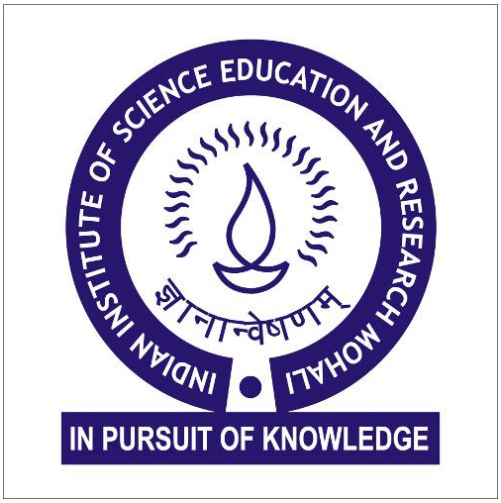
Genetics of Female Mate Preference in *Nasonia*

Amruta Rajarajan

A dissertation submitted for the partial fulfilment of
BS-MS dual degree in Science



Indian Institute of Science Education and Research Mohali
April 2018

Certificate of Examination

This is to certify that the dissertation titled "**Genetic Basis of Female Mate Preference in *Nasonia***" submitted by Ms. Amruta Rajarajan (Reg. No. MS13119) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recomends that the report be accepted.

Dr. Rhitoban Ray Choudhury (Supervisor)

Dr. Nagaraj Guru Prasad

Dr. Ram Yadav

Dated: April 20, 2018

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Rhitoban Raychoudhury at the Indian Institute of Science Education and Research, Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a confide record of original work done by me and all sources listed within have been detailed in the bibliography.

Amruta Rajarajan
April 20, 2018

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Rhitoban Raychoudhury
(Supervisor)

Acknowledgement

I would like to thank my supervisor, Dr. Rhitoban Raychoudhury for his unwavering guidance and support throughout this project, as well as for his mentorship during my stay at IISER Mohali. I would also like to thank Babita for her inputs during this project and all the other lab members – Garima, Alok, Renuka, Manisha and Pooja for their generous help and the cordial interaction they indulged me with.

I would also like to thank my parents for their loving and firm encouragement throughout my five-year stay. Finally, this thesis and the four years of coursework I completed before it would not have been as fulfilling without the company of my closest friends Simran Tinani and Vaishnavi Sidharthan, who became my extended family at IISER Mohali.

Contents

List of Figures

List of Tables

Abstract

Diverging mate preferences promote incipient speciation in a diverse range of taxa. The young and sympatric sister species pair of the parasitoid wasp genus *Nasonia* - *N. giraulti* (NG) and *N. oneida* (NO) show asymmetric assortative mating and no evidence of any post-zygotic barrier. In particular, NO females reject NG males while NG females do not. This suggests that the evolution of mate discrimination could have initiated speciation in this species pair.

The present study investigates the inheritance of female mate preference behaviour in hybrids of the species pair, with the ultimate aim of discovering the genes responsible for the same. This was achieved by **(1)** Conducting no-choice mating trials on parental, F1 and F1 backcross hybrid females, **(2)** Setting up recombinant inbred isofemale lines homozygous for NG male acceptance phenotype and **(3)** Conducting no-choice mating trials on females from each of the obtained isofemale lines.

(1) NO females accept NG males in a significantly less percentage of trials (7%, n=65) than their conspecific males (88%, n=33) ($p < 0.01$, Fisher Exact Test). O/G[G] and G/O[O] F1 hybrids accept NG males in 85% (n=23) and 95% (n=32) of mating trials respectively showing no discrimination between NO and NG males, while the G/O[O] F1 backcross females show 0% (n=20) acceptance of NG males compared to 90% (n=14) acceptance of NO males. **(2)** A total of 91 isofemale lines were set up which were derived from 12 F2 hybrid males. **(3)** The progeny are expected to emerge during the last two weeks of April, after which they will be assessed for their mate preference.

Since F1 hybrid females showed the NG acceptance phenotype in heterozygous condition, the phenotype is tractable in a genetic study. The reappearance of the NG male rejection phenotype in F2 hybrid females suggests the preference trait could be polygenic.

8. Introduction

8.1 Mate choice and Speciation

Mayr (1942) proposed the biological species concept, in which he defined species to be a “group of interbreeding (or potentially interbreeding) individuals that are reproductively isolated from other groups of interbreeding individuals”. Going by this definition, speciation is the evolution of reproductive isolating barriers between populations that were previously freely interbreeding. Reproductive barriers may be either pre-zygotic or post-zygotic and the process of speciation involves the accumulation of multiple isolating barriers until complete reproductive isolation is achieved. The sequence of appearance of these barriers between diverging populations in the face of gene flow remains is unclear, since "good" species show multiple barriers. Studies in a wide range of taxa have discovered genes responsible for post-zygotic isolation (Orr, Masly and Presgraves, 2004). However, post-zygotic isolation occurs between populations that have diverged enough to generate hybrid genic incompatibilities - so the genes responsible contribute to maintaining speciation, but not necessarily initiating it. On the other hand, increasing evidence suggests assortative mating, a pre-zygotic isolating barriers may exist in the absence of post-zygotic ones (Raychoudhury *et al.*, 2010; Yukilevich *et al.*, 2018), suggesting their role in initiating speciation. These would be *speciation genes* in the true sense, since they initiated the process.

This chapter presents case studies of the mechanistic roles of differential mate choice in various stages of sympatric speciation across taxa and their genetic bases, beginning with ‘good’ species and working backward towards incipient species and suggests the possibility of differential mate choice alone causing speciation.

Classical Reinforcement: Assortative mating may evolve as an adaptive consequence when the two sister species have diverged to large extent. This may be caused by **classical reinforcement** (Dobzhansky, 1937): highly diverged lineages show multiple post-zygotic isolating factors, making their hybrids either intrinsically unfit or poorly adapted to their environments, or both. This reinforces any existing intrinsic pre-zygotic barrier in regions of sympatry. The butterfly Mullerian mimics *Heliconius cydno* and *H. melpomene* are sister species that mimic different toxic models resulting in divergent wing coloration patterns. However, these patterns also act as visual cues in male mate choice and cause assortative mating between the two species (Jiggins *et al.*, 2001). Hybrids of the two species

are maladapted as they are not effective Mullerian mimics. Reinforcement likely played a role in the divergence of the two species, since they show reproductive character displacement: sympatric populations exhibit higher assortative mating than allopatric populations. (Naisbit, Jiggins and Mallet, 2001). As Mullerian mimicry is controlled by a single 'supergene', the trait of wing coloration may be linked with the preference trait, as found in *H. cydno* and *H. pachinus* (Kronforst *et al.*, 2006).

One possible mechanism for the evolution of pre-zygotic isolation is demonstrated by the parasitoid wasps *Nasonia vitripennis* and *N. giraulti*. These sympatric species harbor a host post-zygotic isolating factors (table 1.2) and differ in the composition of their cuticular hydrocarbons: *N. vitripennis* females discriminate against males that do not contain 4(*R*),5(*R*)-5-hydroxy-4-decanolide, a stereoisomer of an existing pheromone in the mixture which exists in addition to 4(*R*),5(*S*)-5-hydroxy-4-decanolide and 4-methylquinazoline only in *N. vitripennis* males (Niehuis *et al.*, 2013; Ruther *et al.*, 2016). QTL mapping implicated the role of five short-chain dehydrogenases/ reductases (SDRs) in the biosynthesis of the pheromone components (Ruther *et al.*, 2016). Thus the evolution of a new component in pheromone blends allows for species recognition, and could be reinforced in sympatry.

Evidence for reinforcement has also been found in several *Drosophila* species pairs and butterflies (Coyne and Orr, 1997; Lukhtanov *et al.*, 2005).

Mate Choice in Incipient Speciation

Environment-Dependent Sexual selection: Several case studies provide evidence for mate choice promoting speciation in conjunction with traits that are under natural selection ("magic traits") (Servedio *et al.*, 2011; Servedio and Kopp, 2012). These may either be under both natural and sexual selection, or be genetically correlated with a preference trait (Maan and Seehausen, 2012; Thibert-Plante and Gavrilets, 2013). The composition of CHCs in the widespread *Drosophila serrata* and its sister species *D. birchii* causes assortative mating between the two species (Blows and Allan, 1998). Additionally, the CHCs are produced in large quantities in *D. serrata* and contribute to its tolerance towards desiccation. This adaptation may have allowed its dispersal to arid habitats. On the other hand, the low-quantity CHC producing *D. birchii* is desiccation-sensitive and its biogeographic range is limited to rainforests with high humidity. Thus, an adaptive change in CHC composition played a dual

role that also affected mate choice, contributing to speciation (Chung *et al.*, 2014). The production of these CHCs was mapped to a single gene, *mFAS.*

The three-spined stickleback *Gasterosteus aculeatus* inhabiting Paxton Lake, Canada exists in larger-sized benthic and smaller-sized limnetic forms. Each form is adapted to its food source and local environment and the females of both forms show assortative mating. A study that investigated the inheritance of mate preference in hybrids of the two forms found that F2 hybrid females accepted mates showing the same body size as their own, suggesting that the female preference is genetically linked with these traits of adaptive value (Bay *et al.*, 2017). Such environment-dependent sexual selection has been implicated in sympatric forms of *G. aculeatus* inhabiting a lake near Bern, Switzerland as well. Interestingly, these forms are estimated to have diverged only a few decades ago, suggesting a possible role of environment-dependent sexual selection on incipient speciation (Marques *et al.*, 2017).

Sensory Drive: Alternatively, mate choice may lead to sympatric speciation by **sensory drive** in which a pre-existing disposition in the sensory system of the choosy sex is exploited by potential mates to gain a reproductive advantage. In such cases, selection may be on the sensory perception involved in mate choice. This is demonstrated by the sympatric Lake Victoria cichlids *Pundamilia pundamilia* and *P. nyererei.* These sympatric sister species show no evidence of a post-zygotic isolating barrier. However, strong assortative mating exists in mixed populations: females of both species prefer males of conspicuous coloration. Furthermore, male coloration is the only factor causing assortative mating: mating becomes random in turbid water habitats, i.e. when differences in coloration cannot be perceived by females at all. However, the perception of colour conspicuousness depends on ambient light, which varies with depth in Lake Victoria (light scattering by particulate matter shifts the ambient light towards red at lower depth) (Maan, Seehausen and Van Alphen, 2010) and also on the absorption maximum of the *LWS* opsin protein in the females' visual system, which also varies among cichlid fishes. The result is that in shallow waters, males with blue coloration are perceived as conspicuous, while at greater depth those with red coloration are favoured (Seehausen *et al.*, 2008). A study investigating the mating preferences of F1 and F2 hybrid females between this species pair rules out the possibilities of imprinting and mate choice copying. Also, the mating preferences of the parental species is regained in a large fraction of F2 hybrid female progeny, suggesting that female mating preference is an oligogenic trait (Svensson *et al.*, 2017), likely controlled by multiple opsin coding genes in the population. Sensory drive has also been implicated in a wide range of taxa including the cricket frogs, warblers, lizards, moths and wolf spiders (Boughman *et al.*, 2002).

Coupling of mate choice and life history traits: Differential mate choice may evolve upon secondary contact as a result of reinforcement between sympatric sister species pairs that have diverged due to a shift in host preference or food source (Mullen and Shaw, 2014). However, some instances of diverging mate preferences need not be adaptive and instead showcase an 'accidental' correlation of mate choice and a life history trait which upon divergence have been implicated in speciation. In insects, a shift in food source can alter CHC profiles that in turn influence mate choice (Chung and Carroll, 2015). *Drosophila mojavensis* CHCs which play an important role in species recognition and mate choice are altered by feeding on different cactus substrates, leading to substantial pre-mating isolation between its sympatric races and its sister species *D. arizonae* in the complete absence of post-zygotic isolating barriers (Etges, 1992). Similarly, the sympatric, sibling flea beetles *Altica fragaria* and *A. viridicyanea* show assortative mating on the basis of CHC profiles which are altered by the host plant on which they are reared – since F1 hybrid matings are predicted entirely by their host plant (Xue *et al.*, 2016).

Two sympatric sibling orchid bees *Euglossa dilemma* and *E. viridissima* show odour-based assortative mating. Males of these species collect odoriferous compounds from flowers that are displayed to females during courtship and are used for species recognition (Eltz *et al.*, 1999; Eltz, Sager and Lunau, 2005). In this case, the same sensory inputs (olfaction) govern both the trait under sexual selection and the preference for it: and genomic signatures of divergence were found in the chemosensory receptor genes of these two species, implicating their role in incipient speciation (Eltz *et al.*, 2008; Brand *et al.*, 2015).

Summary: Mate choice generally involves an integration of multiple sensory inputs and has a polygenic basis. Diverging mate preferences can facilitate, promote and maintain speciation in sympatry under a wide variety of conditions and are observed in many incipient species. They are either adaptive or correlated with a different life history trait or both. However, in the presence of any additional barrier other than assortative mating, which one emerged first remains elusive – one would be required to study sympatric sister species that show no isolating barrier other than assortative mating. An increasing number of species pairs have been reported to satisfy all of the above criteria and present opportunities to investigate the possibility of differential mate choice alone initiating speciation and to study its genetic basis.

8.2 The Model System *Nasonia*

Phylogeography*: Nasonia* (Chalcidoidea) is genus of ectoparasitoid wasps that includes four species: *N. vitripennis, N. longicornis, N. giraulti* and *N. oneida* (Darling and Werren, 1990; Raychoudhury *et al.*, 2010). The wasps infest cyclorrhaphous Dipteran hosts (Calliphoridae, Sarcophagidae) and are sometimes superparasites, as their host flies in turn infest birds' nests. Natural populations of *Nasonia* are found to co-occur with the distribution of their hosts; *N. vitripennis* is a generalist infesting *Protocalliphora* and *Sarcophaga* flies. It has a cosmopolitan distribution and is sympatric with both *N. longicornis* and *N. giraulti* in the western and eastern regions of North America respectively. *N. oneida* distribution was recently discovered to be embedded within that of its sister species', *N. giraulti*. Excluding *N. oneida*, all species pairs have also been found in microsympatry, i.e. infesting the same fly host.

Figure 8.1 Phylogeny of *Nasonia* (Chalcidoidea), Adapted from Werren *et. al.,* (2010), *Science 327(5963): 343–348*

Life history: *Nasonia* wasps are haplodiploid (females are diploid, males arise from unfertilized eggs and are haploid) and have a short life cycle of 14 days at 25° C (Fig 8.2). Females use their ovipositor to drill a hole through the puparium of their host fly and lay eggs inside after stinging the pupa with a venom. This venom acts on the developing host fly in three ways: it permanently paralyzes it, suppresses its immune response and reallocates lipid reserves in the fly from its haemolymph to the fat reserves on its surface, which is where the hatched *Nasonia* larvae feed on it (Rivers, Hink and Denlinger, 1993; Martinson *et al.*, 2014). After pupation and eclosion, fully developed *Nasonia* adults emerge from the host by chewing a hole through the puparium wall (6 to 60 individuals per host, see appendix) (Darling and Werren, 1990). The sex ratio of a clutch laid by a mated female is by default female biased (80-99% females, see appendix), if the female was presented with a fresh, unparasitized host. Females can assess whether or not a host pupa has already been parasitized and control the sex ratio of their clutch accordingly, by selectively fertilizing eggs with sperm stored their spermatheca

after mating (Steven H Orzack and E. D Parker, 2013). Males emerge from the host before their sisters do; they station themselves at the exit hole on the pupa and immediately court females as they emerge – with the exception of *N. giraulti* males which mate with their sisters within the host, before emergence (Hardy, 1994).

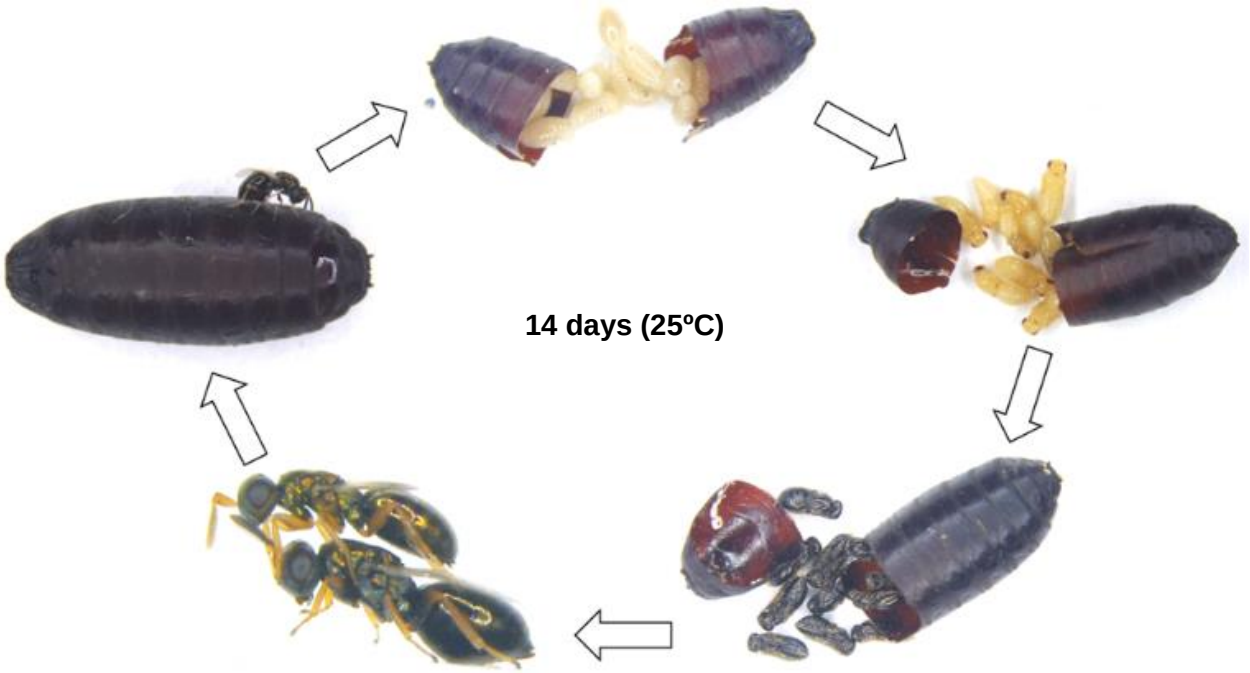


Figure 8.2 *Nasonia* Life Cycle

Courtship behavior: Courtship behavior in *Nasonia* is stereotypic (Vernel, 1979; Van Den Assem and Werren, 1994; van den Assem and Beukeboom, 2004) and consists of three phases: pre-copulation, copulation and post-copulation (table 8.1, fig 8.3, 8.4). Generally, females are choosy and mate only once*, while males are polygynous (Jachmann and Van Den Assem, 1996). Mating between a pair occurs only if the female signals receptivity to mate at the end of the pre-copulatory phase; forced copulations are not possible. Once mated, the females disperse in search of new hosts.

Phase	Component of Phase	Description	Remarks
Pre-copulatory (fig 1.4A)	Chasing	The male chases the female until he approaches close to her	After mounting, the male performs head nods, wing movements and mouth extrusions simultaneously. Multiple head nods are performed in a head nod series followed by a pause before the male begins another series. The time interval between two corresponding head nods of consecutive head nod series is called a cycle. Multiple such cycles constitute the pre-copulatory phase. (fig 1.3)
	Mounting	Male mounts the female and orients himself to the female's head	
	Antennating Female	While mounting, the male repeatedly taps his antennae over the female's body	
	Head nods	Male performs stereotypical nods of his head over the antennae of the female	
	Wing Movement	The male occasionally flutters his wings	
	Mouth Extrusion	The male extrudes his mouthparts in synchrony with nodding while secreting pheromones	
Copulatory (fig 1.4B)	Copulation	Mating occurs	If the female signals receptivity, the male backs up and copulates.
Post-copulatory (fig 1.4C)	Antennating Female	The individual components are similar to those of the pre-copulatory phase. This phase also consists of multiple cycles.	
	Head nods		

Table 8.1 Ethogram of *Nasonia* Courtship behavior

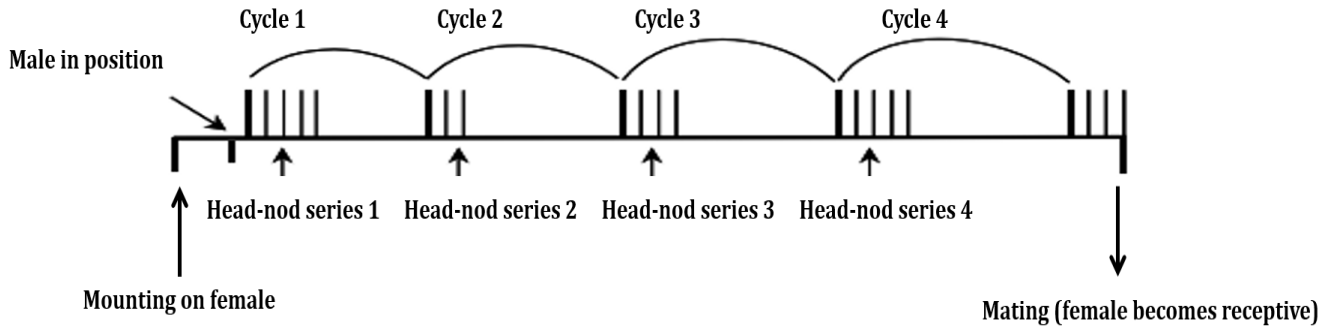


Figure 8.3 Nasonia courtship cycle, Adapted from Van den Assem and Beukeboom 2004 Proc. Neth. Entomol. Soc. Vol 15

Figure 8.4A: Pre-Copulatory Phase

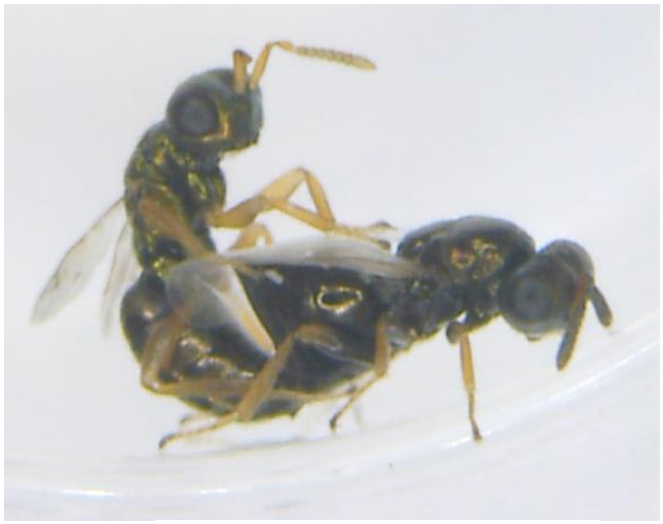


Figure 8.4B: Copulatory Phase

Figure 8.4C Post-Copulatory Phase

Genetics: The four species are interfertile once cured of their endosymbiotic *Wolbachia* infections. The genomes of *N. vitripennis, N. longicornis* and *N. giraulti* have been sequenced and annotated, and a wealth of genetic markers and recombination maps are available.

Speciation: Each species pair displays intrinsic pre- and/or post-zygotic isolating barrier(s) of varying degrees (Table 8.2). The most intriguing speciation event in this genus is that of the youngest sister species pair used in the present study, *N. giraulti* and *N. oneida.* There are no known post-zygotic isolating barriers between the two species – all hybrids are viable and fertile. Due to introgression in their evolutionary history (Raychoudhury *et. al.,* 2010), they harbor compatible *Wolbachia* infections in the wild. Asymmetric assortative mating forms the only isolating barrier between them – females of the derived species *N. oneida* reject *N. giraulti* males whereas *N. giraulti* females do not (Raychoudhury *et al.*, 2010; Buellesbach *et al.*, 2014). Thus, divergence in the genes responsible for female mate preference possibly explains their speciation event and also allows investigation of the possibility of differential mate choice alone causing speciation.

** N. giraulti females frequently mated multiple times with both NO and NG males in this study (pg 30)*

Species pair	Pre-zygotic barrier(s)	Reference	Post-zygotic barrier(s)	Reference
NV-NL	Mate discrimination by females: Both females discriminate against heterospecific males. Males do not discriminate between con- and heterospecific females.	Buellesbach *et al.* 2014 *Ethology 120 (2014) 834–843.*	Infrequent behavioural sterility in F2 hybrid males	Beukeboom *et. al.* 2001 *Behavior Genetics, 31(2), 167-177*
NV-NG	Asymmetric mate discrimination by females: NV females reject NG males, but NG females show no preference. Males do not discriminate between con- and heterospecific females.	Buellesbach *et al.* 2014 *Ethology 120 (2014) 834–843.*	(1) Behavioural sterility (2) Lowered sperm count (3) OX-PHOS pathway malfunction and (4) Phylosymbiotic breakdown with gut microbiota in F2 hybrid males	**(1)** O'Hara *et. al.* 2010 *Heredity (2010) 104, 289–301* **(2)** Gadau *et. al.* 2008 *J. Evol. Biol. 2 1 (2008) 1844–1851* **(3)** Bordenstein
NV-NO	Both males and females discriminate against heterospecific mates.	Raychoudhury *et. al.* 2010 *Heredity (Edinb). 2010 Mar; 104(3): 278–288*	Breakdown in F2 hybrid males	Raychoudhury *et. al.* 2010 *Heredity (Edinb). 2010 Mar; 104(3): 278–288*
NL-NG	Asymmetric mate discrimination by females: NL females reject NG males, but NG females show no preference. Males do not discriminate between con- and heterospecific females.	Buellesbach *et al.* 2014 *Ethology 120 (2014) 834–843.*	*Wolbachia* infection in natural populations, no post-zygotic isolating barrier in its absence	Bordenstein *et. al.* 2001 *Nature vol 409, pg 707–710*
NL-NO	Asymmetric mate discrimination by females: NO females reject NL males, but NL females do not discriminate significantly between con- and heterospecific males. Males do not discriminate between con- and heterospecific females.	Raychoudhury *et. al.* 2010 *Heredity (Edinb). 2010 Mar; 104(3): 278–288*	Asymmetric breakdown in F2 hybrid males, compared with NL F2 males	Raychoudhury *et. al.* 2010 *Heredity (Edinb). 2010 Mar; 104(3): 278–288*
NG-NO	**Asymmetric mate discrimination by females: NO females reject NG males but NG females show no preference. Males do not discriminate between con- and heterospecific females.**	Raychoudhury *et. al.* 2010 *Heredity (Edinb). 2010 Mar; 104(3): 278–288*	**No known post-zygotic isolating barrier**	Raychoudhury *et. al.* 2010 *Heredity (Edinb). 2010 Mar; 104(3): 278–288*

Table 8.2 All known pre- and post-zygotic barriers between *Nasonia* species pairs. (NV – *N. vitripennis*, NL – *N. longicornis*, NG – *N. giraulti*, NO – *N. oneida*)

9. Aim and Experimental Methods

9.1 Aim: This study aims to investigate the inheritance of female mate preference in hybrids of the species pair *N. girault*i and *N. oneida*. The objectives were to (1) conduct mating trials on parental, F1 and F1 backcross females, (2) set up recombinant inbred isofemale lines homozygous for *N. girault*i male acceptance and (3) conduct mating trials on females from each of the obtained isofemale lines.

9.2 Experimental Methods:

9.2.1 Collecting virgin wasps: Parasitized pupae were cracked open 10 to 13 days after being parasitized and virgins were collected and stored in separate vials at 25° C. The two sexes show morphological differences from the early pupal stage onwards. *N. oneida* and *N. girault*i sexes can be distinguished in

(1) The white and champagne stages of development when females lack the posterior-most visible segments on the ventral side of their abdomens, while the males have visible segments across their entire abdomen (fig 9.1) and (2) The black stage, when females have a distinct, white ovipositor on their posterior ventral side, which the males do not (fig 9.2).

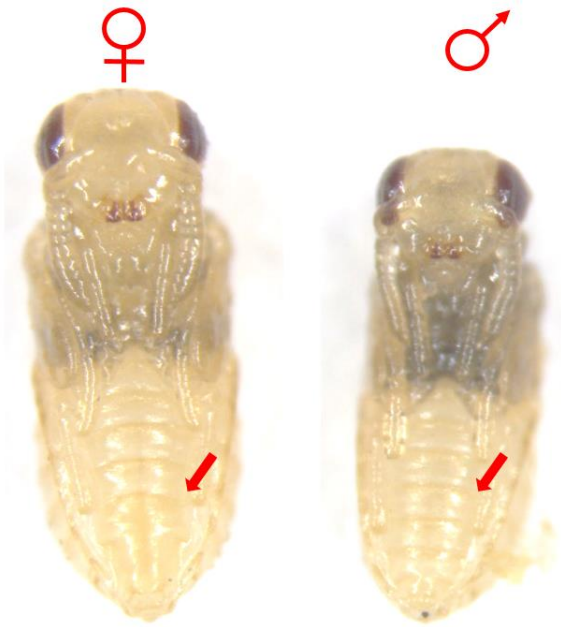


Figure 9.1 Sex differences in the white stage

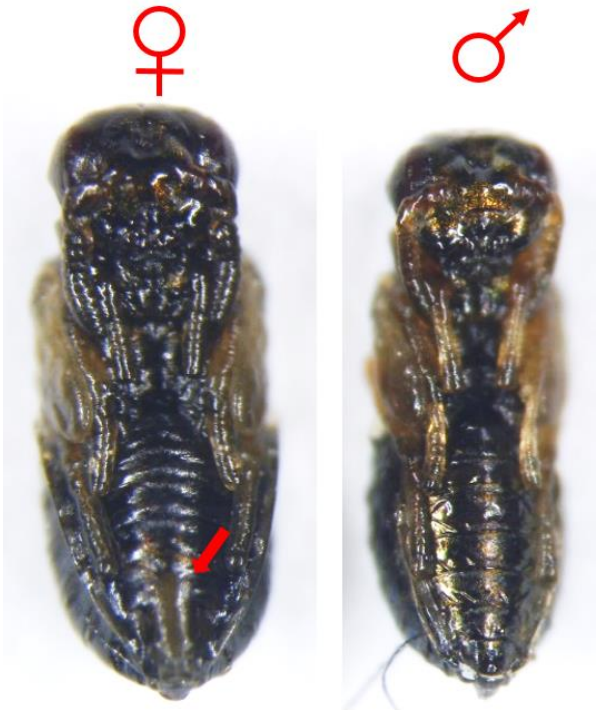


Figure 9.2 Sex differences in the black stage

9.2.2 Mating trials: All males and females used in the trials were naïve virgins, aged between 6 and 48 hours post-emergence. For the trial, a female was added into the mating chamber (fig 9.3). Next, a male was added to the vial and the pair was observed for 5 minutes. Mating latency in conspecific pairs is generally upto 3 min (data not shown). The outcomes of each trial were placed in one of three categories:

Sr. no	Score	Definition
1	Female Acceptance	The female is observed signaling receptivity (lowering her antennae and raising her abdomen) in response to the male's pre-copulatory activities (chasing the female, mounting and pre-copulation) and copulation is completed within the observation period. Post-copulation by the male is not necessary, but was generally found to occur.
2	Female Rejection	Female does not become receptive despite one or more courtship attempts by the male. Courtship attempts include at least one of chasing, mounting and pre-copulatory head nods.
3	Male Rejection/ Stationary	The male does not attempt courting the female even after making physical contact with her OR both the male and female are stationary throughout the trial and no courtship attempts occur.

Table 9.1. Scores of Mating Trials

This was repeated for parental, F1, F2 and F4 hybrid females (fig 9.5). The "percent acceptance", or the percentage of mating trials in which NO and NG males were accepted by the females of all types was subjected to statistical analysis. Observations falling in Category (3) were excluded from this analysis since female mate preference cannot be scored if the female is not courted by the male.

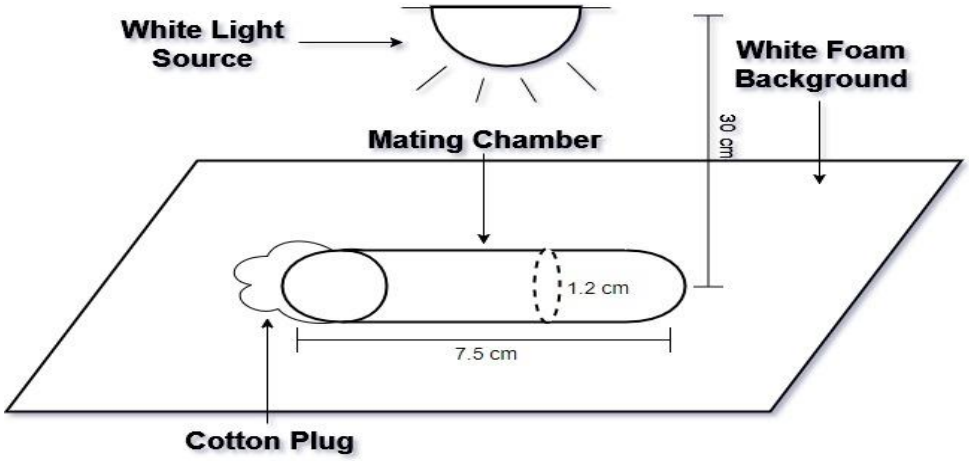


Figure 9.3 Mating chamber used for behavioural assays

9.2.3 Statistical Analysis: Acceptance of *N. giraulti* males was compared to that of *N. oneida* males for females of each genotype. Statistical significance was established using the default Fisher Exact test in R v ($\alpha = 0.01$). The contingency tables were of the form:

For female of Genotype X	# accepted	# rejected	Row Total
NG males			
NO males			
Column Total			

Table 9.2 Contingency Tables used in Fisher Exact Test

Error Bars: Since all graphs represent the percentage of trials in which mating occurred, the error bars are a simple function of sample size. They were calculated as the square root of sample size.

9.2.4 Genetic crosses: (1) The inheritance of *N. giraulti* male acceptance was checked with an F1 backcross (fig 9.4) and (2) Recombinant inbred isofemale lines were set up (fig 9.5)

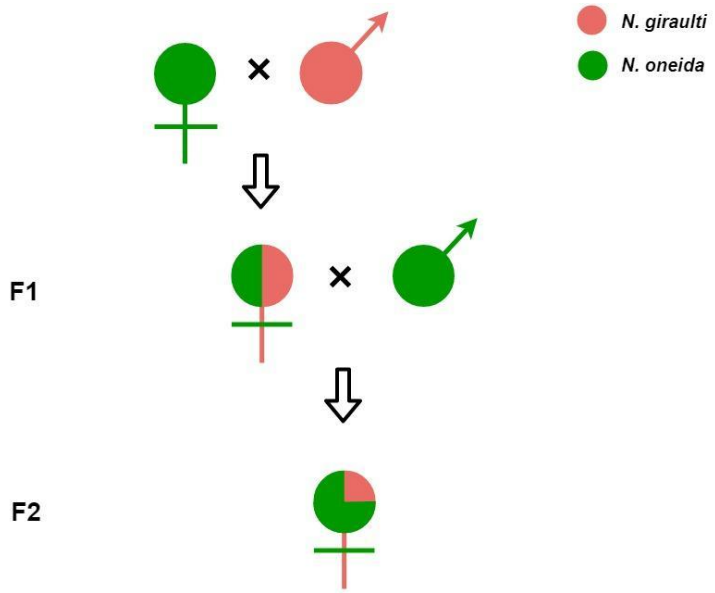


Figure 9.4 Genetic crosses to obtain F1 hybrid females and F1-backcross females

a. F1 hybrids in both directions were tested for *N. girault* male acceptance.
b. F1-*oneida* backcross females were checked for *N. girault* male acceptance.

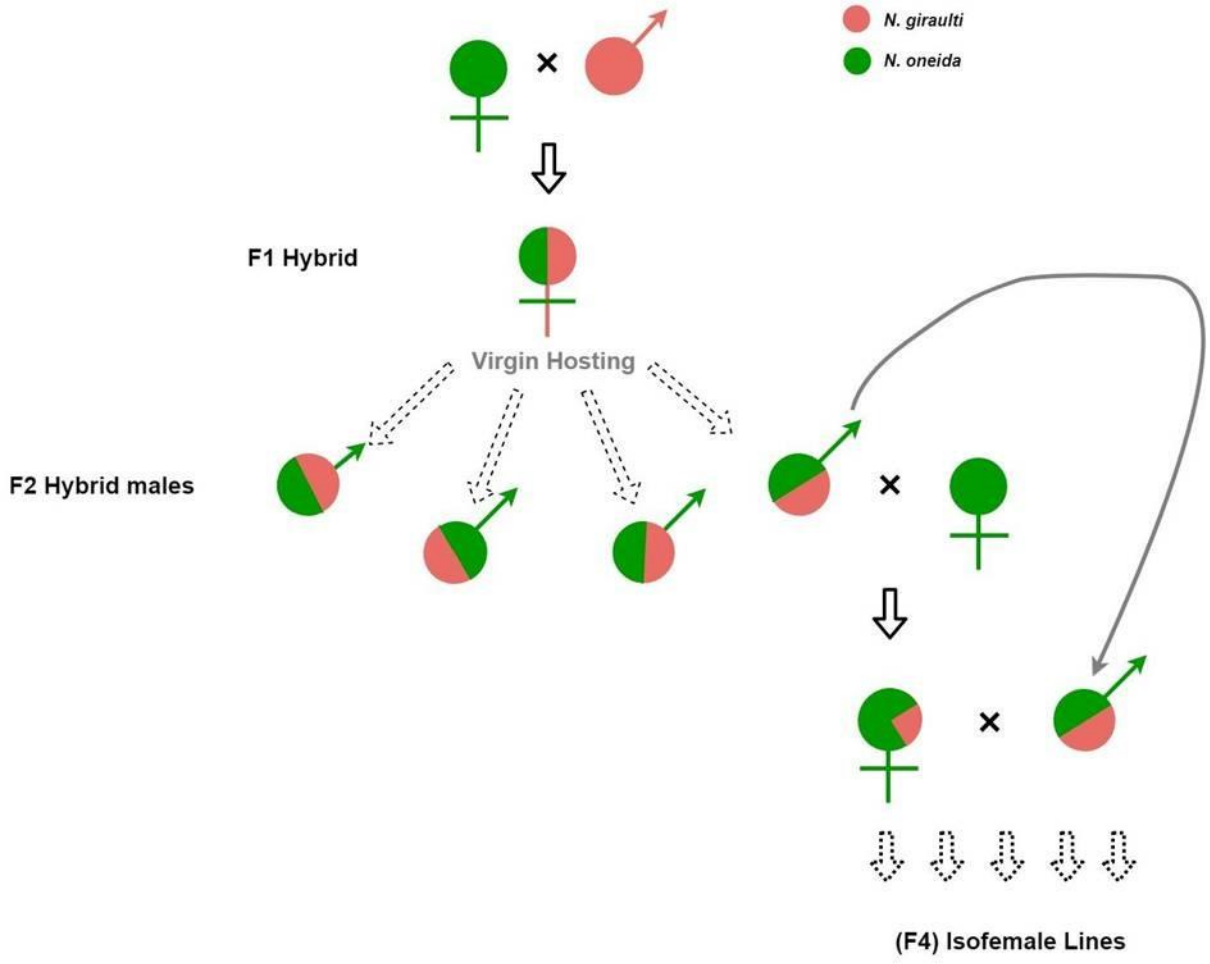


Figure 9.5 Genetic Crosses to obtain recombinant inbred isofemale lines

a. F1 Hybrid females were hosted as virgins to obtain recombinant F2 hybrid males.
b. Each male was backcrossed with an *N. oneida* female. The male was stored at 4 degrees C after providing honey, and was incubated at room temperature for 30 minutes to 1 hour every three days.
c. F3 females from each cross were backcrossed with their respective fathers to establish multiple isofemale lines, homozygous for the *N. giraulti* regions present in the F3 female parent.
d. Mating trials were conducted on F4 hybrid females from each isofemale line.

9.2.5 Curing of *Wolbachia* infection in *N. oneida:* The antibiotic used for curing was rifampicin, with a dosage of 1 mg per mL of 50% v/v honey-water solution stored at room temperature (25° C). A fresh aliquot of antibiotic solution was prepared every six weeks.

1. Mated *N. oneida* females were separated in Ria vials, with 10 to 25 individuals in each vial. The antibiotic solution was smeared on the wall of the vial using a paintbrush and incubated at 25° C for 24 hours to allow the wasps to feed.
2. The surviving females were then hosted: with three female wasps and three *Sarcophaga gus* pupa hosts per vial.
3. The progeny were screened for infection (refer next section) 3-4 days post emergence.
4. If found to be infected, all steps from (1) onwards were repeated for the progeny until a cured strain was obtained.

9.2.6 Screening for *Wolbachia* infection: This included (A) DNA extraction and (b) *w*spec PCR

9.2.6A DNA extraction protocol:

1. 3-4 wasps were pooled in one 1.5 mL microcentrifuge tube and surface sterilized using 70% molecular grade ethanol.
2. Samples were washed with autoclaved water thrice.
3. 200 µL lysis buffer was added and the samples were crushed using an autoclaved pestle.
4. 2 uL of 22mg/mL Proteinase K solution was added. The sample was incubated in a 37° C water bath for 7-8 hours.
5. The sample was removed from the water bath and 250 µL phenol alcohol was added. The microcentrifuge tube was inverted to mix the solution properly.
6. The tube was centrifuged at 12700 rpm and 4° for 5 min.
7. The top aqueous layer was collected carefully and any debris as well as the organic layer were discarded.
8. 250 µL of 24:1 chloroform:isoamyl alcohol was added and step (6) was repeated.

9. Step (7) was repeated.
10. 70% of the existing solution volume of chilled isopropanol and 10% of the existing solution volume of sodium acetate were added.
11. The sample was incubated at room temperature on the work bench for 2-2.5 hours.
12. Step (6) was repeated.
13. The liquid was discarded and step (6) was repeated after adding 70% mol grade ethanol.
14. The ethanol was discarded and the microcentrifuge tubes were laid to air dry upside-down on tissue paper for 15-30 min.
15. 100 µL TE buffer was added and the sample was stored at 4° C overnight before checking extracted DNA concentration using a Nanodrop.
16. The samples were transferred to -20° C for permanent storage.

9.2.6B *w*spec PCR:

1. DNA extracted from *N. oneida* wasps to be tested for infection having a minimum concentration of 25 ng/µL was used as template in a PCR containing primers designed against a conserved region of *w*spec, a surface protein common to all *Wolbachia* supergroups.
2. Reagents were added in the following proportions per sample:

Sr no	Reagent	Volume (µL)
1	Water	14.5
2	$MgCl_2$ Buffer	2
3	dNTPs	0.4
4	Forward primer (10 µM)	0.5
5	Reverse primer (10 µM)	0.5
6	*Taq* polymerase	0.1
7	Template	2
Total		20

Table 9.3 PCR Reagents and volumes

3. A PCR with 28S-specific primers was run simultaneously for all samples to check the quality of DNA.

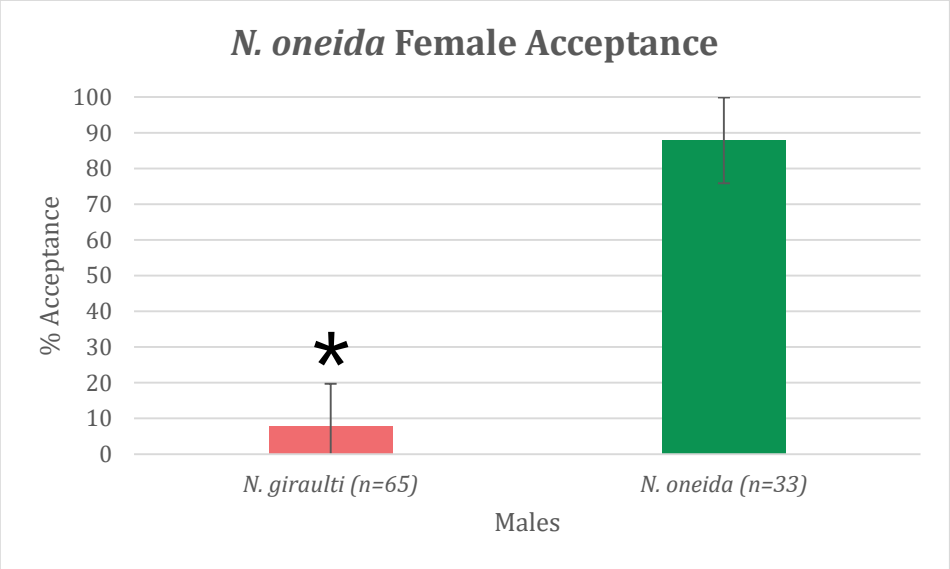
4. Previously verified *Wolbachia* infected sample DNA was used as a positive control and a negative control comprised of all reagents minus the template.
5. The default 28S protocol was run on the PCR machine. (56.5° C hybridizing temperature, 39 cycles).
6. The PCR products were visualized using agarose gel electrophoresis: 1% agarose gel with 3 µL EtBr was prepared.
7. 5 µL of the PCR products (+1.5 µL loading dye) and 2 µL DNA ladder was loaded and run at 80V for 40 min.

10. Results and Discussion

10.1 Results

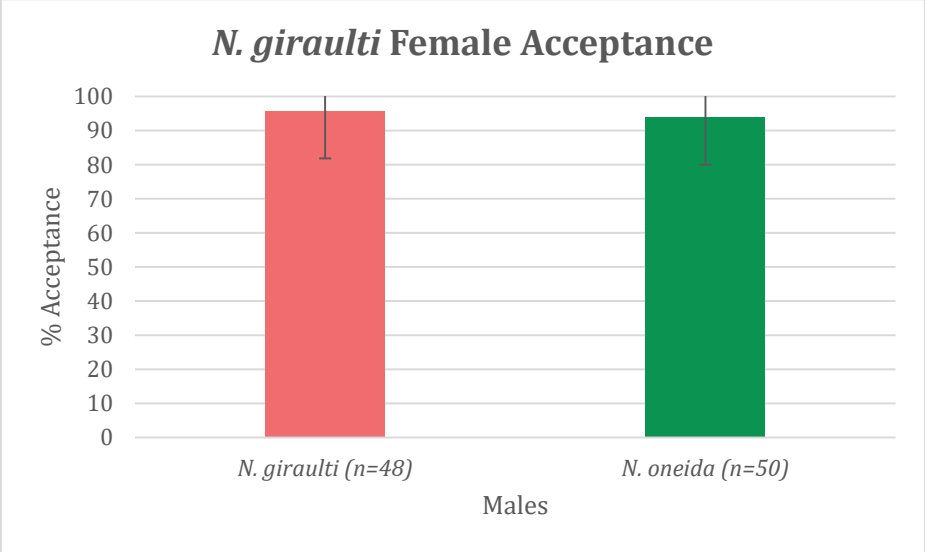
Goal 1: To conduct mating trials on parental, F1 and F1 backcross females

10.1.1A Parental mate preference (*N. oneida* individuals have *Wolbachia* infection)



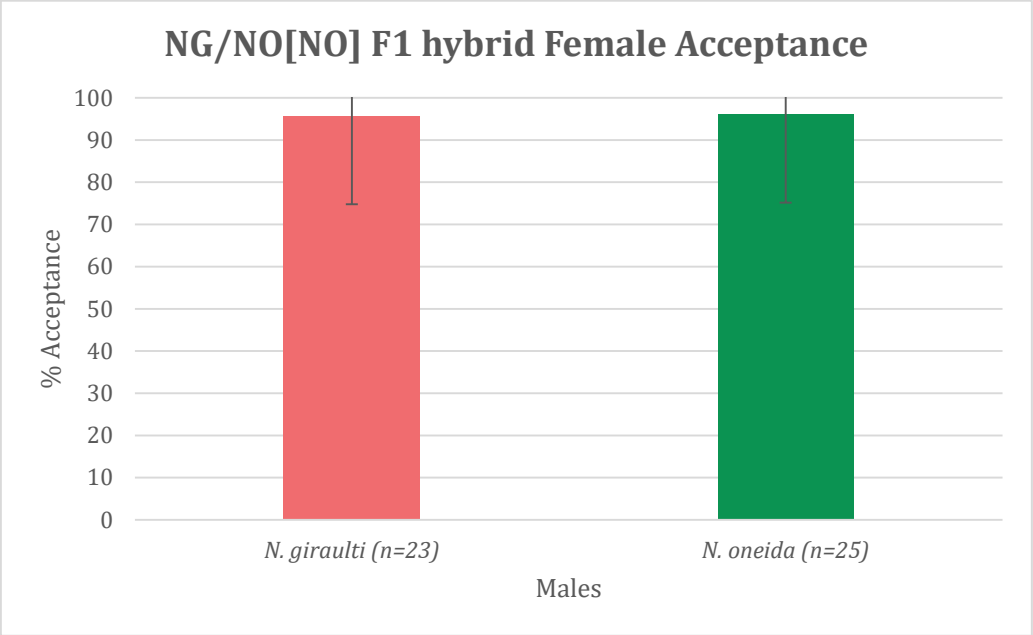
(* $p < 0.01$, Fisher Exact Test)

NO females accepted NG males in 7.69% of the trials, whereas accepted NO males in 88.3% of the trials. The difference in acceptance is statistically significant, showing that NO females discriminate significantly against heterospecific males.

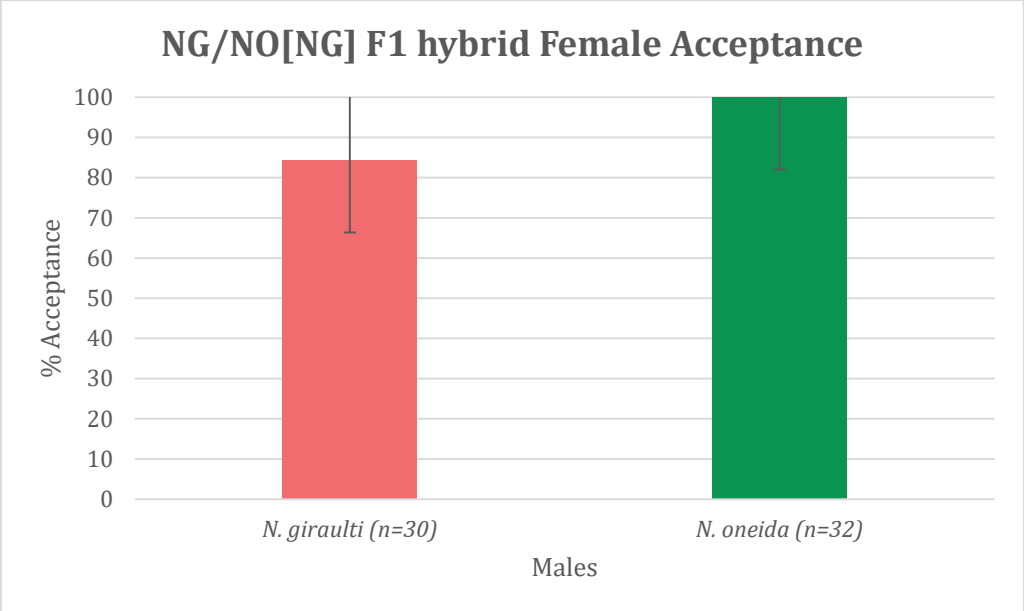


NG females accepted NG males in 95.83% of the trials, and accepted NO males in 94% of the trials, and there is no statistical difference between the two. Thus, NG females do not discriminate significantly between con- and heterospecific males.

10.1.1B F1 hybrid mate preference

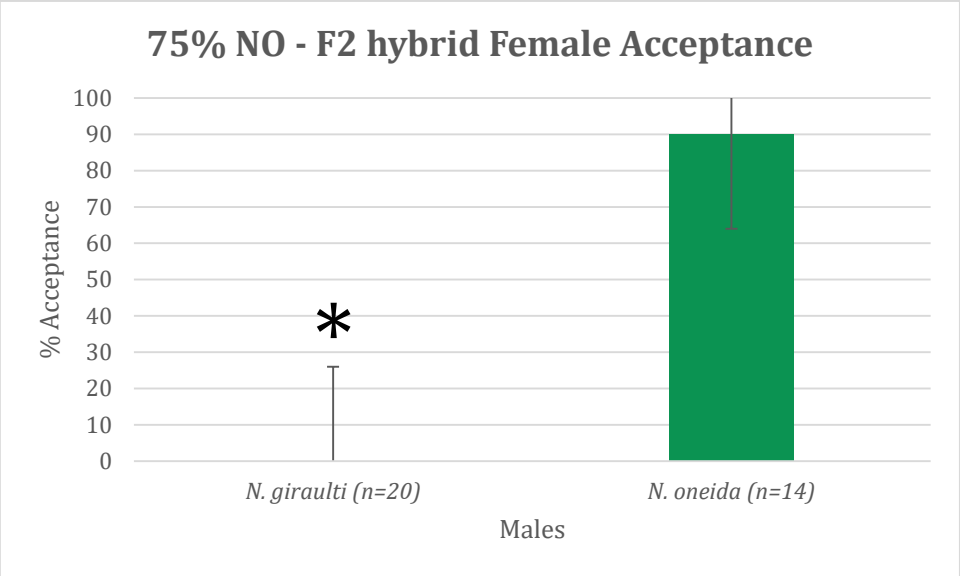


F1 hybrid females in NO background accepted NG males in 95.65% of trials and NO males in 96% of trials. They do not discriminate between NO and NG males.



F1 hybrid females in NG background accepted NG males 84.37% of trials and NO males in 100% of trials, and do not discriminate between them statistically. F1 hybrid females in both directions do not discriminate against NG males, showing that the NG rejection phenotype is lost.

10.1.1C F2 Hybrid mate preference



(* $p < 0.01$, Fisher Exact Test)

(Refer Fig. 2.4 for cross) F1 hybrid females in NO background were backcrossed with NO males: These F2 hybrid females accepted NG males in 0% of trials and NO males in 90% of trials. The difference in acceptance is statistically significant. Additionally, their acceptance of NG males was statistically indistinguishable from the acceptance of NG males by pure-bred NO females, suggesting the NO preference in regained entirely in their F2 hybrid offspring.

Goal 2: To set up recombinant inbred isofemale lines homozygous for NG male acceptance

10.1.2 91 Recombinant Inbred isofemale lines were set up following the protocol in fig (9.5).

Sr. no	**Line ID (F2 hybrid male)**	**Number of Daughter matings**
1	64-2	10
2	53-3	8
3	50-2	8
4	60-2	1
5	63-1	10
6	56-1	10
7	51-3	7
8	55-2	7
9	52-3	4
10	54-3	10
11	58-3	10
12	57-2	6
Total	**12 F2 hybrid males**	**91**

Table 10.1: Summary of the number of recombinant inbred isofemale lines set up

Goal 3: To conduct mating trials on females from each of the obtained isofemale lines

10.1.3 Females from the recombinant inbred isofemale lines are expected to emerge during the first two weeks of April, when they will be tested for their mate preference to meet goal (3).

Additional Work

10.(A). *N. oneida* was cured of *Wolbachia* infection for setting up of crosses

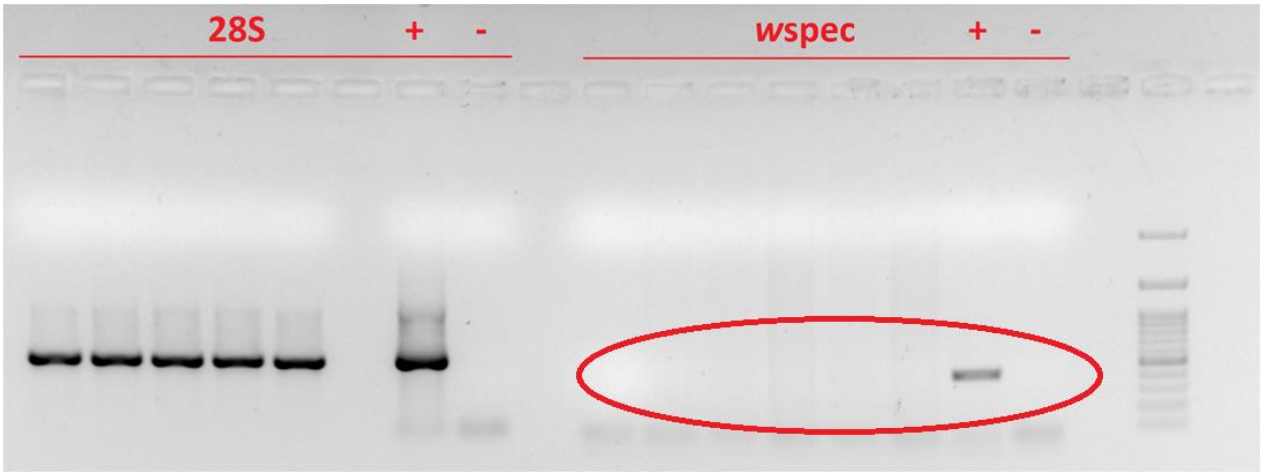
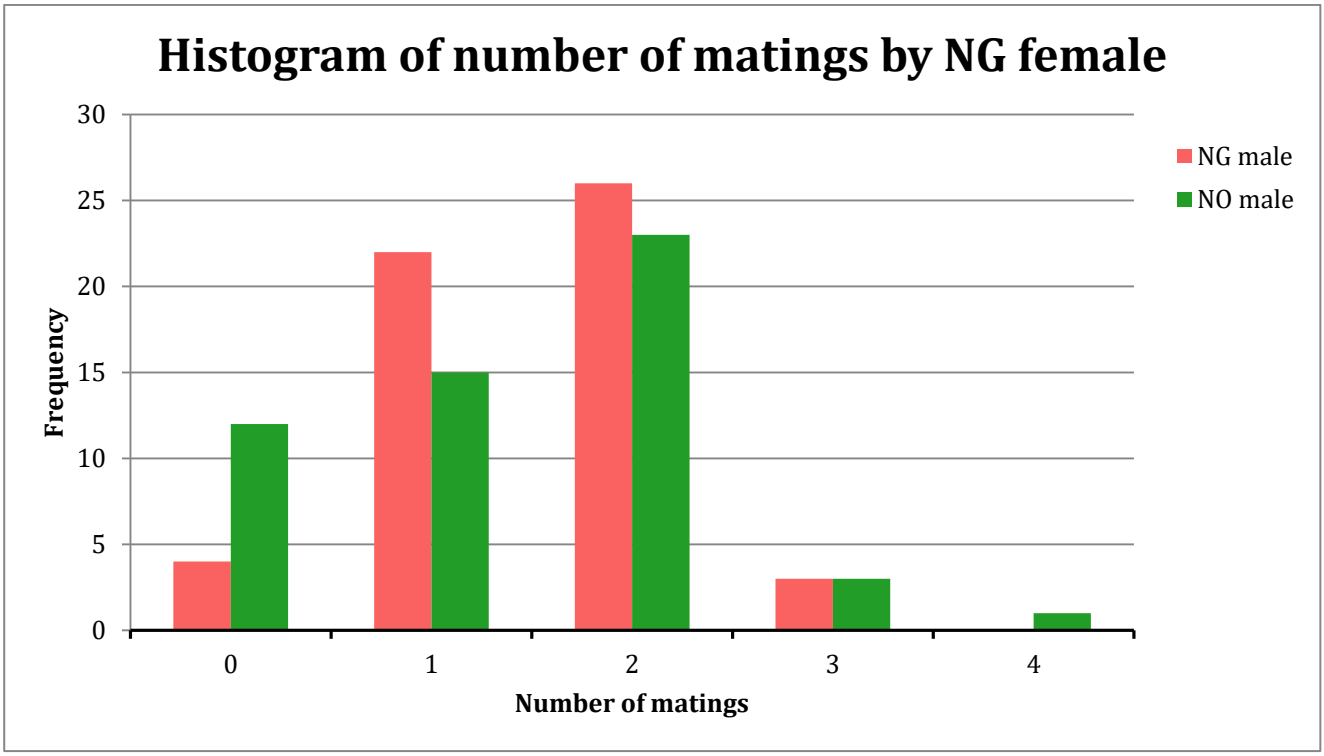


Figure 10.1 Gel Checking for *Wolbachia* infection in *N. oneida*

NO females were screened for *Wolbachia* infection using the procedure 2.2.5, pg 15. The absence of 400bp *w*spec bands suggests the *Wolbachia* titer was low enough to evade detection by PCR. The infection status of NO strain was verified by setting up an expected cytoplasmic incompatibility cross: a putatively cured NO male was mated with an NG female known to be uninfected. If NO was still infected, cytoplasmic incompatibility would occur and only male progeny would be obtained. However, female progeny were obtained from this cross and checked for mate preference, confirming that the NO strain was cured of *Wolbachia* infection.

10.(B). Number of matings performed by NG females during observation period



Generally, females of *Nasonia* are choosy of males and mate only once. However, most NG females mated twice with both NO and NG males; some mated up to three times with NG males and up to four times with NO males, within a 5-minute observation period.

10.2 Discussion

This study included studying mate preference in parental, F1 and F2 hybrids of the young sibling species pair *N. girault*i and *N. oneida* and setting up recombinant inbred isofemale lines with the ultimate aim of discovering the genes governing female mate preference behavior.

(1) Asymmetric assortative mating between NG and NO is maintained even after extensive lab rearing. F1 hybrids in both directions show no preference: therefore, a single round of hybridization with NG was enough to cause the disappearance of the rejection phenotype. This implies that NO's "rejection" alleles are recessive to NG's "acceptance" alleles, making the NG male acceptance phenotype a tractable one in a genetic study. F2 hybrid females (75% NO, 25% NG with NO cytoplasm) would be expected to show a 1:1 phenotypic ratio of NG male acceptance:rejection, assuming the Mendelian one locus – two alleles model. However, the F2 hybrid females entirely regain the NG male rejection phenotype that was present in their NO grandmothers. This suggests that the preference trait could be polygenic. It also means that the acceptance trait cannot be retained by simple introgression, since a single round of hybridization with NO in the F1 hybrid females caused the "acceptance" phenotype to disappear.

Female	% NG male acceptance	% NO male acceptance
Parental: NO	**7.69* (n=65)**	88.3 (n=33)
Parental: NG	95.83 (n=48)	94 (n=50)
F1 hybrid: NG/NO[NO]	95.65 (n=23)	96 (n=25)
F1 hybrid: NG/NO[NG]	84.37 (n=30)	100 (n=32)
F2 hybrid: NG/NO[NO]f X NOm	**0* (n=20)**	90 (n=14)

Table 10.2: % Acceptance of NO and NG males by all females. Bold and *$p < 0.01$, Fisher Exact test

(2) The F2 hybrid males contain recombinant NO and NG genomes, with an expected 50% of each. F3 progeny resulting from a backcross with an NO female would give F3 hybrid females that are heterozygous for the NG loci inherited from F2 hybrid fathers. Finally, a father-daughter mating would result in the F4 female progeny inheriting a subset of the NG loci present in their father. If a single F2 hybrid male is mated with multiple daughters, each cross would yield progeny homozygous for a unique subset of NG loci that were present in the father - this would be because of recombination in the female when producing gametes. It is possible for some isofemale lines to lose the phenotype entirely: however, this would also be advantageous as it could allow genotypic comparison with isofemale lines having the phenotype and ruling out some loci as causative. The establishment of a

total of 91 isofemale lines derived from 12 F2 hybrid males is a step ahead towards arriving at the genes responsible for the trait.

Appendix

I. Morphological differences between *N. giraulti* and *N. oneida*

IA. Males: NO males have a green hue on their dorsum, while NG males have a red hue. The hues persist in the adult stage.

Figure A1 *N. oneida* male

Figure A2 *N. giraulti* male

IB. Females: The stigma vein of the NG female's forewing is arched, while that of NO female's is angular.

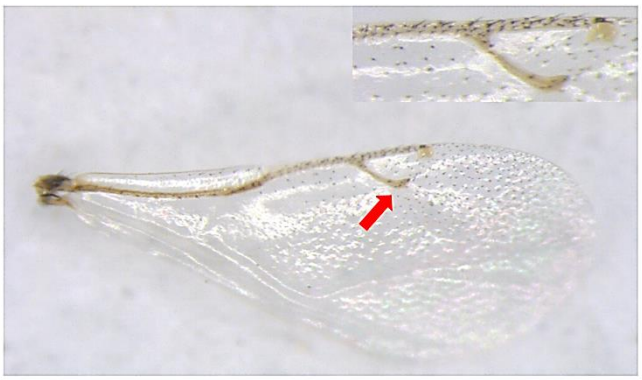


Figure A3: NG female forewing

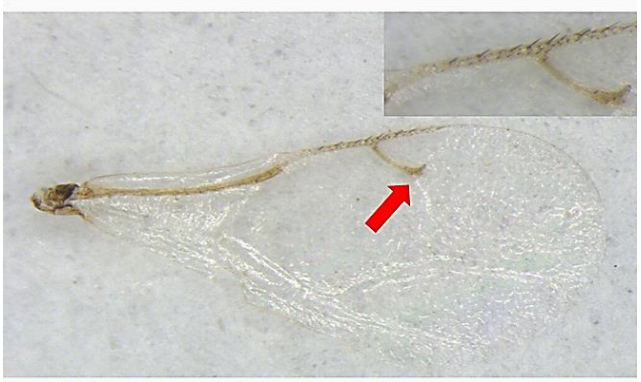


Figure A4: NO female forewing

F1 hybrid females were verified to be hybrids by the hue on the dorsum of their sons. F2 hybrid males show both intermediate and parental hues on their dorsum.

II Family sizes and sex ratios

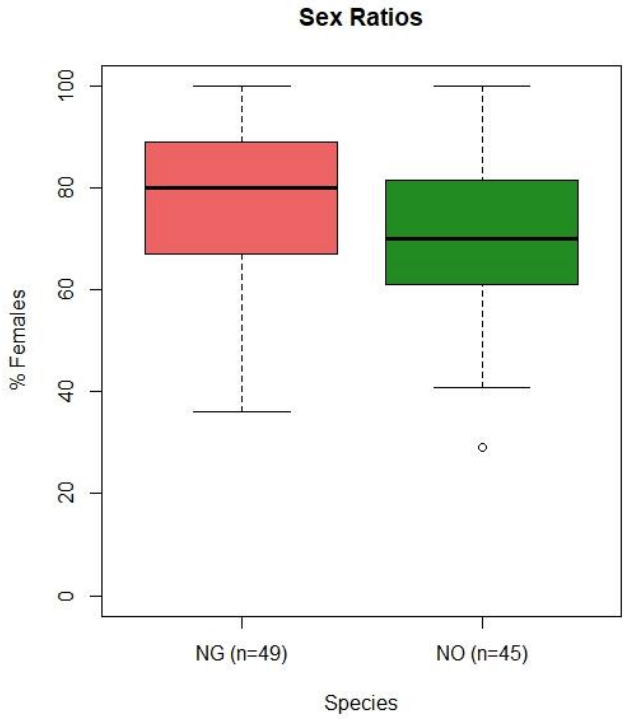


Figure A3: Sex Ratios of clutches obtained from a single infested host

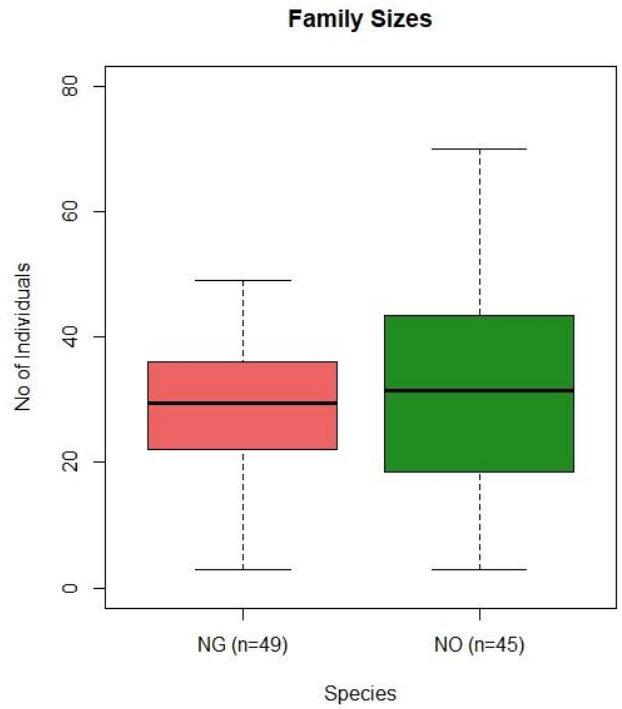


Figure A4: Number of individuals obtained from a single infested host

Bibliography

- van den Assem, J. and Beukeboom, L. (2004) ‘A review of Nasonia (Chalcidoidea , Pteromalidae) courtship and mating behaviour, with some additional, new observations’, *Proceedings of the Netherlands Entomolical Society Meeting*, 15, pp. 123–132.

- van den Assem, J. and Jachmann, F. (1996) ‘A Causal Ethological Analysis of the Courtship Behaviour of an Insect (the Parasitic Wasp Nasonia Vitripennis, Hym., Pteromalidae)’, *Behaviour*, 133(13), pp. 1051–1075. doi: 10.1163/156853996X00594.

- van den Assem, and Jachmann F, Simbolotti P (1980), 'Courtship Behaviour of Nasonia vitripennis (Hym., Pteromalidae): Some Qualitative, Experimental Evidence for the Role of Pheromones. *Behaviour*, 75 (3), pp. 301–307

- Van Den Assem, J. and Werren, J. H. (1994) ‘A Comparison of the Courtship and Mating Behavior of Three Species of Nasonia (Hymenoptera: Pteromalidae)’, *Journal of Insect Behavior*, 7(1).

- Bay, R. A. *et al.* (2017) ‘Genetic Coupling of Female Mate Choice with Polygenic Ecological Divergence Facilitates Stickleback Speciation’, *Current Biology*, 27(21), p. 3344–3349.e4. doi: 10.1016/j.cub.2017.09.037.

- Beukeboom, L., van den Assem, J. (2001) 'Courtship and mating behaviour of interspecific Nasonia hybrids (Hymenoptera, Pteromalidae), *Behavior Genetics 31(2)*

- Beukeboom, L., van de Zande, L. (2010), Genetics of sex determination in the haplodiploid wasp Nasonia vitripennis (Hymenoptera: Chalcidoidea), *J. Genetics 83(3) p 333-339*

- Blows, M. W. and Allan, R. A. (1998) ‘Levels of Mate Recognition within and between Two Drosophila Species and Their Hybrids’, *The American Naturalist*, 152(6), pp. 826–837. doi: 10.1086/286211.

- Bordenstein, S. R., O'hara, F. P., Werren J. H. (2001) '*Wolbachia* induced incompatibility precedes other hybrid incompatibilities in *Nasonia', Nature 409(6821) p 707-710*

- Boughman, J. W. (2002) ‘How sensory drive can promote speciation’, *TRENDS in Ecology & Evolution*, 17(12), p. 571–577 ST–How sensory drive can promotespeciat. doi: 10.1016/S0169-5347(02)02595-8.

- Brand, P. *et al.* (2015) ‘Rapid evolution of chemosensory receptor genes in a pair of sibling species of orchid bees (Apidae: Euglossini)’, *BMC Evolutionary Biology*. doi: 10.1186/s12862-015-0451-9.

- Brock, Chad D., Cummings M. E., Bolnick D. I (2017), ' Phenotypic plasticity drives a depth gradient in male conspicuousness in threespine stickleback, *Gasterosteus aculeatus*' *Evolution 71(8) p 2022-2036*

- Brucker R. M., Bordenstein R. S. (2013), 'The Hologenomic Basis of Speciation: Gut Bacteria Cause Hybrid Lethality in the Genus Nasonia', *Science 341, p 667-669*

- Buellesbach, J. *et al.* (2014) ‘Asymmetric assortative mating behaviour reflects incomplete pre-zygotic isolation in the Nasonia species complex’, *Ethology*, 120(8), pp. 834–843. doi: 10.1111/eth.12250.

- Chung, H. *et al.* (2014) ‘A single gene affects both ecological divergence and mate choice in Drosophila’, *Science*, 343(6175), pp. 1148–1151. doi: 10.1126/science.1249998.

- Chung, H. and Carroll, S. B. (2015) ‘Wax, sex and the origin of species: Dual roles of insect cuticular hydrocarbons in adaptation and mating’, *BioEssays*, 37(7), pp. 822–830. doi: 10.1002/bies.201500014.

- Clark M. E., O'Hara F. P., Chawla A., Werren, J. H. (2010) Behavioral and spermatogenic hybrid male breakdown in Nasonia', *Heredity (2010) 104, 289–301*

- Coyne, J. (1992), 'Genetics and Speciation', *Nature 355(6360) p 511-515*

- Coyne, J. A. and Orr, H. A. (1997) ‘“Patterns of Speciation in Drosophila” Revisited’, *Evolution*, 51(1), pp. 295–303. Available at: http://www.jstor.org/stable/2410984.

- Darling, D. C. and Werren, J. H. (1990) ‘Biosystematics of Nasonia (Hymenoptera: Pteromalidae): Two New Species Reared from Birds’ Nests in North America’, *Annals of the Entomological Society of America*, 83(3), pp. 352–370. doi: 10.1093/aesa/83.3.352.

- Desjardins *et. al.* (2013) 'Fine-Scale Mapping of the Nasonia Genome to Chromosomes Using a High-Density Genotyping Microarray' *G3: Genes, Genomics, Genetics 2013 3(2) p205-215*

- Drapeau, M. D., Werren, J. H., (1999), 'Differences in mating behaviour and sex ratio between three sibling species of Nasonia', *Evolutionary Ecology Research, 1999, 1: 223-234*

- Ellison, C. K., Niehuis, O., Gadau, J. (2008), 'Hybrid breakdown and mitochondrial dysfunction in hybrids of parasitoid wasps *Nasonia', J. EVOL. BIOL . 21 (2008) 1844–1851*

- Eltz, T. *et al.* (1999) ‘Fragrance collection, storage, and accumulation by individual male orchid bees’, *Journal of Chemical Ecology*, 25(1), pp. 157–176. doi: 10.1023/a:1020897302355.

- Eltz, T. *et al.* (2008) ‘An Olfactory Shift Is Associated with Male Perfume Differentiation and Species Divergence in Orchid Bees’, *Current Biology*. Elsevier Ltd, 18(23), pp. 1844–1848. doi: 10.1016/j.cub.2008.10.049.

- Eltz, T., Sager, A. and Lunau, K. (2005) ‘Juggling with volatiles: Exposure of perfumes by displaying male orchid bees’, *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, 191(7), pp. 575–581. doi: 10.1007/s00359-005-0603-2.

- Etges, W. (1992) ‘Premating isolation is determined by larval substrates in cactophilic Drosophila mojavensis’, *Evolution*, 46(6), pp. 1945–1950. Available at: http://www.jstor.org/stable/2410042.

- Flaxman, S. M., Sherman P. W., Paul, W., (1987), 'Offspring Sex Ratios in Parasitoid wasps', *Quarterly Review of Biology, 64(2) p 113-148*

- Hardy, I. C. W. (2016) ‘Nordic Society Oikos Non-Binomial Sex Allocation and Brood Sex Ratio Variances in the Parasitoid Hymenoptera Author (s): Ian C . W . Hardy Published by : Wiley on behalf of Nordic Society Oikos Stable URL : http://www.jstor.org/stable/3544897 REFERENCES’, 65(1), pp. 143–158.

- Ingram, T., Jiang, Y., Rangel, R., Bolnick, D. I. (2015), 'Widespread positive but weak assortative mating by diet within stickleback populations', *Ecology and Evolution 2015; 5(16): 3352–3363*

- Jiggins, C. D. *et al.* (2001) ‘Reproductive isolation caused by colour pattern mimicry’, *Nature*, 411(6835), pp. 302–305. doi: 10.1038/35077075.

- Kronforst, M. R. *et al.* (2006) ‘Linkage of butterfly mate preference and wing color preference cue at the genomic location of wingless’, *Proceedings of the National Academy of Sciences*, 103(17), pp. 6575–6580. doi: 10.1073/pnas.0509685103.

- Lukhtanov, V. A. *et al.* (2005) ‘Reinforcement of pre-zygotic isolation and karyotype evolution in Agrodiaetus butterflies’, *Nature*, 436(7049), pp. 385–389. doi: 10.1038/nature03704.

- Maan, M. E. and Seehausen, O. (2012) ‘Magic cues versus magic preferences in speciation’, *Evolutionary Ecology Research*, 14(6), pp. 779–785.

- Maan, M. E., Seehausen, O. and Van Alphen, J. J. M. (2010) ‘Female mating preferences and male coloration covary with water transparency in a Lake Victoria cichlid fish’, *Biological Journal of the Linnean Society*, 99, pp. 398–406.

- Marques, D. A. *et al.* (2017) ‘Genomic landscape of early ecological speciation initiated by selection on nuptial colour’, *Molecular Ecology*, 26(1), pp. 7–24. doi: 10.1111/mec.13774.

- Martin, S. H., Dasmahapatra, K. K., Nadeau, N. J., (2013) 'Genome-wide evidence for speciation with gene flow in Heliconius butterflies' *Genome Research 23:1817–1828*

- Martinson, E. O. *et al.* (2014) ‘Nasonia vitripennis venom causes targeted gene expression changes in its fly host’, *Molecular Ecology*. doi: 10.1111/mec.12967.

- Mayr, E. (1942), 'Systematics and The Origin of Species', *Nature 139(3515) p 431-432*

- Mullen, S. P. and Shaw, K. L. (2014) ‘Insect Speciation Rules: Unifying Concepts in Speciation Research’, *Annual Review of Entomology*, 59(1), pp. 339–361. doi: 10.1146/annurev-ento-120710-100621.

- Naisbit, R. E., Jiggins, C. D. and Mallet, J. (2001) ‘Disruptive sexual selection against hybrids contributes to speciation between Heliconius cydno and Heliconius melpomene’, *Proceedings of the Royal Society B: Biological Sciences*, 268(1478), pp. 1849–1854. doi: 10.1098/rspb.2001.1753.

- Niehuis, O. *et al.* (2013) ‘Behavioural and genetic analyses of Nasonia shed light on the evolution of sex pheromones’, *Nature*, 494(7437), pp. 345–348. doi: 10.1038/nature11838.

- Nosil, P., Shluter, D. (2011), 'The genes underlying the process of speciation' *Trends in*

Ecology and Evolution, 26(4), doi:10.1016/j.tree.2011.01.001

- Orr, H. A., Masly, J. P. and Presgraves, D. C. (2004) 'Speciation genes', *Current Opinion in Genetics and Development*, 14(6), pp. 675–679. doi: 10.1016/j.gde.2004.08.009.

- Raychoudhury, R. *et al.* (2010) 'Behavioral and genetic characteristics of a new species of Nasonia', *Heredity*. doi: 10.1038/hdy.2009.147.

- Rivers, D. B., Hink, W. F. and Denlinger, D. L. (1993) 'Toxicity of the venom from Nasonia vitripennis (Hymenoptera : Pteromalidae) toward fly hosts, nontarget insects, different developmental stages, and cultured insect cells', *Toxicon*. Pergamon, 31(6), pp. 755–765. doi: 10.1016/0041-0101(93)90381-R.

- Robertson, H. M., Gadau, J., Wanner, K. W. (2010), The insect chemoreceptor superfamily of the parasitoid jewel wasp Nasonia vitripennis, *Insect Molecular Biology (2010), 19 (Suppl. 1), 121–136 Insect*

- Rogers, S. M., Bernatchez, L. (2006) 'The genetic basis of intrinsic and extrinsic post-zygotic reproductive isolation jointly promoting speciation in the lake whitefish species complex (Coregonus clupeaformis' *J. Evol. Bio 1979–1994*

- Ruther, J. *et al.* (2016) 'Epimerisation of chiral hydroxylactones by short-chain dehydrogenases /reductases accounts for sex pheromone evolution in Nasonia', *Scientific Reports*. doi: 10.1038/srep34697.

- Seehausen, O. *et al.* (2008) 'Speciation through sensory drive in cichlid fish', *Nature*, 455(7213), pp. 620–626. doi: 10.1038/nature07285.

- Servedio, M. R. *et al.* (2011) 'Magic traits in speciation:"magic"but not rare?', *Trends in ecology & evolution*, 26(8), pp. 389–397. Available at: http://www.elsevier.com/copyright.

- Servedio, M. R. and Kopp, M. (2012) 'Sexual selection and magic traits in speciation with gene flow', *Current Zoology*, 58(3), pp. 510–516. doi: 10.1093/czoolo/58.3.510.

- Smadja, C., Butlin, R. K. (2009), ' On the scent of speciation: the chemosensory system and its role in premating isolation', *Heredity (2009) 102, 77–97*

- Steven H Orzack and E. D Parker, J. (2013) 'Sex-Ratio Control in a Parasitic Wasp , Nasonia vitripennis *Evolution*, 40(2), pp. 331–340.

- Svensson, O. *et al.* (2017) 'The genetics of mate preferences in hybrids between two young and sympatric Lake Victoria cichlid species', *Proc. R. Soc. B*, 284(1849), pp. 1–9. doi: 10.1098/rspb.2016.2332.

- Thibert-Plante, X. and Gavrilets, S. (2013) 'Evolution of mate choice and the so-called magic traits in ecological speciation', *Ecology Letters*. doi: 10.1111/ele.12131.

- Vernel, C. (1979) 'Courtship Behaviour Of *Nasonia vitripennis* (Hym: Pteromalidae): Observations And Experiments On Male Readiness To Assume Copulatory Behaviour', *Behaviour*, 682139227(1), pp. 118–135. Available at: http://www.jstor.org/stable/4533945.

- Werren *et. al. (2010)* Functional and Evolutionary Insights from the Genomes of Three Parasitoid Nasonia Species *Science 327(5963): 343–348*

- Whiting, A. R. (1967), 'The Biology of the Parasitic Wasp Mormoniella vitripennis', *The Quarterly Review of Biology, 42(3) p 333-406*

- Xue, H. J. *et al.* (2016) 'Contact pheromones of 2 sympatric beetles are modified by the host plant and affect mate choice', *Behavioral Ecology*, 27(3), pp. 895–902. doi: 10.1093/beheco/arv238.

- Yukilevich, R. *et al.* (2018) 'Rapid sexual and genomic isolation in sympatric Drosophila without reproductive character displacement', *Ecology and Evolution*, 8, pp. 2852–2867. doi: 10.1002/ece3.3893.