Chapter 6

Summary, discussion and perspective

Summary

Insects gut microbiomes influence host health and development, with effects varying from harmful to beneficial in different systems and contexts (1, 2). In order for insects to reliably maintain the beneficial associations they have with their symbionts, insects have evolved diverse methods to acquire their gut mutualists, from strict vertical transmission from parents to offspring, to horizontal transmission that relies on a form of environmental transfer (3, 4). In addition to mutualists, other microbes can also invade insect gut systems that can be pathogenic, or produce toxins that may harm their hosts (5–7). Insects have evolved a multiplicity of behavioural and antimicrobial strategies to compete against these microbial competitors, such as the expression of insect immunity derived antimicrobial peptides (AMPs) (8–10). In addition, insect symbionts can also help their hosts exclude invading microbes via a process called colonization resistance (11–13). This type of antimicrobial resistance takes place within the host gut community, and benefits both the hosts and their gut symbionts (12, 14). However, it is still unknown if and how insect hosts influence this type of colonization resistance, and whether it is caused by the specific composition of host microbiomes. In this thesis, by studying the burying beetle *Nicrophorus vespilloides*, I was able to test the associations between insect ecology and its microbiota, along with the effects of the competitive interactions within the host gut community on host ecology. My study will help us to better understand the complex interactions between insects and their microbiota.

The burying beetle *N. vespilloides* (Coleoptera, Silphidae) is a holometabolous insect, which undergoes a complete metamorphosis. These insects are reared on decomposing carcasses where they encounter dense and diverse bacterial populations (15). The carrion-borne microbes that are encountered by beetle larvae can invade the beetle gut and reduce beetle fitness throughout their development (16). Notably, parental beetles provide different types of parental care (pre-hatch and post-hatch care)

to the offspring that can reduce the microbial challenges experienced by larvae. These behaviours include, among others, the modification of the carcass by secreting antimicrobials and changes to larval nutrition via direct regurgitation from parents to offspring (15, 17). In this thesis, I tested if the microbial interactions within the *N.vespilloides* gut microbiota could also contribute to the antimicrobial strategies of beetles. I further clarified how parental care facilitates the transmission of gut microbiota to larvae during their development in the presence of high bacterial densities on the carcass. In addition to microbial interactions, *N.vespilloides* is also associated with mites and nematodes. By examining interactions with nematodes, in this thesis I was able to broadly examine a multiplicity of interspecific interactions during insect development and to assess their effects on *Nicrophorus* ecology.

N.vespilloides fitness is challenged by environmental microbes during the egg stage

In **Chapter 2**, I assessed how microbial challenge influences the survival of *N.vespilloides* eggs by varying the levels of environmental exposure to bacteria. I show that egg survival is reduced following exposure to the carrion environment. The negative effect from environmental microbes can be offset by sterilizing the eggs and can be reintroduced by exposure to environmental bacteria. I further studied if *Nicrophorus* eggs express any intrinsic immunity. First, I determined that *Nicrophorus* eggs possess the immunologically active serosa. I next quantified the immune response mediated by the serosa after bacterial injections. Surprisingly, and in contrast to other insects (18), I found that the serosa doesn't appear to regulate AMP production, which indicates an absence of immune response in eggs. In addition, *Nicrophorus* eggs show limited defence against desiccation—another serosa-dependant trait. I finally consider these results in an evolutionary context. Similar to *Drosophila melanogaster* (19), *N.vespilloides* eggs seems to trade-off rapid development for an explicit immune response.

Parental care facilitates the gut microbiota transmission of *N.vespilloides*

In **Chapter 3**, I manipulated the types of *N.vespilloides* parental care to offspring larvae, and I monitored the dynamics of gut microbiome colonization in terms of bacterial density and composition through development. By using a combination of MALDI-TOF Biotyping and 16s rDNA sequencing,

I show that the larval gut microbiome undergoes similar dynamics, in terms of density, regardless of the duration of parental care; however, bacterial composition is strongly determined by parental care. I also discovered that there is an aposymbiotic stage during larval pupation (where pupae become bacterially sterile), after which pupae are recolonized at eclosion with bacteria similar to those found on the molted larval cuticle and on the wall of the pupal chamber. I determined that pre-hatch care facilitates and ensures the colonization and transmission of *N.vespilloides* gut microbiota from parents to offspring. Additionally, I find that environmental bacteria predominantly colonize the larval gut when parental care is completely absent. These results together suggest that competitive interactions within the *N.vespilloides* gut community might be a factor in the colonization and persistence of *N.vespilloides* gut microbiota.

Colonization resistance functions in the persistence of gut bacteria and provides resistance against pathogens

In **Chapter 3**, we concluded that the endogenous microbiota outcompete the carrion-associated bacteria for colonization of the larval gut niche. In **Chapter 4**, I was able to test this hypothesis directly by using competition assays in vivo. I set up experiments with four bacterial species, of which *Providencia rettgeri* and *Morganella morganii* are abundant species in the *Nicrophorus* gut, and conversely *E.coli* and the pathogen *Serratia marcescens* (20) are commonly found in the environment. I first inoculated these four strains alone into *N.vespilloides* larvae, and the results show that the bacterial species vary in their colonization capability within the beetle gut. I next co-inoculated larvae with different bacterial combinations simultaneously or in series. I show that endogenous species significantly outcompete foreign species within the *Nicrophorus* gut, regardless of the inoculation order. Therefore I confirm that *N.vespilloides* gut bacteria provides colonization resistance against the pathogen *S. marcescens*. To further determine and illuminate the potential benefit of this colonization resistance, I quantified the fitness effects of native gut flora in developing larvae. Results show that the gut microbiota benefit *N.vespilloides* by increasing both parental and larval fitness. I discuss these results in both ecological and evolutionary perspectives, and suggest that parental behaviour and microbial competition interact to influence the transmission and colonization of *Nicrophorus* gut microbiota.

Influence of phoretic nematodes on *N.vespilloides* fitness

The last experimental part of my thesis (**Chapter 5**) focused on the interspecific interactions between *N.vespilloides* and nematodes. Using direct microscopy counts, I first quantified the number of nematodes in both wild and lab beetle samples. I find no significant difference of nematodes in densities between males and females. Next, I determined that the phoretic nematode species of *N.vespilloides* is *Rhabditoides regina*. I characterized the efficacy of nematode transmission across partners and generations. My results show that nematode transmission occurs within the *N.vespilloides* breeding context between mating partners and also from parents to offspring. Notably, nematode transmission from parents to offspring can start with an extremely low inoculation of ~ 10 worms. In contrast, nematode transmission across mating adults shows a threshold of ~ 100 worms. Finally, I estimated the effect of different starting nematode densities on the larval fitness, and I showed that the negative effects of nematodes on *N.vespilloides* larvae are seen even with a very low inoculum sizes.

Discussion and perspective

In this thesis, I have illuminated the evolution and ecology of gut symbionts of the social insect, *N. vespilloides*, and examined some of their benefits to their host. I linked the extensive parental care of *Nicrophorus* beetles with their gut microbiota colonization ecology.

Nicrophorus beetles are reared on highly decaying carcasses, which exposes larvae to severe microbial challenge that begin at the egg stage (**Chapter 2**) (17). However, neither direct antimicrobial provisioning from parents nor intrinsic immunity can be found in *Nicrophorus* eggs to cope with these microbial threats. This is similar to other insects such as *D. melanogaster*, which feeds on rotting fruits. From an evolutionary perspective, our results suggest that *Nicrophorus* eggs might be selected for rapid growth and this results in a trade-off between developmental speed and immune competence (21, 22). Still, these results need to be interpreted carefully, because *Nicrophorus* eggs may be not be as unprotected as we believe. Many potential forms of protection, such as the antiseptic volatiles from parents or the effects of bacterially produced antimicrobial regents in soil have yet not been tested (23–25), and this will require a more complex experimental set-up in future

research. This work will extend our knowledge about the animal parental strategies on maintaining the offspring fitness and developments.

Although parents do not apparently protect *Nicrophorus* eggs, parental beetles provide a comprehensive set of caring behaviours for their hatched larvae, including carcass preparation (pre-hatch care) and direct larval feeding (post-hatch care). Throughout both stages of parental care, parent beetles transmit their endogenous gut microbiota to offspring larvae (Chapter 3), and we showed that these native microbes can increase larval fitness under pathogen challenge (**Chapter 4**). Interestingly, with pre-hatch care alone, offspring larvae can also acquire and assemble many of the prominent members of their endogenous gut community. We found this was not due to the inability of environmental bacteria to colonize larvae, because larvae reared without parental care are still colonized by the carcass-borne bacteria at high densities. Also, larvae receiving pre-hatch care alone are partially colonized by the environmental bacteria (**Chapter 3**). We thus suggest that endogenous bacteria outcompete foreign species within the larval gut, which is an essential factor in stable microbiota transmission. The route by which parents manipulate the carcass during pre-hatch care to bacterial facilitate transmission to larvae are only partly understood. Parents open the carcass abdomen and coat the carcass with lysozyme-like secretions. These behaviours may bias carcass microbiota towards aerobic and Gram-negative species, respectively (17, 25, 26), and further influence gut microbiota establishment. Previous research has shown that *Nicrophorus* suffer a dramatic fitness cost when reared on an aged carcass (16). In future work, it would be interesting to more closely examine the differences between fresh and aged carcass microbiomes after parental preparation, and carry out further work on larval microbiota transmission from aged carcasses, where parental care is dramatically challenged. In **Chapter 3**, I also show a clear aposymbiotic stage during beetle pupation, which is consistent with several fly and mosquito species (27, 28). Interestingly, following this aposymbiotic stage, eclosed beetles recover their gut bacteria in density and the composition of this microbiota significantly overlap with those present prior to pupation. This indicates a reliable mode of parent-offspring transmission of *Nicrophorus* gut microbiota, and further suggested that there may be beneficial functions of this microbiome that potentially limit larval infections derived from the carcass environment. I address this question in this thesis in the laboratory, and in the future it will be exciting to extend our research to field derived beetles that are exposed to more complex bacterial communities. This part

of work will help us to better understand the mechanisms and associations of maternally transmitted symbionts in other insects e.g. Aphid or mammals.

In **Chapter 4**, I examined the hypotheses proposed in **Chapter 3**. I demonstrate that native bacteria outcompete foreign species within the larval gut, which could play an important role in the stability of *Nicrophorus* gut microbiota (29). I also show that even after 24 hours of head-start colonization by *S. marcescens*, *P. rettgeri* can still successfully invade the larval gut and replace *Serratia*. This outcome differs from the priority effects observed in other models (30), although why this is the case is not fully clear. One possibility is that endogenous species are better colonizers of the larval gut than non-native species, a result confirmed in **Chapter 4**. This implies that bacterial colonization within larval gut is highly specific. However, we still have little understanding about what factors between bacteria, their host and the interactions between these factors drive this specificity in *N.vespilloides*. One of the factors could involve specific co-evolved genes between *Nicrophorus* and certain bacterial species that favour colonization. For example, the cell adherence gene ccf in Bacteroides fragilis is essential for the association with colonic mucus, and thus plays an important role in Bacteroides colonization in the mice gut. The mice colonic crypts meanwhile represents a stable niche even after microbial disruption by other bacteria or antibiotic treatments (31). In addition, around 15% of protein-coding genes in *Snodgrassella alvi* are found to be essential for gut colonization in honeybees, and some factors like cell O-antigens and type IV pili (T4P) function in attachment on the hindgut epithelium. Another factor could be derived from host metabolic and biosynthesis pathways, which promote and favour the specific colonization of bacteria. This has been also found in the specific colonization of S. alvi within the honey bee gut (32). Genome-wide screening and transcriptome targeting could address these questions in our system, for example, non-endogenous strains with transformed genes of specific colonization factors might offset prior competitive advantages in the endogenous species. Other factors such as host triggered direct bacterial killing (33), microbiota-mediated host immune responses (34) and the physical structure of host intestinal tract (35) might influence the process of colonization and colonization resistance. Further, the specificity mediated bacterial colonization resistance will shed light on the host-symbionts cooperated therapeutics in the future medical research.

In **Chapter 5,** I provide evidence of an association between the phoretic

nematode *R. regina* and *N.vespilloides*, I further show that transmission of this nematode relies on *N.vespilloides* social behaviour and thus persists through larval development. I also show a strong negative effect of *R. regina* on *N.vespilloides* fitness.

The reason for the negative effects of *R. regina* to beetles could be diverse: 1) nematodes that reach high densities might directly compete with beetle larvae for food resources; 2) because nematodes feed on bacteria and potentially associate with pathogens, this could convert nematodes into incidental vectors of pathogens (36). Further research will be required to determine the mechanisms underlying the negative effects of *R. regina* on *Nicrophorus* beetles.

In nature, other phoretic mites and nematodes might simultaneously interact with *Nicrophorus* beetles. Due to the potential of mite predation on nematodes (37) and the density-dependent effects of mites on *Nicrophorus* fitness (38), there might be an ecological balance in terms of the number of associated species within the *Nicrophorus* living context. All these would be interesting to further examine in the future.

Conclusions

In conclusion, I demonstrate vertical transmission of gut microbiota by parental *N.vespilloides* that persists across larval development. I next demonstrate the apparent mutualism between *N.vespilloides* and their gut symbionts. And interestingly, I show that colonization resistance plays an important role in the transmission and colonization of *N.vespilloides* gut microbiota. Finally, I report a new species of phoretic nematode, *R. regina* that associates with *N.vespilloides* and which is significantly reduces beetle fitness. My data contributes to an increased understand of the relationships between insect parental care and the social transmission of beneficial gut bacteria. Future studies will shed light on the mechanisms that regulate host-symbiont mutualism in this model system.

References

1. Macke E, Tasiemski A, Massol F, Callens M, Decaestecker E. 2017. Life history and eco-evolutionary dynamics in light of the gut microbiota. Oikos 126:508–531.

2. Engel P, Moran NA. 2013. The gut microbiota of insects - diversity in structure and function. FEMS Microbiol Rev 37:699–735.

3. Drown DM, Zee PC, Brandvain Y, Wade MJ. 2013. Evolution of transmission mode in obligate symbionts. Evol Ecol Res 15:43–59.

4. Russell J a, Moran N a. 2005. Horizontal Transfer of Bacterial Symbionts : Heritability and Fitness Effects in a Novel Aphid Host Horizontal Transfer of Bacterial Symbionts : Heritability and Fitness Effects in a Novel Aphid Host. Appl Environ Microbiol 71:7987–7994.

5. Ruiu L. 2015. Insect Pathogenic Bacteria in Integrated Pest Management. Insects 6:352–367.

6. Miyoshi S, Shinoda S. 2000. Microbial metalloproteases and pathogenesis. Microbes Infect 2:91–98.

7. Wong ACN, Luo Y, Jing X, Franzenburg S, Bost A, Douglas AE. 2015. The host as the driver of the microbiota in the gut and external environment of *Drosophila melanogaster*. Appl Environ Microbiol 81:6232–6240.

8. Zhukovskaya M, Yanagawa A, Forschler B. 2013. Grooming Behavior as a Mechanism of Insect Disease Defense. Insects 4:609–630.

9. Dimarcq JL, Zachary D, Hoffmann J a, Hoffmann D, Reichhart JM. 1990. Insect immunity: expression of the two major inducible antibacterial peptides, defensin and diptericin, in *Phormia terranovae*. EMBO J 9:2507–2515.

10. Hui-Yu Yi, Munmun Chowdhury, Ya-Dong Huang and X-QY. 2014. Insect antimicrobial peptides and their applications. Appl Microbiol Biotechnol 98:5807–5822.

11. Sant'Anna MR, Diaz-Albiter H, Aguiar-Martins K, Al Salem WS, Cavalcante RR, Dillon VM, Bates PA, Genta FA, Dillon RJ. 2014. Colonisation resistance in the sand fly gut: *Leishmania* protects *Lutzomyia longipalpis* from bacterial infection. Parasit Vectors 7:329.

12. Mohanraj P, Subramanian S, Muthuswamy M. 2009. Assessment of colonization resistance in silkworm , Bombyx mori L . using molecular marker tagged *Escherichia coli*. Karnataka J Agric Sci 22:519–520.

13. Dillon RJ, Vennard CT, Buckling A, Charnley AK. 2005. Diversity of locust gut bacteria protects against pathogen invasion. Ecol Lett 8:1291–1298.

14. Charlie G. Buffie, Pamer EG. 2013. Microbiota-mediated colonization

resistance against intestinal pathogens. Nat Rev Immunol 13:790–801.

15. Eggert A-K, Reinking M, Mu JK, Ller. 1998. Parental care improves offspring survival and growth in burying beetles. Anim Behav 55:97–107.

16. Rozen DE, Engelmoer DJP, Smiseth PT. 2008. Antimicrobial strategies in burying beetles breeding on carrion. Proc Natl Acad Sci U S A 105:17890–17895.

17. Scott MP. 1998. The ecology and behaviour of burying beetles. Annu Rev Entomol 43:595–618.

18. Jacobs CGC, Spaink HP, van der Zee M. 2014. The extraembryonic serosa is a frontier epithelium providing the insect egg with a full-range innate immune response. Elife 3:1–21.

19. Jacobs CGC, Van Der Zee M. 2013. Immune competence in insect eggs depends on the extraembryonic serosa. Dev Comp Immunol 41:263–269.

20. Flyg C, Kenne K, Boman HG. 1980. Insect pathogenic properties of *Serratia marcescens*: phage-resistant mutants with a decreased resistance to *Cecropia* immunity and a decreased virulence to *Drosophila*. J Gen Microbiol 120:173–181.

21. Siva-Jothy MT, Moret Y, Rolff J. 2005. Insect Immunity: An Evolutionary Ecology Perspective. In Advances in Insect Physiology. 32nd edition. Edited by Simpson SJ. London, UK: Academic Press 1–48.

22. Diamond SE, Kingsolver JG. 2011. Host plant quality, selection history and trade-offs shape the immune responses of *Manduca sexta*. Proc R Soc B-Biological Sci 278:289–297.

23. BLUM MS, NOVAK AF, TABER S. 1959. 10-Hydroxy-Δ2-Decenoic Acid, an Antibiotic Found in Royal Jelly. Science (80-) 130:452 LP-453.

24. Degenkolb T, Düring RA, Vilcinskas A. 2011. Secondary Metabolites Released by The Burying Beetle *Nicrophorus vespilloides*: Chemical Analyses and Possible Ecological Functions. J Chem Ecol 37:724–735.

25. Arce AN, Smiseth PT, Rozen DE. 2013. Antimicrobial secretions and social immunity in larval burying beetles, *Nicrophorus vespilloides*. Anim Behav 86:741–745.

26. Duarte A, Welch M, Wagner J, Kilner RM. 2016. Privatization of a breeding resource by the burying beetle *Nicrophorus vespilloides* is associated with shifts in bacterial communities 44.

27. K. ROCHON, T. J. LYSYK ALBS. 2004. persistence of *E.coli* in housefly and stable fly. J Med Entomol 41:1082–1089.

28. Moll RM, Romoser WS, Modrzakowski MC, Moncayo AC, Lerdthusnee

K. 2001. Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. J Med Entomol 38:29–32.

29. Coyte KZ, Schluter J, Foster KR. 2015. The ecology of the microbiome: Networks, competition, and stability. Science (80-) 350:663–666.

30. Devevey G, Dang T, Graves CJ, Murray S, Brisson D. 2015. First arrived takes all: inhibitory priority effects dominate competition between co-infecting *Borrelia burgdorferi* strains. BMC Microbiol 15:381.

31. Lee SM, Donaldson GP, Mikulski Z, Boyajian S, Ley K, Mazmanian SK. 2013. Bacterial colonization factors control specificity and stability of the gut microbiota. Nature 501:426–429.

32. Powell JE, Leonard SP, Kwong WK, Engel P, Moran NA. 2016. Genome-wide screen identifies host colonization determinants in a bacterial gut symbiont. Proc Natl Acad Sci.

33. Jarosz J. 1979. Gut flora of *Galleria mellonella* suppressing ingested bacteria. J Invertebr Pathol 34:192–198.

34. Kamada N, Seo S-U, Chen GY, Núñez G. 2013. Role of the gut microbiota in immunity and inflammatory disease. Nat Rev Immunol 13:321–335.

35. Ohbayashi T, Takeshita K, Kitagawa W, Nikoh N, Koga R, Meng X-Y, Tago K, Hori T, Hayatsu M, Asano K, Kamagata Y, Lee BL, Fukatsu T, Kikuchi Y. 2015. Insect's intestinal organ for symbiont sorting. Proc Natl Acad Sci.

36. Jiménez-Cortés JG et al. 2016. Microbiota from *Rhabditis regina* may alter nematode entomopathogenicity. Parasitol Res 115:4153–4165.

37. Bilgrami A, Tahseen Q. 1992. A nematode feeding mite, *Tyrophagus putrescentiae* (Sarcoptiformis: Acaridae). Fundam Appl Nematol.

38. Wilson DS, Knollenberg WG. 1987. Adaptive indirect effects: the fitness of burying beetles with and without their phoretic mites. Evol Ecol 1:139–159.