Though the founders of the modern synthesis theorized different ways that Darwinian evolution and Mendelian genetics worked together, they converged on the premise that natural selection acts on variations brought about by random, genetic mutations (Mayr and Provine 1980). When genetic and phenotypic variations result in populations that can no longer interbreed, these reproductively isolated populations form new species.

The modern synthesis’ focus on random and simple genetic mutations dominated the evolutionary axiom for decades despite numerous works on the power and potential of host-microbial symbioses to influence and ultimately unite different species’ genomes together in a manner that causes phenotypic variation (Kozo-Polyansky, 2010; Martin and Kowallik 1999; Wallin 1923). Among the most conceptual scholars in this field, Lynn Margulis championed the evolutionary significance of symbiosis more so than typically appreciated. In this article, we present and review the case system that she long envisioned, one in which layers of microbial symbiosis reduce species interbreeding and assist species diversification among a closely related group of small, metallic green, parasitoid wasps from the genus Nasonia.

1 Introduction

“To understand the true complexity of life - the main source of evolutionary novelty Charles Darwin sought - one must understand how organisms come together in new and fascinating ways” (Margulis and Dorion 2002).

Charles Darwin and Alfred Wallace first proposed that natural selection acts as the driving evolutionary force underpinning organismal varieties and species (Darwin 1859; Darwin and Wallace 1858). Their theory revealed how natural selection operates on variation to drive organismal change, yet the precise nature of this variation was not resolved at the time. The origin of new variants and species largely remained shrouded in mystery until the modern synthesis merged Darwin’s theory of evolution with Mendel’s principles of genetics (Dobzhansky 1974; Huxley 1942). Though the founders of the modern synthesis theorized different ways that Darwinian evolution and Mendelian genetics worked together, they converged on the premise that natural selection acts on variations brought about by random, genetic mutations (Mayr and Provine 1980). When genetic and phenotypic variations result in populations that can no longer interbreed, these reproductively isolated populations form new species.

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time, her seminal manuscript on symbiogenesis purportedly circulated through over 15 journal submissions before it was published. Margulis steadfastly promoted and defended symbiogenesis over several decades (Sagan 2012), and her conviction was rewarded at multiple timepoints along the way. Experimental evidence now demonstrates that chloroplast and mitochondrial organelles share genomic ancestry with certain cyanobacteria and proteobacteria, respectively (Schwartz and Dayhoff 1978). Such findings directly supported the symbiogenesis theory, thus securing its foundation in the life sciences and the roles other endosymbioses play in the evolution of biological complexity (Margulis 1970, 1993).

Margulis’ thoughts emboldened as she expanded her ideas in evolutionary symbiosis, namely that “random mutation is wildly overemphasized as a source of hereditary variability... rather the important transmitted variation that leads to evolutionary novelty comes from the acquisition of genomes” (Margulis and Dorion 2002). While the relative influence of cumulative host nucleotide mutations versus symbiont variation on novelty differs between organismal systems, phenotypes, and timescales, one of Margulis’ many legacies was to postulate and popularize a vision of the biological world in which symbiotic interactions between macroorganisms and microorganisms are central to the two main processes of evolutionary biology: adaptation and speciation.

With this Margulian view that symbiosis is an enduring engine of biodiversity, research on host-microbe interactions and evolution flourished, driving the rapid adoption of a lexicon and concept that emphasizes eukaryotic hosts are not simply singular, autonomous species but rather a multispecies consortium consisting of a host and its associated microbiome in and on the body, referred to as a holobiont (Margulis 1991; Meyer-Abich 1943). The collective genomes of a holobiont constitute a hologenome wherein variation the host genome and/or associated microbiome can cause phenotypic change subject to selective or neutral forces (Theis et al. 2016). Margulis originally used the term holobiont to refer to a symbiotic compound of bionts, and this terminology is more widely used today to refer to the whole host-microbiome community without presuppositions of stability levels or interaction types (Bordenstein and Theis 2015).

The notion that symbiosis can spur new adaptations and evolutionary novelty is now commonplace. Symbiotic interactions span harmful, helpful, or harmless outcomes that can be environmentally dependent, transient, stable, and subject to mixed transmission modes spanning vertical, horizontal, or mixed inheritance modes. The relative influences of these varied parameters can change with the type and timescale of the system, but it is increasingly clear that host-microbe and host-microbiome aggregates are subject to neutral or selective forces under such varied conditions (Fitzpatrick 2014; Huitzil, Sandoval-Motta, Frank, & Aldana, 2018; Kopac and Klassen 2016). As such, there are several paths by which symbiosis can promote adaptation and speciation Brucker and Bordenstein 2012b; Miller et al. 2021; Telschow et al. 2005). For example, in herbivorous insects, families with obligate symbionts have 19-fold more species than families without obligate symbionts, suggesting nutritional symbioses drive spectacular diversification rates of insects (Cornwallis et al. 2021). In termites, a change in composition of digestive symbionts from gut protozoa to gut bacteria accommodated the evolution of new dietary niches by the family Termitidae, likely enabling it to become the most diverse family of extant termites (Bucek et al. 2019). In subspecies of Drosophila paulistorum flies, reproductive symbionts of the genus Wolbachia cause hybrid male sterility by aberrantly proliferating in the testes (Miller et al. 2010). These exiguous instances only touch on the multitude of ways that microbial symbionts can sculpt major evolutionary patterns and processes.

The symbiotic basis of reproductive isolation: Progress, patterns, and prospects in Nasoniawasps.

When discussing how microorganisms contribute to diversification of host macroorganisms, it is important to note that the requisites for defining different species are not complicated. Based on the Biological Species Concept that was originally derived during the modern synthesis, a species is defined as a group “of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups” (Mayr 1942). Dobzhansky is also credited with defining the concept; particularly, he emphasized the importance of reproductive isolation in three main categories: (1) premating isolation, (2) postmating, prezygotic isolation, and (3) postzygotic isolation (Dobzhansky 1937). Speciation is thus equivalent to the evolution of reduced interbreeding between populations or reproductive isolation.

To assess the relative importance of Margulis’ postulates on symbiosis and speciation, biologists can ask how often and by which mechanisms do symbioses contribute to host speciation? This is a large question without a vast literature, and it is thus in need of increased attention and unification across meetings, teams, lexicons, and literature (Miller et al. 2021). Here, we will focus attention on the exemplar system of four species of tiny, emerald green wasps in the genus Nasonia. Nasonia vitripennis diverged from a common ancestor of the three younger species about one million years ago, while N. giraulti diverged less than 400,000 years ago from N. longicornis and N. oneida (Raychoudhury et al. 2010; Werren et al. 2010). Each Nasonia species naturally harbors Wolbachia, a reproductive tissue bacteria that
is the most widespread endosymbiont in animals (Kaur et al. 2021). Multiple and often divergent Wolbachia strains naturally occur in each Nasonia species (Raychoudhury et al. 2009). Moreover, Nasonia harbor a gut microbiome, and the recent evolutionary relationships between the four host species are recapitulated in the relationships of the four gut microbiomes, a common pattern termed phylosymbiosis (Brucker and Bordenstein 2013; Kohl 2020; Lim and Bordenstein 2020; Song et al. 2020). In other words, more similar Nasonia species have more similar microbiomes, and this pattern holds across developmental stages and sexes of the four species (Brooks et al. 2016; van Opstal and Bordenstein 2019). In summary, the four closely-related and evolutionary young Nasonia species harboring reproductive endosymbionts and gut microbiomes make this wasp holobiont a well-suited model (Dittmer et al. 2016) for evaluating the Margulian perspective of symbiotic speciation.

**Endosymbiont-mediated postzygotic isolation and genomic evolution in Nasonia.**

Intracellular, endosymbiotic bacteria of the genus Wolbachia (Alphaproteobacteria: Rickettsiales) are the most widespread endosymbionts in animals. Wolbachia are primarily maternally inherited and estimated to occur in 52-60% of terrestrial arthropods and 52% of aquatic arthropods (Sazama, Bosch, Shouldis, & Ouellette, 2017; Weinert, Araujo-Jnr, Ahmed, & Welch, 2015), as well as various filarial- and plant-parasitic nematodes (Taylor et al. 2005; Wasala et al. 2019). Wolbachia commonly function as reproductive parasites in arthropods by manipulating sexual reproduction through selfish mechanisms such as cytoplasmic incompatibility, male killing, and parthenogenesis (Kaur et al. 2021), each of which enhance the maternal spread of Wolbachia throughout host populations. In Nasonia, these bacteria cause cytoplasmic incompatibility and can also (in)directly assist speciation by causing postzygotic reproductive isolation and evolutionary diversification of the wasp genome. Below we describe examples of these two illustrative processes related to symbiotic speciation.

### 1.1 Embryonic lethality by cytoplasmic incompatibility

In Nasonia, Wolbachia induce a host reproductive manipulation called cytoplasmic incompatibility (CI) in two basic ways. The first is unidirectional CI, which results in embryonic lethality when sperm from a male wasp infected with Wolbachia fertilizes eggs of a female lacking the bacteria. This gametic incompatibility results in embryonic death of diploid fertilized embryos (females in the haplodiploid system of Nasonia), while haploid males from unfertilized eggs are viable. The reciprocal cross of an uninfected male crossed to infected female is compatible, hence the term unidirectional CI. Moreover, CI is rescued in fertilized embryos containing the same bacterial strain (Yen & Barr, 1973), thus Wolbachia impart a relative fitness advantage to infected females that transmit the bacteria transovarially to their offspring. The fitness advantage from CI can be strong enough to deterministically spread Wolbachia-infected matrilines against uninfected ones (Turelli 1994).

The second main CI type is bidirectional when the male and female harbor divergent, incompatible strains of Wolbachia wherein reciprocal crosses yield embryonic inviability (J Dylan Shropshire, Leigh, & Bordenstein, 2020). In contrast to unidirectional CI, bidirectional CI notably can reduce or outright eliminate gene flow between species of Nasonia (Bordenstein, O’Hara, & Werren, 2001; Breeuwer and Werren 1990) because crosses in both directions suffer F1 hybrid lethality. Bidirectional CI between the allopatric species N. giraulti and N. longicornis importantly evolved before other reproductively isolating mechanisms between these two closely-related species, strongly supporting the model that CI-causing symbionts can principally cause reductions in gene flow between incipient species if and when they contact each other in eventual sympatry or in the laboratory (Bordenstein et al. 2001; Bordenstein and Werren 2007; Breeuwer and Werren 1990).

One interesting feature of CI-mediated reproductive isolation is that it can strongly prevent gene flow by causing F1 hybrid lethality, whereas the more conventionally studied nuclear-based incompatibilities often evolve from recessive genetic factors that typically are expressed in the F2 generation; thus, CI causes an earlier and more complete restriction of gene flow between populations or young species (Bordenstein 2003). Mathematical models also support the premise for the stable existence of two Wolbachia strains in parapatric host populations: the maximum rate of migration between populations while stably maintaining two Wolbachia strains is substantially high, justifying wider possibilities in which bidirectional CI can drive speciation in many host systems (Telschow et al. 2005). Thus in addition to CI acting at the F1 generation compared to recessive nuclear incompatibilities, bidirectional CI is also able to remain stable at much higher migration rates than nuclear incompatibilities, further advocating for bidirectional CI as a robust speciation driver (Telschow et al. 2005). Finally, even transient CI Wolbachia may seal off gene flow between host populations long enough for host genetic divergence to evolve as the principal cause of reproductive isolation.

Each of the four Nasonia species are double or triple infected with divergent Wolbachia strains from the A and B supergroups, totaling 11 unique strains: N. vitripennis is doubly infected with one strain from both supergroups, N. giraulti and N. oneida are both triple infected each with two strains from supergroup A and the third strain from
supergroup B, finally *N. longicornis* is infected with a single A supergroup strain and two strains from the B supergroup (Raychoudhury et al. 2009). Almost all of these 11 strains were acquired through independent horizontal transfer events, though it is notable that the B Wolbachia in *N. giraulti* and *N. longicornis* were acquired before the hosts’ speciation and have since co-diverged with their respective host (Raychoudhury et al. 2009). The species-specific A infections of *N. vitripennis*, *N. giraulti*, and *N. longicornis* were indeed acquired independently, and thus each strain diverged into distinct incompatibility types as seen in the bidirectional incompatibility amongst the infections due to an inability to rescue one another. Conversely, the natural co-divergence of the B infections of *N. giraulti* and *N. longicornis* is showcased by a difference in CI levels (Raychoudhury et al. 2009). Moreover, closely related B Wolbachia strains in *N. longicornis* can cause unidirectional or bidirectional CI against each other (Raychoudhury and Werren 2012), the outcome of which is dependent on interactions between Wolbachia and the host genotype. Thus, bidirectional CI can emerge within or between *Nasonia* species.

### 1.2 Diversification of the wasp genome by symbiont accommodation

Symbionts do not always directly cause reproductive isolation between host species, yet they may (in)directly assist reproductive isolation through a process termed host accommodation (Raychoudhury and Werren 2012). As hosts and symbionts may co-evolve in an arms race, for example, they have the potential to affect each other’s evolutionary fate. Selective pressures posed by the symbiont on the host can drive host genetic divergence that accommodates or controls the presence of the symbiont. This outcome is especially relevant for Wolbachia that can alter important aspects of host fecundity, survival, gametogenesis, embryogenesis, mitosis, and cytonuclear conflict. As such, Wolbachia may leave an evolutionary footprint of its presence via host genetic changes, some of which can hypothetically be maladaptive in hybrids of diverging species and potentially lead to reproductive isolation. Testing this prediction is not simple as it requires either fine-scale mapping of Nasonia genes that diverged in response to the Wolbachia symbiosis or multi-omic analyses of features that vary in the presence/absence of Wolbachia. Moreover, it’s difficult in practice to know if host accommodation genes evolved during or before the symbiosis. Below we detail two examples reflective of these types of investigations in *Nasonia* parasitoid wasps.

Each *Nasonia* species predominantly acquired their Wolbachia infections through independent horizontal transfer events leading to a variety of Wolbachia strains amongst the four sister species (Raychoudhury et al. 2009). Several of these Wolbachia lineages have been investigated to showcase the intricate relationships between host genotype and native endosymbiont. For instance, the native strain wVitA from *N. vitripennis* proliferates up to 100-fold higher in the sister species *N. giraulti*, which diverged about a million years ago from the most recent common ancestor (Chafee et al. 2011). The increased densities occur in reproductive and somatic tissues, the latter of which do not occur in the native host species. Notably, high bacterial densities affect the foreign *N. giraulti* host by reducing its fecundity and ability to discriminate against interspecific mates, while increasing levels of cytoplasmic incompatibility and male-to-female transfer of the Wolbachia (Chafee et al. 2011). Introgression of wVitA back into *N. vitripennis* returns Wolbachia density and host fecundity to native levels. Considering that both *N. giraulti* and *N. vitripennis* possess identical cytotypes when infected with wVitA, the variation in density and resulting phenotypes are attributable to interspecific variance in the host genome. These central findings indicate selective pressures likely shaped the *N. vitripennis* host genome to evolve suppression of the wVitA strain, with the question remaining - what host gene(s) evolved to enable suppression of Wolbachia?

Recent genetic analyses identified the maternal effect gene Wolbachia density suppressor (Wds) as one of two loci regulating Wolbachia wVitA densities (Funkhouser-Jones et al. 2018). Interestingly, Wds is a taxon-restricted gene under accelerated evolution and positive selection. Wds between the two species differs by ten amino acid differences, three of which are in a region estimated to be under strong positive selection (Funkhouser-Jones et al. 2018). Though involvement of Wds in reproductive isolation has not been evaluated, this study serves as proof of principle for host-Wolbachia interactions driving rapid evolution of natural variation - a precursor to speciation.

Wolbachia genes can also directly contribute to diversification of the Nasonia genome via horizontal gene transfer, e.g., insertion of Wolbachia genes into the nuclei of Nasonia (Hotopp et al. 2007; Pers and Lynch 2018; Werren et al. 2010). This is especially relevant for germline bacteria like Wolbachia whose DNA may aberrantly integrate into host genomic DNA for inheritance and evolutionary persistence. If the insertions from different Wolbachia occur in different host populations or incipient species, then host genomic divergence is underway that could lead to functional divergence and reproductive isolation.

### 1.3 Gut microbiome-mediated postzygotic isolation and hologenomic evolution in *Nasonia*

While Wolbachia that affect Nasonia gametogenesis and embryogenesis cause CI-mediated F$_1$ hybrid lethality, gut
bacteria have been shown in reproductive isolation assays to contribute to \(F_2\) hybrid lethality. Moreover, experimental evolutionary studies are a tool in this system to select for hologenomic divergence within species in which both the host genome and gut microbiome change, thus highlighting the capacity for selection to drive variation that may in theory set the stage for reproductive isolation to ensue. The interplay between host divergence and microbial community changes can also directly underpin the broader eco-evolutionary pattern of phyllosymbiosis that is widespread in plants and animals, including in *Nasonia* (Lim and Bordenstein 2020). Below we highlight two exemplars on the impacts of gut microbiomes on *Nasonia* holobiont divergence.

### 1.3.1 The gut microbiome and Nasonia speciation

Upon elimination of *Wolbachia* by antibiotic curing, *Wolbachia*-free *N. vitripennis* and *N. giraulti*, which harbor different gut microbiome compositions, readily produce viable and fertile hybrid \(F_1\) females. However, the \(F_2\) hybrid male offspring produced by these \(F_1\) females suffer catastrophic death: \(\sim 90\%\) of \(F_2\) hybrid males die during larval development in association with a hypermelanization response that is used by insects to encapsulate and kill pathogens (Bordenstein et al. 2001; Breeuwer and Werren 1995; Nakhlehe et al. 2017). Notably, the larval stage is a period of host consumption and digestion; hence it is the first intake of microbes (either from mother deposits or hosts) that may impact the assembly of the gut microbiome. All *Nasonia* species are typically reared in the lab on *Sarcophaga bullata* flesh fly pupae (Welren and Loehlin 2009a, b). The female wasp stings the fly pupa and oviposits her eggs through the puparium where they eventually develop within and hatch from. Thus, the majority of the *Nasonia* lifecycle, from embryo to early-stage adult, is carried out within the fly host environment, making it difficult to separate the conventionally-reared wasp host from its microbial gut symbionts (Whiting 1967).

An *in vitro*, germ-free, rearing protocol for *Nasonia* (Brucker and Bordenstein 2012a; J. Dylan Shropshire, van Opstal, & Bordenstein, 2016) made possible a discovery about the aforementioned \(F_2\) hybrid lethality. When reared in the absence of a microbiome, the \(F_2\) hybrid males of *N. vitripennis* and *N. giraulti* remarkably exhibit near-complete viability and thus rescue of conventional hybrid lethality that typically results in \(90\%\) death of offspring. Moreover, re-exposing germ-free \(F_2\) hybrids to specific native gut bacteria recapitulates the wild-type lethality (Brucker and Bordenstein 2013), supporting the premise that antagonistic interactions between the microbiome and host hybrid genotype causes the lethality. These results were the first to demonstrate that gut bacteria can serve as a cause of reproductive isolation.

Studying specific microorganisms present in the *Nasonia* gut microbiome and their microbe-microbe or microbe-host interactions reveals more details of the intertwined processes between gut bacteria and hosts. The intestinal microbiomes of male larvae from *N. vitripennis* and *N. giraulti* contain a dominant strain of the Gammaproteobacteria *Providencia rettgeri* and a less abundant strain of the related genus *Proteus mirabilis*. Notably, these bacterial abundances are inverted in \(F_2\) hybrid male larvae (Brucker and Bordenstein 2013), and *in vitro* co-culture of *Proteus* and *Providencia* demonstrates *Proteus mirabilis* can out-compete *Providencia rettgeri* during biofilm formation and make up more than \(90\%\) of the co-culture biofilm due to a unique swarming behavior (Brucker and Bordenstein 2013; Cross et al. 2021). As such, the *in vivo* and *in vitro* evidence supports a model in which *Nasonia* regulation keeps resident *Proteus mirabilis* numbers in check within species, whereas the \(F_2\) hybrids that catastrophically suffer from lethality lose this regulation, causing an imbalance in the gut microbiome and ultimately triggering hybrid lethality. Indeed, the *Proteus mirabilis* genome in parental *Nasonia* species and their hybrids are the same (Cross et al. 2021). Notably, the immune system is hyperexpressed in conventional as well as bacteria-inoculated larval hybrids that die (Brucker and Bordenstein 2013), suggesting the shift in resident gut microbiome dynamics causes or responds to a shift in *Nasonia* immune system expression. Quantitative genetic analyses demonstrate the *Nasonia* genome is also involved in the postzygotic isolation as \(F_2\) hybrid incompatibility factors in *Nasonia* map to several of the five chromosomes (Gadau, Page, & Werren, 1995; Gibson et al. 2013; Niehuis, Judson, & Gadau, 2008). Notably, the presence or absence of the gut microbiome and thus hybrid lethality associates with whether or not there are marker ratio distortions at these quantitative trait loci for hybrid lethality, which suggests a hologenomic interaction between the host hybrid and associated microbiome governs the \(F_2\) hybrid lethality (Brucker and Bordenstein 2013).

### 1.3.2 Hologenomic adaptation in response to experimental selection pressure

The *Nasonia* microbiome has been experimentally demonstrated to provide fitness advantages to its host. When artificial selection is applied to a population of *Nasonia* holobionts, the microbiome responds by rapidly adapting to the new environment, and this in turn exerts pressure on the host to handle the altered microbiome (Wang et al. 2020; Wang, Dittem, Douglas, Huang, & Brucker, 2021). Subtoxic levels of the widely used atrazine pesticide were used...
to induce a selective pressure on *N. vitripennis*. Remarkably within eight generations, the gut microbiome composition shifted in favor of bacteria members that efficiently metabolize the chemical, which in turn conferred atrazine resistance. Indeed, when the altered microbiome was transplanted to a control population, fitness was reduced, which implies the experimental *N. vitripennis* population endured a period of adjustment in order to tolerate the new and otherwise costly microbiome (Wang et al. 2021). Additionally, several *N. vitripennis* genomic regions were identified as having signatures of selection within the exposed population, though it cannot be discerned whether this selection came about from the atrazine exposure, the changed microbiome, or a combination of the two.

2 The margulian view of symbiotic speciation

Perhaps the most recognizable body of literature in *Nasonia* is on speciation, and that literature is in part a deep, honorary look into the past of Margulis’ conceptual creativity as well as a window into the future since *Nasonia* reveals the broader, unifying vistas by which symbiotic, genetic, and cytonuclear interactions can all potentially evolve to cause divergence in incipient species and initiate speciation (Wang et al. 2021). In this light, speciation biologists restricted to genomic regions were identified as having signatures of selection within the exposed population, though it cannot be discerned whether this selection came about from the atrazine exposure, the changed microbiome, or a combination of the two.

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