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### Of moths, mites and microbes - The role of bacteria in the life history of two arthropod herbivores

Staudacher, H.

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## General discussion

Virtually all macro-organisms are associated with microbes. If microbe-host associations persist across generations, a host and its associated microbes (holobiont) can be viewed as a unit of selection (Margulis & Chapman, 1998; Rohwer *et al.*, 2002; Zilber-Rosenberg & Rosenberg, 2008; Guerrero *et al.*, 2013). Natural and sexual selection acts on the holobiont as if it were an integrated unit and the formation of microbe-host associations is often considered a driver of evolution (Zilber-Rosenberg & Rosenberg, 2008; Oliver *et al.*, 2010; Guerrero *et al.*, 2013). Hosts containing beneficial microbes may gain a selective advantage compared to hosts which do not contain these microbes (Zilber-Rosenberg & Rosenberg, 2008). One of the most prominent examples of beneficial microbes in arthropods is the obligate intracellular bacterium *Buchnera aphidicola*, which provides essential amino acids to its aphid host (Buchner, 1965; Shigenobu *et al.*, 2000; Baumann, 2005). Microbes are further known to support the breakdown of recalcitrant food in termites (Cleveland, 1923; Inoue *et al.*, 1997; Warnecke *et al.*, 2007) and have been shown to protect their host against pathogens (Teixeira *et al.*, 2008; Moreira *et al.*, 2009; Koch & Schmid-Hempel, 2011). Microbes with detrimental effects on their hosts promote the evolution of host defences, such as an immune system or behavioural adaptations that reduce the frequency or impact of infection (Schmid-Hempel, 2011). Since immune defence is costly, having and using an immune system can negatively affect other life history traits (Sheldon & Verhulst, 1996).

In my thesis, I investigated bacterial communities of two arthropod herbivores and the impact of bacteria or immune challenge on the life history of their hosts. In the following I will discuss the findings of my thesis in the light of present literature on (1) non-pathogenic host-microbe interactions and (2) pathogenic effects of microbes on arthropods.

### ***1. Non-pathogenic associations of bacteria with herbivores and their role in plant utilization***

Herbivore-associated bacteria may support the host plant use of their herbivore hosts via different mechanisms, such as the breakdown of nutrients (1.1) and the alteration of plant physiology to the host's benefit (1.2) (Dillon & Dillon, 2004; Douglas, 2009; Engel & Moran, 2013; Hansen & Moran, 2014). In this section, I will discuss these aspects in relation to my findings in *Heliothis virescens* and *Tetranychus urticae* (CHAPTERS 2 and 5). As it is unlikely that microbes in these hosts synthesize and/or provide nutrients, I will not discuss the possibility that bacteria can synthesize and provide nutrients to the host that are lacking its diet.

#### ***1.1. Breakdown of plant chemicals and nutrients by herbivore-associated bacteria***

In *H. virescens* there was high variability in bacterial communities depending on diet, developmental stage, rearing background (laboratory or field population) and even between biological replicates, indicating that the bacterial community of this moth is only transiently associated with its host (CHAPTER 2). In Lepidoptera,

evidence that bacteria may enhance host fitness in a long-term, stable symbiosis is scarce, even though bacterial communities of many lepidopteran species have been investigated. High alkalinity and the tubular gut structure without pouches, which are characteristic of lepidopteran guts, may impede a stable colonization by microbes (Broderick *et al.*, 2004; Engel & Moran, 2013). Moreover, holometabolous metamorphosis of Lepidoptera, during which the gut is purged, may further complicate the establishment of stable bacterial communities (Moll *et al.*, 2001; Rani *et al.*, 2009; Hammer *et al.*, 2014). Nevertheless, Lepidoptera may benefit from bacteria that are present in their guts. For instance, bacteria isolated from lepidopteran guts possess enzymes with cellulolytic, pectinolytic or xylanolytic activity which can break down plant material (Pinto-Tomas *et al.*, 2007; Anand *et al.*, 2010; Belda *et al.*, 2011). Similar metabolic capabilities can also (at least partly) be expected for the bacterial community of *H. virescens*, because many of the bacterial genera that were found associated with this moth have been identified before in Lepidoptera (CHAPTER 2).

Irrespective of the stability of an association, insects that take up bacteria from the environment may benefit from traits that bacteria have evolved independently of their host associations (Oliver *et al.*, 2010). For example, some phyllosphere bacteria can use methanol as carbon source, fix nitrogen or break down secondary plant metabolites (Sy *et al.*, 2005; Frnkranz *et al.*, 2008; Vorholt, 2012; Mason *et al.*, 2014). As an example of the latter, the growth of larvae of the gypsy moth, *Lymantria dispar*, was enhanced in the presence of bacteria from its host plant aspen, when phenolic glycosides, the defensive metabolites of aspen, were present in the larval diet (Mason *et al.*, 2014). The bacteria that were detected in *H. virescens* could have been taken up from the plants on which the caterpillars were reared (CHAPTER 2). In line with benefitting from plant-associated bacteria, the variability of bacterial communities in *H. virescens* could be viewed as flexibility, which may support this generalist herbivore in feeding on different host plants.

*Wolbachia* are intracellular bacteria that are commonly found in arthropods and nematodes. They are well known as reproductive parasites, but they are also able to deliver metabolic benefit to their hosts (Duron *et al.*, 2008; Werren *et al.*, 2008; Zug & Hammerstein, 2015). For instance, in *Drosophila melanogaster*, *Wolbachia* appears to be involved in iron homeostasis (Brownlie *et al.*, 2009). Furthermore, *Wolbachia* in filarial nematodes was suggested to provide nutrients or ATP to the host (Foster *et al.*, 2005; Darby *et al.*, 2012), and in the bedbug *Cimex lectularius*, *Wolbachia* likely provides its host with vitamin B (Hosokawa *et al.*, 2010). In the mite *T. urticae*, digestion- and detoxification-related genes of the mite were upregulated in the presence of *Wolbachia* (CHAPTER 5). One possible explanation for this finding is that *Wolbachia* performs early catabolic steps, which could alter digestion processes in the mites. Notably, in contrast to some of the above-mentioned examples, *Wolbachia* is not an obligate symbiont of the *T. urticae* strains that were tested, because mites were viable and could reproduce without this

bacterium (Staudacher and Schimmel, personal observation). Nevertheless, *Wolbachia* may increase mite fitness by performing metabolic steps that reduce the costs of digestion for the mite.

### *1.2. Effects of herbivore-associated bacteria on host plant physiology*

Herbivore-associated microbes can affect plant physiology. For instance, the presence of *Wolbachia* in the lepidopteran leafminer *Phyllonorycter blancardella* is associated with the preservation of photosynthetically active and nutrient rich regions (so-called ‘green-islands’) in otherwise senescing leaves (Kaiser *et al.*, 2010). Furthermore, herbivore-associated microbes may alter induced plant resistance in favour of their herbivore hosts (Chung *et al.*, 2013; Su *et al.*, 2015). Bacteria can reduce the jasmonic acid (JA) defence responses of plants, that are commonly induced by herbivore feeding (Schoonhoven *et al.*, 2005; Karban & Baldwin, 2007). Herbivores are susceptible to JA-responses, which can cause reduced larval growth, amount of feeding, survival and oviposition of the herbivores and may increase the attraction of their natural enemies (Howe *et al.*, 1996; Li *et al.*, 2002; Thaler *et al.*, 2002). JA-responses can be suppressed by salicylic acid (SA)-responses, which may be induced by microbes (JA-SA crosstalk) (Pieterse *et al.*, 2012; Thaler *et al.*, 2012). For instance, the Colorado potato beetle, *Leptinotarsa decemlineata*, secretes in its oral secretions *Pseudomonas*, *Stenotrophomonas* and *Enterobacter* to the plants, which suppresses JA-related plant responses via JA-SA crosstalk (Chung *et al.*, 2013). The moth *H. virescens* contained bacteria that belonged to the same genera as those in the Colorado potato beetle (CHAPTER 2). Importantly, the beetles also contained Enterobacteriaceae and one *Pseudomonas* strain that did not suppress defences, so that the capacity to suppress plant defences seems to be strain specific (Chung *et al.*, 2013). Plant defence suppression may thus not necessarily occur in other herbivores that contain bacteria of the same genus as bacteria in the Colorado potato beetle, such as *H. virescens*.

In the mite *T. urticae*, we tested whether the presence of *Wolbachia*, *Spiroplasma* and *Cardinium* affected induced plant responses after feeding of two strains of mites: a plant defence inducer strain (Santpoort-2), which induces JA- and SA-responses upon feeding, and a plant defence suppressor strain, which suppresses such defences (Alba *et al.*, 2015; CHAPTER 5). The suppressor strain contained *Wolbachia* and *Spiroplasma*, whereas the inducer strain contained *Cardinium* and *Spiroplasma*. Both mite strains were cleared from these bacteria with antibiotics. Additionally, there was one group of suppressor mites that only contained *Spiroplasma*. When testing the suppressor strain of plant defences, SA accumulated significantly more in leaflets infested with mites containing only *Spiroplasma* compared to leaflets infested with mites containing *Wolbachia* and *Spiroplasma* or none of the two bacteria. This suggests that *Spiroplasma* induced SA-responses, while *Wolbachia* antagonizes this effect. SA induction did not result in suppression of JA-responses via JA-SA crosstalk. However, for the precursor of JA, 12-*oxo*-

phytyldienoic acid (OPDA), the pattern that we found for SA was nearly reversed, suggesting that *Spiroplasma* suppressed OPDA-responses, while *Wolbachia* seemed to induce it. Even though OPDA may also mediate plant resistance, independently from JA (Stintzi *et al.*, 2001; Bosch *et al.*, 2014; Scalschi *et al.*, 2015), the differences in plant responses depending on bacteria in the mites were not correlated to mite fitness in the suppressor strain. Possibly, changes in plant physiology are primarily beneficial for the bacteria, but not for the herbivorous host: bacteria that suppress plant defences may be exposed to fewer secondary plant metabolites that are ingested by their host herbivores, which often have antimicrobial activity (Wallace, 2004; Karamanoli *et al.*, 2005; Karban & Baldwin, 2007).

In the plant defence inducer strain of *T. urticae*, the presence of *Cardinium* and *Spiroplasma* in the mites was associated with higher transcript accumulation of SA marker genes. This observation was in line with the finding that *Cardinium* and/or *Spiroplasma* negatively affected mite fitness (CHAPTER 5). Bacteria such as *Wolbachia*, *Cardinium* and *Spiroplasma* secure their persistence in a host population by manipulating host reproduction to increase the number of infected females and/or by increasing the fitness of the host (Werren *et al.*, 2008; Himler *et al.*, 2011; Zug & Hammerstein, 2015). Beneficial effects of reproductive manipulators are expected when reproductive manipulation is weak in a population (Hoffmann *et al.*, 1998; Fry *et al.*, 2004). Thus, these non-beneficial or even detrimental bacteria in the inducer strain may increase their prevalence in the mite population via reproductive manipulation, but we did not test this in our mites.

### 1.3. Interactions between multiple bacteria in one arthropod host

Bacteria can shape their environment by affecting abiotic factors, such as pH, and as such are true ecosystem engineers (Madigan *et al.*, 2009). Moreover, bacterial strains can utilize and even depend on breakdown products of other strains in a metabolic cross-feeding network (Flint *et al.*, 2007). For instance, bacteria that are unable to use complex carbohydrates depend on the presence of primary degraders (Flint *et al.*, 2007). Furthermore, *in vitro* experiments with human gut bacteria showed that lactate utilizing bacteria grow on starch only in the presence of particular strains of lactate producing *Bifidobacterium adolescentis*, which are able to metabolize starch (Belenguer *et al.*, 2006). Because of their strong influence on community structure, the presence of bacterial strains with keystone function could underlie high variability between bacterial communities. Another common type of interaction between bacterial strains is competition for resources and space both at the intra- and inter-specific level (Hibbing *et al.*, 2010). The outcome of competition may depend on environmental factors that promote or inhibit the growth of particular bacterial strains.

The moth *H. virescens* and the spider mite *T. urticae* harbour multiple bacterial strains (CHAPTERS 2 and 5). Interactions between these strains are expected in the hosts and may in part explain the variability of bacterial communities in *H. virescens* and the combination effects of *Spiroplasma* and *Wolbachia* in *T. urticae* that we

observed. For example, competition could underlie some of the variation in bacterial communities *H. virescens* in relation to diet, life stage and rearing background (CHAPTER 2). Such competition may explain the fact that *H. virescens* larvae, collected in the field and reared in the laboratory for four generations, were colonized with enterococci that were absent in field larvae. Enterococci may thus be strong competitors in larvae under laboratory conditions. Another possibility is that under laboratory conditions different bacterial strains disappeared because of a lack of replenishment of new individuals from the environment. Enterococci could thus have replaced these strains without competition.

In *T. urticae*, the combined presence of *Wolbachia* and *Spiroplasma* affected induced plant responses and mite gene expression. This observation suggests that either the two bacteria affected plant responses and mite gene expression independently, or that the bacteria interacted. Based on sequencing data, we speculated that *Wolbachia* negatively affects the abundance of *Spiroplasma* in the mites (CHAPTER 5). For many maternally transmitted bacteria, colonization of the ovaries is important; in the ovaries co-infecting bacteria may compete for space or nutrients. For instance, *Wolbachia* impeded *Asaia* bacteria from colonizing of reproductive organs in mosquitos (Rossi *et al.*, 2015). In *T. urticae*, *Wolbachia* was shown to reside in the ovaries (Zhao *et al.*, 2013). Possibly, *Spiroplasma* also colonizes the ovaries in the mite, because this bacterium stably infected our mite populations and is likely vertically transmitted. Thus, *Wolbachia* and *Spiroplasma* may compete in the ovaries.

*Wolbachia* not only resides in the ovaries but also in the gnathosoma (i.e., mouth and feeding parts of mites, including salivary glands, amongst others) of *T. urticae* (Zhao *et al.*, 2013). From the gnathosoma, *T. urticae*-associated *Wolbachia* possibly affects plant physiology, e.g., SA and OPDA related plant responses after mite feeding (CHAPTER 5). *Wolbachia* seems to affect plant responses in an opposite way of *Spiroplasma*. Competition for space in the gnathosoma could underlie this result.

## **2. Effect of immune challenge on life history traits**

### **2.1. Cost of immune defence**

Activation and maintenance of the immune system in animals is costly and can trade off against other life history traits such as reproductive traits (Sheldon & Verhulst, 1996). The trade-off between immunity and reproduction can depend on sex and the investment of each sex into offspring (Rolf, 2002; Zuk & Stoehr, 2002; Roth *et al.*, 2011; Vincent & Gwynne, 2014). Males and females of *H. virescens* differ in their investment strategy into immunity (CHAPTER 3). Non-challenged males had higher expression levels of immune-related genes than non-challenged females. This suggests that males have higher immune maintenance than females, which implies physiological costs to keep the immune system at a certain level of readiness. Females showed higher immune deployment than males after immune challenge, which implies physiological costs of immune system activation to combat pathogens

(see Schmid-Hempel, 2011). These findings are in line with the result that bacteria-challenged females had lower mating success than unchallenged females, but in males there was no difference between bacteria-injected and non-injected mates. Thus, in *H. virescens*, immune response is negatively correlated to mating success in females but not in males.

In addition to the differences between the sexes in immune deployment and maintenance, the differences between wounding and bacterial challenge were more pronounced in females than in males, suggesting that the female immune system differentiates more specifically between wounding and bacterial challenge than the male system. Together, female immune response may be more cost efficient than the male immune response, because different types of immune responses differ in their costliness: non-specific and constitutive immune responses are assumed to have higher (continuous) energy costs, with a higher risk of self-reactivity, than induced and specific responses (Schmid-Hempel, 2011).

In general, immunity is hypothesized to be more important for females than for males, because males maximize their fitness by increasing mating rate, while females maximize their fitness by increasing longevity (Rolff, 2002). However, in moths longevity is important for both sexes as discussed in CHAPTER 3. As in moths females are sexual signalers to which males respond (Nesbitt *et al.*, 1979; Vetter & Baker, 1983; Tumlinson *et al.*, 1986; Groot *et al.*, 2014), female fitness may not only depend on longevity, but also on access to males. A (cost) efficient immunity may therefore be necessary to uphold a high quality of the sexual signal. To assess how exactly males and females differ in their type of immunity, additional immune responses should be measured, such as phagocytosis (or encapsulation) (i.e., a not delayed, constitutive response with little specificity) and levels of antimicrobial peptides (i.e., a delayed, induced and more specific response) (Mallon *et al.*, 2003; Schmid-Hempel & Ebert, 2003).

The sex pheromone blend of *H. virescens* females seems to be condition dependent, as the blend of bacteria-challenged females was less attractive than that of wounded or unchallenged females (CHAPTER 3). Thus, infection could possibly affect attractiveness and therefore mating success of female *H. virescens* in the field. The costs of the quality of female sex pheromones in moths has scarcely been assessed, but may be high (Harari *et al.*, 2011; Xu *et al.*, 2014). In addition, our result suggests that the quality of the female sex pheromone of this moth could be an honest signal that is costly and indicates to the males the condition of the females (Zahavi, 1975; Hamilton & Zuk, 1982; Zahavi & Zahavi, 1997). Possibly, there is a physiological trade-off between immune defence activation and regulation of biosynthesis of the female sex pheromone of *H. virescens*.

In insects, juvenile hormone (JH) plays a central role in the regulation of development and reproduction and was shown to have an immunosuppressive effect, which may thus mediate the trade-off between immunity and reproduction (Nijhout & Williams, 1974; Wyatt & Davey, 1996; Rolff & Siva-Jothy, 2002;

Rantala *et al.*, 2003). JH could also play a role in the trade-off between attractiveness and immunity, a situation that was encountered in mealworm beetles, in which JH increased the attractiveness of male sex pheromone which coincided with lower phenoloxidase (PO) activity in response to the JH treatment (Rantala *et al.*, 2003). The altered sex pheromone blend in *H. virescens* upon immune system activation in bacteria-challenged females could thus possibly result from reduced JH levels after an immune challenge. Unlike in beetles, female sex pheromone production in moths is regulated by pheromone biosynthesis-activating neuropeptide (PBAN) and not directly by JH (Raina *et al.*, 1989; Jurenka, 1996, 2004; Jurenka & Rafaeli, 2011). In migratory moths, JH seems to regulate the release or synthesis of PBAN (Cusson & McNeil, 1989; Gadenne, 1993; Picimbon *et al.*, 1995), while the role of JH in non-migratory moth species is less clear. In the non-migratory moth *Helicoverpa armigera*, JH primes the sex pheromone gland to respond to PBAN, indicating that JH is involved in the initiation of female sex pheromone biosynthesis (Fan *et al.*, 1999; Rafaeli *et al.*, 2003). Thus, even though the role of JH in an immunity-sex pheromone trade-off may not be as straightforward as in beetles, JH remains to be a candidate for mediating such a trade-off in moths. Since the sex pheromone change in *H. virescens* in response to bacterial challenge concerned pheromone composition and not total amounts of the sex pheromone (CHAPTER 3), we expect that later steps than the initiation of pheromone synthesis are (also) involved in the trade-off.

### *2.2 Behavioural changes in response to immune challenge*

Many organisms have evolved behavioural adaptations in the face of infection (Schmid-Hempel, 2011; de Roode & Lefèvre, 2012). Prior to infection, animals can show infection avoidance behaviour, i.e., spatial and temporal avoidance of contaminated food or contaminated co-specifics such as mates (Christe *et al.*, 1994; Kavaliers & Colwell, 1995; Penn *et al.*, 1998; Hutchings *et al.*, 2001; Alma *et al.*, 2010). We found that *H. virescens* males mated less with bacteria-challenged than with unchallenged females (CHAPTER 3), indicating that males avoid bacteria-challenged females. As an alternative explanation, bacteria-challenged females could have been less apt to mate than unchallenged females. However, we found no difference in calling behaviour (extrusion of the sex pheromone gland to emit pheromone) between bacteria-challenged, wounded and unchallenged females, which makes this alternative explanation less likely. Avoidance of mating with infected mates has been shown in vertebrates (Kavaliers & Colwell, 1995; Penn & Potts, 1998; Penn *et al.*, 1998; Deaton, 2009). However, in experiments testing avoidance of mating with infected mates in invertebrates, infected and uninfected mates did equally well (with exception of infection with reproductive parasites; Vala *et al.*, 2004) (Abbot & Dill, 2001; Luong & Kaya, 2005; Burand & Tan, 2006; de Roode & Lefèvre, 2012). We thus show for the first time that avoidance of infected mates can also occur in invertebrates.

Once infection has been contracted, adaptations may either take the form of curative behaviours, such as grooming and therapeutic medication, or behaviour that leads to an increase in fecundity to maximize fitness in the short lifespan that remains, such as terminal investment (Schmid-Hempel, 2011; de Roode & Lefèvre, 2012; Abbott, 2014). Terminal investment may involve an increase in courtship activity, oviposition rate or parental care (Minchella & Loverde, 1981; Part *et al.*, 1992; Polak & Starmer, 1998; Adamo, 1999; Bonneaud *et al.*, 2004; Creighton *et al.*, 2009). Bacteria-challenged *H. virescens* females had a higher oviposition rate than control females one night after the challenge (CHAPTER 4). Surprisingly, even though immune-challenged females had shorter life spans than control females, the total amount of eggs females produced was not significantly different between the two groups of females. This suggests that bacteria-challenged females may be able to compensate for shortened life span by higher oviposition rate. However, the increase in oviposition rate was linked to reduced oviposition site selectivity between plants that were damaged by *H. virescens* larvae and undamaged plants (CHAPTER 4), which increases the chance that hatching larvae have lower survival and/or develop more slowly than larvae hatching from eggs laid on undamaged plants. The costs incurred by an increased oviposition rate in infected females are thus likely to become apparent in the offspring generation, indicating that not only immune system activation, but also behavioural changes due to infection may be costly (see also Minchella & Loverde, 1981; Minchella, 1985).

The concept of terminal investment implies that the actual strategy after an infection may depend on the acuteness of the threat for survival and thus on the dose and virulence of an infecting agent (Williams, 1966; Clutton-Brock, 1984). Females that are in acute mortal danger may follow a terminal investment strategy and spend all the resources they have into reproduction, while females that are not in acute life danger may invest in immune system activation or curative behaviours and thus survival. Possibly, there is an ‘acuteness threshold’ at which species switch from a curative to a terminal investment strategy.

Finally, the immune response may depend on the mating status of an animal (Rolff & Siva-Jothy, 2002). In several insect species, including *H. virescens*, juvenile hormone levels rise significantly after mating compared to virgin adults (Loher *et al.*, 1983; Couche *et al.*, 1985; Shu *et al.*, 1998; Rolff & Siva-Jothy, 2002). Since the juvenile hormone can have an immunosuppressive effect (Rolff & Siva-Jothy, 2002), it is possible that immune response is stronger in virgin than in mated females in this moth. Consequently, the immune challenge with *S. entomophila* could have been a stronger and more acute life threat for mated females in the oviposition assay (CHAPTER 3) than for the virgin females in the mating assay (CHAPTER 4). This is in line with our finding that immunity is negatively correlated to reproduction, indicating a trade-off (CHAPTER 3), while we found mated females to make a terminal investment after bacterial challenge (CHAPTER 4). Moreover, while all tested virgin females survived for at least three days (as our experiment

ended after three days) (CHAPTER 3), 38.5% of the mated females of the oviposition experiment died after two nights (CHAPTER 4). It will be interesting to determine whether virgin and mated *H. virescens* females differ in their immune system activation.

Together, the data presented in this thesis underline that arthropods may be exposed to and colonized by multiple bacteria during their lifetime and that bacteria may have a wide variety of effects on their arthropod hosts, ranging from beneficial to lethal. These effects may be due to the effect of bacteria on host physiology, which may extend to induced plant responses after herbivore feeding. In addition, immune challenge via bacterial pathogens can activate costly immune defences which may trade off with other traits or may result in terminal investment behaviour by which a shortened lifespan can be compensated.

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