The Genetics of Speciation in the Parasitoid Wasp, *Nasonia*

by

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ABSTRACT

Speciation is the fundamental process that has generated the vast diversity of life on earth. The hallmark of speciation is the evolution of barriers to gene flow. These barriers may reduce gene flow either by keeping incipient species from hybridizing at all (pre-zygotic), or by reducing the fitness of hybrids (post-zygotic). To understand the genetic architecture of these barriers and how they evolve, I studied a genus of wasps that exhibits barriers to gene flow that act both pre- and post-zygotically. *Nasonia* is a genus of four species of parasitoid wasps that can be hybridized in the laboratory. When two of these species, *N. vitripennis* and *N. giraulti* are mated, their offspring suffer, depending on the generation and cross examined, up to 80% mortality during larval development due to incompatible genic interactions between their nuclear and mitochondrial genomes. These species also exhibit pre-zygotic isolation, meaning they are more likely to mate with their own species when given the choice. I examined these two species and their hybrids to determine the genetic and physiological bases of both speciation mechanisms and to understand the evolutionary forces leading to them. I present results that indicate that the oxidative phosphorylation (OXPHOS) pathway, an essential pathway that is responsible for mitochondrial energy generation, is impaired in hybrids of these two species. These results indicate that this impairment is due to the unique evolutionary dynamics of the combined nuclear and mitochondrial origin of this pathway. I also present results showing that, as larvae, these hybrids experience retarded growth linked to the previously observed mortality and I explore possible physiological mechanisms for this. Finally, I show that the pre-mating isolation is due to a change in a single pheromone component in *N. vitripennis* males, that this change is under simple genetic

control, and that it evolved neutrally before being co-opted as a species recognition signal. These results are an important addition to our overall understanding of the mechanisms of speciation and showcase *Nasonia* as an emerging model for the study of the genetics of speciation.

DEDICATION

To my parents, Janet and Joseph, who have never stopped encouraging my curiosity about the world. Your help, whether it was in looking for more critters under a log or in considering the direction of my life, has meant the world to me.

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CHAPTER 1

INTRODUCTION

Speciation

Charles Darwin, quoting Sir John Herschel, referred to the origin of species as "that mystery of mysteries" as he set out to explain the processes that resulted in the diversity of life that he observed on his trip aboard the H.M.S. Beagle (Darwin, 1859). How these processes, collectively known as speciation, produce new species and keep existing species separated continues to be one of the most fundamental questions in evolutionary biology today. Despite this enduring interest, and while much headway has been made, there are still limited examples of the processes that lead to evolution of new species. This is particularly true of genetic mechanisms that serve to keep incipient species isolated. However, recent advances in molecular and genetic techniques are opening new avenues for fruitful research in speciation genetics (Maheshwari & Barbash, 2011).

Speciation, by its definition, is the process of forming biological species and therefore it is necessary to define what I mean by "species" for this dissertation. I utilize the modified Biological Species Concept of Coyne & Orr (2004). This concept is an adaptation of the Biological Species Concept (BSC), which defines a species as "a group of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" (Mayr, 1942). The modification of Coyne & Orr (2004) relaxes the requirement of complete reproductive isolation to allow for speciation with limited gene flow. The core component of this concept is that there is more reproductive isolation between the groups than within each group.

Reproductive isolation is the result of barriers to gene flow between the groups, or incipient species. Many barriers that separate these incipient species are either part of or are heavily influenced by the external environment. These barriers include geographic or temporal barriers (Williams & Simon 1995; Hasegawa *et al*. 2006), infection by endosymbionts (Bordenstein *et al.,* 2001) and differential adaptation of life history traits (Kay & Schemske, 2003; Soliva & Widmer, 2003; Funk *et al.,* 2006; Nosil, 2006) or any environmental variable that reduces gene flow between subpopulations of a species. These barriers serve to keep the incipient species genetically separated from one another, but as extrinsic factors (or adaptations to extrinsic factors) they may change in such a way that gene flow between the incipient species is reestablished. Consequently, if these factors change, there is the possibility that the species will interbreed and genetic admixture will occur, resulting in reversion to a single species. This reversion, however, may not occur if the species have evolved intrinsic genetic incompatibilities that result in decreased fitness of hybrids between the species.

Any incompatibility that impacts the fitness of interspecific hybrids will reduce gene flow between the parental species and, depending on the extent of the impact, may result in the species remaining as independent populations that continue to diverge. Once the incompatibilities are sufficiently deleterious to allow the two populations to continue diverging, the incipient species will be on the path toward complete genetic isolation. During the evolution of these species, any reduction in hybrid fitness can also serve as selective pressure for the evolution of species recognition signals that allow for assortative mating in order to reduce the occurrence of costly interspecific mating (Servedio & Saetre, 2003). Assortative mating, therefore, keeps the incipient species

further isolated and results in more genetic incompatibilities evolving over time (Orr & Turelli, 2001). The speciation process at this point can become a feedback loop with assortative mating allowing further incompatibilities to evolve, which in turn puts more selective pressure for prezygotic isolating mechanisms until the species cease all interspecific matings.

Intrinsic Isolation Barriers:

The intrinsic genetic incompatibilities that occur in hybrids cannot evolve specifically as barriers to gene flow, because the maladaptive nature of the barrier would be opposed by selection in the parental species. Rather, the pathways that ultimately serve as barriers to gene flow in hybrids evolve independently within each parental species, either due to genetic drift or selection (Coyne & Orr, 2004). The "evolutionary byproduct" nature of these barriers is because they are due to a breakdown in compatibility between interacting partners that make up gene complexes that have been co-adapted to one another in each parental lineage (Mayr, 1942). This co-adaptation occurs because, within each parental lineage, the proper interaction of the genes making up these complexes needs to be maintained in order for the complex to function properly as a whole. The incompatibility that occurs in hybrids, therefore, is due to independently evolved orthologous copies of these genes being brought together in the hybrid genome. The evolutionary process of co-adaptation leading to dysfunction in hybrids is illustrated by the Bateson-Dobzhansky-Muller (BDM) model (Figure 1.1; Bateson, 1909; Dobzhansky, 1937; Muller, 1942). The BDM model shows that the independent evolution of the interacting genes in each parental lineage *can* lead to incompatibilities in hybrids simply because the novel pairing between these genes has never been "tested" by

selection. The model, however, is agnostic to the evolutionary forces that lead to divergence between the genes.

In the absence of selection (i.e. neutrality), the interacting genes will eventually diverge in each parental lineage to an extent that is sufficient to result in incompatible interactions when they are brought together in hybrids. However, incompatible interactions between two (or more) genes in hybrids doesn't necessarily mean that they result in any reduction in hybrid fitness (Naisbit *et al.,* 2001). Indeed, genes that evolve neutrally or nearly neutrally should be less likely to cause disruptions in hybrids than more constrained genes because their function in the parental lineages is more tolerant of variability in their sequences (as evidenced by their neutral evolution). The complexes that, when disrupted, are most likely to result in hybrid inviability are those with constituent genes that have very close interactions, evolve quickly and that serve important cellular functions (Mishmar & Gershoni, 2007). This is because the orthologues will diverge quickly in each parental lineage if the substitution rates are high. Incompatible interactions in hybrids will be more likely to occur if these fast-evolving genes normally interact closely within the complex. And finally, the effect of the resultant "untested" interactions in hybrids will be more likely to have a major impact on fitness if the complex is involved in an important cellular function. These considerations indicate that there may be particular molecular pathways that are more likely to evolve hybrid incompatibilities and to therefore serve as drivers of speciation.

The Oxidative Phosphorylation Pathway

The oxidative phosphorylation (OXPHOS) pathway is a system that has qualities that may make it a likely candidate for driving speciation. The pathway is composed of

protein subunits that interact tightly with one another to form five complexes. These complexes, taken together, are responsible for establishing a proton gradient within the mitochondria that is then used to produce cellular energy in the form of ATP (Scheffler, 2008). The energy generating function of the pathway is conserved across most eukaryotes, which rely on this energy to maintain basic cellular functions (Porcelli *et al.,* 2007). This conservation of function indicates that disruption of this pathway in hybrids is likely to cause significant deleterious effects. The proteins that make up the complexes of the OXPHOS pathway are known to interact tightly, and yet they evolve quickly due to the unique composition of these complexes. Four of the five complexes are composed of both nuclear- and mitochondrial-encoded proteins, and these mitochondrial genes have been shown to evolve much more quickly than nuclear genes (Hatefi, 1985; see also Blier *et al.,* 2001; Oliveira *et al*., 2008). The high substitution rate is hypothesized to cause slight disruptions in the function of the pathway due to the introduction of slightly deleterious substitutions. This slight disruption places increased selective pressure on the mitochondrial genome to remove these deleterious substitutions, but the ability of selection to act on the mitochondrial genome is reduced relative to the nuclear genome due to its reduced population size and lack of recombination (Rand *et al.,* 2004). The inability of selection to effectively remove deleterious substitutions from the mitochondrial genome faster than they arise results in an accumulation of these substitutions. The accumulation of these substitutions places compensatory selective pressure on the nuclear-encoded genes that interact with the mitochondrial-encoded genes in order to rescue the function of the pathway. This ongoing process in the OXPHOS system between accumulating deleterious substitutions in the mitochondrial-

encoded genes and compensatory evolution in the nuclear-encoded genes, known as compensatory co-adaptation (Rand *et al.,* 2004), provides a potentially very strong mechanism by which barriers to gene flow evolve and, ultimately, by which speciation occurs.

Nasonia

Nasonia is a genus of parasitoid wasps (Hymenoptera: Pteromalidae) that parasitize flesh fly and blow fly pupae that are found on animal carcasses as well as in nests of cavity nesting birds. There are four species currently known in the genus; *N. vitripennis*, *N. longicornis, N. giraulti* and *N. oneida*. *N. vitripennis* is the most distantly related species in the genus, separated from the other species by ~1 million years (MY), has a cosmopolitan distribution across North America, Europe and Asia and is found in sympatry with the other species . *N. longicornis* and *N. giraulti* are separated by 0.2-0.4MY and are found only in western and eastern North America, respectively. *N. oneida* (sister species to *N. giraulti*) has only been found at one location in upstate New York and is sympatric with *N. giraulti* (Werren *et al.,* 2010). Females of this genus use their ovipositor to sting the host pupa through its puparium, arresting development and altering the host body composition to suit the *Nasonia* larvae (Rivers & Denlinger, 1994a, 1994b, 1995). The females then lay eggs within the host puparium, but on the exterior of the host pupa and the larvae hatch and begin feeding within 36 hours after oviposition. After feeding, the larvae pupate, eclose and then emerge from the host puparium ~14 days after oviposition (Whiting, 1967). *N. giraulti* is known to mate within the host before emerging, while the other species will mate upon emergence (Drapeau & Werren, 1999). In nature, the species are isolated by differential *Wolbachia* infections

that result in complete cytoplasmic incompatibility between the species, with the exception of *N. giraulti* and *N. oneida*, which share the same *Wolbachia* strains and therefore don't experience this cytoplasmic incompatibility (Bordenstein *et al.,* 2001; Raychoudhury *et al.,* 2010).

Nasonia is a good system in which to study the genetics of speciation for a number of reasons. In the laboratory, *Wolbachia*-free strains can be produced by treating the strains with antibiotics (Breeuwer & Werren, 1993). Once cured of their *Wolbachia*, the species can be crossed with one another to produce hybrids that are fertile and partially viable, with the extent of the inviability varying between crosses. Importantly, the sex of *Nasonia* individuals can be determined at the pupal stage (before mating), allowing virgin individuals to be collected easily for genetic crosses. As in all Hymenoptera, *Nasonia* has a haplo-diploid genetic system; diploid females develop from fertilized eggs and haploid males develop directly from unfertilized eggs. In this genetic system, the first hybrids that are produced (F_1) are all females that have a single copy of each of the parental species chromosomes (i.e. they are heterozygous at all loci), and any males that develop are pure species individuals with the maternal genotype. The first hybrid males are the F_2 individuals that are produced by virgin F_1 hybrid females and, due to recombination during meiosis in these females, they each have a unique nuclear "mosaic" composed of chromosomal regions from both parental species. The haploid genetics of these males also greatly increases their utility in the lab, as genetic incompatibilities can be identified that might otherwise be masked by dominance in heterozygous diploids. Finally, the genome sequences of all *Nasonia* species are available along with a large suite of molecular and genomic tools, and strains are

available of each species that have been collected across their known ranges (Werren *et al.,* 2010).

Once cured of *Wolbachia*, the *Nasonia* species can be crossed with one another to produce F_1 hybrid females, which don't exhibit marked decreases in either fertility or viability in any cross within the genus (Breewer & Werren 1995, Koevoets *et al.,* 2011). When these F_1 females are from the widest crosses (*N. vitripennis x N. giraulti* or *N. vitripennis* x *N. longicornis)* and are given hosts as virgins, however, the resultant F_2 male offspring show a significant difference in the number of surviving adults relative to the number of newly hatched larvae, indicating mortality during larval development. The extent of the larval mortality depends on the species that are crossed as well as the cytotype of the hybrid and ranges from ~25-80% (Breewer & Werren 1995, Koevoets *et al.,* 2011). The cytotype specificity of the mortality indicates that nuclear-mitochondrial incompatibilities contribute to the mortality.

The nuclear-cytoplasmic nature of the mortality in *Nasonia* argues that the OXPHOS pathway may be impaired in these hybrids. This is both because of the hypothesis outlined above of compensatory co-adaptation acting as a driver of hybridization barriers as well as the fact that all 13 protein coding genes in the mitochondrial genome encode subunits of the OXPHOS complexes. Indeed, the adult F_2 hybrid males of the *N. vitripennis* x *N. giraulti* cross show less activity of their OXPHOS complexes than males of the parental species (Ellison *et al.,* 2008). The OXPHOS complexes that show a reduction differ between the two cytotypes and Complex II, the only wholly nuclear-encoded complex, showed no reduction in activity. Both of these

findings further help to link the mortality observed in this cross to nuclear-cytoplasmic incompatibilities that impact the OXPHOS pathway.

In order to determine the evolutionary forces that have led to this hybridization barrier in *Nasonia*, it is necessary to first identify the genes that underlie the mortality. Several nuclear genomic regions that underlie the mortality have been identified in previous studies by evaluating, in a large sample of adult F_2 hybrid males, the proportion of alleles of each species at markers spread across the genome and comparing this to the expected segregation ratio (i.e. 50:50 for each parental allele). Areas that deviate from this expected ratio are referred to as transmission ratio distorting loci (TRDL). Given that these areas don't show any distortion in embryos of these crosses, the distortion seen in TRDLs is therefore attributable to mortality during larval development, linking these regions to the mortality that was previously identified. Four TRDL were identified in the *N. vitripennis* x *N. longicornis* cross and an additional four were identified in the *N. vitripennis* x *N. giraulti* cross (Niehuis *et al.,* 2008; Koevoets *et al.,* 2011).

Overview of Dissertation

The OXPHOS impairment in adult F_2 hybrid males, combined with the identificaiton of TRDL implicated in the larval mortality, opens the door to assess the genes that contribute to the incompatibility as well as the evolutionary forces that have acted on them. In Chapter 2 of this dissertation, I present results of the manual annotation of the nuclear-encoded OXPHOS genes from *N. vitripennis* and their orthologues in *N. giraulti* and *N. longicornis* in order to determine both their coding nucleotide sequences for evolutionary analysis (Gibson *et al.,* 2010). I aligned the sequences with their orthologues from six other holometabolous insects and assessd the role of positive

selection in their evolution, as predicted by the compensatory co-adaptation hypothesis. I also analyzed the amino acid changes that occurred within *Nasonia* for evidence of substitutions that are predicted to have a negative impact on the function of the protein they encode. I used these techniques to determine nuclear-encoded OXPHOS genes that may have evolved compensatory adaptations and that may lead to incompatibilities in the pathways' function in *Nasonia*. In order to compare the positions of these genes to the previously identified TRDL, it was necessary to map the scaffolds of the *Nasonia* genome. I designed species-diagnostic markers that were used for a single nucleotide polymorphism (SNP) genotyping microarray and ran this array with GV[V] F_2 hybrid embryos in order to map the *Nasonia* genome (Niehuis *et al.,* 2010; Werren *et al.,* 2010). I determined candidate genes for the incompatibility by assessing their position within the genome against the known positions of TRDL in the genome. While this "candidate pathway" approach allowed the assessment of genes across the genome, it relied on the most current genomic map and the data available for proper annotations and may not capture the true causal gene due to missing information.

The data gathered for the TRDL analysis can be used to determine whether there are potentially more regions that weren't detected. This is done by determining the proportion of the total observed mortality for which each locus can account (Niehuis *et al.,* 2008). The TRDL found in the *N. vitripennis* x *N. giraulti* cross with a *N. vitripennis* cytoplasm (hereafter referred to as GV[V]; the letters outside the brackets designate the nuclear composition while the letter in the brackets designates the cytotype, G = *N. giraulti* and V = *N. vitripennis*) could explain all of the mortality in that direction of the cross, but the single TRDL in the VG[G] direction could not explain 40% of the observed

mortality in that cross. This large proportion of unexplained mortality may indicate that there were genomic regions that were not included in the initial analyses.

Utilizing the genomic resources currently available in *Nasonia*, it is possible to assess whether there were regions missing in previous analyses and whether these regions harbor any TRDL. In chapter 3, I present results from genotyping 331 VG[G] adult F_2 hybrid males for 1134 SNPs spread across the largest genomic scaffolds in the *Nasonia* genome that indicate that there is a previously unidentified region that leads to larval mortality in ~98% of individuals that have the *N. vitripennis* allele. I also present results indicating that hybrid larvae with the *N. vitripennis* allele in this region appear to halt their growth very early in development relative to their siblings with the *N. giraulti* allele (Gibson *et al.,* 2013). Using the updated genomic resources, I also identified a previously unannotated OXPHOS gene that resides in the same region as the putative causal allele for the mortality and larval developmental defects. In total, there are four nuclear-encoded OXPHOS genes that reside within this new region

The question remains of whether the observed mortality and associated TRDLs are causally associated with the reductions in OXPHOS enzyme activity. This is an important consideration, as previous studies assessed the activity of these enzymes at the organellular level, with isolated mitochondria that are normalized for protein concentration. It is possible that individual animals that experience reduced activity of their OXPHOS complexes may simply produce more mitochondria or more of the deficient complex to compensate for the lack of efficacy, in which case there could be very little impact of the deficiency on the fitness of the animal as a whole. To address this, in Chapter 4 I present results of physiological measurement of whole animal CO2

production and isolated mitochondrial O2 consumption of hybrids as well as their parental species equivalent. VG[G] F_1 hybrid adult females, despite the lack of apparent incompatibilities, showed a reduction in the O2 consumption of their mitochondria relative to females of either parental species. These results may indicate that there is organellular compensation occurring in these wasps, however it is not clear if this is due to differential allele expression between the compatible and incompatible alleles or if there are differences in the number of complexes produced. However, the adult F_2 hybrid males have a different metabolic scaling rate in relation to mass than males of either parental species. This result is interesting in light of the genotype dependent larval sizes discussed in Chapter 3 because it may indicate that these surviving males, of which ~5% have the incompatible allele in the newly identified region, were individuals that were over a critical size threshold to maintain metabolism at a sufficient level to fully develop whereas those that died were under this threshold. This implies that the smallest larvae should have an even lower metabolic rate (corrected for mass) than the smallest adults. I also include in Chapter 4 results of O2 consumption rates of VG[G] larvae that were collected to assess whether there is a difference in metabolic rates between the largest and the smallest larvae. The O2 consumption was not different from the levels attained when testing only the mitochondrial isolation medium and I discuss reasons for this finding.

Given the severity of the incompatibilities that are suffered by the *N. vitripennis* x *N. giraulti* hybrids, as well as the cytoplasmically-isolating *Wolbachia* infections in nature, it is expected that there has been selective pressure for species recognition signals in order to reduce the chances of interspecific mating. Indeed, the *Nasonia* species are

known to differ in courtship behavior and mating location preference (Van den Assem & Werren, 1994; Drapeau & Werren, 1999). In Chapter 5 I present results of an investigation into a sex pheromone that *Nasonia* males utilize to attract conspecific females. I show that the male sex pheromone of all *Nasonia* species is composed of three major components, except in *N. vitripennis*, which has an additional component. This additional component [4(R),5(R)-5-hydroxy-4-decanolide; hereafter *RR*] is produced through the conversion of a preexisting pheromone component [4(R),5(S)-5-hydroxy-4-decanolide: hereafter *RS*] by the action of three tandemly arranged genes. I also show that the ancestral *Nasonia* likely did not respond to this new component when it first evolved, which explains how new pheromones can evolve without being selected against by the pheromone receivers.

This dissertation has leveraged the biology of *Nasonia* and the continually growing molecular and genomic techniques available for the study of these wasps in order to assess the genetic basis of speciation. I have identified candidate speciation genes between *N. vitripennis* and *N. giraulti* that cause both pre-and post-zygotic isolation and have assessed them to determine the potential evolutionary forces that have shaped them in each parental species. While much work remains to be done, the results so far have helped to elucidate potentially broadly-acting mechanisms of speciation as they evolve in incipient species. This dissertation, as well as excellent work being performed at the University of Rochester, Vanderbilt University and the University of Groningen demonstrates that *Nasonia* is an emerging model organism for the study of the genetics of speciation.

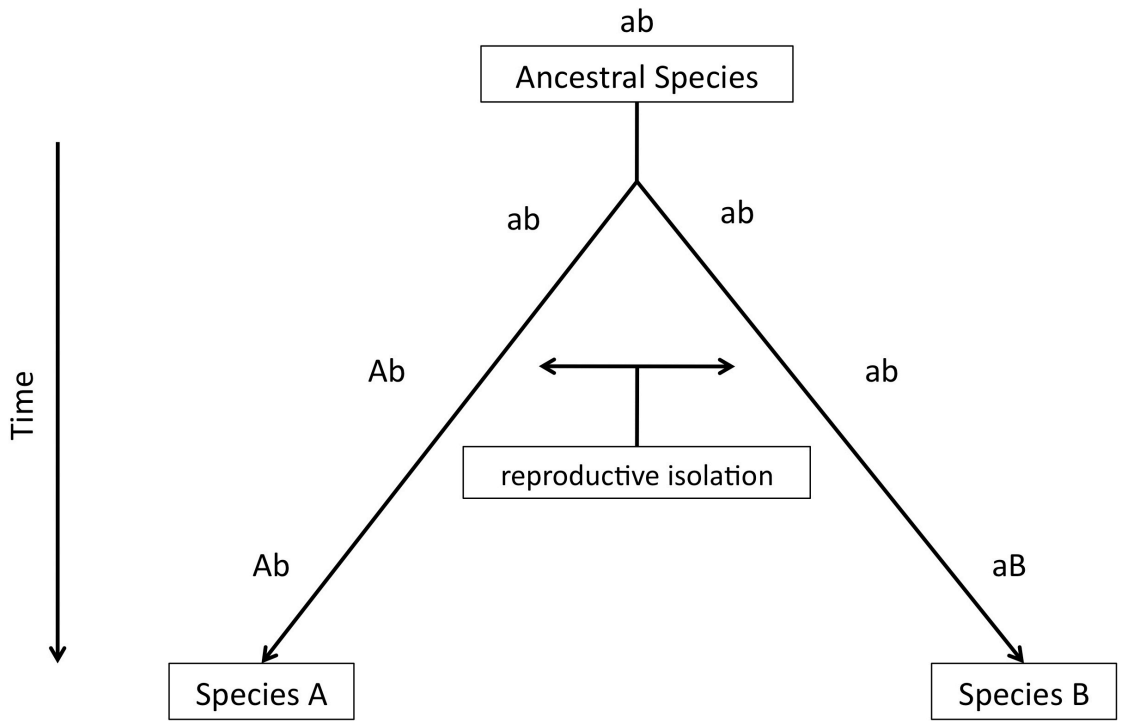

Figure 1.1. Bateson-Dobzhansky-Muller (BDM) model of the evolution of genic incompatibilities. The BDM model demonstrates how incompatibilities between two interacting genes ("a" and "b") can evolve in two reproductively isolated species without either parental species (Species A or Species B) experiencing the incompatibility. The genotypes "ab", "Ab" and "aB" have all been subjected to selection and should not show incompatibilities, while the "AB" genotype has not been tested and may be an incompatible combination in hybrids between these two species.

CHAPTER 2

CONTRASTING PATTERNS OF SELECTIVE CONSTRAINTS IN NUCLEAR ENCODED GENES OF THE OXIDATIVE PHOSPHORYLATION PATHWAY IN HOLOMETABOLOUS INSECTS AND THEIR POSSIBLE ROLE IN HYBRID BREAKDOWN IN *NASONIA*

Abstract

The principal energy generating system in animals is the oxidative phosphorylation (OXPHOS) pathway, which depends on the tight interaction of nuclear and mitochondrial encoded genes to function properly. Mitochondrial genes accumulate substitutions more quickly than nuclear genes, yet the impact of selection on mitochondrial genes is significantly reduced relative to nuclear genes due to the non-recombining nature of the mitochondrial genome and its predicted smaller effective population size. It has therefore been hypothesized that the nuclear encoded genes of the OXPHOS pathway are under strong selective pressure to compensate for the accumulation of deleterious nucleotide substitutions in mitochondrial encoded OXPHOS genes; a process known as compensatory co-adaptation. We evaluated this hypothesis by analyzing nuclear encoded OXPHOS genes for signatures of positive selection as well as evolutionary constraints at amino acid sites. We considered OXPHOS genes of six holometabolous insects and their orthologs from three *Nasonia* parasitoid wasps; hybrids of which suffer from an increased mortality rate caused by cytonuclear genic incompatibilities. Although nuclear OXPHOS genes are typically highly conserved, we found significant evidence for elevated amino acid divergence in four of the 59 studied nuclear encoded OXPHOS genes. We also found that three of these four genes, as well as

six other OXPHOS genes, contain amino acid substitutions between *Nasonia* species at evolutionarily constrained sites. It is possible that these genes account for the reported incompatibility in *Nasonia* hybrids and their characterization may lead to a better understanding of the role of positive selection in the genetics of speciation.

The oxidative phosphorylation pathway (OXPHOS) is the principal energy generating system in animals. Its composition is unique in combining both nuclear and mitochondrial encoded proteins, and the pathway is highly conserved in insects and vertebrates (Porcelli *et al*., 2007). At the same time there is growing evidence that hybrids between closely related species suffer from a reduction in their OXPHOS pathway efficiency, indicating an accumulation of incompatible substitutions in nuclear and mitochondrial encoded OXPHOS genes between even closely related species (Ellison & Burton, 2006; Ellison *et al*., 2008; Rand *et al*., 2004). We set out to assess the hypothesis that positive selection has guided the evolutionary changes found in this otherwise highly conserved pathway.

Mitochondria, in which oxidative phosphorylation occurs, are organelles that are likely derived from a single bacterial endosymbiont ancestor (Gray *et al.*, 1999). While this ancestor is assumed to have contained between 3,000 and 5,000 genes (Boussau *et al.*, 2004), mitochondrial genomes harbor only a fraction of these, typically less than 100 (Ballard & Rand, 2005). The majority of these presumably have been lost because they were no longer required within the eukaryotic environment. The function of others, however, has been maintained through translocation to the nuclear genome or by exaptation of existing nuclear genes for functions within the mitochondria, followed by a deletion of the functionally corresponding mitochondrial genes (Rand *et al.*, 2004, Gross & Bhattacharya, 2009). Over 1,000 nuclear genes have been estimated to encode proteins that are targeted to the mitochondria (Calvo *et al.*, 2006). As a result, mitochondria critically depend in their function on the proper and tight interaction of both nuclear and mitochondrial gene products.

Research on xenomitochondrial cybrid cells (i.e., chimeric cells with a nucleus and mitochondria from two different species) from primate and murid species provided direct evidence for the disruption of mitochondrial functions due to nuclear-cytoplasmic ("cytonuclear") incompatibility (King & Attardi, 1989; Kenyon & Moraes, 1997; McKenzie *et al.*, 2003). The severity of the disruption was thereby found to positively correlate with the divergence time of the analyzed species. Cytonuclear incompatibility has indeed not only been found in hybrid individuals from crosses between species (i.e., *Drosophila*, Sackton *et al.*, 2003; *Nasonia*, Niehuis *et al.*, 2008; Ellison *et al.*, 2008), but also from crosses between diverged populations of the same species (i.e., *Drosophila simulans*, Sackton *et al.*, 2003; *Tigriopus californicus*, Ellison & Burton, 2006, 2008). For this and other reasons, cytonuclear genic incompatibility has been implicated as an important postzygotic barrier to hybridization (Sackton *et al.*, 2003; Ellison & Burton, 2008; Ellison *et al.*, 2008).

The OXPHOS pathway is a prime candidate for cytonuclear genic incompatibilities, given that four of its five complexes (i.e., I, III, IV, and ATPase) are composed of subunits from both the nuclear and mitochondrial genomes (Blier *et al.*, 2001; Rand *et al.*, 2004). In vertebrates as well as insects, all 13 protein coding genes found in the mitochondrial genome are involved in the OXPHOS pathway. This pathway shows reduced ATP generation capacities in interpopulation hybrids of the copepod *Tigriopus californicus* as well as in interspecific hybrids of *Drosophila*, and most recently, in those of the parasitoid wasp *Nasonia* (Sackton *et al.*, 2003; Ellison & Burton, 2006; Ellison *et al.*, 2008). Because ATP is the primary source of energy for cellular processes, these hybrids likely have a reduced fitness due to their deficit of energy.

Breeuwer & Werren (1995) found that interspecific hybrid males of *Nasonia giraulti* and *Nasonia vitripennis* indeed exhibit significantly increased mortality (i.e., F_2 hybrid breakdown) during larval development, a process that has high energetic demands. Niehuis *et al.* (2008) mapped four loci in the nuclear genome of these hybrids that are incompatible with an allospecific cytoplasm and that account for a major fraction of the observed hybrid mortality. Finally, Ellison *et al*. (2008) measured OXPHOS enzyme efficiency in *Nasonia* F_2 hybrid males directly and found that they have a significantly reduced efficiency relative to the parental species in each OXPHOS complex except for complex II, which contains only nuclear encoded subunits.

Given that there are far more possibilities for genic incompatibilities to exist between nuclear genes than between nuclear and mitochondrial genes, why do cytonuclear genic incompatibilities seem to play a major role in the early evolution of postzygotic reproductive isolation? One reason could be the fundamentally different way that the nuclear and mitochondrial genomes are processed and transmitted during reproduction. The nuclear genome is, with the notable exception of the sex chromosomes, inherited from both parents with recombination during meiosis, while the mitochondrial genome is usually exclusively maternally inherited and non-recombining (e.g. Hutchinson *et al*., 1974; Birky, 1995; but see male inheritance in mussels, Zouros *et al.*, 1992). As a consequence, the mitochondrial genome has a theoretically smaller effective population size than the nuclear genome, which significantly reduces the efficacy of natural selection on nucleotide substitutions in the mitochondrial genome (Rand *et al.*, 2004). For this and other reasons (e.g., higher risk of oxidative damage), the mitochondrial genome tends to accumulate nucleotide substitutions at a higher rate than

the nuclear genome (Montooth & Rand, 2008). In the genus *Nasonia,* for example, the substitution rate for mitochondrial genes is, on average, estimated to be approximately 30 times higher than that of nuclear genes (Oliveira *et al.*, 2008).

It has been hypothesized that the accumulation of slightly deleterious nucleotide substitutions in the mitochondrial genome leads to positive selection for compensatory nucleotide substitutions in the nuclear genome to maintain mitochondrial functionality (Rand *et al.*, 2004). If there are nuclear genes of the OXPHOS pathway that compensate for deleterious changes in the mitochondrial encoded OXPHOS genes with which they interact, we would expect to find evidence for positive selection in these nuclear encoded OXPHOS genes. However, to the best of our knowledge there is no study published in which such an analysis has been conducted.

Nasonia is a good model in which to begin the assessment of the role of positive selection in compensating for the accumulation of slightly deleterious nucleotide substitutions in mitochondrial encoded OXPHOS genes for several reasons. First, only the OXPHOS complexes composed of both nuclear and mitochondrial encoded genes show reduced efficiency in (haploid) F_2 hybrid males relative to (haploid) males of the parental species (Ellison *et al*., 2008). Second, we can utilize the newly sequenced genomes of the genus *Nasonia* (Werren *et al*., 2010) to annotate the nuclear encoded genes of the OXPHOS pathway in *N. vitripennis*, *N. giraulti*, and *N. longicornis*. Third, the incompatibility leading to hybrid breakdown in *Nasonia* has been mapped to regions of the genome (Niehuis *et al*., 2008), allowing us to narrow down candidate genes that are potentially involved in the incompatibility.

In comparing the *Nasonia* nuclear encoded OXPHOS genes with orthologs from other holometabolous insects (Porcelli *et al.*, 2007), we here exploit the evolutionary divergence between these taxa to search for positively selected amino acid sites by analyzing the ratio of nonsynonymous (amino acid replacing) to synonymous (silent) substitutions (ω). Given the high functional constraint on genes that is typically seen in this pathway, we would expect to find high levels of historical purifying selection on the OXPHOS amino acid sequences. In addition, historical compensatory evolution in this pathway is not expected to target all genes, but rather, due to the tight and localized interaction of nuclear and mitochondrial genes, that adaptation has occurred at potentially only a few candidates. Therefore, only a few genes are expected to exhibit such signatures of positive selection, and thus, any signs of adaptation would be interpreted as significant in light of this highly conserved pathway. Further utilizing this divergence, we examine evolutionary constraint on individual amino acid sites in genes of the OXPHOS pathway across the holometabolous insects. We then identify substitutions at these sites within the three *Nasonia* species that violate this constraint and therefore may impact the protein structure and ultimately the function of the gene. The obtained data enable us to assess the hypothesis that positive selection has guided some of the evolutionary changes in the nuclear genes of the OXPHOS pathway. They also allow us to provide a list of candidate genes that are potentially involved in F_2 hybrid breakdown in *Nasonia* and that will set the stage for future model studies addressing the role of co-adaptation between nuclear and mitochondrial genes for the evolution of intrinsic postzygotic reproductive isolation in this species group.

Methods

Annotation of nuclear encoded OXPHOS genes in *Nasonia.* For each nuclear encoded gene of the five OXPHOS complexes, we obtained the *Drosophila melanogaster* protein sequence from the MitoDrome database (D'Elia *et al*., 2006; http://www2.ba.itb.cnr.it/MitoDrome/). These sequences were used as queries to search the *Nasonia* genome assembly 1.0 (Werren *et al*., 2010) using the program TBLASTN and applying the SEG filter for low complexity sequences (Altschul *et al.*, 1997). The predicted gene models from the National Center for Biotechnology Information databases (NCBI RefSeq and/or NCBI *ab initio*) with the highest similarity for a given OXPHOS gene was then manually annotated using the APOLLO genome annotation curation tool (Lewis *et al.*, 2002). Intron-exon boundaries, start and stop sites, and 5' and 3' untranslated regions (UTRs) were confirmed or updated based on expressed sequence tags (EST) from the three sequenced *Nasonia* species (i.e., *N. vitripennis*, *N. giraulti*, and *N. longicornis*; Werren *et al*., 2010). We also considered NCBI-curated EST data from three additional Hymenoptera taxa (i.e., *Vespula squamosa, Lysiphelbus testaceipes*, and *Solenopsis invicta*) as well as Swissprot curated protein sequences from the honey bee (*Apis mellifera*), *Drosophila melanogaster*, and the red flour beetle (*Tribolium castaneum*).

The *N. vitripennis* DNA sequences of annotated OXPHOS genes (Supp I) were used as queries to search the *N. giraulti* and *N. longicornis* NCBI trace sequence archives with the program BLASTN, applying an *E* value cutoff of 10^{-10} (Altschul *et al.*, 1997). The orthologous DNA sequences from *N. giraulti* and *N. longicornis* were manually aligned to the *N. vitripennis* sequences with the aid of BioEdit (Hall, 1999). In addition,

we considered pre-aligned 45-bp long Illumina short-read sequences from *N. giraulti* and *N. longicornis* from the *Nasonia* genome sequencing project (Werren *et al*., 2010). The coordinates of the start and stop codon sites, as well as the intron/exon boundaries of the OXPHOS genes annotated in *N. vitripennis* were used to trim the corresponding *N. giraulti* and *N. longicornis* sequences. All alignments were visually checked for inconsistencies between the trace and the short-read sequences for both *N. giraulti* and *N. longicornis*. In cases where short-read and trace sequences were contradictory at a single site, the nucleotide with the majority of support (i.e., present in larger number of short-read/trace sequences) was assigned. If equal support was found for contradictory nucleotides, the nucleotide site was considered ambiguous.

OXPHOS gene sequences from other holometabolous insects. Coding sequences of the nuclear encoded OXPHOS genes from *Anopheles gambiae, Aedes aegypti*, *Drosophila melanogaster, Bombyx mori, Apis mellifera* and *Tribolium castaneum* were obtained from the MitoComp2 database (Porcelli *et al.*, 2007; http://www.mitocomp.uniba.it/). These sequences were translated and aligned to the orthologus amino acid sequences of *N. vitripennis, N. giraulti*, and *N. longicornis* with the program CLUSTAL W (Thompson *et al.*, 1994), employing the BLOSUM62 substitution matrix. The alignment of the corresponding coding sequences was deduced from the amino acid sequence alignments. To ensure that all analyses are based on the same amount of sequence information, we excluded gene alignments for which the OXPHOS gene sequence of one or more taxa was missing. If genes had multiple transcripts in a given species, we considered only the transcript with the highest number of conserved nucleotides (to be conservative in subsequent analyses involving tests for

positive selection). The exception to this was if multiple transcripts for a gene were found across all studied holometabolous insects, in which case the transcripts were retained in *Nasonia*.

To ensure comparable data analyses, and to remain conservative in subsequent tests for positive selection, we applied several criteria to remove highly divergent sequences: we manually edited the alignments in BioEdit, removing unalignable N- and C- termini up to the first set of completely conserved amino acid residues. Similarly, if any amino acid residue within the remaining sequence was missing or ambiguous in any species or not reliably aligned across all taxa, that residue was removed from all sequences (unedited alignments are available upon request). Finally the resulting gene alignments were concatenated into a single dataset and the position of each gene was recorded as a code sequence (using a-z, A-P, R-Z, 0 and 1, see Supplement 2.1 & II) to facilitate identification of the genes after concatenation. The results reported here are from this concatenation of genes instead of examining them on a simple single gene-by-gene model. This was done because many of these genes are short (e.g., 40% are less than 100 amino acids in length) and thus, parameters such as codon frequencies and neutral substitution rates can have large variances, which can greatly impact the power of a likelihood analysis, leading to erroneous acceptance of the null hypothesis (Anisimova *et al.,* 2001). The concatenation enabled us to estimate these parameters globally to identify patterns in the independent genes and provide us with more statistical power to evaluate the null hypothesis.

Test for positive selection. To assess the impact of positive selection on the evolution of the nuclear encoded OXPHOS genes, we examined the ratio of

nonsynonymous (amino acid replacing) substitutions per nonsynonymous site to synonymous (silent) substitutions per synonymous site (ω) with the codon-based maximum-likelihood method (codeml) proposed by Goldman & Yang (1994) and implemented in the software package PAML 4 (Yang, 2007). We performed this analysis on the sequences of the distantly related holometabolous insects to increase the power of the test in order to determine whether historical positive selection has acted on any of the genes in this pathway. We applied the site models M7 and M8, accounting for transitional rate bias (κ estimated) and unequal codon frequencies (F3x4 matrix), and assessed the differences in their likelihood values with the aid of a likelihood ratio test (df = 2). The M7 model assumes a beta distribution of ω values ranging from 0 to 1 (i.e. no positive selection allowed). The M8 model also assumes a beta distribution of ω values, but includes an additional ω class with values > 1 (i.e. positive selection allowed). For both models, we tested a wide range of start parameters for ω (0.01-5.0) and κ (0.5-5.0). Both models require a tree topology, which specifies the phylogenetic relationships between the investigated taxa and which we inferred from the amino acid sequence data in a maximum likelihood framework using the program phyML (Guindon & Gascuel, 2003). We applied three different empirical models of amino acid substitution: JTT (Jones *et al.*, 1992), Dayhoff (Dayhoff *et al.*, 1978), and WAG (Whelan & Goldman, 2001). Substitution rate heterogeneity was approximated using a discrete gamma distribution with four categories. We obtained the same topology for all three substitution models, which proved to be consistent with the phylogeny of the holometabolous insects hypothesized by Savard *et al.* (2006). The posterior probabilities

of individual sites belonging to the class $\omega > 1$ was calculated using the Bayes empirical Bayes approach implemented in PAML (Yang *et al.*, 2005)

Mapping of nuclear-encoded OXPHOS genes. We assessed the possibility that nuclear-encoded OXPHOS genes play a role in cytonuclear genic incompatibility by comparing their position in the *Nasonia* genome relative to previously identified transmission ratio distorting loci (TRDL; Niehuis *et al*., 2008). We mapped nuclear encoded OXPHOS genes to chromosomes of *Nasonia* using positional information from a published high-density linkage map for *Nasonia* (Niehuis *et al*., 2010).

Amino acid substitutions in nuclear encoded OXPHOS genes. We used the program MAPP (Stone & Sidow, 2005) to assess the potential negative impact of amino acid replacements in the nuclear encoded genes of the OXPHOS pathway in *Nasonia*. The program calculates the physicochemical constraints at each amino acid position based on the observed distribution of amino acids across distantly related taxa and then generates an impact score and an associated *p* value for every potential amino acid substitution. The impact scores are based on the prior distribution (found across many evolutionarily diverged taxa) of physicochemical properties at each amino acid site (i.e. evolutionary constraint), with higher impact scores resulting from greater violations of this constraint (Stone & Sidow, 2005). We again utilized the evolutionary divergence of the holometabolous insects by using MAPP to calculate the evolutionary constraint at each studied amino acid site in the OXPHOS pathway. The substitutions found within *Nasonia* were then compared with the calculated impact score for each amino acid.

Results

OXPHOS gene annotation in *Nasonia.* We identified 59 of the 65 OXPHOS pathway subunit genes known from *Drosophila* (MitoDrome database; D'Elia *et al*., 2006) in the *Nasonia* genome (Table 2.1; Supplement 2.1). It is possible that the remaining genes were not identified because our conservative search strategy parameters did not allow us to reliably identify orthologs that have rapidly diverged. For 9 of the 59 genes (i.e., two of complex I, two of complex III, and five of complex IV), EST data suggested alternate transcripts (Table 2.1; Supplement 2.1, Werren *et al*., 2010). Utilizing the high-density linkage map for *Nasonia* (Niehuis *et al.,* 2010), we were able to map 56 of the 59 identified nuclear-encoded OXPHOS genes in the *Nasonia* genome (Figure 2.1, Supplement 2.1).

Test for positive selection. After removing OXPHOS genes for which we were missing sequences in one or more taxa, the concatenated sequence alignment included 53 of the 59 nuclear encoded OXPHOS genes identified in *Nasonia*, composed of a total of 8,739 codons (Supplement 2.2). The likelihood ratio test indicated a significantly better fit of the M8 sites model, which accounts for positive selection, than the M7 model to the holometabolous insect dataset ($-2 \log \Delta = 8.491$, df = 2, $p = 0.011$). Bayes empirical Bayes revealed four genes (Table 2.2, Fig 1, Supplement 2.3) containing sites with posterior probability values >80%, corresponding with ω values >1 when accounting for the standard error.

When we examined patterns of divergence on a gene-by-gene basis in comparison with the concatenated dataset (using models M7 and M8, results not shown), only one of the four genes previously identified (51 KDA subunit) still showed a significant

likelihood ratio test. However, as previously discussed, this is expected given that the power of the likelihood ratio test is greatly decreased when using shorter sequences (Anisimova *et al.,* 2001). Similarly, we also evaluated the effect of divergence time on our results. For this purpose we performed an analysis of ω for each branch of the tree (using the branch model (model=1) in PAML, Supplement 2.4), which indicated that variation in dS associated with recent vs. deeper divergence times does not explain the significant estimates of dN/dS in our dataset.

Analyses of amino acid substitution patterns. Of the 56 mapped OXPHOS subunit genes, we found that within *Nasonia,* 36 genes contain amino acid substitutions. Of these 36 genes, 22 were located within or near the estimated position of previously identified TRDL (Figure 2.1; Niehuis *et al.*, 2008). Since the four TRDL had been identified in hybrids of *N. giraulti* and *N. vitripennis*, we subsequently restricted our analysis to amino acid substitutions between these two species. This resulted in the examination of 53 amino acid substitutions found in 19 genes. The MAPP analysis assigned a statistically significant impact score to 17 of these amino acid substitutions across 9 of the 19 genes located near TRDL (Table 2.2, Fig 1, Supplement 2.5).

Discussion

Dobzhansky (1937) and Muller (1942) envisioned the evolution of intrinsic postzygotic reproductive isolation as a consequence of the incompatibility of interacting genes that have diverged between taxa (e.g., Brideau *et al*., 2006). These interactions are not limited to nuclear genes, but can include mitochondrial genes as well. A prime candidate for the evolution of genic incompatibility between nuclear and mitochondrial genes is the OXPHOS pathway, whose enzyme complexes are composed of both nuclear

and mitochondrial encoded gene products. While the pathway itself is highly conserved across taxa in respect to both the number and the function of involved genes, the accumulation of slightly deleterious nucleotide substitutions in the non-recombining mitochondrial genome seems to quickly lead to an array of divergent mitochondrial OXPHOS gene variants (e.g. Oliveira *et al*., 2008). One possible explanation for the maintenance of these variants, and thus overall OXPHOS functionality, is compensatory selection in nuclear encoded OXPHOS genes, which may also result in the rapid divergence of these particular genes. As a result, genic incompatibilities are expected to quickly develop between nuclear and mitochondrial genes that evolve independently from each other in individuals from different populations and species. Cytonuclear genic incompatibility thus may play a significant role in incipient speciation. It is this aspect that makes the OXPHOS pathway a particularly attractive model to study the co-evolutionary dynamics of nuclear and mitochondrial encoded genes.

Our analysis of the substitution pattern in 53 nuclear encoded OXPHOS genes across nine holometabolous insect species finds that an evolutionary model that accounts for positive selection fits the substitution pattern in the nuclear genes of the OXPHOS pathway significantly better than one that does not. It should be noted that our gene sample is not a random one with respect to the genome in general, in which case we may expect that a large sampling would include a certain proportion reflecting different constraints, i.e., classes of genes each evolving under neutral, positive, and purifying selection. Instead, our sample of genes here is selected with respect to a specific hypothesis regarding selective constraints on OXPHOS genes, which have been shown to be highly conserved (Porcelli *et al*., 2007). This premise, together with our likelihood

modeling, implies that the patterns of elevated divergence are not well explained by simply neutrality or sampling variance (i.e., "chance"), but may represent potential candidates for positively selected genes.

Given this rationale, the observation of even a few genes exhibiting significantly elevated divergence would be surprising without an a priori hypothesis providing an explanation for diverged genes in a conserved pathway. It is possible that our data are consistent with the hypothesis that nuclear genes of the OXPHOS pathway compensate for the accumulation of slightly deleterious nucleotide substitutions in their mitochondrial counterparts. While this is one potential explanation, it is clearly not the only one that may explain elevated divergence and patterns of positive selection, especially since many of the genes still show strong patterns of purifying selection. Thus, it is clear that further studies are needed to distinguish among these competing alternative hypotheses.

It is also possible that the ω value of 2.44 for sites estimated by PAML to be under positive selection (site class -11; Supplement 2.3) could be considered as a low estimate. First, we are missing six OXPHOS pathway genes in our analysis that are known from *Drosophila*. While we cannot exclude the possibility that these genes are not present in *Nasonia* (or in the *Nasonia* genome assembly), it is possible that we did not recognize them because they are sufficiently divergent from their *Drosophila* orthologs. We also excluded six genes for which we were missing orthologous sequences in one or more taxa of the dataset other than *Nasonia*. These genes could likewise be absent from those datasets because they evolve rapidly. Finally, we discarded sequence sections from the alignment that could not be reliably aligned across all taxa, because they seemingly have

diverged sufficiently to preclude meaningful analysis. Overall, these measures resulted in a relatively conservative analysis that removed genes that may be undergoing rapid sequence evolution and may be erroneously interpreted as positive selection.

Our analysis resulted in the identification of four candidate genes that show patterns of significantly elevated divergence across holometabolous insects (Figure 2.1, Table 2.2, Supplement 2.5). In addition to the conservative data-trimming measures outlined above, we also expect that this set of genes is further reduced in number given that the estimates of ω are averaged across many lineages because our analysis involved multiple taxa that diverged, in some cases, > 300 million years ago (Krauss *et al*., 2008). We would not expect the same sites to be selected in all lineages, and thus, the number of sites suggested here by PAML to be under positive selection could conservatively also be considered as a low estimate. It should be noted that these results indicate that positive selection may potentially act on the nuclear OXPHOS genes, but the current methods are not able to determine which lineages are responsible for these elevated divergence estimates. As a result, this analysis provides an initial view of the gene and protein subunit regions that reflect long-term evolutionary changes in the OXPHOS pathway among holometabolous insects.

Due to the breadth of the previous analysis, our MAPP results are likely more informative for comparisons within *Nasonia*, which indicate amino acid changes that are predicted to have a significant impact on the encoded protein. We found that 53 of the amino acid substitutions in our comparison between *N. giraulti* and *N. vitripennis* took place in OXPHOS genes that are within the confidence intervals that Niehuis *et al*. (2008) provided for the position of incompatibility loci in the nuclear genome of *Nasonia*

(Figure 2.1, Table 2.2, Supplement 2.5). Because these intervals do reflect a large proportion of the overall genomic content, the fact that many of our genes reside within or near these TRDL reflects only a preliminary inspection of their actual link to hybrid incompatibility. Nonetheless, the combination of the present data with those published by Niehuis *et al*. (2008) does narrow the list of candidate genes for additional hypothesis testing. For example, although *N. giraulti* and *N. vitripennis* have split very recently, with an estimated divergence time of approximately one million years (Campbell *et al*., 1993), they have already accumulated substantial protein changes in the otherwise highly conserved nuclear OXPHOS genes that could potentially result in hybrid cytonuclear incompatibilities.

This substitution pattern is even more impressive given that despite our conservative approach, the MAPP analysis predicts that over 30% of the 53 amino acid substitutions located at TRDL may impact protein structures. The possibility remains that this substitution pattern could be the result of relaxed purifying selection due to reduced effective population sizes, especially for the mitochondrial genome. This could lead to deleterious substitutions in both nuclear and mitochondrial genes that could produce the observed hybrid breakdown in *Nasonia* analogous to synthetic lethals (Hartman *et al*., 2001). However, a reduced effective population size and reduced selection efficacy would be expected to impact all genes similarly (with some stochastic variance aside). Thus, it is not readily obvious why only certain genes would be subjected to relaxed selection while others show strong purifying selection. Nonetheless, further studies that examine within population diversity and recent evolutionary changes in selective

constraints at these genes may address this question in distinguishing between competing selection models.

Based on the present results, it appears possible that some of the genes and possibly even single amino acid residues identified here are involved in the observed F_2 hybrid breakdown in *Nasonia*. We speculate that these loci may reflect "speciation gene" or even "speciation site" candidates. The *Nasonia* model system will allow us to test the phenotypic effects of these amino acid substitutions in more detail through future studies. This is one of the more attractive aspects of our study on *Nasonia*; that is, identifying genes and specific sites with statistically elevated divergence and potential signatures of positive selection is much more compelling when their functional effects can be further validated *in vivo*. This combination of statistical and functional approaches is likely to provide new insights into the intimate interactions of mitochondrial and nuclear gene products that form the genetic basis of intrinsic postzygotic reproductive isolation in *Nasonia* and other species. This may also lead to a better understanding of the functioning of the OXPHOS system and the genetics of speciation.

Table 2.1

Annotated nuclear OXPHOS genes across different complexes.

	Number of Genes		
OXPHOS Complex	***Nasonia*[1]**	***Drosophila***	**Alternate transcripts[2]**
I	27	31	2 (2)
II	4	4	0
III	9	9	0
IV	6	8	2 (2)
V	13	13	4 (2), 1 (4)
Total	59	65	8 (2), 1 (4)

[1] Number of *Nasonia* genes identified is the result of comparisons with *Drosophila* OXPHOS gene alignments (see Methods).
[2] Number of genes identified from EST data with alternate transcripts in *Nasonia*, with the number of alternate transcripts shown in parentheses.

Table 2.2

Nuclear OXPHOS genes showing patterns of elevated divergence and amino acid substitutions with potentially disruptive effects on protein structure.

Gene Name	Complex	Position	P.P. (ω +/- SE)[1]	Amino acid replacement impact (*N. vit/ N. gir*)[2]	Residue[3]								
					Ng	Nl	Nv	Am	Tc	Bm	Ag	Aa	Dm
39 KDA SUBUNIT	1	316	0.913 (1.424 +/-0.252)	n.s.	P	P	A	I	H	H	Y	L	T
51 KDA SUBUNIT	1	415	0.837 (1.355 +/-0.337)	n.s.	N	N	S	A	E	R	S	A	K
CORE PROTEIN 2	3	305	0.838 (1.356 +/-0.334)	n.s.	L	L	L	L	V	A	V	L	V
		51	n.s.	0.005 / < 0.001	R	R	H	C	N	N	T	T	N
		277	n.s.	0.010 / 0.680	Q	Q	E	F	K	V	K	Q	K
D CHAIN	5	123	0.879 (1.390 +/-0.302)	n.s.	K	K	R	S	R	Y	K	R	R
		89	n.s.	0.460 / 0.022	V	A	A	K	E	A	D	E	E
		102	n.s.	0.455 / 0.029	T	A	A	A	S	A	S	A	Q
30 KDA SUBUNIT	1	166	n.s.	0.949 / 0.003	T	T	A	A	A	A	A	A	A
B12 SUBUNIT	1	5	n.s.	< 0.001 / <0.001	E	E	K	P	P	P	P	P	P
		15	n.s.	0.902 / < 0.001	E	E	A	A	A	A	A	A	G
YT B SMALL SUBUN	2	47	n.s.	0.040 / 0.978	V	V	L	T	V	V	T	T	V
CYT B560 SUBUNIT	2	75	n.s.	0.002 / 0.949	A	A	V	A	A	A	A	A	A
		104	n.s.	< 0.001 / <0.001	R	R	K	S	A	S	T	S	S
SULFUR SUBUNIT	3	11	n.s.	0.008 / 0.052	G	G	N	S	S	S	S	S	S
		181	n.s.	0.109 / 0.022	V	V	A	P	Y	P	H	H	T
		188	n.s.	0.119 / 0.022	V	V	I	T	T	L	L	L	L
E CHAIN	5	2	n.s.	0.009 / 0.999	V	V	I	V	V	V	V	V	V
F CHAIN	5	24	n.s.	< 0.001 / <0.001	K	K	R	P	A	P	A	P	A
		51	n.s.	0.949 / 0.003	T	A	A	A	A	A	A	A	A
		86	n.s.	0.025 / 0.999	F	F	L	F	F	F	F	F	F

Positions are amino acid sites within each gene of the concatenated dataset (Supp. II). [1] Posterior probabilities (P.P.) of sites predicted to be subject to positive selection and ω values (both were calculated using the PAML program, Yang, 2007). [2] Significance values for the impact scores of amino acid substitutions were calculated for each *Nasonia* species using the program MAPP (Stone and Sidow, 2005). Shaded areas indicate genes suggested by PAML analyses to have putative positively selected sites and disruptive amino acid substitutions. [3] The residues present at each position for each taxon are abbreviated: Ng = *Nasonia giraulti*; Nl = *Nasonia longicornis*; Nv = *Nasonia vitripennis*; Am = *Apis mellifera*; Tc = *Tribolium castaneum*; Bm = Bombyx mori; Ag = *Anopheles gambiae*; Aa = *Aedes aegypti*; Dm = *Drosophila melanogaster*

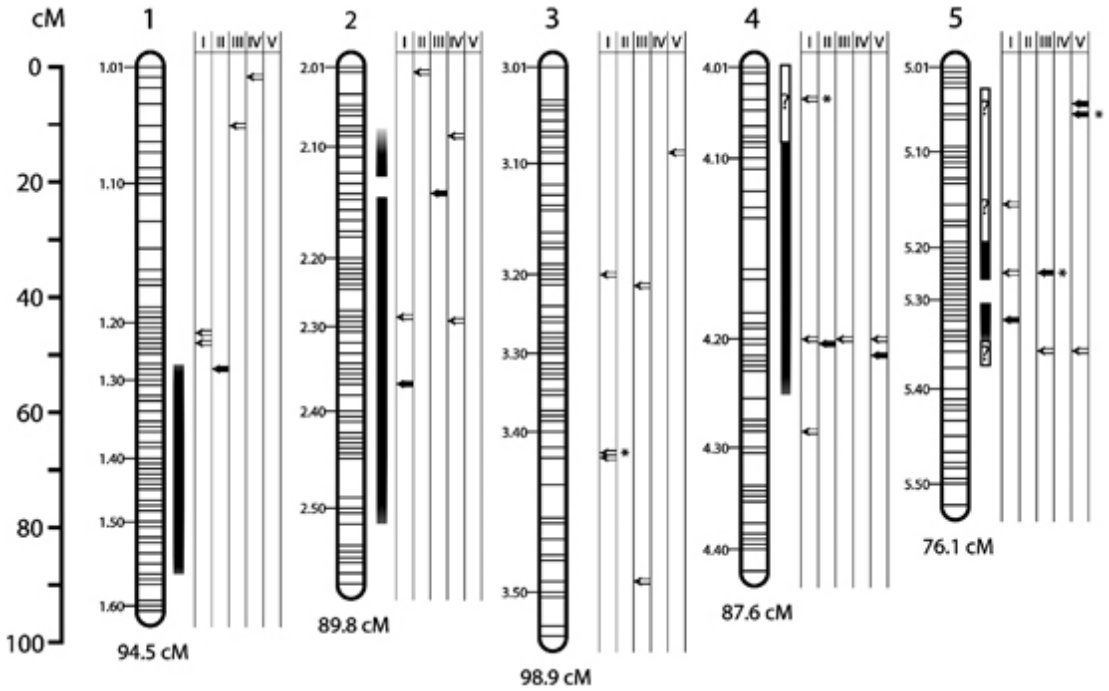

Figure 2.1. Nasonia linkage map adapted from Niehuis *et al*. (2010). The leftmost scale shows the genetic distance in centimorgans. The horizontal bars in each chromosome (1.01-5.51) represent groups of markers showing no recombination between them, while the space between bars represents recombination. Dark gradient bars to the right of the chromosomes represent incompatibility loci identified by Niehuis *et al*. (2008); question marks designate locus boundaries that are unclear due to recent expansions of the ends of the linkage groups on the linkage map. The columns to the right of the chromosomes designate the complex (I-V) of each OXPHOS gene. Empty arrows = genes with amino acid substitutions with non-significant impact scores. Filled arrows = genes with amino acid substitutions predicted to significantly disrupt protein structure in *Nasonia*. stars = genes containing sites that show the signature of positive selection ($\omega > 1$).

CHAPTER 3

GENETIC AND DEVELOPMENTAL BASIS OF F_2 HYBRID BREAKDOWN IN *NASONIA* PARASITOID WASPS

Abstract

Speciation is responsible for the vast diversity of life, and hybrid inviability, by reducing gene flow between populations, is a major contributor to this process. In the parasitoid wasp genus *Nasonia*, F_2 hybrid males of *N. vitripennis* and *N. giraulti* experience an increased larval mortality rate relative to the parental species. Previous studies indicated that this increase of mortality is a consequence of incompatibilities between multiple nuclear loci and cytoplasmic factors of the parental species, but could only explain ~40% of the mortality rate in hybrids with *N. giraulti* cytoplasm. Here we report a locus on chromosome 5 that can explain the remaining mortality in this cross. We show that hybrid larvae that carry the incompatible allele on chromosome 5 halt growth early in their development and that ~98% die before they reach adulthood. On the basis of these new findings, we identified a nuclear-encoded OXPHOS gene as a strong candidate for being causally involved in the observed hybrid breakdown, suggesting that the incompatible mitochondrial locus is one of the six mitochondrial-encoded NADH genes. By identifying both genetic and physiological mechanisms that reduce gene flow between species, our results provide valuable and novel insights into the evolutionary dynamics of speciation.

The vast diversity of life on earth is the result of countless episodes of speciation, in which populations diverge and become reproductively isolated from one another. While successful mating may never take place between many species due to various prezygotic isolating mechanisms, interspecific hybridization has been estimated to occur in up to 10% of known animal species (Mallet, 2005; Schwenk *et al.,* 2008). When this happens, reproductive isolation may still occur if the hybrid offspring are sterile or inviable due to intrinsic factors, such as deleterious interactions between genes derived from the two parental species, *i.e.*, due to Bateson-Dobzhansky-Muller incompatibilities (Bateson, 1909; Dobzhansky, 1937; Muller, 1942). The case in which the hybrid F_1 generation is largely unaffected by these incompatibilities while subsequent generations suffer severely from them is referred to as F_2 hybrid breakdown (= hybrid breakdown). Intrinsic postzygotic isolation, such as hybrid breakdown, has historically been difficult to study in many traditional model organisms because crosses between species often produce no viable or fertile offspring, hindering genetic analysis of the isolating mechanisms (Coyne & Orr, 2004). Despite this difficulty, some progress has been made, and a number of genes have been implicated in such cases of isolation, referred to as speciation genes (see Maheshwari & Barbash, 2011 for examples). However, the proximate causes for hybrid breakdown are still poorly understood and more examples in experimentally tractable organisms are needed in order to determine what general principles might be involved in this process (Maheshwari & Barbash, 2011).

Interspecific hybrid breakdown has been well documented within the pteromalid wasp genus *Nasonia* (Darling & Werren, 1990; Breeuwer & Werren, 1995; Gadau *et al.,* 1999; Niehuis *et al.,* 2008; Ellison *et al.,* 2008; Koevoets *et al.,* 2011). *Nasonia* is well-

suited for genetic studies in the laboratory due to its haplo-diploid genetics, the ease with which virgins of both sexes can be collected before they eclose, and the availability of genomic resources (Werren *et al.,* 2010; Muñoz-Torres *et al.,* 2011). *Nasonia vitripennis* is the only species of its genus with a cosmopolitan distribution and is widely sympatric with *N. giraulti* in eastern North American (Darling & Werren 1990). Species-specific strains of *Wolbachia* endosymbionts prevent gene flow between all known *Nasonia* species through bidirectional cytoplasmic incompatibility (Breewer & Werren, 1990; Bordenstein & Werren, 1998; Bordenstein *et al.,* 2001). However, *Wolbachia*-free strains can be produced from each species through antibiotic treatment and they can then be crossed to produce F_1 hybrid females (Breeuwer and Werren 1990). It is important to note that *Nasonia* males develop parthogenetically from unfertilized eggs, and thus the first hybrid males are produced in the F_2 generation from unfertilized eggs produced by the F_1 hybrid females.

Breeuwer & Werren (1995) found that in crosses between *Wolbachia*-free strains of *N. vitripennis* and *N. giraulti*, F_1 hybrid females do not suffer any major genic incompatibilities that manifest as sterility or inviability. They discovered, however, that F_2 hybrid males suffer from an increased mortality rate during larval development. Mortality ranges between ~53% and ~82%, depending on whether the hybrids possess a *N. vitripennis* or *N. giraulti* cytoplasm (the two types of hybrids are subsequently denoted GV[V] and VG[G], respectively, with the letter in brackets specifying the origin of the cytoplasm: [G] = *N. giraulti* and [V] = *N. vitripennis*). Although it has been known that incompatibilities in GV[V] and VG[G] F_2 hybrid males lead to larval mortality, the specific growth rates and developmental trajectories of these larvae have so far not been

studied. To gain a better understanding of the developmental and physiological deficiencies that F_2 hybrid males suffer from, we examined the number of larvae and their sizes at two stages of development and genotyped them for markers in the region of a newly identified incompatibility locus on the left arm of chromosome 5.

Niehuis *et al. (*2008) mapped regions of the genome involved in larval mortality in hybrids of *N. vitripennis* and *N. giraulti* by searching for loci that exhibit a conspicuous bias in the frequency of the parental alleles in adult wasps. The authors showed that there is significant, cytoplasm-specific marker transmission ratio distortion (MTRD) of nuclear markers in the F_2 hybrid adults, but not in hybrid embryos, suggesting that the additional mortality of hybrids occurs during their larval development. They also mapped loci that could explain all of the additional mortality that GV[V] hybrids suffer from, but discovered only one locus that explained mortality in VG[G] hybrids, leaving approximately 60% of the observed mortality in VG[G] hybrids unexplained (Niehuis *et al.,* 2008). One hypothesis put forth to explain this discrepancy was that regions accounting for the remaining observed mortality of VG[G] hybrid larvae lie in parts of the genome that were unmapped at that time. Indeed, the map only covered approximately 70% of the genome (Niehuis *et al.,* 2008). To address this, Niehuis *et al. (*2010) remapped the *Nasonia* genome using the newly available genomic resources and found a region of ~10Mbp on the left arm of chromosome 5 that was missing from previous genetic maps. Werren *et al. (*2010) assessed whether this new map revealed any additional regions exhibiting MTRD, by pooling 100 adult F_2 hybrid males for each reciprocal cross and hybridizing their DNA onto a competitive genotyping microarray (Desjardin *et al.,* 2013). They found a locus within the new region on the left arm of

chromosome 5 that had nearly 100% *N. giraulti* alleles in VG[G] hybrids, but with a sample size of one (due to the pooling of the samples' DNA) it was not possible to statistically evaluate the result. In addition to this statistical problem, another shortcoming of this approach was that it did not allow for assessment of the genotype of each marker within a single individual. As a result, it was not possible to use individual recombination events in hybrid males to narrow down the genomic region that harbors the genetic factor causally involved in the mortality. In the present investigation, we utilize a microarray capable of genotyping individual samples across 1,536 single nucleotide polymorphism (SNP) markers to statistically assess the strength of MTRD in VG[G] F_2 hybrid males and to analyze recombination events in individual hybrids in order to better map and characterize the region and genes on chromosome 5 that exhibit and cause MTRD (Goldengate Genotyping Assay; Illumina, Inc. San Diego, CA; Niehuis *et al.,* 2010).

A genotyping approach is one method of narrowing down the causal genetic elements underlying mortality. A complementary approach is to determine, *a priori*, pathways that may be involved in mortality and to identify and investigate candidate genes within this pathway, both in terms of their role in the pathway and their position within the genome. Ellison *et al. (*2008) showed that adult VG[G] F_2 hybrid males exhibit reduced activity of their oxidative phosphorylation (OXPHOS) enzymes relative to that of parental males, making this pathway a strong candidate for the deleterious interactions in these *Nasonia* hybrids due to genic incompatibility. In addition, incompatibilities of individual components of the OXPHOS pathway could produce a pattern of cytoplasm-specific MTRD, since it is the only pathway to incorporate both

nuclear- and mitochondrial-encoded proteins. Using this candidate pathway approach, we hypothesize that the OXPHOS system is involved in hybrid breakdown in *Nasonia* and we therefore predict that either a single gene or multiple genes belonging to the OXPHOS pathway lie within the above mentioned region of chromosome 5.

Here we present results demonstrating that VG[G] F_2 hybrid male larvae show a genotype-dependent size distribution. We found that larval size is strongly related to the genotype of a small genomic region on the left arm of chromosome 5 and we show that this region contributes to a large proportion of the observed mortality during larval development, resulting in an extreme paucity of *N. vitripennis* alleles within this region. Finally, we evaluated candidate gene(s) that could be involved in the observed mortality of VG[G] F_2 hybrid larvae by studying individual recombination events at the distal-most end of the left arm of chromosome 5 in adult hybrid males, and we propose a nuclear-encoded OXPHOS gene in this region as a likely candidate for the incompatibility**.**

Methods

Stocks. We used two *Wolbachia*-free *Nasonia* strains, AsymCX and RV2X(U), in our cross experiments. AsymCX is derived from a wild-type strain of *N. vitripennis* (LBii or LabII) collected in Leiden, The Netherlands (Breeuwer & Werren, 1995). RV2X(U) is derived from a wild-type strain of *N. giraulti* (RV2) collected in Rochester, New York (Breeuwer & Werren, 1995). Both strains were chosen because they are highly inbred, their genomes are sequenced (Werren *et al.,* 2010), and they have been previously shown to suffer from hybrid inviability (Niehuis *et al.,* 2008). These strains, as well as their hybrids, were reared under constant light in an incubator (25°C) on pupae of the flesh fly, *Sarcophaga bullata*.

Cross experiments. We produced F_2 hybrid males following the procedures described by Niehuis *et al. (*2008). Briefly, virgin wasps were collected and allowed to mate with one heterospecific partner, producing two types of F_1 hybrid females (*i.e.*, GV[V] and VG[G]). These F_1 females were set as virgins on hosts to produce F_2 males, which were collected at either their embryo stage (12–16 hours old) or their adult stage (2 days post eclosion). For larval measurements, females were kept individually and were initially given a single host for feeding purposes. After this initial host was discarded, the females were left without a host for twelve hours and were then given hosts in twelve-hour intervals interspersed with twelve hours without a host for a maximum of two hosts per female. All samples were stored at -70°C until DNA extraction was performed. Note that we utilized DNA of embryos from a previous study by Niehuis *et al. (*2008). See supplementary information for more specific information on sample sizes (Supplement 3.1).

Molecular procedures. DNA from larvae and adult wasps was extracted using a Chelex extraction protocol described by Niehuis *et al. (*2007). Chelex extracted DNA quantity and quality was assessed with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Products, Wilmington, DE). We studied two types of molecular markers: microsatellite and SNP markers. Microsatellite markers were amplified using polymerase chain reaction (PCR) and were designed specifically for this study (Supplement 3.2). The PCR products were separated and visualized on a Li-Cor 4300 DNA Analyzer (Li-Cor, Lincoln, NE) and analyzed with the SAGA Generation 2 software (Li-Cor). We additionally analyzed 1,134 SNP markers spanning the entire genome and described in detail by Niehuis *et al. (*2010).

Larval size measurements. Infected hosts were opened either three or six days post-oviposition (*i.e.*, halfway through or at the end of normal larval development time of *Nasonia*; Whiting 1967), and all larvae were placed over a size standard and photographed using an 11.2 Color Mosaic digital microscope camera (Diagnostic Instruments Inc,; Sterling Heights, MI) connected to a Leica MZ125 stereomicroscope (Leica Microsystems; Heerbrugg, Switzerland). We measured the body width of each larva (Figure 3.1) and subsequently pooled larvae based on their size (within each female/host cohort). Larval size differences between hybrids and their parental species were tested using a Wilcoxon signed-rank test with a Bonferroni-corrected critical *P*-value to reject the null hypothesis of < 0.0042.

Analysis of marker transmission ratio distortion. We performed a X^2 goodness of fit test (Zar, 1999) to test whether or not the segregation ratios of the markers in the data set were consistent with an expected 1:1 Mendelian segregation ratio of parental alleles. Yates' correction for continuity was applied (Yates, 1934) as well as Bonferroni correction for multiple testing (241 markers on chromosome 5 resulted in a critical value to reject the null hypothesis of $P < 0.00021$).

Analysis of the number of recombinant individuals between markers. In order to fine-map the genomic region causally involved in hybrid mortality on the left arm of chromosome 5, we analyzed pairs of markers of chromosome 5 and searched for a significant difference between the expected and actually observed number of recombinant adult VG[G] hybrid males . We determined the expected number of recombinant VG[G] hybrid males by accounting for the mortality incurred during development. Thus, our population of 333 adult hybrids are the result of ~18% survival

(Breeuwer & Werren, 1995) of an original population of ~1,850 individuals. Based on the genetic distances of markers given by Niehuis *et al.,* 2008, we then calculated the number of recombinant individuals for individual pairs of markers that we would have expected to observe in the population if there was no hybrid-specific mortality (Supplement 3.3). We focused on two markers, M1 and M3 (Supplement 3.3), that are separated by ~3Mbp in order to ensure a sufficient number of recombinant individuals to test, though we utilized an additional 15 SNP markers that span the entire distance (9.49cM) between M1 and M3 (see Supplement 3.3). There are two classes of individuals that result from a recombination event between markers M1 and M3. Each class was, *a priori*, expected to occur at equal frequency. We designate these two classes as M1v:M3g and M1g:M3v, differing in whether the *N. vitripennis* allele is distal (M1v:M3g) or proximal (M1g:M3v) to the centromere of chromosome 5. We expected that individuals with a recombination between these markers that still possess the *N. vitripennis* allele of a gene that is causally involved in hybrid mortality will be more likely to die during development, but that individuals with a recombination that brings in the compatible *N. giraulti* allele will be rescued and will be observed at the expected number of instances. By comparing the observed and expected number of individuals of each recombinant class, we were able to better assess the exact location of the incompatibility locus on the left arm of chromosome 5. We performed a X^2 goodness of fit test (Zar, 1999) to test whether the observed number of recombinant individuals significantly deviated from the expected number. Bonferroni correction for 16 different tests in this interval resulted in a critical value to reject the null hypothesis of $P < 0.003$. We used this method to reduce the size of region that we subsequently searched for

candidate genes using the official gene set for *Nasonia* (OGS 1.2; Muñoz-Torres *et al.,* 2011).

Analysis of evolutionary rate of candidate genes. Candidate genes were analyzed for evidence of selection with the program MEGA5 (Tamura *et al.,* 2011). We aligned the complete coding sequence of candidates (from translation start to stop) from *N. vitripennis* and *N. giraulti* and calculated the proportions of both synonymous (dS) and nonsynonymous (dN) substitutions using the Nei-Gojobori model (Nei & Gojobori, 1986).

Results

Timing and genetic basis of larval inviability. *N. vitripennis* females produced more larvae per host than *N. giraulti* or either type of hybrid (VG[G] and GV[V]) when compared at both three and six days post-oviposition (Table 3.1). F_2 hybrid males with either cytoplasm were significantly smaller than either parental species at both three and six days post-oviposition (Figure 3.1; Wilcoxon signed-rank test, $W > 532$, $P < 0.0042$).

At three days post-oviposition the *N. giraulti* larvae have a unimodal size distribution, whereas the *N. vitripennis* larvae as well as the F_2 hybrid larvae may have bimodal distributions (Figure 3.1, top panel). As these larvae develop from three to six days, the GV[V] hybrid larvae and the larvae of both parental species continue to grow in size and their distributions appear to shift toward unimodality. Despite this growth, the GV[V] hybrid larvae remain smaller than age-matched larvae of either parental species. In VG[G] F_2 hybrid males, however, the two modes of the distribution become more distinct from one another, with the initially larger mode continuing to get larger over

time (similar to what is seen in GV[V] F_2 hybrids) and the initially smaller mode remaining similar in size (Figure 3.1, bottom panel).

MTRD on chromosome 5. Analysis of SNP markers in 333 adult VG[G] F_2 hybrid males confirmed a region of very strong distortion on the left arm of chromosome 5, with the distal-most marker on this chromosome exhibiting less than 5% *N. vitripennis* alleles in adult hybrids (observed = 14, expected = 165.5; $X = 277.4$, $P < 0.00021$; Figure 3.2A and Supplement 3.1). The bias was not detected in embryos of the same cross using five evenly spaced markers spanning the whole chromosome (Supplement 3.2). Neither embryos nor adults from the reciprocal cross show significant MTRD in this region (Niehuis *et al.,* 2010). However, Niehuis *et al. (*2008) reported MTRD in GV[V] on the opposite arm of this chromosome that we are able to confirm here as well (Supplement 3.1).

MTRD in larval classes. We analyzed MTRD at the distal end of the left arm of chromosome 5 in the small and large class of VG[G] larvae at day six post-oviposition. In the class of large larvae, only 5.4% of the individuals carried the *N. vitripennis* allele at marker M1 (X^2 test, $P < 0.05$; Figure 3.2A), a percentage similar to that observed in adult F_2 hybrid males. In contrast, in the class of small larvae, 80% of the individuals carried the *N. vitripennis* allele of this marker (X^2 test; $P < 0.05$; Figure 3.2A).

Recombination on chromosome 5. We found no significant difference between the observed and expected number of recombinant individuals (between markers M1 and M3) when the distal-most marker (M1) carried the *N. giraulti* allele (M1g:M3v; observed = 83, expected = 87.8; $X = 0.26$, $P > 0.003$; Figure 3.2B). In contrast, we found significantly fewer recombinant individuals than expected when the distal-most marker

carried the *N. vitripennis* allele (M1v:M3g; observed = 2, expected = 87.8; $X = 83.8$, $P < 0.003$; Figure 3.2B). This pattern held statistically for 13 of the remaining 15 tested pairs of markers (Supplement 3.3), with the *N. giraulti*-distal genotype observed at the expected frequency and the *N. vitripennis*-distal genotype found far less often than expected. We found more individuals of the *N. giraulti*-distal type than expected with a recombination between marker M1 (Figure 3.2B) and a marker ~1.81Mbp (~2.9cM) closer to the centromere (observed = 45, expected = 27.2; $X = 11.65$, $P < 0.003$; Supplement 3.3). The other marker pair not showing statistical significance was the most distal one of the chromosome, M1 and M2 (Figure 3.2B). We found fewer individuals than expected of both recombinant types (M1g:M2v: 1 vs. 2; M1v:M2g: 0 vs. 1). The counts are too low to be statistically compared, but this pattern is exactly what would be expected if the causal gene were located between these two markers as opposed to being more distal than marker M1.

Candidate genes in the MTRD-affected region of chromosome 5. Given that the OXPHOS pathway is likely impaired in hybrids (Ellison *et al.,* 2008), we assessed genes (OGS 1.2) on the left end of chromosome 5 in order to determine potential candidates for being causally involved in hybrid mortality. While three nuclear-encoded OXPHOS genes were identified near this end of the chromosome in a previous attempt to identify candidate genes, these do not fall within the most heavily biased region of the chromosome (Gibson *et al.,* 2010). However, we subsequently identified a fourth nuclear-encoded gene (*Ndufa11*) involved in the OXPHOS pathway and that is located in the region of chromosome 5 that is most strongly affected by MTRD (OGS ID: *NV13533*; Scaffold14: 121419–122670; Supplement 3.4). This gene is located ~120kb

from the left end of the assembled chromosome and is nested between markers M1 and M2 (Figure 3.2B). The gene comprises three predicted exons and exhibits a total of 14 substitutions between *N. vitripennis* and *N. giraulti*; nine of which result in amino acid changes (Supplement 3.5 and 3.6). Based on these data, we estimated a dN/dS ratio of 0.73 between *N. vitripennis* and *N. giraulti*, which is considerably higher than that for any other nuclear-encoded OXPHOS gene analyzed to date (previous highest ratio was 0.45 for *Atp5h*; Gibson *et al.,* 2010).

Discussion

The evolution and co-existence of species in sympatry depends on the establishment of barriers to gene flow (Mayr, 1942). Hybrid incompatibility represents one important postzygotic barrier to gene flow and is the strongest intrinsic barrier acting in hybrids between *Nasonia vitripennis* and *Nasonia giraulti* (Breeuwer & Werren, 1995). The foremost goal in speciation genetics is to identify the genes and genic interactions that underlie barriers to gene flow, because it allows us to unravel patterns in the genetics of hybrid breakdown (*e.g.*, to identify pathways that might be particularly prone to disruption in hybrids) and to investigate the evolutionary forces that have driven the divergence of genes, ultimately leading to incompatible interactions in hybrids. Taken together, these answers from diverse species groups will help to determine what, if any, commonalities there are in the speciation processes that have led to the diversity of life seen today. Here we have begun answering these questions in *Nasonia* F_2 hybrid males.

To date, the incompatibilities of F_2 hybrid males between *N. vitripennis* and *N. giraulti* have been inferred to mainly involve mortality during larval development.

However, the physical characteristics and genetic makeup of hybrid larvae remained to be investigated. Our result from genotyping larvae confirms that "large" hybrid larvae almost always carry the *N. giraulti* allele in the candidate region on chromosome 5 and that these individuals tend to survive to adulthood, whereas the small larvae almost always carry the *N. vitripennis* allele in this region and tend to die before eclosion. These results imply that the incompatibility locus involved in the observed mortality is also very likely to be involved in the observed larval growth. It is not clear whether this locus causes the larvae to stop growing, which in turn prevents them from surviving due to other extrinsic environmental factors, or if it causes both retarded growth and mortality independently of one another. Koevoets *et al. (*2012) recently showed that F_2 hybrid male larvae from crosses between *N. vitripennis* and *N. longicornis* (a species closely related to *N. giraulti*) suffer from an elevated mortality rate and that this mortality rate is further elevated when the hybrids experience temperature stress. This indicates that, at least in that particular cross, the genic incompatibilities can depend on environment. However, it is not known how environmental factors affect the mortality in hybrids of *N. vitripennis* and *N. giraulti*. Future studies should determine how the larval size and mortality are connected.

The pattern and frequency of specific recombinant VG[G] F_2 hybrids demonstrates the natural rescue effect of a recombination event that replaces the incompatible *N. vitripennis* allele with the compatible *N. giraulti* allele on the distal end of the left arm of chromosome 5. This pattern across successively closer marker pairs indicates that the causal locus is located near the distal-most marker that we studied (*i.e.*, M1; Figure 3.2B). Interestingly, we observed fewer individuals than expected of both

types of recombinants between the two most distal markers and we identified a nuclear-encoded OXPHOS gene, *Ndufa11*, very near the distal end of chromosome 5, about halfway between these final two markers (Figure 3.2B). The pattern of recombination discussed above is fully consistent with the hypothesized involvement of this OXPHOS gene in the observed hybrid mortality.

An incompatibility in the OXPHOS pathway could produce a pattern of incompatibilities like that observed in these *Nasonia* hybrids. As mitochondrial biogenesis proceeds and energy demands increase during early ontogeny, more OXPHOS complexes will be produced using incompatible gene products, which in turn might amplify the effects of the genic incompatibilities as development proceeds, resulting in larval instead of embryonic mortality. These incompatibilities could also potentially explain the larval growth patterns observed because individuals with a reduced capacity to produce cellular energy would likely suffer from delayed development/growth.

Previous work has hypothesized that nuclear-encoded genes that interact with mitochondrial-encoded genes may be subjected to directional selection in order to compensate for deleterious mutations that occur in the mitochondrial genome (Blier *et al.,* 2001; Rand *et al.,* 2004). This is hypothesized to occur because of the limited ability of selection to act on the mitochondrial genome (due to its small effective population size and lack of recombination) in conjunction with the typically elevated substitution rate of the mitochondrial genome. Indeed, Oliveira *et al. (*2008) showed that the substitution rate of protein-coding genes of the mitochondrial genome in *Nasonia* is ~35 times higher than that of nuclear-encoded genes. While the dN/dS ratio of 0.73 for *Ndufa11* is below the

strict cutoff value to infer directional selection (*i.e.*, dN/dS > 1), this elevated ratio could indicate relaxed selective constraint on this gene relative to other genes in the pathway. A signature of relaxed selective constraint acting on a gene is consistent with a hybrid incompatibility gene, because this type of gene would be expected to be more divergent than others in the pathway (thereby increasing chances for being incompatible with other genes in hybrids) while still being constrained in its function within the pathway (*i.e.*, the gene is still essential to the pathway and therefore the genic incompatibilities may lead to defects in its functioning). Based on our results, *Ndufa11* is a promising candidate for being involved in both the larval mortality and the reduced larval growth of VG[G] F_2 hybrid males, though future studies will be required to determine the mechanistic connection between these two phenotypes and to ascertain the specific role of this gene in hybrid breakdown in *Nasonia*.

Table 3.1

Number of male offspring per host produced by females of Nasonia vitripennis, N. giraulti, and hybrids of these two species.

Species/Hybrid	Age (days)	Average number of offspring (± SD)	Sample size (N)
N. vitripennis	3	20.2 ± 3.0	12
	6	20.1 ± 4.0	9
N. giraulti	3	13.9 ± 3.3	11
	6	10.8 ± 5.1	12
GV[V]	3	13.0 ± 5.6	8
	5	9.4 ± 4.6	10
VG[G]	3	14.4 ± 3.7	25
	6	12.1 ± 4.7	22

Abbreviations: *N.* = *Nasonia*; GV[V] = *N. giraulti* × *N. vitripennis* F_2 hybrid males with *N. vitripennis* cytoplasm; VG[G] = *N. vitripennis* × *N. giraulti* F_2 hybrid males with *N. giraulti* cytoplasm.

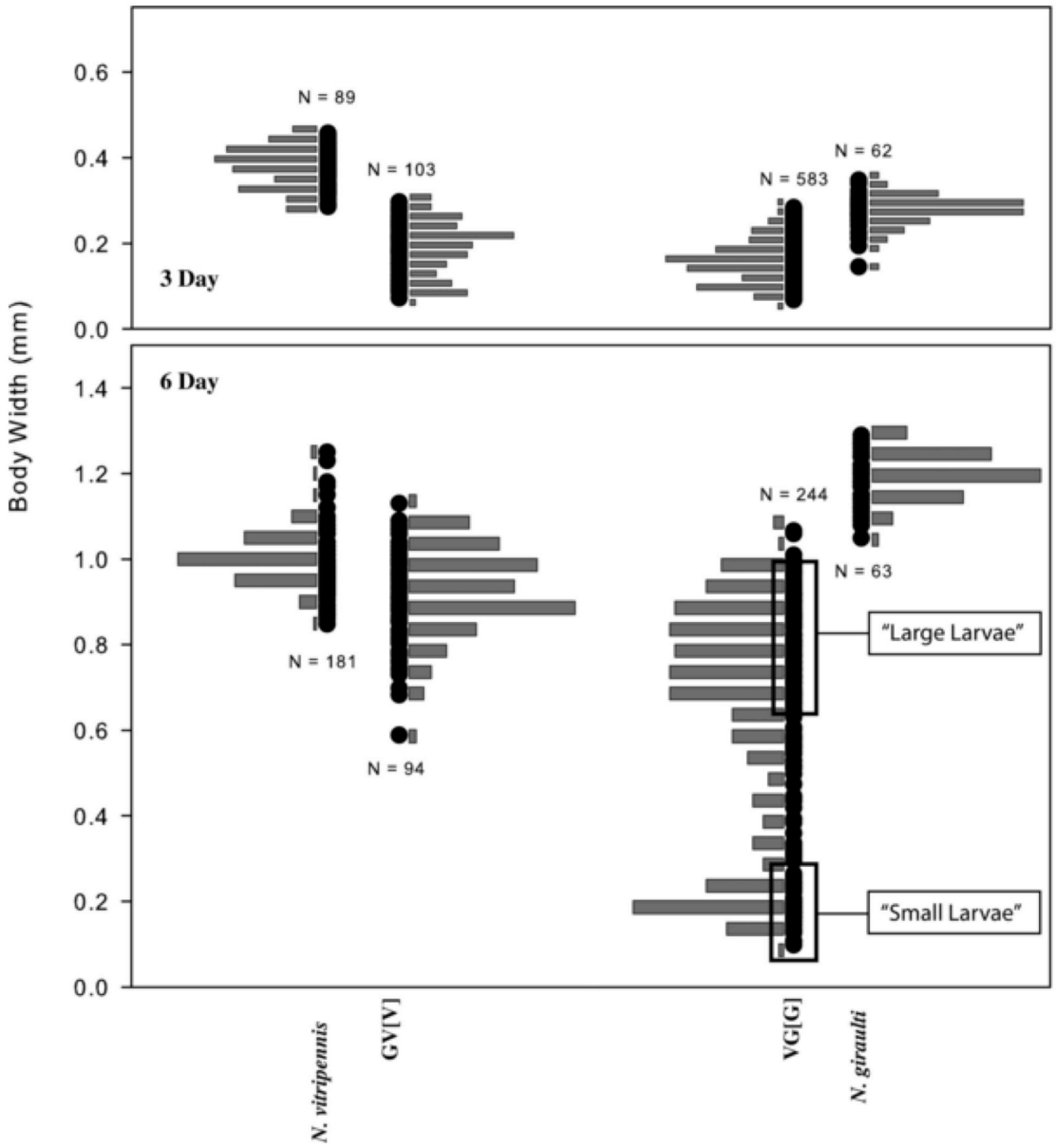

Figure 3.1. Body sizes of larval male *Nasonia*. *N. vitripennis*, *N. giraulti* and F_2 hybrids of these two species *N. vitripennis*, *N. giraulti* and F_2 hybrids of these two species are shown. The hybrids have either *N. vitripennis* or *N. giraulti* cytoplasm (GV[V] and VG[G], respectively) at day three (top panel) and day six (bottom panel) of their development. Histograms show proportion of total number of individuals (given as N) for different body widths. Black boxes in bottom panel for VG[G] hybrids designate size ranges for larval classes (large and small) that were subsequently genotyped.

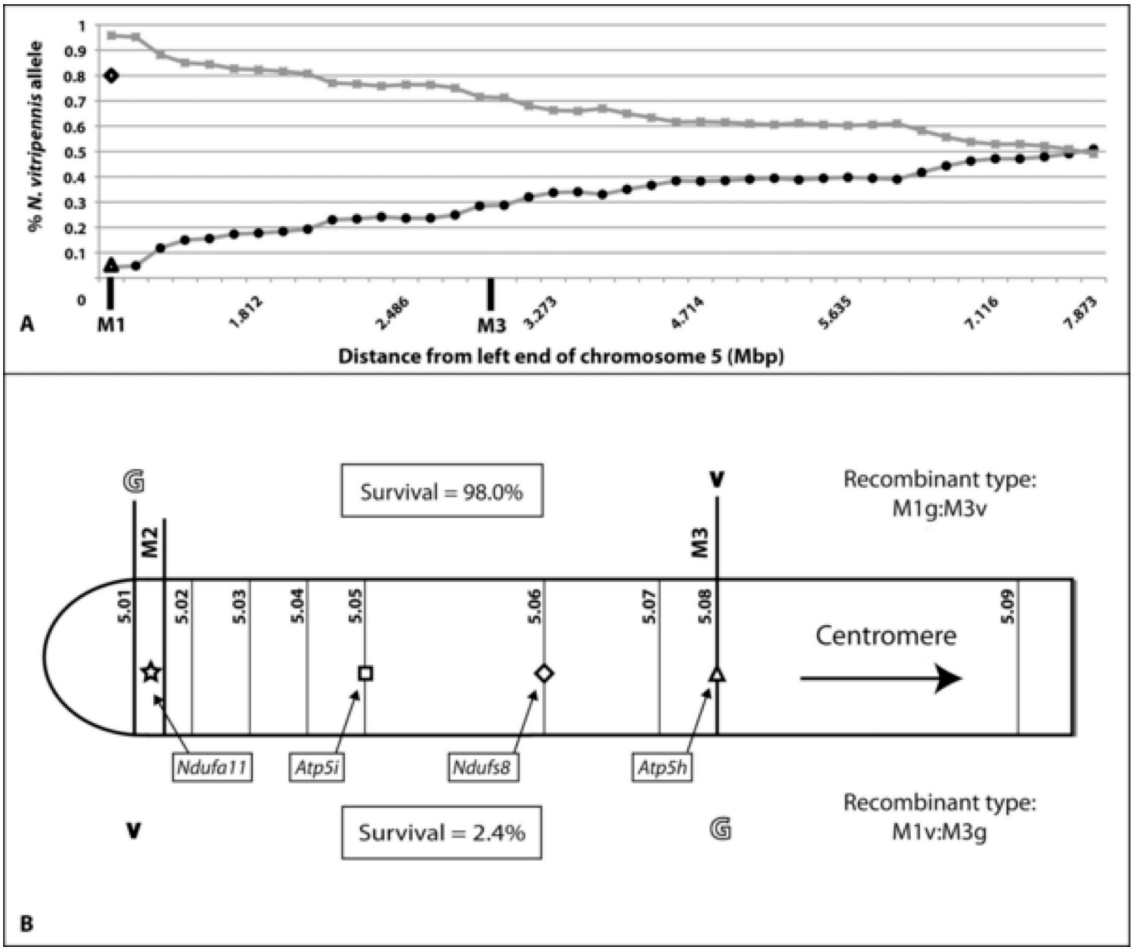

Figure 3.2. Diagram of the distal left arm of chromosome 5. **A**. Percentage of *Nasonia vitripennis* alleles along the distal part of the left arm of chromosome 5 in *N. vitripennis* x *N. giraulti* F_2 hybrid males with *N. giraulti* cytoplasm (= VG[G]). Black circles represent the proportion of alleles found in surviving adults and grey boxes represent the proportion of alleles expected to be found in individuals that die during development (*i.e.*, the inverse proportion of the survivors). Triangle = proportion of *N. vitripennis* alleles in the "large larvae" class (see Figure 3.1), diamond = proportion of *N. vitripennis* alleles in the "small larvae" class. The X-axis shows the distance of the represented SNP markers (in Mbp) from the left end of the chromosome. Bolded lines on X-axis designate positions of markers M1 and M3. **B.** Distal part of left arm of chromosome 5. Vertical lines within the chromosome represent map positions 5.01-5.09 defined by Niehuis et al. (2010). Markers M1 and M3 (~3Mbp apart) as well as marker M2 are shown as solid lines extending above the chromosome. The two reciprocal recombinant types are represented above (M1g:M3v) and below (M1v:M3g) the chromosome. The survival rate of the specific type of recombinant individuals is given in enclosed boxes as observed percentage of total expected number of individuals of each recombinant type. Symbols in the chromosome represent candidate genes described here (star = *Ndufa11*) and from Gibson et al. (2010) (square = *Atp5i*, diamond = *Ndufs8* and triangle = *Atp5h*).

CHAPTER 4

METABOLIC PHYSIOLOGY OF F_1 AND F_2 HYBRIDS OF *NASONIA* VITRIPENNIS AND *NASONIA* GIRAULTI

Mitochondria are organelles that are responsible for a wide range of cellular functions, but they are most notable for their role as the principal source of cellular energy in Eukaryotes. This energy, in the form of adenosine triphosphate (ATP), is produced by the oxidative phosphorylation (OXPHOS) pathway. The OXPHOS pathway operates through several large protein complexes (Complex I-IV) that shuttle electrons from their donors to a final electron acceptor, Oxygen, while using the energy from this electron transport to produce a proton gradient across the inner mitochondrial membrane. This gradient is then used by a fifth complex (Complex V, or ATP Synthase) to produce ATP from adenosine diphosphate (ADP; Scheffler, 2008). One of these complexes (Complex II) is composed of four nuclear-encoded protein subunits while the remaining four complexes (Complex I, III, IV and V) are composed of protein subunits encoded by both the mictochondrial and nuclear genomes (Figure 4.1; Hatefi, 1985; see also Blier *et al.,* 2001; Rand *et al.,* 2004). The tight interaction of these subunits within each complex is essential to maintain the proper function of the pathway to ensure adequate ATP production to fuel cellular processes. However, the mitochondrial genome evolves at a much faster rate than the nuclear genome (Boore 1999; Oliveira *et al.,* 2008), which is expected to lead to incompatible interactions between subunits (Mishmar & Gershoni 2007). Due to the decreased efficiency of selection acting on the mitochondrial genome vs the nuclear genome, selection is expected to favor substitutions in nuclear subunits

that can compensate for deleterious substitutions in the mitochondrial subunits that would otherwise lead to incompatibilities in the pathway (Rand *et al.,* 2004).

Nuclear compensation for deleterious mitochondrial substitutions is hypothesized to maintain the function of the pathway within each species as they evolve (Rand *et al.,* 2004). However, hybrids from interspecific crosses will posses genes from both parental species, leading to potential incompatible interactions between genes that have evolved independently in each parental species. Indeed, there is increasing evidence for disruption of mitochondrial function and OXPHOS activity in hybrids. In "cybrid" cell lines that have their nucleus from one species and their mitochondria from another, the mitochondrial disruption increases with the divergence time between the species supplying each cellular components (King & Attardi, 1989; Kenyon & Moraes, 1997; McKenzie *et al.,* 2003). In animal crosses, there is evidence of mitochondrial disruption in both intraspecific wide crosses (Sackton *et al.,* 2003; Ellison & Burton 2006, 2008; Montooth *et al.,* 2010; Meiklejohn *et al.,* 2013) and in interspecific crosses (Sackton *et al.,* 2003; Ellison *et al.,* 2008; Niehuis *et al.,* 2008). These studies all point to a breakdown in interactions between nuclear- and mitochondrial-encoded genes as the basis of hybrid incompatibilities.

The parasitoid wasp genus *Nasonia* is composed of four morphologically similar species that are largely reproductively isolated from one another in nature, despite occurring in sympatry, due to differential infection with *Wolbachia* endosymbionts (Darling & Werren, 1990; Breeuwer & Werren, 1990; Bordenstein *et al.,* 2001; Raychoudhury *et al.,* 2010). These wasps, however, can be cured of their *Wolbachia* and mated interspecifically, producing largely fertile and viable F_1 hybrid females (Breeuwer

& Werren, 1990, 1995; Koevoets *et al.,* 2011). It should be noted that, due to haplo-diploid sex determination in the Hymenoptera, the only hybrids produced in the first generation are females because any males that occur have developed from unfertilized eggs (and are haploid) and therefore are of the maternal species type. These hybrid females have only minor differences from the parental species in terms of fertility and viability, but if allowed to lay male destined eggs (unfertilized) the F_2 hybrid male progeny suffer from significant mortality during their larval development (Breeuwer & Werren, 1995; Koevots *et al.,* 2011). The extent of the mortality is cytoplasm-dependent, indicating that nuclear-mitochondrial incompatibilities underlie this inviability. These F_2 hybrid males have reduced activity of some of their OXPHOS complexes, and which complex is disrupted is cytoplasm-specific (Ellison *et al.,* 2008). This work was performed on isolated enzyme complexes in vitro and hasn't been extended to include effects on either whole mitochondria or whole animals.

Here I use the *Nasonia* system to investigate the effects of nuclear-cytoplasmic incompatibilities on respiration at both the level of whole mitochondria as well as the whole animal. I utilize *Nasonia* not only because of the incompatibilities that have been documented, but because it is a good system in which to investigate these incompatibilities. There are extensive genomic resources available for the entire *Nasonia* genus, which allows the genes underlying incompatibilities to be more easily assessed (Werren *et al.,* 2010). As mentioned, *Nasonia* has a haplo-diploid sex determination system. This system is highly amenable to genetic analysis because males are all haploid, which allows the assessment of genetic incompatibilities without any effects of

dominance, while the diploid F_1 females can be used to assess how dominance or heterozygosity may affect these incompatibilities.

I use hybrids from the *N. vitripennis* x *N. giraulti* cross due to the previous work on their incompatibilities and to complement the work presented in previous chapters of this dissertation (these hybrids are hereafter referred to as VG[G]; the letters outside the brackets designate the nuclear genotype and the letter in the brackets denotes the cytotype; V = *N. vitripennis* and G = *N. giraulti*). I measure intact F_2 hybrid male CO_2 production to determine how the previously identified OXPHOS deficiencies scale to the level of the whole animal (Ellison *et al.,* 2008). I measure the O_2 consumption of mitochondrial isolates from VG[G] F_2 hybrid male larvae in order to test whether there is a connection between the larval mortality of these hybrids and their OXPHOS deficiencies. And finally, I measure the O_2 consumption of mitochondrial isolates from heterozygous F_1 females relative to the parental species to determine if there are effects of the incompatibility at the organellular level that are not apparent at the whole animal level.

Methods

Stocks and cross experiments. We used two *Wolbachia*-free strains of *Nasonia*. AsymCX is a strain of *N. vitripennis* that originated in Leiden, The Netherlands and RV2X(U) is a strain of *N. giraulti* that originated in Rochester, New York (Breeuwer & Werren, 1995). These strains are highly inbred and have had their genomes sequenced (Breeuwer & Werren, 1995) and have been shown to suffer from interspecific hybrid breakdown (Gadau *et al.,* 1999, Niehuis *et al.,* 2008, Ellison *et al.,* 2008, Gibson *et al.,* 2013). We produced F_2 hybrid males by following the procedures of Niehuis *et al.* (2008)

and modified as in Chapter 3. Briefly, we collected virgin *N. vitripennis* males and *N. giraulti* females and allowed them to eclose before pairing them together for 24 hours without a host. The females were then given hosts on which to lay their eggs. The resulting F_1 hybrid females were then collected as virgins and were provided, individually, with a host to allow them to feed and begin egg production. After 24 hours, the feeder hosts were removed and the females were left without a host for 12 hours. The females were then given a single host, which was removed after a further 12 hours. These hosts, containing F_2 hybrid males, were then reared at 25°C for 72 hours to match the age distribution of Gibson *et al.* (2013). Larvae of AsymCX and RV2X(U) were reared from virgin females treated identically to the F_1 females described above.

Mass of wasps. The mass of adult wasps used for the CO_2 production assays as well as the mass of pooled larvae for the mitochondrial O_2 consumption assays was determined using an MX5 microbalance (Mettler-Toledo, Columbus, OH). Individual wasps were placed into a microcentrifuge tube and initial mass of the wasp and the tube was determined. The wasps were then removed from the tube and placed in the respiration chamber. The mass of the tube alone was then measured and the mass of the wasp alone was calculated.

Real-time CO_2 production rate of adult males. Adult males of *N. vitripennis, N. giraulti* and VG[G] hybrids were placed singly into individual respiration chambers connected to an infrared CO_2 analyzer (LI6262, Li-Cor, Lincoln, NE). Dry, CO_2-free air was flowed flowed at a rate of 1ml/min at standard temperature and pressure through a MFC-4 mass flow control (Sable Systems International, Las Vegas, NV, USA). This air was then passed through the chamber containing the wasp for a period of 10 minutes.

This procedure was repeated for each wasp at 25°C, 30°C, 35°C and 40°C. The order of temperatures was randomized for each wasp, and every wasp was given a rest period of several hours, with moist filter paper as a water source, between tests. No more than two temperatures per wasp were tested on a single day.

Isolation of mitochondria. Larvae were placed in a room temperature petri dish with moistened cotton to maintain humidity during extraction from the host puparia. Once all individuals were extracted, larvae were pooled into a dounce homogenizer and kept on ice for the remainder of the mitochondrial isolation, modified from the protocol described by Makinen and Lee (1968). To the dounce homogenizer was added 400ul of a solution containing (in mmol l^{-1}) 100 KCl, 40 Tris HCl, 10 Tris base, 5 MgCl2, 1 EDTA and 1 ATP, pH 7.4 (solution I) (Makinen and Lee 1968). The larvae were then homogenized to uniform consistency. Protease (subtilisin A; Sigman-Aldrich, St Louis, MO, USA) was added (500mg in 100ul solution I), and the homogenate was incubated on ice with continual mixing for 7 min. 600ul of solution I was then added to halt the digestion. The digested homogenate was then centrifuged at 200 g for 10 min. in a 4°C centrifuge (Allegra X022R, Beckman Coulter, Brea, CA) to pellet cellular debris and cuticle from the wasps. The supernatant was removed, placed in a new tube and centrifuged at 9000 g for 10 min. at 4°C. After centrifugation, the supernatant was discarded and the pelleted mitochondria were resuspended in 50ml of a solution containing (in mmol 1^{-1}) 220 D-mannitol, 70 sucrose, 10 TRIS-hydrochloride and 1 ethylene glycol tetraacetic acid (EGTA) using a wide bore pipet tip to ensure mitochondria were not damaged. All mitochondrial isolates were kept on ice until O_2 measurements were performed.

Mitochondrial content. Mitochondrial content of the isolates was estimated by determining the total protein content of each sample using a BCA protein assay (Thermo Scientific, Rockford, IL). The protein content was used to normalize between samples.

Oxygen consumption of mitochondria. Maximal mitochondrial oxygen consumption rate (J_{O2}) was measured polaragraphically as in Messor, Jackman and Willis (2004). Measurements were made in a respiration chamber (Oxygraph system, Hansatech Instruments, Norfolk, UK) maintained at 25°C in a volume of 250ul of respiration medium (RM), adapted from Wanders *et al.* (1984). The RM contained (in mmol 1^{-1}) 100 KCl, 50 MOPS, 20 Glucose, 10 K2PO4, 10 MgCl2, 1 EGTA and 0.2% BSA and was at pH 7.0. Resuspended mitochondria, 20ul, were then added to the chamber along with Pyruvate and Proline, (100 mmol 1^{-1} each) as oxidative substrates. Maximal respiration (State 3) J_{O2} was initiated with the addition of 12.5 mmol adenosine diphosphate (ADP).

Analysis of data. Maximal mitochondrial O_2 consumption (State 3) rates (J_{02}) were analyzed using the Mann-Whitney U test to test for differences between the F_1 hybrid females and the parental species. Mann-Whitney U tests were also used to test for differences in mass between F_2 hybrid males and males of the parental species. Given that there is very little overlap in the range of mass between *N. giraulti* males and F_2 hybrid males, comparisons were made between the hybrids and *N. vitripennis* and between *N. vitripennis* and *N. giraluti.* Within these comparisons, only CO_2 production rates from individuals of comparable masses were assessed (Figure 4.3). Mann-Whitney U tests were utilized to test for differences in mass and CO_2 production rates in the adult males. CO_2 production rates from all temperatures were standardized to 25°C using the

Q10 measurement described by Lighton (2008) and values for mass and CO_2 production rate were averaged for each individuals across all temperatures.

Results

CO_2 production by whole adult male wasps. Adult VG[G] F_2 hybrid males were significantly smaller than adult males of either parental species (Figure 4.2; *N. vitripennis* and VG[G], U = 258, P < 0.05; *N. giraulti* and VG[G], U = 269, P < 0.05). Males of comparable mass were used to compare CO_2 production rates (Figure 4.3, mass differences; VG[G] x *N. vitripennis*, U = 30, P > 0.05; *N. vitripennis* x *N. giraulti*, U = 65, P > 0.05). After normalizing all temperatures to 25°C, *N. giraulti* males have a higher CO_2 production rate than *N. vitripennis* males (Figure 4.3; U = 0, P < 0.05), and *N. vitripennis* males have a higher CO_2 production rate than VG[G] F_2 hybrid males (Figure 4.3; U = 22, P < 0.05).

O_2 consumption of mitochondria isolated from larvae. Mitchondria isolated from larval VG[G] F_2 hybrid males as well as larvae of both parental species did not consume any more O_2 than respiration chambers with RM alone (Figure 4.4). The sample sizes are too small to test for significance (N = 2 for *N. vitripennis*, *N. giraulti* and RM only and n = 4 for VG[G] hyrids), but when the protein content is taken into account, the apparent O_2 consumption drops even further due to the high protein content of these samples.

O_2 consumption of mitochondria isolated from adults. Mitochondria isolated from adult F_1 hybrid females consumed less than half the amount of O_2 as the parental species (Figure 4.5). This was significant for the test between the VG[G] hybrids and *N.*

vitripennis females (N = 5, U = 23 $P < 0.05$), but not for the test between the hybrids and *N. giraulti* (N = 3, U = 13 $P > 0.05$) due to the small sample size in *N. giraulti.*

Discussion

Nuclear- and mitochondrial-encoded subunits of the OXPHOS pathway have to interact properly to be functional and provide the necessary energy for an organism. (Hatefi, 1985). In order to maintain this function, the fast accumulation of substitutions in the mitochondrial genome is predicted to lead to compensatory selective pressure on the nuclear-encoded genes, leading to an overall increased evolutionary rate of the subunits of this pathway (Rand *et al*., 2004; Mishmar & Gershoni, 2007). This increased evolutionary rate within species is, in turn, predicted to lead to incompatible interactions in hybrids, a prediction that is consistent with the increasing evidence on hybrid dysfunction (King & Attardi, 1989; Kenyon & Moraes, 1997; McKenzie *et al.,* 2003; Sackton *et al.,* 2003; Ellison *et al.,* 2008; Niehuis *et al.,* 2008). Previous work has shown that *Nasonia* VG[G] F_2 hybrid males suffer from nuclear-cytoplasmic incompatibilities that result in reduction of the activity of some of their OXPHOS complexes (Ellison *et al*., 2008). It is not clear from this work, however, whether this reduction in activity translates to any impairment for these wasps as whole animals, and whether the reduction is causally related to the mortality experienced by hybrids (Breeuwer & Werren, 1995; Gadau *et al*., 1999; Niehuis *et al*., 2008) and/or the larval growth patterns described in Chapter 3. In this chapter I have begun to address these possibilities through a combination of assays of mitochondrial physiology and whole animal respirometry.

Ellison *et al.* (2008) found that the enzyme activity of individual OXPHOS complexes from VG[G] hybrids had reduced activity, per mg of protein, relative to the

activity of complexes from males of the either *N. vitripennis* or *N. giraulti,* and yet nothing is known about the metabolic rates of adult animals from which the complexes were isolated. It is subsequently not apparent whether this mass-corrected reduction in enzyme activity translates into an overall reduced metabolic rate for these hybrids, or if they are able to compensate by either increasing the number of complexes incorporated into the mitochondria, increasing the number of mitochondria, or both. My results from examining the CO_2 production rates of intact adult males show that the VG[G] hybrid males have an overall lower rate than either parental species when comparable sized individuals are compared (Figure 4.3). This indicates that these hybrids are likely not able to completely compensate for the reduced activity of their OXPHOS enzymes. In addition, the small adult size of these hybrids relative to the parental species is consistent with my findings in Chapter 3 that larval size appears to be tied to the genetic locus implicated in the larval mortality previously observed in these hybrids (Figure 4.2; Breeuwer & Werren, 1995). However, my results from Chapter 3 show that less than 5% of the surviving males from this cross carry the incompatible allele at this locus, which means that there are likely other incompatibilities that contribute to both the reduced activity of the OXPHOS enzymes and the reduced metabolic rates in intact adults. This also indicates that the surviving adults are not the ideal setting in which to assess the effects of the incompatibility based on the locus on chromosome 5.

In Chapter 3 I show that VG[G] F_2 hybrid males show a bimodal distribution of larval sizes during their development that is dependent on the genotype of the larvae at a locus on the left arm of chromosome 5. The finding that these larvae are still alive even late in larval development opens the door to assess the impact of this incompatibility on

respiration because now approximately 50% of the individuals should carry the incompatible allele. My results from measuring O_2 consumption by mitochondria isolated from these VG[G] larvae as well as larvae of the parental species, however, did not differ from the background levels (Figure 4.4). While it could be argued that these rates are simply very low due to the lack of flight muscle in larvae, the results showed no relationship between the total amount of animal tissue used for the experiment and the rate of O_2 consumption. This indicates that the rates found for each larval type are likely just variations in the background noise of the assay. Moreover, the rates I present in Figure 4.4 have not been corrected for the amount of protein in the sample, which is used as a proxy for the total mitochondrial content of these samples. This is because these samples may have protein concentrations well above the range of the assay used (these samples caused the colorimetric assay to quickly change color without incubation instead of taking ~30 minutes while being incubated). The first round of assays used the entire remainder of the sample, so further dilution was not possible. Using the values obtained from the assay, the protein concentrations appear to be 2-3 times higher than that found for samples from adult wasps, but are likely much higher. These extremely high protein concentrations are likely not due to metabolically active tissue, however. Rather, these larvae feed on fly pupae and have not defecated at the point of collection for these assays, so a large portion of the protein may be attributable to non-metabolic peptides derived from the fly host.

Given that most of the haploid VG[G] F_2 males that carry the incompatible allele on chromosome 5 have died before eclosing and therefore can't be assayed for mitochondrial O_2 consumption, and as living larvae they don't appear to have enough

mitochondria to be assessed, is it at all possible to determine if there is an effect of the incompatible allele on mitochondrial respiration? We can attempt to address this question by leveraging the haplo-diploid genetics of *Nasonia* by assessing the VG[G] F_1 hybrid females. These hybrid females have been shown to survive to adulthood in comparable proportions to the parental species (Breeuwer & Werren, 1995) and yet each individual, being diploid and heterozygous at all loci, carries a copy of the incompatible allele at the locus on chromosome 5. Moreover, mitochondrial isolates from these adult wasps are typically enriched for mitochondria relative to the larval samples due to the high proportion of flight muscle. My results from measuring O_2 consumption of mitochondria isolated from adult F_1 hybrid females shows that these females, despite not suffering from any outwardly apparent deficiencies (e.g. mating success, number of offspring, etc…), have a reduced capacity for mitochondrial respiration relative to the parental species (Figure 4.5).

Taken together, my results show that the locus on chromosome 5 may lead to decreased mitochondrial respiration, though current assays don't appear to be sufficiently sensitive to accurately test groups of larvae. This is the most rigorous way to address this question, as the larvae can be sorted by size and larval size correlates with genotype at this locus. This means that groups of individuals with the compatible allele can be tested against groups with the incompatible allele. Future studies, however, will be required to address this in these larvae. I have also shown that adult VG[G] F_2 hybrid males suffer from a mass-specific depression of their metabolic rate relative to males of the parental species and that this is likely independent of the locus on chromosome 5. This finding can lead to studies of other incompatibilities in these males, and given that the VG[G] F_1

females carry all possible incompatibilities, could be used to help account for any cumulative effects of these incompatibilities. Finally, my results on the mitochondrial O_2 consumption from these F_1 females is also a first step in understanding how defects at the molecular level can be mitigated by compensation at higher levels of organization. Future studies will be required to determine how effective this compensation is as well as the mechanisms that underlie it.

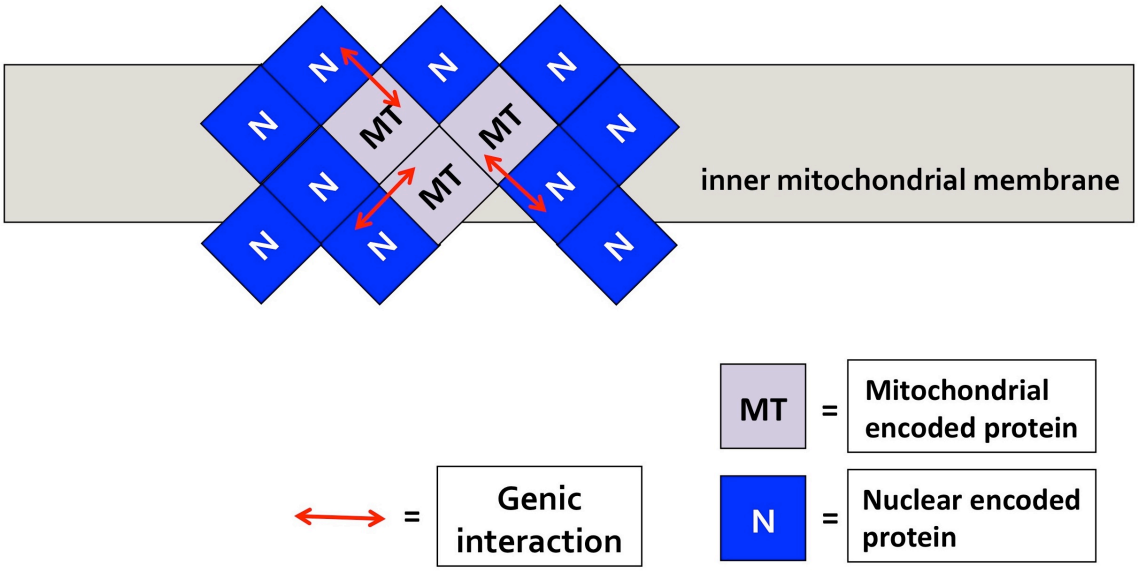

Figure 4.1. Diagram representing an oxidative phosphorylation (OXPHOS) complex. Four of the five complexes are composed of both nuclear- (N, dark blue) and mitochondrial- (MT, light blue) encoded subunits. Red arrows indicate a subset of genic interactions between subunits that must occur in order to maintain the energy generating function of this pathway.

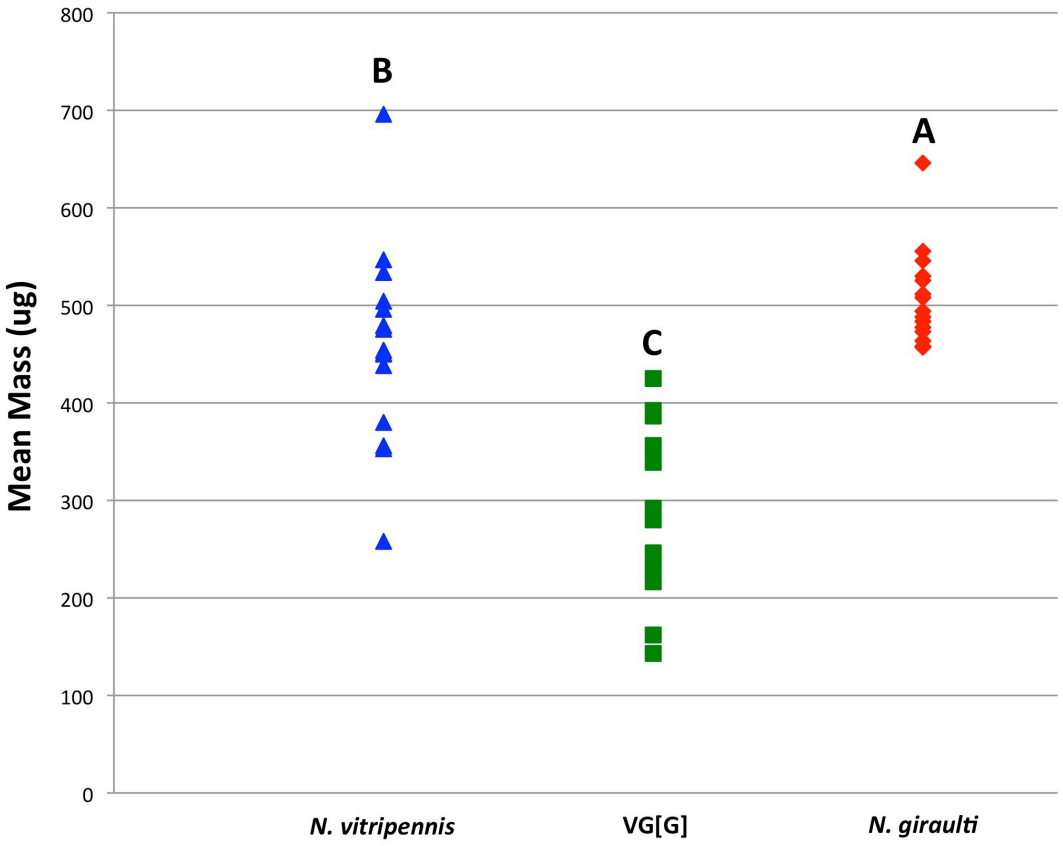

Figure 4.2. Mass of adult male *Nasonia*. Mean mass of individual adult males of *N. vitripennis* (blue), *N. giraulti* (red) and adult F_2 hybrid males from these two species with a *N. giraulti* cytoplasm (VG[G], green). The letters above each data cluster designate significant differences.

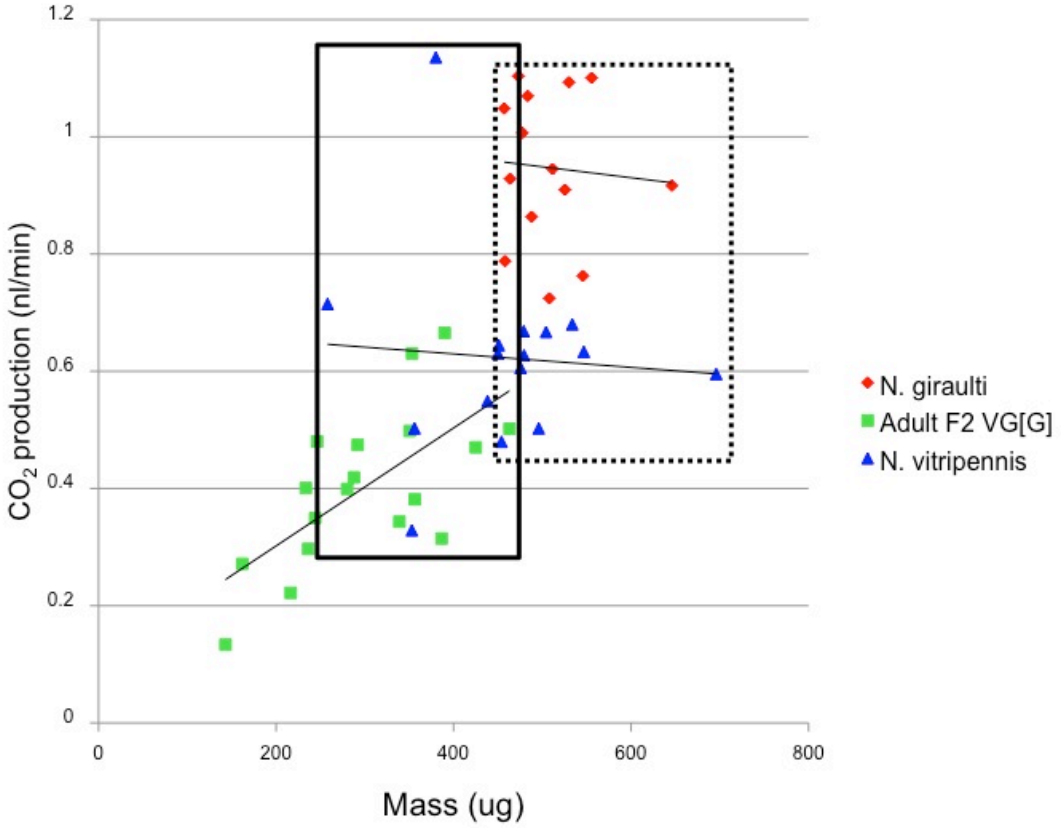

Figure 4.3. CO_2 production versus mass in adult male *Nasonia*. *N. giraulti* (red), *N. vitripennis* (blue) and F_2 hybrid males from these two species with a *N. giraulti* cytoplasm (VG[G], green) are shown. The CO_2 production rates from all temperatures are standardized to 25°C for comparisons. The solid black box shows the individuals that were used to compare *N. vitripennis* and the F_2 VG[G] hybrids (*N. vitripennis*, n = 9; VG[G], n = 11). The dashed black box shows the individuals that were used to compare *N. vitripennis* and *N. giraulti* (*N. vitripennis*, n = 11; *N. giraulti*, n = 14).

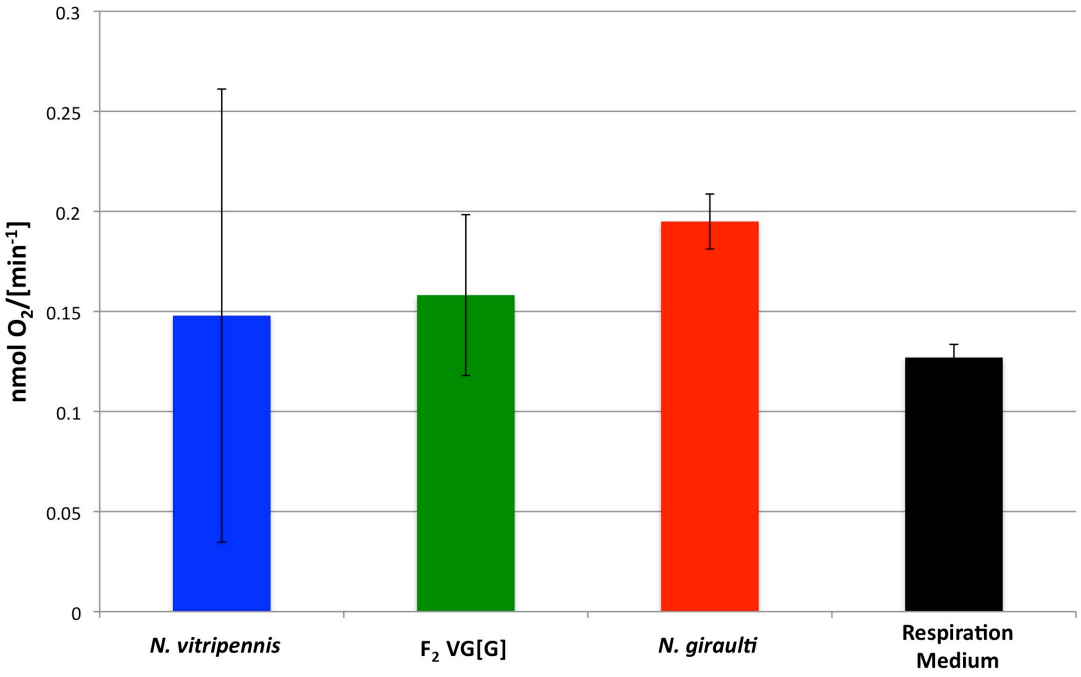

Figure 4.4. O_2 consumption of mitochondrial isolates from *Nasonia* larvae. *N. vitripennis* (blue), *N. giraulti* (red) and F_2 hybrid males from these two species with a *N. giraulti* cytoplasm (VG[G], green) are shown. Error bars indicate standard error. Sample sizes are too small for statistical testing (N = 2 for *N. vitripennis*, *N. giraulti* and RM only and n = 4 for F_2 VG[G] hyrids).

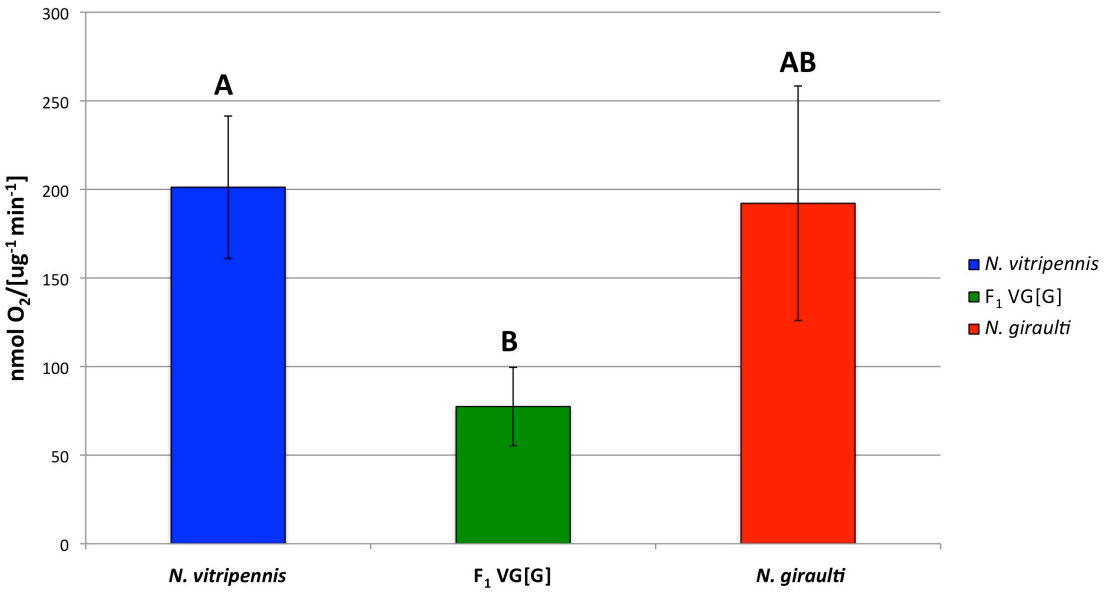

Figure 4.5. O_2 consumption of isolated mitochondria from adult female *Nasonia*. *N. vitripennis* (blue), *N. giraulti* (red) and F_1 hybrid females from these two species with a *N. giraulti* cytoplasm (VG[G], green) are shown. Error bars indicate standard error and the letters above each column indicate significant differences.

CHAPTER 5

BEHAVIOURAL AND GENETIC ANALYSES OF *NASONIA* SHED LIGHT ON THE EVOLUTION OF SEX PHEROMONES

Abstract

Sex pheromones play a pivotal role in the communication of many sexually reproducing organisms (Wyatt, 2003). Accordingly, speciation is often accompanied by pheromone diversification enabling proper mate finding and recognition (Smadja & Butlin, 2009). Current theory implies that chemical signals are under stabilizing selection by the receivers who thereby maintain the integrity of the signals (Butlin & Trickett, 1997). How the tremendous diversity of sex pheromones seen today evolved is poorly understood (Symonds & Elgar, 2008; Steiger *et al*., 2011). Here we unravel the genetics of a newly evolved pheromone phenotype in wasps and present results from behavioural experiments indicating how the evolution of a new pheromone component occurred in an established sender–receiver system. We show that male *Nasonia* vitripennis evolved an additional pheromone compound differing only in its stereochemistry from a pre-existing one. Comparative behavioural studies show that conspecific females responded neutrally to the new pheromone phenotype when it evolved. Genetic mapping and gene knockdown show that a cluster of three closely linked genes accounts for the ability to produce this new pheromone phenotype. Our data suggest that new pheromone compounds can persist in a sender's population, without being selected against by the receiver and without the receiver having a pre-existing preference for the new pheromone phenotype, by initially remaining unperceived. Our results thus contribute valuable new insights into the evolutionary mechanisms underlying the diversification of sex

pheromones. Furthermore, they indicate that the genetic basis of new pheromone compounds can be simple, allowing them to persist long enough in a population for receivers to evolve chemosensory adaptations for their exploitation.

Sexually reproducing organisms depend on their ability to localize and recognize conspecific mates reliably (Wyatt, 2003). Many insects, for example, make use of sex pheromones to attract mates. The signal integrity of sex pheromones has been postulated to be maintained by pheromone receivers selecting against modifications of a given pheromone composition. It is unclear, however, how sex pheromones diversify among species and how new pheromone phenotypes evolve if stabilizing selection is acting against pheromone modifications (Symonds & Elgar, 2008; Steiger *et al*., 2011). Studies on *Drosophila* and moths have provided important insights into the evolution of quantitative pheromone changes (shifts in the relative abundance of individual pheromone components). Notably, they have highlighted the importance of pre-existing pheromone preferences and biosynthetic enzymes in the evolution of pheromone diversity (Roelofs & Rooney, 2003; Ferveur, 2005; Xue *et al*., 2007; Shirangi *et al*., 2009; Smadja & Butlin, 2009; Lassance *et al*., 2010; Liénard *et al*., 2010; Albre *et al*., 2012). To shed light on the evolution of qualitative pheromone alterations, we studied sex pheromones in the wasp genus *Nasonia*, an emerging model system for investigating the genetics of speciation and complex traits (Werren & Loehlin, 2009; Werren *et al*, 2010).

Male *Nasonia vitripennis* attract conspecific virgin females by releasing a sex pheromone consisting of 4(*R*),5(*S*)-5-hydroxy-4- decanolide (referred to here as *RS*), 4(*R*),5(*R*)-5-hydroxy-4-decanolide (referred to here as *RR*) and 4-methylquinazoline (referred to here as *MQ*) (Figure 5.1a, b, d; Ruther *et al*., 2007, 2008; Abdel-latief *et al*, 2008; Steiner & Ruther, 2009). We compared this pheromone with those of all other known *Nasonia* species and with that of the closely related wasp *Trichomalopsis sarcophagae* and found

N. vitripennis to be the only species whose males biosynthesize *RR* (Figure 5.1b and Supplementary Figure 5.1 in Supplementary Information Chapter 5). The pheromone of all other *Nasonia* species and of *T. sarcophagae* consists only of *RS* and *MQ*. Therefore the ancestral sex pheromone of *Nasonia* males probably consisted solely of *RS* and *MQ*, and the additional occurrence of *RR* is probably a derived state that evolved in the *N. vitripennis* lineage (Figure 5.1c).

To assess the contribution of *RR* as an active component of the *N. vitripennis* sex pheromone, we exposed virgin females of *N. vitripennis* and *Nasonia giraulti* (representative of the sister lineage of *N. vitripennis* occurring in sympatry with *N. vitripennis* (Werren & Loehlin, 2009; Werren *et al*, 2010); Figure 5.1c) in a two-choice olfactometer to natural pheromone extracts of males of these two species and to synthetic pheromone components. Our experiments showed that females are attracted by natural pheromone extracts of both con- and heterospecific males and do not differentiate between natural extracts and synthetic pheromone blends (Figure 5.1e, f and Supplementary Tables 5.1 and 5.2 in Supplementary Information Chapter 5). Females of both species were also attracted by 100-ng doses of synthetic *RS* (Figure 5.1e, f). However, females of neither species responded to 100-ng doses of synthetic *RR* alone (Fig. 1e, f). We subsequently manipulated sex pheromone extracts of *N. giraulti* males by adding synthetic *RR* at naturally occurring dosages, thereby creating a pheromone phenotype almost identical to that of *N. vitripennis* males. When given the choice between manipulated and genuine pheromone extract, *N. giraulti* females did not discriminate between them (Figure 5.1g and Supplementary Figure 5.2 in Supplementary Information Chapter 5). *N. vitripennis* females, however, clearly preferred the

manipulated *N. giraulti* extract containing synthetic *RR*. Hence, *RR* influenced the behaviour of *N. vitripennis* females only in the presence of the other pheromone components. In fact, their response to the *N. giraulti*- manipulated pheromone extract did not differ significantly from their response to *N. vitripennis* pheromone extract (Figure 5.1g). This notable result opens up the possibility that *N. vitripennis* females did not respond to *RR* when it first occurred as a new component of their males' sex pheromone—a behaviour still found today in the sister lineage of *N. vitripennis*. Thus, instead of having had a predisposition to prefer the new pheromone blend, even before it existed, *N. vitripennis* females may have evolved this preference later, fostered by the co-occurrence of *RR* and *RS*.

To determine the genetic basis of *RR* biosynthesis, we studied recombinant F_2 hybrid males of *N. vitripennis* and *N. giraulti*. We found the *RR*/*RS* ratio in the sex pheromone of the hybrids to vary across a wide range and exploited this variation in a quantitative trait locus analysis, studying molecular markers distributed across all five chromosomes of *Nasonia*. The analysis indicated two quantitative trait loci (QTL) at a genome-wide significance level of 0.01: one on chromosome 1 (log of odds (lod) score = 42.7; $P < 0.001$) and the other on chromosome 4 (lod score = 5.2; $P < 0.001$) (Figure 5.2a and Supplementary Figure 5.3 in Supplementary Information Chapter 5). The predicted QTL on chromosome 1 explained 81.5% of the total phenotypic variance and was indicative of whether or not the *N. vitripennis* pheromone phenotype was observed, whereas the QTL on chromosome 4 explained only 3.9% of the variance.

We exploited the strong association between the genotype of the QTL on chromosome 1 and the presence of *RR* in the hybrids' sex pheromones for fine-scale mapping of this

locus. We screened 1,007 F_2 hybrid males for evidence of recombination on genome sequence scaffold 1 between 1,900 and 2,100 kilobases (kb) (Muñoz-Torres *et al*., 2011; Niehuis *et al*., 2010). We then searched by genotyping additional markers across this interval for the smallest mappable region for which the association between the *N. vitripennis* genotype and the presence of *RR* held across all recombinant hybrids. This allowed us to narrow down the location of the QTL to a 34.1 kb region (Figure 5.2b). The official gene set for *N. vitripennis* (Muñoz-Torres *et al*., 2011) predicted nine genes in the candidate region (Figure 5.2c). To learn more about their potential functions, we searched their amino acid sequences against the Pfam protein family database (Punta *et al*., 2012). Intriguingly, five predicted genes showed high similarity to short-chain dehydrogenases/reductases (SDRs), especially 15-hydroxyprostaglandin dehydrogenases: *NV10125*, *NV10127*, *NV10128*, *NV10129* and *NV21627*. SDRs are oxidoreductases with epimerase and/or racemase activity that catalyse the oxidation of hydroxyl groups to the respective carbonyls and vice-versa (Tanner, 2002; Kavanagh *et al*., 2008). Given that *RS* and *RR* differ only in the chirality of their hydroxyl group, we considered these genes promising candidates for mediating a conversion of *RS* to *RR*.

Gene expression data provided evidence for the expression of only four of the five candidate genes in adult *N. vitripennis* males: *NV10125*, *NV10127*, *NV10128* and *NV10129*. Of these, *NV10127*, *NV10128* and *NV10129* showed an astonishingly high similarity at the nucleotide sequence level (87.4–98.7% identity). Phylogenetic analysis subsequently indicated that this tandem array of genes—all of which are present in *N. giraulti* and similarly expressed in *N. vitripennis* and *N. giraulti* (Supplementary Figure 5.4 and Supplementary Table 5.3 in Supplementary Information Chapter 5)—was subject

to gene conversion (the information of one gene was partially or completely replaced by that of another) in the *N. vitripennis* lineage (Figure 5.3a and Supplementary Figure 5.5 in Supplementary Information Chapter 5), which is a possible explanation for changes in gene function linked to the evolution of a novel pheromone compound. We therefore decided to test the involvement of *NV10127*, *NV10128* and *NV10129* in the biosynthesis of *RR* by means of double- stranded RNA (dsRNA)-mediated gene knockdown experiments. We injected dsRNA of *NV10127* (treatment A) and NV10128 (treatment B) as well as a mixture of both (treatment AB) into male pupae of *N. vitripennis*. We did not synthesize or inject dsRNA of *NV10129* as the coding sequence of the gene was almost identical to that of *NV10128* in the 539-base pair target region for the dsRNA of *NV10128*. Gene expression analysis in the treated males subsequently confirmed significant (71–93%) knockdown of *NV10127*, *NV10128* and *NV10129* relative to controls in which we injected dsRNA derived from green fluorescent protein (GFP)-encoding sequence (treatment C) (Figure 5.3b–d). None of the treatments significantly altered expression of *NV10125*, the only remaining candidate gene expressed in adult males ($P = 0.296$; Figure 5.3e). Intriguingly, treatments A, B and AB caused a significant shift in the *RR*/*RS* ratio towards *RS* in the pheromone blend of adult males compared to controls (Figure 5.3f). This establishes that at least one of the three predicted SDR-encoding genes is required for the ability to produce the *N. vitripennis* pheromone phenotype. We found no statistically significant differences in the knockdown and *RR*/*RS* ratio change between the different treatments that could have given further insights into the importance of each of the three putative SDRs ($P \geq 0.275$).

This is the first study, to our knowledge, on the genetics of pheromone evolution in Hymenoptera (wasps, ants and bees) and the first characterization of genes that cause a qualitative change in an insect pheromone by triggering the stereochemical inversion of a pre-existing signalling molecule. The results shed new light on the biosynthesis and evolution of chemical signals in sender–receiver systems. The high similarity of the identified putative SDRs with 15-hydroxyprostaglandin dehydrogenases (oxidoreductases with carbonyl reductase activity targeting the 15(S)-hydroxy group of prostaglandins; Cho *et al*., 2003) and the structural resemblance between 15-hydroxyprostaglandins and 5-hydroxy-4-decanolide (a five-membered oxygenated ring with a hydroxylated side chain) suggest that the putative SDRs catalyse the direct conversion of *RS* to *RR*. Specifically, we assume that the hydroxyl group at carbon atom five of *RS* is oxidized by one of the three putative SDRs and that the resulting ketone is subsequently reduced with inversion of stereochemistry by either the same or one of the other two putative SDRs, leading to *RR* as a new pheromone component (Figure 5.1a). Our data thus provide support for the idea that some new pheromone compounds arise as modifications of existing signalling molecules (Symonds & Elgar, 2008), and show that the genetic basis of these modifications can be relatively simple. They further indicate that sex pheromone receivers do not automatically respond to new pheromone compounds within an existing pheromone blend. Thus, a new pheromone component may remain initially uncoupled from selective constraints exerted by the receiver. This and a simple genetic basis underlying the biosynthesis of a new compound would give a receiver time to associate the new compound with conspecific mates and to recognize it

as part of the species-specific chemical signal, thus illustrating how new pheromone compounds can evolve within established sender–receiver systems.

Methods Summary

We screened several strains of each *Nasonia* species to infer the composition of the male-specific sex pheromone using coupled gas chromatography–mass spectrometry. The stereochemistry of 5-hydroxy-4-decanolide isomers was established by enantioselective gas chromatography (Ruther *et al*., 2007). We tested the response of virgin *N. vitripennis* and *N. giraulti* females to naturally occurring doses of purified pheromone extracts of either species and to synthetic pheromone blends containing *RS*, *RR* and *MQ* using a two-choice olfactometer (Steiner & Ruther, 2009). Virgin *N. vitripennis* and *N. giraulti* females were also given the choice between natural *N. giraulti* pheromone extract and manipulated *N. giraulti* pheromone extract that contained synthetic *RR*. Finally, we tested the response of virgin *N. vitripennis* females when exposed simultaneously to experimentally designed (natural *N. giraulti* extract plus synthetic *RR*) and genuine *N. vitripennis* pheromone. Bioassay data (residence times in test and control area) were statistically analysed with the Wilcoxon matched-pairs test (n = 20 per test), and differences between treatments were assessed with the Kruskal–Wallis test followed by pairwise comparisons with Holm-corrected Mann–Whitney U tests. Genetic cross experiments were conducted as described previously (Niehuis *et al.*, 2008). The QTL analysis was done with R/qtl (Broman *et al*., 2003), applying a multiple-QTL model and genotyping 71 molecular markers listed in Niehuis *et al*. (2011) and in Supplementary Table 5.4 (in Supplementary Information Chapter 5) in 106 F_2 hybrid males. We followed the protocol described previously (Lynch & Desplan, 2006) for gene

knockdown and measured gene expression by quantitative reverse transcriptase PCR (qRT–PCR), applying the oligonucleotide primers listed in Supplementary Tables 5.5 and 5.6 (in Supplementary Information Chapter 5). Gene expression differences were statistically analysed with the Kruskal–Wallis test followed by pairwise comparisons with Holm-corrected Mann–Whitney U tests (for qRT–PCR experiments n = 8–10 per locus, treatment and sex; for *RR*/*RS* ratio experiments n = 24–52 per treatment). Phylogenetic analyses of DNA sequences were executed with MEGA5 (Tamura *et al*., 2011) and applying the maximum likelihood optimality criterion. Node support was statistically assessed from 1,000 bootstrap replicates.

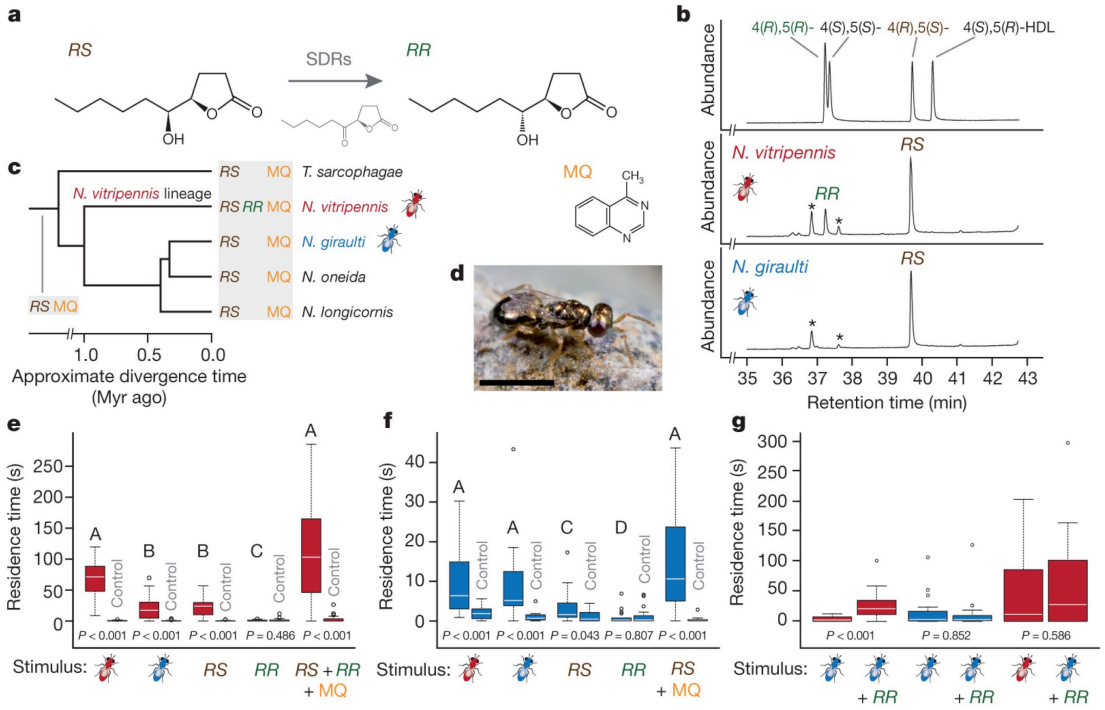

Figure 5.1. Evolution of sex pheromone diversity and behavioural response in *Nasonia* parasitoid wasps. **a**, Chemical structures of *Nasonia* male-specific sex pheromone compounds. In grey: proposed biosynthesis of 4(R),5(R)-5- hydroxy-4-decanolide (*RR*) by oxidation and successive reduction of 4(R),5(S)- 5-hydroxy-4-decanolide (*RS*) catalysed by short-chain dehydrogenases/ reductases (SDRs). **b**, Enantioselective gas chromatography–mass spectrometry of synthetic samples of all four stereoisomers of 5-hydroxy-4- decanolide (HDL) (top) and sex pheromone extracts of *N. vitripennis* (middle) and *N. girault* males (bottom). Asterisks indicate contaminants. **c,** Phylogenetic relationships of *Nasonia* wasps and evolutionary history of male-specific sex pheromone components. **d**, Adult *N. vitripennis* male (scale bar, 1 mm). **e**, **f**, Box-and-whisker plots showing median (horizontal line), interquartile range (box), maximum/minimum range (whiskers) and outliers (>1.5X upper quartile) of the residence time spent by virgin *N. vitripennis* (**e**) and *N. girault* (**f**) females in the two odour fields of a static two-choice olfactometer when given the choice between a solvent control, pheromone extracts of *N. vitripennis* males (red wasp) and *N. girault* males (blue wasp) and enantiopure synthetic pheromone components (*RS*, *RR* and *MQ*) (Wilcoxon matched-pairs test; n = 20 per test). Differences between treatments (residence time in the test field) were assessed with the Kruskal–Wallis test followed by pairwise comparisons with Holm-corrected Mann–Whitney U tests (different uppercase letters indicate statistical differences at $P < 0.05$). **g**, Response of *N. vitripennis* (red boxes) and *N. girault* (blue boxes) females to sex pheromone extract (representing 100 ng of *RS*) of male *N. vitripennis* and male *N. girault*, and to the same amount of male *N. girault* sex pheromone extract to which 60 ng of synthetic *RR* had been added (blue wasp 1 *RR*). Statistical analysis and illustration as in **e** and **f**.

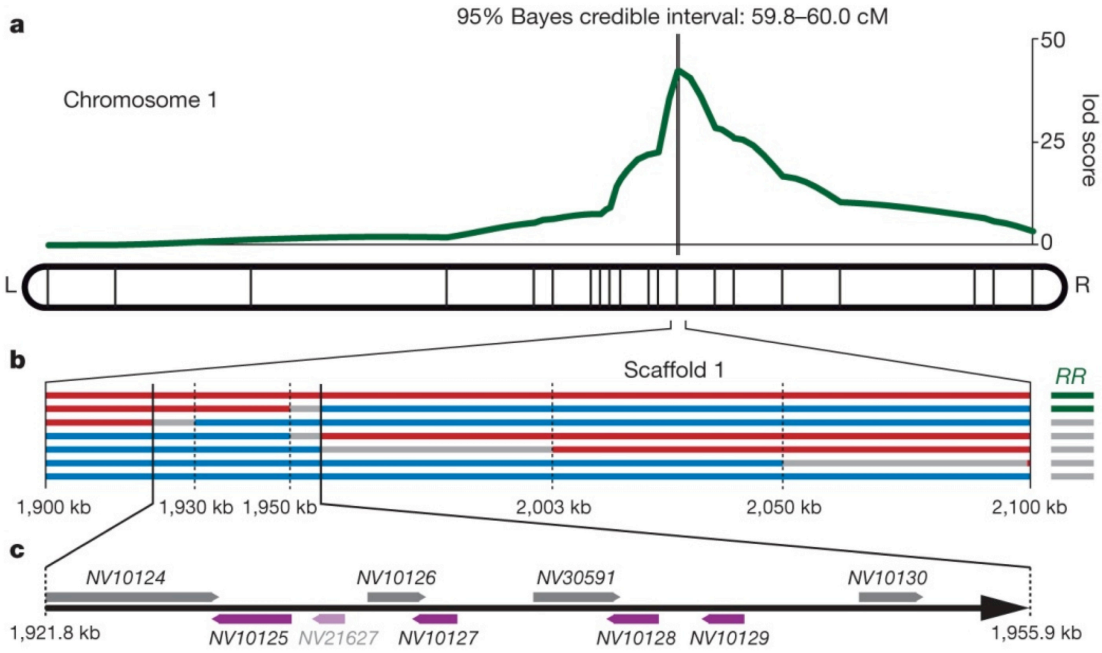

Figure 5.2. Genetics of sex pheromone differences between *N. vitripennis* and *N. giraulti* males. **a**, Mapping of QTL that explain the RR/RS ratio in the sex pheromones of *N. vitripennis* X *N. giraulti* F_2 hybrid males. The curve specifies the lod score along chromosome 1 for the presence of a genetic factor responsible for the *RR*/*RS* ratio of hybrid offspring. **b**, Fine mapping of the genetic factor on genome sequence scaffold 1 of chromosome 1 that controls for the presence of *RR* in the males' sex pheromone. Hybrids with the *N. vitripennis* genotype (red) between nucleotides 1,921.8 kb and 1,955.9 kb on scaffold 1 have *RR* present in the sex pheromone, those with the *N. giraulti* (blue) genotype in this region lack *RR*. In grey are regions with unknown genotype. **c**, Predicted genes in the candidate region of genome sequence scaffold 1 of chromsome 1. In violet are predicted SDRs (the predicted gene *NV21627* is not supported by extrinsic evidence, for example, transcript data).

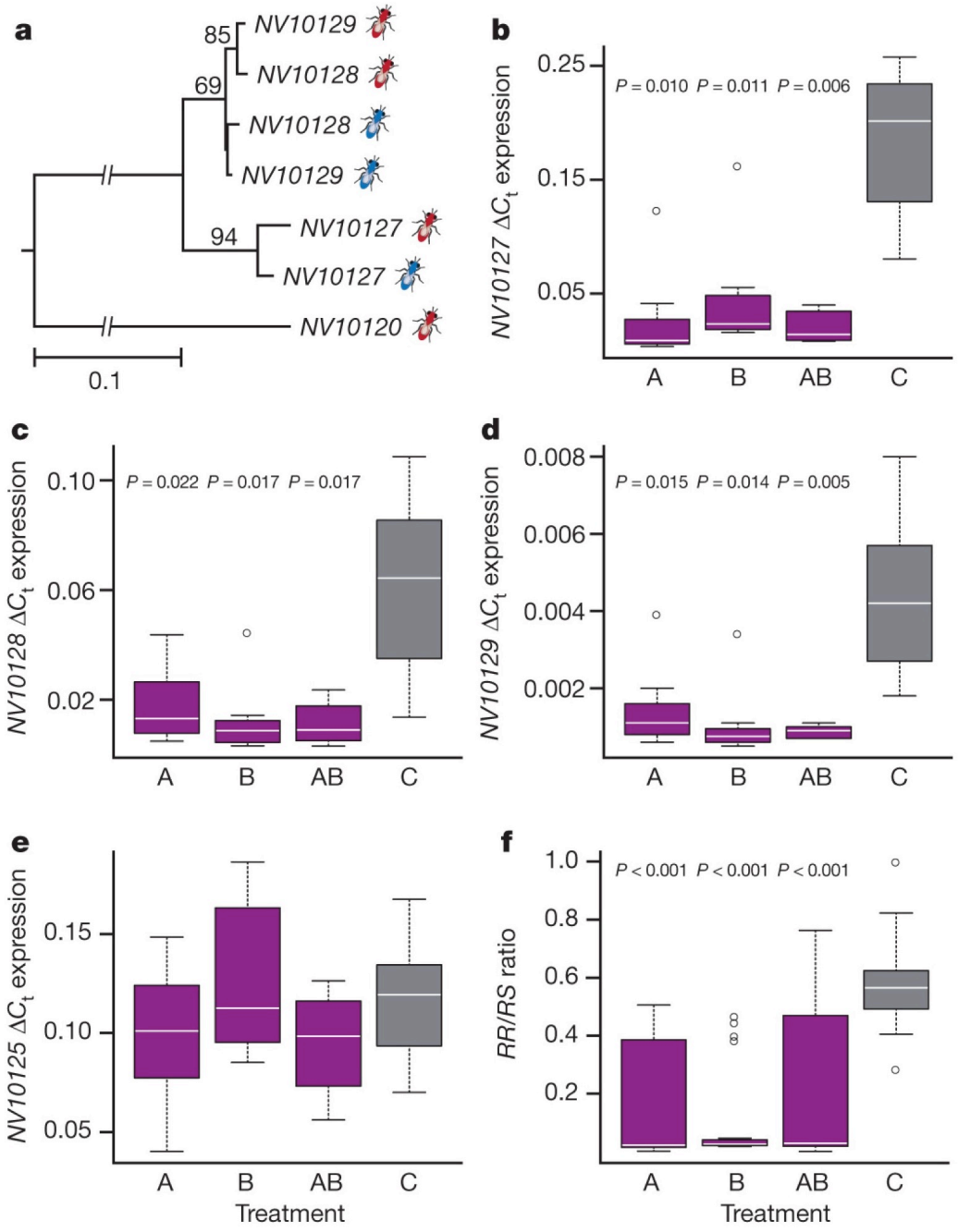

Figure 5.3. Phylogeny and dsRNA-mediated knockdown of candidate genes. **a**, Phylogenetic relationships of the predicted SDR-encoding genes *NV10127*, *NV10128* and *NV10129* of *N. vitripennis* (red wasps) and *N. giraulti* (blue wasps) inferred from coding sequence information. *NV10120* of *N. vitripennis* was used for outgroup comparison. Numbers above branches indicate statistical bootstrap support. **b**, Box-and-whisker plot showing median (horizontal line), interquartile range (box), maximum/minimum range (whiskers) and outliers (>1.5X upper quartile) of the expression of *NV10127* in male *N. vitripennis* after injecting dsRNA of *NV10127* (treatment A), *NV1028* (treatment B),

equimolar mixture of *NV10127* and *NV1028* (treatment AB), or dsRNA derived from GFP sequence (treatment C) (Kruskal–Wallis test followed by pairwise comparisons with Holm-corrected Mann–Whitney U tests; n = 8 per treatment). **c–e** All samples treated, statistically analysed and illustrated as in **b**. **c**, Expression of *NV10128* in male *N. vitripennis*. **d**, Expression of *NV10129* in male *N. vitripennis*. **e**, Expression of *NV10125* in male *N. vitripennis*. (P = 0.296; Kruskal–Wallis test; n = 8 per treatment.) **f**, The *RR*/*RS* ratio in sex pheromone extracts of *N. vitripennis* males treated and illustrated as in **b** (Kruskal–Wallis test followed by pairwise comparisons with Holm-corrected Mann–Whitney U tests; n = 24–52 per treatment.)

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

Taken as a whole, this work explores the genetics of speciation in *Nasonia*. Within this work, I describe the nuclear-encoded OXPHOS genes within *Nasonia* and show that, despite the functional constraint of the system, there is evidence of both directional and purifying selecting acting on the pathway, which may be indicative of the contrasting selective pressures on nuclear and mitochondrial genes (Chapter 2). In chapter 3 I identify a genomic region on the distal left arm of chromosome 5 in VG[G] F_2 hybrid males that, when individuals carry the incompatible allele, results in retarded growth throughout larval development and, ultimately, ~98% mortality. I explore the effect of nuclear-mitochondrial incompatibilities on both mitochondrial function and whole animal respiration in chapter 4 and I describe the issues encountered with the samples needed for these analyses. In chapter 5 I describe the genetics and evolution of a *Nasonia* male sex pheromone component that acts as a pre-zygotic barrier to gene exchange between the sympatric species pair, *N. vitripennis* and *N. giraulti* .

I would not have been able to complete this work without the information gathered from several other projects that I was engaged in throughout my work on this dissertation. For example, to perform any of the genetic work described here would have been impossible without a sequenced genome and the genomic tools that have been developed for *Nasonia* (Werren *et al.,* 2010). Equally important to the availability of the genome sequences is the genetic linkage map that enabled me to localize the OXPHOS genes that I annotated, the regions of incompatibility in F_2 hybrids, and the genes involved in the synthesis of the *Nasonia* male sex pheromone (Niehuis *et al*., 2010). And

finally, I would not have been able to identify the gene (*Ndufa11*) at the end of chromosome 5 in *Nasonia* had I not also annotated the OXPHOS genes in three ant species to identify orthologs in more closely related species (red harvester ant, C.R. Smith *et al.,* 2011; Argentine ant, C.D. Smith *et al.,* 2011; leaf-cutter ant, Suen *et al*., 2011).

Within the field of speciation, this work is significant in that it describes the genetics of both pre- and post-zygotic isolation in a non-traditional model organism. To date, much of the work on speciation has involved a handful of model organisms, including yeast, Drosophilid flies and moths, and within this, only the flies have been utilized to study both pre-and post-zygotic mechanisms (Smadja & Butlin, 2009; Maheshwari & Barbash, 2011). The use of emerging model organisms, like *Nasonia*, is essential to our understanding of the general processes that lead to the generation and maintenance of species as they allow us to test hypotheses that are generated in more extensively studied organisms. This work has identified candidate speciation genes that appear to be responsible for larval growth defects and mortality in hybrids, further implicating nuclear-mitochondrial evolutionary dynamics as a driver of speciation (Rand *et al*., 2004; Mishmar & Gershoni,2007). I have also identified the genes underlying a sex pheromone difference that acts as a pre-zygotic barrier and have shown that this difference could evolve without being selected against in spite of the longstanding hypothesis that this should not occur (Symonds & Elgar, 2008; Steiger *et al*., 2011). This finding underscores the importance of addressing major, overarching questions in speciation by examining multiple species groups to determine which aspects of their evolution represent generalizable rules and which represent caveats to these rules.

Finally, this work demonstrates that *Nasonia* is an excellent model organism for the study of the genetics of speciation and, like all scientific inquiry, provides many questions to fuel further study both within this system as well as in other species groups.

The work presented here answers many questions about the genetics underlying speciation in *Nasonia*, but there are still many to be considered. The chief candidate gene in Chapter 3 (*Ndufa11*) has yet to be confirmed as the causal gene in stunted larval development and/or larval mortality. Future studies should examine the effect of the incompatible allele of this gene (i.e. the *N. vitripennis* copy) in the genetic background of *N. giraulti*, either through introgression experiments or, if the technology becomes available, transformations of this allele into *N. giraulti*. This would allow assessment of the gene in isolation from other genetic barriers and would allow the mitochondrial respiration of these individuals to be measured (surviving adults with the allele could be assessed instead of small, dying larvae). Similarly, there are many open questions regarding the VG[G] F_1 females. Future work should focus on the mechanisms that allow the females to develop normally even though they seem to have impaired mitochondria. This should include work both to determine how the composition of the mitochondria differs from that of the F_2 males as well as how their total mitochondrial content differs. Finally, there are still many questions regarding the female response to the male sex pheromone discussed in Chapter 5. Future work should focus on whether the females that do not respond to the new pheromone simply ignore the signal that they receive, or if they are incapable of receiving the signal at all. The answers to these questions will provide a much fuller understanding of the evolution of speciation genes than is currently possible.

This work demonstrates the evolution, and the impact, of mechanisms of speciation in *Nasonia*. It is my sincere hope that this work will improve our understanding of speciation across many other organisms and contribute to our growing understanding of the diversity of life.

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APPENDIX A

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