traits, WHM and the duration of male courtship, though these two traits were independent of each other. First, the duration of the first courtship and female remating were negatively correlated (Figure 4.3A). This correlation was in the expected direction based on the parental lines. However, the other correlation, a positive relationship between WHM and female remating, was in the opposite direction than expected. This observation has several possible explanations. One is that the breeding design resulted in selection for WHM and female remating. However, this was not possible because females were never allowed to mate inside the host or more than once during production of the RILs. A second explanation is that the parental lines were accidentally switched during laboratory culture. This is highly unlikely because we retested the parental lines for remating late in the experiment and found identical results to those at the start. A third explanation is that that the P2 phenotype is not recoverable in the RILs. For example, if the P2 parental line maintained heterozygosity at a single locus that has a major effect on both WHM and female remating (genotype AC, Figure 4.5) and parental line P1 was homozygous for a different allele (genotype BB, Figure 4.5), the resulting RILs ( which can be AA, BB or

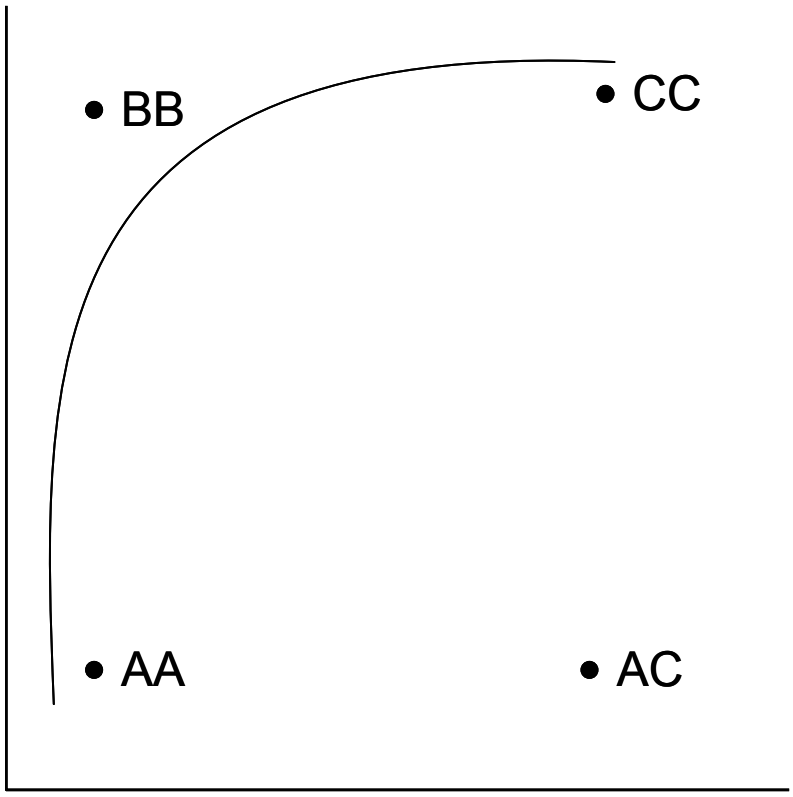


Figure 4.5. The relationship between correlated traits that are affected by a single locus with three alleles after fixation due to inbreeding. Genotypes AC and BB represent the parental lines and the curved line indicates the distribution of RILs. The initial cross between parental lines can never yield genotype AC.

CC) would all be homozygous after forced inbreeding and fixation of alleles (Figure 4.5). In this scenario, it is not possible to recreate the heterozygous genotype of parental line P2 and our results are consistent with this hypothesis. However, parental line P2 had been in continuous culture for over 4 years (~90 generations) so the likelihood of this line remaining heterozygous is low unless extremely strong selection in the laboratory was maintaining heterozygosity.

Six of the ten traits studied showed significant genetic variation (Table 4.1). For example, as mentioned previously, the post-copulatory display has no current function in *N. longicornis,* but in *N. giraulti* is strongly correlated with female remating frequency (Leonard and Boake 2008). Additionally, six *N. longicornis* RILs were transgressive for the duration of the post-copulatory display: three lines that had shorter durations than those in either parental line, and three lines that had longer durations than those in either parental line (Table 4.1). These data suggest that the genetic variation exists for populations of *N. longicornis* to increase (or decrease) the duration of this behavior should it be functionally relevant, as it is in *N. giraulti* (Leonard and Boake 2008).

*Transgressive Traits*

Transgressive traits are common in studies involving RILs but few studies have found transgressive behavioral traits (reviewed in Rieseberg et al. 1999). Multiple hypotheses have been proposed to explain transgressive segregation in hybrid populations. For example, the extreme phenotypes could be due to 1) the complementary

action of additive alleles at multiple loci, 2) the expression of rare recessive alleles normally masked in heterozygotes, 3) nonadditive effects within a single locus (overdominance), 4) nonadditive effects between loci (epistasis) and 5) chromosome number variation (Grant 1975, Voigt and Tischler 1994). Two of the three traits that showed transgressive phenotypes in *Nasonia* are male traits (copulation duration and post-copulatory display duration). Male haploidy eliminates several of the hypotheses above (1,2 and 3) as there can be no overdominance and rare recessive alleles are immediately expressed every generation and are likely to be purged from the population. Additionally, complementary genes with additive effects can not be an effective explanation because additive genetic variance is a construct of diploid populations. Variation in the number of chromosomes is common property of plants through polyploidization and is not a common feature in animals. This leaves epistatic interactions between alleles as the most likely source of transgressive traits in males, which provides evidence that epistatic interactions may play a more important role in evolutionary divergence than previously thought (Reiseberg et al. 1999).

Only one RIL was transgressive for WHM, line number R187, which with a value of 53% was much higher than either of the parental lines. This RIL had several characteristics in common with *N. giraulti* despite being derived from *N. longicornis*. Line R187 was developmentally the most similar to *N. giraulti* in egg-to-female and egg-to-eclosion times. The transgressive nature of this RIL suggests the potential for the right combination of alleles to result in a shift from the intermediate levels of WHM seen in *N. longicornis*, to a more *N. giraulti*-like state.

*Defining "a trait" in social interactions*

The expression of a behavior pattern is subject to environmental inputs especially when the environment is the behavior of another individual (Moore et al. 1997). The genotypes of the interacting pair create indirect genetic effects on the expression of a behavior pattern (Moore et al. 1997, Wolf et al. 1998). In many cases, defining the behavior as a strictly male or female trait becomes difficult because the interaction (called interacting phenotypes) between the sexes during reproductive behaviors dictates the outcome (Moore et al. 1997). In this case, selection can then act on the interaction, and not simply the individual components of the interaction. In *Nasonia*, several of the behaviors described here may not simply be male or female traits. For example, the duration of male courtship is composed of male persistence to remain in the courtship position and continue his head nodding sequence, but it could also be a female trait in that she can dictate how long he needs to court her in order to get her to produce a receptivity signal. Selection on the interaction should not change the covariance structure and can even strengthen the correlation because selection is acting on the interaction (Moore et al. 1997). The reduction in remating frequencies that is correlated with the duration of courtship could have developed in this manner. Factorial mating tests between divergent RILs could show to which sex this behavior could be assigned, or if the interaction is most important.

*Developmental Shifts*

The age of onset of female receptivity could potentially have a significant influence on a suite of mating behaviors. In the *N. vitripennis* and *N. giraulti* stocks that we tested, all females were immediately receptive to courtship following eclosion. In the third species, *N. longicornis*, females varied in whether receptivity was delayed or immediate (Table 4.3). Our RILs exhibited consistent differences between lines in timing, indicating the presence of a genetic component to the onset of female receptivity.

We found that the age of onset of female receptivity and the frequency of female remating were positively associated genetically (Table 4.3). This pattern was observed in both *N. longicornis* parental stocks and their RILs, as well as in our population of *N. vitripennis*. *Nasonia giraulti* appears to contradict this pattern in that females are immediately receptive yet also have a low frequency of female remating (Leonard and Boake 2008). However, we must note that the lower frequency of remating in *N. giraulti* appears to be associated with the duration of the male post-copulatory display, by which males appear to have a direct effect on the continued receptivity status of the female (Leonard and Boake 2008). Thus this species appears to operate under different rules from the others and raises the possibility that *N. giraulti* males are manipulating female receptivity.

The relationship, if any, between the age of onset of female receptivity and WHM remains unclear. Interestingly, a positive correlation was observed between delayed

female receptivity and low levels of WHM in the RILs being tested (Table 4.3). The association is opposite that in the two *N. longicornis* parental lines from which the RILs were derived. That is, P2 exhibited delayed receptivity in all individuals tested, while the line also exhibits high levels of WHM. Correspondingly, P1 exhibited immediate receptivity in 70% of females tested, while the line also exhibits low levels of WHM. Further, females from lines of the other species, NV1 and NG1, all exhibited immediate receptivity. Line NV1 displayed almost no WHM, whereas NG1 displayed near-complete WHM (Drapeau and Werren 1999, Leonard and Boake 2006). Our data suggest that WHM is not dependent solely upon the timing of initial female receptivity and that other factors are involved in successful copulation inside the host puparium.

The functional relationship between behavioral traits and development in *Nasonia* is a complex one. It appears that the majority of traits act independently of each other, all for the common outcome of mating, but are dependent on the receptivity status of the female. However, because the majority of traits are independent, the genetic relationship between this suite of traits seems very simple. Because we tested RILs, they can now be used to identify regions of the genome involved in these behavioral differences, as well as to identify genes involved in genetic and phenotypic divergence between species.

# Chapter 5 - Ecological associations between *Protocalliphora* blowflies and three species of the parasitoid wasp *Nasonia*.

## Introduction

The experiments described in previous chapters gave results that need an ecological context for explanation. The behavioral differences in *Nasonia* may have evolved due to overlapping ecological requirements. In particular, within-host mating (WHM) has been proposed as being related to species isolation because this behavior is hypothesized to have evolved as a by-product of selection against hybridization in areas of sympatry (Drapeau and Werren 1999, Leonard and Boake 2006). This hypothesis requires that two species of wasp would need to parasitize the same host at the same time. The incidence of nest and host sharing would need to be compared between the two species that show strong differences for WHM (*N. vitripennis* and *N. giraulti*) and the two species that show little or no difference in WHM (*N. vitripennis* and *N. longicornis*).

All three species of *Nasonia* are pupal parasitoids of cyclorraphous flies belonging to the genera *Protocalliphora*, *Calliphora* and *Sarcophaga*. Flies of genera *Protocalliphora*, which prey on nestling birds, are the natural host of *Nasonia* (Darling and Werren 1990, Whitworth 2006). However, little is known about natural populations as evidenced by the discovery of two new species in 1991 (Darling and Werren 1991). *Nasonia vitripennis* is a cosmopolitan species, whereas *N. longicornis* and *N. giraulti* are restricted to the western and eastern portions of the United States respectively where they share their ranges with *N. vitripennis* but not each other. All three species are small gregarious ectoparasitoids and can lay as many as 140 eggs in a single *Sarcophaga* pupa (personal observation). These wasp species can interact with both their host species and

with each other, which could influence how *Nasonia* are distributed across the United States. *Nasonia vitripennis* is a generalist and will feed on multiple different genera of fly pupae whereas *N. longicornis* and *N. giraulti* are thought to be more specialized as these two species have only been found in bird nests (Darling and Werren 1990). Where *Nasonia* range overlap occurs (see Figure 5.1), in both the eastern and western U.S., two species can be found in the same bird nest and even in the same host (Darling and Werren 1990). However, the frequency of nest and host sharing is critical to evolutionary change in WHM because of the potential for both competition and hybridization.

Investigating current patterns of species distributions can help identify present selection pressures and allow us to infer a plausible adaptive mechanism for the evolution of WHM. Recently collected populations of *N. giraulti* show high levels of WHM while populations of *N. vitripennis* show low levels of WHM (Drapeau and Werren 1999), which suggests that these two species share the same nests and hosts where their ranges overlap in the eastern United States (Darling and Werren 1990). Additionally, *N. girualti* may have evolved high WHM because it was initially the less common species and therefore at higher risk of mating with the wrong species, *N. vitripennis* (Leonard and Boake 2006). This would in turn increase the strength of selection favoring WHM, allowing its frequency to increase in *N. giraulti*. Thus, the intensity of selection in favor of WHM in *N. giraulti* is hypothesized to be proportional to the frequency with which the two species share hosts. Because *N. vitripennis* and *N. longicornis* overlap in the western U.S., but show only a weak difference in WHM, I would hypothesize that these two species would share fewer nests and hosts than *N. vitripennis* and *N. giraulti*.

Dr. Jack Werren at the University of Rochester provided me with a data set in which he had examined bird nests collected across the United States to describe the distribution of all three species of *Nasonia* (Darling and Werren 1990). I used these data to develop initial answers to some of the hypotheses above. First, I wanted to determine why there is an apparent barrier between *N. longicornis* and *N. giraulti* in the central portion of the U.S. (Figure 5.1). Does this gap occur because *N. longicornis* and *N. giraulti* are closely tied to the distribution of their host resource or because *N. vitripennis* is excluding these two species from the central U.S.? I hypothesized that because these two species are restricted to bird parasites there would be a direct relationship between the distribution of both blowflies and wasps across the U.S., in particular that bird blowflies would be less common in the central U.S. Second, I wanted to determine the degree to which the three species share bird nests. I predicted that the proportion of nests with two species should be greater for *N. vitripennis* and *N. giraulti* than for *N. vitripennis* and *N. longicornis* (see description above). Finally, I wanted to determine how frequently two species could be found in the same host puparium.

**Materials and Methods**

All data were collected by Dr. Jack Werren and his students at the University of Rochester, N.Y. for the years 1987-1994 and 1997-2000. In 2002, I examined nests collected in 2000, focusing on species identification of wasps from individual hosts.

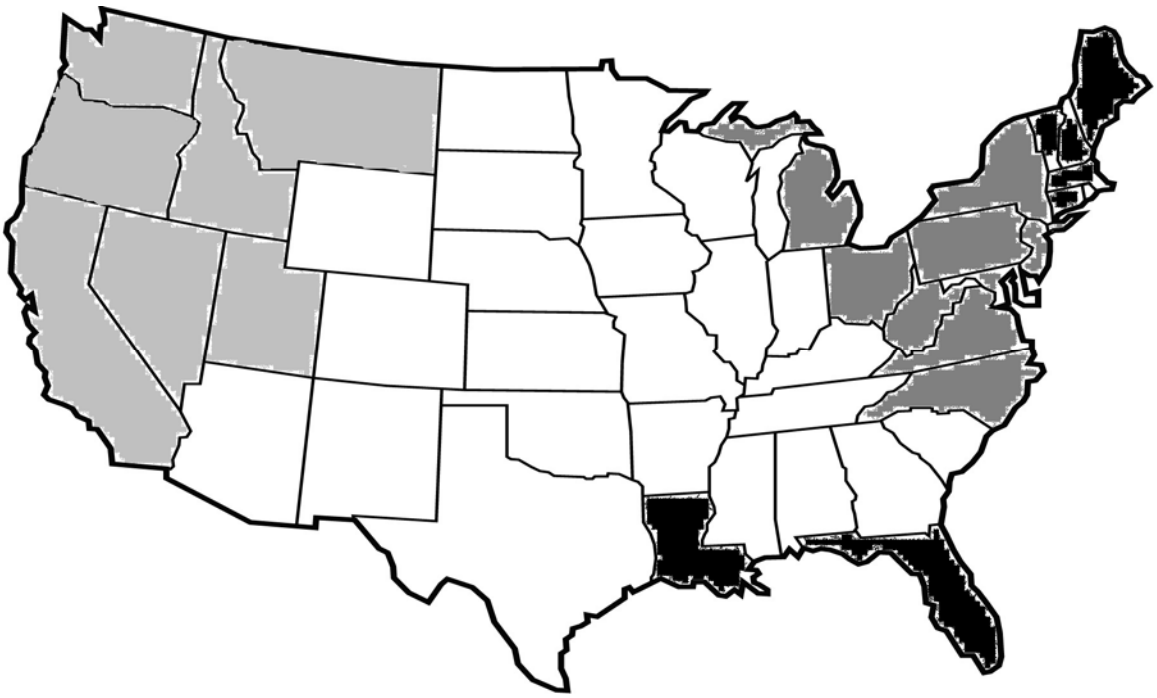

Figure 5.1. Species distributions of three species of *Nasonia*. White areas are where *N. vitripennis* only has been found, light grey areas are where *N. longicornis* has been found and dark grey areas are for *N. giraulti*. *Nasonia vitripennis* has been found in all the states where *N. longicornis* and *N. giraulti* live. States colored in black have not been sampled. Based on data found in Darling and Werren (1991).

For the years 1987-1994 and 1997, data were collected as follows. Bird nests were collected immediately after the baby birds had fledged and were sent individually to the University of Rochester. The nests were left in individual bags at room temperature upon arrival. Each bag was checked twice daily for wasp emergence. When wasps had emerged, all were removed from the bags and a small sample of males (when available) was taken and identified to species using wing size and antennal length as diagnostic traits. When no more wasps were found in bags, bird nests were dissected and all fly pupae and dead flies were collected and counted. Fly pupae were categorized as 1) a fly had emerged (the pupal case had a large hole in one end), 2) wasps had emerged (the pupal case had a very small hole), 3) parasitized but no adult wasps (wasp larvae were still inside the fly pupal case), or 4) bad host (the fly died during development and was not available to be parasitized). Flies were not identified to species. I used these data to calculate mean values for fly infestation (total number of flies per nest), as well as habitat and range overlap for the species of *Nasonia*.

The nests collected for the years 1998 to 2000 in New York were evaluated in more detail than other collections in that Dr. Werren had asked his student employees to keep track of the wasp species emerging from each host rather than each nest. Accordingly, the bird nests were dissected upon arrival and each fly pupa was placed into an individual vial. Pupae were kept in an incubator and watched daily for wasp or fly emergences. When wasps emerged, a sample of males was taken from the vial and identified to species. For 1998 to 2000, the same data were calculated as for the other years.

To evaluate fly infestation in bird nests, I needed to combine all years and bird species for each state into a single data set. This was because the sample sizes for each bird species were extremely inconsistent and the same bird species was not sampled from every state. In many states, nests were only sampled for one or two years and neither the specific years nor the number of years was consistent across all states sampled. Finally, the numbers of nests sampled from each state varied dramatically (Figure 5.2); in some cases pooling the data within a state was the only method for obtaining a large enough sample size to identify distribution patterns.

I was able to address my question of the number of wasp species per bird nest for only two states, New York and Utah. These two states had the largest sample sizes across multiple years and had the most thoroughly collected data. All other states sampled for wasp species had too many cases of "unknown wasp species" found in the nests. Additionally, as stated above, many states were sampled only in one or two years, and in at least one case only *N. vitripennis* was found even though *N. longicornis* was known to inhabit the same area (Darling and Werren 1990).

Finally, for the evaluation of the frequency of host sharing, only the data from New York for the years 1998 to 2000 were adequate for analysis. Data were collected from Utah in the year 2000, however, no *N. longicornis* were found in the 12 nests that were dissected.

I used chi-squared tests to identify differences between states for the abundances of each species and their degree of nest sharing and host sharing. For each test, alpha = 0.05.

## Results

### *Blowfly patterns across the United States*

I examined whether there was regional variation across the U.S. in the incidence of blowfly infestations in bird nests because blowflies are the most important known resource for two species of *Nasonia, N. giraulti* and *N. longicornis.*. I pooled data for all bird species and years to estimate an overall infestation rate within each state sampled and found substantial variation between states (Figure 5.2). States in the eastern U.S. north of North Carolina had an average of greater than 50% of nests infested with blowflies, and the same trend is evident west of Colorado, and the central U.S. north of Illinois. Several states had infestation rates higher than 90%, including Colorado, Montana, Minnesota and Virginia. In contrast, the central and south eastern portions of the U.S. from Texas, Louisiana, and Georgia north to Nebraska, Illinois and Iowa had an average of fewer than10% of nests infested with blowflies (Figure 5.2).

It is possible that the absence of fly infestations in the central portion of the country was due to inadequate sampling. This would be suggested if a single nest had a large number of flies, while the rest had very few. I examined the frequency

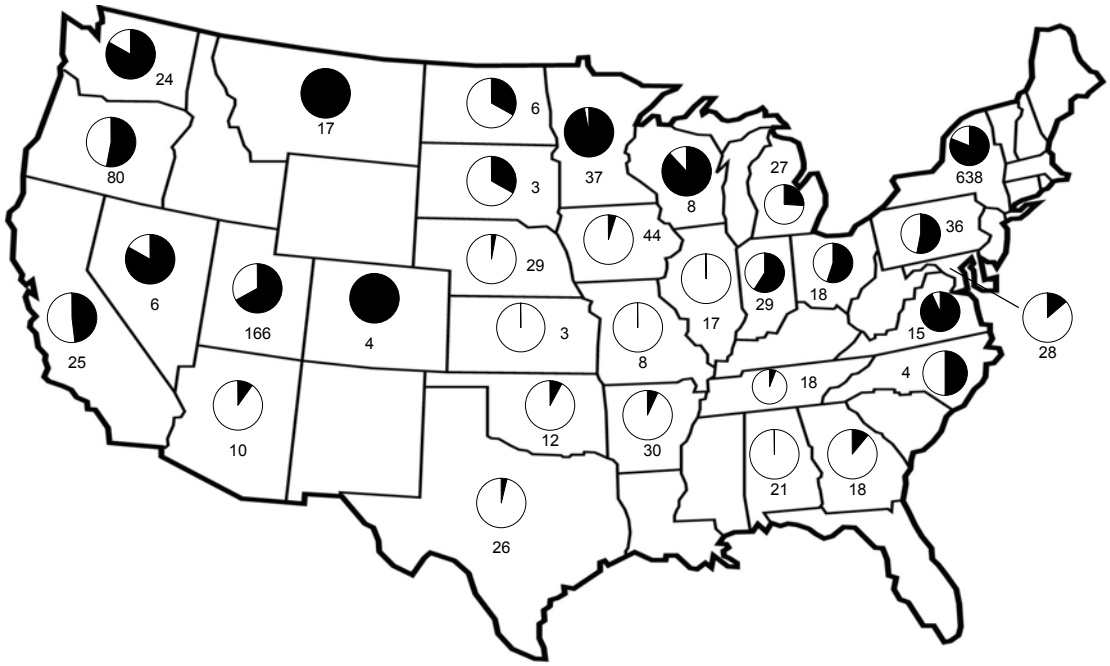


Figure 5.2. Proportions of bird nests inhabited by blowflies across North America. Data from all years and from multiple bird species were pooled within each state. Black areas are proportions of nests that had flies and white areas are proportions of nests that did not have flies. Numbers within each state are the number of nests sampled.

distribution of infestation for each state by using seven categories of fly pupal densities, ranging from 1-25 (smallest) to 300-399 (largest; Figure 5.3). In the central U.S. the few nests that were infested with blowflies belonged to the lowest category of fly pupal densities (Figure 5.3) indicating that blowflies are uncommon rather than that sampling error affected the data. States that had high infestation rates (Figure 5.2) also showed variation for the numbers of fly pupae found in the nests (Figure 5.3). For example, over 95% of the nests sampled from Minnesota were infested with flies, and the numbers of fly pupae in the nests ranged from 5 to 311. The variation in pupal densities seen in Minnesota was common in states where infestation rates were high (Figures 5.2, 5.3).

In summary, the absence of *N. longicornis* and *N. giraulti* from the central portion of the U.S. (Figure 5.1) appears to be associated with the absence of *Protocalliphora* blowflies in the same portion of the country (Figures 5.2-5.3). These two species of *Nasonia* are found in states in which a large proportion of nests are infested with blowflies (Figure 5.1); New York, Pennsylvania, Ohio and Virginia are common areas for *N. giraulti* whereas Oregon, Washington, Utah, and Montana are common areas for *N. longicornis*. This result suggests that *N. longicornis* and *N. giraulti* are far more dependent on *Protocalliphora* as hosts than *N.vitripennis*.

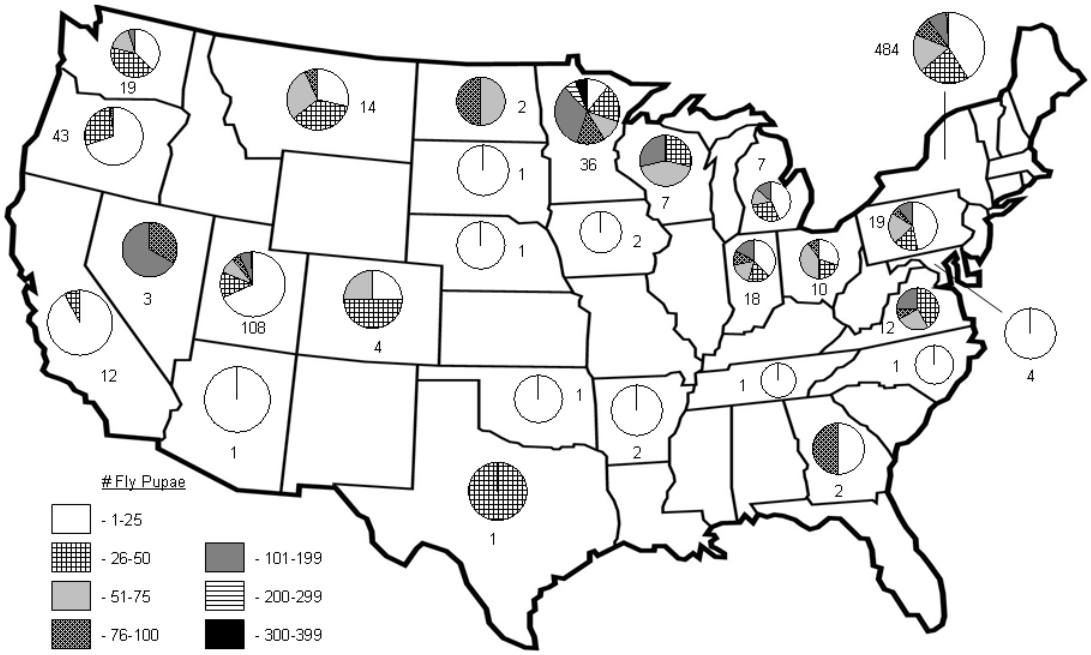


Figure 5.3. Proportion of bird nests with different densities of blowfly pupae across the United States of America. Numbers within each state are the numbers of infested nests examined.

*Nest overlap in Nasonia*

For nests where *Nasonia* could be identified, only two states had sample sizes sufficiently large to calculate the proportions of nests with one or two species of *Nasonia*, Utah (43 nests) and New York (167 nests). In New York state, *N. vitripennis* was frequently found by itself in birds nests (Figure 5.4A). Overall, 119 nests (71%) had only *N. vitripennis*, 9 nests (5%) had only *N. giraulti*, and 39 (24%) nests had both species. In only two of the eight years was *N. giraulti* found by itself (Figure 5.4A). Over all years, the proportion of total nests that contained *N. giraulti* was the lowest for all three species (Table 5.1) and never exceeded 50% in any year. The majority of nests that contained *N. giraulti* also contained *N. vitripennis* (Figure 5.4A, Table 5.1).

In Utah, the pattern was much different than for New York. Over the six year sampling period, *N. longicornis* was the more common species (Figure 5.4B). Overall, 7 nests (16%) had only *N. vitripennis*, 23 nests (53%) had only *N. longicornis* and 13 nests (31%) had both species. For five of the six years, *N. longicornis* was found by itself in at least 40% of the nests sampled.

The two states differed significantly for all comparisons of wasp species composition (Table 5.1). New York state had a higher proportion of total nests that contained *N. vitripennis* as well as a much higher proportion of nests with only *N. vitripennis* (Table 5.1). Additionally, the overall proportion of nests that contained *N. longicornis* in Utah (83%) was much greater than the overall proportion of nests that

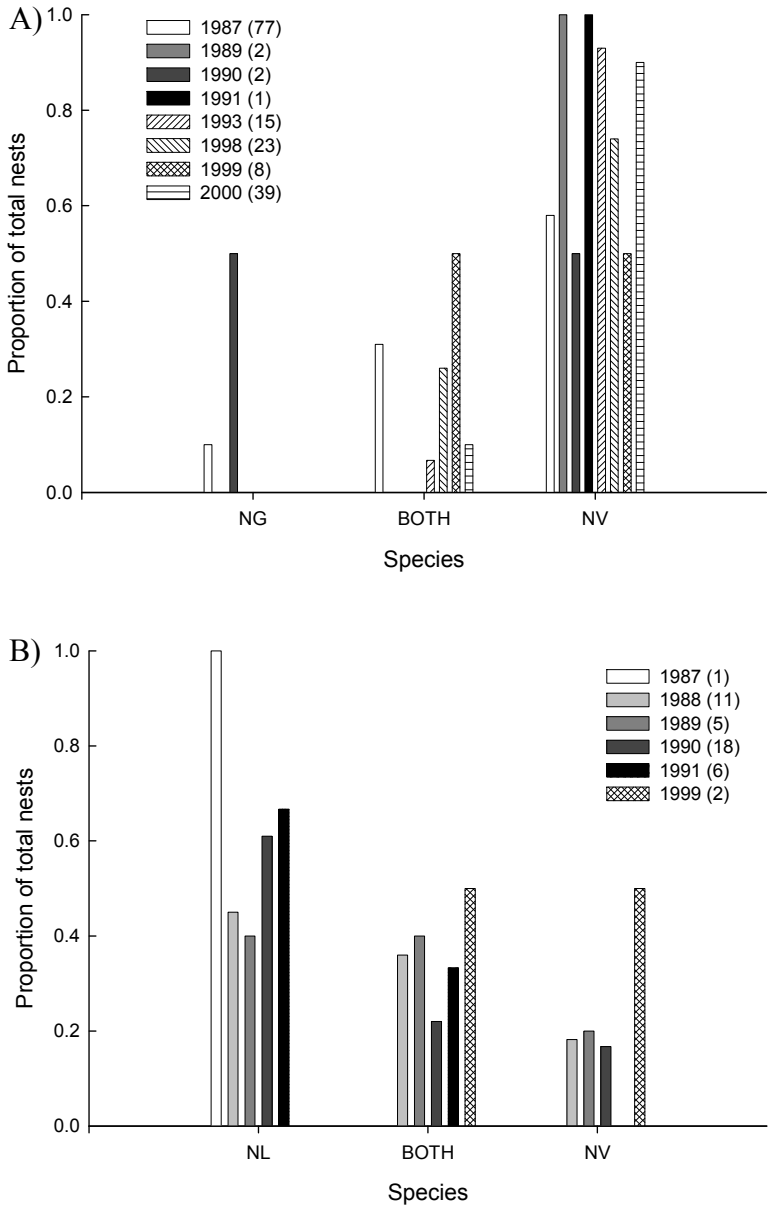


Figure 5.4. Proportion of total bird nests occupied by one or two species of *Nasonia* in A) New York, U.S.A. and B) Utah, U.S.A. Bars are coded identically by year for each figure. Numbers in parentheses are numbers of nests sampled for that year. A) NG = *N. girault*i; NV = *N. vitripennis*. B) NL = *N. longicornis*; NV = *N. vitripennis*.

Table 5.1. Proportion of bird nests inhabited by one or two species of *Nasonia* in two states in the U.S. across all years and bird species sampled. Total proportions are for any nest that contained that species.

| Species in Nests | New York (N = 167) | Utah (N = 43) | $X^2$ | p value |
|---|---|---|---|---|
| Total NV | 0.946 | 0.465 | 70.4 | <0.0001 |
| Total Other sp. | 0.287 | 0.837 | 112.0 | <0.0001 |
| NV Only | 0.713 | 0.163 | 27.7 | <0.0001 |
| NV and Other sp. | 0.234 | 0.302 | 42.1 | <0.0001 |
| Other sp. Only | 0.054 | 0.535 | 71.5 | <0.0001 |

NV = *N. vitripennis*
Other sp.: New York = *N. giraulti* ; Utah = *N. longicornis*

contained *N. giraulti* in New York (29%) indicating that of all three species, *N. giraulti* was the least common, at least in the states that were sampled (Table 5.1).

Were *N. longicornis* and *N. giraulti* found co-inhabiting bird nests with *N. vitripennis* with a higher probability than expected by chance? As a test I used the observed proportions of nests containing each species and multiplied the proportions to obtain an expected proportion of co-occurence, then compared that to the observed proportion of nests that contained both species. I did this for each state. For New York, the expected proportion of finding both species in the same nest (0.27) was not significantly different from the observed proportion (0.23, $X^2 = 1.72$, $p>0.19$). In Utah, there was also no significant difference between the expected proportion (0.38) and the observed proportion (0.30, $X^2 = 1.67$, $p<0.19$). These data indicate that the species neither avoided nor were attracted to each other.

*Incidence of Host Sharing*

Only New York for the years 1998 to 2000 provided data to allow me to assess the frequency of host sharing, as opposed to sharing nests for *N. vitripennis* and *N. giraulti*. For all years, 18 nests contained both species and 767 fly pupae were evaluated ($\overline{X} = 42.6$ fly pupae/nest). On average, these two species did not share an appreciable number of hosts (Figure 5.5). The data from these years are similar to the nest sharing distribution pattern above where *N. vitripennis* was the more common species and *N. giraulti* was found by itself or with *N. vitripennis* with equal probability. However, no

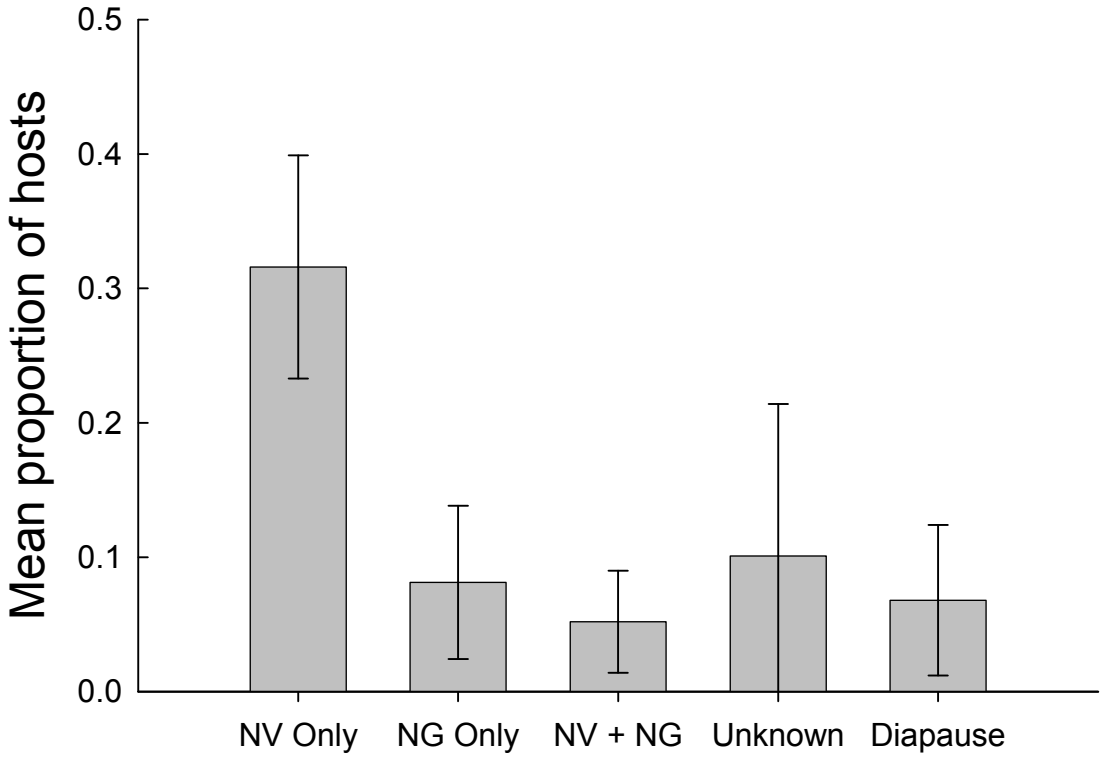


Figure 5.5. Mean proportion of hosts (± 95% CI) occupied by one or two species of *Nasonia*, where they were identified and in nests with both species, for the years 1998 to 2000 in New York, U.S.A. *Nasonia vitripennis* was found most frequently by itself within a single host puparium while *N. giraulti* was found by itself or with *N. vitripennis* with equal probability.

more than 21% of hosts from a single nest contained both species. Of the 24 fly pupae in that nest, a total of 17 was parasitized: 10 were parastized by *N. vitripennis* only, 2 held *N. girault*i only, and 5 held both species.

I calculated the probability of joint occupancy by multiplying the observed mean probability of finding *N. vitripennis* in a host (0.314) with the probability of finding *N. girault*i in a host (0.083) to obtain an expected probability of joint occupancy (0.026). This value does not differ from the observed probability of finding both species in a single host (0.052, $X^2 = 1.45$, $p>0.20$) indicating that each species was parasitizing hosts independently of the other.

**Discussion**

The species *N. longicornis* and *N. girault*i are physically isolated from each other by a large gap in the central portion of the U.S. (Darling and Werren 1990, Figure 5.1). Bird nests in this region of the country do not have an abundance of the blowfly pupae that these wasp species use as a resource (Figures 5.2 and 5.3). Of the approximately 28 species of *Protocalliphora* in North America, the majority are found in eastern and western North America (Whitworth 2006). It appears that the absence of *Protocalliphora* species creates a geographic barrier for these two wasp species as they may be specialized to feed only on this genus of flies. To date, only *N. vitripennis* has been found to attack flies outside the genus *Protocalliphora* in natural populations (Wylie 1958, Whiting 1967, Quicke 1997). In the laboratory, I have reared both species on two other

fly genera, *Sarcophaga* and *Calliphora*. *Nasonia longicornis* and *N. giraulti* do not perform as well as *N. vitripennis* on these hosts, in that they are less likely to parasitize the host, are more likely to enter diapause, and in *N. giraulti* have reduced adult longevity (J.L. personal observation).

Previous studies (Drapeau and Werren 1999, Leonard and Boake 2006) had hypothesized that the incidences of nest and host sharing should be different in the eastern and western portions of the U.S. The species of *Nasonia* differ in several reproductive behaviors, most notably the location of mating. *Nasonia vitripennis* females mate almost exclusively after emergence outside the host puparium while *N. giraulti* females mate almost exclusively inside the host puparium before emergence (Drapeau and Werren 1999, Leonard and Boake 2006). This large behavioral difference is thought to have arisen as a by-product of selection against hybridization as there is an immense cost to mating with the wrong species due to hybrid inviability and *Wolbachia*-mediated incompatibilities (Drapeau and Werren 1999, Brodenstein et al. 2001, Leonard and Boake 2006). This hypothesis was predicated on the idea that *N. vitripennis* and *N. giraulti* should share more nests and hosts than *N. vitripennis* and *N. longicornis*, in effect creating a stronger selective environment for *N. giraulti*. Furthermore, Leonard and Boake (2006) had suggested that in addition to the difference in range overlap, *N. giraulti* had to be the less common species because the within-host mating behavior was the derived state within the phylogeny (see Chapter 6, Figure 6.1). These hypotheses were supported by our data in that *N. giraulti* appears to be the least common species of

*Nasonia* and had a higher probability of being found within the same nest as *N. vitripennis* than *N. longicornis* did.

While I was unable to compare the probability of *N. giraulti* and *N. longicornis* sharing a host with *N. vitripennis*, the data indicate that in the eastern U.S., *N. giraulti* and *N. vitripennis* share hosts and could interact before they emerged from the host puparium. It remains to be seen if there is a higher frequency of host sharing in the eastern U.S. than in the western U.S. but I predict that *N. longicornis* and *N. vitripennis* would not share a higher proportion of hosts than *N. vitripennis* and *N. giraulti.* This hypothesis is supported by the nest data where *N. longicornis* and *N. vitripennis* share fewer nests than *N. giraulti* and *N. vitripennis* (Figure 5.4).

The phylogenetic analysis (Chapter 6, Figure 6.1) indicates that WHM is a derived trait. If it evolved in response to selection to avoid hybridization, then the two species would need to share hosts as well as nests, and hybrid inviability would have to exist. Bordenstein et al. (2000, 2001) found that *N. vitripennis* and *N. giraulti* had the strongest barriers to gene flow due to hybrid breakdown and *Wolbachia*-induced incompatibilities. Their data support the hypotheses presented in this chapter because the reproductive barriers demonstrate an immense cost to mating with the wrong species for *N. giraulti* and *N. vitripennis* (Figures 5.4, 5.5). In contrast, the pre-zygotic barriers between *N. vitripennis* and *N. longicornis* are unidirectional and incomplete and hybrid breakdown is not as strong (Bordenstein et al. 2000, 2001). It is possible that there is not as severe a cost to mating with the wrong species for *N. vitripennis* and *N. longicornis*.

These data, along with the preliminary nest data suggest that in the western U.S. selection may not be as strong on *N. vitripennis* and *N. longicornis*, and hybridization may be preventing *N. longicornis* from evolving higher levels of WHM.

## Chapter 6 - Synthesis

In order to truly understand the evolution of a behavior, it is important to look at its phylogenetic history (Whitman 1899, Foster 1995). Phylogenetic history can provide information on the question of whether traits are homologous or homoplasious, which in turn can provide information on how the behaviors have been modified through evolutionary time (Foster 1995). Also, when a suite of traits in a species are found to be derived, there is the question of whether derived characters evolved together or independently (Huey and Bennett 1987, Lauder 1990, Maddison 1990).

The phylogenetic analysis of *Nasonia* estimated that the initial divergence occurred approximately 1.2-1.5 mya (Campbell et al. 1993, Figure 6.1). Furthermore, the most recent divergence occurred approximately 0.8 mya between the ancestors of *N. longicornis* and *N. giraulti*. From a phylogenetic perspective, *N. giraulti* represents the derived state for at least three of the behaviors; WHM, male post-copulatory display and male site-fidelity. For WHM, additional evidence for *N. giraulti* representing the derived state is that no other member of the family Pteromalidae has been found to mate inside their host (Quicke 1997). The ancestor to *N. giraulti* and *N. longicornis* probably had an intermediate and/or variable level of WHM because this behavior is nearly absent in *N. vitripennis*, and is variable in *N. longicornis* (Figure 6.1).

Another trait for which *N. giraulti* represents the derived state is the male post-copulatory display. The evolution of a display behavior typically involves the

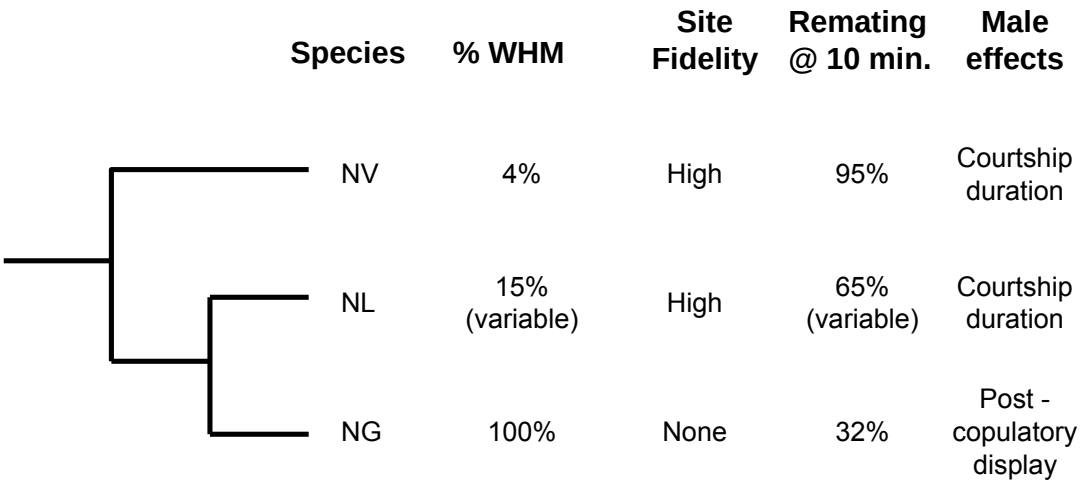


Figure 6.1. A cladogram of the three species of *Nasonia* and their general behavior patterns for four different behaviors. Male effects refers to the component of the courtship display that affects female remating frequency. Data are based on the phylogeny reported from Campbell et al. (1993).

modification of an existing behavior or motor pattern to suit a new function (Lorenz 1950). Additionally, new displays often undergo changes in form over time, so they rarely retain their original form (Baerends 1975, Hinde and Tinbergen 1978). My study (Chapter 3) identified the post-copulatory display behavior as a potential mechanism for inhibiting female remating in *N. giraulti* only; I was unable to detect an effect of the post-copulatory display in the other two species. In this case, each species is capable of performing the behavior, but the function differs in one of them. Based on the phylogenetic location of *N. giraulti* (Figure 6.1) it appears that the effect of the post-copulatory display on remating is a derived condition. One problem that exists for this family of Hymenoptera (Pteromalidae) is that many wasps have been placed in this family have little or no behavioral data (Quicke 1997). Broad genetic and behavioral comparisons would be valuable in determining the frequency of some of these behaviors and identifying the phylogenetic history of some of these behaviors.

The male post-copulatory display in *Nasonia* appears to be a modification of the courtship cycle because males return to their original courtship position and perform another series of head nods as they do during courtship. However, there is no apparent difference in the motor pattern of the post-copulatory display among the three species, and the only visible difference lies in the duration of the display. This raises several questions. First, what has allowed *N. giraulti* to obtain a new function for this behavior? *Nasonia* males are thought to release a pheromone during their normal courtship sequence (in conjunction with their first head nods; van den Assem et al. 1980) and I hypothesize that *N. giraulti* males have either retained their ability to release this

pheromone during the post-copulatory display, which could result in sensory overload in females, or have a different pheromone that makes them more successful at inhibiting female remating. Second, has the function of the post-copulatory display been lost for *N. vitripennis* and *N. longicornis*? Female remating frequencies have been shown to increase over time in laboratory populations of *N. vitripennis* (Burton-Chellew et al. 2007) so it is possible that with the evolution of female remating in the laboratory, there was a corresponding loss of function of the post-copulatory display for this species. However, remating frequencies have not changed in my *N. longicornis* populations for over 3 years, so the mechanism of change does not appear to hold across species. And third, is it possible that the post-copulatory display has another function? Males of *N. vitripennis* and *N. longicornis* are highly competitive for females, and a lingering male after copulation could be seen as a type of mate guarding provided he can keep other males from successfully mounting the female.

I attempted to explain the evolution of several behaviors in *N. giraulti* by using the genetic covariance structure of a closely related species, *N. longicornis*. This idea has two major assumptions, which are that the genetic covariance structure (G-matrix) is stable (will not change over time), and that the covariance structure is similar across species. These assumptions have been the subject of contention. For example, Phillips et al. (2001) showed that the covariance structure in wing size components changed over two generations of inbreeding in lines of *Drosophila melanogaster* when compared to an outbred control population. Conversely, Whitlock et al. (2002) demonstrated the constancy of the G matrix after a population bottleneck in *Drosphila melanogaster*,

although genetic drift did produce several lines that were not consistent. Also, Begin and Roff (2003) showed that the genetic covariances for five morphological traits were fairly conserved across three different species of *Gryllus* that showed divergent morphologies. Jones et al. (2003) suggest that the G-matrix for certain suites of characters like morphological traits will remain stable over other types of traits. However, few studies have strictly investigated behavioral genetic correlations (Via 1986, Brodie 1993) and more studies are needed to determine if the covariance structure between behaviors would remain constant.

Changes in the timing of developmental events, or heterochrony, can create the variation needed for a response to selection (West-Eberhard 2003). Shifts in developmental events have been well documented in animals (Gould 1977), most of them dealing with morphological characters (Wray and McClay 1989). Behavior is no exception to heterochronic shifts and the majority of behavioral modifications can be considered changes in developmental timing (see Tinbergen 1951). However, the relationship between shifts in life-history characters and the associated changes in behavior remains unexplored. Studies that explore this relationship have used species that show a diversity of morphologies, for example termites and aphids, that result in distinct behavior patterns for each type of morph (Stern and Foster 1996, Nalepa and Bandi 2000). I found that a simple change in the timing of the onset of female sexual receptivity in *Nasonia* is associated with the frequency of female remating within the RILs and across species. However, the parental lines and the RILs showed opposite patterns, mostly because females from the RILs that were not receptive immediately resembled the

parental line (P2) that had a low remating frequency. Nonetheless, within the RILs, as remating frequency decreased, the age of the onset of female receptivity was delayed developmentally. The delay appears to affect a behavioral trait that is expressed later in life because remating would not occur until after the initial courtship from the male. This complex relationship was unexpected because I had hypothesized that the timing of female receptivity would be correlated with WHM, not remating. However, the factors that influence the probability of WHM remain elusive.

Transgressive segregation is common in studies using hybrid F2, backcross and RIL progeny (Rieseberg et al. 1999). The majority of studies that identify propose that complementary action of additive alleles is the causative agent of these extreme hybrids and that nonadditive effects between loci are negligible (reviewed in Rieseberg et al. 1999). I was able to identify three behavioral traits for which the RILs showed transgressive segregation. Six RILs were transgressive for the duration of the post-copulatory display, which we have defined as a male trait. By identifying these transgressive lines, I have potentially uncovered the initial step for the evolution of this behavior. The duration of the post-copulatory display in *N. giraulti* is approximately twice as long as it is in *N. longicornis* and the initial step in increasing the duration of this behavior may be through transgressive segregation. Additionally, the RILs demonstrate that epistatic interactions may have a far more important role in the evolution of any male trait in the Hymenotpera because males are haploid.

A major issue that exists for any scientist that studies the hymenoptera is haplodiploidy. The fact that males develop from unfertilized eggs while females develop from fertilized eggs make evolutionary quantitative genetic studies difficult. Quantitative genetic studies in the Hymenoptera are few, and most of them deal with social Hymenoptera where females are the predominant sex; males are typically ignored (Moritz et al. 1985, Page et al. 2000, Ruepell et al. 2001). One possible reason for the lack of quantitative genetic studies on male traits is that the proper mathematical estimates for coefficients of relatedness and additive genetic variation have not been worked out. Previous studies that looked at quantitative traits have used traditional full and half-sib analyses (Falconer and MacKay 1996). However, most of these studies have failed to realize that the mathematical estimates need to be adjusted for haploid males. For example, males only contribute genes (all of them) to the daughters of the next generation, and in an outbred population, would not be related to the males produced in that generation. This creates a problem for a sib analysis because an additional generation of breeding needs to be performed to look at how male traits would run in families, and even then, those males that were produced would only on average have ¼ of the genes from the initial male, not the typical ½ in a sib analysis. Also, haploid males cannot show additive genetic variation or dominance variation, and thus male traits cannot be said to have narrow-sense heritabilities. A question that can then be asked is whether or not males have breeding value? Males undoubtedly contribute to the phenotypes of their daughters as they provide all of their genes to the next generation of females, but provide no genes to males. Quantitative genetic estimates in sex chromosome theory may provide some answers, but the values used to calculate breeding values are still based on diploid

species (Caballero 1995). These types of mathematical irregularities were one of the primary reasons for why I did not perform a strict quantitative genetic analysis in *Nasonia*, and why I designed (with the help of Dr. Sergey Gavrilets) a breeding design for the RILs that allowed me to investigate the genetics of behavior without having to develop new theory.

The behavioral differences in *Nasonia* may have evolved due to overlapping ecological requirements. The nest data supported the hypothesis that at least WHM could have evolved as a by-product of selection against hybridization in areas of sympatry, but the level of detail was not available to make a full assessment. More detailed data are needed from the western U.S. to determine the frequency of host sharing between *N. vitripennis* and *N. longicornis* to further support this hypothesis. Additionally, it would be informative to determine if there are other fly species on which *N. girualti* and *N. longicornis* prey, which in turn may affect the amount of habitat overlap between the species. Personal conversations with Dr. Jack Werren suggest that *N. longicornis* may not be the host specialist we think because these wasps will readily go to bait traps that hold *Sarcophaga* hosts.

*Nasonia* is a very difficult species to work with because the timing of the expression of behavioral traits is so important. Some of the larger issues raised here could be examined in other Hymenoptera, for example the covariances between behaviors in a phylogenetic context and the nature of inheritance in haploid males, and my hope is that

my work will spark an interest in resolving some of these issues. After all, for every species of insect, there may be a parasitoid (Godfray 1994).

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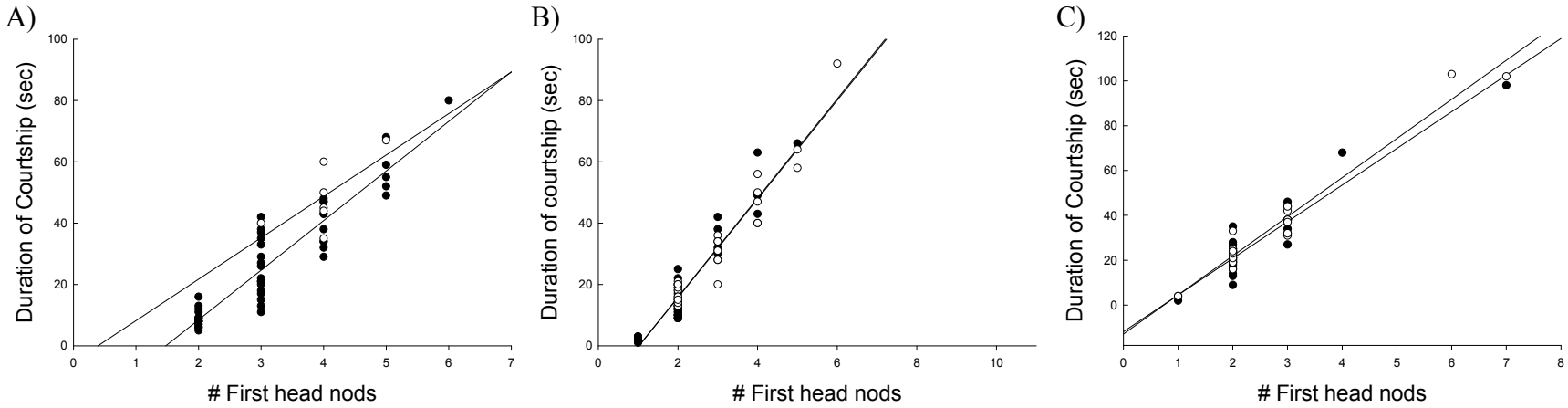
## Appendix

*Courtship duration and number of head nods*

We were able to count the number of first head nods (cycles) for each courtship bout in only about 65% of tests. Because we had limited data on the number of first head nods, we present the actual times instead of the number of first head nods, although we included head nod data when possible for statistical analyses. We found a significant positive relationship within all species between the courtship duration and the number of first head nods performed by the first male in tests when remating did and did not occur (ANOVA, $p<0.0001$; Appendix Figure 1) and $r^2$ values for each regression exceeded 0.74 except in *N. vitripennis* (see below). This indicated that our use of courtship duration should allow comparison with previous studies.

For tests of remating 24 hours after the first copulation, we had sufficient data to test the effect of first head nods rather than courtship duration on remating probabilities. The results of the models did not change for two of the species (data not shown). In the only species with different results, *N. vitripennis*, the number of first head nods was a weaker predictor of remating than courtship duration, because the overall model that used head nods was not significant. A more detailed look at the relationship between number of first head nods and courtship duration showed that these two variables are highly correlated in trials where remating occurred, but they are not as strongly correlated in trials where

remating does not occur (Appendix figure 1). The probable reason for these results is that the number of first head nods is a categorical variable whereas courtship duration is continuous: individuals that produce the same number of first head nods can have as much as a 20 sec difference in courtship duration (Appendix figure 1), which is anywhere from 33% to 100% of first courtships. Thus courtship duration provides more useful information than the number of first head nod




Appendix Figure 1. The relationship between courtship duration and the number of first head nods for the first male presented to the female for all three species of *Nasonia*. Data from tests 10 minutes and 24 hours after the first copulation were pooled. Each line is the linear regression for tests in which females did (●) and did not (○) remate. Correlation values are as follows: A) *N. vitripennis* ($r^2$ = 0.87(●) and 0.48(○)), B) *N. longicornis* ($r^2$ = 0.90(●) and 0.93(○)), C) *N. girault*i ($r^2$ = 0.88(●) and 0.95(○)).

Appendix Table 1. Temporal components of the male courtship sequence for both first and second males in trials where remating occurred. Values are in seconds (± SE).

| | | Male 1 | | | | | Male 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain/Trial Type | N | Latency to Mount | Latency to Court | Courtship Duration (1st interval) | Copulation Duration | Post Copulatory Display Duration | Latency to Mount | Latency to Court | Courtship Duration (1st interval) | Copulation Duration | Post Copulatory Display Duration |
| NV1 | | | | | | | | | | | |
| 10 min. | 29 | 52.62 ± 7.35 | 4.76 ± 0.55 | 8.72 ± 0.82 | 14.72 ± 0.52 | 18.90 ± 1.21 | 50.37 ± 6.17 | 4.10 ± 0.33 | 22.52 ± 4.18 | 14.03 ± 0.34 | 12.24 ± 0.61 |
| 24 hr. | 30 | 68.56 ± 8.67 | 4.77 ± 0.51 | 9.87 ± 1.00 | 14.83 ± 0.49 | 18.30 ± 0.98 | 59.77 ± 6.34 | 3.63 ± 0.27 | 15.37 ± 1.72 | 14.60 ± 0.36 | 14.53 ± 0.72 |
| NV2 | | | | | | | | | | | |
| 10 min. | 23 | 95.82 ± 15.93 | 8.00 ± 0.75 | 26.91 ± 2.12 | 14.21 ± 0.28 | 20.82 ± 2.64 | 102.26 ± 19.66 | 8.47 ± 1.07 | 52.65 ± 6.97 | 13.61 ± 0.41 | 21.26 ± 1.75 |
| 24 hr. | 23 | 115.56 ± 16.84 | 5.60 ± 0.45 | 40.56 ± 3.16 | 15.22 ± 0.34 | 22.30 ± 1.26 | 135.57 ± 20.18 | 6.57 ± 0.42 | 96.97 ± 9.80 | 13.82 ± 0.29 | 20.00 ± 1.22 |
| NL1 | | | | | | | | | | | |
| 10 min. | 26 | 54.11 ± 7.45 | 5.81 ± 0.29 | 8.04 ± 1.29 | 12.81 ± 0.34 | 18.81 ± 1.31 | 67.12 ± 8.18 | 5.96 ± 0.25 | 29.77 ± 4.69 | 13.77 ± 0.31 | 17.96 ± 1.01 |
| 24 hr. | 29 | 89.79 ± 15.16 | 6.31 ± 0.41 | 9.93 ± 1.23 | 13.48 ± 0.32 | 18.17 ± 0.89 | 119.31 ± 17.07 | 5.55 ± 0.34 | 25.03 ± 3.92 | 13.52 ± 0.24 | 17.69 ± 1.04 |
| NL2 | | | | | | | | | | | |
| 10 min. | 14 | 75.79 ± 9.19 | 11.35 ± 1.71 | 24.36 ± 5.35 | 14.57 ± 0.46 | 17.92 ± 1.05 | 69.42 ± 8.53 | 10.21 ± 1.38 | 82.86 ± 14.74 | 14.21 ± 0.66 | 20.50 ± 6.65 |
| 24 hr. | 12 | 105.75 ± 14.86 | 6.42 ± 0.39 | 45.08 ± 11.78 | 16.00 ± 0.65 | 17.08 ± 1.10 | 91.67 ± 17.68 | 9.17 ± 1.78 | 118.33 ± 17.50 | 15.25 ± 0.28 | 17.08 ± 1.23 |
| NG1 | | | | | | | | | | | |
| 10 min. | 10 | 69.00 ± 15.66 | 4.50 ± 0.45 | 26.6 ± 2.76 | 12.00 ± 0.65 | 38.40 ± 2.02 | 99.90 ± 11.22 | 5.10 ± 0.50 | 76.60 ± 12.80 | 11.60 ± 0.65 | 41.20 ± 4.60 |
| 24 hr. | 21 | 109.24 ± 21.97 | 4.09 ± 0.24 | 28.05 ± 2.98 | 11.62 ± 0.39 | 36.14 ± 2.61 | 133.33 ± 20.58 | 4.05 ± 0.47 | 53.29 ± 5.22 | 12.42 ± 0.36 | 42.09 ± 2.81 |
| NG2 | | | | | | | | | | | |
| 10 min. | 8 | 89.13 ± 15.68 | 7.50 ± 2.04 | 19.00 ± 4.18 | 12.00 ± 0.18 | 30.00 ± 3.56 | 143.38 ± 26.64 | 7.13 ± 0.99 | 62.25 ± 12.18 | 12.00 ± 1.00 | 32.25 ± 2.30 |
| 24 hr. | 22 | 141.86 ± 16.81 | 4.96 ± 0.41 | 22.91 ± 3.69 | 13.64 ± 0.29 | 32.18 ± 1.89 | 177.68 ± 33.12 | 4.41 ± 0.35 | 72.23 ± 11.24 | 13.45 ± 0.35 | 34.18 ± 2.06 |

**Vita**

Jason Edward Leonard was born in Lewiston, N.Y. on August 14, 1975. He was raised all over the eastern United States, went to grade school at Underwood elementary school in Raleigh, N.C., middle school at Penwood middle school in Newtown, PA. and graduated from Cheshire High School in Cheshire, CT. in 1993. From there he went to Tunxis Community College in Farmington, CT. and received an A.S. in liberal arts in 1996. He then went on to Central Connecticut State University and received a B.S. in biology with a minor in history in 1999. Jason then came to the University of Tennessee, Knoxville and received a Ph.D. in ecology and evolutionary biology in 2008.

Jason is currently an Assistant Professor at Hiwassee College in Madisonville, TN.