# SPECIATION MICROBIOMES: CONSEQUENCES OF GUT BACTERIA ON HYBRID MORTALITY IN THE GENUS *NASONIA*

By

Robert M. Brucker

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Approved:

Professor Seth R. Bordenstein

Professor Patrick Abbot

Professor Eric P. Skaar

Professor Antonis Rokas

Professor Jeurgen Gadau

# DEDICATION

To my wonderful partner Bojana Jovanovic for all her support,

and my family, who do not understand what I do, but believed in me anyway.

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LIST OF ABBREVIATIONS

| | |
|---|---|
| aa | amino acid |
| AMP | antimicrobial peptide |
| ANOVA | analysis of variance |
| AR | antibiotic resistant |
| BDM | Bateson-Dobzhansky-Muller |
| bp | base pair |
| BLAST | basic logic alignment search tool |
| BSA | bovine serum albumin |
| C | centigrade |
| cDNA | complementary DNA |
| CFU | colony forming unit |
| CI | cytoplasmic incompatibility |
| CM | centimeter |
| Cv | conventional |
| DNA | deoxyribonucleic acid |
| dsDNA | double-stranded DNA |
| FISH | fluorescent *in situ* hybridization |
| GF | germ-free |
| GFP | green fluorescent protein |
| h | hour |
| HI | hybrid incompatibility |
| HMM | hidden Markov models |
| IIID | Insect Innate Immunity Database |
| In | inoculated |
| ITS | internal transcribed spacer |
| kb | killobase |
| LB | Luria broth |
| mb | megabase |
| mg | milligram |
| µg | microgram |
| MgCl2 | magnesium chloride |
| MHz | mega hertz |
| µL | microliter |
| µm | micrometer, micron |
| µM | micromolar |
| mm | millimeter |
| min | minute |
| mRNA | messenger RNA |
| MTRD | marker transmission ratio distortion |
| NCBI | National Center for Biotechnology Information |
| NGS | next generation sequencing |
| NRM | *Nasonia* rearing medium |
| OTU | operational taxonomic unit |
| OXPHOS | oxidative phosphorylation |

| | |
|---|---|
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| QIIME | quantitative insights into microbial ecology |
| QPCR | quantitative polymerase chain reaction |
| rDNA | ribosomal DNA |
| RDPII | Ribosomal Database Project II |
| RI | reproductive isolation |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| RNAseq | RNA sequencing |
| RPKM | reads per killobase per million mapped reads |
| SEM | scanning electron microscope |
| ssDNA | single-stranded DNA |

# CHAPTER

# I. INTRODUCTION

*"It is a rather startling proposal that bacteria, the organisms which are popularly associated with disease, may represent the fundamental causative factor in the origin of species." (Ivan E. Wallin, 1927)*

## Background and significance

Debates about the types of heritable elements that promote speciation began as early as the 1920s among evolutionary biologists and geneticists. The main demarcation of these debates is best represented by two books - Ivan Wallin's *Symbionticism and the Origin of Species* (Wallin, 1927) and Theodosius Dobzhansky's *Genetics and the Origin of Species* (Dobzhansky, 1937) published ten years later. Wallin's central thesis was that the universality of bacterial-derived mitochondria reflected the importance of bacterial symbionts as building blocks of evolutionary change and, ultimately, of speciation. Despite Wallin's efforts to incorporate symbiosis into mainstream evolutionary biology, only Dobzhansky's book would have a lasting influence throughout the century. From the Biological Species Concept to the Dobzhansky-Muller model of postzygotic isolation, Dobzhansky laid a solid foundation for future study of nuclear genes as the agents of speciation (Orr, 1996).

With the advent of next-generation sequencing techniques that make the identification of bacterial symbionts simple and thorough, as well as a growing understanding of the ubiquity of microbial symbiosis, a reassessment of Wallin's ideas on

the symbiotic origin of species is needed. The emergence of *Wolbachia* in topical discussions of invertebrate speciation (Bordenstein et al., 2001; Presgraves, 2010; Werren et al., 2008) is just a start to reviving Wallin's silenced ideas on the role of bacterial symbionts in speciation. A dynamic exploration of their role beyond *Wolbachia* will reveal the diverse ways in which microbiotas potentially contribute to speciation.

**Speciation genes and the Bateson-Dobzhansky-Muller model**

The Bateson-Dobzhansky-Muller (BDM) model of speciation postulates that when two populations of a species evolve in isolation from each other, substitutions in genes that epistatically interact within those populations fail to interact properly in hybrids when the populations come back into contact (Dobzhansky, 1937; Muller, 1942). These so called "speciation genes" or "hybrid incompatibility genes" evolve as a by-product of natural selection, genetic drift, or selfish genetic elements (Coyne and Orr, 2004; Orr et al., 2004). As a consequence, hybrids can be unfit and rendered either sterile or inviable due to negative, epistatic gene interactions (Orr and Turelli, 2001). Once established between species, hybrid incompatibility is irreversible (Gavrilets, 2003; Nei, 1975; Presgraves, 2010).

The breakdown of normal epistatic interactions in hybrids has been well characterized in many organisms. In *Saccharomyces* yeast, for example, nuclear-mitochondrial hybrid incompatibilities that have evolved between *S. cerevisiae* and *S. bayanus* which cause a failure of sporulation and a respiratory defect in hybrids (Lee et al., 2008). Hybrid sterility occurs between *Drosophila melanogaster* and *Drosophila simulans* because one of the duplicated copies of the male fertility gene, *JYalpha*, has

degenerated under relaxed selection and can cause sterility in a recombinant hybrid background that lacks the functional copy (Masly et al., 2006). In varieties of *Arabidopsis thaliana*, coevolutionary arms races with pathogens have driven the evolution of Dangerous-Mix1 and Dangerous-Mix2 alleles that induce an autoimmune-like response in hybrid progeny called hybrid necrosis (Bomblies et al., 2007). Although these examples support the DM model, the latter example is most applicable to my research since immunity genes are implicated in hybrid breakdown.

**Speciation microbiotas and the Bateson-Dobzansky-Muller model**

In the decades since Wallin first proposed microbes as a cause of speciation, symbiosis has taken a central position within the biological sciences. In eukaryotes, microbes play important roles in nutrition (Hastings, 1944; Kudo, 2009; Russell et al., 2009; Shigenobu et al., 2000), immunity and defense (Gerardo et al., 2010; Haine, 2008; Lauer et al., 2008), development (Gilbert et al., 2010; McFall-Ngai, 2002), and reproduction (Orzack et al., 1991; Wade, 2001), among other processes. Despite this renaissance in how biologists view symbiosis, microbial symbionts are still considered a distant cousin to nuclear genes as a cause of speciation. I suggest this perspective may be unnecessarily narrow because the nuclear genes that underlie hybrid inviability may evolve as a direct consequence of interacting with a rapidly evolving microbiota.

There is mounting empirical evidence that supports the plausibility of the microbiota as a speciation agent. First, hybridization can result in the breakdown of immunity gene regulation. This is evident in the hybrid necrosis studies that yielded a hypersensitive, autoimmune response (Bomblies and Weigel, 2007). Here, a BDM-

incompatibility between two nuclear alleles leads to an abnormal suppression of the native microbial flora in non-hybrids. Second, hybrid dysfunction can result as a consequence of microbial interactions with the host. Reproductive symbionts such as *Wolbachia* and *Cardinium* can cause cytoplasmic incompatibility between populations of the same or closely related species without any nuclear divergence (Bordenstein et al., 2001; Werren et al., 2008). Similarly, a bacterial pathogen induces severe $F_1$ male sterility in hybrid subspecies of *Drosophila paulistorum* and also in hybrids of *Heliothis virescens* and *H. subflexa*; wherein, the bacterium persists naturally in the guts of parental populations, but over-proliferates in hybrid testes (Miller et al., 1995; Miller and Miller, 1996). Third, immunity genes evolve rapidly compared to the rest of the genome (Moran and Plague, 2004; Staley, 2006). For instance, in *Drosophila* species, immunity genes undergo positive selection at a rate approximately twice that of the genomic average (Obbard et al., 2009; Sackton et al., 2007; Schlenke and Begun, 2003). Similarly, in humans and chimps, the majority of the known functional genes that are under positive selection are immunity or defense-related genes (Nielsen et al., 2005). The rapid evolution of immunity genes potentially increases their likelihood as incipient speciation genes, prior to the evolution of other hybrid dysfunctions. Fourth, the immunity genes are misexpressed in hybrids compared to their parental species, suggesting that the genes subject to high rates of positive selection within species are also the ones most likely to change their expression patterns in hybrids. For example, in hybrids of *D. melanogaster* and *D. simulans*, 93% of the immune genes were misexpressed when compared to parental expression levels, whereas only 57% of the non-immune genes showed changes in expression (Ranz et al., 2004).

Few model systems, however, have been used to explore how microorganisms drive animal speciation, with the exception of *Wolbachia* (Bordenstein, 2003). In comparison to this single reproductive symbiont, the general microbiota may be an unexpected and even more significant cause of reproductive isolation. For instance, a simple extension of the BDM model that includes interactions with microbial symbionts shows that hybrid dysfunctions can evolve more readily than gene-gene interactions based on a network of incompatible gene-gene, gene-microbe, or microbe-microbe interactions, as discussed in detail in Chapter II. Therefore, speciation microbiomes are the "infectious" equivalent to speciation genes, but with more opportunities to enforce postzygotic isolation than gene-gene dysfunctions alone.

Hybrid sterility and inviability have been well documented in animals, though the underlying causes are not well understood. Negative interactions between the nuclear genome, organelles, and/or the endogenous microbiota may disrupt the processes that underlie normal development and lead to reproductive isolation. In insects, hybrid inviability is commonly observed between the embryonic and larval development stages. This early mortality, in theory, could be linked to infections because the innate immunity pathways breakdown in hybrids, resulting in an inability to regulate coadapted microbial complexes and defend against pathogenic challenges.

**The genus *Nasonia*, a model system**

The jewel wasp, *Nasonia*, is a member of the order *Hymenoptera.* The wasp is a parasitoid of several fly species including the carrion associated blowfly *Sarcophaga bullata*. The wasp parasitizes the developing pupae of *S. bullata* by paralyzing the

developing fly and depositing its own eggs into the puparium of its host where they will hatch, feed, and develop upon the host (Figure I.1a). The three *Nasonia* species (*N. vitripennis, N. longicornis,* and *N. giraulti*) used in this work are estimated to have diverged in North America within the last 400,000 to one million years ago (Figure I.1b)(Werren et al., 2010). While *N. longicornis* (found primarily in the North Western part of North America) and *N. giraulti* (found in the North Eastern part of North America) are allopatric, their ranges are sympatrically embedded within the range of the widely distributed *N. vitripennis* species. While these sympatric species may mate in the wild, it is not likely that these species form hybrids due to interspecific cytoplasmic incompatibility induced by the reproductive parasite *Wolbachia*. An obligate, intracellular bacterium, *Wolbachia* has strain specific infections that cause cytoplasmic incompatibilities between *Nasonia* that do not share the same *Wolbachia* infection (Bordenstein and Werren, 2007). To circumvent the effects of cytoplasmic incompatibility between species, we can use strains of the three *Nasonia* species that have been antibiotically cured of *Wolbachia*.

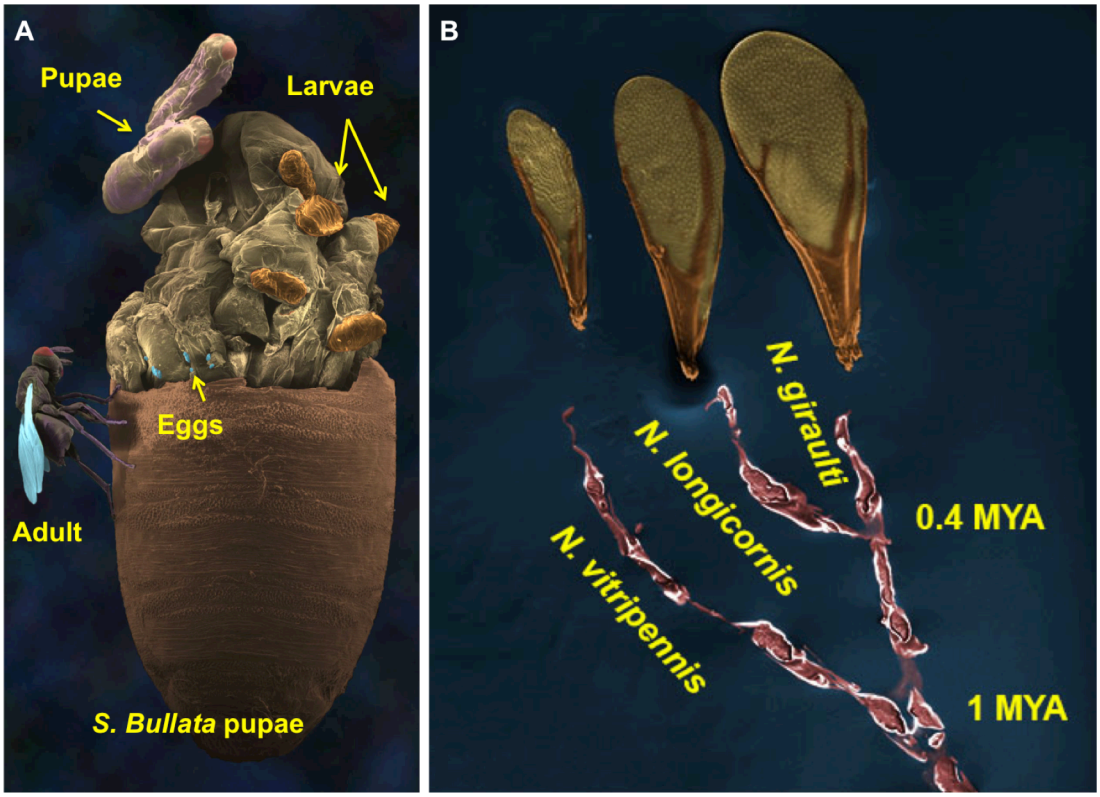


Figure I.1 The genus *Nasonia*. (a) An SEM depicting the lifecycle of the *Nasonia* parasitoid wasp. The adult female ovipositing eggs into the *S. bullata* fly puparium. The upper part of the puparium is removed to see eggs, larvae and pupae. The whole life cycle takes approximately 14 days from egg to reproductive adult. (b) An SEM of the wings from male *Nasonia* with the phylogenetic tree drawn in on the substrate. Each of the three species used in this dissertation are represented. Male and female *Nasonia* are sexually dimorphic and each species is identified by the size and patterning of the male wing.

**Using *Nasonia* as a model system for speciation microbiomes**

Although not historically known as a model organism, *Nasonia* now has full genome sequences (Werren et al., 2010), interfertile species, RNAi, genotype and transcriptome microarrays (Desjardins et al., 2010; Werren et al., 2010), and mapped chromosomal regions of hybrid inviability (Gadau et al., 1999; Niehuis et al., 2011; Niehuis et al., 2008; Werren et al., 2010). The biological advantages of studying interspecific differences in this system and their genetic toolkit make *Nasonia* one of the best insect genetic models apart from *Drosophila* and an ideal model system for this thesis.

There are several unique advantages to studying speciation in the *Nasonia* species complex. The recent period of speciation permits hybridization between the species after elimination of *Wolbachia* (Bordenstein, 2003; Bordenstein et al., 2001; Breeuwer and Werren, 1995; Niehuis et al., 2008). In *Nasonia*, females are diploid and males are haploid, providing an excellent study system for inducing hybrid incompatibilities. Some deleterious alleles may still function normally or at partial capacity within a heterozygous hybrid (females of the F1 generation). However, when these F1 females are hosted as virgins, their F2 haploid male offspring allow for a direct assessment of all recessively interacting loci, including those that cause hybrid mortality and immune dysfunction. Hybrid mortality varies among the species. For example, hybridization between the older species pair, *N. vitripennis* and *N. giraulti*, yields severe $F_2$ mortality, while hybridization between the younger species pair, *N. giraulti* and *N. longicornis*, yields less $F_2$ hybrid

mortality. The relative differences in hybrid mortality between the "older" and "younger" species pairs are reproducible and permit investigations of how variation in interspecific hybrid inviability associates with changes in hybrid microbiotas, immune gene expression, and pathogenesis. F2 hybrid mortality is most severe during the larval-pupal transition (66-77% mortality) (Breeuwer and Werren, 1995), when the microbiota is at its lowest diversity. Finally, an assessment of published genome data specifies that 63% of the currently annotated *Nasonia* immunity genes are located within the four regions of the genome linked to hybrid mortality (Niehuis et al., 2008).

### *Nasonia* hybrid mortality

In recent years, causes for F2 postzygotic hybrid mortality in *Nasonia* have been attributed to host-cytonuclear interactions and host gene-by-environment interactions (Breeuwer and Werren, 1995; Gibson et al., 2013; Koevoets et al., 2012a; Koevoets et al., 2012b; Niehuis et al., 2008). Indeed, mapping hybrid incompatibilities to host chromosomes and organelles is the conventional approach to genetic analyses of speciation loci. For example, in Gibson et al. (2013), ~40% of hybrid mortality was explained by cytonuclear incompatibilities in the OXPHOS pathway, resulting in arrested growth and development. While their findings primarily focused on mortality during late larval to pupal development, this thesis discusses the early larval stages of hybrid mortality and how improper host-microbiota interactions can be a causative factor in larval death. Together, these two mechanisms may explain the majority of hybrid mortality and could very well be linked in their mechanisms of killing.

**Chapter Previews**

In this dissertation, I assess the roles of microbial symbionts in hybrid mortality within the genus *Nasonia*. In Chapter II, I provide the theoretical framework by which the microbiome can establish reproductive barriers, such as hybrid mortality, that ultimately define a species. By exploring evolutionary theory in light of microbial symbiosis, I modernize the early 20$^{th}$ Century viewpoint of Ivan Wallin, who originated the concept of bacterial symbionts as the causative agents of speciation.

In Chapter III, I describe the bacterial variation observed in the three *Nasonia* species: *N. vitripennis, N. longicornis,* and *N. giraulti.* I observed species-specific microbial community complexes in the pupal and adult life stages of development, which also paralleled the evolutionary history of the genus. Larval microbiota for all three species was the least diverse and the least similar to the evolutionary relationships of the host. These results did indicate that species-specific microbiotas are a consequence of the hosts' genetic background and thus reflect the evolutionary changes of its host.

Next, in Chapter IV, I reannotated the *Nasonia* innate immune genes by first establishing a database of other insect immune genes and then searching the *Nasonia* genome for genes homologous to those in the database. The results implicated 489 putative innate immune genes for the *Nasonia* genus, one of the largest repertoires of insect immune genes identified to date. Defining the *Nasonia* immune genes was required in order to examine hybrid gene expression. In *Drosophila*, approximately 57% of all genes in hybrids were under or over expressed relative to parental expression. However, approximately 93% of all the immune genes were misexpressed in hybrids relative to parentals (Ranz et al., 2004). The bias towards missexpression in hybrid immunity genes

indicates larger pathological issues with microbial regulation. Extrapolation to *Nasonia* provided the basis for transcriptional analyses that are discussed in Chapter VI.

In Chapter V, I describe the development of a technical assay for rearing *Nasonia* in a germ-free environment, thereby regulating the microbial community that can potentially establish within *Nasonia.* This tool was an important achievement for the larger success of my thesis. One important issue within the field of microbial ecology is the lack of experimental techniques available to manipulate host-microbe systems. However, with this germ-free assay, I am able to manipulate the *Nasonia* system to my advantage; thus, I am able to ask research questions that can be backed by experimental and functional tests. This outcome is especially important for determining the role that the microbiome plays in *Nasonia* hybrid mortality.

Finally, in Chapter VI, I describe two basic studies that elucidate the role of the microbiota in the development of larval-pupal hybrid mortality. First, I used germ-free *Nasonia* to establish if the removal of the microbiota would ameliorate hybrid mortality rates, thereby implicating a pathogenic microbe and/or beneficial symbionts as a causative factor in hybrid mortality. For the second set of experiments, I used controlled inoculations of native and foreign bacteria into germ-free *Nasonia* hybrids and non-hybrids to test if hybrid mortality in caused by the colonization of germ-free individuals with bacteria. Monitoring changes in gene expression and the microbiota during development and rearing conditions, with and without bacterial challenges, indicated that indeed there is an inability of hybrids to regulate their microbiota due to aberrant immune gene expression.

## CHAPTER

# II. SPECIATION BY SYMBIOSIS*


### Abstract

In the *Origin of Species*, Darwin struggled with how continuous changes within a species lead to the emergence of discrete species. Molecular analyses have since identified nuclear genes and organelles that underpin speciation. In this review we explore the microbiota as a third genetic component that spurs species formation. We first recall Ivan Wallin's original conception from the early 20th century on the role that bacteria play in speciation. We then describe three fundamental observations that justify a prominent role for microbes in eukaryotic speciation, consolidate exemplar studies of microbe-assisted speciation, and incorporate the microbiota into classic models of speciation.


### Speciation by symbiosis

The fields of microbial symbiosis and speciation have achieved astonishing advances during the last two decades. The universality and significance of microbial symbionts in multicellular life is now unmistakable (Douglas, 2010; Moran, 2006). Concurrently, our understanding of the genetic underpinnings of how one species becomes two is maturing in a wide array of eukaryotic species (Johnson, 2010; Presgraves, 2010; Rieseberg and Blackman, 2010). Symbiosis and speciation are not commonly discussed together and can seem to be odd partners in their capacity to operate


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synergistically in nature. Indeed, microbial symbiosis is a process by which two or more distinct organisms interact as one entity, whereas speciation is the diversifying process by which one species splits into two. Yet since the earliest hypotheses of the symbiotic nature of organelles within the eukaryotic cell (Kozo-Polyanksy, 1924; Mereschkowsky, 1905; Wallin, 1927), microbial symbiosis has been put forth as an engine of novelty owing to its capacity to confer new traits (Margulis and Sagan, 2006), and to augment the rate of evolution of genetically-based reproductive barriers between incipient species (Bordenstein, 2003; Werren, 1998). In this review, we synthesize recent studies that suggest microbe-assisted reproductive isolation is widespread, and we propose how symbionts can be more formally considered in theoretical and empirical studies of reproductive speciation.

The idea of a synergistic effect of symbiosis on speciation was first introduced almost a century ago and nearly forgotten. In 1927, microbiologist Ivan E. Wallin hypothesized in his book, *Symbionticism and the Origin of Species*, that the origin of new species primarily occurs through the acquisition of bacterial endosymbionts. The hypothesis was put forth several decades before it's time as the tools to sample bacterial symbiont diversity and host-microbe interactions were not yet developed. Although Wallin's hypothesis elicited appropriate skepticism at the time, modern biology recognizes the universality of symbiosis in shaping eukaryotic life. First, advances in profiling the microbial symbionts of hosts have lowered the technical hurdles from a century ago. Second, a new outlook is underway that places microbial symbiosis as a central discipline within the reticulated set of biological sciences. Third, and most importantly, there are a number of cases in which either microbes are the causal factors in

reproductive isolation (RI) or chromosomal "speciation genes" evolved by interactions with microbes. The latter cases serve as a reminder that while speciation geneticists frequently map RI traits to nuclear genes, it is not an automatic justification to rule out microbial-assisted speciation. If one were to map speciation loci to genes involved in immunity, it would strongly implicate host-microbe interactions in the underlying processes of species formation.

The goal of this review is to complement the 70 years of research that fortified nuclear genes as a critical agent of species formation (Coyne and Orr, 2004) with a comprehensive assessment of microbial symbiosis in eukaryotic speciation. We will demonstrate that if one views the microbiome of any given species as an extension of that host's genome, as described within the hologenome theory (Zilber-Rosenberg and Rosenberg, 2008), then it becomes intuitive that symbionts can be openly incorporated into speciation models such as the evolution of Bateson-Dobzhansky-Muller (BDM) incompatibilities. Given the technical and experimental progress since 1927, Wallin's theory on the symbiotic origin of species is primed for a new assessment.

**Symbionticism and the Origin of Species: A brief history of Ivan Wallin's concepts**

In making his case for the bacterial origin of mitochondria in *Symbionticism and the Origin of Species*, Wallin argued that microbial symbiosis can "*determine morphologic and physiologic changes in organs and cells*," ultimately leading him to no other conclusion than that "*symbionticism is a fundamental causative factor in the origin of species*." To Wallin, microbial symbiosis was the process of evolutionary change by which biologists could explain animal, plant, and fungal diversity. Considering the

universal presence of bacterial-derived mitochondria within the cells of eukaryotes, he was left to derive that bacteria were fundamental in generating new biodiversity. A decade later, the similarly titled *Genetics and the Origin of Species*, was published by geneticist Theodosius Dobzhanksy. These two books epitomized the debates of the early 1900's about what types of heritable elements promote speciation. Over time, Dobzhansky's legacy to the Biological Species Concept and BDM incompatibilities would solidify Mendelian genes into the foundation of speciation studies (Coyne and Orr, 1998; Coyne and Orr, 2004; Noor and Feder, 2006; Presgraves, 2010; Rieseberg and Blackman, 2010).

In retrospect, what happened to Wallin's theory? Why did it not share the same prominence as Dobzhansky's work? There are three likely reasons as to why Wallin was almost lost to obscurity. First and foremost, testing Wallin's concept on the symbiotic origin of species was certainly beyond the capabilities of the biological sciences at the time. Due to the technological limitations of his era, the best a microbiologist could hope for was a culturable microorganism that had easily identifiable morphological features. Even more of a limitation was the impossible task of determining the complexity of symbiotic communities in animals and plants, and how the host's biology was altered by the symbionts. Second, while his views on microbial symbiosis and speciation were received as plausible, they were overshadowed by T. H. Morgan's discovery of chromosomal genes (Morgan et al., 1915; Sapp, 1990) and the subsequent rise of the modern synthesis that married evolution and genetics. Despite Wallin's effort to put symbiosis into the mainstream of evolutionary biology, only Dobzhansky's legacy would have a lasting influence. The third reason that Wallin's theory failed to hold water is that

it has been difficult to convincingly link symbiont-induced changes within species to RI between species. In contrast, a large number of studies map RI to eukaryotic chromosomes (Nosil and Schluter, 2011).

## Three general observations

Before we consider specific cases of symbiont-assisted speciation, it is important to summarize the recent and compelling reasons for why the processes of speciation and symbiosis are intertwined. At least three major observations can be made from the standing experimental evidence. First, microbial symbionts are universal in eukaryotes. Second, hosts typically exhibit strong specificity for microbial symbionts and their functions. Third, host immune genes are rapidly evolving in response to microbial symbionts and represent a gene family frequently involved in hybrid incompatibilities (HI). These unifying principles are discussed below.

### Observation 1 - Microbial symbionts are universal in eukaryotes

Microbial symbionts are universal and comprise the major fraction of the cellular and genetic machineries in eukaryotes. For example, if one summarizes the bacterial endowment of the average human, there are ten bacterial cells for every one human cell and 100 bacterial genes for every one human gene (Hooper and Gordon, 2001; Qin et al., 2010; Whitman et al., 1998). Likewise, insects harbor dense prokaryotic populations in their hindguts (Brucker and Bordenstein, 2012b). Plant roots and leaves are also highly populated with microbes (Hirano and Upper, 2000; Lugtenberg and Kamilova, 2009). Terms such as superorganism (Sleator, 2010; Wilson and Sober, 1989) or hologenome

(Zilber-Rosenberg and Rosenberg, 2008) are used to reflect a composite view of a eukaryotic species as the sum of its genes and cells from the eukaryotic and microbial components.

### Observation 2 - Host Specificity

The majority of microbial entities in eukaryotes are not transient passengers randomly acquired from the environment, but instead function with specific roles in eukaryotic nutrition (McCutcheon et al., 2009), immunity (Chow et al., 2010; Lee and Mazmanian, 2010; Oliver et al., 2009), development (Fraune and Bosch, 2010), and reproduction (Bordenstein, 2003; Perlman et al., 2008; Werren et al., 2008), for instance; many of these functions can only be sustained in the presence of specific host-microbe combinations (Medina and Sachs, 2010). In humans, for example, the existence of three host–microbial enterotypes (characteristic microbiota structure of the gut community (Arumugam et al., 2011)) are strongly host-dependent (Fierer et al., 2010; Turnbaugh et al., 2009) and, once established, are relatively stable over time (Costello et al., 2009). Specificity between host and microbiota could be due to host diet, geography, and/or phylogenetic histories (Hongoh et al., 2005; Ley et al., 2008b; Mouton et al., 2012; Ochman et al., 2010). When diet and environment are strictly controlled for, the microbial community relationships between different species of *Nasonia* parasitoid wasps reflect the host species phylogenetic relationships (Brucker and Bordenstein, 2012b). Therefore, parallel divergence between host eukaryotic genes and members of the general microbiota might be a common phenomenon (Figure II.1).

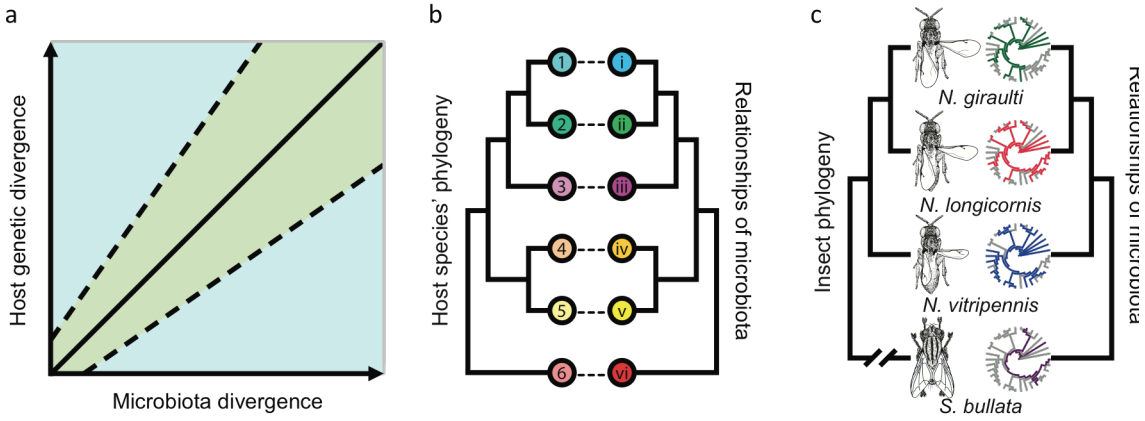


Figure II.1 The predicted relations between divergence in host nuclear genes, divergence in microbiotas and host speciation. (a) The positive relation between nuclear genetic divergence and speciation is a major tenet of genetic studies of speciation. Here, we extend this trend by theorizing that, during a host speciation event, divergence in host genes (y-axis) positively correlates with divergence in microbial communities (x-axis) and the timing of speciation (denoted by the unbroken black line). Environmental variables (the upper and lower bounds in the broken lines) can skew the correlation if they are not controlled for while sampling the host microbiota. Divergence in microbial communities can occur because of changes in both microbial species richness and/or abundance. (b) If the aforementioned relations operate in nature, then they can be tested in practice by comparing whether the phylogeny between host species (denoted by numbers) parallels the dendrogram of community relations between the symbiotic microbiotas (denoted by roman numerals). Note that this conceptual model assumes that, for each generation, the structure of intraspecies microbial communities will be more similar than of interspecies microbial communities and that the levels of genetic divergence between host species will associate with the relative relations of their microbial community structures. It does not presume that microbial communities are stable or even vertically transmitted from generation to generation, as endosymbionts are. (c) A real data example of the parallel relations between a phylogeny of insect species based on mitochondrial genes (the parasitoid species Nasonia and their host fly Sarcophaga bullata) and a dendrogram of the microbial community relations based on a bacterial gene. The schematic trees are not scaled. Circular trees at the tips of the microbial community dendrogram depict the bacterial taxa present (bold) out of the total microbial community.

### Observation 3 - Immune genes are rapidly evolving and underpin changes in the microbiota

Components of host immunity genes are in an arms race with components of the microbiota. This gene family is in a constant struggle between managing beneficial or commensal symbionts while turning on host defenses to prevent pathogenic infections. These dynamics can generate rapid coevolutionary changes between the host genes and microbes, particularly if a change in one causes selective pressure in the other. In *Drosophila*, humans, and chimps, defense and immunity genes evolve more rapidly and are under more positive selection than the rest of the genome (Nielsen et al., 2005; Obbard et al., 2006; Obbard et al., 2009). The conflict and compromise between host genes and microbes can ultimately give rise to reproductive barriers. Hybridization between two host species can lead to immune related incompatibilities that we have categorized as The Large Immune Effect on HI.

## Premating isolation and microbes

By adhering to the widely accepted Biological Species Concept, most biologists equate speciation with the evolution of RI (Dobzhansky, 1937; Mayr, 1963). Under this definition, RI simply refers to those mechanisms that prevent or reduce interbreeding between populations or species. In general, two forms of RI hinder gene flow; processes acting before (premating) or after (postmating) mating. Several key studies on symbiosit-assisted speciation are discussed below as well as in the supplemental material in Brucker and Bordenstein, 2012c.

**Behavioral isolation**

We begin with behavioral isolation because some of the most recent evidence on microbe-assisted RI comes from research on mate preferences. Behavioral isolation is a reproductive barrier that bars mating between species because of differences in courtship behavior or sexual attraction. In a striking example of symbiont-induced behavioral isolation, genetically identical *D. melanogaster* flies that were reared on different diets (i.e., molasses and starch) acquired different microbiotas, which in turn led to strong mate discrimination between them (Sharon et al., 2010). This effect occurred after one generation of maintenance on the two diets and persisted for dozens of generations. The mate discrimination was elegantly 'cured' when the flies were treated with antibiotics. Likewise the trait could be introduced into naïve *Drosophila* populations by inoculating them with the associated bacteria. Ultimately, the microbiota was the genetic component underlying the mating preference. In a similar experiment, mate discrimination evolved in long-term cage populations of *D. melanogaster* flies derived from a single maternal line and was partially cured by antibiotic treatment (Koukou et al., 2006). The putative culprit in this experiment was the widespread invertebrate symbiont, *Wolbachia*, though transfection experiments were not performed to establish a causal role of this bacterium. Recent studies also reveal that *Wolbachia* is associated with enhanced mate discrimination in *D. paulistorum* (Miller et al., 2010) and reduced mate discrimination in *Nasonia* wasps (Chafee et al., 2011).

At least two mechanisms could underlie these symbiont-associated mating preferences. First, the bacteria could infect the host tissues involved in mate discrimination, thereby causing a behavioral pathology that alters mate discrimination.

For instance, infection of the brain or other sensory organs, as in *Nasonia* (Chafee et al., 2011), could have detrimental side effects on how interspecific mate discrimination is regulated at the cellular and signaling levels. Second, symbiotic bacteria could either contribute to or alter the level of sex pheromones produced by the host. The nature by which sex pheromones are altered by microbes could be explained by either bacterial-specific molecules that act as sex attractants or by bacterial-induced effects on nuclear genes that code for sex pheromones (i.e., cuticular hydrocarbons), the latter of which has been recently demonstrated in *D. melanogaster* (Ringo et al., 2011). Further, males of the grass grub beetle *Costelytra zealandica* are attracted to products generated from bacteria located in the colleterial glands, an outpocketing of the vagina of this species (Leal, 1998). Additionally, associations with microorganisms can affect immunocompetence and perhaps mitigate sexual selection based on mating decisions related to immunocompetence. For instance, female mealworm beetles have a preference for males with pheromones that indicate immunocompetence (Rantala et al., 2002). Overall, microbial-induced sex attractants have been generally unexplored, but could prove significant in speciation given the extensive distribution of normal bacterial flora of animals.

An example of a symbiont-derived courtship alteration that leads to RI in animals is observed with parthenogenesis-inducing (PI) bacteria. PI bacteria span three genera, including *Wolbachia* (Bordenstein, 2003), *Rickettsia* (Adachi-Hagimori et al., 2008), and *Cardinium* (Zchori-Fein et al., 2004). They typically manipulate haplodiploid sex determination in virgin mothers, leading to the conversion of haploid eggs into diploid eggs that develop into females. Thus, all-female species can exist without any male input

to reproduction.

What are the consequences of PI bacteria on species formation?

The process by which an asexual population splits from a sexual population is a form of cladogenesis that can be termed asexual speciation. This process falls neatly under the Biological Species Concept because it is concerned with the severing of gene flow between sexual and asexual populations (Bordenstein, 2003; Werren, 1998). Specifically, the onset of microbial-induced parthenogenesis does not immediately prohibit gene flow between the asexual and sexual populations because asexual females still retain the ability to mate with males from a sexual population, as in *Trichogramma* wasps (Stouthamer et al., 1990). However, speciation can be achieved once asexuality is established because asexual females frequently suffer from an accumulation of mutations that degenerate characters involved in sexual reproduction, including mating behaviors, secondary sexual characteristics, and fertilization processes (Gottlieb and Zchori-Fein, 2001). Out of six cases of reproductive degradation associated with bacterial-induced parthenogenesis, five showed female-specific degradation and the sixth exhibited both female and male sexual degradation (Bordenstein, 2003). Thus, female traits degrade more rapidly than male traits, suggesting that mutations in genes encoding female sexual traits might be selected for because they pleiotropically increase the fitness of asexual females. An alternative explanation to this pattern is that there is a substantially, higher fraction of female sexual genes than male ones in the genome, which could also lead to more rapid female degradation by genetic drift. Either way, the enhancement of decay in female sexual traits strengthens the possibility for asexual speciation due to females becoming the majority of individuals in an asexual population; the result is they will

become locked into parthenogenesis. Dozens of cases of bacterial-induced parthenogenesis have been characterized within the Hymenoptera (Luck et al., 1992), suggesting microbial-induced asexual speciation is not uncommon. However, studies on whether bacterial-induced parthenogenesis evolves prior to other isolation barriers is needed as the alternative is that speciation of the asexual lineages occurred before the evolution of bacterial-induced parthenogenesis.

**Ecological isolation**

Many organisms complete their entire life cycle in a single habitat, and adaptation of incipient species to different habitats is an important engine of allopatric and sympatric speciation (Rundle and Nosil, 2005). Given enough time, it is generally accepted that bouts of positive adaptation to new habitats will drive speciation and comparative analyses across taxa support this theory (Funk et al., 2006). Genetic analyses of ecological isolation have focused largely on the nuclear basis of habitat specificity (Hawthorne and Via, 2001; Michel et al., 2010; Naisbit et al., 2003). However, there is extensive evidence that, in addition to nuclear genes, bacterial symbionts play a crucial role in resource exploitation and specificity (Janson et al., 2008; Margulis and Fester, 1991; Zientz et al., 2004). In fact, the use of new niches is one of the very hallmarks that intrigued Paul Buchner, a pioneering authority on bacteriome-associated symbionts of insects (Buchner, 1965). He estimated that approximately 10% of insect species harbor vertically transmitted nutritional mutualists, and along with their hosts' genes, these microbes extend the heritable genetic variation present in one species. Notably, symbiont

genomes encode pathways for amino acid and vitamin synthesis that fit closely with the expected nutritional needs of their hosts (Zientz et al., 2004).

Much of the evolutionary success of arthropods is attributable to the fact that they harbor endosymbionts that permit the use of a wide array of nutrient deficient or imbalanced habitats. For instance, in the pea aphid *Acyrthosiphon pisum,* a symbiont from the gamma-proteobacteria class confers an increase in fitness on white clover plants (*Trifolium repens*) in comparison to aphids that lack this symbiont (Tsuchida et al., 2004). This adaptation theoretically could confer a niche expansion that leads to geographic isolation between an aphid population that makes use of white clover and an allopatric, aphid population that utilize other plants such as vetch. In weevils, the genus *Sitophilus* is ecologically isolated from its closest relatives because its symbiotic bacteria are enclosed in a specialized structure, called a bacteriocyte, that allow it to be the only member of Rhynochophorinae subfamily to feed exclusively on cereal grains as opposed to the roots and stems of monocotyledons (Nardon and Grenier, 1991). The nutritional endosymbiont *Buchnera* is functionally important to more than 4400 aphid species across different plants in sympatry or allopatry (Adams and Douglas, 1997; Guildemond and Mackenzie, 1994). Furthermore, variation in plants that aphids use with the aid of their *Buchnera* symbionts is tightly correlated with instances of aphid speciation (Favret and Voegtlin, 2004; Lozier et al., 2007). Finally within *Acyrthosiphon pisum* aphids, populations exhibit variation in their amino acid requirements (Janson et al., 2008), suggesting coevolution between the amino acid supply of the symbiont-aphid combination and the amino acid deficiency of their plant diet. The aphid fossil record implies an approximate minimal date of 100–200 million years for the original infection (Janson et al., 2008) and

codiversification ever since. Similar results of ancient infection and codiversification have been reported for many other bacteriome-associated symbionts as reviewed in (Moran et al., 2008). Codiversification patterns are not necessarily an indicator of symbiont-induced speciation, as functional work on RI must be coupled with these studies. However, if it were not for the origin of these ancient host-microbe mutualisms, major groups of arthropods would simply not exist. In the aforementioned cases, the symbionts are transmitted maternally and have been closely associated with host cells for long periods of time. Extracellular bacteria that are passed from one generation to the next can also exhibit host-symbiont cospeciation, as observed in the family Plataspidae (Hosokawa et al., 2006).

In most of the examples mentioned above, the microbial symbiont supplements the host genome with functional genes that open up nutritional opportunities that would otherwise not be available to the host. Nutritional symbiont adaptations can confer ecological isolation between host races or species that have the symbiont and those that lack it. Any disruption of these nutritional symbioses through hybridization with other species could lead to a breakdown in genomic complementarity between the animals' genes and symbiont genomes, and ultimately hybrid inferiority.

**Postmating isolation and microbes**

Recent studies identifying genes involved in postmating isolation such as hybrid sterility and inviability indicate that these genes can sometimes spread within populations as a consequence of genetic conflict (Johnson, 2010; Presgraves, 2010); these genes then cause epistatic interactions in hybrids, i.e., BDM incompatibilities (Figure II.2a and b).

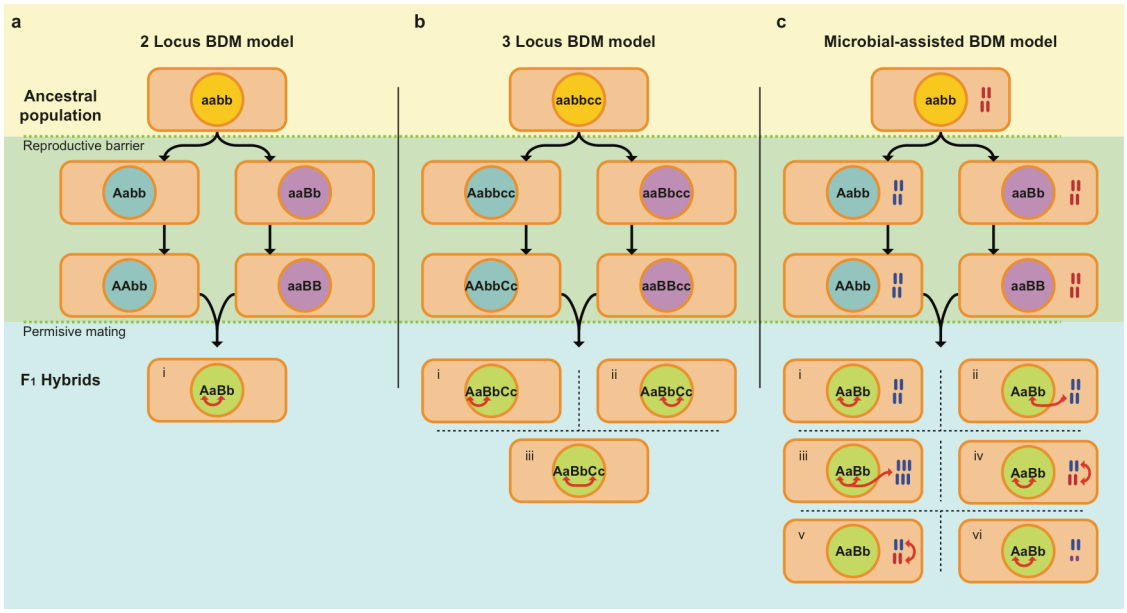


Figure II.2 Bateson–Dobzhansky–Muller (BDM) Models with and without microbes. A classic, two-locus BDM model of speciation (a) arises when substitutions in locus 'a' and locus 'b' negatively interact in hybrids (red arrows indicate potential negative epistasis) to cause one hybrid incompatibility (HI). In a three-locus BDM model of speciation (b), the number of negative epistatic interactions increases from one to three. By extending these models, one can ask, when a bacterial symbiont replaces the third nuclear loci, how many new interactions can evolve? (c) Divergence in the two nuclear loci and a single symbiont produces six possible incompatibilities, twice as many as when there are three nuclear loci. These six HIs include: (i) a typical BDM that does not impact the microbial community; (ii) a single derived allele from one population negatively interacting with the derived bacterial symbiont of the other population; (iii) a three-locus interaction between the two derived mutations and the derived symbiont genotype; (iv) a microbe–microbe interaction between two symbiont genotypes that occurs only when there is also a negative interaction between the two derived nuclear alleles; (v) a microbe–microbe interaction between the two symbiont genotypes that is detrimental to the host because the two bacterial genotypes can be incompatible with each other (i.e. cytoplasmic incompatibility); and (vi) a two-way nuclear interaction that leads to a breakdown in the immune system and the instantaneous acquisition of a new bacterial species (purple) that causes pathology, an outcome of the Hybrid Susceptibility Hypothesis. Alternatively, the two nuclear interactors can abnormally suppress beneficial bacteria and lead to hybrid problems owing to the lack of beneficial bacteria, such as an autoimmune response.

How do microbes fit into a standard model of postmating isolation? The BDM model postulates that when two populations of a species evolve in isolation from each other, at least two genetic changes between the species must occur to cause negative, epistatic interactions and thus incompatibility in hybrids (Coyne and Orr, 2004; Dobzhansky, 1937). These HI genes evolve as a by-product of selection and/or genetic drift (Coyne and Orr, 2004; Orr et al., 2004). Once established between species, HI is almost always irreversible.

Following Orr and Turelli (Orr and Turelli, 2001), population genetic theory shows that when *k* number of nuclear substitutions are fixed between two populations, there are

$$\binom{k}{2} = \frac{k(k-1)}{2}$$

possible HI. We extrapolate this model by showing that when $k = 2$, and thus there are two nuclear loci (a single nuclear HI), but the number of symbionts is allowed to increase, then the number of possible incompatibilities is

$$\binom{S_{k=2}}{2} = \frac{s(s+1)}{2}$$

where *s* is equal to the two nuclear loci plus the number of symbionts being considered. In other words, in the simplest case of when *k* and *s* equal three, and there are three nuclear loci versus two nuclear loci plus a symbiont, respectively, the model with symbionts produces more incompatibilities than the nuclear model (Figure II.2).

A simple, microbial extension of the BDM model is to replace one of the nuclear genes with that of a microorganism (Figure II.2c). In other words, how many more HI can evolve when comparing a model of two nuclear loci and a symbiont (Figure II.2c) versus a model of three nuclear loci (Figure II.2b). Effectively, the number of loci is

equal in both cases, three. However, this schema shows that the number of potential negative epistatic interactions underlying HI is higher in the symbiont model because a HI could now arise from an expanded network of incompatible gene-gene, gene-microbe, or microbe-microbe interactions, whereas the standard nuclear BDM model only generates gene-gene interactions. Within each of the following sections we will describe the various cases of microorganisms that cause postmating isolation.

### Cytoplasmic incompatibility

Cytoplasmic incompatibility (CI) is a postmating incompatibility that typically leads to $F_1$ inviability between infected males and uninfected females, or females harboring a strain of bacteria different than that of the male. When we last reviewed the topic, *Wolbachia*-induced CI was the only phenomenon known to prevent host gene flow by a microbe-microbe interaction (Bordenstein, 2003). Expanding research in other invertebrate-microbe associations over the last decade demonstrates that the unrelated *Cytophaga*—Flavobacterium–Bacteroides *Cardinium* can also cause CI (Gotoh et al., 2007; Perlman et al., 2008). In addition, there have been a number of important experimental and theoretical advancements that continue to support various roles for *Wolbachia*-induced CI in arthropod speciation. In addition to bacterial-induced CI, cytoplasmic organelles of bacterial origin (mitochondria and chloroplasts) are capable of causing hybrid maladies through epistatic interactions with genes of the host nucleus.

Based on the Biological Species Concept, populations with identical genetic backgrounds are considered different species if they are isolated by bidirectional CI (Figure II.3). CI reduces or eliminates the production of $F_1$ hybrids and hinders gene flow

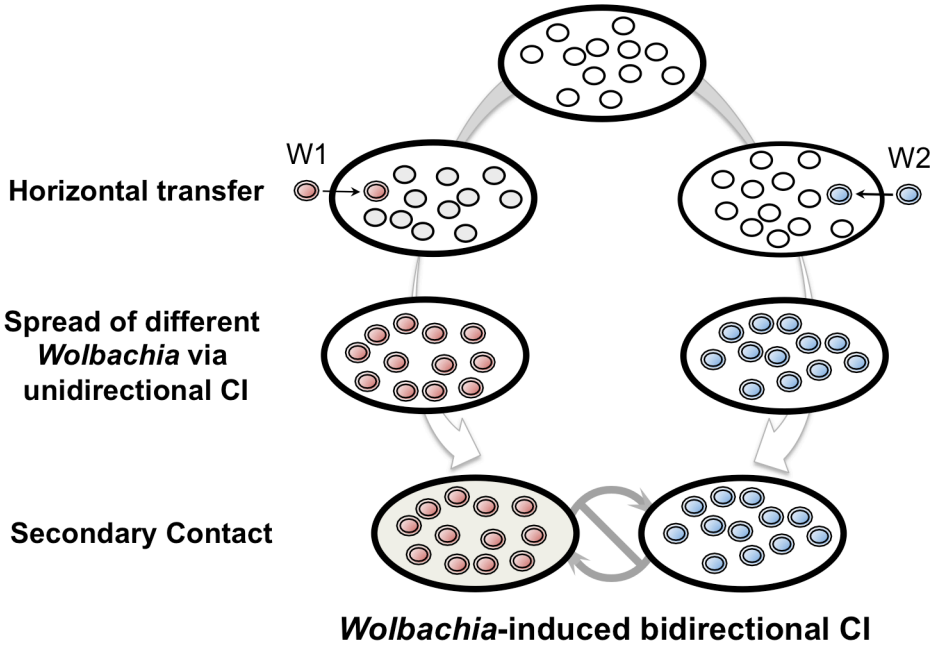


Figure II.3 Schematic of speciation by bacterial-induced cytoplasmic incompatibility. An ancestral population (large circle) of uninfected individuals (small open circles) splits into two populations that subsequently acquire different cytoplasmic, Wolbachia infections (colored circles labeled W1 and W2) by horizontal transfer. These single infections spread to fixation within each population by unidirectional cytoplasmic incompatibility (CI), a phenomenon that imparts a relative fitness advantage to infected females by causing embryonic death in crosses between infected males and uninfected females. Because infected females (the transmitting sex of cytoplasmic bacteria) are compatible with either infected or uninfected males, they do not suffer this fitness reduction; therefore, unidirectional CI can rapidly spread the bacteria through host populations. Upon secondary contact of these populations in sympatry or parapatry, bidirectional CI causes reciprocal incompatibility in both cross directions. Thus, species can arise without morphological or genetic divergence. Furthermore, F1 bidirectional CI can select for the additional evolution of pre-mating isolation by reinforcement.

between hybridizing populations. Thus, infection status can form the basis of a species diagnosis. Empirical evidence on bidirectional CI is taxonomically widespread, and occurs within species in *Culex pipiens* mosquitoes, various species of *D. simulans* flies (reviewed in (Bordenstein, 2003)), and between sympatric species of mites and species of *Nasonia* wasps (Bordenstein and Werren, 2007; Gotoh et al., 2005). Further, theoretical evidence indicates that bidirectional CI can stably persist in populations that undergo high rates of migration (Telschow et al., 2005a; Telschow et al., 2005b).

The expression of CI can select for additional forms of RI through reinforcement - the process by which postmating isolation acts as a direct selective pressure for the evolution of premating isolation in areas of sympatry (Coyne and Orr, 2004; Dobzhansky, 1937; Jaenike et al., 2006). Premating isolation is selected for because postmating isolation is a wasteland for parental gametes: since hybrid offspring are dead or sterile, they cannot pass on genes themselves. *Wolbachia*-induced CI will have a strong effect on reinforcement. Consider a simple model in which hybrid fitness is reduced due to CI in one scenario and a simple two-locus genetic incompatibility in the other. Because CI halts gene flow at the F1 generation, whereas most genes involved in early genetic incompatibilities are recessive and limit gene flow in some F2 genotypes or the heterogametic F1 genotype (in accordance with Haldane's rule), *Wolbachia* has a higher likelihood of driving reinforcement (Bordenstein, 2003). The upshot is twofold. First, the F1 isolation caused by CI reduces more gene flow by eliminating hybrids irrespective of their sex or genotype; second, F1 isolation prevents recombination from slashing the required linkage disequilibria between the incompatibility locus and the mate-discrimination locus (Bordenstein, 2003). Recessive incompatibilities do not share this

luxury because more fit hybrids will be produced and recombination in the previous generations can break down the required linkage disequilibria. Theoretical treatment supports this prediction (Telschow et al., 2005a) and empirical evidence from mushroom-feeding flies strongly supports reinforcement of premating isolation by *Wolbachia*-induced CI (Jaenike et al., 2006).

**Hybrid susceptibility**

Hybridization in plants and animals can result in either (i) genetic novelty by joining new combinations of genes from different species or (ii) breakdown of coadapted gene complexes causing reduced fitness, such as sterility or inviability, as reviewed in (Arnold and Hodges, 1995; Burke and Arnold, 2001). The outcome of hybridization is determined by many factors. As the immune system is subject to frequent bouts of positive selection and rapid evolution to combat a pathogenic microbiota and maintain a beneficial one, hybridization could spur negative epistasis between immunity genes from different species and cause an inferior level of resistance than either parental species. This phenomenon is generally referred to as the hybrid susceptibility hypothesis, in which hybrids are more susceptible to infection by pathogens than non-hybrids.

If pathogens have a detrimental effect on hybrid fitness, then they could be important in reducing hybrid fertility and viability. Three examples of $F_1$ viral activation in plant hybrids have recently been characterized, in which pathogenicity is rare or asymptomatic in parents but pathogenic in hybrids (Lheureux et al., 2003; Lockhart et al., 2000). In addition, a comparative study of 162 plant and animal hybridizations found that even in the absence of controlling for environment or genetic divergence between parents,

hybrid susceptibility was observed in 10–20% of the cases for insect herbivores on hybrid plants, 29% of the cases for fungal parasites on hybrid plants, and 50% of the cases in animal studies (Fritz et al., 1999).

One of the most famous cases of hybrid susceptibility is from Dobzhansky's work on *Drosophila paulistorum* (Dobzhansky and Pavlovsky, 1966). Dobzhansky describes several, morphologically indistinguishable, "*races or incipient species*" that show varying degrees of sexual isolation and $F_1$ hybrid male sterility. He postulated, based on Lee Ehrman's and David Williamson's unpublished work at the time, that the sterility of these incipient species is "*due to its having acquired and become adapted to a new commensal or symbiont*" (Dobzhansky and Pavlovsky, 1966). Recently *Wolbachia* was identified as the resident inhabitant of the testes and ovaries of the fly semi-species (Miller et al., 2010). In hybrid males, however, these infections essentially become severe pathogens and cause $F_1$ male sterility. Further, the original mate discrimination observed between the semi-species can be depleted by antibiotically treating the *Wolbachia*. A similar observation is true for *Heliothis virescens* and *H. subflexa* moths, which harbor a bacterium that persists naturally in the guts of parental populations but over-proliferates in hybrid testes and causes male sterility (Krueger et al., 1993; Miller and Miller, 1996). Taken together, these data specify that selective pressures related to host-pathogen conflict or symbiont maintenance can cause the breakdown of immunocompetence in hybrids and the evolution of gene flow barriers.

**Hybrid autoimmunity**

Negative epistasis between immunity genes in hybrids can also upregulate the

immune system, suppress beneficial bacteria, and cause hybrid maladies. Thus, a hybrid can turn its immune system on itself (i.e., autoimmunity), even in the absence of pathogens. The genes induced during hybrid autoimmunity correspond to the genes induced during the immune response to pathogen infection.

While this subject has recently received attention, it is a very common, incipient incompatibility described in the plant literature (Bomblies et al., 2007; Bomblies and Weigel, 2007; Coyne and Orr, 2004; Ispolatov and Doebeli, 2009; Mizuno et al., 2010). In a genetic dissection, plant defense genes in varieties of *Arabidopsis thaliana* were found to induce an autoimmune response in hybrid progeny, known as hybrid necrosis, as well as an abnormal suppression of inoculated pathogens (Bomblies et al., 2007). These rapidly evolving genes encode nucleotide-binding domain and leucine-rich repeat (NB-LRR)-type immune receptors that function to specifically recognize a wide range of effector proteins from different pathogens and initiate downstream immune responses. Likewise, in a subspecies of *Oryza sativa* rice, the NB-LRR gene family causes an autoimmune like phenotype (Yamamoto et al., 2010). Finally, *Rin4* is an immunity gene underlying HI in hybrids between the lettuce species *Lactuca sativa* and *L. saligna* (Jeuken et al., 2009). The incompatibility gene *Rin4* is a membrane-associated protein that is a negative regulator of basal defense and a target of effectors secreted by *Pseudomonas syringae* (Belkhadir et al., 2004).

In summary, studies of hybrid autoimmunity reinforce the idea that features of the immune system can predispose defense genes to rapid evolution and ultimately the evolution of negative epistasis in hybrids. While more research is needed, a bias of effector-triggered immunity genes in hybrid weakness would be persuasive evidence for

symbiont-assisted speciation. For example, our own assessment of a 4450 gene study by Ranz et al., 2004 (Ranz et al., 2004), demonstrated that approximately 93% of the immune genes in hybrid *Drosophila* species are irregularly expressed in comparison to 59% of the whole genome. HI between immune genes is essentially a speciation footprint of symbiosis.

**Evaluating speciation by microbes**

The challenge ahead for those studying symbiont-assisted speciation is to formulate a coherent theory of speciation that includes both genes and symbionts, with evidence from empirical investigations and theoretical evaluation. We propose three ways in which these advances are likely to be made. First, comparative investigations will assess the relative role of symbionts in speciation using well-studied species pairs that permit a dissection of isolation barriers caused by symbionts and genes. Such studies could address the relative fraction of isolation due to symbionts and whether taxa infected with certain microbial species show higher rates of speciation than uninfected taxa. Second, empirical studies in the laboratory that test the strength of RI in conventionally-reared and germ-free (i.e., antibiotically-cured) hosts could determine the dependence of RI on the microbiota. For instance, an intriguing question would be is hybrid lethality curable? Third, population genetic studies will need to assess if symbionts accelerate the evolution of reproductive barriers and how their contribution compares to other causes of reproductive barriers. Our example in Figure II.2 highlights that symbionts can accelerate the evolution of HI, and this topic could be a fruitful area of research.

## Conclusion

In this review we organized and critically synthesized the literature on the microbiology of speciation to answer the following questions: Why has symbiosis lurked in the background of most speciation research? Does the microbiota of a host directly induce RI and if so, how frequently? Do microbial symbionts shape the evolution of nuclear-based RI, such as HI between immune genes? The data presented in this review equip microbiologists and evolutionary biologists with evidence on where symbiosis fits into the speciation field and vice versa. Arguably, the study of evolution is experiencing a significant fusion with microbial symbiosis. Multicellular organisms cannot exist in nature without their symbionts. The 20th century pioneers of evolutionary biology would have been astonished to see what roles the microbiota play in eukaryotic evolution. We conclude with a suggestion; as biologists weave symbiosis into classical models of speciation, Wallin's 1927 synthesis (Wallin, 1927) should be recognized for its rightful position as the initial and imaginative work on the microbiology of speciation.

## CHAPTER

# III. THE ROLES OF HOST EVOLUTIONARY RELATIONSHIPS (GENUS: *NASONIA*) AND DEVELOPMENT IN STRUCTURING MICROBIAL COMMUNITIES[†]


## Abstract

The comparative structure of bacterial communities among closely related host species remains relatively unexplored. For instance, as speciation events progress from incipient to complete stages, does divergence in the composition of the species' microbial communities parallel the divergence of host nuclear genes? To address this question, we used the recently diverged species of the parasitoid wasp genus *Nasonia* to test whether the evolutionary relationships of their bacterial microbiotas recapitulate the *Nasonia* phylogenetic history. We also assessed microbial diversity in *Nasonia* at different stages of development to determine the role that host age plays in microbiota structure. The results indicate that all three species of *Nasonia* share simple larval microbiotas dominated by the γ-proteobacteria class; however, bacterial species diversity increases as *Nasonia* develop into pupae and adults. Finally, under identical environmental conditions, the relationships of the microbial communities reflect the phylogeny of the *Nasonia* host species at multiple developmental stages, which suggests that the structure of an animal's microbial community is closely allied with divergence of host genes. These findings highlight the importance of host evolutionary relationships on microbiota composition



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and have broad implications for future studies of microbial symbiosis and animal speciation.

## Introduction

It is increasingly apparent that microbial symbionts in eukaryotes are not transient passengers randomly acquired from the environment. Rather, their varied roles in nutrition (McCutcheon et al., 2009), immunity (Lee and Mazmanian, 2010), development (Fraune and Bosch, 2010; McFall-Ngai, 2002), reproduction (Perlman et al., 2008; Werren et al., 2008) and speciation (Bordenstein, 2003) indicate that symbiosis is a major component of eukaryotic fitness and evolution. However, it is less apparent whether the general microbial community, i.e., the richness and abundance of multiple microbial species, diverges in parallel with the host during the formation of new host species. Simply put, can the relationships between microbial communities predict the evolutionary relationships of the host species and vice versa?

A recent study of wild hominids demonstrated that the relatedness of several primate species was correlated to the relationships of the microbial communities that they harbored in fecal samples (Ochman et al., 2010). Likewise, in a study focusing on wild termite populations, the relationships of the microbiota for each termite species recapitulated the phylogenetic relationships of the hosts (Hongoh et al., 2005). The findings suggest that the majority of the gut bacteria are species-specific symbionts that codiverged as a community with their host termite species. Finally, a study comparing the gut microbiota within and between two species of scarab beetle larvae (genus: *Pachnoda*) showed that the variation in microbial community structures within species is reduced

relative to the microbial variation observed between species (Andert et al., 2010). Of these studies, no attempt was made to determine if the observed microbiota variation was due to host genotype or numerous environmental factors (e. g. diet, temperature, humidity). For instance, what proportion of the variation in the microbiota is due to the environment as opposed to intrinsic, genetic factors of the host? Thus, our study aims to explore the microbial communities between closely related species to determine if the host evolutionary history is recapitulated in the structure of their microbial communities when environmental variables do not confound the analysis.

Figure II.1 illustrates the basic concept of how host nuclear genes and microbial communities as a whole may diverge in parallel during the process of speciation. This conceptual model does not presume that microbial communities are stable or vertically transmitted from generation to generation; rather it only assumes that for each generation, host species will accumulate communities of microbes that are more closely related to each other than to their sibling species, and that the levels of genetic divergence between host species will also associate with the amount of divergence between their microbial communities. If true, this model would suggest that the speciation process leads to parallel divergence in both nuclear genes and microbial species composition/abundance.

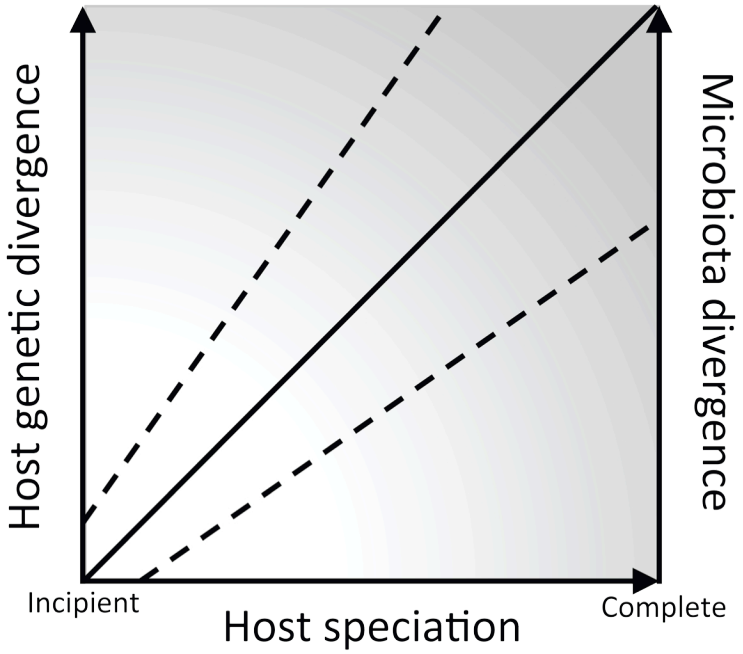


Figure III.1 A hypothetical plot of the relationships between divergence in host nuclear genes, divergence in microbial communities, and host speciation. The relationship between nuclear genetic divergence and speciation is a major tenet of genetic studies of speciation. Here we hypothesize that during a host speciation event, from the incipient to complete stages (x-axis), divergence in in host nuclear genes (y-axis) positive correlates with divergence in microbial communities (z-axis) and the timing of speciation (denoted by the solid black line). Environmental variables can skew the correlation if they are not controlled for while sampling the host microbiota. Thus, a portion of the variation in the microbial community would not correlate with host genetic divergence (denoted by the upper and lower bounds in the dashed lines). The relationship of both microbiota divergence and host speciation is proposed to be linear to host genetic divergence, but may take alternative forms in practice. Microbiota divergence may occur in regards to both microbial species richness and abundance, since hosts may select for different microbial species, as well as different abundances of shared species.

To investigate the evolutionary relationships between closely related species and their bacterial microbiota, and the effects of development on these relationships in a controlled environment, we selected three young species from the *Nasonia* genus of parasitoid wasps: *N. vitripennis*, *N. giraulti*, and *N. longicornis*. *N. vitripennis* diverged approximately one million years ago from the ancestor of *N. giraulti* and *N. longicornis,* which diverged from each other less than 400 thousand years ago (Raychoudhury et al., 2010). *Nasonia* is becoming a powerful model system for the biological sciences owing to the vast number of genomic resources (Werren and Loehlin, 2009b; Werren et al., 2010) and the benefits of haplodiploid sex determination, in which haploid males can be exploited to ubiquitously express recessive genes.

Although decades of research have characterized the role of reproductive endosymbionts in *Nasonia* such as *Wolbachia* (Saul, 1961) and *Arsenophonus* (Werren et al., 1986), little attention has been given to the general bacterial community in *Nasonia.* We report six key findings: (i) The *Nasonia* microbial community diversifies as development proceeds from larva to adult. (ii) Microbial communities differ between host species, despite identical rearing conditions. (iii) The differences in the species' microbiotas mirror the evolutionary relationships of the host species during the pupal and adult developmental stages. (iv) The primary bacterium harbored in the wasps' blow fly host (Diptera: *Sarcophaga bullata*) is prevalent in all three species, especially in larvae, suggesting an initial horizontal transmission of microbiota from fly host to *Nasonia*. (v) The dominant class of bacteria in adults colonizes the hindgut of all three *Nasonia* species. (vi) Finally, of the bacterial taxa discovered in this study, 32% are novel to

sequence databases, indicating *Nasonia* form symbioses with bacterial species that are new to science. Our findings demonstrate that the general microbiota of animal species can diversify in a manner that parallels host phylogenetic relationships. Further, as a consequence of identifying the microbes present in the *Nasonia* genus, this work will allow genetic studies of interspecific differences in an animal model to be combined with microbiological studies of resident bacteria in a controlled laboratory setting.

## Methods

### *Nasonia* strains and collection

Three species of *Nasonia* were used throughout the experiment: *N. vitripennis* strain 13.2 was derived from the inbred strain R511 that was collected in the Rochester, NY area. It underwent prolonged larval diapause for two years at 4°C to cause a spontaneous loss of the *Wolbachia* infection (Perrot-Minnot et al., 1996). *N. giraulti* strain RV2xU was derived from the isofemale inbred strain RV2 collected in the Rochester, NY area. This strain was created from an individual mother to son mating and cured of *Wolbachia* using rifampin for 3 generations in 2005. The RV2xU strain has been in standard laboratory culture in our lab since October 2008 and its original description is in Werren *et al.* (2010). *N. longicornis* strain IV7R3-1b was produced from the standard inbred strain IV7 and cured of *Wolbachia* using rifampin for three generations in 2000. The absence of *Wolbachia* from our experimental strains is important as the endosymbiont occurs in high abundance and would ultimately mask less abundant microbial species during the cloning and sequencing procedure. In addition, another goal

of this investigation was to characterize the microbiota of *Nasonia* strains that are regularly used in the research community. All strains were reared under identical conditions: 25°C, with constant light, on *Sarcophaga bullata* pupae from the same brood. The *S. bullata* were reared on beef liver until their last larval instar (about 4 days old) under 25°C and constant light; then the larvae were transferred to a 30°C incubator under constant dark for two days before their use in experiments.

Virgin *Nasonia* females were collected and serially hosted onto two unparasitized *S. bullata* pupae from the same brood every 48 hours over a period of 14 days. Three life stages from each species were collected in pools of ten males: final instar larvae, yellow/red eye pupae, and newly emerged adults. We used male *Nasonia* for four reasons. First, there are no available techniques that easily determine the sex of the larval stages. Second, female *Nasonia* are diploid while males are haploid; therefore, males would represent all the potential genetic variation that might underlie recessive traits that could contribute to microbial variation between *Nasonia* species. Third, when fertile *Nasonia* mothers oviposit their eggs they do not produce a 1:1 ratio of male to female offspring. Often the number of females produced outweighs the number of males or vice versa. Eliminating this sex ratio variable reduces the likelihood that our variation in our observations is due to factors that are not associated with species' specific variation. Fourth, the mothers of the male *Nasonia* used in this study were collected and isolated as pupae to ensure virginity. This isolation circumvents interactions between *Nasonia* reducing the likely number of unknown variables such as horizontal transfer of microbes. Before DNA extraction, all wasps were serially washed in sterile water (nucleotide-, DNAse-, RNAse-free) and molecular grade 70% ethanol to rinse the body surfaces of

environmental microbial contamination. DNA was extracted from the pooled samples using the Qiagen DNeasy Blood & Tissue extraction kit according to manufacturer's protocol and prepared for PCR amplification of the bacterial 16S rRNA gene. An additional 15 adult male wasps of each species were collected for fluorescent in situ hybridizations described below. All *Nasonia* were flash frozen in liquid nitrogen and stored at -80°C until further processing, except for four adult and four larval *N. vitripennis* that were used in the identification and culturing of bacteria as described below.

**Cloning and sequencing to characterize bacterial communities**

16S rRNA sequences were amplified using the universal primers 5′-AGAGTTTGATCCTGGCTCAG-3′ (27F) and 5′-GGTTACCTTGTTACGACTT-3′ (1492R) (Weisburg et al., 1991) and the following PCR protocol: (1) 94°C for 10 min; (2) 30 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 2 min; (3) 72°C for 5 min. The 16S amplicon was cloned into an *E. coli* vector (pCR®-TOPO4) via the Invitrogen TOPO-TA cloning kit with TOPO10 One Shot ® Chemically Competent cells. Transfomants were plated onto Luria-Bertani plates containing kanamycin monosulfate (Research Products International) and X-Gal (ChromoMax™, Fisher Scientific) at a final concentration of 50 μg/ml. Plasmid-containing bacterial colonies were isolated and sequenced by Genewiz, Inc. (South Plainfield, NJ). Sanger sequencing was conducted on ~90 clones from each sample. A total of 882 clones were sequenced and checked for short, ambiguous, and chimeric sequences using the GreenGenes chimera checker program (DeSantis et al., 2006). The remaining 807 sequences were trimmed to

approximately 550bp in length and aligned to three genomic databases (NCBI, RDPII, and Hugenholtz) using GreenGenes to identify bacterial operational taxonomic units (OTUs; (DeSantis et al., 2006). All OTUs were determined using a 97% pairwise identity cutoff for each 16S rRNA sequence to a known member of the database. Sequences are available at NCBI (http://www.ncbi.nlm.nih.gov/) accession numbers: JN594818 - JN595784

**Statistical analysis of the microbial communities**

To determine if the microbial species richness and abundance of *Nasonia* are significantly different across development or host species, we utilized the UniFrac metric in the program Fast UniFrac (Hamady et al., 2010). The analysis begins by making a maximum likelihood phylogeny of all OTUs. Using a phylogeny based on PhyML, which was supported by an independent RaxML predicted phylogeny, the UniFrac metric and significance is then calculated by measuring the differences between two samples in terms of the tree branch length that is unique to one sample or the other. The metric ranges from 0.0 to 1.0, indicating that every OTU in one sample is represented in the other sample or no OTUs are shared between the two samples, respectively. Because the relative abundance of different kinds of bacteria can be critical for describing community changes, weighted UniFrac weighs the branches based on abundance information during the calculations. Therefore, the analysis detects changes in how many organisms from each lineage are present, as well as detecting changes in which organisms are present. Samples are significantly different if the UniFrac value for the real tree is greater than would be expected if the sequences were randomly distributed between the samples,

using 1000 permutations. The reported p-value is the fraction of permuted trees that have UniFrac values greater or equal to that of the real tree. The UniFrac tree that depicts relationships between microbial communities is generated from a hierarchical clustering analysis on the samples based on the distance matrix between each pair of sequence sets from the maximum likelihood tree. For example, the length of the branch differences on the ML tree between *N. vitripennis* adults and *N. vitripennis* adults is zero, whereas it will be greater than zero for any comparisons that have OTU differences.

**Quantitative PCR (qPCR) confirmation of relative abundance of OTUs**

As an independent means to verify the cloning and sequencing libraries we designed bacterium specific 16S primers for two of the dominant and one rare OTUs of the *Nasonia* microbiota. Specifically, primers were designed to anneal to the 16S rRNA genes of *Providencia*, *Acinetobacter*, and *Brevundimonas*. Two samples consisting of pools of 10 individuals were analyzed for each of the following three sample types: *N. vitripennis* adults, *N. giraulti* adults, and *N. longicornis* pupae. All samples were run in duplicate in a BioRad CFX-96 Real-Time System, using iQ™ SYBR® Green Supermix (BioRad) after the initial template DNA concentrations were normalized between samples. Each experimental plate of samples was run under the same PCR conditions: 30 cycles with an annealing temperature of 55°C for 30s with a melting curve observed at the end of each run. Standards were prepared for each of the three bacterial primer sets consisting of a seven step 1:10 dilution series that was run with each qPCR to establish a standard curve. Cycle threshold (CT) values of each sample were then compared to the

standard curve to approximate the copy numbers (CN) for each bacterium in a given sample.

**Fluorescent in situ hybridization (FISH):**

Following a modified protocol outlined in Hugenholtz (Hugenholtz et al., 2002), whole insects were flash-frozen in liquid nitrogen, mounted for sectioning using tissue freezing medium (Triangle Biomedical Sciences, Inc) and sectioned with a cryostat into 20μm transverse cross sections. Approximately 10 cross sections of the midgut were made on nine individuals per *Nasonia* species, for a total of 27 individuals. Using the established eukaryotic probes for EU338 (Amann et al., 1990), γ-proteobacteria (Manz et al., 1992), and *Acinetobacter* (Wagner et al., 1994), hybridization was conducted in a 46°C humid chamber with a 35% formamide solution. For each hybridization, a control slide without the addition of a probe was made to compare sections for background autofluorescence. All sections were then conditioned with Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc) before imaging and analysis. All sections were imaged and analyzed on the Nikon Eclipse 90i with 20X and 40X objective lenses.

**Evaluation of *Sarcophaga bullata* host microbiota**

Four samples of four to ten unparasitized blow fly hosts were collected at the time the *Nasonia* were hosted on the *S. bullata* pupae. These samples were washed, flash frozen, and extracted in the same conditions as the *Nasonia* samples for cloning and sequencing analysis (see above) or kept unfrozen and used in the identification of culturable bacteria (see below).

### Identification of cultured bacterial species:

Four larvae and four adult *N. vitripennis,* as well as one unparasitized *S. bullata* pupae, were individually rinsed in 0.5 ml of sterile PBS, then submerged in 70% ethanol for 1 min for surface sterilization, rinsed again in sterile PBS and homogenized in 0.5 ml of sterile PBS. The homogenate solutions were subsequently diluted 1:10 in sterile PBS, and 1 ml was plated on Nutrient agar plates (standard media type composed of 0.5% peptone, 0.3% yeast extract, 1.5% agar, and 0.5% NaCl; by volume, at a pH of 6.8) in replicates of three. The plates were incubated at 25°C for 48 hr. Colony counts for each plate were tabulated as colony forming units (CFUs) and five morphologically unique colonies were selected and recultured from each of the sample types onto new Nutrient agar plates for identification via sequencing and gram staining. A single discrete colony for each of the cultures was suspended in 50 µl of sterile PBS; 25 µl were streaked and fixed to sterile glass slides for gram staining and cell morphology analyses, while 1µl was used as PCR template (see above). The resulting 16S rRNA amplicon was sequenced at Genewiz, Inc to assign the OTU. Additional cultures of the external surfaces of the *S. bullata* puparium, pupae, and pupae gut were also made to determine where the developing *Nasonia* acquire any host-derived microbiota.

## Results

### The *Nasonia* microbial community

A total of 882 clones containing 16S rRNA amplicons were sequenced across the larvae, pupae and adults of *N. vitripennis*, *N. giraulti*, and *N. longicornis*, as well as the

unparasitized *Sarcophaga bullata* pupae. The combined clone library had 44 different bacterial OTUs based on a 97% or greater sequence identity cutoff (Figure III.2), of which 14 did not have a significant match at the species level in the Ribosomal Database Project (http://rdp.cme.msu.edu/) or NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) databases. These 14 unknown bacterial species occur at low frequencies within our dataset, yet nine are shared between at least two *Nasonia* species (Figure III.2). We have assigned these unknown bacterial species to a genus or family based on the closest match to the online databases. The bacteria in the datasets are predominantly from the γ-proteobacteria class (Figure III.2). Of the sequences, 67.0% were identified as *Providencia rettgeri*. The second most abundant OTU was that of *Acinetobacter* sp. isolate AU3560, constituting 14.8% of the clones. Rarefaction analyses indicate that the bacterial diversity is simple in the larvae and more complex in the pupal and adult stages (See supplemental material in Brucker and Bordenstein, 2012b). As each *Nasonia* species develops from larvae to adult, the microbial diversity markedly increases.

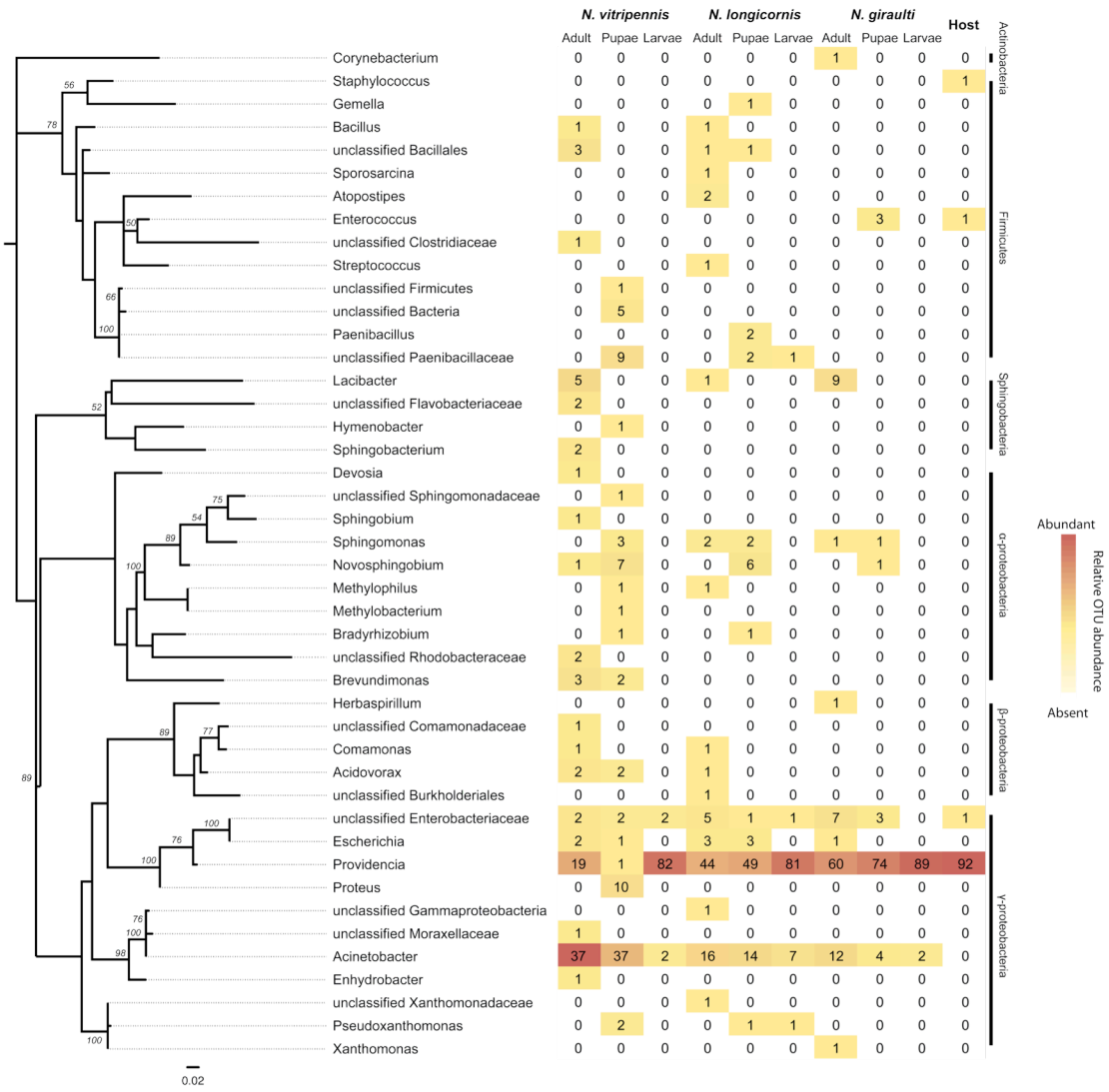


Figure III.2 Bacterial OTUs from the three Nasonia species and the *S. bullata* host. The Best PhyML tree is based on 16S rRNA nucleotide sequences and inferred using the General Time Reversible model. It depicts the microbial diversity across larvae, pupae, and adults of three *Nasonia* species and their pupal host, *S. bullata*. The adjacent matrix indicates the number of incidences that each bacterial OTU occurred within any given sample. The heat map indicates the fraction of each bacterial OTU relative to the total number of OTUs within a sample's microbial community. Branch labels denote bootstrap support and unlabeled branches indicate bootstrap support below 50%.

In fourth instar larvae, the microbial diversity is simple and nearly identical in all three *Nasonia* species. *P. rettgeri* constitutes 95.3% of the sequences in *N. vitripennis*, 97.8% of the sequences in *N. giraulti*, and 89.0% of the sequences in *N. longicornis*. The remaining sequences in both *N. vitripennis* and *N. giraulti* larvae align to *Acinetobacter* sp. AU3560 and an unclassified *Enterococcus*. The *N. longicornis* larvae have the most diversity, comprised of five bacterial OTUs spanning *P. rettgeri*, *Acinetobacter* sp. AU3560, a *Psudoxanthomonas* sp., an unknown *Paenibacillaceae* sp., and an unknown *Enterococcus* sp. Overall, the larval microbial community is very similar to that of the *S. bullata* fly host, in which the dominant species is *P. rettgeri* (96.8% of the clones sequenced), suggesting that the larvae may have acquired the dominant members of their microbial community from their food source.

A microbial succession is evident during wasp development as the microbial communities diversify from larvae to pupae. The pupal OTU diversity is more than twice that of the larval diversity in *N. giraulti* and *N. longicornis* and six times greater in *N. vitripennis* (Table III.1). All of the bacterial OTUs in the larval stages of the three *Nasonia* are observed in the pupal stage. The two dominant OTUs in this life stage are *P. rettgeri* and *Acinetobacter* sp. AU3560. There is, however, a *Nasonia* species-specific difference in the abundance of these two microbes. *P. rettgeri* remains the most dominant taxa in *N. giraulti* and *N. longicornis* (87.9% and 66.2% of clone sequences, respectively), yet is a rare taxa in *N. vitripennis* (1.1% of the clone sequences). *Acinetobacter* sp. AU3560 is the most common OTU in *N. vitripennis* (representing 52.7% of the clones sequenced) followed by a *Proteus* sp. (12.3%) that is closely related to the genus

Table 0.1 Microbial community analysis.

| | | No. of Shared OTUs[a] | | | | | | | | | | No. of bacterial OTUs[b] | Chao1 (no. of OTUs)[c] | Coverage[d] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ***N. vitripennis*** | | | ***N. longicornis*** | | | ***N. girualti*** | | | | | | |
| | Stage | Adult | Pupae | Larvae | Adult | Pupae | Larvae | Adult | Pupae | Larvae | Host | | | |
| ***N. vitripennis*** | Adult | | 7 | 3 | 9 | 6 | 3 | 5 | 3 | 2 | 2 | 21 | 38 | 0.55 |
| | Pupae | | | 2 | 6 | 8 | 5 | 5 | 5 | 2 | 2 | 18 | 25 | 0.72 |
| | Larvae | | | | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | 3 | 1.00 |
| ***N. longicornis*** | Adult | | | | | 4 | 4 | 4 | 3 | 2 | 2 | 17 | 27 | 0.63 |
| | Pupae | | | | | | 5 | 4 | 3 | 2 | 2 | 12 | 14 | 0.86 |
| | Larvae | | | | | | | 3 | 3 | 2 | 2 | 5 | 6 | 0.83 |
| ***N. girualti*** | Adult | | | | | | | | 3 | 2 | 2 | 9 | 14 | 0.64 |
| | Pupae | | | | | | | | | 2 | 3 | 6 | 7 | 0.86 |
| | Larvae | | | | | | | | | | 2 | 3 | 3 | 1.00 |
| | Host | | | | | | | | | | | 4 | 5 | 0.80 |

[a]Number of shared operational taxonomic units (OTUs) for cloning and sequencing results based on the 16S rRNA gene clone library.

[b]Number of OTUs within a given sample, based on 3% sequence divergence.

[c]Chao1, ACE, Shannon index, and Simpson's evenness was calculated for each sample (only Chao1 species richness estimator shown).

[d]The proportion of OTUs observed/estimated in a given sample based on the Chao1 estimate of community richness.

*Providencia*. The bacterial OTUs shared in the larval and pupal stage are also shared in the adult stage with the exception of *N. longicornis*, which only contains 3/5 of the OTUs observed in the earlier life stages. Approximately half of the OTUs are shared from pupae to adult (7/18 for *N. vitripennis*, 6/12 for *N. longicornis*, and 3/6 for *N. giraulti*). In adults, each of the three *Nasonia* species has both unique and shared taxa (Figure III.2) comprised of 21 (*N. vitripennis*), 17 (*N. longicornis*), and 9 (*N. giraulti*) observed OTUs. Species richness estimators indicate that all three *Nasonia* species harbor more bacterial species than sequenced (Table III.1). For example, *N. vitripennis*, *N. longicornis*, and *N. giraulti* are estimated to harbor 38, 27, and 14 OTUs based on the Chao1 species richness estimator (Table III.1).

To determine if the variation in OTU diversity in adults is significantly different between the three species, we compared the bacterial diversity and abundances using the program Fast UniFrac (See supplemental material in Brucker and Bordenstein, 2012b). The sister species *N. longicornis* and *N. giraulti*, which diverged approximately 400,000 years ago (Campbell et al., 1993; Werren and Loehlin, 2009b), have significantly different microbiotas ($p < 0.001$) and are also significantly different from *N. vitripennis* ($p < 0.001$ for *N. longicornis* and $p = 0.024$ for *N. giraulti*).

### Relationships between the microbial communities recapitulate the *Nasonia* phylogeny

To test the basic prediction that when environment is controlled for, the relationships of the host's microbial communities reflect the phylogenetic relationships of the host species, we compared the OTU richness and relative abundance between each of

the *Nasonia* species and displayed the relationships in two separate UniFrac generated distance trees (not weighted and weighted to abundance differences). First, when the data is analyzed with OTU diversity alone, without regard to abundance variation, we observe a notable clustering of the microbial communities at each developmental stage (Figure III.3a). As expected, the larvae and *S. bullata* host bacterial communities cluster together because they have a simple microbiota dominated by *P. rettgeri*. With the increase in microbial diversity in the later stages of development, the pupal and adult microbiotas from each of the three species each exhibit community relationships that recapitulate the evolutionary relationships of the three *Nasonia* species (Figure III.3a). For instance, the adult microbiotas of the sibling species *N. giraulti* and *N. longicornis* are more closely related to each other than either is to the microbiota of *N. vitripennis*; this same pattern holds for pupal microbiotas. The probability of observing this pattern in the distance tree for six taxa (the sum of the three pupal and adult taxa) is 0.009. Additionally, the probability of observing that each developmental stage reflects the *Nasonia* phylogeny compared to any possible or unresolved tree with three taxa is ¼ (pupal microbiota tree) x ¼ (adult microbiota tree), or 0.0625. The microbial community relationships are also evident when we compare the shared bacterial OTUs (Table III.1). For instance, the two sister species *N. giraulti* and *N. longicornis* share 35% of their adult bacterial OTUs, while *N. vitripennis* shares only 24% and 18% with each of them, respectively. Thus, the composition of the bacterial communities in regards to OTU richness is strongly development specific and the relationships directly reflect the host's evolutionary relatedness. Next, when taking into account OTU diversity and abundance differences by weighting the Unifrac analysis to the proportion of each OTU, Figure III.3b continues to

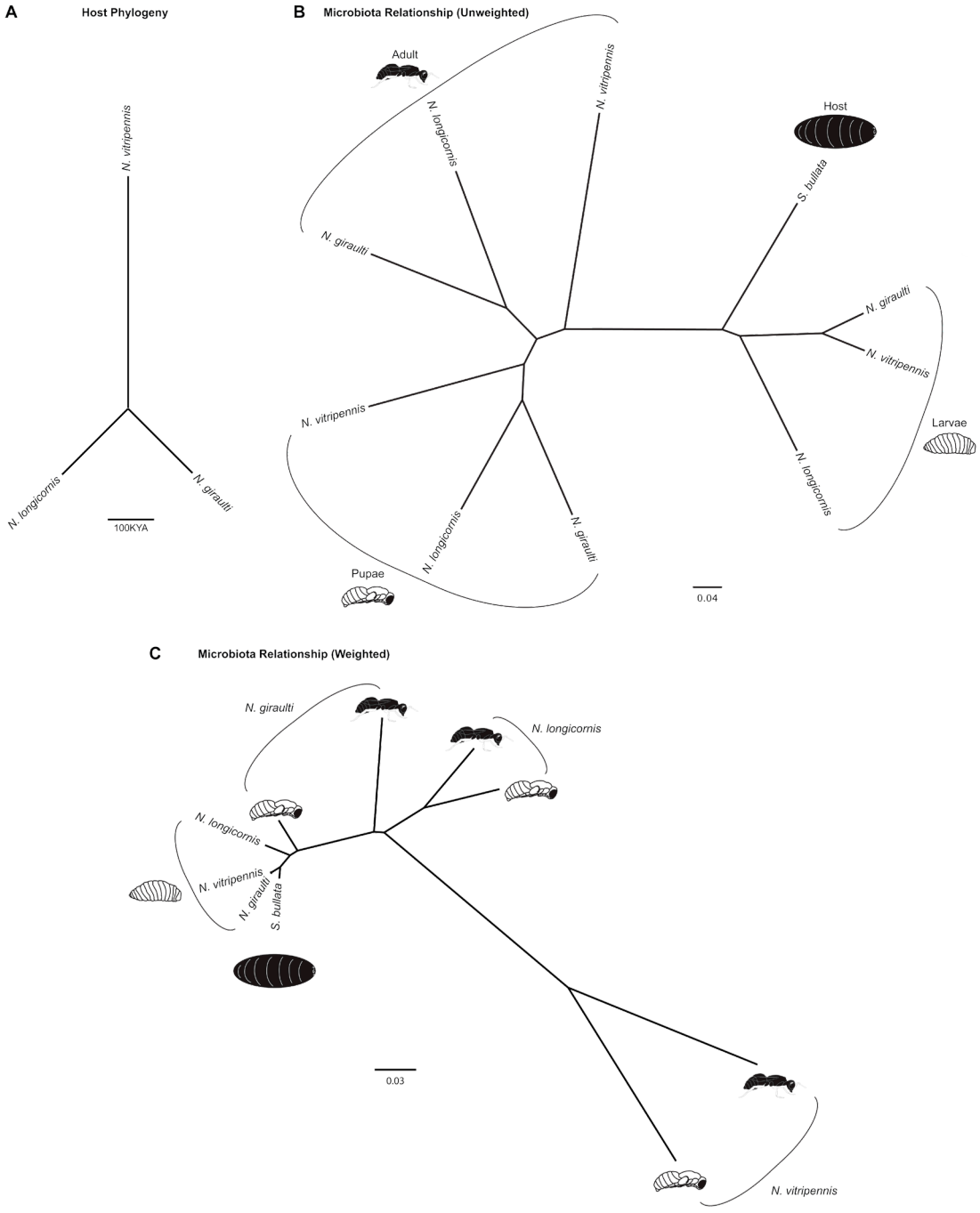


Figure III.3 The phylogenetic relationship of the three *Nasonia* species compared to a cluster analysis of the microbiota across all samples. (A) Schematic of the evolutionary relationships of the three *Nasonia* species based on nuclear divergence (estimated time scale in thousands of years) (Campbell et al., 1993; Werren and Loehlin 2009). (B) Unweighted UniFrac cluster analysis depicting the relationships of the bacterial microbiotas generated from the three *Nasonia* species during larval, pupal, and adult life stages, as well as the unparasitized *Sarcophaga* host. (C) A weighted UniFrac cluster analysis.

show that host species has a significant effect on how much of an OTU is present. In particular, the weighted Unifrac distance tree again clusters the larvae and host microbiota because of their simple community dominated by *P. rettgeri*. However, the *N. vitripennis* adult and pupal microbiotas, dominated by *Acinetobacter* sp. AU3560, cluster independently from the *N. longicornis* adult and pupal microbiotas, as well as the *N. giraulti* adult microbiota. The *N. giraulti* pupal microbiota clusters with the simple larval microbiotas because 87.9% of the sequences in the *N. giraulti* pupae are from *P. rettgeri*. In sum, developmental stage has a profound effect on which microbes colonize and host species background has an effect on how much of each species is present.

**qPCR confirmation of interspecific variation in microbial diversity**

To confirm the major differences in OTU presence and abundance, we used quantitative PCR (qPCR) with species-specific primers to the 16S rRNA gene in independent samples of the three *Nasonia* species. We targeted the two dominant bacterial species in the three *Nasonia* species, *P. rettgeri* and *Acinetobacter* sp. AU3560, and the bacterium *Brevundimonas diminuta* strain zjs01 that was only observed in *N. vitripennis*. Between the two samples of *N. vitripennis*, the copy numbers observed of *P. rettgeri* and *Acinetobacter* sp. AU3560 are 2,249 ± 90 and 6,173 ± 579, with a relative ratio of 1:2.75. This ratio is remarkably similar to the ratio determined from the cloning and sequencing results of 1:2.5. Furthermore, when we compare *P. rettgeri* to *B. diminuta* (7 ± 2 copies) we observe a ratio of 1:0.003 using qPCR, which is similar to the 1:0.03 ratio from the cloning and sequencing results. Finally, the two *N. longicornis* and two *N. giraulti* samples had average proportions of *P. rettgeri* to *Acinetobacter* sp. AU3560 that

were identical to what was observed in cloning and sequencing with relative ratios of 14:1 (1,341 ± 354 : 95 ±15) and 6:1 (2,954 ± 305 : 537 ± 60), respectively. These samples were, as expected, negative for *B. diminuta*. Taken together, the qPCR findings on multiple, independent samples indicate a high degree of repeatability for the relative OTU abundance differences.

**Identification of cultured bacterial species**

Culturing on nutrient agar and 16S rRNA sequencing of a subset of bacterial colonies from three *N. vitripennis* larvae and three adults, as well as three *S. bullata*, also confirm that *Providencia rettgeri* strain YL is common in the dataset (E-value = 0, 99.7% pairwise identity, see supplemental material in Brucker and Bordenstein, 2012b). This species is the same taxon identified in the cloning and sequencing results. From the larvae, a colony also aligned to *Morganella morganii* strain Sam123-6 (E-value = 0, 100% pairwise identity) that was not previously detected in the cloning and sequencing results. The remaining bacterium detected was the gram-positive *Bacillus* sp. (strain 1P01SE) that was cultured from both larvae and adults. This *Bacillus* sp. was previously observed in the cloning and sequencing results of the adult *N. vitripennis* and *N. longicornis* (E value=0, 100% pairwise identity). The bacteria sequenced from the unparasitized *S. bullata* pupae were predominantly *P. rettgeri* and a single colony of the gram-positive *Staphylococcus lentus* strain CICCHLJQ29 (E value = 0, 100% pairwise identity); both were previously observed in the cloning and sequencing results from *S. bullata*. Cultured bacteria from the exterior and interior of the fly puparium, in addition to

the gut of the developing fly pupa, indicated a high abundance of *Providencia* in all areas of the fly host.

Despite the parallels between specific members of the bacterial cloning and culturing, we did not culture the dominant bacterium cloned in the adult *N. vitripennis* (*Acinetobacter* sp. isolate AU3560). This OTU is not known to be cultured with the growth conditions used in this study, but other *Acinetobacter* species have been cultured on clinical blood agar plates (La Scola et al., 2001).

**Localization of bacteria using florescent *in situ* hybridization**

We conducted fluorescent *in situ* hybridizations (FISH) on 20 μm cross sections of the three adult *Nasonia* species with a general probe for γ-proteobacteria and a specific probe for the *Acinetobacter* genus. The first probe targets the dominant class of bacteria that was identified in *Nasonia* and the latter probe targets a specific bacterial genus within this class. Results indicate that the hindgut is the primary organ harboring these bacteria within the mature insect (Figure III.4A). Approximately 10 cross sections of the hindgut were made on nine individuals per *Nasonia* species, for a total of 27 samples. Total area of fluorescence was measured using the Nikon software NIS-Elements Basic Research (Nikon Instruments Inc.) to determine pixel intensity and overlay. A ratio of the average area of the two florescent markers was determined for each species. Compared to the cloning and sequencing results, these hybridizations again confirm that there is a significant difference in the abundance of *Acinetobacter* in the hindgut of *N. vitripennis* compared to *N. longicornis* or *N. giraulti* (Figure III.4C, Mann-Whitney U; $p<0.01$ respectively).

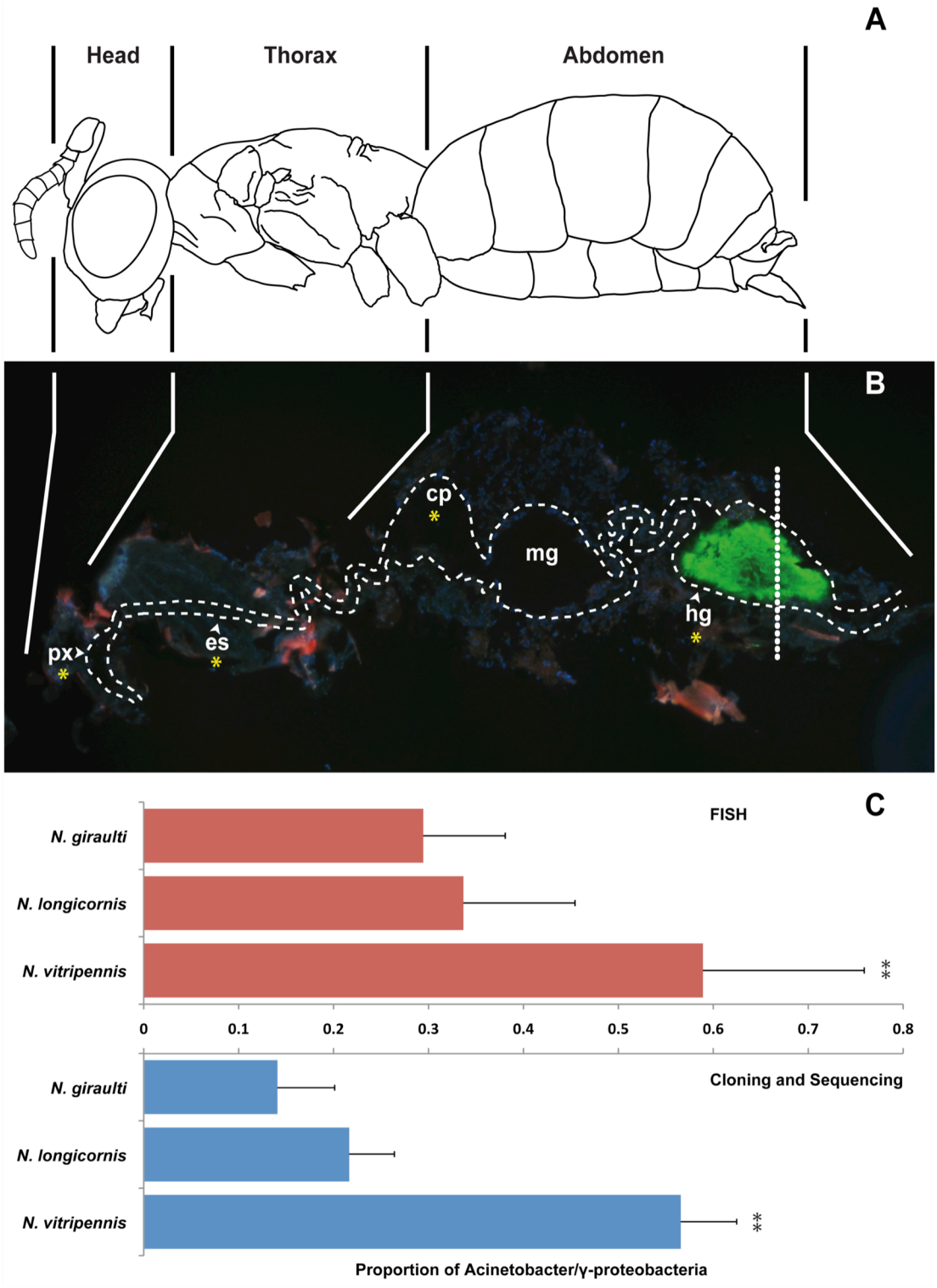


Figure 0.4 Fluorescent *in situ* hybridization (FISH) of γ-proteobacteria and *Acinetobacter* in adult Nasonia. (A) A schematic of the sagittal view of an adult male Nasonia. (B) A sagittal cross section of an adult *N. vitripennis* with the γ-proteobacteria probe (green). The digestive tract is outlined in dashed lines with its main tissues labeled; cr-crop, es-esophagus, hg-hindgut, mg-midgut, and px-pharynx. Tissues containing γ-proteobacteria have been indicated with a yellow asterisk. All sections were co-stained with DAPI (blue), which localizes to the nucleus. To compare colocalization of γ-proteobacteria and *Acinetobacter* probes, transverse cross sections were taken of the abdomen for all three species (approximate location indicated with the dotted line), as this is the region in which the hindgut is located. (C) Red bars indicate the proportion of *Acinetobacter* to γ-proteobacteria ± standard error within the transverse cross sections of the hindgut for each of the three species (n = 9 per species; red bars). ** $p < 0.01$, Mann-Whitney U test. Blue bars indicate the proportion of *Acinetobacter* to all γ-proteobacteria ± standard error that are observed in the cloning and sequencing results.

**Discussion**

*Nasonia* parasitic wasps harbor a diverse community of bacteria that undergo several successions during the insects' development. Our results demonstrate that the three species of *Nasonia* begin their larval life with a similar, simple microbiota. However, as development progresses to adulthood, the observed species richness of the microbiota increases yielding a more complex community of bacteria. The Chao1 richness estimates for the three adult species of *Nasonia* range from 38 to 14 bacterial OTUs and are within the range of species richness in other insects: 49 OTUs in the emerald ash borer *Agrilus planipennis* (Vasanthakumar et al., 2008), 9.5-49 OTUs in the fruit fly *Drosophila melanogaster* (Corby-Harris et al., 2007; Cox and Gilmore, 2007), 45 in the termite *Reticulitermes speratus* (Ohkuma and Kudo, 1996), 10 in the ant lion *Myrmeleon mobilis* (Dunn and Stabb, 2005), 30-46.5 OTUs in the fire ant *Solenopsis invicta* (Lee et al., 2008). The expansion of OTU diversity between the *Nasonia* larval microbiota and the later developmental stages could be due to the final meconium that is released by the larvae before pupation. Though the microbiota of the fecal matter was not explored, such a large expulsion of microbial cells from the gut would likely have a large affect on turn over and succession of the microbial community.

By comparing the microbial communities across the three species of *Nasonia* reared under the same environmental conditions, we were able to test the association of the relationships of microbiota to that of the host genotype. Of the three developmental stages observed, two (pupae and adults) harbored development-specific microbial

communities that each reflect the *Nasonia* species-level divergence (Figure III.3), indicating a relationship between host genotype and the types of bacteria that persist in the latter stages of development. Of the three species, the more divergent *N. vitripennis* harbors the most divergent microbial community compared to the other two sister species, *N. longicornis* and *N. giraulti*. In particular, the dominant bacterium observed in *N. vitripennis* is from the *Acinetobacter* genus (40% and 42% in the pupae and adult, respectively) while the other two species of *Nasonia* were dominated by the *Providencia* genus (53% for both *N. longicornis* developmental stages, and 64% and 86% for *N. giraulti* adults and pupae, respectively).

We speculate that the differences in microbial communities among closely related species are likely due to rapidly-evolving interactions between the host immune system and the microbes that it encounters. Recent studies indicate that immunity genes are subject to higher rates of adaptive evolution compared to the rest of the genome (Obbard et al., 2009; Schlenke and Begun, 2003). It is likely that future systems of animals and plants will show that the divergence of the host species affects the microbial symbiont community structure. This concept supports the same basic observation in a recent analysis of the microbiotas of wild hominids(Ochman et al., 2010), indicating that host phylogeny shapes the gut microbiota.

A core of two γ-proteobacterial species is shared among all the developmental stages: *Providencia rettgeri* and *Acinetobacter* sp. AU3560 (Figure III.2). γ-proteobateria is the dominant class in a number of different insects (Babendreier et al., 2007; Dunn and Stabb, 2005; Lee et al., 2008; Vasanthakumar et al., 2008; Yoshiyama and Kimura, 2009). Interestingly, other insects from the order Hymenoptera, of which *Nasonia* belongs,

exhibit a high prevalence of γ-proteobateria (Lee et al., 2008; Yoshiyama and Kimura, 2009). Much like *Nasonia*, the larvae of the fire ant, *Solenopsis invicta*, have *Providencia* as the dominant bacteria (Lee et al., 2008).

The *Providencia* genus is implicated in many symbiotic roles in different insects such as nutrition (Sasaki-Fukatsu et al., 2006) and pathogenesis (Jackson et al., 1995), but the function of *Providencia* within *Nasonia* is not yet clear. This genus has been isolated from the gut of the honey bee and is implicated as a natural control against the insect pathogen *Paenibacillus larvae* (Yoshiyama and Kimura, 2009). *P. larvae* is a gram-positive spore forming pathogen that is the cause of the honey bee disease, American Foul Brood (Genersch et al., 2006). It is highly contagious and destructive to the honey bee colony as it over-replicates inside the gut of young larvae, killing them before they can pupate. Recent propositions have suggested using endogenous gut bacteria, like *Providencia,* as control agents against American Foul Brood to manage the disease without the use of antibiotics (Evans and Armstrong, 2006; Yoshiyama and Kimura, 2009). It is possible that the bacteria within the gut of *Nasonia* are serving a similar protective function, particularly in the early stages of development. An alternative function of *Providencia* could be that it plays a behavioral or olfactory role in locating fly hosts for *Nasonia* to parasitize. An example of this is the adult screwworm fly *Cochliomyia hominivorax*, which have a rich community of bacteria including several species of *Providencia* (Caballero et al., 1996). These bacteria infect bovine wounds and provide a chemical cue for additional screwworm females to locate the wound and oviposit their eggs (Chaudhury et al., 2010; Chaudhury et al., 2002; Hammack et al., 1987).

Interestingly the other dominant microbe, *Acinetobacter*, has also been widely observed in multiple insect species, including the human body louse (La Scola and Raoult, 2004), Fomicidae species of ants (Lise et al., 2006), and the honey bee (Evans and Armstrong, 2006). This bacterial genus is thought to be a beneficial symbiont associated with the digestive track and nutrient acquisition, though the exact mechanisms by which it benefits its host are unknown.

As indicated by the FISH analysis, a high density of bacteria occurs in the hindgut of all three species of adult *Nasonia*. A less dense microbial community was distributed across the gastrointestinal tract with the exception of the midgut, in which no signal was observed. In other insects, the reduced microbiota in the midgut is attributed to the high levels of enzymatic activity in the organ, which limits the types of microbes that can thrive there (Andert et al., 2010; Terra and Ferreira, 1994). The high density of the microbes within the hindgut, whose main function is to store fecal matter, suggests that the bacteria could be functioning in a number of different manners of which we can only speculate. In the hindgut, bacteria are not competing directly with the host for nutrient acquisition of digested compounds and the host's immune defenses are typically the weakest. The microbiota of the hindgut has been implicated in several functions in insects such as carbohydrate utilization – as seen in the cricket *Acheta domesticus* (Kaufman and Klug, 1991), nitrogen fixation and prevention of pathogens – as seen in the termites *Coptotermes lacteus* (French et al., 1976; Veivers et al., 1982), and pheromone signaling – as seen in the desert locust *Schistocerca gregaria* (Dillon et al., 2000). Exploitation of the hindgut bacterial community has been attempted as a means of vector control in human disease transmitting insects. For example, a transgenic hindgut symbiont in the

reduviid bug, *Rhodnius prolixus*, was generated to produce a pore-forming peptide that kills *Trypanosoma cruzi*, the causative agent of Chagas disease, (Durvasula et al., 1997).

Since the simple microbiota of the *S. bullata* blow fly that *Nasonia* parasitizes is similar to the *Nasonia* larvae, it is uncertain as to how the microbial diversity in *Nasonia* pupae and adult arises. We suggest three scenarios. (1) First, *Nasonia* foundresses could include a seed culture of bacteria by defecation or other means when she oviposits into the *S. bullata* host, thereby inoculating the developing offspring with the bacterial consortia observed in our study. This seed culture could exist at very low titers inside or on the skin of the blow fly, and it would be undetectable with the methods utilized in this study. Maternal deposition of symbionts is seen in other insects such as the human body louse *Pediculus humanus* (Sasaki-Fukatsu et al., 2006) or the European beewolf *Philanthus triangulum* (Kaltenpoth et al., 2005). (2) Second, the bacteria could endogenously exist within the *S. bullata* host at very low titers but were not detected in our study. However, as the *Nasonia* larvae feed on the fly pupae over time, they would be inoculated with an increasing number of bacterial OTUs. (3) Finally, there is a possibility that the bacteria within the developing *Nasonia* are derived from the lab environment. Since the fly pupal casings in which *Nasonia* are developing are not completely isolated from environmental microbes, new bacterial species could be acquired from the air or surfaces within the laboratory. As these possibilities are not mutually exclusive, some or all could contribute to the overall bacterial diversity within the *Nasonia*. One final consideration in the genesis of the microbiota is the fact that the *Nasonia* used in this study lacked a *Wolbachia* infection. The obligate intracellular bacterium naturally infects all three species of *Nasonia.* It has been shown in other arthropods to associate with

colonization resistance against RNA viruses (Teixeira et al. 2008; Hedges et al. 2008) and eukaryotic parasites (Kambris et al. 2009; Moreira et al. 2009; Hughes et al. 2011). *Wolbachia*-free *Nasonia* were purposely used in this study for two reasons. First, the abundance of the endosymbiont would overshadow rare members of the microbial community and make the phylogenetic variation between strains more difficult to detect. Second, the goal of this study was to understanding the microbiotas of *Nasonia* that are widely used in the laboratory community for studies of interspecific differences.

This research is the first investigation of the microbial community within the closely related species of the emerging *Nasonia* model system. The results observed in the four lines of experimentation independently confirm the species-specific microbiotas and their relatedness. Future sampling of additional strains and species will extend and bolster this basic observation. The high proportion of γ-proteobacteria observed across all three species of *Nasonia* during their development is reflective of the microbial communities in other insects. Further, by comparing bacterial communities across species between 400,000 and 1,000,000 years old, we have shown that bacterial microbiotas may essentially serve as phylogenetic markers for host evolution. Since *Nasonia* is a tractable animal system with interfertile species it is an ideal model for testing interconnected questions in development, microbial symbiosis, and speciation.

## CHAPTER

# IV. INSECT INNATE IMMUNITY DATABASE (IIID): AN ANNOTATION TOOL FOR IDENTIFYING IMMUNE GENES IN INSECT GENOMES[‡]


## Abstract

The innate immune system is an ancient component of host defense. Since innate immunity pathways are well conserved throughout many eukaryotes, immune genes in model animals can be used to putatively identify homologous genes in newly sequenced genomes of non-model organisms. With the initiation of the "i5k" project, which aims to sequence 5,000 insect genomes by 2016, many novel insect genomes will soon become publicly available, yet few annotation resources are currently available for insects. Thus, we developed an online tool called the Insect Innate Immunity Database (IIID) to provide an open access resource for insect immunity and comparative biology research (http://www.vanderbilt.edu/IIID). The database provides users with simple exploratory tools to search the immune repertoires of five insect models (including *Nasonia*), spanning three orders, for specific immunity genes or genes within a particular immunity pathway. As a proof of principle, we used an initial database with only four insect models to annotate potential immune genes in the parasitoid wasp genus *Nasonia*. Results specify 306 putative immune genes in the genomes of *N. vitripennis* and its two sister species *N. giraulti* and *N. longicornis*. Of these genes, 146 were not found in previous annotations of *Nasonia* immunity genes. Combining these newly identified immune genes with those in previous annotations, *Nasonia* possess 489 putative immunity genes, the largest


[‡] This chapter is published in *PLOSone*, 7(9):e45125, 2012

immune repertoire found in insects to date. While these computational predictions need to be complemented with functional studies, the IIID database can help initiate and augment annotations of the immune system in the plethora of insect genomes that will soon become available.

## Introduction

The innate immune system evolved early in the evolution of multicellular life, while the adaptive immune system evolved in the ancestor of the vertebrate lineage (Cooper and Alder, 2006). Thus, in insects and other invertebrates, the innate immune system not only combats foreign invaders, but it is also employed in wound healing, stress responses, and the management of microbial symbiont populations (Beckage, 2008). The versatility of the insect innate immune response is in part championed by the ability of insects to colonize diverse ecological niches across the planet while defending against pathogens that inhabit those niches (Loker et al., 2004). Indeed, immunity genes in general evolve at a faster rate than the genome as a whole (Lazzaro and Little, 2009), which is in part explained by the persistent selective pressures posed by a flux of new pathogens.

With the advent and growth of next-generation sequencing technology, rapid genome sequencing of non-model organisms is now feasible. The "i5k" initiative, launched in 2011, aims to sequence 5,000 insect genomes by 2016 (Robinson et al., 2011), generating vast amounts of data for comparative studies among insects. Annotation of immunity genes in these novel insect genomes will not only provide valuable insight into the diverse mechanisms insects employ for defense, but may also

contribute to the development of new insecticides for the control of agricultural pests. To facilitate the annotation of immunity genes in insects, including our own model system of *Nasonia* parasitoid wasps, we have generated an open-access database called the Insect Innate Immunity Database (IIID, http://www.vanderbilt.edu/IIID) to serve as a starting point for researchers interested in using comparative biology to identify potential immune genes in insects. The database contains the immune repertoires of five insect models (including *Nasonia*) that span several orders, and each gene is categorized based on the pathway it participates in and the role it plays in that pathway. The intuitive web interface allows researchers to search for specific immunity genes by name, retrieve all immunity genes in the database for a particular species, pathway or class, and find putative homologs for a gene of interest using an internal BLAST tool.

The jewel wasp *Nasonia* is a genus of haplodiploid, parasitoid wasps composed of four closely related species (Order: Hymenoptera): *N. vitripennis, N. giraulti, N. longicornis*, and *N. oneida*. *Nasonia* is a model system to study the genetics of interspecific differences including host-microbe interactions (Bordenstein and Werren, 2007; Brucker and Bordenstein, 2012b; Chafee et al., 2011), development (Keller et al., 2010; Loehlin and Werren, 2012; Lynch et al., 2012), and behavior (Blaul and Ruther, 2011; Clark et al., 2010; Desjardins et al., 2010; Niehuis et al., 2011). Recently, the genomes of the first three species mentioned above were sequenced (Werren et al., 2010). An initial characterization of immune genes in *N. vitripennis* was conducted as part of the *Nasonia* genome project (Werren et al., 2010) using two sets of Hidden Markov Models (HMMs). The first set of HMMs was generated based on alignments of select immune-related protein families from *Aedes aegypti, Anopheles gambiae* and *Drosophila*

*melanogaster* (Waterhouse et al., 2007), and the second set was compiled using *A. aegypti* immune genes as seeds to find orthologous genes from five vertebrate and five insect species (Werren et al., 2010). Scanning the *N. vitripennis* gene set with these HMMs produced a total of 270 putative immunity genes (http://cegg.unige.ch/Nasonia_genome). This number is likely an underestimate given that not all immune genes from the three Dipteran species above were used to generate the first set of HMMs. The second set of HMMs expanded the number of species incorporated in the models but only for those immune genes present in *A. aegypti*. Furthermore, only the *N. vitripennis* genome was examined; no study has attempted to identify immune genes in the sequenced sister species, *N. giraulti* and *N. longicornis*. Using the genes within the IIID to perform homology searches against the *Nasonia* genomes, we independently describe 306 putative immune genes in each of the *Nasonia* species, of which 146 genes were not found in previous annotations of *N. vitripennis* [16].

## Methods

### Initial construction of the IIID

To facilitate the annotation of innate immunity genes in insects, we initially created an Insect Immunity Database (IIID) composed of the published immune repertoires of four insect models spanning several different orders: *Drosophila melanogaster,* Diptera (Obbard et al., 2009; Pappas, 1980), *Anopheles gambiae*, Diptera (Parmakelis et al., 2010; Werren et al., 2010), *Apis mellifera*, Hymenoptera (Evans et al., 2006; Waterhouse et al., 2007), and *Acrythosiphon pisum*, Hemiptera (Gerardo et al.,

2010). Our criteria for inclusion were that the species have a complete, publicly-available genome sequence, that the innate immune genes have been previously identified in computational or molecular studies, and that each species has an extensive review of its global immune pathways available as a resource. Sequence information was obtained through NCBI for the 105 immunity genes described for *Acrythosiphon pisum* (Gerardo et al., 2010), 317 genes for *Anopheles gambiae* (Christophides et al., 2002; Parmakelis et al., 2010), 379 genes for *Drosophila melanogaster* (Obbard et al., 2009; Pappas, 1980), and 174 genes for *Apis mellifera* (Evans et al., 2006; Waterhouse et al., 2007). In total, 975 genes were included in the dataset used to analyze the *Nasonia* genomes. Each gene was categorized into its primary, secondary and tertiary pathways of putative function (i.e. Toll pathway, IMD pathway, humoral response, JAK/STAT, and cell cycle regulation) and into finite classes of function based upon its putative role in an immune response. Such classes include recognition (identifying potential pathogens and stressors), signaling (communicating between recognition and response), and response (molecules that interact with the pathogen or stressor).

**Comparative analysis of *N. vitripennis* immunity genes**

To validate the utility of this database, we used a sequence similarity BLASTx approach to mine for putative homologs of the 975 protein sequences in the IIID within the *N. vitripennis* transcriptome (OGS v1.2). A total of 18,941 unique transcripts were obtained from NasoniaBase (http://hymenopteragenome.org/Nasonia/). For the BLASTx analyses, we used the BLOSUM62 matrix with a word size of 3 and a gap cost of 11, -1. The results were filtered to only contain hits with an *E*-value < 1e-10, a bit score ≥ 30. A

total of 1206 *N. vitripennis* transcripts were similar to entries in the IIID. To eliminate redundancies in the dataset, a reciprocal BLASTx analysis for each of the 1206 *Nasonia* transcripts was conducted against each of the four insect immunity gene datasets. This analysis resulted in 306 unique immune gene identifiers in *Nasonia vitripennis* (Table S2).

### Analysis of *N. giraulti* and *N. longicornis* immunity genes

Since the immune genes in the sister species *N. giraulti* or *N. longicornis* had not yet been evaluated, we conducted independent BLASTn analyses of the 489 *N. vitripennis* immunity genes (IIID predictions and previously annotated immune genes) against the *N. longicornis* (NCBI assembly name Nlon_1.0) and *N. giraulti* (NCBI assembly name Ngir_1.0) scaffolds (Werren et al., 2010). The parameters for the BLASTn search are as follows: *E*-value < 1e-10, word size 11, low complexity filter, and a gap cost 5, -2. For each species, best hits for the 489 genes were manually assessed as to the *E*-value and bit score, as previously described above, and nucleotide sequences were compiled for each gene in *N. giraulti* and *N. longicornis.*[§]

## Results

The initial IIID was compiled using the immune repertoires of *D. melanogaster*, *A. gambiae*, *A. pisum*, and *A. mellifera* for a combined total of 975 genes. Using this dataset to perform homology searches against the *N. vitripennis* transcriptome, we identified 306 putative immune genes. 138 of these genes were previously reported as immune genes in the *Nasonia* genome (Nvit_1.2) paper, which identified a total of 270 putative immune

[§] Due to the nature of the data, all tables are available, in their entirety, in the original publication: Brucker et al., *PLOSone*, 7(9):e45125, 2012

genes using HMMs for protein domains common in immunity gene families (Werren et al., 2010). We also manually searched the *N. vitripennis* official gene set (v1.2) and the *Nasonia* literature (Tian et al., 2010a; Tian et al., 2010b; Ye et al., 2010) for genes with annotations similar to those of conserved immunity genes in other insect species. In total, we found 66 genes from our manual search that were not reported in Werren *et al.,* (Werren et al., 2010). Importantly, 146 of the 306 genes identified using the IIID were not previously described in any of the *Nasonia* literature. Furthermore, using the IIID, we were able to assign names to 28 genes that were not previously annotated in the *N. vitripennis* gene set (Nvit_1.2). Conversely, a total of 183 immune genes identified previously in the *Nasonia* literature are absent from the IIID analyses of the *N. vitripennis* genome (see discussion).

Combining the immune genes identified using the IIID with the additional genes described in the literature, *N. vitripennis* possesses a total of 489 putative immunity genes. This is the largest predicted immune repertoire found in insects to date. None of the genes found in *N. vitripennis* were missing in either *N. giraulti* or *N. longicornis*.

**Discussion**

Using the IIID, we increased the putative *Nasonia* immune repertoire by 58% in comparison to the number of immune genes originally published in the *Nasonia* genomes (Werren et al., 2010), while only finding 46% of the immune genes originally published. The missing genes are of interest. It is important to note that the *Nasonia* immune gene set in the genome sequence [16] was identified using Hidden Markov Models (HMMs) that search for genes with protein domains common in immunity genes. One problem

with this approach is that all members of a gene family with an immunity-related protein domain may not have a biological role in innate immunity if this domain can also function in other processes. Thus, using only HMMs to find immune genes will increase the likelihood of false positives for any given protein family in which only a subset of its members are involved in immune pathways. For example, sixty-four of the innate immunity genes in the original *Nasonia* genome annotation are not found in our annotation using the IIID; these genes are classified as serine proteases. Several serine proteases play important roles in insect innate immune pathways, specifically the Toll pathway and the prophenoloxidase signaling cascade leading to melanization (Jang et al., 2006; Katsumi et al., 1995; Leclerc et al., 2006; Ligoxygakis et al., 2002; Tang et al., 2006; Waterhouse et al., 2007; Zou et al., 2010). However, the serine protease family is highly diverse, and most of its members function in other aspects of insect physiology (Chasan and Anderson, 1989; Moussian and Roth, 2005; Muhlia-Almazan et al., 2008; Schneider et al., 1994). A HMM that identifies conserved serine protease domains may simply find any serine protease, regardless of its biological function or relevance to insect immunity. Using the IIID for sequence similarity searches partially avoids this source of error because the search is performed using an entire gene, not just a protein domain, which has been identified as part of the innate immune system in another insect species. For example, the IIID predictions identified only 38 serine proteases while the HMMs found 97 serine proteases. Nevertheless, further experimental approaches are needed to determine whether the genes that we have identified actually function in the *Nasonia* immune system.

The other obvious limitation of using a sequence similarity based approach to find immune genes in a specific gene set is that the analysis misses any species-specific genes. For example, thirty-nine genes from our manual search of the literature (that were not detected by the BLASTx analysis) are antimicrobial peptides (AMPs) unique to the *Nasonia* genus, which were predicted computationally based on structural properties common to AMPs (Tian et al., 2010a; Tian et al., 2010b). Sequence similarity searches are also constrained by the reference species used to generate the database. Genes in the *Nasonia* immune repertoire present in an insect species not in the IIID would also be missed, although they are not unique to *Nasonia.*

In total, 489 unique genes have been described as potential immune genes in *N. vitripennis* when all previously published studies (Chasan and Anderson, 1989; Moussian and Roth, 2005; Tian et al., 2010a; Zou et al., 2010), manual annotations, and sequence similarity searches using the IIID are combined. To our knowledge, this list is the most complete set of insect immunity genes currently available and the first to include those from *N. giraulti* and *N. longicornis*. While future studies are needed to confirm the functionality of these genes in the *Nasonia* immune response, the list will provide a stepping-stone for comparative analyses within the *Nasonia* genus and between *Nasonia* and other insect species. More importantly, the IIID will provide one more tool in the efforts to annotate complete immune gene repertoires in other insect genomes. Based on our investigation, we recommend the use of multiple annotation tools that will provide the most comprehensive set of predictions *in silico*, which can then be analyzed for their biological role *in vivo*.

## CHAPTER

# V. *IN VITRO* CULTIVATION OF THE HYMENOPTERA GENETIC MODEL, *NASONIA*[**]


## Abstract

The wasp genus *Nasonia* is a genetic model with unique advantages for the study of interspecific differences, including haplodiploidy and interfertile species. However, as a parasitoid, *Nasonia* is confined within a fly host, thus restricting direct observations and manipulation of development over time. Here, we present the first *in vitro* cultivation method for this system that decouples *Nasonia* from its host, allowing continuous observations from embryo to adulthood. Using transwell plates and a simple *Nasonia* rearing medium, we demonstrate a technique that will significantly expand the utility of the *Nasonia* model.


## Introduction

The genus *Nasonia* is a genetic model for research in evolution, behavior, development, and symbiosis (Muers, 2010). *Nasonia* is a versatile system with four interfertile species (*N. vitripennis*, *N. giraulti*, *N. longicornis*, and *N. oneida*), three completely sequenced genomes (Werren et al., 2010), two-week generation times, and simple rearing methods. The haploid genetics afforded by haplodiploid sex determination - in which diploid females develop from fertilized eggs and haploid males develop from

[**] This chapter is published in *PLOSone*, 7(12):e51269, 2012

unfertilized eggs - is a major advantage for dissecting complex traits, epistasis, and recessive genetic factors (Werren and Loehlin, 2009b).

As parasitic wasps, *Nasonia* require an insect host within the Dipteran family (typically *Sarcophaga bullata* flesh flies in the laboratory (Werren and Loehlin, 2009c)) to complete its lifecycle. Female *Nasonia* sting the pupa of their Dipteran host to paralyze it, and then oviposit eggs through the fly puparium and directly onto the surface of the developing fly pupa. Eggs hatch and the emerging larvae ectoparasitically feed and develop within the host puparium. This intimate association between the wasp and fly host is a challenge for researchers who focus on biological processes throughout *Nasonia* development because there is a hindrance to studying features of the animal's lifecycle that are restricted to within-host development and therefore not amenable to continuous observation. Typically, the total time for *Nasonia* development from egg to adult takes 14–17 d at 25°C. The first 7–10 d are periods of larval feeding and growth. The remaining time is spent as pupae until eclosure as mature adults within the host fly puparium. Opening the fly puparium before the end of larval development leads to desiccation of the *Nasonia* larvae (Lynch and Desplan, 2006; Werren et al., 2009). As such, it is difficult to conduct continuous, developmental studies of the same individual from embryonic development through the larval instar stages.

To tackle the limitation of the wasp's lifestyle and strengthen the utility of *Nasonia* as a biological model, we developed a novel technique for rearing *Nasonia* outside of its fly puparium, as well as a method for *in vitro* culturing the fly without its carrion food source. The *Nasonia* rearing technique requires the simple use of transwell plates (Figure V.1a) and a liquid medium containing processed fly pupae

(hereafter referred to as *Nasonia* rearing medium). Previous investigations have cultivated parasitoids outside their hosts under a variety of nutrient conditions and as liquid, gel, or encapsulated media environments (as reviewed in (Grenier, 2012)). Although there are similar methodologies for rearing parasitoids, with the nutritional composition of the media being the major limiting factor for parasitoid rearing, our rearing method is unique for its use of a transwell rearing chamber, and it is the first *in vitro* cultivation method of the *Nasonia* model.

## Methods

### *Nasonia* Rearing Medium and Setup

*Nasonia* rearing medium was made using approximately 55 g of *S. bullata* pupae (approximately 1.5 cm in length) that were submerged for 5 min in a 10% bleach solution. The pupae were then rinsed with sterile millipore water before incubating in a final volume of 100 ml sterile millipore water at 36°C for 25 min. The pupae were then homogenized and filtered using a 75 µm nylon mesh sterile cell strainer (Fisherbrand, Fisher Scientific) to remove large, unhomogenized particles. The filtrate was then centrifuged for 5 min at 4°C (25,000×G). The centrifuged filtrate separates into three distinct layers, the bottom and top layers consisted of insoluble lipids and exoskeleton debris that were discarded while the middle layer is pipetted into a sterile beaker. Then 50 ml of Schneider's *Drosophila* Medium 1× with L-Glutamine (GIBCO, Invitrogen) and 10 ml of Fetal Bovine Serum (GIBCO, Invitrogen) were combined with the liquid *S. bullata* (from the middle layer). The mixture was then filtered using a 0.44 µm pore,

sterile filter. The flow-through was filtered again using a sterile 0.22 μm pore filter. Finally, 2 ml each of carbenicillin and penicillin/streptamycin (Research Products International Corporation, Mt. Prospects, IL) was added at 100 μg/ml. The completed medium was stored at 4°C for up to 2 weeks or frozen at −20°C for up to 2 months. Upon thawing, the medium needs to be warmed to 34°C and filtered again as particulates will form.

### *Nasonia* Rearing Medium Growth Assays

Each virgin *Nasonia* female was hosted on 2–3 pupae of the fly *S. bullata* for 8 hr. Eggs were then collected using a blunt probe, placed on a Transwell Permeable Support 6.5 mm polyester membrane with a 3.0 μm pore in 24 well plates (Costar, Corning Incorporated, Corning, NY), rinsed with a 10% bleach solution for 1 min, and then rinsed with sterile millipore water. Collecting *Nasonia* eggs for the rearing medium is the most challenging aspect of this assay. Finally, 250 μl of *Nasonia* rearing medium was aliquoted into the well so that the media touches the bottom of each membrane and forms a meniscus around the eggs without completely submerging them. Each well was photographed and the final numbers of eggs were tallied (an average of 18 eggs/well). All transwell plates were stored in a sterile chamber kept at 25°C and around 40% humidity. After 10–12 d, the fourth instar larvae on the transwell basket membrane were removed from the media and placed in a clean sterile well, with sterile water in empty wells to maintain high humidity. See references for descriptions on maintenance, virgin collection and egg collection of *Nasonia* (Werren and Loehlin, 2009a, d, e).

Each well was tallied and/or photographed every other day for 14–22 d throughout the experiments. Anterior-posterior lengths were calculated using Adobe Photoshop CS5.1 analytic tools. Confirmation of living individuals was done visually by confirming either peristaltic contraction of the visible, grey gut or through observation of body movement.

### Conventional *Nasonia* Rearing

Concurrent to the *Nasonia* rearing medium growth assays, sister virgin female *Nasonia* were likewise hosted on fly pupae to observe growth and development compared to their media-reared cousins. For each observation of growth and/or development, a total of 10–15 hosts were opened and individual *Nasonia* were counted (approximately 400 individuals per stage). To estimate the average proportion of conventionally-reared *Nasonia* that develop to a specific developmental stage, a cohort of fly pupae were parasitized on the same day and a subset were selected at random at 0 d (n = 12 hosts) to determine the average number of eggs laid. Different random subsets of hosts were used to calculate the average number of first instar larvae (d 2, 12 hosts), fourth instar larvae (d 8, 10 hosts), yellow pupae (d 12, 10 hosts) and adults (d 18, 10 hosts) that developed. The average proportion of *Nasonia* that survived to each stage was calculated by dividing the average number of *Nasonia* per host at a particular stage by the average number of eggs per host.

### Maternal Venom Experiment

Two containers of fly pupae were weighed out at 60 g each. Virgin *N. vitripennis* females were distributed among one container (2–3 hosts per female) for 8 h at 25°C before being removed. The second container was maintained in the same conditions but without being exposed to*Nasonia*. To confirm that the batch exposed to *Nasonia* was parasitized, a random subset of fly pupa was visually inspected for envenomation (black mark on the fly pupal surface). Each batch of hosts was processed into the *Nasonia* rearing medium as described above.

Concurrently, virgin *N. vitripennis* females were hosted on fly pupa for egg collection and set up for growth assays as previously described. Approximately 15 eggs were placed in per transwell across 12 wells containing one of the two media. Each media type (*Nasonia* rearing medium and *Nasonia* rearing medium with venom) was replicated six times.

Rate of the gut peristaltic movement was determined by counting the number of contractions in 30 s. Individuals were chosen at random (four/well) across the six replicates for each experimental treatment. Any dead larvae were excluded from the peristaltic assessment.

### *Nasonia* Strains

In developing the *Nasonia* rearing medium, *N. vitripennis* (strain 13.2), *N. giraulti* (strain RV2x(u)) and *N. longicornis* (strain IV7R3-1b) were used. Growth assays for *N. giraulti* were replicated for a total of 11 times with 6–12 transwells per

replication; *N. vitripennis* was replicated eight times with 6–12 transwells per replication; and *N. longicornis* was replicated six times with 6–12 transwells per replication. A control transwell of phosphate saline buffer (pH 7.4) was maintained in each replicated experiment, though the first instar larvae from eggs placed in these wells die within 24–48 hours of eclosing. All strains have been maintained in our laboratory at 25°C in constant light.

***In vitro* cultivation of *Sarcophaga bullata* flesh flies.**

All laboratory-reared *Nasonia* strains are traditionally reared on the pupae of *S. bullata*, a fly species that lays its eggs in rotting meat for its larvae to feed upon. These flesh flies are reared in the laboratory using beef liver as an ovipositioning platform and larval food. It takes approximately seven days of larval feeding on the liver before they are mature enough to pupate. The pupae are aged for two to three additional days before being harvested for use in *Nasonia* rearing (see Werren and Loelin 2009, *Cold Spring Harbor Protocols* for additional details). While effective and regularly used by *Nasonia* labs, this method of rearing *S. bullata*does not afford a controlled condition for studying *Nasonia* immunity, development, or microbial symbiosis. The basic reason is that being a carrion feeder, the fly acquires microorganisms from its environment that the *Nasonia* acquire when they feed on the fly (see Brucker and Bordenstein 2011). Here, we report a technique for *in vitro* rearing of *S. bullata* to pupation on a sterile diet.

The *S. bullata* rearing medium is agar based and can be used to successfully induce ovipositioning of adult female flies. Fresh medium was made by adding 18.5 g of BBL™ Brian Heart Infusion (Becton, Dickinson and Company, Spark, MD, USA) and

3.75 g of Agar (Fisher Scientific) to 450 ml of sterile millipore water. After mixture, the solution is autoclaved at 121°C for 15 min. After cooling to 50°C, 50 ml of room temperature defibrinated sheep's blood (HemoStat Laboratories) was gently mixed into the suspension. 2 ml each of carbenicillin and penicillin/streptamycin (Research Products International Corporation, Mt. Prospects, IL) was then added at 100 μg/ml; 10 ml of the antifungal Methyl p-Hydroxybenzoate (Fisher Scientific) was also added at 20% concentration by volume in 200 proof ethanol. 12 ml aliquots of the mixture were poured into 90 mm sterile petri dishes (Fisher Scientific) and allowed to cool overnight. Plates can be stored, covered in a plastic bag in the dark at 4°C for up to one month.

Before use, the *S. bullata* rearing medium plates are warmed to room temp. Fly eggs and newly eclosed larvae are collected from the liver used in culturing the insect. Additionally, when *in vitro* cultivated *S. bullata* adults were provided without any liver, they willingly oviposited onto the rearing medium plate over an 8 hr period, though qualitative observations indicated fewer overall egg deposits compared to liver. The eggs and larvae are then submerged in a 9:1 water to household bleach solution for 4 min to remove any transient bacteria or fungi on their surface before transferring onto the medium plate. Approximately 15–30 eggs or larvae are maintained on a single plate. The egg or larval plates are then stored in a sterile lidded container and incubated at 30°C. Checked daily, the larvae grew and tended to burrow into the medium. Venting the chamber by regularly opening the container in a UV sterilized hood was important as we noted that ammonia buildup adversely affected the developing larvae. Larvae were transferred to sterile containers with sterile paper towel at the bottom on the eighth day while continuously incubated. Pupation occurred within one to three days and were

viable for *Nasonia* rearing. When *N. vitripennis* females were given access to a cohort of 24 *S. bullata* that were reared conventionally, an average of 33±13 viable adults per host emerged. Likewise, when the flies were reared on *S. bullata* rearing medium, a cohort of 24 parasitized fly pupae had an average of 36±15 adult *Nasonia* emerge ($p = 0.453$, t-test).

Interestingly, when the *S. bullata* were reared on medium with antibiotics, PCR and culturing methods of detecting bacteria (using 16S rDNA primers and culture techniques described in Brucker and Bordenstein. 2011) indicated no detectable levels of bacteria present in the system. However, when medium was tested without the use of antibiotics, the eggs and larvae still contain bacteria and fungus which quickly overgrew the dish, killing the developing larvae. The microbial contamination was mitigated, however, by regularly transferring the developing larvae onto new medium every day, with subsequent rinses in 10% bleach solution between transfers.

**Protocol**

**Preparing Nasonia Rearing Medium**

1. Weigh out 55 g of *S. bullata* hosts into an autoclaved beaker. (Note: Discard any hosts or dead larvae that are not good quality before weighing.)
2. Fill the beaker with household bleach diluted to 10%, by volume, in water, to cover the hosts. Gently agitate the hosts in the solution for 5 min.
3. Pour off the bleach solution and rinse hosts with sterile water to remove the remaining bleach.

4. Add sterile water to the hosts in the beaker to approximately 2/3 the volume of hosts.

5. Cover the beaker and place in a 36°C water bath for 25 min.

6. Homogenize the host pupae. (Note: A sterilized and warmed kitchen blender is an efficient means of evenly and rapidly homogenizing the hosts.)

7. Quickly, pass the homogenate through a 75 μm nylon mesh sterile cell strainer into sterile 50 ml conical tubes to remove large particulates.

8. Centrifuge the filtered homogenate for 5 min at 4°C (25,000×g).

9. The homogenate will separate into three distinct layers; pipette the middle layer into a sterile beaker and discard the top and bottom layer.

10. Combine the homogenate with 50 ml of Schneider's *Drosophila* Medium 1× and 20% Fetal Bovine Serum.

11. Filter the mixture with a 0.44 μm pore, sterile filter. (Note: Larger pore sizes can be used to prefilter the mixture, passaging it through smaller pore sizes each time; prefiltering can decrease the total handling time of the mixture.)

12. Filter the flow-through using a sterile 0.22 μm pore filter. The final filtrate should be a translucent honey color. (Note: the *Nasonia* rearing medium is ready for use, however we recommend adding antibiotics to the medium as bacteria can rapidly overgrow and kill *Nasonia* in the assay.)

13. Add 200 µg each of carbenicillin and penicillin/streptamycin. (Note: Alternative antibiotics, chemicals, and concentrations can be supplemented in this step.)

14. Store at 4°C for up to 2 weeks or frozen at −20°C for up to 2 months. (Note: Upon thawing, the medium needs to be warmed to 34°C and filtered again as particulates will form.)

**Setting up *Nasonia* Rearing Medium Growth Assays**

1. Working in a sterile laminar flow hood, allow *Nasonia* rearing medium to warm to room temperature.

2. Collect *Nasonia* embryos or larvae to be used in the assay.

3. Place *Nasonia* into the transwell basket(s) and rinse with 10% bleach solution to sterilize their surface for 45 s.

4. Remove bleach solution and rinse *Nasonia* with sterile water, removing excess water when *Nasonia* are fully rinsed. (Note: Prolonged exposure to the bleach solution is harmful to the *Nasonia*, thus it is important to work quickly to rinse away any remaining bleach.)

5. Pipette 250 µl of *Nasonia* rearing medium into the well. The media should contact the bottom of the transwell basket containing the *Nasonia*. (Note: The volume of media needed will vary depending on the size of the well; in this study we used 24 well plates.)

6. Using sterile water, fill space around the wells or in empty neighboring wells to increase humidity.

7. The assay is now ready. Transfer the transwell baskets to new, sterile, wells containing fresh medium every 24–48 hrs based on bacterial contamination or evaporation.

**Experiments**

We demonstrate that *in vitro* cultivation of *N. vitripennis* on the rearing medium yields larval sizes comparable to that of larvae conventionally reared on the fly pupae. Measurements of anterior-posterior length over time indicate that growth rate of the larvae on the *Nasonia* rearing medium is similar to that of larvae from a fly host at the same time point (Figure V.1b-d; t-test for days 0–14, $p \geq 0.158$). However, development differs slightly. Specifically, there is a lengthening of the developmental time to reach pupation and adulthood on *Nasonia* rearing medium (Figure V.1e). The most notable difference occurs in the larval stages in which *in vitro* cultivated *Nasonia* experience an extended larval stage 4 instar ($p<0.001$, t-test). There is also a reduction in number of adults that eclose *in vitro*, from 70.3% emerging in conventional rearing compared to 53.5% in *Nasonia* rearing media (Figure V.1e, $p<0.001$, t-test). The other two species, *N. giraulti* and *N. longicornis,* were reared successfully on the *Nasonia* rearing medium as well. 69.7% of *N. giraulti* eclose as adults on the rearing medium compared to 74.6% on conventional host rearing ($p = 0.957$, t-test). Similarly, 61.6% of *N. longicornis* eclose on the rearing medium compared to 74.8% on conventional hosts ($p = 0.908$, t-test).

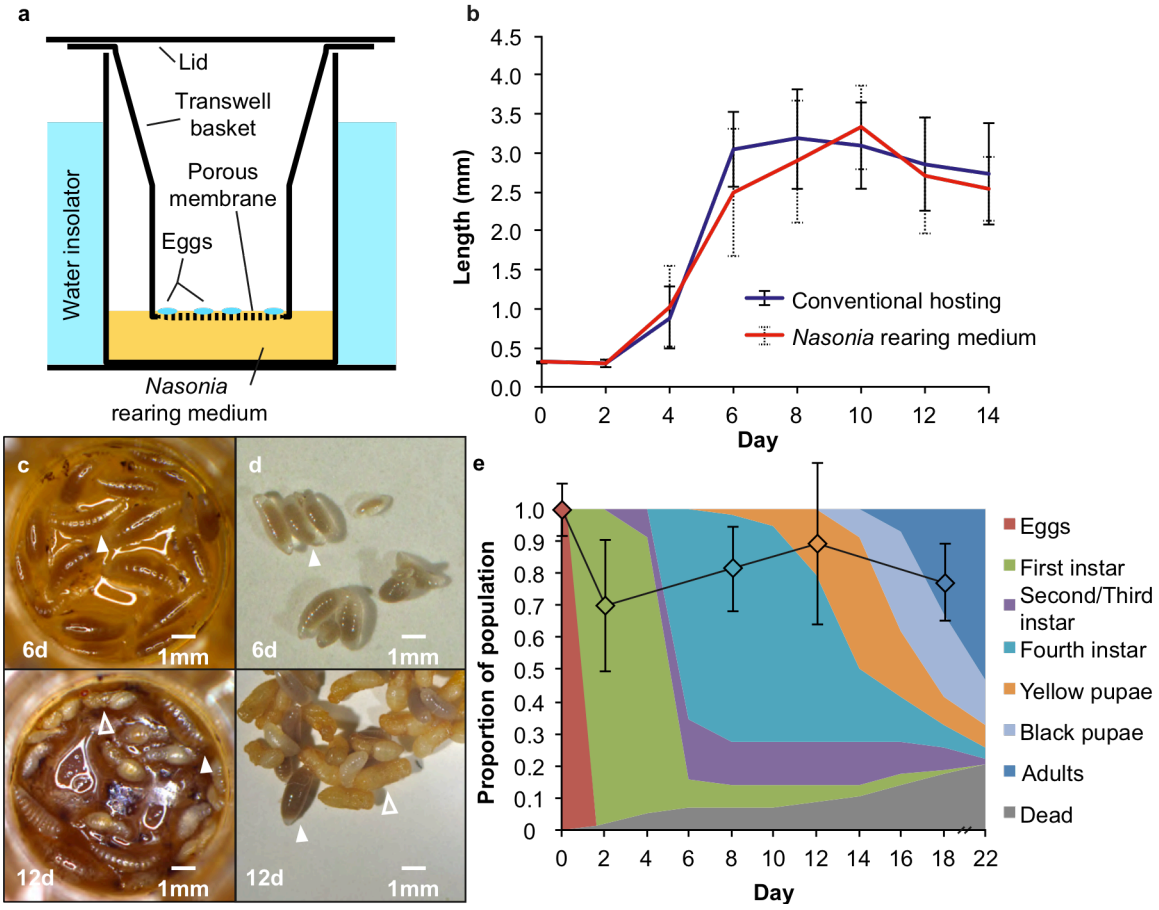


Figure V.1 Comparative analysis of conventionally reared *Nasonia* and the artificial rearing assay. a) Schematic of *Nasonia* rearing chamber. Eggs are deposited onto the three micron porous membrane of a transwell basket; the basket is then placed within the well containing 25µl of *Nasonia* rearing medium so that the two surfaces come in contact with each other, bathing the eggs in nutrients. b) A plot of anterior-posterior measurements during *N. vitripennis* development in conventional rearing conditions (blue line) and in the *Nasonia* rearing medium (red line). Each data point is an average of 150 individuals with standard deviations. c) Photos of *N. vitripennis* larvae in the rearing chamber with *Nasonia* rearing medium 6 d and 12 d post hatching, fourth instar larvae at solid arrow and pupae at outlined arrow. d) Photos of conventionally reared *Nasonia* 6 d and 12 d post hatching. e) Proportional distribution of developmental stages in *N. vitripennis* reared conventionally and on the liquid medium. The *Nasonia* rearing medium allows for direct observation of individuals over time; thus, actual proportions of the population in any given developmental stage are displayed in the stacked area graph, n=58 individuals. The average number at each stage as a proportion of the average number of eggs laid is depicted by the line graph with standard deviations. The color inside the diamond indicates which developmental stage was counted on a particular day.

The delay in larval development on *Nasonia* rearing medium is due to at least two variables: availability of nutrients early in development and level of humidity within the chamber. The difference in developmental time observed in other *in vitro* cultivated parasitoid species has been associated with restricted nutritional components of the artificial diet (Grenier, 2012). When developing this protocol, we first provided fresh media every third day. Under these conditions, we observed fratricide by *Nasonia* larvae eating their well-mates, and those larvae that did survive never developed past their fourth instar. The fratricidal cannibalism has never previously been observed in *Nasonia* and could be a strategy for the parasitoid in periods of limited resources. Increasing the frequency at which media was replaced from every three days to two days improved development and eliminated fratricide. Also, removing the availability of *Nasonia* rearing medium shortly after the larvae enter their final instar can improve the rate of pupation. However, without an increase in humidity, larvae lose mass and desiccate as early pupae. Thus, keeping humidity high increased successful pupae development.

When *Nasonia* females first parasitize a host pupa, they inject venom that arrests the development of the fly host. To test whether the utility of the *Nasonia* rearing medium is enhanced by the presence of the venom, the *Nasonia* rearing medium was made from parasitized fly hosts after 8 hr of exposure to virgin female *Nasonia*. Results demonstrated that larval development arrested in all 1$^{st}$ instar larvae after they hatched from their egg casings and began to feed. Peristaltic action of larval gut was observed in these larvae at an average rate of 3.8±3.0 contractions in 30 s (Figure V.2). This rate is

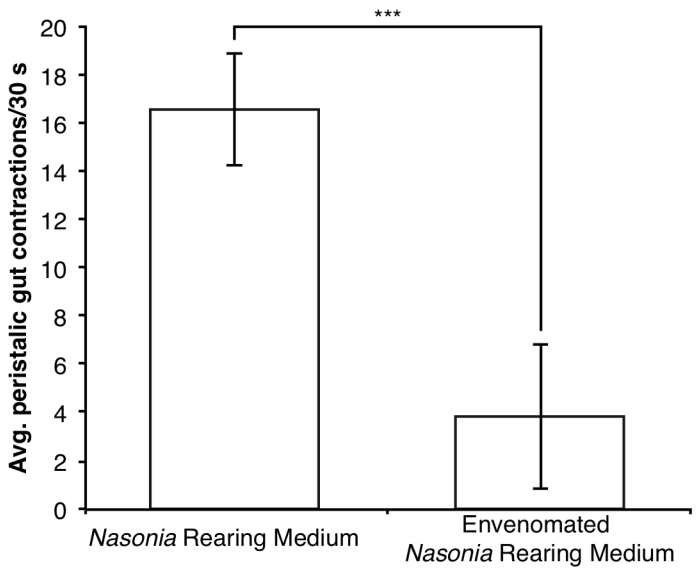


Figure V.2 Peristaltic action of larval gut reared on *Nasonia* rearing medium with and without maternal venom. Average rate (±SD) of gut peristalses as observed in first instar larvae (1-2 d post eclosion) for 30 s. For each rearing condition, 35 individual larvae were observed between six replicates (*** = $p < 0.001$, t-test).

more than five-fold slower than the rate of their brothers reared on *Nasonia* medium that lacked the venom, 16.6±2.3 contractions in 30 s ($p<0.001$, t-test). Ultimately, all larvae were dead by the sixth day of observation. This result indicates that components of the *Nasonia* venom in the rearing media are non-specific and arrest *Nasonia* larval growth and development. Considering that *Nasonia* is an ectoparasitoid, conventionally raised *Nasonia* larvae may have little contact with the components of the maternal venom. However, the *Nasonia* rearing medium itself could alter or concentrate the venom compounds and increase non-specificity of the venom.

**Conclusions**

Overall, the ability to chronicle individuals' development from embryo to adulthood is a feature of this model system that was missing prior to this technique. In conventionally-reared *Nasonia*, obtaining the developmental stages for a given time point would kill the wasps and terminate the samples if they were not already in the pupal stage. This approach required additional replicates of an experiment if one wanted to observe responses in development throughout the *Nasonia* lifecycle, in particular larval development and early pupation.

The *Nasonia* rearing medium untethers the model parasitoid from its host. In conjunction with already established molecular techniques, such as RNA interference (Lynch and Desplan, 2006; Werren et al., 2009) to alter specific gene regulation, this technique can expand the range of *Nasonia* investigations. Regular inspections of the wells for larval eclosure, growth, behavior, and development are easily performed without physically disturbing the larvae or exposing them to conditions

outside the well. Additionally, muscular kinetics, gut morphology/peristalsis, neurological development, tracheal systems, and fat bodies can be documented due to the translucent tissues of the larvae. Further development of molecular and histological techniques for *Nasonia* will increase the utility of this rearing method and the model system to broader research interests.

## CHAPTER

# VI. THE PHYLOSYMBIOTIC BASIS OF SPECIATION: GUT BACTERIA ARE REQUIRED FOR HYBRID LETHALITY


## Abstract

Although the gut microbiome influences numerous aspects of organismal fitness, its role in animal speciation is largely unknown. Here we present evidence that gut bacterial communities among closely related animal species form phylosymbiotic assemblages that cause hybrid mortality in the parasitoid wasp genus *Nasonia*. Bacterial constituents and abundance are irregular in hybrids relative to parental controls, and antibiotic curing of the communities significantly recovers hybrid survival. Moreover, inoculation of bacterial strains into germ-free hybrids reinstates mortality and causes misexpression of innate immune genes also observed in conventionally-reared hybrids that die. We conclude that in this animal complex, the gut microbiome is as important as host genes in promoting hybrid mortality and thus speciation.


## Introduction

The superorganismal, metaorganimsal, and hologenome concepts of Life define an animal or plant as a singular community of eukaryotic cells and microbial symbionts (Bosch and Mcfall-Ngai, 2011; Sleator, 2010; Wilson and Sober, 1989; Zilber-Rosenberg and Rosenberg, 2008). Under these concepts, the microbiome functions as an integrated extension of the host genome in which immune genes maintain an adaptive or commensal flora. While this understanding has been applied to the study of infectious adaptations

across various animals and plants, little attention has been given to how evolution shapes the superorganism over periods of time that span host speciation events and how changes in the microbiome affect host speciation. For example, how might the Biological Species Concept (Mayr, 1963) and Bateson-Dobzhansky-Muller model of hybrid incompatibilities (Orr, 1996) generally incorporate the microbiome? We have recently scrutinized these questions and presented theoretical evidence that negative epistasis between host genes and the microbiome can accelerate the evolution of hybrid incompatibilities such as lethality and sterility (Brucker and Bordenstein, 2012c).

As isolated species or populations diverge, evolutionary processes such as mutation, selection, drift, and symbiont acquisition can spur the evolution of reproductive isolation and consequently initiate speciation. Changes to the host-associated microbial community may occur rapidly, as demonstrated in *Drosophila melanogaster* in which flies acquire diet-specific changes to their gut microbiota that remarkably cause behavioral isolation (Sharon et al., 2010). We recently established that when environmental factors such as diet are controlled for, species of the *Nasonia* wasp complex harbor *phylosymbiotic* gut microbiotas - a term introduced here to denote microbial community relationships that recapitulate the phylogeny of their host (Brucker and Bordenstein, 2012b). The implication is that similar to a nuclear genome, the entire gut bacterial assemblage retains an ancestral signal of the host's evolution across time scales of a speciation event. That phylosymbiotic signal could be derived from immune genes that are rapidly evolving in a constant arms race with components of the microbiota. Indeed, defense and immunity genes evolve more rapidly and are under more positive selection than the rest of the genome in *Drosophila*, humans, and chimps (Lazzaro and

Clark, Oxford Press, Oxford, UK, 2012; Nielsen et al., 2005; Obbard et al., 2009). As a consequence of this Large Immune Effect, interactions between immune gene products and the microbiota can in principle lead to correlated evolutionary changes as new host species arise, and hybrids can exhibit immune related incompatibilities (Brucker and Bordenstein, 2012c). Such incompatibilities can be due to negative epistasis between host immune genes and the microbiome or between different host incompatibility genes as in hybrid autoimmunity (Bomblies et al., 2007; Jeuken et al., 2009; Yamamoto et al., 2010).

Here we present evidence, for the first time to our knowledge, that the gut microbiota breaks down in interspecific hybrids and causes lethality using the speciation model, *Nasonia*. This Hymenopteran genus consists of several young species of parasitoid wasps including: *N. vitripennis* (*v*), which diverged approximately one million years ago from the ancestor of *N. giraulti* (*g*) and *N. longicornis* (*l*), which diverged from each other less than 400 thousand years ago (Werren et al., 2010). We previously demonstrated that this species complex is reproductively isolated by gonadal infections of *Wolbachia* that cause cytoplasmic incompatibility (Bordenstein et al., 2001). In the absence of these infections, interspecific crosses between *v* males and *g* females are fertile and produce F1 hybrids, but F2 hybrid males suffer from ~90% lethality during larval development (Bordenstein et al., 2001; Breeuwer and Werren, 1995; Koevoets et al., 2012a; Koevoets et al., 2012b; Niehuis et al., 2008). Since *Nasonia* are haploidiploid organisms, F2 haploid males derived from hybrid mothers have the advantage of expressing all recessive, hybrid incompatibilities.

## Methods

### *Nasonia* strains and collection

The genus *Nasonia* consists of relatively young species that readily interbreed in the laboratory. A key genetic advantage is that their haplodiploid sex determination allows for the generation of F2 recombinant hybrid males that are haploid and thus express all recessive incompatibilities (Niehuis et al., 2008). Three species of *Nasonia* were used throughout the experiments: *N. vitripennis* strain 13.2, *N. giraulti* strain RV2xU, and *N. longicornis* strain IV7R3-1b. Each had been cured of *Wolbachia* for over a decade of laboratory rearing. Hybrid *Nasonia* were generated by collecting virgin females and males from each parental species during early pupae development. Upon eclosion, parental adults were crossed in single-pair, observed matings. Since *Nasonia* is haplodiploid, only the diploid F1 females are hybrids. The resuting F2 generation are haploid recombinant males. Control parental strains were also reared concurrently with the hybrids under identical conditions: 25°C, constant light, on *Sarcophaga bullata* pupae from the same brood. Virgin *Nasonia* females were collected and serially hosted onto two unparasitized *S. bullata* pupae from the same brood every 48 hours over a period of 14 days.

### Conventional survival assays

Virgin F1 females of 32-48 hours old were individually hosted in sterile glass test tubes (12 x 75 mm; Fisher Scientific, Fair Lawn, NJ) without food or water. Single *S. bullata* hosts were inserted into sterile foam plugs that allowed the female access to the

upper half (mid thorax and head) of the fly pupae, enclosed within the puparium. Females were hosted in this way for eight hours before being transferred into new tubes with a fresh fly host. All counts and collections of F2 *Nasonia* were done on the second, third, and/or fourth hosting of a single mother in order to encourage higher egg production. Fly hosts were reared in our laboratory to ensure that all fly pupae within the study came from a single brood. Eggs of *Nasonia* were counted for each opened host, opened using a sterile pin, within the first 2-4 hours of the mother being removed from the fly host. First instar larvae (L1) were counted within 28-36 hours of the mother being removed from the fly host while fourth instar larvae (L4) were observed seven days after. All pupae were observed 9-12 days of the mother being removed from the fly host and adults were placed into new tubes while they eclosed into adulthood (14-16 days). Survival estimates were calculated by hosting 96 replicates for each cross type, using 48 replicates to count eggs of a single hosting period and then using the remaining 48 replicates to count the number surviving to a given life stage. Each observation was performed in triplicate for each mother by rehosting a mother a total of four times and discarding the first hosting.

An average number of eggs per host was calculated for each cross. Percent survival calculations were tabulated using the average number of pupae to adults that emerged per host. Each experimental condition (germ-free NRM, germ-free hosts, and all inoculation experiments) had conventionally reared control groups set up in parallel. All hosting and *Nasonia* development were done in constant conditions as described above.

**Collecting samples for microbial 16S rDNA analysis and *Nasonia* genomic/transcriptomic analysis**

Twenty *Nasonia* second instar larvae of each cross were collected and placed in individual sterile microcentrifuge tubes; individuals were collected from fly hosts in the conventionally reared experiments and from the transwell assay baskets in the germ-free and inoculation studies. All *Nasonia* were flash frozen in liquid nitrogen and stored at -80°C until further processing, except for the *Nasonia* used to culture bacteria as described below. Additional *Nasonia* were collected at the end of each experiment in the pupae or adult stage for genotyping and MTRD analyses. Before DNA and mRNA co-extractions, all wasps were serially washed in sterile water (nucleotide-, DNAse-, RNAse-free) and molecular grade 70% ethanol to rinse the body surfaces of environmental microbial contamination. DNA and RNA was extracted from the samples using the ZR-Duet DNA/RNA MiniPrep (Zymo Reseach, Irvine, CA, USA) kit according to manufacturer's protocol. The output DNA was stored at -80°C until it was genotyped for marker transmission distortion ratios and microbial 16S rDNA sequencing while the RNA was stored at -80°C before being sent to the Vanderbilt Technologies for Advanced Genomics for RNAseq, see below.

**PCR and 454-Roche pyrosequencing of 16S rRNA**

Each sample was assessed for DNA concentration using a Qubit® 2.0 Fluorometer with the dsDNA HS Assay kit (Invitrogen, Carlsbad, CA, USA) and normalized, via dilution, to 2 ng/µl of template DNA. The resulting template DNA then underwent PCR amplification for 16S rDNA sequences using the universal primers (27F

and 338R) that included Roche adaptor sequences (lowercase) and a 10nt 'barcoded' sequence for multiplexing samples (represented as N in the sequence) 5′-cgtatcgcctccctcgcgccatcagNNNNNNNNNNAGAGTTTGATCMTGGCTCAG-3′ (Adapter A, 27F) and 5′-ctatgcgccttgccagcccgctcagGCWGCCTCCCGTAGGAGT-3′ (338R) (Mattila et al., 2012). Each sample PCR was performed in triplicate using a high-fidelity polymerase (Phusion Hot Start, Finnzymes, Lafayette, CO, USA), 2–50 ng of DNA template in 25 μL volumes and 25 cycles at an annealing temperature of 50°C. Amplicons from the three replicates were purified and pooled using the Qiagen PCR Cleanup kits (Qiagen, Valencia, CA, USA). Each barcoded sample was then quantified using a Qubit® 2.0 Fluorometer with the dsDNA HS Assay kit (Invitrogen, Carlsbad, CA, USA). The final barcoded amplicons from all samples were combined and used as templates for sequencing on a GS Junior 454-pyrosequencer (Roche, Basel, Switzerland).

**Statistical analysis of the microbial communities**

The raw sequence data from the GS Junior 454-pyrosequencer was trimmed, filtered, and analyzed using the statistical software package Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). A total number 21,543 sequences passed a quality filter with a minimum score of 25 and an average length of 329 base pairs. The parental lines had 1,360 – 5,333 sequences while the hybrids had a markedly higher number of reads, 5,996 & 6,119. The fly host, *S. bullata,* only had a total of 274 reads; since the quantification of the genomic DNA was normalized for the amount of input DNA, the variation in the number of 16S amplicon sequences are an indicator of bacterial density relative to the host genomic DNA. All OTUs were determined by a 95%

pairwise identity cutoff for each 16S rRNA sequence to a known member of the database. Sequences are available at NCBI accession numbers: Available upon submission and review.

### Isolation and selection of antibiotic resistance of *Nasonia* bacterial strains

Two pools of ten whole L4 instar *N. vitripennis* and *N. giraulti* larvae were homogenized in sterile saline buffer and 100 µl was spread onto Luria Broth (Cat: L24041, Miller's LB Broth Capsules, Research Products International Corp., Mount Prospect, IL) agar as well as MacConkey agar plates (Cat: M7408-250G, Sigma-Aldrich, St. Louis, MO). Each plate was prepared in triplicate and placed in an incubator at 32°C for 8 hours. Individual colonies were selected and restreaked onto new plates. To define taxonomy, colonies of isolates' were picked with a sterile toothpick and suspended in 1 ml of sterile nuclease free water and were subsequently diluted 1:100 in fresh water and boiled at 99°C for 2 min. PCR was performed for the 16S rRNA gene using the Phusion Hot Start enzyme as stated above. The resulting amplicons were sequenced at Genewiz Inc. (South Plainfield, NJ), and blasted against the NCBI nucleotide database. Sequences are available at NCBI accession numbers: Available upon submission and review. To develop antibiotic resistant strains, we subcultured isolates onto their respective agar media containing 100 µg/Ml Carbenicillin Disodium Salt (Cat: C460001, Research Products International Corp., Mount Prospect, IL). Two out of the three isolates, *Providencia rettgeri* strain IITRP2 (99.8% nucleotide identity to dominant 16S rRNA) and *Enterococcus faecalis* str. XJALT-127-2YG1 (99.8% nucleotide identity to dominant

16S rRNA) grew on the Carbenicillin plates, while the *Proteus mirabilis* strain SNBS (100% nucleotide identity) did not.

**Germ-free *Nasonia* and inoculation assays**

We previously developed a technique that allowed for *Nasonia* to be reared without the *S. bullata* host on a liquid media diet called the *Nasonia* Rearing Medium (NRM) (Brucker and Bordenstein, 2012a). This method was developed with the intention of rearing *Nasonia* germ-free. With the addition of antibiotics to the NRM, we can prevent further colonization of the *Nasonia* L1 larvae as they eclose from their egg corion; we also take precaution to surface sterilize the embryos with a 10%, by volume, commercial bleach solution and subsequently rinse them with sterile water. All germ-free and inoculation experiments were set up with the same procedures. When egg counts were taken for conventionally reared *Nasonia*, these embryos were collected and placed on the transwell membrane with the NRM. The NRM was replaced daily for the germ-free rearing and inoculations with the *Proteus mirabilis* strain SNBS and *Providencia rettgeri* strain IITRP2, non-antibiotic resistant isolates. Inoculations of L1-L2 instar larvae were done 24 hr embryo collection. For each experimental condition, we set up replicates of 24 wells containing 12-52 surface sterilized embryos per cross type. A total of three replicates were set up for each of the inoculation experiments alongside which parallel, germ-free experiments. The inoculation of the larvae was conducted by first isolating a single colony of a bacterial culture with a sterile loop, suspending it in 500 µl of NRM, and diluting it 1:10 into fresh NRM for a final volume of 3 ml. Co-inoculations used 25 µl of each inoculum per well, bringing the final volume of NRM in the well to

300 µl. The control, germ-free cohorts were treated with just NRM in the same manner as the inoculation *Nasonia*. To determine the amount of bacteria inoculated onto the *Nasonia* larvae, 50 µl of the inoculum were spread onto LB agar plates (for *Providencia*) and MacConkey agar plates (for *Proteus*) and incubated as per above. The average number of colony forming units (CFUs) were then tabulated for each inoculum at an average of 320 CFUs ±104 (SD) and 260 CFUs ± 58 respectively.

The inoculation experiments with antibiotic resistant (AR) *Providencia rettgeri* strain IITRP2 and *Enterococcus faecalis* str. XJALT-127-2YG1 as well as the GFP-*E. coli* had NRM replaced on 12 hr intervals due to the rapid proliferation of the bacteria in the medium. The developing *Nasonia* and transwell baskets were rinsed daily with 10% bleach, sterile water, and blotted dry with sterile lab tissue before being placed into a new, sterile well with fresh NRM. Likewise, the germ-free NRM parallel experiments were handled in the same manner. An average of 546 ±53 (SD) and 145 ±62 CFUs of AR *Enterococcus* and *Providencia* CFUs respectively was applied to the three replicates for this set of mono-inoculation experiments.

Using a green fluorescent protein (GFP) expressing *E. coli* with antibiotic resistance (Cat: 211080 Green Gene Colony Transformation Kit, Carolina Biological, Burlington, NC) we conducted inoculation studies to diagnose bacterial tropism/pathogenicity within the dying larvae. Experimental set up was the same as the above mentioned inoculation experiments, with an average of 135 CFUs ±76.

Mortality was tabulated for each individual *Nasonia* on a 24 to 48 hr cycle. Observation of larval motility, a benefit of the NRM condition, was conducted via a

stereoscope. Lack of movement and subsequent lack of peristaltic movement of the gut was used to distinguish living and dead larvae. Prodding of presumed dead larvae with a sterile dissecting probe was used to confirm an individuals' death. Final tabulations of surviving larvae took place on the 12$^{th}$ day post eclosure of the larvae from the corion; this time period was selected because the majority of larvae would have pupated by then. Used NRM was plated on LB and MacConkey agar throughout the germ-free and inoculation experiments to confirm sterility or bacterial colonization status. A subset of larvae and pupae, living and dead, were also homogenized and plated onto LB and MacConkey agar plates, as per above protocol, to confirm sterility or infection status (approximately 3-5 individuals per well). Germ-free wells that were positive for bacterial contamination were excluded from the analysis. Finally, a t-test was used to compare pairs for statistical differences of intra- and inter-specific cross types under their respective rearing condition.

**Germ-free *S. bullata* with *Nasonia* hybrids**

We previously developed a technique for rearing the fly host *S. bullata* germ-free, although there were greater challenges in maintaining germ-free fly larvae and pupae (Brucker and Bordenstein, 2012a). We utilized this technique to test for host-associated hybrid mortality by having antibiotically treated *Nasonia* F1 virgin females (v/v, g/g, v/g, and g/v crosses) parasitize germ-free fly hosts. We hosted female *Nasonia* as we had done with the conventionally reared *Nasonia*, only with 48 fly hosts per cross and two replicate hostings. Of the 48 flies per cross, 24 were used to calculate average number of eggs per host and the remaining were allowed to develop into pupae before being

calculated. We observed an increase in hybrid survival under these germ-free rearing conditions; however we note that there was a reduction in the total number of eggs laid in the germ-free hosts (27 ± 12) relative to the conventionally reared hosts (46 ± 21). We suspect that the lack of bacteria within the hosts may increase the search time of the female *Nasonia* before she begins ovipositing her eggs. Alternatively, the female egg laying behavior or fecundity may be altered from a lack of enough bacteria in the host or from antibiotic feeding. The rearing of germ-free *Nasonia* hybrids on germ-free flies provided significant rescue of hybrid survival; however, the variation in survival of hybrids relative to control species on germ-free fly hosts versus the liquid NRM raises questions as to whether the parasitized, conventional hosts contribute maternal factors such as venom or nutrients that in turn mitigates the extent of the hybrid rescue. When developing the NRM, we tested differences in NRM derived from envenomed hosts vs. unparasitized hosts (Brucker and Bordenstein, 2012a), and we observed significant decline in larval survival on envenomed hosts.

**RNAseq | Transcriptome analyses**

A total of 54 individual L2 *v/g* larvae spanning the three rearing conditions (conventional (N=14), germ-free (N=20), and inoculated (N=20)) were RNAseqed. *Nasonia* were inoculated with *Providencia* and *Proteus*. The RNA samples were processed at the Vanderbilt Technologies for Advanced Genomics core for library prep and sequenced on a single lane of the Illumina HiSeq 2000, PE-150. The sequence reads were assembled, and analyzed in the RNA-Seq and expression analysis application of CLC Genomics workbench 3.7. (CLC Bio, Aarhus, Denmark). The *Nasonia* genome 2.0

(http://www.ncbi.nlm.nih.gov/genome/449) (Werren et al., 2010) was used for reference assembly and annotation. Data was normalized by calculating reads per kilo base per million mapped reads (RPKM) for each gene (Mortazavi et al., 2008). For the statistical analysis, a genome wide average RPKM was calculated for each rearing condition; they were not statistically different from each other. Of the innate immune gene set that we previously reported (Brucker et al., 2012), there was a significant difference in the immune gene expression between germ-free and the other two conditions. A t-test was performed on $\log_2$-transformed data to identify the immune genes with significant changes in expression ($p \leq 0.05$) between the average germ-free and conventional/inoculation average. Prior experimental studies proposed a breakdown of the oxidative phosphorylation pathway (OXPHOS) as a primary cause of hybrid mortality (Gibson et al., 2010). If these genes were linked to the hybrid mortality, then we expected that the germ-free rearing condition where mortality is rescued would be least like either the conventionally reared *Nasonia* hybrids or the inoculated hybrids that exhibit high rates of lethality; however we observe no significant difference between the conventionally reared *Nasonia* hybrids and those reared germ-free (Table VI.1). We hypothesize that the OXPHOS pathway is an indicator of hybrid dysfunction later in development, after the majority of hybrid mortality has occurred.

**Genotyping and MTRD**

Previous studies of *Nasonia* hybrid mortality determined genome-wide regions of high allelic bias towards one parental type over the other (Ellison et al., 2008; Koevoets et al., 2012a; Niehuis et al., 2011; Niehuis et al., 2008; Werren et al., 2010). Since we

Table 0.1 RPKM summary of the genome wide average, the OXPHOS pathway, and the innate immune genes.

| | | Conventional ( C) | | Germ-free (GF) | | Inoculated (I) | | Chi Square significance | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | genes | RPKM | SD | RPKM | SD | RPKM | SD | C vs. GF | I vs. GF | C vs. I |
| Genome | 12149 | 65 | 41 | 55 | 26 | 59 | 26 | p=0.325 | p=0.529 | p=0.633 |
| Oxphos | 56 | 249 | 78 | 239 | 103 | 299 | 61 | p=0.743 | p=0.032 | p=0.029 |
| Immune | 488 | 86 | 129 | 30 | 28 | 75 | 62 | p<0.001 | p<0.001 | p=0.104 |

RPKM = Reads Per Kilobase of exon model per Million mapped reads

were able to rescue hybrid mortality under germ-free conditions, we hypothesized that these allelic biases associated with hybrid lethality would disappear. To test the rescue of marker transmission ratio distortion (MTRD) under germ-free conditions, we genotyped four markers from across the genome, three with expected allelic biases and one control. We used 60 conventionally reared, individual *Nasonia* hybrid pupae per cross and 40 germ-free *Nasonia* hybrids for the analysis. Individuals were PCR amplified for each of the four markers and their allelic results tabulated (Table VI.2). Statistical significance was calculated using a one-tailed, Chi-square analysis without Yates correction. Expected frequency estimates were determined based upon prior reports (Werren et al., 2010). Some samples did not yield proper PCR amplification of some markers, and they were excluded from the analysis (Table VI.2).

**Results**

In this study, we observed a large cohort of developing F2 hybrid males and determined that 78% of the mortality occurs between the first and the fourth instar larval stages (Figure VI.1a). In contrast, hybrids of the younger sister species *g* and *l* exhibit little to no hybrid mortality, as previously reported (Bordenstein et al., 2001) (Figure VI.2a). The hybrid mortality manifests itself with pathological characteristics including melanization of the body and the loss of the gut lumen as evident by lack of internal peristaltic movement (Figure VI.1b).

We postulated that since parental *Nasonia* have phylosymbiotic gut microbiotas whose community relationships recapitulate the evolutionary history of the *Nasonia* species complex (Brucker and Bordenstein, 2012b) (Figure VI.1c-d), then the lethality,

Table VI.2 RPKM summary of the genome wide average, the OXPHOS pathway, and the innate immune genes.

| | Chr 1 - PRGP | | | | Chr 2 - Nv20 | | | | Chr 4 - MM4.38 | | | | Chr 5 - MM5.03 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | g/v | n | v/g | n | g/v | n | v/g | n | g/v | n | v/g | n | g/v | n | v/g | n |
| Exp. Frequency - Co | 0.75 | | 0.65 | | 0.74 | | 0.72 | | 0.49 | | 0.42 | | 0.60 | | 0.08 | |
| Obs. Frequency - Co | 0.74 | 65 | 0.80 | 32 | 0.71 | 58 | 0.68 | 32 | 0.51 | 64 | 0.36 | 32 | 0.62 | 28 | 0.09 | 28 |
| Obs. Frequency - GF | 0.56 | 32 | 0.60 | 24 | 0.44 | 32 | 0.50 | 22 | 0.54 | 22 | 0.48 | 22 | 0.42 | 24 | 0.20 | 27 |
| *P*-value Exp. vs. Obs. - Co | 0.420 | | 0.079 | | 0.339 | | 0.392 | | 0.362 | | 0.399 | | 0.456 | | 0.320 | |
| *P*-value Exp. vs. Obs. - GF | 0.028 | | 0.289 | | 0.002 | | 0.051 | | 0.311 | | 0.248 | | 0.105 | | 0.103 | |
| *P*-value Obs. - Co vs. Obs. - GF | 0.040 | | 0.030 | | 0.006 | | 0.083 | | 0.405 | | 0.181 | | 0.085 | | 0.206 | |

Expected frequencies are estimated from J. H. Werren et al., *Science* 2010

P-values calculated with Chi-square contingency, one tail test

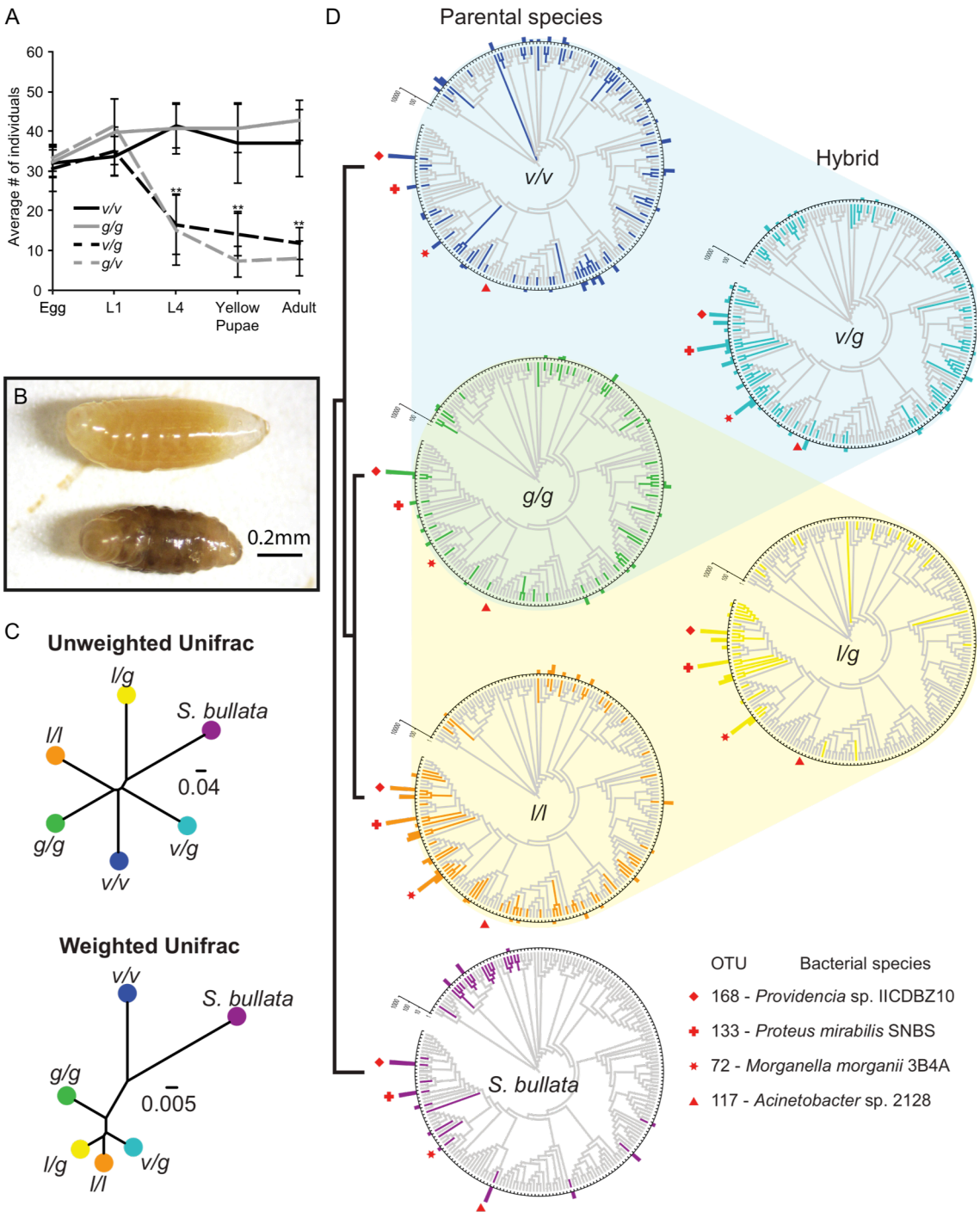


Figure VI.1 Hybrid lethality and the bacterial microbiota (A) Average number of F2 males ± SD during development from egg, 1st instar larvae (L1), 4th instar larvae (L4), yellow red-eye pupae, and eclosed adults conventionally reared on *S. bullata* hosts. The F2 hybrid genotype is indicated as grandfather/grandmother. Mann-Whitney U test, $^{**}P < 0.001$. (B) Top: *N. vitripennis* 3rd instar larvae that is healthy and alive; Bottom: hybrid *g/v* 3rd instar larvae that is melanized and dead. (C) UniFrac cluster analyses of the microbial relationships of the three *Nasonia* species and two hybrids for the second instar larval microbiota, as well as the unparasitized *Sarcophaga* host pupa. Analyses are unweighted and weighted to OTU abundance. (D) A schematic phylogeny of the host insect species in which branch tips lead to 16S rDNA circular trees that denote presence of bacterial OTUs (colored branches) in non-hybrids and hybrids and the abundance of those OTUs on the outer ring (log scale). Absence of a bacterial OTU in a specific host species is indicated by grey branches.

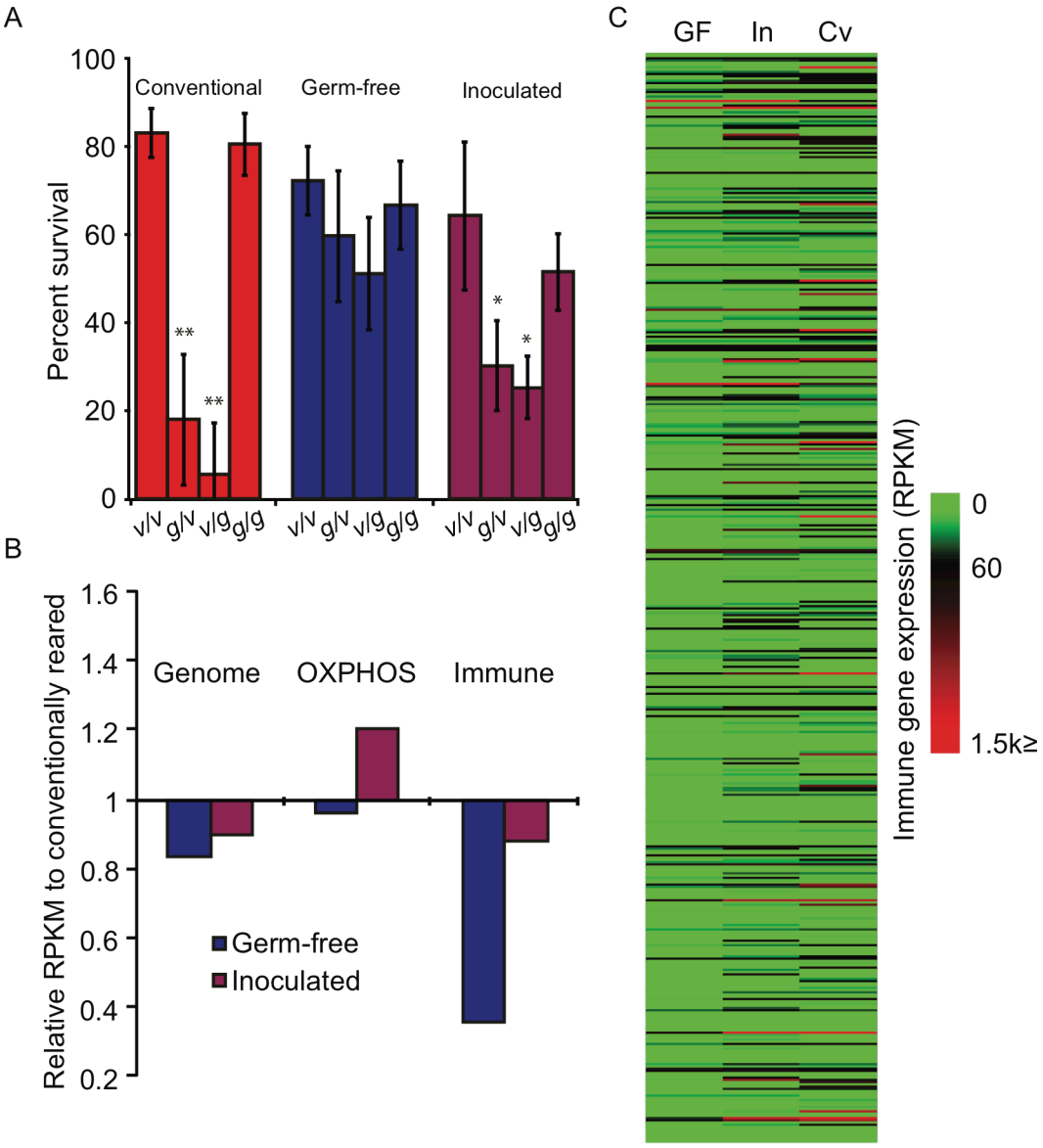


Figure VI.2 The symbiotic and genetic basis of hybrid lethality (A) % survival from egg to pupae of conventionally reared, germ-free, and inoculated *N. vitripennis* (*v*) and *N. giraulti* (*g*) parental species and hybrids. T-test, $^{**}P < 0.001$, $^{*}P < 0.01$. (B) Average hybrid *v/g* gene expression relative to conventionally reared hybrids for the total genome, oxidative phosphorylation genes, and immunity genes. RPKM denotes Reads Per Kilobase per Million mapped reads. (C) Heat map depicting RPKM values of innate immune gene for germ-free (GF), inoculated (In), and conventional (Cv) rearing conditions.

loss of the larval gut lumen, and melanization in *v/g* hybrids (F2 hybrid genotype denotes grandfather/grandmother) results at least in part from an altered gut microbiota. As a negative control, we reasoned that the *l/g* hybrids will not show a distorted microbiota because there is little to no hybrid lethality in this interspecific cross (Bordenstein et al., 2001).

First, we set out to determine the bacterial community differences between larval hybrids and nonhybrids during the 2nd instar larval stage, just prior to the point of hybrid mortality. As expected, the microbiota of *v/g* hybrids was unlike that of either parental species (Figure VI.1c-d). The major differences in the *v/g* hybridization were (i) a shift in the dominant bacterial symbiont from *Providencia* sp. IICDBZ10 in pure species controls (81% and 96% of the reads in *v/v* and *g/g*, respectively) to *Proteus mirabilis* SNBS in the *v/g* hybrid (86% of reads), (ii) an expansion of *Morganella morganii* 3B4A and *Acinetobacter* sp. 2128 from 1.2 % and 0.04% of the reads in *v/v* and *g/g*, respectively, to 7.2% of the reads in *v/g* hybrids, and (iii) an increase in microbial species richness from 71 and 50 OTUs observed in *v/v* and *g/g* controls, respectively, to 85 observed OTUs in hybrids. Of these 85 OTUs, half (47.1%) were unique to the *v/g* genotype (Figure VI.1d). In contrast, *l/g* hybrids elicited no major shifts in the abundant microbial species in comparison to *l/l* and had fewer OTUs compared to *g/g* and *l/l* controls, of which only 30% were unique to the hybrid microbiota.

Second, to test if the lethality in hybrids (Figure VI.2a) is conditional on their bacterial microbiota, we reared conventional, germ-free, and bacterial inoculated hybrids and non-hybrids. For germ-free rearing of *Nasonia*, we used a liquid media that we developed for *Nasonia* development without its *S. bullata* fly host, called *Nasonia*

Rearing Medium (NRM) (Brucker and Bordenstein, 2012a). Treated with antibiotics, NRM supports germ-free *Nasonia* development from egg to adulthood. If hybrid mortality is intrinsically based on negative epistasis between host incompatibility genes, then we expect *v/g* hybrid mortality to occur. However, if the mortality is conditional upon the microbial flora, then we expect that germ-free rearing of *v/g* hybrids will "rescue" hybrid mortality. Results indicate a near complete rescue of hybrid mortality by comparing the survival of germ-free hybrids to pure species controls (Figure VI.2a, t-test, $P = 0.47$ and $P = 0.09$). Therefore as expected, germ-free *v/g* hybrids have a significant increase in survival in comparison to that of conventionally-reared *v/g* hybrids (Figure VI.2a, t-test, $P < 0.001$). Similarly, when we reared hybrids on germ-free *S. bullata* fly hosts, there was a marked increase in hybrid survival relative to those that were conventionally reared (Figure VI.3b, t-test, $P < 0.05$). In the *l/g* negative controls, survival values of germ-free hybrids and non-hybrid controls are both high and not significantly different (Figure VI.3a, t-test, $P > 0.5$), as expected.

It is possible that the rescue of hybrid lethality could be related to the *in vitro* rearing conditions rather than the absence of the microbiota. This concern is unsubstantiated for at least two reasons. First, if the NRM generally increased survival, it should affect parental species survival as much as hybrids. However, average survival rates of control parental species insignificantly decreased on the NRM. Second, germ-free hybrids that were reared on the NRM and inoculated with bacterial strains once again showed higher mortality in comparison to controls. The bacteria *Providencia rettgeri* strain IITRP2 and *Proteus mirabilis* strain SNBS were isolated from *Nasonia* and used in co-inoculation experiments. Upon feeding a 1:1 co-inoculation of these bacteria to germ-

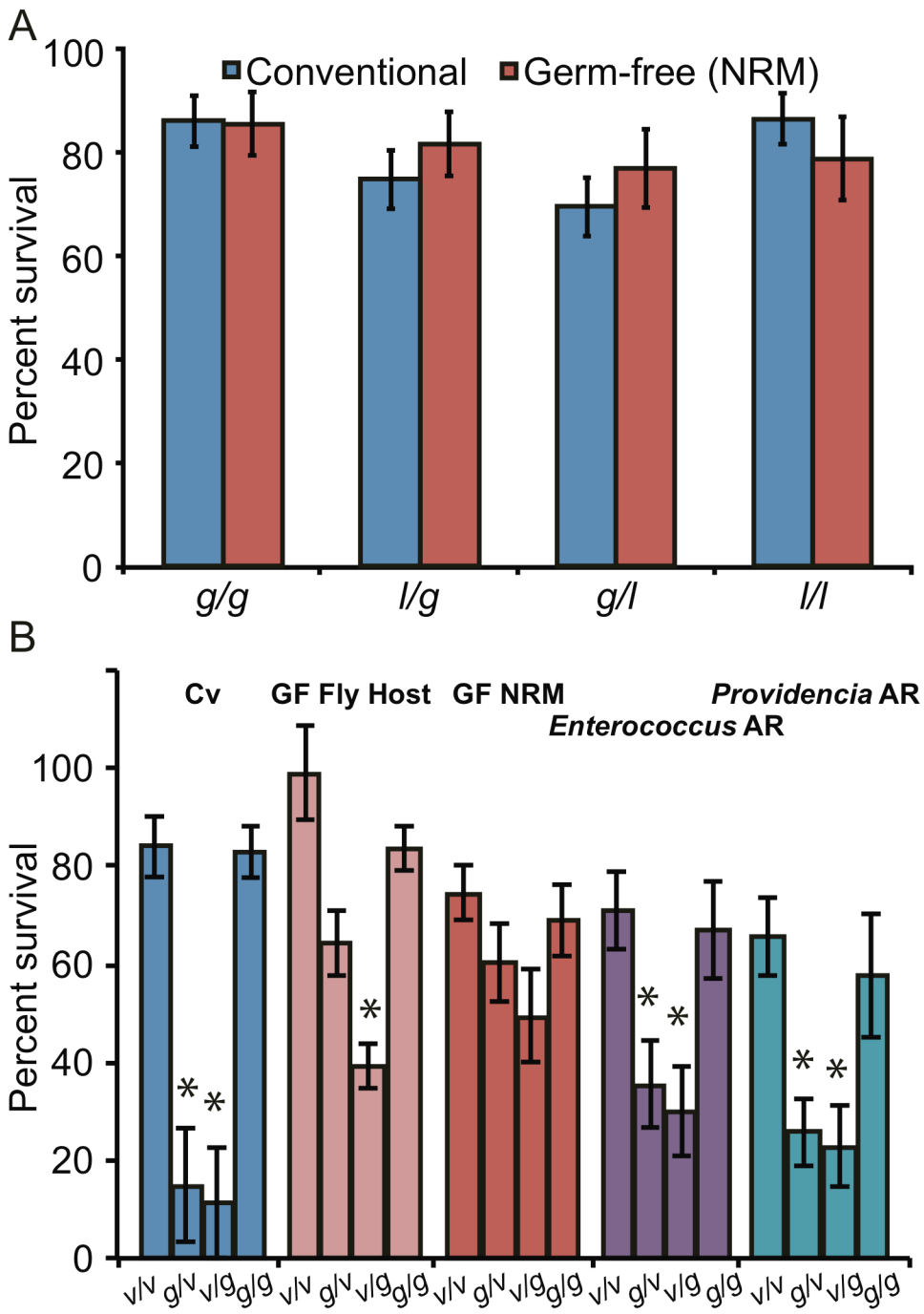


Figure VI.3 Comparative analyses of survival between conventionally reared and germ-free hybrids. (A) F2 hybrid survival for a control cross between the younger species pair *N. longicornis* (*l*) and *N. giraulti* (*g*) species that do not exhibit hybrid mortality. (B) F2 hybrid survival for interspecific crosses between the older species pair *N. vitripennis* (*v*) and *N. giraulti* (*g*) using five different rearing conditions - conventionally reared, germ-free *S. bullata* fly hosts, germ-free *Nasonia* on rearing medium (NRM), and inoculations of antibiotic resistant (AR) *Enterococcus* sp. and *Providencia*. % survival is based on the ratio of the number of pupae to eggs. T-test, **P* < 0.01.

free hybrids in the L1 instar, we observed a severe induction of hybrid mortality in comparison to inoculated parental controls (Figure VI.2a, t-test, $P < 0.01$) and at levels similar to those in conventionally reared hybrids (Figure VI.2a, t-test, $P > 0.4$). Furthermore, mono-inoculants of antibiotic resistant (AR) *P. rettgeri* and an *Enterococcus faecalis* str. XJALT-127-2YG1 isolated from *Nasonia* also recapitulated significant hybrid mortality (Figure VI.3b, t-test, $P < 0.01$).

Under conventional rearing conditions, hybrid mortality exhibits varying larval pathologies including melanized bodies (Figure VI.1b), lack of gut definition, or punctuated/segmented guts. Similar observations were made for the larvae reared on NRM with bacterial inoculums. To further characterize this pathology and demonstrate the gut immune incompetence of hybrids, we conducted inoculation experiments with a green fluorescent protein (GFP) expressing *E. coli*, a member of the family *Enterobacteriaceae* that are common gut symbionts in *Nasonia*. As with the previous inoculation experiments, larval hybrids suffered from significant hybrid mortality while parental crosses survived (Figure VI.4a, t-test, $P <0.01$). We observed several pathologies of dead hybrid larvae. First, systemic GFP throughout the larval body cavity indicated that the *E. coli* was able to escape the gut and proliferate within the dead *Nasonia* larvae (Figure VI.4b, solid arrow). Second, we observed high GFP expression around the mouthparts and early gut of the larvae (Figure VI.4b, hollow arrow). Third, punctuated pockets of the gut were observed with and without GFP expression (Figure VI.4b, solid and hollow circles).

The requirement of gut bacteria for interspecific hybrid mortality in *Nasonia* is remarkable given that several studies previously mapped the genetic factors associated

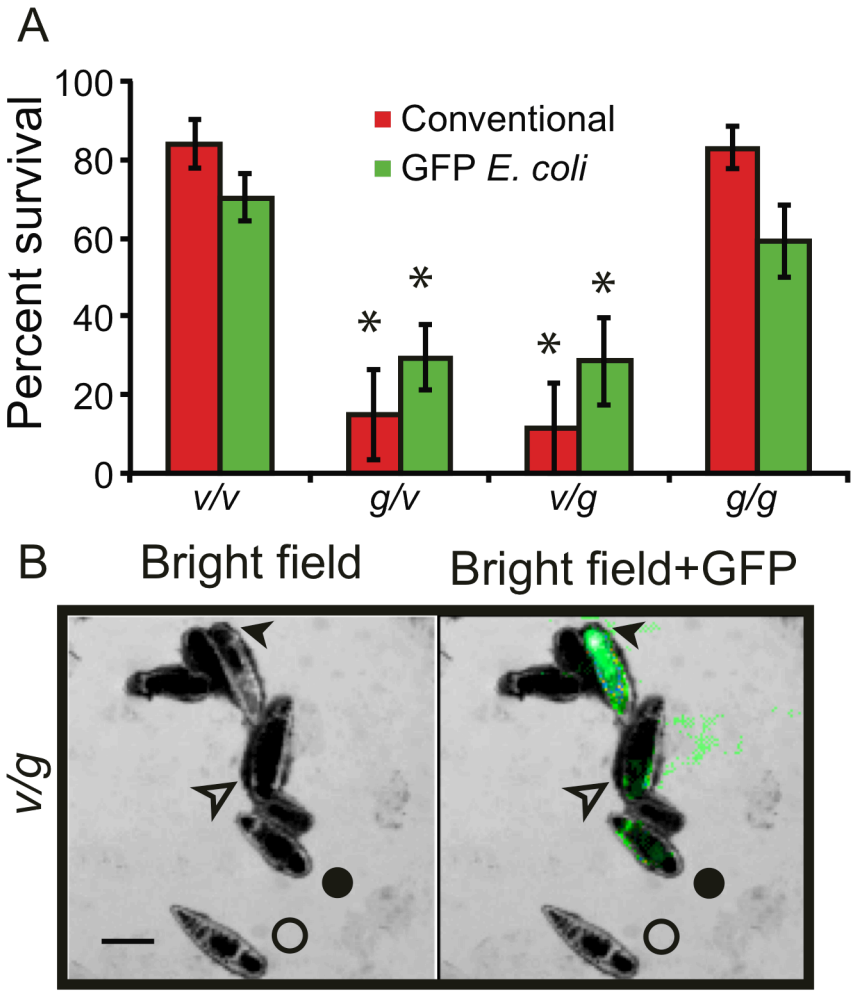


Figure VI.4 Hybrid mortality and gut pathology. (A) Percent survival from egg to pupae of conventionally reared and GFP/*E. coli* inoculated larvae. T-test, $*P < 0.01$. (B) Hybrid L2 instar larvae inoculated with GFP *E. coli* during L1 instar. Solid arrow indicates the anterior of dead larvae with systemic GFP expression and a lack of defined gut (scale bar indicates 0.2 mm). Hollow arrow indicates the anterior of dead larvae with GFP expression around the mouthparts and early gut of the larvae. Solid circle indicates the anterior of dead larvae with a segmented gut and pockets of GFP expressed within the punctuated gut structures. Hollow circle is placed at the anterior of dead larvae that has a highly punctuated gut with no observable GFP expression at this magnification.

with hybrid mortality to the wasp chromosomes and mitochondria (Breeuwer and Werren, 1995; Koevoets et al., 2012b; Niehuis et al., 2008; Werren et al., 2010). Indeed, marker transmission ratio distortion (MTRD) analyses in hybrids demonstrate an allelic bias towards one parental genome (Koevoets et al., 2012a; Koevoets et al., 2012b; Werren and Loehlin, 2009b; Werren et al., 2010). Based on our observation that germ-free hybrid *Nasonia* exhibit a significant increase in survival, we predicted that MTRD would revert to near Mendelian inheritance in the germ-free hybrids. We selected four markers - three within loci associated with hybrid mortality and one from a locus that is expected to have 50:50 inheritance ratios. Genotyping *v/g* hybrid *Nasonia* in L4 instar larvae that were reared conventionally yielded expected frequencies for each of the MTRD markers (Werren et al., 2010) (Table VI.2). However, rescue of hybrid mortality in germ-free hybrids simultaneously rescued the frequencies of MTRD from their expected values under conventional rearing (Table VI.2). All genes in the germ-free rearing condition were within typical Mendelian inheritance, except for *v/g* MM5.03 marker. A large bias against *v* alleles remained (*v* frequency of 0.08), though the frequency was significantly higher from the MTRD under conventional-rearing (*v* frequency of 0.20, Fisher's exact test, $P < 0.001$).

One explanation for discovering an infectious basis to the larval hybrid mortality is that negative epistasis occurs between chromosomal genes and the microbiome. We recently discussed that these interactions accelerate the number of hybrid incompatibilities that can potentially evolve (Brucker and Bordenstein, 2012c). To validate host-microbe interactions that underscore hybrid mortality, we benchmarked the transcriptomes of germ-free L2 instar hybrid larvae (just prior to mortality) against

conventionally-reared and bacterial-inoculated larvae. We report three results. First, genome-wide expression patterns were similar across all three-rearing conditions (Figure VI.2b). Second, the oxidative phosphorylation (OXPHOS) enzyme complex that was previously observed in F2 hybrid males to have reduced ATP production (Ellison et al., 2008) did not show any major transcriptome changes that correlated with the rescue of hybrid survival in germ-free conditions (Figure VI.2b). Third and finally, the 489 innate immune genes in *Nasonia* that we previously annotated (Brucker et al., 2012) yielded an average, significant decrease in transcript levels in germ-free individuals relative to conventional or inoculated hybrids (Figure VI.2b, t-test, $P < 0.001$). 39.7% of the immune genes were under-expressed by 2-fold or greater in germ-free hybrids relative to conventional and inoculated hybrids, while only 4.9% were over-expressed (Figure VI.3c). Conventionally-reared and inoculated hybrids in turn have similar immune gene expression (Figure VI.2c, t-test, $P = 0.104$) that associates with the hybrid breakdown.

**Discussion**

Causes for postzygotic hybrid mortality in *Nasonia* have been attributed to host cytonuclear interactions or host gene-by-environment interactions (Breeuwer and Werren, 1995; Koevoets et al., 2012a; Koevoets et al., 2012b; Niehuis et al., 2008). Indeed, mapping hybrid incompatibilities to host chromosomes and organelles is the conventional approach to genetic analyses of speciation. The evidence presented here shows for the first time that gut bacteria and host incompatibility genes are required for severe hybrid mortality between closely-related animal species. Coupling this evidence with the vast effects that the host-associated microbiome can have on fitness suggests that studies of speciation should be routinely done with germ-free and conventionally-reared organisms

that can untether the multi-way interactions between symbionts and host genes. This superorganism concept of speciation is underexplored and ripe for study that may have the potential to explain variation observed in previous studies of speciation. Based on the mounting evidence for speciation by symbiosis (Brucker and Bordenstein, 2012c), it is clear that a synthetic theory of speciation must consider eukaryotic genes and microbial symbionts as unified components in the formation of new species of superorganisms.

# CHAPTER

# VII. CONCLUSIONS AND FUTURE DIRECTIONS

The dynamic roles in which the microbiota can influence its host are vastly underexplored. This body of work demonstrates the utility of using both comparative genomics and microbial ecology to address the definition of a species. Using the genus *Nasonia* as a model organism allowed for a combinatorial approach for host and microbial analyses with tractable molecular tools. My research goes beyond the current paradigm of speciation genes by testing the contribution of species-specific microbial community to the survival and fecundity of the host. Indeed, intrinsic hybrid lethality between species can be due to an irregular microbial community shaped by hybrid breakdown in immunocompetence.

**Testing the phylosymbiotic hypothesis.**

This work can be applied broadly to evaluate the extent of phylosymbiosis in other clades of host species. In previous reports, the association between a host and its microbiome has been attributed to a complex amalgam of phylogeny and diet (Chandler et al., 2011; Ley et al., 2008a; Ochman et al., 2010; Ridley et al., 2012). These studies differ from the work done in this thesis (Chapter III) owing to the fact that their host sampling did not control for confounding variation among the diet, age, and gender of each species—contributing to the potential variation in microbial communities observed. It is important to define the role of the host genome in the establishment of a species' microbial community; however, carefully controlled experimental measures must be taken to confirm the codependency of microbial establishment and the host genome.

## Future directions

To test the phylosymbiotic relationship of species we must first establish clades that can be reared under identical conditions with controlled diet. I have identified three differing genera that, within each genus, can be reared under identical conditions; six species of the genus *Peromyscus* (deer mice), six species of the genus *Drosophila*; thirteen species of *Anopheles* (mosquitoes); and four species of the genus *Nasonia*. By using age- and gender-matched individuals feeding on the same diet, within a genera, one can then sequence the microbiota of these host species and perform comparisons like those used for interrogating *Nasonia*. If the evolution of the host species dictates the structure of the microbiota, one would expect to observe the host phylogenetic relationship replicated in the phylosymbiotic relationship between microbial communities.

## Deeper mining of the microbial diversity in *Nasonia* and hybrids

The microbial community is not limited to bacteria. *Nasonia* likely harbor a diverse set of viruses and fungi. Additional sequencing of these communities may yield interesting patterns of phylosymbiosis yet to be discovered as well as potential pathologies and phenotypes conveyed to the host. For example, while constructing the original draft of the *Nasonia* genome, cDNA sequencing revealed three novel single-stranded RNA viruses: NvitV-1, -2, -3. (Oliveira et al., 2010). These viruses were not previously found in other insect hosts, though they are related to *Picornavirales*—a known order of insect pathogens.

Furthermore, NGS allows us to explore the diversity of microorganisms as well as the genes expressed by both bacteria and host that contribute to the microbe-host and

microbe-microbe interactions that shape the phenotype and fitness of the host organism. You can imagine that a host organism needs to maintain a modest equilibrium of form and function with its microbial community, while the microbial community may do some self regulating - something I am extrapolating from observations I made when working in conservation biology - the host genome acts as a regulator of the microbial complex. Recent studies have shown that the function of bacterially-derived genes in the human microbiome maintain a relative homeostasis compared to the species diversity of the microbiome (HMB Consortium, 2012). Now every gene of a host and every gene of the microbes are not likely to play a factor in establishing their host-microbe relationship. So, could we expect to see an impact of symbiosis in the genome of any microbe that is associated with a host organism and, with enough time, could the genome of the host organism also undergo changes due to the microbe associated with it? Within the *Nasonia* system, would we expect to see functional genes maintained within a species that break down in hybrids?

**Future directions**

The fungal diversity within *Nasonia* is largely unknown, as is the case with most animal microbiome studies. However, sequencing of the fungal ITS gene sequence in a similar fashion to taxonomic screens with the bacterial 16S rDNA, would provide a window into the fungal diversity within *Nasonia*. The implications of fungal symbionts within an insect host is not well documented beyond pathogens and as a source of food (Hajek, 2004). Although evidence suggests that fungal symbionts of insects could provide detoxifying enzymes that allow for the host to feed on plants laden with

defensive toxins (Dowd, 1992). *Nasonia* could also benefit from such detoxifying properties as they feed on putrefying fly pupae.

Examining the viral diversity within *Nasonia* and their hybrids will be relatively simple. Using the transcriptional data collected in preliminary analyses for the hybrid gene expression (Chapter VI, unpublished) in conjunction with the isolation, sequencing and cataloguing of many viruses in the lab (work done by Sarah Bordenstein), we are able to estimate the viral loads of each *Nasonia* species through development, as well as in hybrid *Nasonia.* Preliminary results indicate that the viral loads in *Nasonia* do differ between certain species and developmental periods. These species-specific viral associations break down in hybrids but do not vary with the germ-free rearing of hybrids or conventionally-reared hybrids. Further exploration, using techniques like QPCR and antiviral drug treatments, could reveal commonality and function of the *Nasonia* specific viruses.

**Immune gene evolution in *Nasonia***

To determine the consequences of hybridization on the *Nasonia* immune response, we reannotated the immune genes of *N. vitripennis* genome and annotated, for the first time, the immune genes of *N. giraulti* and *N. longicornis* genomes (Chapter IV). The original immune gene annotation of *N. vitripennis* did not have the *Apis melifera* immune genes available at the time for homology comparisons. Thus, the inclusion of this gene dataset as well as the other species provided additional resources as we defined putative innate immune genes (IIID). With these putative immune genes, we can now explore immune gene evolution and regulation, as well as experimentally manipulate immune

gene expression to rescue microbial-based hybrid mortality from the breakdown in immune gene expression.

**Future directions**

One aspect of the IIID that has yet to be developed is the ability to estimate evolutionary divergence for given genes between species. Considering that the innate immune genes are rapidly evolving due to selective pressures from the microbiota (see Large Immune Effect in Chapter II), it would be interesting to estimate substitution rates for the genes and plot that against the divergence of microbiotas and their functional genes. With the inclusion of the *N. oneida* genome, the four *Nasonia* species would be a simple model of host genome evolution and microbiota establishment. These data could then be followed with experimental evolution studies using specific bacteria, fungi, and viruses to select upon the innate immune genes within lab strains with the intent of observing gene family evolution under microbial pressures.

The innate immune genes of *Nasonia* are useful in understanding hybrid breakdown and mortality. Genes that have been observed in conventionally-reared hybrids at higher than normal expression levels can be targeted by RNAi to knock down gene expression to the expression level in parental species levels or in germ-free individuals. We can then monitor gene expression in each and the consequences on the microbiome and survival. This method has the potential to parse out the underlying mechanism that links hybrid mortality to the microbiota.

**Other roles of the microbiome in *Nasonia* reproductive barriers**

The observation of microbiome-genome interactions as a cause of hybrid mortality in *Nasonia* is a unique case of a post-fertilization reproductive barrier. Chapter II details additional examples of how changes in the microbiome induce other reproductive isolation events such as development time, behavior, and fecundity. The role of microbial-induced reproductive barriers could extend to similar biologies of the *Nasonia* system. For example, we know that the germ-free *Nasonia* develop at a rate that is slower than conventionally-reared *Nasonia* (Brucker and Bordenstein, 2012a). A delay in development changes the availability of potential mates and invokes temporal isolation between populations, which could implicate a role for the microbes in ensuring synchrony of adult emergences.

Behavioral changes associated with bacterial communities have been observed in several insect species. For example, *Drosophila* are able to distinguish between mates that have a more similar or dissimilar microbiota to themselves (Sharon et al., 2010). Male field crickets, *Gryllus integer,* are behaviorally more aggressive due to their microbial communities and fight for mates (Kortet et al., 2012; Polkki et al., 2013). Mate selection within scarab beetles is dependent upon immune competence that the females sense in the bacterial-derived male pheromones secretions (Andert et al., 2010; Leal, 1998; Vasanthakumar et al., 2008). The behavioral issues that arise in hybrids (Clark et al., 2010) may have microbial underpinnings.

### Future directions

Experiments to explore the microbimes's effects on *Nasonia* development can easily be tested with the germ-free assay I established in Chapter V. Germ-free *Nasonia* that have been inoculated with bacteria from *Nasonia* parentals and hybrids would be one means to determine the influence specific microbial species have on hybrid development. Further experiments could compare how longevity, fecundity, and overall fitness are impacted in germ-free versus inoculated insects.

Additional experiments in behavior could be conducted using multi-parasitism (when two or more parasitoids parasitize the same fly host). Allowing two or more *Nasonia* species to co-parasitize a host would allow for a comparison of the effects that the same developmental environment has on mate selection and microbiota composition. Theoretically, the shared environment could allow for cross-colonization of parental microbiota from each species to the offspring of both species. Behavioral observations as well as microbial assessment would provide an interesting perspective to how a species is equipped with information to select a mate.

## Summary

As a whole, the work described in this dissertation has broadened our understanding of the role in which microbial symbionts impact speciation. Based on this rationale, my dissertation addresses three tenants for phylosymbiotic relationships as a factor in evolutionary biology. First, the microbiota and host influence each others' genetic repertoire. Second, the evolutionary relationship between host species can be reflected in their microbial communities. Third, the microbiota can establish reproductive

barriers and therefore underpin speciation. With this framework, we can begin to explore new concepts that elevate phylosymbiosis as a theme in modern evolutionary biology.

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