Trends in
Parasitology

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Review

Nasonia–microbiome associations: a model for evolutionary hologenomics research

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In recent years, with the development of microbial research technologies, microbiota research has received widespread attention. The parasitoid wasp genus *Nasonia* is a good model organism for studying insect behavior, development, evolutionary genetics, speciation, and symbiosis. This review describes key advances and progress in the field of the *Nasonia*–microbiome interactions. We provide an overview of the advantages of *Nasonia* as a model organism for microbiome studies, list research methods to study the *Nasonia* microbiome, and discuss recent discoveries in *Nasonia* microbiome research. This summary of the complexities of *Nasonia*–microbiome relationships will help to contribute to a better understanding of the interactions between animals and their microbiomes and establish a clear research direction for *Nasonia*–microbiome interactions in the future.

Highlights

Parasitoid wasps of the genus *Nasonia* are an emerging model for research on animal development, pest control, (co)evolutionary genetics, speciation, phylosymbiosis, and endosymbiosis.

The biological advantages of *Nasonia* wasps include rapid development, simple husbandry, haplodiploid genetics, functional and classical genetics, and interbreedable species.

Recent findings indicate that *Nasonia* endosymbionts and the gut microbiome are involved in interspecific reproductive isolation and thus speciation.

Nasonia is a model for research on animal–microbiome interactions

Microbiota exists both inside and on the surface of organisms. As a result of long-term coexistence with microbiota, various host physiological and biochemical functions (development, physiology, ecological interactions, and evolutionary diversity) are closely tied to microbes [1]. Thus far, many insect systems have been developed to study the microbiome [2]. Compared with mammals, insects are easy to rear, have short life cycles, large numbers of offspring, and few ethical concerns for research. This makes insects ideal systems to study and further develop research in host–microbiome interactions.

The parasitoid wasp genus *Nasonia* (also referred to as the 'jewel wasp') is a good model organism for insect development, evolutionary genetics, **speciation** (see Glossary), and insect–microbiome interactions [3]. The life history of *Nasonia* is divided into four stages [4]. Adult female wasps inject venom to kill the host fly and the wasps lay their eggs within the fly pupae. The egg, larval, and pupal stages of *Nasonia* wasps all develop within the fly pupae. The emerging adults mate and lay their eggs within fly pupae (Figure 1). In the process of growth and development, *Nasonia* is in a relatively closed system, and hosts are easily available commercially (Calliphoridae, Sarcophagoidea, *Musca domestica* L., etc.) for standardized rearing. In addition, the low cost of rearing, low resource consumption, large numbers of offspring, and the short (about 2 weeks) and non-overlapping generations contribute to *Nasonia* wasps being a well-established, laboratory-ready, and manageable insect [5]. The **diapause** period of *Nasonia* lasts for up to 2 years and can be influenced by various environmental factors, such as the photoperiod and temperature [6,7]. Diapause not only increases the lifespan of insects but is also convenient for research on the inheritance of adaptive traits because of the capability for long-term storage. The genomes of several *Nasonia* species (*Nasonia vitripennis*, *Nasonia longicornis*, and *Nasonia giraulti*) have been sequenced [8], and functional genetic tools of RNA interference (RNAi) and clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) are available [9]. These characteristics make *Nasonia* wasps an excellent genetic model organism for the study of evolution

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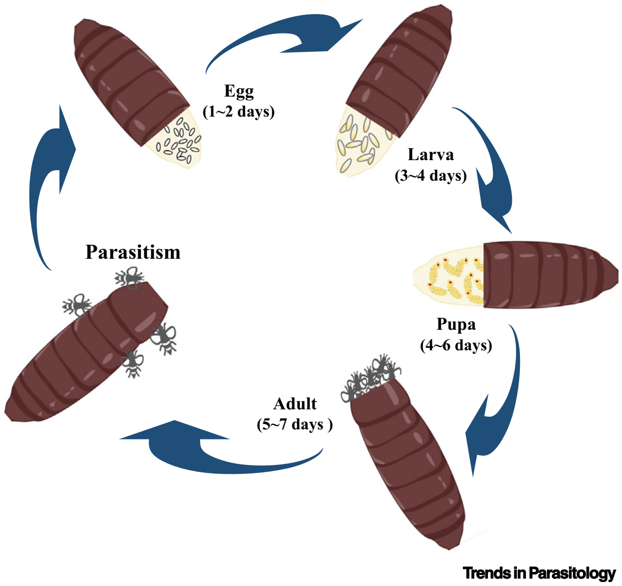


Figure 1. Life cycle of *Nasonia* wasps. The number of days indicates the duration of the corresponding developmental period of *Nasonia* wasps. Brown pupae are host fly pupae. Yellow caps on the pupae indicate the internal tissue of the developing flies inside the pupae. Shapes on the pupae indicate wasps at different stages of life.

and development [9]. Furthermore, *Nasonia* has a **haplodiploid** sex-determination system in which unfertilized eggs develop into haploid males, while fertilized eggs develop into diploid females [10]. This haploid genetic characteristic is amenable to effective genotyping, mutation screening, and evaluation of gene interactions (epistasis), without the increased complexity of genetic dominance [11].

The biological advantages of *Nasonia* detailed above have been critical to the significant research progress that has been made on insect biology using this model. Furthermore, **germ-free wasps** can be obtained through established protocols (Box 1), which makes *Nasonia* a dynamic model system to explore host–microbiome interactions [12]. Germ-free *Nasonia* wasps are essential for research on the effects of the microbiome on the host [13]. Using germ-free techniques, it was reported that the microbiome is involved in speciation and hologenomic evolution in *Nasonia* [11]. The *Nasonia* model system has already been used to investigate several important questions related to the function and evolution of the host-associated microbiome. Here, we present an overview of the significant progress in this system.

Microbial characteristics of *Nasonia* wasps

The bacterial diversity of *Nasonia* wasps has been described for larvae, pupae, and adults for the three *Nasonia* species *N. vitripennis*, *N. giraulti*, and *N. longicornis* [14]. Larval wasps feed on fly pupal tissue with a relatively simple microbial community. When wasps mature into pupae and adults, the diversity of bacterial species increases [4]. The overall bacterial diversity of the genus *Nasonia* is similar to that of most insects, with about 74.4% Proteobacteria, 15.7% Actinomyces, and 9.5% Firmicutes [15]. Analysis of microbial communities at different developmental stages of *Nasonia* wasps demonstrated that the two genera, *Providencia* and *Proteus*, were the most dominant operational taxonomic units (OTUs) observed in three *Nasonia* wasp species [4]. To explore the spatial distribution of bacteria, fluorescence *in situ* hybridization (FISH) of three adult *Nasonia* species was conducted. The FISH analysis indicated that the hindgut is the main organ that contains a high density of bacteria in the three species of adult *Nasonia* [15]. There has also been extensive study of the two main heritable symbionts, ***Wolbachia*** *pipientis*, and

Glossary

Cytoplasmic incompatibility (CI): a phenomenon that prevents sperm and eggs from forming viable offspring depending on the infection status of the parents. This effect is caused by changes in gamete cells caused by intracellular parasites such as *Wolbachia*.

Diapause: an ability induced by environmental conditions to temporarily stop embryonic development to avoid the effects of harsh environments.

Experimental evolutionary biology: an approach to the study of microevolutionary dynamics explored by studying populations that span multiple generations under controlled conditions.

Germ-free wasps: *Nasonia* wasps that are sterile or have microorganisms that are undetectable by existing methods.

Haplodiploid: a genetic system in which unfertilized eggs develop into haploid males, and fertilized eggs develop into diploid females.

Holobiont: the ecological community comprised of the host and all its symbiotic microbiota.

Hologenome: the entire genomic content of the host and the associated microbial community.

Phylosymbiosis: an evolutionary pattern in which evolutionary genetic changes in the host associate with ecological changes in the microbial community.

Speciation: the evolutionary process by which populations evolve to become distinct species.

***Wolbachia*:** an intracellular symbiotic bacterium that is widely found in arthropods and some nematodes. It can affect the reproduction and development of the host.

Box 1. The germ-free *Nasonia* system

Germ-free rearing technology has promoted the rapid development of research on insect microbiomes and human gut microbiomes [67]. A sterile animal body is defined as a special organism that does not have any microorganisms inside or outside the body, and microbes cannot be detected by existing detection methods [67]. Pasteur originally believed that germ-free animals could not exist in society or nature. But at present, sterile systems obtained by artificial methods in laboratories have been used on a large scale in mice and many insects (Figure I) [68]. The *Nasonia in vitro* sterile feeding system was first developed in 2012 and was optimized as an accessible technology in subsequent years, which has contributed to insights into the study of *Nasonia*–microbiome interactions and enabled the discovery of specific microbial functions in *Nasonia* [69]. The creation of germ-free *Nasonia* is done in two main steps: (i) sterilization of embryos and (ii) provision of sterile food for larvae *in vitro*. However, the *in vitro* culture process developed by Brucker and Bordenstein in 2012 introduced antibiotics and substances such as bleach and fetal bovine serum, all of which are potentially harmful to *N. vitripennis* [69]. Shropshire *et al*. used filter sterilization instead of antibiotics and removed residual bleach and fetal bovine serum to optimize the sterile feeding program, which did not affect larval growth and development [70]. Unfortunately, this approach still has some disadvantages, such as high workload and medium consumption, and leads to drowning, underfeeding or desiccation of embryos, larvae, and closed pupae [12]. Recently, the *Nasonia* rearing medium version 3 [12], created by Wang and Brucker, not only reduced the cost (replacing the 3 mm pore transwell polyester membrane with a polypropylene mesh sheet) and reduced media consumption (only 50 μl of fresh media per well per day instead of 250 μl) but also removed substances potentially harmful to *N. vitripennis* and further increased the survival rate of larvae to adults. This refinement of the approach contributes to the development of *N. vitripennis* as a model for microbiome research.

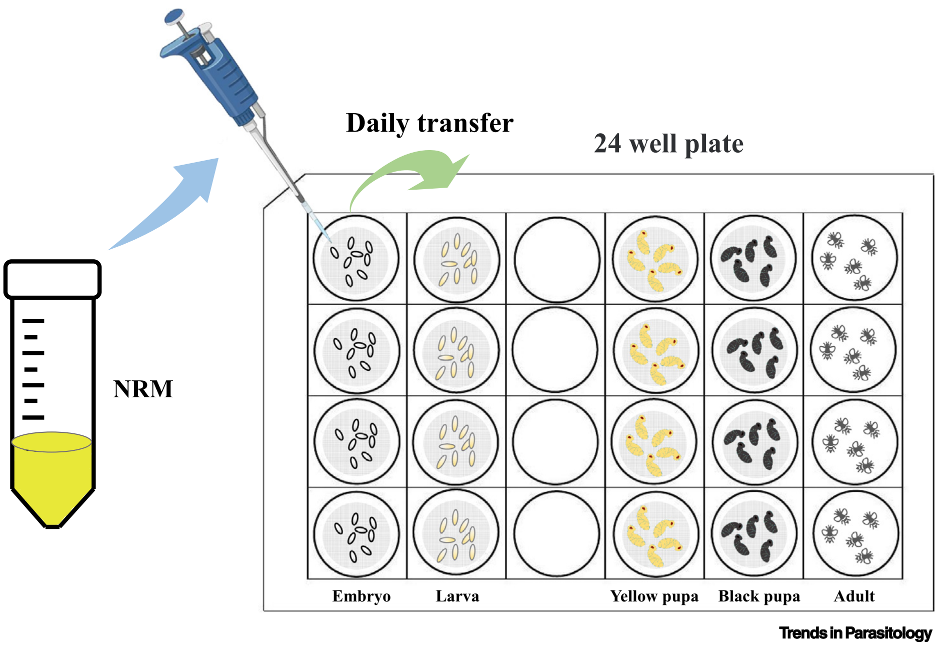


Figure I. Germ-free *Nasonia* feeding process. Sterile, bleach-treated *Nasonia* embryos (gray oval) are placed in 24-well plates to which are added configured *Nasonia* Rearing Medium (NRM, yellow). The organisms are then transferred into new wells with fresh NRM daily as the wasps develop. After around 16 days, germ-free *Nasonia* adults emerge.

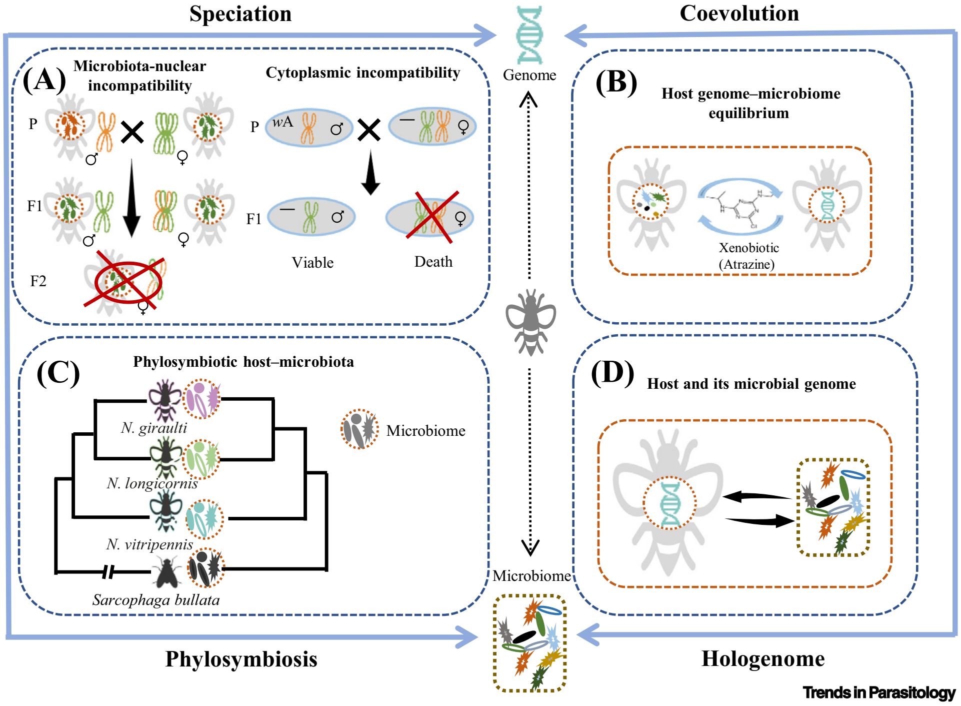
Arsenophonus nasoniae, currently found in *Nasnia* wasps. *Nasonia* wasps in the wild are systemically infected with *Wolbachia* and can also be coinfected with *A. nasoniae* [16]. *Wolbachia* can cause **cytoplasmic incompatibility** (CI, death of offspring in specific crosses depending on maternal and paternal infection status), and *A. nasoniae* can cause male killing (MK, specific death of infected male offspring) [17]. Their modes of transmission are different, with *Wolbachia* spreading through the ovary, while *A. nasoniae* has a great variety of transmission methods. Some strains of *A. nasoniae* are transmitted through the environment, some transfer the microbe to the egg surface, and others transfer via the egg cytoplasm by maternal inheritance [18].

The impact of the microbiota on the fitness of *Nasonia*

Host-associated microbiota studies have led to the understanding that microbes can influence many aspects of host fitness characteristics [19]. Due to advances in sterile wasp rearing and other biological research techniques, the roles of some microorganisms in the *Nasonia* wasp were identified, such as *Wolbachia*-mediated CI [20], microbiota-mediated pesticide resistance [21], and the role of microbiota on nutrient allocation during diapause [22]. In the subsequent text we discuss microbial influences on *Nasonia* fitness.

Wolbachia–Nasonia interactions

Wolbachia belongs to the phylum α-Proteobacteria, which exists widely in more than ten orders of 1.5 million to 5 million species of insects, including Diptera, Hemiptera, Homoptera, Hymenoptera, Lepidoptera, and Orthoptera, as well as rodents [23]. *Wolbachia*, broadly distributed in nature, often contributes positively to female fitness specifically because maternal transmission is its main method of host population spread [24]. At the same time, *Wolbachia* can manipulate the reproduction of its host (Figure 2A) [25]. Various *Wolbachia* strains may manipulate hosts in different ways, including CI, parthenogenesis (PI, asexual reproduction of females), MK, and feminization (genetic males physically develop and reproduce as females) [20]. Among them, CI is the most common type of reproductive manipulation by *Wolbachia* on hosts, and two types of CI



Trends in Parasitology

Figure 2. Recent progress in the study of *Nasonia*–microbe interactions. (A) Microbial-nuclear incompatibility and cytoplasmic incompatibility contribute to *Nasonia* speciation by preventing the generation of viable offspring between certain individuals carrying specific genetic elements. *w*A, *Wolbachia* carriers; –, *Wolbachia*-uninfected individuals. Different color chromosomes represent different genes in the cell nucleus. Microbiota wrapped in different colors represents individual microbial differences between species. (B) Coevolution of the host genome and its microbiota is demonstrable under multigeneration continuous exposure of *Nasonia* to low-concentration atrazine. Atrazine inside the circular bidirectional arrow leads to coevolution of the wasp genome with the wasp microbiome. (C) Three species of *Nasonia* exhibit a phylogeny that mirrors that of their microbiota, also known as phylosymbiosis (fly rearing host *Sarcophaga bullata* used as an outgroup). (D) Hologenome represents the sum of the host and its microbial genomes. Brown boxes indicate the genomes of all microbiota of an individual.

(unidirectional CI and bidirectional CI) have been identified. Unidirectional CI refers to the mating between *Wolbachia*-infected males and uninfected females, in which the fertilized eggs cannot develop [26]. In contrast, mating between *Wolbachia*-infected females and uninfected males can produce normal, viable offspring [27]. Bidirectional CI refers to mating between male and female individuals infected with different, incompatible *Wolbachia* strains, which produces cytoplasmic sterility [28,29]. In other words, male and female gametes infected with different *Wolbachia* strains fuse to form fertilized eggs that then exhibit varying degrees of viability [30]. The underlying cause of both unidirectional CI and bidirectional CI is impaired fertilized egg development caused by the endosymbiotic bacterium *Wolbachia*.

Previous work found that *Nasonia* species naturally carry 11 different *Wolbachia* strains from supergroups A and B. *N. vitripennis* carries two *Wolbachia* strains, while the other three *Nasonia* species (*N. longicornis, N. giraulti,* and *Nasonia oneida*) are triple infected (infected by three different *Wolbachia* strains) [31]. Fertilization barriers between species of the genera *Nasonia* with different *Wolbachia* strains are closely tied to the rapid formation of new host species in this clade. This speciation is due to the presence of intraspecific sperm dominance, which creates an interspecific fertilization barrier and leads to blocked gene exchange, thus facilitating the rapid formation of new species. For example, studies on *N. giraulti* and *N. longicornis* showed that when their respective *Wolbachia* strains were present, hybrids were largely sterile. However, when *Wolbachia* infection was cured with antibiotics, fertile hybrid offspring emerged, suggesting that *Wolbachia* in *Nasonia* is capable of causing reproductive isolation. This indicates that *Wolbachia*-induced reproductive isolation in *Nasonia* can impede gene flow between species, which is likely to occur at an early stage of species formation.

Microbiota mediate pesticide resistance

Some hosts and their associated ‘microbiomes’ are known to be so closely linked that they are often described as a ‘**holobiont**’ unit [32]. Wang *et al*. demonstrated that exposure to low concentrations of atrazine pesticide through multiple successive generations induced changes in the microbiota and that such changes conferred pesticide resistance to the host of *Nasonia* [21]. Experimental exposure to exogenous heterotrophs leads to an altered *Nasonia* microbiome that is maternally transmitted and contributes to atrazine resistance (Figure 2B). Once atrazine exposure induces heritable changes in the microbiome, the altered microbiome is unlikely to revert to the ancestral microbial community. This may be due to a lack of environmental reservoirs of the ancestral microbial community or a lack of selective costs of maintaining the new microbial community.

Two microorganisms (*Serratia marcescens* NVIT01 and *Pseudomonas protegens* NVIT02) from these resistant hosts were successfully isolated (Box 2) using microbial isolation and culture. Further experiments showed that atrazine resistance was conferred by the metabolic capabilities of at least these two rare bacteria [21]. This work used *Nasonia* as a model to show that microbial communities can be altered by external environmental stress and that altered microbial communities can help hosts to adapt to the stress. In addition, it has been previously demonstrated in agriculture that pest resistance is closely related to the microbiome [33]. A crucial question worth considering and solving in the future is whether continuous exposure to pesticides induces continuous changes in the microbial community and leads to the ensuing enhancement of drug resistance.

The role of microbial nutrient allocation during diapause

Nasonia wasps may undergo diapause for up to 2 years. This feature is not only useful for long-term preservation of samples but also helps to solve problems related to storage, transportation, and developmental control in practical applications of natural enemy insects [7,34]. It is known

Box 2. Isolation, culture, and localization of microorganisms

The process of isolation, cultivation, and localization of microorganisms is the basis for the discovery of new species, genes, and functions of microorganisms. These techniques and tools are indispensable for the study of *Nasonia*–microbe interactions. The main steps in the isolation and culturing of *Nasonia* microorganisms are as follows. First, a certain number of wasps are collected, and their surfaces are disinfected with 70% ethanol for 1 min. Then, 0.01M phosphate-buffered saline (PBS) is used for flushing, and the samples are resuspended in 20 μl 1*PBS. Finally, suspended individuals are homogenized and inoculated in the corresponding medium [21,71]. Cross *et al*. [71] used this method to isolate and culture the gut bacteria *Providencia rettgeri* and *Proteus mirabilis*, which are abundant in *Nasonia*. The characteristics of this new isolate show that *P. mirabilis* forms a stronger biofilm than *P. rettgeri*, and when growing in coculture, *P. mirabilis* outcompetes *P. rettgeri.* Similarly, Wang *et al*. obtained *N. vitripennis* strains resistant to pesticides by experimental evolutionary biology and isolated two rare bacteria that can metabolize atrazine, *S. marcescens* NVIT01 and *P. protegens* NVIT02 [21]. These recent examples reflect the applicability and importance of this research method. In addition, the most common strategy for microbial localization is fluorescence *in situ* hybridization (FISH) [72]. In this technique, a characteristic region of the 16S rRNA gene is targeted using species-specific fluorescent DNA probes to match the target species. This technique can help not only to longitudinally compare the relative abundance of different bacteria in the host [72] but also to visualize multiple bacterial species simultaneously through multiple fluorescent markers and observe their spatial relationships [73].

that insects accumulate a large number of low-temperature protective substances and energy substances such as carbohydrates, amino acids, and fats in advance during diapause. This accumulation is accompanied by metabolic inhibition, improvement of stress resistance, and expression of specific genes and proteins [6,35]. However, there are few reports on host–microbe interactions under diapause. To explore the effects of microbiome presence or absence on wasp nutritional status, Dittmer and Brucker [22] studied the role of the microbiome in host nutrient allocation in 4th instar non-diapause larvae, 4th instar diapause larvae, and after 1, 3, and 6 months of diapause under cold conditions (6°C). The nutrient levels of glucose, glycerol, and triglycerides between conventional and axenic non-diapausing and early diapause larvae are similar [36]. By comparison, it was found that the glucose level of conventional larvae increased by 2.5 times at the early stage of diapause, while that of sterile larvae decreased from 1 month after diapause. The glucose and glycerol levels of sterile larvae were significantly lower than those of normal larvae in diapause for 1–6 months. In addition, the protein level of conventional larvae was significantly lower at all time points except 1 month after diapause, while the protein level of diapause sterile larvae gradually increased with time, and the protein level of diapause sterile larvae was higher than that of conventional larvae after 3 and 6 months of diapause. In addition, triglycerides are usually low in conventional and germ-free larvae, and even lower after diapause begins [22]. These results indicate that microbiota plays an important role in the nutrition distribution, mobilization, and metabolism of *N. vitripennis* during diapause, especially when exposed to low temperatures [22].

This study was the first time a functional role was identified for the microbiome in nutrient allocation and mobilization during diapause. It was also the first demonstration that diapause has a transstadial effect on the microbiota, suggesting that the microbiota should be considered as an additional factor in determining diapause physiology [22]. However, the host fitness impact of microbiome alterations induced by larval diapause is unknown, which needs to be confirmed in future studies.

A single *Nasonia* gene suppresses *Wolbachia* density

Regulation of microorganism titer is based on factors such as host heredity, environment, and microbial interactions [37,38]. Given existing knowledge in the field of host–microbiome interactions and the plasticity of gut microbes, it is reasonable to believe that manipulating changes in the microbiota by certain means could allow us to predict microbial function and even help to control changes in host adaptive capacity and therapeutic approaches to related diseases [39]. For example, one potential application would be changing the abundance of specific microorganisms to observe changes in host fitness.

Maternal transmission of intracellular microbes is the key to the establishment of some long-term, intimate symbioses [40]. Effective maternal transmission and host reproductive control of *Wolbachia* usually depend on a sufficiently high *Wolbachia* density in the host [41], but *Wolbachia* overpopulation will affect host longevity [42]. Due to the difference in *Wolbachia* titer among closely related *Nasonia* species, Funkhouser-Jones *et al*. mapped two quantitative trait loci that inhibit bacterial titers through maternal effect by forward genetic, transcriptomic, and cytology. And the subsequent fine mapping and knockdown experiments identified the gene *Wolbachia density suppressor* (*Wds*), which mainly controls *Wolbachia* titers [40]. The discovery of this maternal effector gene proved that relatively small genetic differences between alleles of a single gene can govern the maternal transmission of a symbiont. This work further demonstrated that a gene that regulates bacterial maternal transmission underwent a simple genetic change through Darwinian selection in less than a million years. The maternal transmission of intracellular symbionts can also have a simple genetic basis, and natural selection can rapidly shape the evolution of density suppression of maternally effect genes symbionts in invertebrates [40]. Future research needs to focus on the specific mechanism by which this gene affects *Wolbachia* density and further explore whether this mechanism exists in other insect–*Wolbachia* symbioses.

Phylosymbiosis between *Nasonia* and the microbiome

Phylosymbiosis is defined as the ecological relatedness of host-associated microbial communities that parallels the phylogeny of host species and can be summarized as a host phylogenetic–microbial community relationship [3]. Previous work found that relationships between microbial communities reflect the phylogenetic relationships of their *Nasonia* hosts at multiple developmental stages (Figure 2C), and the composition and function of microbial communities are closely related to host evolution [3]. Sterile *Nasonia* larvae were exposed to heat-inactivated microbiota from conventionally reared intraspecific or interspecific larvae, and their resulting life history changes were measured through development and survival rates. Fitness among larvae exposed to intraspecific microbiota was higher than that in those exposed to the microbiota of other hosts. This work provides evidence that phenotypic selection pressure generated by host–microbiota interactions may underlie the pattern of phylosymbiosis in this genus [43]. Given the recognition of the significance and complexity of host–microbiome interactions, the term ‘holobiont’ is now frequently used to refer to a host together with its microbial symbionts, while the ‘**hologenome**’ encompasses the genomes of the holobiont (Figure 2D). In addition to host nucleus interactions and host gene–environment interactions [44], gene–microbe interactions with beneficial members of the symbiotic gut microbiota can cause severe lethality in interspecific hybrids of *Nasonia* larvae (Figure 2A) [11]. We can therefore understand the systemic symbiotic microbiome as complementary to the coadaptive genome of the host organism, rather than an arbitrary amalgam [11]. This also supports the idea that the microbiome and host genome represent a kind of coadaptive ‘hologenome’.

Many studies have demonstrated that host genetics and gut microbes are not fully independent, and instead have extensive and complex interactions with each other [45]. The ‘hologenome’ theory provides us with new perspectives and paradigms to understand organisms (Figure 2D): all plants and animals are ‘holobionts’ composed of a host and related microorganisms, and their symbiotic, cometabolic and coevolutionary relationships can jointly influence host traits [46]. By exposing *N. vitripennis* to atrazine for 85 generations, Wang *et al*. [21] proved that the pesticide not only mediated the adaptive changes of microbiome and made the host acquire new traits (atrazine resistance), but also induced the selection pressure of the host genome and changed the gene expression and immune response of the host (Figure 2B). For instance, the candidate genomic regions with signatures of selection in the atrazine-exposed population were identified [13]. It cannot be discerned whether this selection is due to the atrazine exposure, the changed microbiome, or a combination of the two. The microbiota of the atrazine-exposed group was transplanted to the control group (Box 3), resulting in a significant decrease in the survival rate of the *Nasonia* [21]. These results imply the *N. vitripennis* population endured a period of adaption to tolerate the new microbiome and form a new host–microbiome equilibrium [13]. This work validates host–microbiome coevolution using experimental evolution methods in *Nasonia* for the first time and further advances the development of holobiont evolutionary dynamics. Nonetheless, current symbiotic total genomics studies mostly focused on simple representative organisms, and there are comparatively fewer systematic studies and clear experimental evidence in mammals. Symbiotic total genomics studies in other organisms will be an important research direction.

Similarly, studies on systemic symbiosis in *Nasonia* have demonstrated that the composition and function of the microbial community are closely related to host evolution [3]. How host microbiota aggregate and evolve is one of the prominent questions in microbial ecology. Research on this question has repeatedly demonstrated a model of phylosymbiosis, which can also be expressed as phylogenetic signals (the tendency for related species to resemble each other) in host-phase microbial composition [47]. Simply put, the higher the phylogenetic signal, the higher the host–microbial phylogenetic ecological correlation. This pattern suggests that phenotypic selection pressure caused by host–microbiome interactions may be one potential basis of systemic symbiosis [43]. In terms of viruses, Leigh *et al*. [14] discovered a complete phylosymbiosis between virus metagenomes and insect phylogeny by sequencing the metagenomes of purified virus communities from three different parasitic wasp *Nasonia* species, one cytonuclear introgression line of *Nasonia*, and the flour moth outgroup *Ephestia kuehniella*. It reveals the first complete evidence for phylosymbiosis in viral metagenomes, which extended phylosymbiosis beyond the gut bacteria to the level of the host virome. In summary, these *Nasonia* phylosymbiosis studies provide new evidence for the concept of ‘hologenome’, suggesting that species can evolve guided by host–microbiome interactions in the presence of specific phenotypic traits, and this process is enhanced by natural selection [48]. However, some scholars believe that the microbiota cannot be inherited by the next generation entirely, and the hologenome cannot be used as an independent unit of natural selection in the process of speciation. This is the reason why this concept is controversial, but it can be explored in future studies using **experimental evolutionary biology** methods [21]. This approach plays an important role in the enrichment and development of the unified theory of evolution.

In recent years, phylosymbiosis has been reported in other insects, such as cockroaches, termites [49], mosquitoes [50], cephalopod turtle ants [51], and honeybees [52]. Studies of these insect symbionts have found that phylosymbiosis may arise due to stochastic and/or deterministic evolutionary and ecological forces [53]. For example, changes in survival may arise due to the

Box 3. Microbial transplantation

Microbial transplantation is a powerful biological validation method for the study of *Nasonia* systemic symbiosis and the mechanisms of microbiome–host interactions. Using *Nasonia* wasps as a research model, the main steps of microbial transplantation are as follows. First, sterile *Nasonia* wasps are reared using the *in vitro* rearing system. Microbes are purified from *Nasonia* by filtration through a filter and centrifugation. Then the pellet is suspended in a sterile PBS solution and fed to the recipient host [3]. This microbial transplantation method can verify the roles of microbes in the host and is widely used in the study of host–microbiome interactions. For example, this research approach can shed light on the developmental and survival costs of *Nasonia* exposure to interspecific microbes [43]. In addition, microbial transplantation has been used clinically in humans for the treatment of many diseases [74]. In the future, there will likely be more and more research of microbiome–insect interactions using this method.

random dispersal of microbial communities or host migration. Phylosymbiosis can also be formed through changes in the ecological [54] and dietary [55] ecological niches of hosts. It defines a link between host evolutionary relationships and microbial diversity that can be quantified and applied to the whole living system [53]. Darwin's theory of evolution emphasizes that individual differences will form higher-level differences after a long period of accumulation, such as the emergence of reproductive isolation, which leads to population differentiation, followed by speciation. However, Darwin's theory emphasizes the role of natural selection in the accumulation of variation, and modern biological research has found that host evolution processes (such as speciation) are more affected by microbial symbiosis than commonly thought [4]. When external selective pressure acts on the host in the holobiont theory, the microbiome can respond by rapidly adapting to the new environment, which in turn creates new pressure on the host to adapt to the altered microbiome [13]. Although it is controversial, some results suggest that microbiota play an important role in evolution. The host's fitness in a specific environment is not always the only determining factor in its evolutionary path since the microbiome may be able to help the host acquire new traits by responding to the external environment itself. This microbial adaptation may confer a high level of phenotypic diversity at the host population level. Therefore, the study of phylosymbiosis contributes significantly to the understanding of host–microbiome interactions, and future causal studies of phylosymbiosis will be a bridge to further our understanding of its evolutionary, genetic, and molecular bases.

These findings enrich the phylogenetic relationships of *Nasonia*, underscore the importance of host evolutionary relationships in microbiota composition, and provide valuable insights regarding host–microbe interactions [56]. Also, in *Nasonia*, the study of *Wolbachia* provides basic theoretical support for its reproductive manipulation and application to infectious disease control. *Nasonia* was first described in 1948 and has been studied for many decades since [57]. We summarize the above representative *Nasonia*–microbe research results in Figure 3.

Concluding remarks

In light of the ethical and practical limitations of rodents and other mammalian models, insect model systems offer a plethora of alternative options for experimental biological research. In addition to *Nasonia*, many insects have been widely used in the field of host–microbiota interactions. For example, *Drosophila* is an excellent model with established techniques for genetic manipulation of the host and representative commensal bacteria due to its easy laboratory manageability, short generation time [58], and the production of fertile germ-free *Drosophila* models [19]. In addition, the various biological advantages of honey bees and their great economic value have led to extensive research [59], and their simple and specific gut microbial community facilitates microbial *in vitro* culture as well as microbial backfill experiments in sterile individuals [60]. Further, mosquito-borne diseases such as malaria, dengue, yellow fever, zika, and chikungunya are still rampant in many parts of the world [61]. Using highly diverse and dynamic mosquito microbiotas, efforts have been made to develop new safe and efficient tools such as *Wolbachia* to combat various mosquito-borne diseases [62]. The most common models (fruit flies, bees, mosquitoes, etc.) of insect microbiome research have been studied either for their biological advantages or for their applications, but these models also have limitations. Even for *Drosophila*, which is currently very well studied in various fields, there are still some microbial genetic transformation methods that have not been developed [2]. Similarly, the endoparasitic nature of *Nasonia* underlies the inability to pass on sterile individuals, and limitations of host manipulation during developmental stages also limit the field of research.

More and more studies suggest that these symbiotic microbes are not just simple 'passengers' in the host, but play a key role in physiology, including immune response, behavior, metabolism, and diseases [63]. The ongoing discovery of the functions and consequences of the microbiome

Outstanding questions

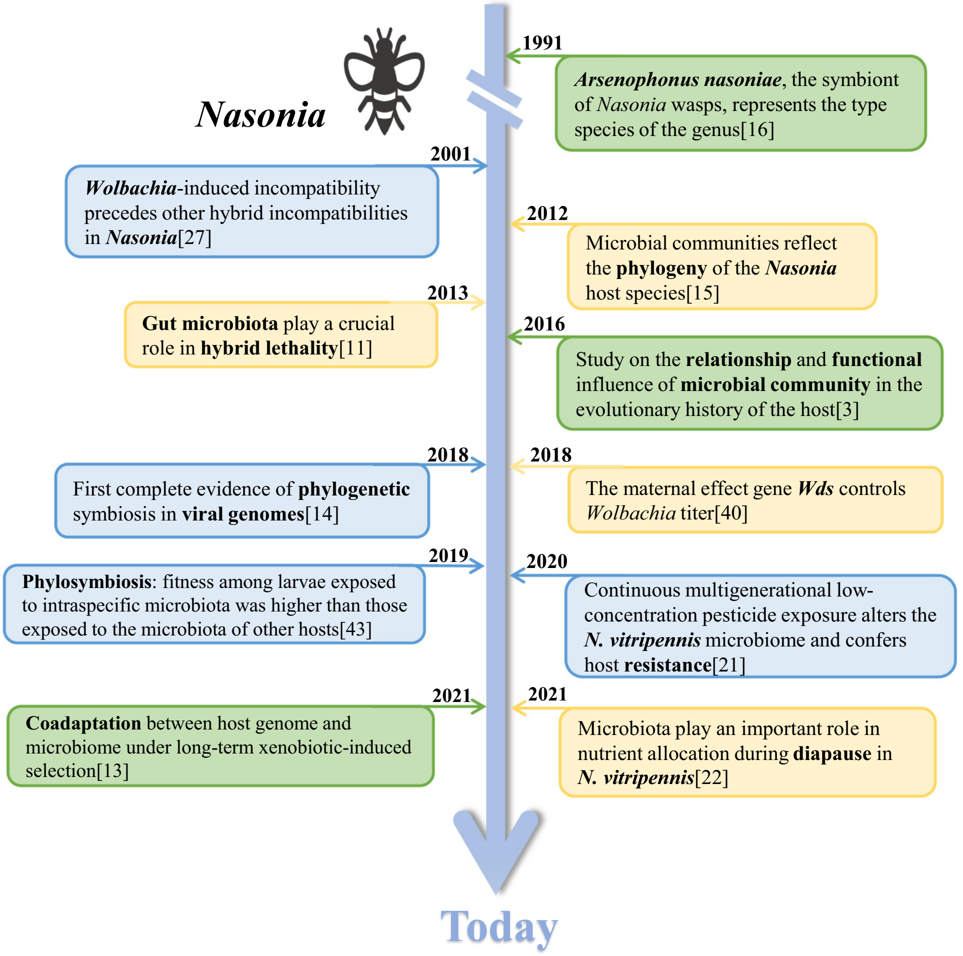
Can the *Nasonia* microbiome rapidly assist and/or complete speciation?

How do interactions among members of the microbial community impact the symbiotic outcome of individual members of the microbiota?

What are the functions of the different microbes in *Nasonia*?

Which factors principally drive the composition and evolution of the *Nasonia* microbiota?

What is the role of fungus in *Nasonia*? Do fungi contribute to host metabolism, or can they alter the phenotype and/or fitness?



Trends in Parasitology

Figure 3. Research history of *Nasonia*–microbe interactions and major research advances. Each box summarizes an important finding from a publication on *Nasonia* that has contributed to our understanding of host–microbe interactions. See also [3,11,13–16,21,22,27,40,43].

forces us to take a systematic approach to the host–microbe system as a whole [64]. This approach corresponds to another bold concept: the holobiont, a new entity composed of thousands of microbes and their host whose collective genomes are called the hologenome. Holobiont theory is still very controversial [65,66], and *Nasonia* is an excellent system in which researchers can perform experimental evolutionary biology experiments. Such studies heretofore have demonstrated that reproductive isolation and speciation can be achieved by exogenous selection pressure. However, research on *Nasonia* microbiota function, evolution, and speciation remains to be advanced (see Outstanding questions). Research on *Nasonia* microbes requires further advancement, and new research directions need to be developed to fully exploit this excellent model organism and make a continuing meaningful contribution to basic theories of the life sciences.

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Author contributions

All authors developed the scope and focus of the review and contributed to the writing of the manuscript.

Declaration of interests

There are no interests to declare.

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