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Author: Wang, Y.

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Chapter 1

Introduction

Overview on insect microbiomes

The microbiome of animals consists of the community of microbes that colonize host organisms, including the complete set of commensal, symbiotic and harmful species (1–3). This ecological community has long been known to affect host biology, and their diverse roles have been further clarified in recent years following numerous studies of animal:microbiota interactions in diverse systems (4–6). The models used to study host-microbiota interactions have covered a broad range of animal taxa, such as nematodes, bobtail squid, insects, zebra fish, mouse and human beings, among others (7). Using these models, scientists are drawing on interdisciplinary approaches and techniques across ecology, bioinformatics and biomedical science to gain deeper knowledge about the biological significance of host-microbe symbioses.

Insects are the largest animal group on earth, and feature varied symbiotic associations with microbial species (8). Insect microbiota colonize the external surface and also reside internally in specialized compartments of insects, such as the external carapace and the bacteriocites inside the body (9–11). The intestinal gut tract provides an ecological niche for microbes. Bacterial densities within insect guts differ broadly across host species ranging from 10^5 - 10^9 cells per gut, with species like the fruit fly *Drosophila* containing a bacterial density of about 10^5 bacteria (9, 12). The guts of some sap-feeding aphids may even be sterile (13). In contrast to the rich microbial diversity in vertebrate guts which can contain > 500 taxa, most insects harbour relatively limited bacterial diversity (14, 15). For example, there are only 1~30 taxa of microbes found in the gut of *Drosophila* (16). By contrast, some eusocial insects i.e. honey bees and wood-feeding termites, contain a more diverse community of bacteria within their gut microbiota, with more than 300 and 367 identified phylogenetic clusters, respectively, in worker honey bees and lower termites (7, 12, 17, 18). It has been suggested that eusociality may enhance opportunities for microbiota transmission between colony members, and thus promote a diverse gut microbiome. In spite of their limited diversity, insect microbiota

can have dramatic effects on their hosts, in terms of nutrition, development, immune response, morphogenesis and behavior, among others (19–23). And in return, these effects might also influence the colonization and composition of insect microbiota (12, 24–26).

For example, the honey bee gut microbiota can promote host physiology by increasing host weight, hormonal signaling and sucrose sensitivity (27). The fungus *Beauveria*, which resides in the breeding environment of dung beetles *Euoniticellus intermedius*, stimulates the immune response by triggering Toll signaling of beetles to fight against microbial infections (28). Other microbial symbionts can modify animal behaviour. For example, the bacteria *Proteus mirabilis* can produce volatiles to attract blow flies, who can transport this bacteria to new food resources; in return, blow flies use the chemical cues from bacteria *P. mirabilis* to locate food resources (29). In addition, some microbes residing in termites guts can help the host to digest lignocellulose and thus play essential roles in nutrient metabolism (30). Other bacteria from *Lactobacillales* and *Acetobacteraceae* activate the TOR pathway in *Drosophila*, which regulate the hormonal signals involved in molting (31). In addition, insect microbiota have been also reported to influence macro-evolutionary processes of hosts by promoting the divergence of host lineages and speciation. For example, symbiont-mediated changes in host behavior, e.g. mate choice, may lead to host reproductive isolation (32). In addition, the long-term colonization of microbiota within hosts can result in host-microbiota co-speciation over millions of years (seen as congruent phylogenies), which has been reported in many insect species, such as aphids, bees and mealybugs (33–35). For instance, Moran (36) has described the co-speciation between sap-feeding aphids and their obligate nutritional mutualist *Buchnera*, and suggests that their symbiotic association began more than 50 million years ago (37). Despite these wide-ranging influences, the effects of insect microbiota may be broader still, and more studies are needed to explore the diverse roles of the microbiota on hosts with different life-histories, which will enable us to better understand general and specific features of insect-microbiota ecology.

In this thesis, I will shed light on the ecological interactions between the burying beetle, *Nicrophorus vespilloides*, and its gut microbiota. I will investigate the potential mechanisms underlying the transmission and colonization of gut microbiota of this species. In addition, I will examine some

of the effects conferred by the beetle's microbiota on its ecology. My research highlights the association between host behaviour and gut microbiota ecology.

Transmission and colonization of insect gut microbiota

Insects can acquire their microbiota through either vertical or horizontal transmission. Vertical transmission refers to cases where parents transmit their gut microbiota to their offspring directly via the egg or egg coat, while insects that rely on horizontal transmission acquire their microbiota from the environment, including from other individuals (38, 39). However, some insects fall in the middle of these strict extremes. For example, honeybee workers *Apis mellifera* acquire and establish their gut communities via different routes including mouth-mouth or anal-mouth transmission between nest members (trophallaxis), and fecal consumption from the environment (coprophagy) (40, 41). In addition, the modes of microbiota transmission can vary significantly even among closely related insects. For instance, many stinkbugs initiate the vertical transmission of their core microbiota by excreting anal secretions to either the egg surface or offspring larvae (42). In contrast, the stinkbug *Riptortus clavatus* acquire their beneficial symbiont *Burkholderia* obligately from the environment (43). A similar mode of transmission might be used by the stinkbug *Megacopta punctatissima*. If the new born nymphs receive no parental provisioning for the gut symbionts, they will show more wandering behaviour which potentially facilitates the acquisition of their gut symbionts (44).

During insect gut microbiota establishment, different insect life styles, e.g. solitary or eusocial, can be an important factor facilitating the transmission and thus the composition of gut microbiota. For example, *Drosophila melanogaster* replenish their obligate symbionts via food consumption (45); bumble bees (*Bombus terrestris*) obtain their gut microbiota via contact with nest mates via trophallaxis, and reduced association with nest mates will result in an alteration of their gut microbiota (46). Although the association between host behaviour and microbiota transmission varies through different systems, host behaviour and microbiota transmission could co-evolve for the benefits of both sides of host-microbiomes (47, 48). In this thesis, I will investigate the transmission mechanisms of *N. vespilloides* gut microbiota during development, and demonstrate how this is associated with host social behaviour and developmental transitions.

Factors that influence the insect gut microbiota

Multiple factors influence the establishment and subsequent maintenance of the insect gut community. Any change of these factors, such as diet, habitat, social interactions and microbial or parasitic infections to insects can alter colonization dynamics and shift the gut microbiota in density and/or diversity (49–51). Another important factor that influences the microbiome is development (20). Holometabolous insects go through several molting stages during their development from eggs to adults that are usually accompanied with significant changes to gut structure and condition (52, 53). Insects with a complete metamorphosis undergo 4 stages of development in general, including egg, larva, pupa and adult. For some insect species, e.g. moth, butterfly and *Coleoptera* beetles, they usually undergo a pre-pupa and an adult-eclosion stage before and after the pupa imago (54–56). Hemimetabolous insects with incomplete metamorphosis go through a transition simply from egg to nymph then to adult, and there is no pupal stage during this metamorphosis (52, 53). Despite the differences in metamorphic types, both hemimetabolous and holometabolous processes require that insects shed their exoskeleton during the molting stage and this includes the lining of the fore and hind gut epithelium (57, 58). Thus, over the course of development, the entire intestinal tract is shed during the process (57, 59). The reformation of the adult intestinal tract prior to adult eclosion results in a series of changes in gut physiology and contents, such as the size of epithelium cells and the activity of metabolic enzymes (12, 60, 61). These physiological changes potentially result in alterations to the insect microbiota in both abundance and structure (12, 62). For example, in the newly emerged adult mosquito *Culicidae*, a nearly complete clearance of bacteria is found in the mid-gut (63). Similarly, a molting mediated reduction of gut symbionts has been also found in bean bugs *Riptortus pedestris* (64). By contrast, gut bacteria persist through stages of housefly development (65). Hence, while developmental shifts can alter gut microbiota dynamics, the affects are neither universal nor predictable. One of the aims of this thesis is to quantify the dynamics of gut bacteria across *N. vespilloides* developmental stages via profiling of cultured microbes, and to illuminate how microbiome dynamics are influenced by host metamorphosis and behaviour.

In addition to host and environmental factors, microbial interactions within the gut could also mediate the variation in composition and abundance

of gut microbiota, and thus play an important role in their maintenance and stability. Coyte et al (2015) suggest that bacterial competition within the gut could increase gut microbiota stability as compared to bacterial cooperative interactions. Their model suggests that hosts could interfere with the interactions taking place between members of their microbiomes and thereby manipulate the microbial communities to their benefit (66). Another example from *Bombus* bees found that the richness of non-core microbiota negatively associates with microbiota abundance in the gut, which also suggests the structure of gut microbiota might be facilitated by host-influenced microbial interactions within the gut (67). Despite the understanding that both insect development and microbial interactions could influence the composition and the maintenance of the gut microbiome, few studies have shown the details about how the colonization of gut microbes changes throughout the entirety of insect development, which is important if we are to comprehend how the microbiota persist.

Colonization resistance of insect gut microbiota

While the microbiota can be helpful to insects, insects will still encounter harmful microbes that can be pathogenic. These harmful species can infect insects and possibly colonize their guts. Such colonization may result in bacterial competition within the gut between resident species and potential pathogens and may cause community-level changes to the gut microbiota (67). Insects have evolved diverse strategies to overcome threats from such harmful microbes. As one of these strategies, colonization resistance has been observed in many animal gut communities. Colonization resistance is a mechanism whereby resident microbiota resist against subsequent microbial colonization, including pathogens, following exposure. The endogenous microbiota of insects such as sand flies, silk worms and desert locusts, help their hosts to resist against pathogen colonization of the gut (68–70), and the ability to resist challenge may scale with the diversity of the gut microbiome (71). For example locusts, *Schistocerca gregaria*, with more diverse gut symbionts are better able to reduce the density of the pathogen *Serratia marcescens* during experimental colonization of the gut (70). Colonization resistance is known to protect hosts from microbial infection and play an important role in the stability of the host gut microbiota and thus host health (72, 73).

The mechanisms of colonization resistance vary in different host systems

and can be driven by either direct or indirect factors. In some cases of colonization resistance, the resident microbiota can directly compete with foreign microbes for the gut niche or inhibit them directly by producing bacteriocins or antibiotics (74). For other cases, resident species mediate host immunity that initiate a type of indirect resistance against the colonizing microbes. These have been found in both mammals and insects. For instance, the native gut microbiota in honey bees have been reported to induce the expression of antimicrobial peptides (AMPs) in host gut tissue (75).

Colonization resistance can also be influenced by two ecological factors: priority effects and specificity effects. Priority effects in gut communities suggests that the first bacterial colonizer within the gut persists challenge by virtue of being there first, which allows it to shape the inner intestinal environment and influence the establishment of later communities (76). Conversely, specific effects indicates that some gut bacterial species are competitively superior in a given host no matter what the colonization sequence is (77). In Chapter 4 of my thesis, I will examine if colonization resistance of bacteria occurs within the *N. vespilloides* gut, and I test which of these two ecological factors most contribute to *N. vespilloides* gut microbiota transmission and colonization, and further how these affect host fitness.

Nicrophorus vespilloides

The burying beetle *Nicrophorus vespilloides* (Coleoptera, Silphidae) is a holometabolous insect, which undergoes a complete metamorphosis (Figure 1). After hatching, larvae transition through several larval moults, which is followed by pupation and then eclosion as an adult. Beetles are reared on decomposing carcasses of small mammals or birds that are detected by breeding adults using volatiles emitted from the carcass (78, 79). These beetles evolved sophisticated parental care behaviors during breeding which is usually divided into two phases: pre-hatch care and post-hatch care (80). Pre-hatch care starts before oviposition, and consists of a series of manipulations that prepare the carcass for burial and the arrival of larvae. Adult beetles first bury the carcass into a shallow grave, then strip off the fur and roll the carcass into a ball, after which they open a hole on the carcass abdomen for access to the offspring (81, 82). At the same time, parental beetles cover the carcass with oral and anal secretions, containing a lysozyme-like compound as well as other compounds that are used to defend against bacterial and fungal competitors

(81, 83). Beetle eggs are laid nearby the carcass, and newly hatched larvae migrate to the prepared carcass for development. After larvae arrive to the carcass, parental beetles continue to provide post-hatch care to their offspring by defending them from other insect predators and feeding the larvae through direct regurgitation (Figure 2A) (79, 80, 84). In the lab, approximately 7 days post-hatching, individual larvae disperse from the carcass and then construct a chamber for pupation. The whole period of pupation usually takes around 2-3 weeks (Figure 2B, C), after which the newly eclosed adults emerge from soil. The entire duration of larval development lasts around one month, but its length varies through seasons in nature (85, 86).

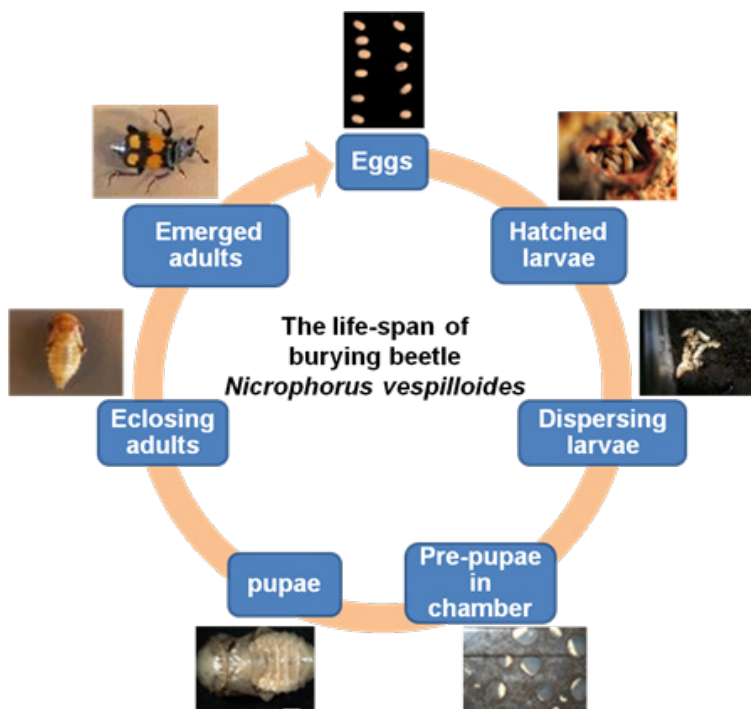


Figure 1. The life-span of burying beetle *N.vespilloides*.

Interspecific interactions across *N. vespilloides* development

Burying beetles *N. vespilloides* associate with diverse species during their life span, including microbes, nematodes and phoretic mites. Beetles feed and reproduce on carrion, and thus are exposed to and compete with diverse communities of bacterial decomposers (79, 87–89). Previous work has shown

that beetle larvae are harmed during these interactions and also that parental pre-hatch care and up-regulated immunity can partly reduce the threats from potentially pathogenic microbes (83, 88). In this thesis I examine the further protective role potentially provided by the beetles' gut microbiota. In addition to microbial interactions, Sloan Wilson examined the associations between *N. vespilloides* and phoretic mites, and shed light on the ecological effects of mites on beetle biology (90). Kilner further examined the diverse species of *N. vespilloides* associated mites and extended knowledge about their influence on the parental care of *N. vespilloides* (91–93). Although nematodes have been reported in association with *N. vespilloides* (94), we still lack an understanding of their effects on beetles social ecology.

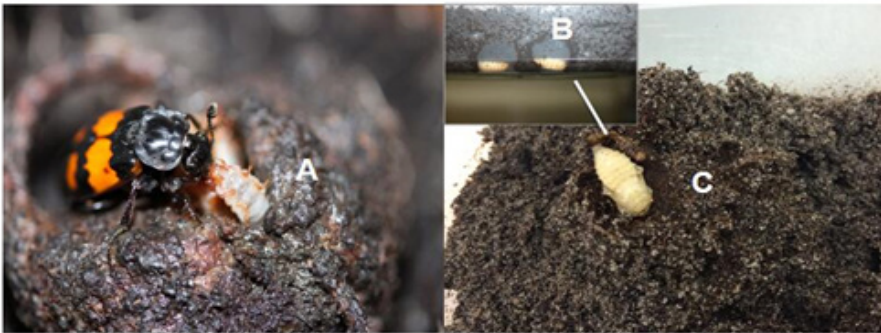


Figure 2. Morphology of the burying beetle *N. vespilloides* in larval and pupa stages of development. (A) parental regurgitation to larvae. (© Per T Smiseth); (B) larval beetles during pupation in their chambers; (C) beetle pupa inside the chamber.

Bacteria: Small vertebrate carcasses utilized by *Nicrophorus* for breeding become populated by saprophytic and pathogenic bacteria and fungi, which can harm *Nicrophorus* beetles and compromise their development and health (81, 88, 95). *Nicrophorus* eggs are initially laid nearby the carcass where microbial densities can be quite high due to nutrient pools that accumulate from the carcass (79). Studies have shown that competition with microbes from highly decomposing carcasses (aged carcasses) reduce both brood size and larval mass of *N. vespilloides* (88). In response, *N. vespilloides* reduces and avoids these threats via diverse antimicrobial strategies. For example, the direct fight against carcass derived microbiota via personal or social immunity occurs during offspring development (83, 96). Parental beetles prepare the carcass and continuously apply exudates, including e.g. lysozyme

onto the carcass surface (83, 88). Meanwhile, offspring larvae contribute to the social immunity by secreting their own anal secretions onto the carcass (83). At present there is limited understanding of how parental manipulations of the carcass influence the composition of the carcass bacterial community (84, 97). In addition, we lack an understanding about the potential interactions between the carcass microbiota and the endogenous gut symbionts of the beetles. The endogenous gut communities of *N. vespilloides* predominantly consist of *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (89). Recent research from Kilner's group, consistent with the work presented in Chapter 3 of my thesis, shows that the most abundant bacterial taxonomic units from both adult and larval beetles are related to *Providencia spp.*, *Morganella morgani*, *Proteus spp.*, *Vagococcus spp.*, *Clostridium spp.* and *Neisseria spp.* (97). These results, based on sequencing, are consistent with my data using culture-based approaches. In my thesis, I look at the dynamics of *N. vespilloides* gut microbiota colonization and clarify when and how parental beetles transmit their bacteria to larval offspring. The potential benefits of endogenous bacteria in *Nicrophorus* will be also examined.

Mites and Nematodes: Phoretic mites and nematodes can be found in association with many insects and in many contexts (98, 99). Phoretic species hitch a ride on insect hosts for transmission and dispersal to new resources (100). *N. vespilloides* associates with many different mite species in nature (90). Notably, these mites species vary from harmful to neutral or even beneficial to *N. vespilloides*, depending on the density and species of mites (90, 92). A recent study indicates that the association with *Poecilochirus carabi* mites interferes with both parental behavior and larval fitness, and high mite densities changes the trade-off between the total brood size and larval mass of the developing larvae (91–93, 101).

In addition to mites, Ritcher in 1993 first reported the association between *N. vespilloides* and the apparently phoretic nematode species *Rhabditis stammeri* (94). Although nematodes have been found to be commonly associated with *N. vespilloides* in the field, it is not known how widespread *R. stammeri* is or if other nematode species may associate with *N. vespilloides*. Furthermore, the potential fitness consequences of the association between beetles and nematodes have never been characterized. Scarab beetles show carriage of different nematode species, including either *necromenic* species or entomopathogenic species (102). *Necromenic* nematodes are species that

consume the microbes growing within the host cadaver. While in some cases they harmoniously coexist with the host until the host dies, others secrete bacteria to speed up host death, which suggests a transition from the neutral towards the entomopathogenic (or parasitic) species in nematodes (103, 104).

Aim and outline of this thesis

The aim of the thesis is to experimentally investigate the origin and consequences of different interspecific interactions within burying beetle *N. vespilloides* social ecology. Parental care is an important factor in this species and thus may impact these interspecific interactions. My thesis will especially help us to better understand how parental behaviour impacts gut microbiota transmission and colonization.

According to the previous results we obtained, the hypotheses of this research are that 1) gut symbionts of *N. vespilloides* will benefit host fitness; and 2) symbiont transmission to offspring is facilitated by parental care. To target our research goals and examine our hypotheses, we thus put forward the following questions:

1. What are the dynamics of transmission of *N. vespilloides* symbionts to larvae during development?
2. What are the fitness effects to larvae of retaining the “endogenous” gut microbiota?
3. How does the “endogenous” microbiota persist in the *N. vespilloides* gut?
4. How do other interspecific associations affect *N. vespilloides* ecology?

In **CHAPTER 2** I evaluate the challenge of the carcass associated soil environment to *N. vespilloides* egg survival and examine potential antimicrobial strategies of *N. vespilloides* eggs. I first examine egg survival with different levels of microbial exposure. I next test the immune response of *Nicrophorus* eggs and newly hatched larvae. Further, I investigate whether the immunologically active physical barrier called the Serosa exists in *N. vespilloides* eggs. In light of these results, I discuss evolutionary consequences of antimicrobial activities of *N. vespilloides* in their early life stages.

In **CHAPTER 3** I clarify the transmission mechanisms of *N. vespilloides*

gut microbiota and demonstrate the role of parental care in this transmission. I first illuminate changes in the density of gut microbiota during the development of *N. vespilloides*, and then manipulate *N. vespilloides* parental care to offspring larvae, and monitor the composition of larval gut symbionts through development. Further, I conclude that *N. vespilloides* undergoes a significant aposymbiotic stage during pupation, after which they are recolonized at eclosion with bacteria similar to those found on the molted larval cuticle and on the wall of the pupal chamber. In addition, I clarify the importance of pre-hatch care on the transmission and colonization of *N. vespilloides* gut microbiota.

CHAPTER 4 focuses on questions about the fitness effects of the indigenous bacteria to parental and larval *N. vespilloides* and the mechanisms underlying the persistence of *N. vespilloides* gut bacteria. I first assess the general effects of endogenous microbiota on larval fitness. Next, I take a closer look at the impact of different bacterial symbionts on larval survival through time. Last, I conduct bacterial competition assays within the larval intestinal environment and estimate the colonization resistance of gut symbionts. I carry out these tests on two “endogenous” species and two environmental bacterial species, including pathogens. First, I show that beetles colonized by their endogenous microbiota produce heavier broods than those colonized with carcass bacteria. Next I show that the endogenous bacterial species are better colonizers within the beetle gut. Finally, I find that the endogenous species outcompete the carcass bacterial species in the larval gut and thus provide beetles with colonization resistance against pathogens. A priority effect is suggested within the bacterial competition in the beetle gut.

The last experimental part of this thesis (**CHAPTER 5**) examines the influence of nematodes on *Nicrophorus* fitness. I first characterize the efficacy of nematode transmission across partners and generations during *Nicrophorus* breeding. I next show that this interspecific interaction significantly harmful to *Nicrophorus* parental fitness. Finally, I provide the first report a new species of nematode symbiont in *N. vespilloides*.

Finally, in **CHAPTER 6** of this thesis, all the findings are summarized. I focus on the interactions between gut symbiont ecology and burying beetle *N. vespilloides* parental behaviour. I also highlight the ecological significance of bacterial competition derived colonization resistance against pathogens in the

beetle gut. I further discuss the potential to better mimic *Nicrophorus* natural conditions for future research, and elucidate potential host-microbiota co-evolved factors that influence *Nicrophorus* gut ecology. Last, I discuss the causes of harmful nematode species to *N. vespilloides* and suggest a further investigation of nematode infections on developing larvae.

My work will significantly advance our understanding of the evolution of mutualistic gut flora in insects, as well as the relevance of social behaviour for the transmission of animal bacterial symbionts. In addition, my results will highlight the need to integrate symbiont microbiology and behavioral ecology to better understand insect ecology and evolution. Detailed analysis of the interplay between *N. vespilloides* and their bacterial symbionts may identify novel mechanisms of colonization resistance, and establish the framework for similar studies in other animal:symbiont associations.

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