OF MOTHS, MITES AND MICROBES

THE ROLE OF BACTERIA IN THE LIFE HISTORY
OF TWO ARTHROPOD HERBIVORES

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THE ROLE OF BACTERIA IN THE LIFE HISTORY
OF TWO ARTHROPOD HERBIVORES

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General introduction

Microbes are omnipresent in the environment of macro-organisms and innumerable examples of interactions between micro and macro-organisms have been described. Microbes may colonize different organs or body cavities of macro-organisms and are then referred to as "symbionts" that reside in a "host". Symbiosis was originally defined as the "living together of unlike organisms" by Anton de Bary (1879). Bacterial symbionts can have beneficial, neutral (commensal symbiont), harmful (parasites and pathogens) or mixed effects on the host (Dillon & Dillon, 2004; Baumann, 2005; Werren *et al.*, 2008; Himler *et al.*, 2011; Engel & Moran, 2013). The role of symbionts in hosts may also change during evolution and may be context dependent and thus differ under different circumstances (Werren *et al.*, 2008; Zug & Hammerstein, 2015). First, I will describe and discuss non-pathogenic microbe-host interactions. Thereafter, I will focus on pathogens and the effects of immune challenge on host life history.

1. Non-pathogenic associations of bacteria with arthropod hosts

Symbioses can be categorized according to the co-dependence between symbionts and their hosts. Long-term, stable associations between symbionts and hosts are more likely to lead to mutualistic symbiosis than short-term, loose associations, because host and symbionts together form a unit of selection during evolution (Zilber-Rosenberg & Rosenberg, 2008; Rosenberg *et al.*, 2009; Salem *et al.*, 2015).

Obligate endosymbionts

Obligate bacterial endosymbionts are strictly necessary for host survival, growth and/ or reproduction. Obligate symbioses are evolutionarily ancient and the phylogeny of these so-called primary endosymbionts is strictly congruent with the phylogeny of their host (Munson *et al.*, 1991; Chen *et al.*, 1999; Baumann, 2005; Dale & Moran, 2006). The co-speciation of the symbiotic partners can result in specializations of the host and the bacterium (Moran & Baumann, 2000; Baumann, 2005; Dale & Moran, 2006). For instance, endosymbionts live inside specialized host cells (bacteriocytes), which can form special structures in the host, the bacteriomes and are maternally transmitted inside the eggs (Buchner, 1965; Baumann, 2005; Moran *et al.*, 2008). This tight coevolutionary living together has resulted in genomes of obligate endosymbionts that can be very small, and that are characterized by the loss of functional genes that are necessary for e.g. recombination, replication or transcriptional regulation, while genes for the synthesis of nutrients that the host diet lacks, such as essential

amino acids, are retained (Moran & Wernegreen, 2000; Baumann, 2005; Dale & Moran, 2006; Moran *et al.*, 2008).

Facultative endosymbionts

Facultative (so-called secondary) endosymbionts are not strictly necessary for survival or reproduction of their host. Secondary endosymbionts are mostly maternally transmitted inside the eggs, although horizontal transmission can also occur (Huigens *et al.*, 2004; Caspi-Fluger *et al.*, 2012). Consequently, there is only a partial congruence between symbiont and host phylogeny (Dale & Moran, 2006; Werren *et al.*, 2008). The prevalence of facultative, maternally transmitted bacteria in a host population depends on the efficiency of vertical transmission of the bacteria, but also on host fitness (Werren, 1997; Oliver *et al.*, 2010). Many facultative endosymbionts were shown to benefit the host e.g. by protecting them against parasitoids (Oliver *et al.*, 2003), or by supporting host plant use (Tsuchida *et al.*, 2011; Su *et al.*, 2015).

Endosymbiotic reproductive parasites, such as *Wolbachia*, "*Candidatus* Cardinium", *Spiroplasma* and *Rickettsia* are known to increase their prevalence in a host population by manipulating the reproduction of their host. Since these bacteria are maternally transmitted, reproductive manipulation increases the proportion of infected females in a population via mechanisms such as feminization, parthenogenesis, male-killing or cytoplasmic incompatibility (Werren, 1997; Stouthamer *et al.*, 1999; Weeks *et al.*, 2001; Werren *et al.* 2008; Duron *et al.*, 2008).

Besides reproductive manipulation, reproductive parasites may increase and strengthen their persistence in a host population by benefitting the host (Hoffmann *et al.*, 1998; Fry *et al.*, 2004; Werren *et al.*, 2008; Himler *et al.*, 2011; Zug & Hammerstein, 2015). Accordingly, reproductive parasites have recently been found to enhance host fitness and to increase host resistance to bacterial and viral pathogens (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Moreira *et al.*, 2009) or parasitoids (Xie *et al.*, 2010; Xie *et al.*, 2014), act as nutritional mutualist (Hosokawa *et al.*, 2010; Nikoh *et al.*, 2014), or alter host physiology to their host's benefit (Kaiser *et al.*, 2010).

Gut bacteria

Gut bacteria do not live intracellularly, but inhabit different regions or compartments of the gut. Associations between gut bacteria and a host range from permanent (core community) to transient (Shade & Handelsman, 2012). Gut bacteria are in some cases necessary for host

survival, development or reproduction (Hosokawa *et al.*, 2007; Salem *et al.*, 2013). When gut bacteria are permanently associated with a host, they are often vertically transmitted, may reside in special compartments or pouches in the guts and provide divers beneficial services to their hosts. If gut bacteria are vertically transmitted, transmission occurs mostly extracellularly via various mechanisms. For instance, gut bacteria maybe smeared on the egg surface by a female after oviposition or, as is the case of the stinkbug *Megacopta punctatissima*, provided in capsules together with the egg mass, which ensures high transmission rates (Fukatsu & Hosokawa, 2002; Engel & Moran, 2013; Salem *et al.*, 2015). Other insects, such as the stinkbug *Riptortus pedestris*, take up bacterial symbionts each generation again from the environment (Kikuchi *et al.*, 2011). Another route of transmission for gut bacteria is coprophagy, in which bacteria are excreted together with feces and can be taken up by conspecifics (Beard *et al.*, 2002). In social insects, gut bacteria maybe taken up during contact with nest mates or hive contents and may be transferred together with food from adults to larvae (Koch & Schmid-Hempel, 2011; Martinson *et al.*, 2012; Marsh *et al.*, 2014; Powell *et al.*, 2014).

Gut bacteria may also only be transiently present in the gut if random uptake from the environment is not followed by stable colonization (Shade & Handelsman, 2012; Engel & Moran, 2013). High variability of bacterial communities between individuals of a host population or variability due to diet or developmental stage may indicate transient association of gut bacteria with a host (Shade & Handelsman, 2012). Nevertheless, the metabolic capabilities that bacteria have evolved independently of the host may suffice to enhance the fitness of an animal that takes these bacteria up into its gut, even if transiently (Mason *et al.*, 2014). For instance, some plant-associated bacteria are able to use methanol as carbon source, fix nitrogen or break down secondary plant metabolites (Sy *et al.*, 2005; Fürnkranz *et al.*, 2008; Vorholt, 2012). The latter was shown to benefit larvae of the moth *Lymantria dispar* as described below (Mason *et al.*, 2014).

1.1 Host plant utilization of herbivores in relation to bacterial symbionts

Many herbivores have colonized and feed on plants and plant parts that are poor or unbalanced in nutrients or are hard to digest (Schoonhoven *et al.*, 2005). Moreover, herbivores have to deal with physical and chemical defences that plants have evolved to reduce the damage inflicted by herbivores (Schoonhoven *et al.*, 2005; Karban & Baldwin, 2007). Symbiotic bacteria can play an important role in the ability of herbivores to use plant

species or plant tissues as food and thus determine their host's ecological niche (Douglas, 2009; Feldhaar, 2011; Hansen & Moran, 2014).

Direct facilitation of host plant use

Symbiotic bacteria may directly facilitate host plant use by providing amino acids, vitamins or co-factors that are absent from the host diet or by producing enzymes that enhance the digestion of plant tissues (Buchner, 1965; Douglas, 2009; Feldhaar, 2011; Engel & Moran, 2013; Hansen & Moran, 2014). Obligate endosymbionts are well known for synthesizing nutrients for their hosts. For instance, plant sap is poor in amino acids. Hence, phloem feeders such as aphids are provided with essential amino acids by their primary endosymbionts, *Buchnera aphidicola* (Shigenobu *et al.*, 2000; van Ham *et al.*, 2003; Baumann, 2005). Nutrient provisioning is not restricted to obligate endosymbionts, although gut bacteria can fulfil this function as well. For instance, the gut symbiont of the firebug *Pyrrhocoris apterus* was shown to provide its host with vitamin B, which proved essential for its ability to reproduce (Salem *et al.*, 2013; Salem *et al.*, 2014). The plataspid stinkbug *Megacopta punctatissima* relies on its gut symbiont *Ishikawaella capsulata* for normal growth, development and reproduction (Fukatsu & Hosokawa, 2002; Hosokawa *et al.*, 2007). In ants, there is strong indication that gut bacteria from the order Rhizobiales may be involved in nitrogen fixation (Russell *et al.*, 2009). Even bacteria that only transiently inhabit the gut may affect host plant use of herbivores. For instance, larval growth of the gypsy moth, *Lymantria dispar*, was enhanced by bacteria that inhabit the foliage of its host plant aspen, when phenolic glycosides, the major defence metabolites of aspen, were added to the larval (artificial) diet (Mason *et al.*, 2014). The authors further showed that these bacteria can reduce the concentration of phenolic glycosides *in vitro* (Mason *et al.*, 2014).

Indirect facilitation of host plant use

Besides the direct nutritional effects mentioned above, herbivore-associated bacteria may manipulate plant physiology to their host's benefit (Kaiser *et al.*, 2010; Chung *et al.*, 2013; Hansen & Moran, 2014; Zhu *et al.*, 2014; Su *et al.*, 2015). *Wolbachia* was shown to enhance the fitness of the lepidopteran leaf-miner *Phyllonorycter blancardella*, by preserving photosynthetically active and nutrient rich (green) islands in senescent leaves, thereby increasing host fitness (Kaiser *et al.*, 2010).

Few examples show that symbionts can also interfere with herbivore-induced plant defences. Phytohormones such as jasmonic acid (JA) or salicylic acid (SA) are central to the

coordination of plant defences. The induction of phytohormone biosynthesis is specific to the type of attacker: in general, feeding by herbivorous insects induces the JA-related plant responses (Karban & Baldwin, 2007; Erb *et al.*, 2012). JA-responses were shown to cause reduced larval growth, amount of feeding and survival of herbivores and may attract their natural enemies (Howe *et al.*, 1996; Li *et al.*, 2002; Thaler *et al.*, 2002). Biotrophic phytopathogens generally induce SA-related responses in plants (Glazebrook, 2005). Importantly, JA and SA responses are interrelated and JA-responses can be suppressed via the induction of SA responses and vice versa (JA-SA crosstalk) (Pieterse *et al.*, 2012; Thaler *et al.*, 2012). *Hamiltonella defensa*, the secondary symbiont of the whitefly *Bemisia tabaci*, was found to suppress JA-responses in tomato, via small non-proteinaceous molecules in the whitefly's saliva (Su *et al.*, 2015). Also, bacteria associated with the Colorado potato beetle, *Leptinotarsa decemlineata*, were shown to reduce herbivore-induced JA-responses and to increase the larval growth of the beetle (Chung *et al.*, 2013). In both examples, JA-suppression did not occur in SA-deficient plants, indicating that induction of SA-related responses caused the suppression of JA-responses (Chung *et al.*, 2013)

The role of bacterial symbionts in agriculture

The ability of bacterial symbionts to determine the host plant range of herbivores can have implications for agriculture, in particular pest management. Many herbivores are destructive agricultural and forest pests. Microbes that are harboured by arthropod pests may affect their pest status by influencing the dietary range of the pest or by affecting pest management. For instance, the pest status of the stinkbug *Megacopta punctatissima* on legume crop was found to be determined by the genotype of its obligate gut symbiont *Ishikawaella capsulata* (Hosokawa *et al.*, 2007). In this study, egg hatch rate of the pest species *M. punctatissima* decreased significantly when its symbionts were replaced for symbionts of a closely related non-pest species (*M. cribraria*). On the other hand, egg hatch rate of *M cribraria* was significantly increased after receiving symbionts of *M. punctatissima* (Hosokawa, 2007). It was demonstrated in aphids that symbiotic bacteria are able to expand host plant range of its insect herbivore host: transfection of the facultative endosymbiont *Regiella insecticola* from the pea aphid (*Acyrthosiphon pisum*) to the vetch aphid (*Megoura crassicaula*) enabled the latter to survive and reproduce on clover (Tsuchida *et al.*, 2011).

Bacteria can also interfere with chemical pest control measures. For example, bacteria of the genus *Burkholderia* have been found to confer insecticide resistance to the bean bug, *Riptortus pedestris*, which is a pest on leguminous crops (Kikuchi *et al.*, 2012). Further, larval

mortality in response to the insecticidal toxins of *Bacillus thuringiensis* was shown to be reduced upon removal of the bacterial midgut community in the gypsy moth, *Lymantria dispar*, and other Lepidoptera (Broderick *et al.*, 2006; Broderick *et al.*, 2009). The authors suggest that benign gut bacteria of these Lepidoptera may breach the midgut barrier after it was damaged by *B. thuringiensis* toxin and become opportunistic pathogens (Broderick *et al.*, 2006; Broderick *et al.*, 2009). The presence of bacterial symbionts may also affect biological control measures, e.g. *H. defensa* rendered its aphid host more resistant to parasitoid wasps (Oliver *et al.*, 2003).

2. Pathogenic effects of bacteria on insect hosts

Besides commensal and beneficial bacteria, insects may be exposed to a suite of pathogenic bacteria during their lifetime. Pathogens cause disease in organisms and pathogenicity of bacteria depends on virulence factors, such as the ability to attach to and invade host cells, the production of toxins and the secretion of proteins that modulate host cellular functions (Farthing, 2004; Galán, 2009). However, whether bacteria cause disease also strongly depends on the host or the host tissue (Ruby *et al.*, 2004).

2.1 The immune system of insects

As a first line of defence against pathogens, insects possess physical and chemical barriers, such as the cuticle or the peritrophic matrix (Dunn, 1990; Lehane, 1997; Kuraishi *et al.*, 2011; Davis & Engström, 2012). Nevertheless, pathogens might gain access to the insect hemocoel via wounds or may be ingested and be able to cross the midgut epithelium (Ashida *et al.*, 2012). As a consequence, insects have evolved an efficient innate immune system to fight pathogenic intruders. Recognition of self and non-self is the first step of immune defence (Janeway, 1992; Royet, 2004). Insects have a wide range of proteins that function as pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) (Medzhitov & Janeway, 1997). PAMPs include surface molecules of bacteria such as lipopolysaccharides, peptidoglycans or lipoteichoic acid.

The insect immune system is a combination of cellular and humoral immune responses. Cellular immune responses include processes like phagocytosis and encapsulation that are mediated by different types of blood cells (hemocytes) (Lavine & Strand, 2002; Strand, 2008). During phagocytosis, biotic or abiotic entities are enclosed by individual hemocytes. Encapsulation response targets large invaders, such as nematodes or parasitoid larvae, that cannot be enclosed by a single cell and are therefore bound by multiple hemocytes

(Strand, 2008). Phagocytized or encapsulated organisms are killed and degraded (Reeves *et al.*, 2002; Stuart & Ezekowitz, 2005; Stuart *et al.*, 2007).

Humoral defences include a broad range of antimicrobial peptides (AMPs) that are produced by the fat body or hemocytes and that are released into the hemolymph (Bulet *et al.*, 1999; Zasloff, 2002). AMPs vary in their antimicrobial spectrum. Most AMPs show activity against a wide range of pathogens, with particular efficiency against certain groups, depending on their mode of action. For example, lysozymes, which destroy bonds between peptidoglycan forming molecules (Rupley, 1967) can efficiently destroy gram-positive bacteria but are also active against gram-negative bacteria. Other AMPs have a more narrow antimicrobial spectrum. For instance, drosomycin only shows activity against filamentous fungi (Bulet *et al.*, 1999; Zhang & Zhu, 2009).

The melanin-synthesis cascade is another important part of the insect immune system. Phenoloxidase (PO) is the key enzyme in this cascade and catalyzes the reactions of phenols to toxic quinones which are polymerized to melanin (Nappi & Vass, 1993; Cerenius & Söderhäll, 2004). Intermediates of melanogenesis as well as melanin itself are cytotoxic and have been reported to be lethal for pathogens (Nappi & Vass, 1993; Nappi *et al.*, 1995; Nappi & Vass, 1998; Söderhäll & Cerenius, 1998; Nappi & Christensen, 2005). Further, melanin and its intermediates have functions in the encapsulation and phagocytosis of pathogens as well as in wound healing processes (Cerenius *et al.*, 2008).

2.2 The cost of immune defence and trade-offs with other life history traits

In life history theory, the concept of trade-offs is based on the assumption that organisms have limited internal resources available that need to be partitioned among the different life history traits (Stearns, 1989; Roff & Fairbairn, 2007). Life history traits are therefore negatively linked to each other: a resource investment into one trait which increases fitness may reduce resources that are available for another fitness-relevant trait (Stearns, 1989; Roff & Fairbairn, 2007).

The immune system may prevent an organism from being colonized by pathogens. However, an immune system implicates different types of costs which may negatively affect other life history traits (Sheldon & Verhulst, 1996; Zuk & Stoehr, 2002; Schmid-Hempel, 2005). Physiological costs include costs of nutrients and energy which are necessary for the maintenance and deployment of the immune system (Schmid-Hempel, 2011). Maintenance costs are incurred to keep the immune system at a level of readiness, even when the organism is not infected (Schmid-Hempel, 2005; Kraaijeveld & Wertheim, 2009; Schmid-Hempel,

2011). Deployment costs are incurred when the immune system is activated (Kraaijeveld & Wertheim, 2009; Schmid-Hempel, 2011). Immune system activation has been shown to trade off with various other life history traits such as life span (Moret & Schmid-Hempel, 2000; Krams *et al.*, 2014), growth (Soler *et al.*, 2003; Brommer, 2004) and reproduction (Gustafsson *et al.*, 1994; Sheldon & Verhulst, 1996; Adamo *et al.*, 2001; Rolff & Siva-Jothy, 2002; Hanssen *et al.*, 2005; French *et al.*, 2007).

Evolutionary costs of immunity arise through the negative genetic covariance between immunity and other fitness-related traits in uninfected organisms (Schmid-Hempel, 2005; 2011). For instance, several studies showed that highly pathogen-resistant insect strains perform worse than less resistant strains, e.g. have longer developmental time, lower larval competitive ability or lower fecundity in the absence of pathogens (Boots & Begon, 1993; Kraaijeveld & Godfray, 1997; McKean *et al.*, 2008).

2.3 Behavioural changes in the face of infection

As an alternative (or in addition) to a costly immune system (activation), organisms may have behavioural strategies that reduce the frequency of infection and minimize adverse fitness effects (Schmid-Hempel, 2011, De Roode and Lefevre 2012). An important behavioural adaptation to possible infections is to avoid contracting them. This behaviour can include spatial or temporal avoidance of parasites (Orr, 1992; Christe *et al.*, 1994), avoiding feeding on contaminated food (Hutchings *et al.*, 2001; Alma *et al.*, 2010) or avoiding infected conspecifics, such as infected mates (Kavaliers & Colwell, 1995; Penn & Potts, 1998; Penn *et al.*, 1998).

Behavioural adaptations to reduce pathogen loads once infection has been contracted include self-medication behaviour, in which organisms consume medicinal substances to reduce pathogen loads and increase their fitness while infected (Huffman *et al.*, 1996; Singer *et al.*, 2009; Manson *et al.*, 2010). In this context, infected females of the butterfly *Danaus plexippus* were found to engage in trans-generational medication behaviour and oviposit more on plants with high levels of toxic secondary metabolites than on plants with lower levels than uninfected females (Lefèvre *et al.*, 2010).

Another adaptive behaviour of infected animals, which does not directly aim to reduce pathogen loads but may enhance life time fecundity of infected organisms, is terminal investment. If life expectancy decreases due to age or infection, organisms may make a “terminal investment”, i.e. invest their remaining resources into current reproduction in order to maximize their fitness in the short time that remains (Williams, 1966; Clutton-Brock,

1984). Shortening of the lifespan can be caused by the pathogen that may kill the organism or by the costs of immune defence which can trade off with lifespan (Sheldon & Verhulst, 1996). A shift of investment from future to current reproduction may imply an increase in courtship activity, parental care, oviposition rate or acceptance of less preferred host plants for oviposition (Minchella & Loverde, 1981; Part *et al.*, 1992; Adamo, 1999; Bonneaud *et al.*, 2004; Javoiš & Tammaru, 2004; Creighton *et al.*, 2009).

3. Study organisms

In this thesis, I investigated possible effects of bacteria on the life histories of two generalist herbivorous arthropods. In the noctuid moth *Heliothis virescens* (Fabricius, 1777) (Lepidoptera: Noctuidae), I determined trade-offs between immunity and reproduction as well as adaptive behavioural changes in the oviposition strategy of this moth in the face of infection. Moreover, I identified bacterial communities that are associated with *H. virescens.*

In the two-spotted spider mite *Tetranychus urticae* (Koch, 1836) (Acari: Tetranychidae), which is well known to harbour various (endo)symbiotic bacteria such as *Wolbachia*, "*Candidatus* Cardinium" and *Spiroplasma* (Breeuwer & Jacobs, 1996; Gotoh *et al.*, 2003; Enigl & Schausberger, 2007; Gotoh *et al.*, 2007a), I investigated effects of these bacteria on mite fitness parameters, mite transcriptome and induced plant responses of tomato leaflets after mite feeding.

3.1. *Heliothis virescens*

The tobacco budworm, *H. virescens* (Figure 1.1), is distributed throughout the American continent (Fitt, 1989). Its larvae are polyphagous and have been reported to feed on at least 37 plant species in 14 families (Barber, 1937; Stadelbacher, 1981; Fitt, 1989; Waldvogel & Gould, 1990; Sheck & Gould, 1993; Blanco *et al.*, 2007). Among the host plants of *H. virescens* are important economic crops like tobacco, cotton and chickpea (Morgan & Chamberlin, 1927; Barber, 1937; Neunzig, 1969; Blanco *et al.*, 2007), which makes this species a major agricultural pest.

Heliothis virescens females can oviposit up to 1500 eggs in their lifetime of about 30 days in the laboratory at 25 °C (Proshold *et al.*, 1982; Willers *et al.*, 1987; Fitt, 1989). Eggs are oviposited singly on plants throughout the night, starting at dusk (Fitt, 1989; Ramaswamy, 1990). *Heliothis virescens* has five larval stages and developmental time from eggs to pupae takes about 17 days at 25 °C (Fye & McAda, 1972). Larvae pupate in the soil for 13 days at 25 °C after which the adults eclose.

Heliothis virescens was reported to have an efficient immune system (Barthel *et al.*, 2014). However, possible effects of immune response on life history traits have not been investigated in this species. Furthermore, not much is known about its microbiome, i.e. bacteria that are associated with *H. virescens*, particularly in the field.

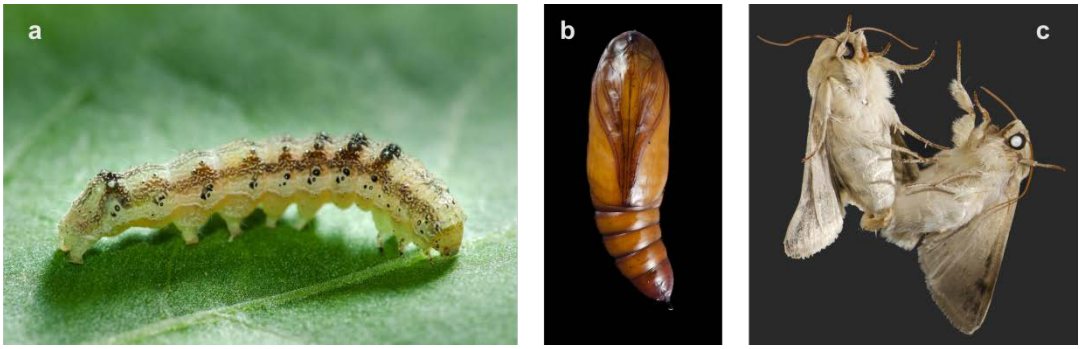

Figure 1.1 *Heliothis virescens* (a) larva, (b) pupa (c) mating adults. © Jan van Arkel.

3.2. *Tetranychus urticae*

The two-spotted spider mite, *T. urticae* (Figure 1.2) is an extremely polyphagous herbivore that occurs worldwide and has been reported to feed on over 1100 plant species, among them are economically important crops like tomato, cucumber, strawberry, bean and cotton (Bolland *et al.*, 1998; Grbic *et al.*, 2011; Migeon *et al.*, 2011) which makes this mite an important agricultural pest.

The life cycle of *T. urticae* includes egg, one larval stage, which is followed by two nymphal stages (protonymph and deutonymph) and the adult stage. The generation time of *T. urticae* takes about two weeks at 27 °C (Chain-ing *et al.*, 1976). Females have been reported to lay over 100 eggs during their lifetime (Carey & Bradley, 1982). Thus this mite can reach large population sizes in a short amount of time.

Spider mites are stylet feeders that pierce plant cells and suck out the cell content. Like other stylet feeders, *T. urticae* has been shown to induce jasmonic and salicylic acid-related plant defences during feeding (Walling, 2000; Kant *et al.*, 2004; Kant *et al.*, 2008; Alba *et al.*, 2015).

Tetranychus urticae was found to harbour different endosymbionts, including *Wolbachia*, "*Candidatus* Cardinium" and *Spiroplasma* (Breeuwer & Jacobs, 1996; Gotoh *et al.*, 2003; Enigl & Schausberger, 2007; Gotoh *et al.*, 2007a). *Wolbachia* and "*Candidatus* Cardinium" were shown to cause cytoplasmic incompatibility in some populations of *T. urticae* (Breeuwer, 1997; Gotoh *et al.*, 2007a; Gotoh *et al.*, 2007b). In other *T. urticae*

populations, *Wolbachia* did not cause cytoplasmic incompatibility (Gotoh *et al.*, 2003). Further, the effects of *Wolbachia* on mite fitness have been assessed with all possible outcomes. i.e. and *Wolbachia* had a negative effect, no effect or a positive effect on female fecundity (Breeuwer, 1997; Vala *et al.*, 2000; Perrot-Minnot *et al.*, 2002; Xie *et al.*, 2011). The presence of *Wolbachia* on mite gene expression has been investigated (Zhang *et al.*, 2015). Less is known about effects of *Spiroplasma* and "*Candidatus* Cardinium" or any combination of endosymbionts on *T. urticae*. Furthermore, the effect of endosymbionts on host-plant use of this mite has not yet been investigated.

Figure 1.2 *Tetranychus urticae* adult female.
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Thesis outline

Chapter 2. As outlined above, bacteria can have diverse effects on their hosts. On the one hand, herbivore-associated bacteria may benefit the host, for instance by facilitating plant utilization of the host (Engel & Moran, 2013; Hansen & Moran, 2014). On the other hand, bacterial pathogens may negatively affect life history traits and thus the fitness of an organism (Grenfell & Dobson, 1995; Poulin, 2007; Schmid-Hempel, 2011). Against this background, we investigated the bacterial community that is associated with field and laboratory populations of *H. virescens* and assessed the spatial and temporal variability of this bacterial community as it depends on diet and life stage.

Chapter 3. The concept of 'Bateman's principle and immunity' predicts that females, who maximize their fitness by increasing longevity, invest more in immunity than males, who maximize fitness by increasing their number of matings (Rolff, 2002). Most moths, including *H. virescens*, do not correspond to the "classical" sex roles in which females invest more into offspring than males. In this species, females and males invest substantially into offspring: females produce up to 1500 eggs whereas males produce a spermatophore that can comprise up to 5% of their bodymass (Fye & McAda, 1972; Proshold *et al.*, 1982; Blanco *et al.*, 2009).

Both sexes produce a sex pheromone, but until now only males have been shown to differentiate between female sex pheromones of different quality, indicating that males are the choosey sex in this species (Vetter & Baker, 1983; Groot *et al.*, 2009; Groot *et al.*, 2014). Because of the deviations in the life history of *H. virescens* from the classical Bateman case, it can be expected that differences in immunity between the sexes do not follow the predictions for 'Bateman's principle and immunity'. To assess the concept of 'Bateman's principle and immunity' in *H. virescens,* we first investigated immune system activation of males and females via immune gene expression experiments using dead cells of the entomopathogen *Serratia entomophila*. Secondly, we assessed the influence of immune activation on mating success of both sexes in mate choice experiments. Thirdly, we tested if immune defence activation would affect the composition of the female sex pheromone as well as female calling behaviour.

Chapter 4. Infection may shorten the life span of an organism. One strategy for organisms to maximize fitness in the face of infection is therefore terminal investment, in which investment into future reproduction is shifted towards current reproduction (Williams, 1966; Clutton-Brock, 1984). In this chapter, we investigated whether terminal investment is part of the life history strategy of *H. virescens* and whether females of this moth adaptively change their oviposition strategy in response to an immune challenge with dead cells of the entomopathogen *S. entomophila.* Specifically, we assessed whether *H. virescens* females would increase their egg output in response to an immune challenge, and whether oviposition site choice of *H. virescens* females would be influenced by an immune challenge.

Chapter 5. Endosymbionts, such as *Wolbachia,* "*Candidatus* Cardinium" and *Spiroplasma,* are well-known reproductive manipulators which may increase the proportion of infected females to increase their prevalence in a host population (Werren, 1997; Duron *et al.*, 2008; Werren *et al.*, 2008; Engelstädter & Hurst, 2009). However, several studies indicate that these bacteria may also positively affect host fitness (Teixeira *et al.*, 2008; Kaiser *et al.*, 2010; Xie *et al.*, 2010; Zug & Hammerstein, 2015). We studied the role of bacterial (endo)symbionts in two strains of the two-spotted spider mite *T. urticae*, one strain is a plant defence inducer and the other strain a plant defence suppressor. We investigated the bacterial community associated with these two strains and tested if the presence of (endo)symbionts was correlated with performance and gene expression profiles of the mites. Further, we assessed if the

symbionts were associated with induction or suppression plant responses in tomato (*Solanum lycopersicum*).

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2

Variability of bacterial communities in the moth *Heliothis virescens* indicates transient association with the host

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Abstract

Microbes associated with insects can confer a wide range of ecologically relevant benefits to their hosts. Since insect-associated bacteria often increase the nutritive value of their hosts' diets, the study of bacterial communities is especially interesting in species that are important agricultural pests. We investigated the composition of bacterial communities in the noctuid moth *Heliothis virescens* and its variability in relation to developmental stage, diet and population (field and laboratory), using bacterial tag-encoded FLX pyrosequencing of 16S rRNA amplicons. In larvae, bacterial communities differed depending on the food plant on which they had been reared, although the within-group variation between biological replicates was high as well. Moreover, larvae originating from a field or laboratory population did not share any OTUs. Interestingly, *Enterococcus* sp. was found to be the dominant taxon in laboratory-reared larvae, but was completely absent from field larvae, indicating dramatic shifts in microbial community profiles upon cultivation of the moths in the laboratory. Furthermore, microbiota composition varied strongly across developmental stages in individuals of the field population, and we found no evidence for vertical transmission of bacteria from mothers to offspring. Overall, the high variability in bacterial communities suggests that the bacteria that we identified are only loosely and temporarily associated with *H. virescens*.

Manuscript submitted

Introduction

Symbiotic bacteria of insects provide diverse beneficial services to their hosts, e.g. upgrading of nutrient-poor diets, digestion of refractory food sources and protection of the host against pathogens e.g. (Dillon & Dillon, 2004; Douglas, 2009; Feldhaar, 2011; Engel & Moran, 2013). Therefore, the study of bacterial communities that are associated with an organism of interest has become an integral part of the fundamental and applied research of, in particular, agricultural pests. Investigating bacterial diversity and insect-bacteria associations is greatly facilitated by high-throughput sequencing methods, especially massive parallel amplicon sequencing, that have been developed in the past decade (Margulies *et al.*, 2005; Rothberg & Leamon, 2008; Bosch & McFall-Ngai, 2011; Loman *et al.*, 2012).

Associations of bacterial communities with a host can be stable or dynamic. Stable associations are characterized by the temporal persistence of bacteria in the host across different life stages and across generations (Zilber-Rosenberg & Rosenberg, 2008; Rosenberg *et al.*, 2009; Shade & Handelsman, 2012; Engel & Moran, 2013; Salem *et al.*, 2015). The stability of the community can be indicative of the relevance of bacteria for the ecology and evolution of the host. If bacteria are transmitted vertically from one generation to the next for many generations, host and bacteria can be viewed as a unit of selection, and bacteria are likely beneficial for the host (Zilber-Rosenberg & Rosenberg, 2008; Rosenberg *et al.*, 2009; Oliver *et al.*, 2010).

Another criterion to assess the relevance or the functional role of bacteria for an organism is the variability of bacterial communities in the different environments in which the insect host resides (Robinson *et al.*, 2010; Chandler *et al.*, 2011; Colman *et al.*, 2012; Gayatri Priya *et al.*, 2012; Sullam *et al.*, 2012). Variability of bacterial communities in relation to host diet is of particular interest in agricultural and forest pest insects, because the flexibility of their bacterial communities may enhance the dietary range of phytophagous insects (Dillon & Dillon, 2004; Hosokawa *et al.*, 2007; Douglas, 2009; Tsuchida *et al.*, 2011; Chu *et al.*, 2013; Engel & Moran, 2013). Bacterial communities have been shown to vary depending on diet in several insect species (Broderick *et al.*, 2004; Belda *et al.*, 2011; Chandler *et al.*, 2011; Gayatri Priya *et al.*, 2012) but may also be stable across diets (Sudakaran *et al.*, 2012). Other factors that may cause variation in bacterial communities in insects are the geographic origin of the host population or rearing history of the insect host (Xiang *et al.*, 2006; Adams *et al.*, 2010; Belda *et al.*, 2011).

Studies of bacterial communities in Lepidoptera have accumulated in the past decade, mostly because larvae of many Lepidoptera are major agricultural or forest pests (Broderick *et*

al., 2004; Xiang *et al.*, 2006; Belda *et al.*, 2011; Gayatri Priya *et al.*, 2012; Xia *et al.*, 2013). Bacteria from the genus *Enterococcus* have been repeatedly found in Lepidoptera (Jarosz, 1975; Broderick *et al.*, 2004; Xiang *et al.*, 2006; Brinkmann *et al.*, 2008; Tang *et al.*, 2012; Hammer *et al.*, 2014) and have been shown to be metabolically active in *Manduca sexta* (Brinkmann *et al.*, 2008). Other bacterial candidates that could serve an ecological role in Lepidoptera were isolated from saturniid butterflies and the silkmoth *Bombyx mori* (Pinto-Tomas *et al.*, 2007; Prem Anand *et al.*, 2010). These bacteria exhibited, among others, cellulolytic, pectinolytic and xylanolytic activities that might help caterpillars to digest plant material (Pinto-Tomas *et al.*, 2007; Prem Anand *et al.*, 2010). Also, a recent metagenomic study of the bacterial midgut community in the pyralid moth *Ostrinia nubilalis* revealed the presence of bacterial cellulase, amylase, β-galactosidase and β-glucosidase encoding genes, indicating the potential of the gut bacterial community to support digestion in its lepidopteran host (Belda *et al.*, 2011). However, when the midgut is damaged by insecticidal toxins in spores of *Bacillus thuringiensis*, formerly benign, resident bacteria may breach the midgut barrier, enter the hemocoel and participate in the destruction of the host (Broderick *et al.*, 2006; Broderick *et al.*, 2009).

The noctuid moth *Heliothis virescens* is a major agricultural pest in North and South America (Fitt, 1989). Its larvae are polyphagous and feed on over 37 plant species from 14 families (Barber, 1937; Stadelbacher, 1981; Fitt, 1989; Waldvogel & Gould, 1990; Sheck & Gould, 1993; Blanco *et al.*, 2007). Among these plants are important economic crops such as cotton (*Gossypium hirsutum*), tobacco (*Nicotiana tabacum*) and chickpea (*Cicer arietinum*) (Barber, 1937; Neunzig, 1969; Fitt, 1989; Blanco *et al.*, 2007). Since bacteria can facilitate host-plant use by herbivorous insects, investigating the bacterial community of *H. virescens* and its variation in relation to diet could lead to the identification of bacterial candidates that enhance or inhibit its development and thereby its impact as an agricultural pest.

In this study, we identified and compared bacterial communities in different developmental stages (eggs, larvae and female adults) of *H. virescens* from the field and laboratory, and reared on different plant species (cotton, chickpea and tobacco). Additionally, we investigated the transmission of the bacterial community from one generation to the next.

Material and methods

Ethics statement

Heliothis virescens was collected in North Carolina, USA, where this species is a pest and not protected by law. Eggs of *H. virescens* were collected on private property, for which no permits were required.

Effect of host plant species on bacterial community composition of laboratory-reared larvae.

To investigate whether the bacterial community composition in *H. virescens* larvae changes depending on the food plant species, we used the long-term laboratory-reared ARS strain. This strain was started in 1971 in Washington County, MS, and since then reared in the laboratory at USDA-ARS in Stoneville, MS, and transferred to the Max Planck Institute for Chemical Ecology (MPI-CE), Jena, Germany in 2010, where the larvae were reared on pinto bean diet containing the antibiotic tetracycline hydrochloride (Sigma-Aldrich, The Netherlands) (Burton, 1970). Adults were provided with a 10% (v/v) honey water solution. All life stages were kept in climate chambers at a temperature of 25 °C, 60% relative humidity and a light/dark cycle of 16h:8h.

We placed 30 first instars of the ARS strain on whole cotton (*Gossypium hirsutum*), chickpea (*Cicer arietinum*) and tobacco plants (*Nicotiana attenuata*), which were grown in the greenhouse of MPI-CE, Jena, Germany. After nine days, when the larvae were in the second, third or fourth instar, they were taken off the plants and starved for eight hours to empty their gut content and to reduce the amount of chloroplasts, as chloroplast DNA may be amplified in the procedure of 16S rRNA amplicon sequencing. To remove bacteria that resided on the outer cuticle, experimental larvae were washed by submerging them for 10 s in 1% (w/v) sodium dodecyl sulfate (SDS) solution and for 10 s in sterile water. Larvae were then put in 1.5-ml Eppendorf tubes, immediately placed on ice and then stored at -80 °C.

Effect of host plant species on bacterial community composition of field larvae

Heliothis virescens eggs were collected in July 2011 in North Carolina, USA, on commercially grown tobacco, *N. tabacum*. Experiments were conducted with larvae, referred to as field larvae, as well as females that developed from these larvae, and eggs that were laid by these females. The field-collected eggs were divided into three groups in plastic cups (100 ml) which were filled with leaf material of one of the three plant species cotton (*G. hirsutum*), chickpea (*C. arietinum*) and tobacco (*N. tabacum*). After emergence, batches of 20-30 first

instar larvae were transferred to whole plants, resulting in 10 batches per host plant species. The plants were kept outdoors until experiments started (i.e. when larvae were placed on them to feed). Each plant was kept in an individual cage made of fine-meshed gauze (diameter: 60 cm, height: 1 m). After larvae had reached the fifth instar, one larva was randomly collected from each plant batch and starved for eight hours. These 30 larvae were washed by submerging them for 10 s in 1% (w/v) SDS solution and for 10 s in sterile water. After the wash step, guts were extracted by cutting the larva between the 1st and 2nd thoracal and between the 7th and 8th abdominal segments. Dissected guts were stored at -80 °C until DNA extraction. The remaining larvae were left to pupate in the soil of the potted whole plant and subsequently used in the bacterial transmission experiment (see below).

Diversity and composition of the bacterial community in adult females and their eggs

To determine bacterial communities in females and their corresponding eggs, a subset of the field larvae that was not used to determine the larval bacterial community was allowed to pupate in the soil and develop into adults. Upon emergence, males and females that had fed on the same plant species were mated in carton cups (200 ml) in single pairs. The cups contained leaves of the same plant species on which the mating pairs had been as larvae to stimulate, and provide a substrate for, oviposition. After four days, we collected abdomens from three females and 30 of their eggs per plant species group, and thus obtained nine female-egg combinations in total. DNA from eggs was extracted in batches of 30 eggs per female. Eggs were collected from the plant leaves with a brush, transferred to 1.5-ml Eppendorf tubes and placed immediately on ice until storage at -80 °C. Female abdomens were cut off and submerged in 1% (w/v) SDS solution for 10 s, followed by a 10 s washing step in sterile water. Abdomens were then transferred to 1.5-ml Eppendorf tubes and placed immediately on ice until storage at -80 °C. All instruments were rinsed with 1% (w/v) SDS solution and sterile water between samples. All tissues were collected in 1.5-ml Eppendorf tubes and placed immediately on ice to prevent changes in the bacterial community composition, after which the samples were stored at -80 °C.

Effect of laboratory-rearing and antibiotics treatment on bacterial communities of field larvae

To assess whether bacterial communities change after transfer from the field to the laboratory and how the antibiotic tetracycline that is normally part of the laboratory diet affects the bacterial gut community, moths that originated from eggs that were collected in the field to

assess microbial communities (see above) were kept in the laboratory at the University of Amsterdam for three generations (in the following referred to as field-lab larvae). Larvae were kept on artificial pinto-bean diet as described above, but without the addition of antibiotics. Females of the third generation were mated in single pairs in empty 200-ml plastic cups. Eggs of the fourth generation hatched in these cups, and the resulting first instars were transferred to cotton leaves with (AB group) or without tetracycline coating (NoAB group). Every other day, cotton leaves were coated with tetracycline by pipetting 1 ml 0.1% (w/v) tetracycline onto the upper leaf surface. Control leaves were treated in the same way with only water. In addition, we wrapped the leaf stems with cotton soaked with 0.1% (w/v) tetracycline or water in the treatment or the control group, respectively. When the larvae reached the fifth instar, they were starved for eight hours, then washed by submerging them for 10 s in 1% SDS solution and for 10 s in sterile water, after which their gut was extracted as described above. The guts were stored at -80 °C until DNA extraction.

DNA extraction

DNA from the field and field-lab population was extracted by grinding samples in 500 µl TES buffer [100 mM tris(hydroxymethyl) aminomethane hydrochloride pH 8, 10 M ethylenediaminetetraacetic acid, 2% sodium dodecyl sulfate] and 4 µl lysozyme from chicken egg white (100 mg/ml) (Sigma-Aldrich), after which the samples were incubated at 37 °C for 30 min. Next we added 2.5 µl proteinase K (20 mg/ml) (Sigma-Aldrich) and incubated the samples for 56 °C overnight. The rest of the extraction procedure was conducted according to the standard CTAB/chloroform protocol as described in Unbehend *et al.* (2013). DNA from the lab-strain larvae was extracted with the Epicenter MasterPure kit (Epicentre Technologies, USA) according to manufacturer's instructions. Additionally, a lysozyme step [4 µl lysozyme (100 mg/ml), incubated at 37 °C for 30 min] was added before the proteinase K step.

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP)

Samples were pooled for sequencing. For the larvae of the laboratory strain, we made three pools, one pool contained DNA of seven larvae that were reared on each of the three plant species (cotton, tobacco or chickpea). For the larvae of the field population, we made six pools of DNA, two pools (biological replicates) for each plant species, to assess the variation between biological replicates; each pool contained the DNA of five larvae. For the field-lab population, we made two pools, one pool of 45 NoAB larvae and one pool of 49 AB larvae. Pooled samples were sent to an external service provider (Molecular Research Lab, MR DNA,

Shallowater, TX, USA) for bTEFAP, using the 16S rRNA primers Gray28F (5'-GAGTTTGATCNTGGCTCA-3') and Gray519R (5'-GTNTTACNGCGGCKGCTG-3') (Ishak *et al.*, 2011). A sequencing library was constructed via one-step PCR with 30 cycles, using a mixture of Hot Start and HotStar hi-fidelity polymerases (Qiagen). Sequencing was performed on a Roche 454 FLX instrument with Titanium reagents and procedures and protocols of Molecular Research LP (http://www.mrdnalab.com/).

Sequence analysis

Analysis of the sequences was performed in QIIME (quantitative insights into microbial ecology), which is a standard pipeline to analyse microbial communities based on 16S rRNA amplicon sequencing (Caporaso *et al.*, 2010a). Raw data were de-noised with denoise_wrapper (Reeder & Knight, 2010). Low-quality reads (quality cut-off = 25) and sequences that were shorter than 200 bp or longer than 600 bp were excluded from further analysis. The remaining sequences were clustered into operational taxonomic units (OTUs) with the open-reference OTU picking command, using uclust (version 1.2.22) (Edgar, 2010) and applying 97% similarity cut-offs. Sequences were first clustered against a reference data set (http://greengenes.lbl.gov/). The sequences that did not cluster with the reference sequences were clustered *de novo* and checked for chimeras with Chimera Slayer (Haas *et al.*, 2011). Identified chimeras were removed from the dataset for downstream analysis.

From each OTU cluster, the most abundant sequence was taken as the representative sequence. Taxonomy was assigned to the representative sequences with the uclust consensus taxonomy classifier. The resulting OTU table was manually edited, and global singletons as well as chloroplasts, mitochondria and OTUs that were classified as moth genomic DNA were removed. Representative sequences of the remaining OTUs were aligned with PyNAST using the Greengenes core set (http://greengenes.lbl.gov/) as a template (Caporaso *et al.*, 2010b). A phylogenetic tree was constructed with the open source software Fasttree 2.1.3, applying the generalized time-reversible (GTR) model of nucleotide evolution (Price *et al.*, 2010). Local support values for tree splits were calculated with the Shimodaira-Hasegawa test (Shimodaira & Hasegawa, 1999). This phylogenetic tree, which included all identified OTUs, was used to calculate the weighted UniFrac metric (Lozupone & Knight, 2005) which was used as the basis for principal component plots (see below).

Diversity and phylogenetic analysis

Diversity analysis of our samples was done with the QIIME pipeline. Since our samples showed unequal sampling depth (see Tables S2.1-2.3), we investigated alpha- and beta-diversity of the bacterial communities of our samples with rarified OTU tables. The number of sequences used for rarefaction corresponded to the number of sequences present in the sample with the lowest number of sequences (339 sequences). To assess alpha-diversity, we calculated the Shannon index with different numbers of sequences (Shannon, 1948). To identify possible clusters based on similarity of bacterial communities among our samples, we produced principal component plots on the basis of weighted non-normalized UniFrac distances and with rarified OTU tables (Lozupone & Knight, 2005). We additionally performed a Jackknife analysis based on weighted UniFrac distances to assess the robustness of our results. Jackknife support is included in the PCA plots as the size of the ellipsoid areas surrounding the data points.

OTU patterns across samples

None of the bacterial OTUs was present in all samples, i.e. present in samples of all origins, rearing plants, and life stages. Most common were three OTUs which were present in 65% of the samples. These OTUs were mainly present in females and eggs, which had a high sample size compared to the other groups. To also consider OTUs that were present in the larvae, we filtered for OTUs that were on average represented more than 1% across all samples, resulting in 18 OTUs (i.e. first the percentage of an OTU in a sample was calculated followed by the average of the percentage of the OTU in all samples, see Table 2.1). To classify and construct a phylogenetic tree of these 18 OTUs, we first aligned the sequences of our dataset and 16S rRNA reference sequences obtained from Genbank (Benson *et al.*, 2011), using ClustalW (Thompson *et al.*, 1994), after which we constructed a maximum likelihood tree in MEGA 6 (Tamura *et al.*, 2013) with the Tamura-Nei model (Tamura & Nei, 1993), using uniform rate variation and 500 bootstrap replicates. To root the tree, we used three Archaea species as outgroups: *Halobacterium salinarum* (NR_113428.1), *Sulfolobus acidocaldarius* (NR_074267.1) and *Pyrolobus fumarii* (NR_102985.1). Outgroup taxa were excluded from the figure for clarity (Figure 2.1).

Table 2.1. Bacterial OTUs with an average presence of more than 1% across all samples.

#	Family (Genus)	Total Average (29)	Larvae Lab (3)	Larvae Field (6)	Larvae Field-lab (2)	Adults (9)	Eggs (9)	Overall presence > 1%
1	Enterobacteriaceae (*Klebsiella/Enterobacter*)	**27.63**	0.00	0.00	35.62	72.34	35.95	68.97
2	Enterococcaceae (*Enterococcus*)	**11.40**	50.55	0.00	61.70	0.00	0.01	17.24
3	Enterobacteriaceae (*Serratia*)	**8.57**	0.00	0.00	0.01	7.20	22.42	44.83
4	Pseudomonadaceae (*Pseudomonas*)	**4.82**	0.00	0.00	0.77	0.69	19.62	55.17
5	Enterobacteriaceae (*Enterobacter*)	**3.97**	0.00	0.00	0.01	5.06	5.29	20.69
6	Acetobacteraceae (*Asaia*)	**2.38**	49.45	0.00	0.00	0.00	0.00	6.90
7	Enterobacteriaceae (unclassified)	**2.15**	0.00	7.75	0.00	0.00	0.00	6.90
8	Mycobacteriaceae (*Mycobacterium*)	**2.15**	0.00	0.00	0.00	0.03	4.13	27.59
9	Acidobacteria (unclassified)	**2.04**	0.00	8.42	0.00	0.00	0.00	3.45
10	Pseudomonadaceae (*Pseudomonas*)	**1.87**	0.00	0.00	0.04	5.32	0.02	3.45
11	Methylobacteriaceae (*Methylobacterium*)	**1.57**	0.00	31.45	0.00	0.00	0.00	13.79
12	Enterobacteriaceae (*Klebsiella/Enterobacter*)	**1.57**	0.00	0.00	0.02	1.92	2.46	13.79
13	Pseudomonadaceae (*Pseudomonas*)	**1.54**	0.00	0.00	0.00	0.17	2.55	20.69
14	Xanthomonadaceae (*Stenotrophomonas*)	**1.39**	0.00	0.00	1.55	4.59	0.20	34.48
15	Paenibacillaceae (*Paenibacillus*)	**1.12**	0.00	18.19	0.00	0.00	0.00	13.79
16	Moraxellaceae (*Acinetobacter*)	**1.04**	0.00	0.00	0.04	0.08	6.53	24.14
17	Sphingomonadaceae (*Sphingomonas*)	**1.02**	0.00	0.00	0.01	0.00	0.16	6.90
18	Comamonadaceae (*Roseateles*)	**1.00**	0.00	8.84	0.07	0.00	0.01	13.79

Taxonomy and percentage of relative abundance in different developmental stages (egg, larva and adult) and populations (laboratory, field and field-lab). Sample sizes are given between brackets.
#: OTU identification numbers

Statistical analysis of similarities between bacterial communities of females and their eggs

To test whether egg bacterial communities resemble the bacterial community of their mother, we performed a linear mixed model in the package lme4 (Bates *et al.*, 2014) and lmerTest (Kuznetsova *et al.*, 2014) in the software R, version 3.0.2 (R Core Team, 2013). Weighted

UniFrac distance values of all 81 female-egg combinations were used as response variable. We used two predictor variables: female-egg combination (eggs belonged to one female or not) and plant species (eggs belonged to the same plant species--cotton, chickpea, or tobacco--as a female or not). To account for repeatedly entering females and eggs into the model, we added egg and female as random factors into the model. Because there was no interaction effect between plant and female-egg combination, we excluded the interaction from the model. Degrees of freedom were approximated using the Satterthwaite method. As a *post hoc* test for the plant effect, we performed planned pairwise comparisons between female-eggs combinations of one plant species group and female-egg combinations in which females did, but eggs did not belong to that respective plant group, by using the multcomp package (Hothorn *et al.*, 2008) in R, applying a Bonferroni correction for multiple comparisons.

Results

Bacterial community composition of *H. virescens*

We received a total of 312,324 sequences, of which 295,289 sequences remained after quality filtering. Other sequences were global singletons or chimeras, derived from chloroplasts and mitochondria or fragments that were identified as part of the moth genome. The bacterial sequences clustered into 566 OTUs, of which 42 were unassigned. An overview of numbers of sequences before and after quality filtering, as well as numbers of OTUs in the different samples is given in Tables S2.1-2.3.

None of the OTUs in *H. virescens* was present in all samples (n = 29), and only three OTUs were found in more than 65% of our samples (65% was the highest percentage of samples in which OTUs were commonly present). When we assessed how many OTUs had an average percentage of more than 1% across all samples, this resulted in 18 OTUs, but only two of these were present with an average percentage of more than 10% across all samples (Table 2.1). The phylogenetic analysis revealed that these 18 OTUs belong to the following bacterial families (bacterial orders in parentheses): Acetobacteraceae (Rhodospirillales), Methylobacteriaceae (Rhizobiales), Sphingomonadaceae (Sphingomonadales), Comamonadaceae (Burkholderiales), Enterobacteriaceae (Enterobacteriales), Pseudomonadaceae, Moraxellaceae (both Pseudomonadales), Xanthomonadaceae (Xanthomonadales), Mycobacteriaceae (Actinomycetales), Paenibacillaceae (Bacillales), Enterococcaceae (Lactobacillales); and one OTU belonged to the phylum Acidobacteria (Table 2.1 and Figure 2.1).

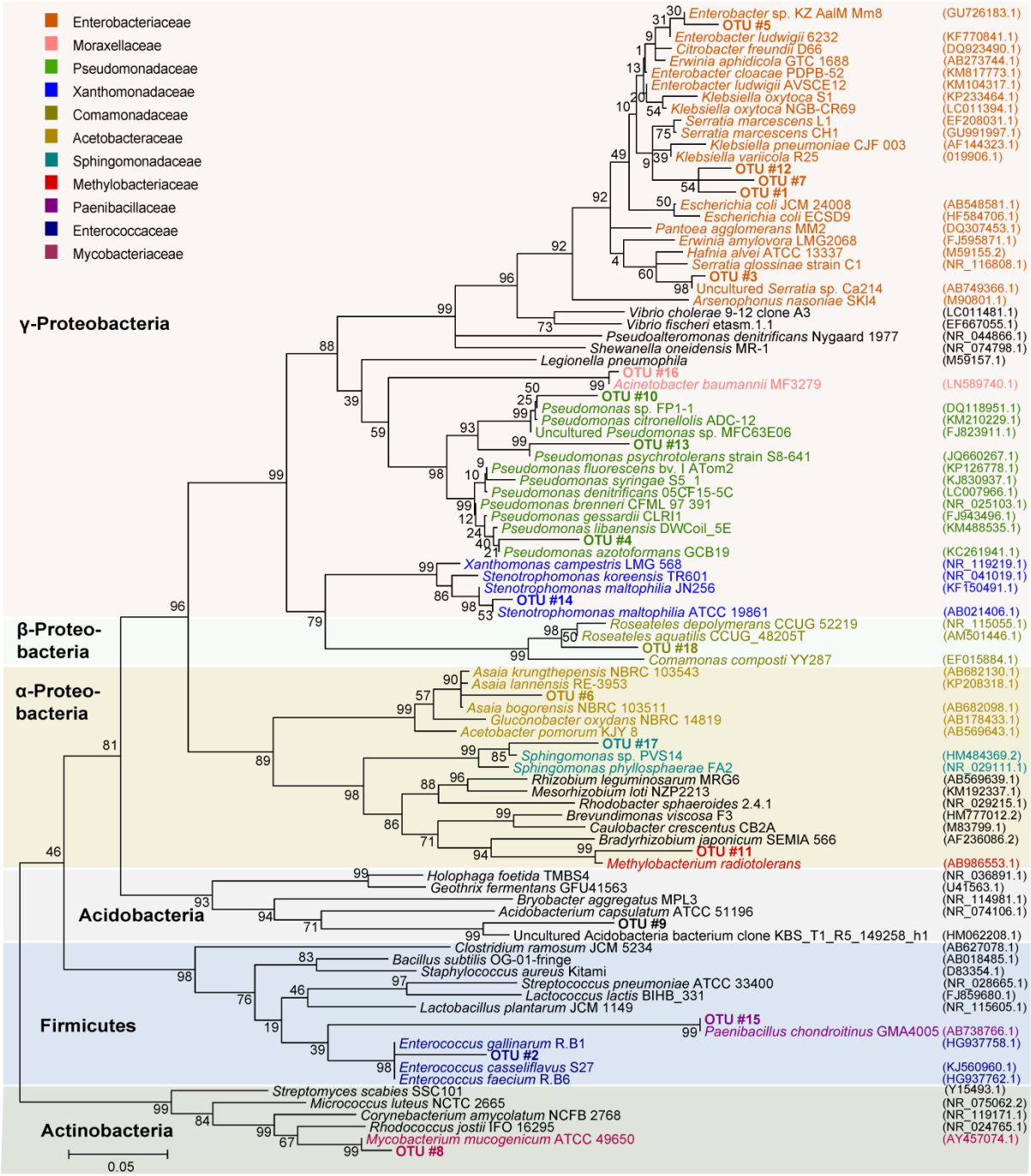

Figure 2.1. Maximum likelihood phylogenetic tree of bacterial OTUs with an average presence of more than 1% across all samples. OTUs that were identified in *H. virescens* are indicated in bold font and labelled OTU #1 to OTU #18. Reference sequences were obtained from Genbank; strain names are given behind the species or genus name. Accession numbers are between brackets. Font colours correspond to different bacterial families, background colours indicate bacterial phyla (or class in the Proteobacteria). The tree was constructed on the basis of partial 16S rRNA gene sequences, applying 500 bootstrap replications. Bootstrap values are indicated above the branches.

Variability of bacterial communities between laboratory and field larvae

We compared bacterial communities between larvae from a long-term laboratory colony and larvae that had been collected in the field as eggs. Laboratory and field larvae had no OTUs in

common. The bacterial community of lab larvae was dominated by two OTUs that were classified as *Enterococcus* sp. (Lactobacillales) (#2) and *Asaia* sp. (Rhodospirillales) (#6), while larvae of the field population did not contain any OTUs affiliated with these genera. In fact, field larvae did not contain clearly dominant OTUs, but harboured Enterobacteriales, Burkholderiales or Rhizobiales in relatively high percentages (see below). Furthermore, bacterial communities in the field-lab larvae differed greatly from the bacterial communities of the field larvae: only 1.4 % of the OTUs (n = 148) of the field larvae were detected in the field-lab larvae that were reared without antibiotics (NoAB). *Enterococcus* sp. (#2) was more abundant in the AB field-lab larvae than in NoAB larvae (Figure 2.2).

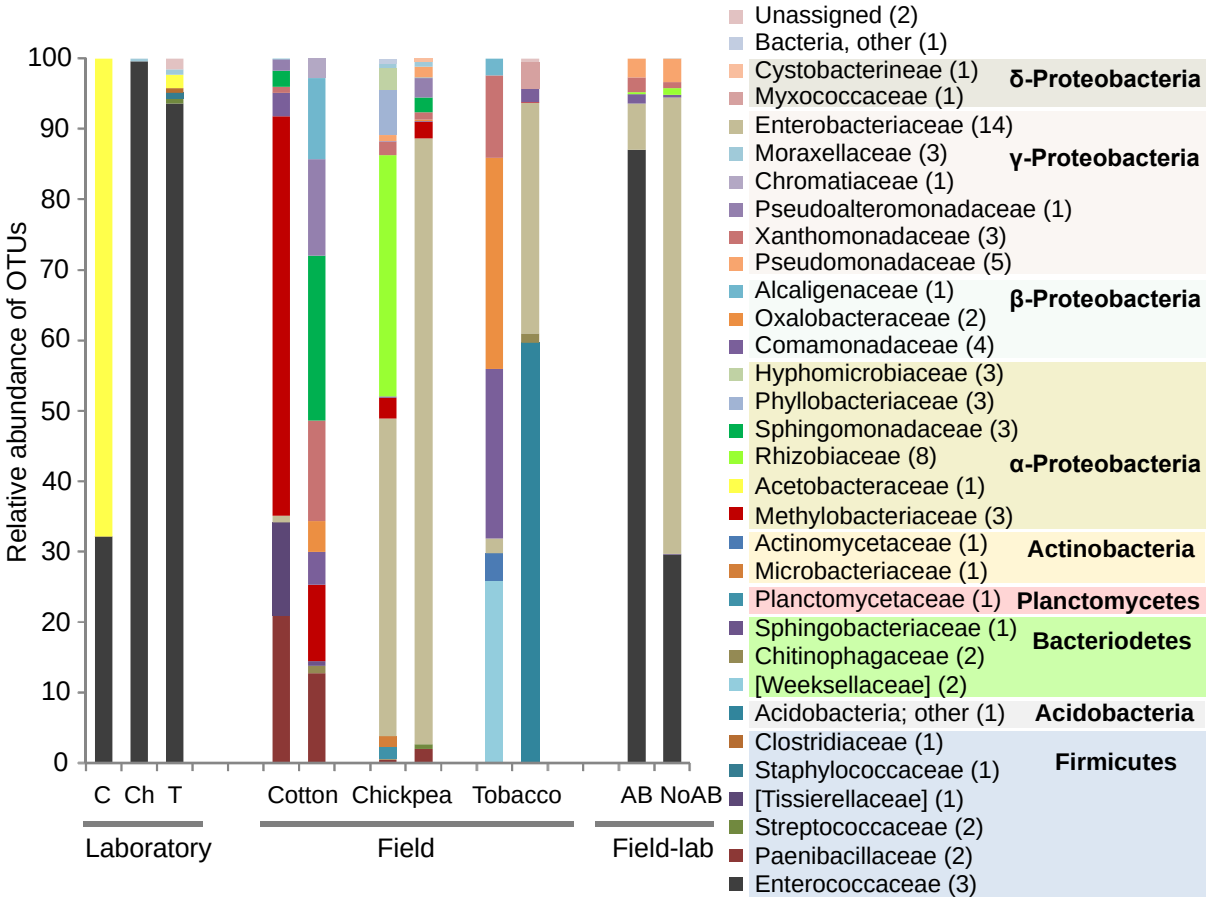

Figure 2.2 Relative abundance of bacterial OTUs in *H. virescens* larvae. Bacterial OTUs were combined on the family level. Numbers in brackets indicate how many OTUs one family contains. Rare OTUs that were represented less than 0.5% in at least one sample are not included. Larvae originated from a laboratory colony, from the field or from the field, after which they were reared in the laboratory for four generations (field-lab): AB: larvae received antibiotics treatment, NoAB: larvae received no antibiotics treatment. Field and laboratory larvae were grown on cotton (C), chickpea (Ch) or tobacco (T); square brackets around taxa indicate that the taxon name is not fully established yet.

Larvae of the field population and the laboratory colony also differed in the diversity of their associated bacterial communities. Larvae from the laboratory colony harboured an average of 25 OTUs, while field larvae had an average of 35 OTUs. Additionally, communities of the field larvae showed a greater evenness than those of the laboratory larvae (Figure 2.2), which was reflected in the higher Shannon index of the field larvae (Figure 2.3).

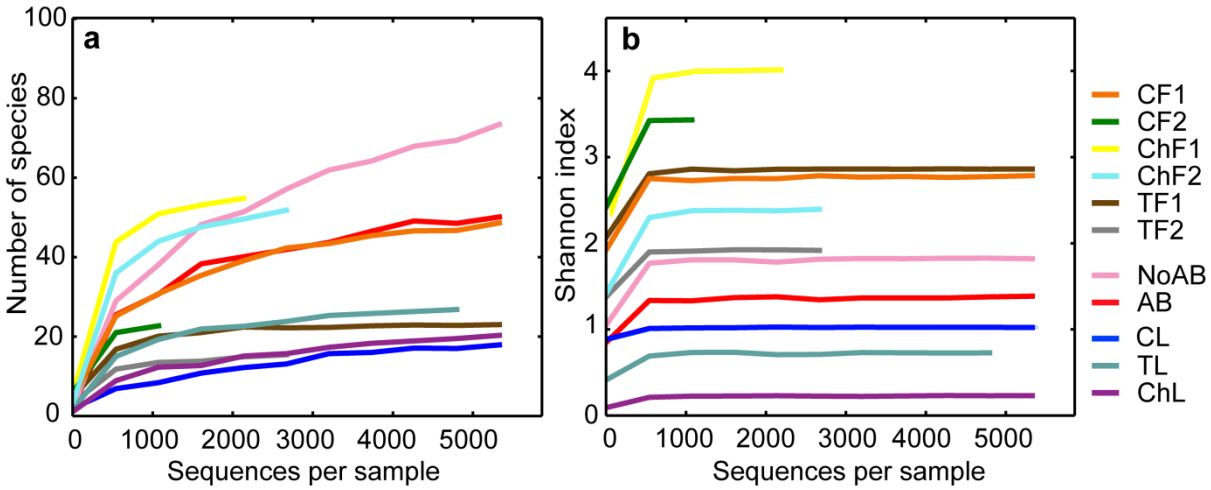

Figure 2.3 Rarefaction curves of alpha diversity of the bacterial communities of *H. virescens* larval guts. **a)** Number of observed bacterial OTUs. **b)** Shannon index of the bacterial communities. Laboratory (L) and field larvae (F) were fed on cotton (C), chickpea (Ch) or tobacco (T). AB: field larvae that were reared for four generations in the laboratory and received antibiotics during one generation; NoAB: field larvae that were reared for four generations in the laboratory and did not receive antibiotics treatment.

Host plant-associated variability of bacterial communities in larvae

The influence of diet on the bacterial communities of laboratory and field larvae was investigated by comparing bacterial communities in larvae that fed on three different host plant species. In laboratory-reared larvae, we found one OTU (#2) from the family Enterococcaceae dominating the bacterial community in the guts of larvae fed chickpea or tobacco, i.e. 97.8% and 91.5% abundant, respectively (Figure 2.2). The closest hits for OTU #2 were *Enterococcus faecium* (HG937762), *E. gallinarum* (HG937758) and *E. casseliflavus* (KJ528946) with 99% similarity, respectively. The larvae fed on cotton also contained OTU #2, but in much lower abundance, i.e. 31.8%, while an OTU of the family Acetobacteraceae (#6) had the highest abundance (67.1%). The closest hit for this OTU was *Asaia lannensis* (KP208318), with 99% similarity.

In the field larvae, we also found differences in bacterial communities depending on the host plant species they fed on. Enterobacteriaceae were the dominant family in the larvae that fed on chickpea and also occurred in larvae that fed on tobacco, while these were rare (<

1%) in the larvae fed on cotton. The larvae fed on tobacco and cotton contained Burkholderiales (Comamonadaceae, e.g. #18, and Oxalobacteraceae), which were rare (< 1%) in larvae fed on chickpea (Figure 2.2). Methylobacteriaceae (e.g. #11) were among the most abundant families in the larvae that fed on cotton, while this family was hardly detected in the other two groups. On the genus (and OTU) level, the two biological replicates from each plant group differed greatly from each other (Figure S2.1).

Variability of bacterial communities across different life stages of the field population

We assessed the temporal persistence of bacterial communities in *H. virescens* by comparing bacterial communities of different life stages of the field population (larvae, adults and eggs). Bacterial communities differed greatly across life stages. Females shared 76% of their OTUs (n = 200) with eggs, and eggs shared 52.1% of their OTUs (n = 292) with females. In contrast, larvae only shared 2.7% of their OTUs (n = 148) with females and 6.1% OTUs with eggs. Accordingly, in a principal component plot, females and eggs partly clustered together, while five of the six larval samples were located in a different area of the plot (Figure 2.4). Females of the field population shared 22% of their OTUs (n = 200) with the field-lab larvae that were reared in the laboratory for four generations (the NoAB group), while the eggs had 18.8% of their OTUs (n =292) in common with the field-lab larvae.

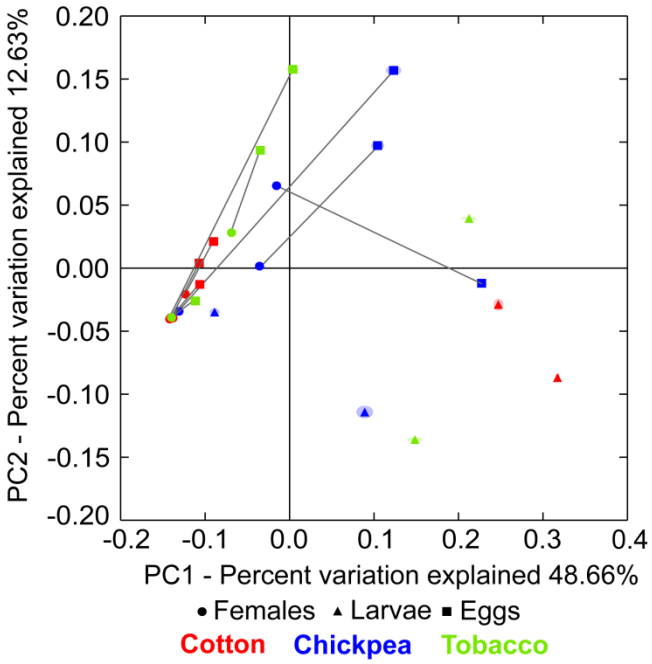

Figure 2.4 Principal component plot of bacterial communities of field larvae, females and their eggs. Absence of confidence ellipsoids around the samples indicates good jackknife support for the location of data points in the PCA plots. Colours indicate food plant; symbols denote developmental stages; lines connect female-egg combinations.

One OTU (#1) from the family Enterobacteriaceae had the highest relative abundance in both females and eggs (Figure 2.5). The closest hits for this OTU in NCBI were *Enterobacter*

ludwigii (KJ767368), *E. cloacae* (KM817773) and *Klebsiella oxytoca* (KM408615), all with 97% similarity; this was confirmed by its position in the phylogeny (Figure 2.1). In the field larvae, OTU #1 was not present, but two other Enterobacteriaceae OTUs were found (#7 and #12), which clustered with OTU #1 in the phylogenetic tree (Figure 2.1 and 2.2).

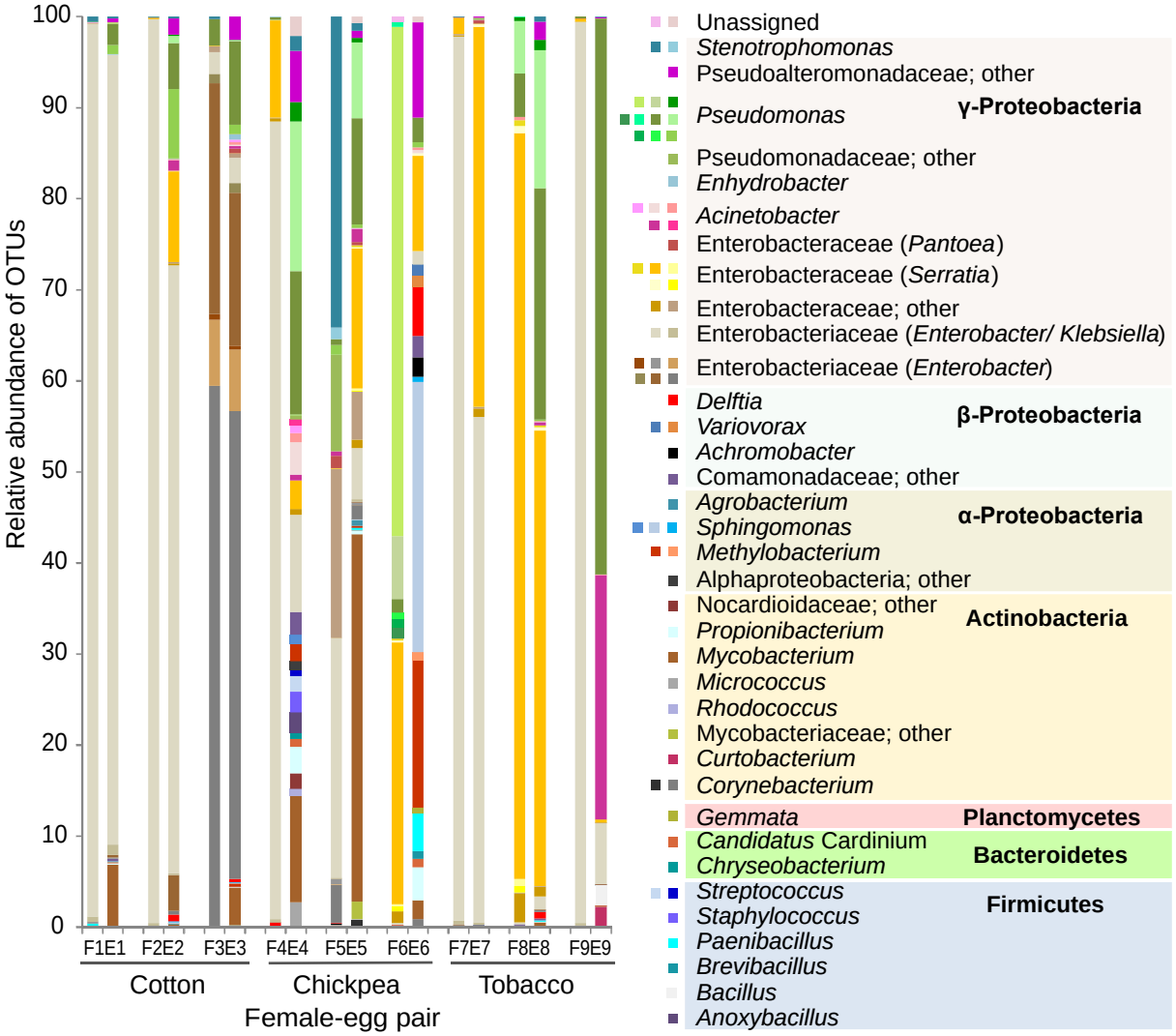

Figure 2.5 Relative abundance of bacterial OTUs in the abdomen of *H. virescens* females (F) and their eggs (E). Females (F1-F9) and their eggs (E1-E9) are shown next to each other. Females had fed on three different plants as larvae: cotton (C), chickpea (Ch), tobacco (T). Rare OTUs that were represented less than 0.5% in at least one sample are not shown. Brackets around genera signify that there were bacterial candidates with > 97% identity, but support in the phylogenetic tree was weak [i.e. nodes that divide branches that contain different genera have bootstrap values equal to or below 10 (see Figure 2.1)].

Vertical transmission of bacterial communities

Based on weighted UniFrac distance values, bacterial communities present in females were not more similar to the bacterial communities of their own eggs than to the bacterial

communities of eggs that were not their own ($F_{1,65.74}$ = 1.28, P = 0.26). However, rearing on different plant species had a significant effect on bacterial communities in female-egg combinations ($F_{5,66.53}$ = 3.17, P = 0.013). None of the comparisons for the variable plant species were significant in planned pairwise comparisons with Bonferroni corrections. However, cotton-reared females tended to be more similar to bacterial communities of eggs of their own plant group than to those of a different plant group (z = -2.31, P = 0.062). This marginal effect was not found for tobacco females and eggs (z = 1.46, P = 0.43) and was even reversed (bacterial communities of females were more similar to bacterial communities of other eggs than to eggs of their own plant group) for chickpea-reared females (z = -2.33, P = 0.060). The principal component plot supports these results (see Figure 2.4).

Discussion

In this study we assessed the variability of bacterial communities in the agricultural pest *H. virescens*. We observed that the bacterial communities of different life stages, of different populations (field population or laboratory colony) as well as of larvae that fed on different host plant species, and even biological replicates, were very different.

Abundant bacterial strains in *H. virescens*

Most of the dominant bacterial families in our study, i.e. Acetobacteraceae, Methylobacteriaceae, Sphingomonadaceae, Comamonadaceae, Enterobacteriaceae, Pseudomonadaceae, Moraxellaceae Xanthomonadaceae, Paenibacillaceae and Enterococcaceae, have been identified in Lepidoptera before (Broderick *et al.*, 2004; Xiang *et al.*, 2006; Robinson *et al.*, 2010; Belda *et al.*, 2011; Pinto-Tomás *et al.*, 2011; Gayatri Priya *et al.*, 2012; Tang *et al.*, 2012; Hammer *et al.*, 2014). This suggests that representatives of these bacterial families may be commonly present in many Lepidoptera and the environments they live in.

Variability of bacterial communities between laboratory and field larvae

Bacterial communities differed greatly between laboratory and field larvae, in fact no OTUs were shared between the two groups. These large differences in bacterial communities underline the importance of including field populations of an organism in studies of the functional role of its bacterial communities. Bacterial communities of field and laboratory insect populations have for instance been studied in the moths *Helicoverpa armigera* (Xiang *et al.*, 2006) and *Ostrinia nubilalis* (Belda *et al.*, 2011), the mosquito *Anopheles stephensi*

(Rani *et al.*, 2009) and several fruitfly species (*Drosophila*) (Chandler *et al.*, 2011). In all these studies, the general finding was that bacterial communities in laboratory colonies are depauperate and dominated by only one (or few) bacterial strain(s), as we also found in *H. virescens*, while communities of field populations tend to be more diverse and contain many bacterial strains with lower and more equal abundances (Xiang *et al.*, 2006; Rani *et al.*, 2009; Belda *et al.*, 2011; Chandler *et al.*, 2011). Differences between laboratory and field populations may be due to differences in environmental conditions. In the field, organisms are probably exposed to a higher diversity of bacterial strains which may vary in space and time, than in the laboratory. For instance, in *H. virescens,* and most moth species, eggs are laid on above-ground parts of plants, where larvae also feed, while the moths pupate in the soil. Emerging adults feed from flowers of many plants and, in the case of the highly polyphagous *H. virescens*, many plant species (Barber, 1937; Stadelbacher, 1981; Fitt, 1989; Waldvogel & Gould, 1990). In contrast, in the laboratory moths are reared in cups of artificial diet, where they also pupate and emerge as adults. Thus, whereas moths in the field may continuously encounter and ingest new strains of bacteria, lab colonies are likely constantly re-infected with the same (small number of) bacterial strains that are dominant under laboratory conditions.

As described in the methods, we used whole (washed) larvae to assess the bacterial communities in the laboratory strain and extracted the guts of larvae to assess bacterial communities in field larvae. Also, laboratory strain larvae were in an earlier instar than field larvae. Even though the differences in treatment and instar might be responsible for part of the differences between field and laboratory larvae in our study, they are unlikely to explain the large qualitative differences with a complete lack of overlap in bacterial communities. Moreover, *Enterococcus* sp. which was the dominant strain in the laboratory larvae was also detected in the field-lab larvae (5^{th} instar), which were kept in the laboratory for four generations and from which only the gut was investigated. Thus the detection of enterococci in laboratory larvae is unlikely to be merely due to methodological differences between laboratory-reared and field larvae.

In our study, *Enterococcus* sp. was the dominant bacterial strain in the laboratory colony and the AB group of the field-lab larvae, and it was the second most abundant strain in the NoAB group of the field-lab larvae. However, this strain was completely absent in the field larvae, suggesting that *Enterococcus* sp. was introduced to the insects in the laboratory. *Enterococcus* sp. has been identified as a dominant microbe in several Lepidopteran gut communities, including *Galleria mellonella*, *Lymantria dispar*, *Helicoverpa armigera*, *Manduca sexta*, *Spodoptera littoralis* and *Heliconius erato* (Jarosz, 1975; Broderick *et al.*,

2004; Xiang *et al.*, 2006; Brinkmann *et al.*, 2008; Tang *et al.*, 2012; Hammer *et al.*, 2014). Interestingly, most of these studies used only laboratory colonies (Jarosz, 1975; Brinkmann *et al.*, 2008; Tang *et al.*, 2012). In *Helicoverpa armigera* and *Ostrinia nubilalis*, where both laboratory and field populations were investigated (Xiang *et al.*, 2006; Belda *et al.*, 2011), single bacterial strains such as *Enterococcus* sp. and other gram-positive cocci (e.g. *Staphylococcus* sp. in *O. nubilalis*) were found to become dominant in laboratory populations, while they were less abundant in field populations. In *H. erato*, enterococci became more abundant in adults after this species had been in captivity for one generation (from adult to adult) compared to field-collected adults (Hammer *et al.*, 2014). Possibly, enterococci are outcompeted in the field by other bacteria (e.g. Proteobacteria such as *Enterobacter, Klebsiella* and *Pseudomonas* species) in the insect gut and/ or on the plants while competition changes in favour of enterococci under (more stable) laboratory conditions. Analogously, the gut of vertebrates is a relatively stable environment. Accordingly, in flycatcher nestlings, which harbour both enterococci and Enterobacteriaceae in their guts, enterococci were the stronger competitors among the two bacterial groups (González-Braojos *et al.*, 2012). Thus, the outcome of bacterial competition may depend on the variability in environmental conditions (Russell *et al.*, 1979; Flint *et al.*, 2007; Hall *et al.*, 2008).

Treating *H. virescens* larvae with tetracycline in the laboratory probably selected for enterococci, as the relative abundance of enterococci was higher in larvae treated with antibiotics than in untreated larvae. This is in line with the finding that many *Enterococcus* strains have developed resistance against tetracycline (Huys *et al.*, 2004; Wilcks *et al.*, 2005; Cauwerts *et al.*, 2007). It is interesting to note, however, that enterococci also became abundant in laboratory-reared larvae in the absence of antibiotics, indicating that antibiotic treatment alone does not account for the shift in microbial communities towards high abundance of *Enterococcus* spp.

Host plant-associated variability of bacterial communities in larvae

In both field and laboratory *H. virescens* larvae, bacterial gut communities varied depending on host plant. Diet has been found to influence bacterial gut community composition in larvae of many insect species, e.g. *Drosophila* (Chandler *et al.*, 2011) and several lepidopteran species (Broderick *et al.*, 2004; Gayatri Priya *et al.*, 2012; Tang *et al.*, 2012). Diet might influence bacterial communities in various ways. For example, different bacteria may be present in or on different host plants, the resources provided by the different plants may promote differential bacterial growth and/or secondary plant metabolites may have a selective

effect on bacterial communities (Yang *et al.*, 2001; Vorholt, 2012). Additionally, host plants can influence the physiochemical conditions of the larval gut, which possibly results in differential bacterial growth (Schultz & Lechowicz, 1986) (Appel & Maines, 1995).

Although we did not sample bacteria from plants on which the larvae had fed, earlier studies have assessed bacterial communities of cotton and tobacco plants, and identified several bacterial families that we also detected in our samples (McInroy & Kloepper, 1995; Brinkmann *et al.*, 2008), suggesting that the experimental *H. virescens* larvae had ingested bacteria from their food plants. For instance, Methylobacteriaceae was the most abundant bacterial family in the field larvae fed on cotton in our study, and this bacterial family has been isolated from different tissues of cotton (McInroy & Kloepper, 1995; Madhaiyan *et al.*, 2012). Also, bacteria from the order Burkholderiales (Comamonadaceae and Oxalobacteraceae), which were mainly present in the field larvae fed on tobacco in our study, were previously isolated from tobacco leaves (Brinkmann *et al.*, 2008). Furthermore, *Enterobacter*, *Pseudomonas* and *Serratia* were the most abundant genera in females and eggs in our study, and have been isolated from, among others, cotton tissues and tobacco leaves (McInroy & Kloepper, 1995; Junker *et al.*, 2011; Lv *et al.*, 2012; Ma *et al.*, 2013).

Interestingly, in *L. dispar*, bacteria that were isolated from leaves of its host plant aspen, have recently been shown to enhance larval growth of this moth in the presence of phenolic glycosides, a defence metabolite of aspen (Mason *et al.*, 2014). Whether *H. virescens* can profit from bacteria that are transient or reside on its host plants still needs to be investigated.

Notably, there were differences in bacterial community composition between the two biological replicates per plant species in the field larvae, particularly at low taxonomic levels (i.e. genus or OTU). This indicates large individual variation in the bacterial gut community of *H. virescens* larvae in the field, and further suggests that the bacterial community is only loosely associated with this moth.

When investigating microbial communities using bacterial tag-encoded FLX pyrosequencing of 16S rRNA amplicons, it is important to keep in mind that this method can lead to incorrect estimates of OTU numbers (Kunin *et al.*, 2010; Schloss *et al.*, 2011; Pinto & Raskin, 2012). Errors that have been described for this method include PCR bias, sequencing errors (particularly in homopolymeric regions) and the production of chimeric sequences during PCR reactions (Chandler *et al.*, 1997; Margulies *et al.*, 2005; Haas *et al.*, 2011; Schloss *et al.*, 2011). Even though we have implemented in our analyses a number of measures to decrease methodological errors (de-noising raw data, quality cut-off, length limitation of

sequences, identification and removal of possible chimeric sequences, removal of global singletons), it is possible that the number of OTUs may still be overestimated and that the diversity patterns that we show on the family level for larvae is actually more realistic than the patterns we encountered on the OTU level (compare Figure 2.2 and Figure S2.1).

Variability of bacterial communities across different life stages of the field population

In *H. virescens*, it seems that bacterial communities are completely restructured or lost during the metamorphosis from crawling larvae to winged adult, because bacterial communities in larvae of the field population differed strongly from those in adult females. One of the few Lepidoptera where bacterial persistence across metamorphosis has been studied is the butterfly *H. erato* (Hammer *et al.*, 2014). In this species, bacterial communities between larvae and adults also differed greatly but more bacterial strains (13%) overlapped between larvae and adults in their study than in our study, in which only 2.7% of OTUs overlapped between larvae and adult females. A similar pattern was found in mosquitoes, in which bacterial composition partly changed during metamorphosis, but many strains were retained (Rani *et al.*, 2009). One reason for the difference in bacterial communities between larvae and adults in Lepidoptera might be that larvae and adults feed on different diets: while larvae usually feed on the foliage of their host plants, adults often feed only on nectar and in this study on honey water. The gut of lepidopteran larvae is less compartmentalized then the gut of many other insect species, mostly has a very alkaline pH, and is purged before metamorphosis e.g. (Nijhout & Williams, 1974; Dow, 1992; Appel & Maines, 1995; Chapman *et al.*, 2013; Engel & Moran, 2013). Together, these characteristics might make it difficult for bacteria to successfully colonize Lepidopteran guts. Moreover, in moths an increase in lysozyme production before and during metamorphosis has been reported, which likely reduces bacterial abundance and diversity especially immediately before and after the pupal stage, i.e. pupating larvae and newly eclosed adults (Russell & Dunn, 1996; Zhang *et al.*, 2009). Upon adult eclosion, bacterial titers may therefore be low and bacterial strains that are different from the larval bacterial community might be able colonize the adults (see also Hammer *et al.*, 2014).

Vertical transmission of bacterial communities

We investigated whether bacteria in *H. virescens* are vertically transmitted by comparing bacterial communities of females and their eggs and tested if bacterial communities of eggs were more similar to bacterial communities of their own mother than to any other female. The fact that we did not find this effect suggests that vertical transmission likely does not occur in

H. virescens at a significant rate. However, since bacterial communities were at least partly similar in females and eggs, we cannot completely exclude the occurrence of vertical transmission. A study using fluorescently-labelled *E. coli* was able to detect a certain amount of mother-offspring transmission in *Galleria mellonella*, although the viability of the bacteria was not assessed (Freitak *et al.*, 2014). Also, a previous study about vertical transmission of *Serratia marcescens* in a laboratory population of *H. virescens* indicated that bacteria can be vertically transmitted in this *H.virescens* (Sikorowski & Lawrence, 1998).

Conclusions

The huge variability of bacterial communities that we found between life stages, diets, biological replicates and field and lab populations indicates that the major part of the bacterial communities that we identified in the gut of *H. virescens* is of a transient nature and only loosely associated with its host. In fact, the bacterial communities seem to be entirely restructured during metamorphosis. Our results further suggest that bacterial communities are not transmitted at a significant rate from mothers to eggs in *H. virescens*. Based on these results, it is doubtful that particular bacterial strains that we have identified form a unit of selection with this moth. It is further unlikely that a long-term mutualistic symbiosis between *H. virescens* and bacteria that could facilitate host plant use and adaptation has evolved. Importantly, we found that enterococci are most probably introduced to *H. virescens* larvae in the laboratory. This finding stresses the importance of including field populations when bacterial communities of an organism are to be characterized.

Acknowledgements

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Supporting information

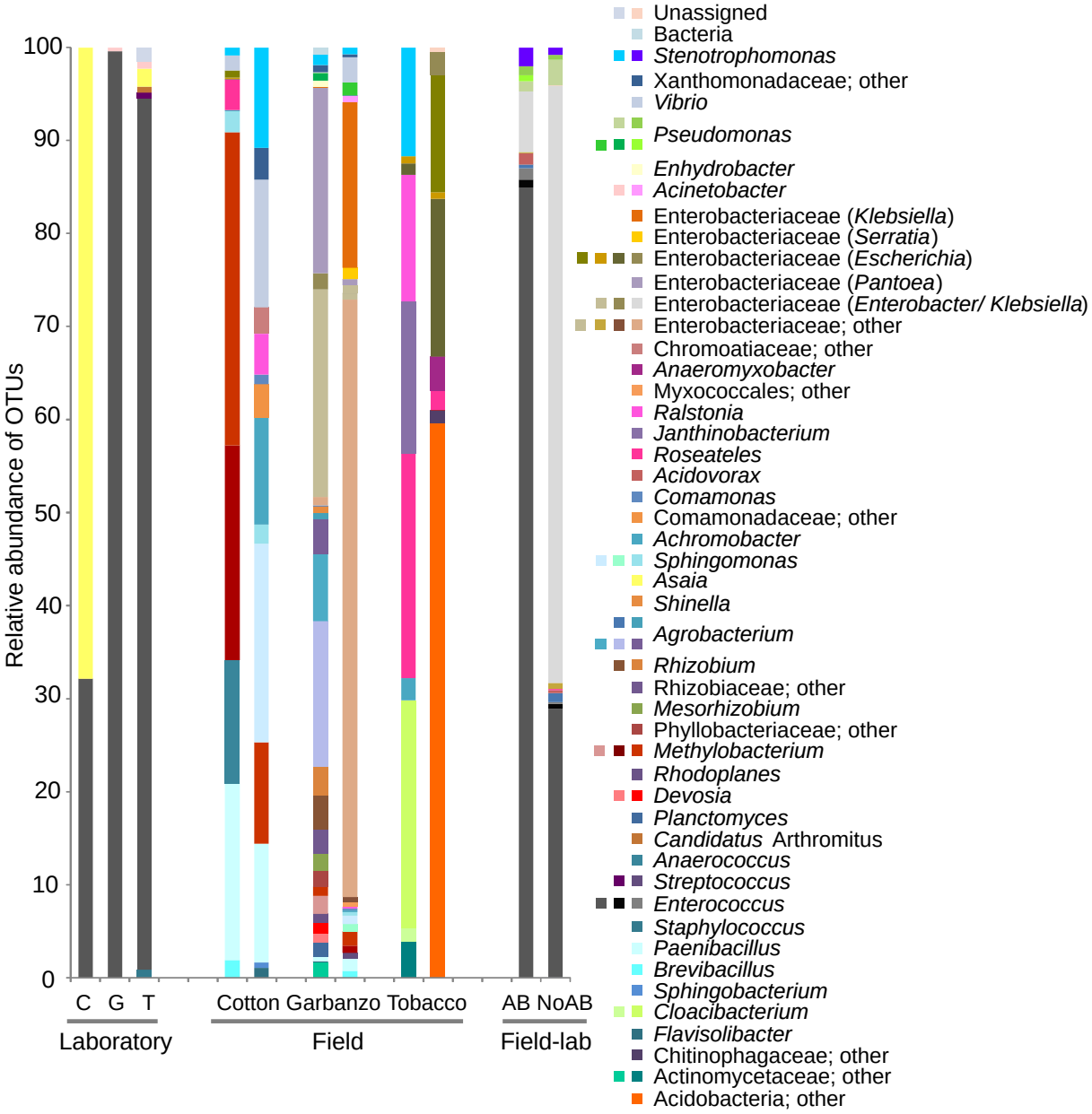

Figure S2.1. Relative abundance of bacterial OTUs in *H. virescens* larvae. OTUs are identified at the genus level. Rare OTUs that were represented less than 0.5% in at least one sample are not included. Larvae originated from a laboratory colony, from the field or from the field, after which they were reared in the laboratory for four generations (field-lab): AB: larvae received antibiotics treatment, NoAB: larvae received no antibiotics treatment. Field and laboratory larvae were grown on cotton (C), chickpea (Ch) or tobacco (T). Brackets around genera signify that there were bacterial candidates with > 97% identity, but support in the phylogenetic tree was weak [i.e. nodes that divide branches that contain different genera have bootstrap values equal to or below 10 (see Figure 2.1)].

Table S2.1. Statistics of bacterial tag-encoded FLX amplicon sequencing and number of OTUs in *Heliothis virescens* larvae. The bacterial communities of field larvae (F), laboratory larvae (L) and field-lab (AB/NoAB) larvae were characterized. The latter was reared in the laboratory for four generations after collection in the field. AB: treated with antibiotics (tetracycline); NoAB: not treated with antibiotics. Larvae of the field and laboratory strain were fed on three different plant species: cotton (C), chickpea (Ch), and tobacco (T). Field larvae: two sequencing pools per plant species; laboratory and field-lab larvae: one pool per plant species; # = number, Qual. seqs = quality filtered sequences.

	Laboratory larvae			**Field larvae**						**Field-lab larvae**	
Sample	**CL**	**ChL**	**TL**	**CF1**	**CF2**	**ChF1**	**ChF2**	**TF1**	**TF2**	**AB**	**NoAB**
# Reads	30475	6917	5614	33880	2226	2590	2835	5356	3189	11464	10344
# Qual. seqs	30400	6750	5112	20963	1520	2318	2791	5333	3189	11443	10341
# OTUs	27	21	27	55	23	55	53	23	16	64	90

Table S2.2. Statistics of bacterial tag-encoded FLX amplicon sequencing and number of OTUs in individual *H. virescens* females. Females fed on three plant species as larvae: cotton (Cot), chickpea (Chi), tobacco (Tob); # = number, Qual. seqs = quality filtered sequences.

Sample	**Cot-fem1**	**Cot-fem2**	**Cot-fem3**	**Chi-fem4**	**Chi-fem5**	**Chi-fem6**	**Tob-fem7**	**Tob-fem8**	**Tob-fem9**
# Reads	7586	16398	7374	15853	13047	9509	13254	2895	16990
# Qual. seqs.	7586	16396	7373	15852	13047	9507	13156	2895	16990
# OTUs	33	44	34	65	72	65	54	38	57

Table S2.3. Statistics of bacterial tag-encoded FLX amplicon sequencing and number of OTUs in *H. virescens* eggs. Mothers of these eggs had fed on three different plant species as larvae: cotton (Cot), chickpea (Chi), tobacco (Tob); # = number, Qual. seqs = quality filtered sequences.

Sample	**Cot-egg1**	**Cot-egg2**	**Cot-egg3**	**Chi-egg4**	**Chi-egg5**	**Chi-egg6**	**Tob-egg7**	**Tob-egg8**	**Tob-egg9**
# Reads	1346	18062	8461	1724	8033	466	27786	8890	19760
# Qual. seqs.	1241	17888	8384	503	7587	339	27784	8855	19746
# OTUs	37	108	81	48	134	30	82	70	124

3

Sex-specific trade-offs between immune response and reproduction in a moth

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**Contributed equally to this study.*

Abstract

Based on Bateman's principle, females are predicted to invest more in immunity than males because they maximize their fitness by increasing longevity, whereas males maximize their fitness by increasing mating frequency. This prediction may hold for species where females invest significantly more into offspring than males, but not when both sexes invest similarly. We investigated investment in immunity and its trade-offs with reproduction in the noctuid moth *Heliothis virescens*, in which both males and females mate only once per night and both sexes invest substantially in offspring. We investigated immune defense in response to challenge by the entomopathogenic bacterium *Serratia entomophila*, and its effects on mating success and the female sexual signal. We found that females had higher expression levels of immune-related genes after bacterial challenge than males. However, males maintained a higher baseline expression of immune-related genes than females. Bacteria-challenged females also experienced reduced mating success. Additionally, the female sexual signal was altered after the challenge. Male mating success was unaffected by bacterial challenge. Our results show that the sexes differed in their investment strategies: females invested in immune defense after a bacterial challenge, indicating immune deployment, whereas males had higher baseline immunity than females, indicating immune maintenance. Interestingly, these differences in investment were reflected in the mate choice assays. As female moths are the sexual signallers, females need to invest resources in their attractiveness. Female moths may thus have evolved a more efficient immune response than males.

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Introduction

The concept of trade-offs is a central component of life history theory (Stearns, 1989; Roff & Fairbairn, 2007). Organisms have a limited amount of internal resources available and face the dilemma of partitioning resources between various fitness-related life history traits. An increased investment in one fitness-related trait may cause a lack of resources available for another fitness-related trait (Stearns, 1989; Roff & Fairbairn, 2007).

Immunity and reproduction are both costly (Sheldon & Verhulst, 1996; Moret & Schmid-Hempel, 2000) and various studies have shown a negative correlation between the two (McKean & Nunney, 2001; Rolff & Siva-Jothy, 2002). Due to differences in life histories of males and females, resource investment strategies to cope with this trade-off and to achieve an optimal fitness following an infection may be sex-specific (Folstad & Karter, 1992; Rolff, 2002; Zuk & Stoehr, 2002). Males of several species have been found to invest fewer resources in immune response than females (Rheins & Karp, 1985; Gray, 1998; Rolff, 2001; Rolff & Siva-Jothy, 2002). These sex-specific differences in immunity have been related to Bateman's principle, i.e. females gain fitness by maximizing their lifespan through immunity investment, assuming that higher immunity increases longevity, which in turn increases the time for egg production and oviposition (Rolff, 2002). Males gain fitness by increasing their mating frequency, and should thus invest in increasing their mating rates instead of immunity (Bateman, 1948; Trivers & Willard, 1973; Zuk, 1990; Rolff, 2002; Roth *et al.*, 2011). Bateman's principle is based on the generalization that females make a large investment into offspring because they have large immobile gametes, whereas males make a small investment into offspring because they produce small and mobile gametes (Bateman, 1948). However, Bateman's principle as well as its consequences for investment in immune defense activation was shown to be reversed in species in which sex-roles are reversed and males invest more into offspring than females, e.g. by investing into parental care (Aisenberg & Peretti, 2011; Roth *et al.*, 2011).

As in other Lepidoptera, both *Heliothis virescens* females and males invest substantially into their offspring (Fye & McAda, 1972; Proshold *et al.*, 1982; Blanco *et al.*, 2009). Female moths spend energetic and nutritional resources into the production of up to 1500 eggs, whereas males invest resources to produce a spermatophore, comprising up to 5 % of their body mass (Fye & McAda, 1972; Proshold *et al.*, 1982; Blanco *et al.*, 2009). Males as well as females in *H. virescens* can mate multiple times in their lifetime, which is about 30 days in the laboratory at 25 °C (Proshold *et al.*, 1982; Willers *et al.*, 1987; Fitt, 1989; Blanco *et al.*, 2009). However, unlike many animal species in which males can mate multiple times

per day every day, *H. virescens* males and females only mate at most once per night (Raina & Stadelbacher, 1990; Heath *et al.*, 1991; Blanco *et al.*, 2009). Consequently, the number of matings is not only limited in females, but also in males. Thus, the male's capacity to increase its number of matings is tightly linked to male longevity, because every night means one more mating opportunity. Therefore, both *H. virescens* males and females can be expected to invest into immunity in response to an immune challenge.

Investment in immunity can trade off with sexual attractiveness. In many species, the attractiveness of a potential mate is determined by the quality of a sexual signal (Domb & Pagel, 2001; Scheuber *et al.*, 2003; Groot *et al.*, 2014). Parasite-mediated sexual selection indicates that sexual signals honestly reflect the quality of the signaler to the receiver (Hamilton & Zuk, 1982). In this scenario, honest signals are costly and condition dependent, and animals in good condition are able to afford to produce a higher quality signal, whereas animals in poor condition cannot invest much in sexual signalling (Zahavi; Sheldon & Verhulst, 1996; Rantala *et al.*, 2003). Most studies that have investigated a trade-off between reproductive success and immune response used species where males are the sexual signalers, competing for the attention of choosy females, e.g. birds, crickets or wolf-spiders (Faivre *et al.*, 2003; Jacot *et al.*, 2004; Peters *et al.*, 2004; Ahtiainen *et al.*, 2005; Spencer *et al.*, 2005; Shaw *et al.*, 2011). In moths, however, females produce a sex pheromone to attract males from a distance, i.e. hundreds of meters up to kilometers (Roelofs *et al.*, 1974; Tumlinson *et al.*, 1975; Klun *et al.*, 1980; Teal *et al.*, 1981; Vetter & Baker, 1983). In *H. virescens*, males have been shown to differentiate between sex pheromone blends that differ in their qualitative and/or quantitative composition (Vetter & Baker, 1983; Groot *et al.*, 2009; Groot *et al.*, 2014). Thus, female fitness in this species is likely also determined by quality of the female signal, because it is crucially important to attract males. Whether immunity may trade off with the quality of female signals in moths has not been determined.

In moths, the female sex pheromone has been shown to be an important sexual selection signal: females with a signal that deviates from the population mean attract significantly fewer males, so that these signals seem to be under stabilizing selection (Lofstedt, 1993; Cossé *et al.*, 1995; Linn *et al.*, 1997; Zhu *et al.*, 1997). However, only a few studies have considered the female sex pheromone as an honest signal that is costly to produce and plastic depending on the condition of the females (Groot *et al.*, 2010; Harari *et al.*, 2011). A decrease in sex pheromone production or quality following infection might provide a further indication that the female pheromone of *H. virescens* is indeed an honest signal that can indicate the quality of the female to the male.

In this study, we investigated Bateman's principle and immunity as well as the trade-off between immunity and mating success in both sexes of *H. virescens* and between immunity and sexual signalling in females. We adapted the predictions for Bateman's principle and immunity to the life history of *H. virescens* as follows. We hypothesize that females and males invest similarly in immunity, because longevity is equally important to the fitness of both sexes, i.e. for females to increase the time to produce and lay eggs and for males to increase their number of matings. We therefore predict that (1) immune gene expression will be similarly induced in males and females in response to an immune challenge. We also hypothesize that sexual attractiveness and immunity compete for the same resource pool and thus trade off with each other. Therefore, we further predict that an induced immune response (2) elicits reduced mating success in both sexes and (3) negatively affects the female sexual signal and sexual signalling behaviour.

Material and methods

Insects and bacterial culture

Heliothis virescens (JEN2; collected in 1988 in Clayton, North Carolina) was reared in environmental chambers at 26 °C, 60 % humidity, with a reverse 16:8 hours light-dark cycle (scotophase starting at 8 am and ending at 16 pm). Larvae of *H. virescens* were fed on artificial pinto bean diet (Burton, 1970). Pupae were collected, separated by sex, and placed in cups individually. Adults were provided with a 10 % honey-water solution. One to four day old virgin adults were used in all experiments of this study.

To induce an immune response in the moths we used freeze-dried cells of the entomopathogenic bacterium *Serratia entomophila*. This strain was shown to be deadly for *H. virescens* larvae and to induce hemocyte apoptosis in *H. virescens* larvae in an earlier study (Barthel *et al.*, 2014). *Serratia entomophila* was obtained from the Department of Bioorganic Chemistry (MPICE, Jena, Germany). Bacteria were grown at 30 °C and 250 rpm in Caso medium. Overnight cultures of *S. entomophila* were centrifuged and the resulting supernatant was discarded. The extracted bacterial pellet was frozen at -20 °C. Samples were then frozen and dried in a lyophilisator at -80 °C for 5 days to kill the bacteria. Lyophilized cells of *S. entomophila* were stored at -20 °C. Dead cells of the entomopathogen were used in this study to measure the effects of immune defense, because this eliminates the confounding effects of metabolism and dynamics of a living pathogen in the moth (Moret & Schmid-Hempel, 2000).

Activation of the immune system response by bacterial challenge in adult moths

For all our experiments, we challenged the moths by injecting adult males and females with bacteria. Injection of bacteria into the abdominal cavity mimics that bacteria enter the body via wounds in the cuticle, and has been commonly used to measure molecules that are involved in the immune response (Shelby & Popham, 2008). By using injections, we ensured a defined and equal immune response induction in all adult moths throughout all experiments. To induce an immune response in adult moths in all experiments of this study, adult moths were injected with 4 µg / 4 µl lyophilized cells of *S. entomophila* diluted in 1x phosphate buffered saline (PBS). As control treatments, adults were either injected with 4 µl pure PBS (referred to as wounded) or were not injected at all (referred to as non-injected or control). All injections were conducted using a 10-µl Hamilton syringe and were performed at the onset of photophase, approximately 16-20 hours before the start of the experiments.

(1) Effect of immune challenge on immune gene expression in females and males

To determine the level and extent of immune response induction in both sexes, we assessed the expression of immune-related genes in female and male adult moths. An induced immune response in insects includes the expression of genes encoding a variety of antimicrobial peptides such as lysozyme, gloverin and hemolin to combat infections (Hoffmann, 1995). Besides antimicrobial peptides, phenoloxidase activating (PO) enzymes and heat shock proteins (Hsp) are important compounds of an efficient immune response in insects (Hoffmann, 1995; Robert, 2003; Cerenius *et al.*, 2008). For our study we measured the expression levels of five immune related genes, i.e. heat shock protein 70, phenoloxidase activating factor, lysozyme, hemolin and gloverin. These genes have been found to be differentially regulated between control and *S. entomophila*-injected *H. virescens* larvae in a preliminary microarray study (unpublished data). We evaluated the expression differences of these 5 genes between *S. entomophila*-injected, PBS-injected and non-injected moths. Injections were carried out as described above. RNA extraction started 3 to 4 hours after the onset of scotophase (20 hours after injections). Moths were flash-frozen in liquid nitrogen and stored at -80 °C until RNA extraction. For each treatment, three replicates of each five bodies were used. Total RNA extraction was performed using TRIzol® (Invitrogen) according to the manufacturer's protocol. To ensure that the isolated RNA was free of genomic DNA we performed a DNase treatment by adding 10 µl of Turbo DNase buffer and 1 µl of Turbo DNase enzyme (Ambion). Samples were then incubated for 30 min at 37 °C. RNA was additionally cleaned with the RNeasy MinElute Cleanup-Kit (Qiagen). To check the quality

and concentration of the total RNA, isolated RNA was measured by ultraviolet (UV) detection using NanoDrop ND-1000 (Thermo Scientific). First-strand cDNA was synthesized using VersoTM SYBRR Green 2-Step QRT-PCRKit Plus ROX Vial (Thermo Scientific, ABgene, UK) according to the manufacturer's instructions, starting with 900 ng of total RNA. Quantitative real-time PCR analysis was performed on a Stratagene Mx3000P QPCR System. Reagents were purchased from ABgene (Thermo Scientific) and used according to the manufacturer's specifications. The PCR reaction for comparative quantification was run at 95 °C for 15 min and 40 cycles at 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s. All PCR reactions were performed in technical duplicates, using three biological replicates for each treatment. The efficiency of each primer pair was calculated using the software program LinRegPCR (Ramakers *et al.*, 2003). To evaluate gene expression, ribosomal protein S18 (RpS18), based on a sequence from an In-house database, was used as a reference. Gene expression is given as copy number per 1000 molecules RpS18. All primers used in this study are shown in Additional file 1, Table S1.

(2) Effect of immune-challenge on the mating success of females and males

To investigate the consequence of an induced immune response on mating success in *H. virescens* females and males, we conducted mate choice experiments. Climate and light conditions in the experimental room were the same as for the rearing, i.e. 26 °C, 60% relative humidity, and a reverse 16:8 hours light-dark cycle (lights off at 8am). Experiments were conducted in square gauze cages (33 x 33 x 33 cm). Each cage contained three adult moths, one chooser and two potential mates of the opposite sex (referred to as potential mate 1 and 2). Mate choice experiments were conducted in six assays, three male and three female choice assays (Additional file 2, Table S2). In assay 1 and 4, one of the two potential mates in one cage was injected with *S. entomophila* to trigger an immune response, whereas the other potential mate was not injected. To test whether wounding alone could influence mating behaviour, we conducted assays 2, 3, 5 and 6 as controls. The choice in these assays was between *S. entomophila*-injected and wounded potential partners (assay 2 and 5) or between wounded and control potential partners (assay 3 and 6). Choosers were not injected in any of the assays (1-6). The different mate choice experiments were randomly spread over several days, and at least two different assays were conducted on a single day, to avoid block or day effects. Fifty cages were observed in one night. To distinguish between the two potential mates in one cage, one was marked with a waterproof black marker, which was alternated between the two differentially treated potential mates to exclude bias due to marking. The

moths were placed in the cages at the end of the photophase and 16 hours after injection. Experiments started 150 minutes after the start of the scotophase, because females and males start to be reproductively active after ~2.5 hours into scotophase (Proshold, 1983; Mistrot Pope *et al.*, 1984; Heath *et al.*, 1991). All cages were checked every 30 minutes for copulation events until 450 minutes into scotophase, after which time point no further activity occurred. Mating behaviour was observed with the use of a red LED light (Sigma LED safety light). Only first matings in a cage were recorded.

To check whether mating behaviour was associated with the survival of adult moths for three days, we also recorded adult mortality daily for three days following the mate choice experiments. For these three days, all moths were kept individually in small plastic beakers (25 ml) and were provided with a 10% honey-water solution.

(3a) Effect of immune-challenge on the female sexual signal

To assess a possible trade-off between an induced immune response and the female sex pheromone profile, we injected virgin *H. virescens* with bacteria, with PBS, or we did not inject them, as described above, and analyzed their sex pheromone profile. Females for this experiment were not used in the mate choice experiments. We extracted sex pheromone glands 20 hours after the injections (see above) when the virgin females were 2 days old, and between 3 and 4 hours after the onset of scotophase. Glands were dissected with microscissors (FST instruments) and incubated for 30 minutes in conical vials containing 50 µl of hexane and 125 ng of the internal standard pentadecane to dissolve the sex pheromone. All pheromone samples were analyzed using a HP7890 gas chromatograph (GC) with a 7683 automatic injector. For the GC analysis, the hexane solution was evaporated with N_2 to 2 µl. A volume of 4 µl (2 µl sample and 2 µl octane) was injected into a HP7890 gas chromatograph (GC) with a splitless inlet. The GC was equipped with a DB-WAXetr (extended temperature range) column of 30 m x 0.25 mm x 0.5 µm and was coupled with a flame ionization detector (FID). For further information on the GC analysis see Groot *et al.* (2010). Sex pheromone peaks were identified by comparing the retention times of our samples with the retention times of synthetic compounds (Pherobank, The Netherlands) of the sex pheromone blend of *H. virescens*. Pheromone peaks were integrated manually.

(3b) Effect of immune challenge on female signalling behaviour

To test whether the frequency and temporal pattern of female signalling, referred to as "calling", was affected by induction of the immune response, we recorded the temporal

patterns of calling behaviour of female moths. As the female sex pheromone is produced in a specialized gland that is located around the ovipositor (Jefferson *et al.*, 1968), females were recorded as calling when the ovipositor with gland was clearly extruded from the female abdomen (see also Figure 3.4). Injection experiments were done as described above. Two-day old virgin females were placed separately in transparent plastic cups (500 ml) at the end of the photophase (16 hours after injection procedure). Calling behaviour experiments were conducted on three consecutive days. Each experimental day, females of all three treatments were tested to avoid block effects. In total, 178 females were observed, 34 at day one, 72 at day two and three, and females were used only once. As female sex pheromone is emitted at night, experiments started at the onset of the scotophase (at 8 am) and the experimental room was kept dark during the experimental period. Calling behaviour (yes or no) was recorded every five minutes until 450 minutes into scotophase, using a red light torch.

Statistical analysis

Expression levels of immune-related genes were calculated relative to a housekeeping gene, the ribosomal protein S18 (RpS18), to adjust for possible differences among the samples which are not due to the treatments. Adjusted expression levels were then analyzed with one-way ANOVAs and consecutive Least-Squares means (LS-means) pairwise comparisons with Tukey adjustment. Data were log-transformed to obtain normality of residuals.

Female and male mate choice were tested with two-sided binomial tests. To statistically test the influence of immune system activation on the female sex pheromone composition, we calculated the relative percentage of each sex pheromone compound in the blend. Females that had a total sex pheromone amount of less than 25 ng were excluded from the analysis, because this was the threshold of accuracy of integration of the pheromone peaks. Moreover, due to a dirt peak in the GC run we had to exclude the minor compound Z7-16:Ald from our data set. Relative amounts of the compounds in the pheromone blend are not independent from each other. We therefore divided them by the minor compounds Z9-16:Ald to be able to perform multivariate analysis (Groot *et al.*, 2010; Groot *et al.*, 2014). We chose Z9-16:Ald as denominator, because this compound is not known to be relevant to attract males and did not differ between treatments in preliminary analyses. We then log10-transformed the data and performed a MANOVA analysis with the ratios of the five remaining compounds as response variable and treatment (*S. entomophila*-, PBS- or non-injected) as predictor variable. Since we detected an overall treatment effect in the MANOVA analysis (Pillai's trace = 0.31, df = 2, $P = 0.00035$), we further analyzed the single compounds using ANOVA, followed by LS-means

pairwise comparisons with Tukey adjustment. We additionally analyzed the ratio between the relative amounts of 16:Ald and Z11-16:Ald, because Z11-16:Ald is the major sex pheromone component and essential to attract males (Vetter & Baker, 1983; Groot *et al.*, 2014) and the ratio 16:Ald / Z11-16:Ald was shown to have biological relevance to attract males as well: females with low ratios of 16:Ald to Z11-16:Ald were more attractive for males than females with high ratio (Groot *et al.*, 2014). To meet the assumption of normally distributed residuals, we log-transformed the ratio. We then conducted LS-means pairwise comparisons between our three treatments with Tukey adjustment for multiple comparisons with the ratio as response and treatment as predictor variable.

The influence of treatments on the calling behaviour of females was tested with a generalized linear mixed model using Poisson distribution in the glmmADMB package version 0.8.0 (Skaug *et al.*, 2011; Fournier *et al.*, 2012) in the software R. To account for temporal autocorrelation, we summed up the calling events of individual females in blocks of 30 minutes, such that female's calling in a period of 30 minutes were counted once for this period (even if they called more than one time in these 30 minutes), which resulted in a smooth parabolic curve (Figure 3.4). We used calling as the response variable and treatment, time and time squared as fixed predictor variables. To account for repeated measurements over time, we added individual females as a random effect to the model, time was added as a random slope (time|individual female). Data were not overdispersed (sum of squared Pearson residuals / residuals degrees of freedom = 0.85). An interaction effect of time and treatment was tested but left out of the final model, because it was not significant. The overall significance of treatment was tested with a Chi-square test, using the car package. All statistical analyses were conducted in the program R, version 3.0.2 (R Core Team, 2013).

Results

(1) Effect of immune challenge on immune gene expression in females and males

Males had higher baseline expression levels of immune-related genes than females (Figure 3.1a). Hsp 70, lysozyme and gloverin exhibited significantly higher expression in control males than in control females, whereas PO activating factor and hemolin were similarly expressed in both sexes. When *S. entomophila* was injected into female and male moths, transcription levels of immune-related genes were induced in both sexes compared to the control (Figure 3.1b and c). In females, a significant increase in transcript levels of all tested immune-related genes was observed after injection of *S. entomophila* compared to wounded and control females (Figure 3.1b). In males, only lysozyme was significantly upregulated in

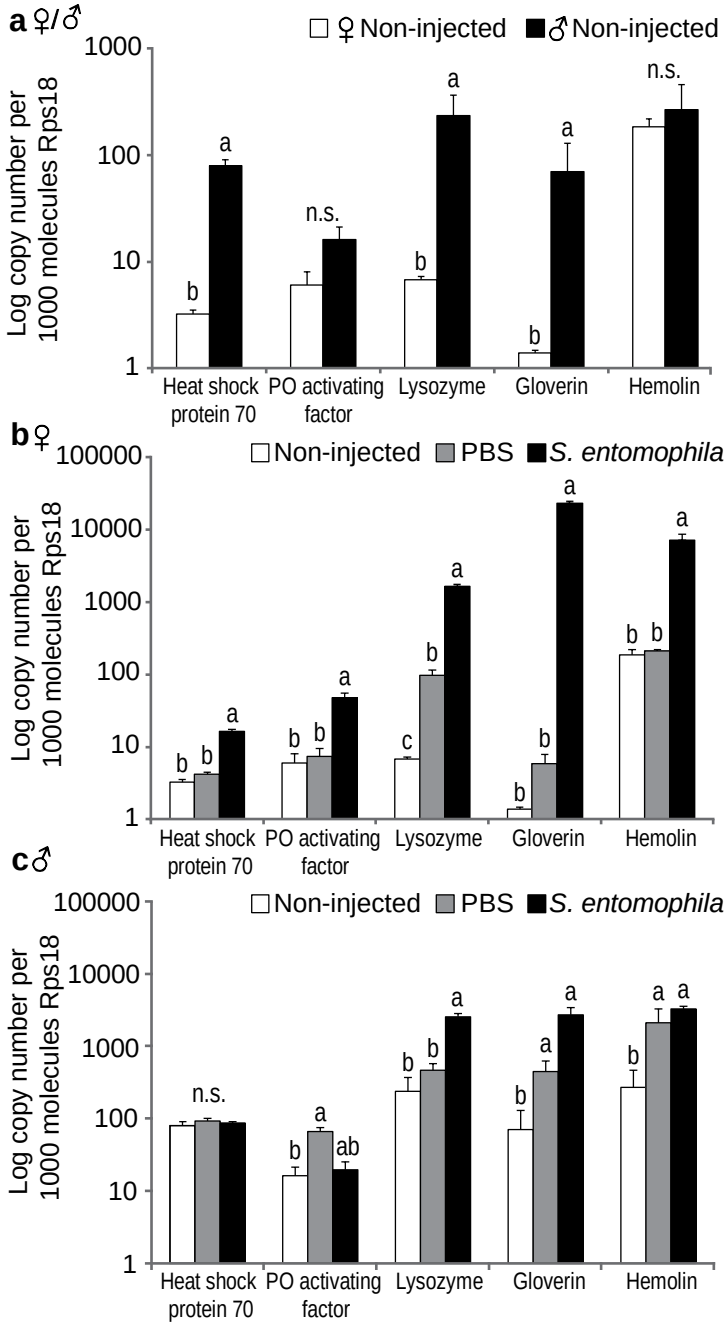

Figure 3.1. Expression level of immune-related genes in non-injected, *S. entomophila*- or PBS-injected *Heliothis virescens* females and males. Expression levels were compared between control females and males (a) and among all treatments in females (b) and males (c). Values are given as logarithmic copy number per 1000 molecules RpS18. Bars represent the mean of 3 biological replicates with corresponding standard errors. Different letters above the bars represent significant at a level of $P < 0.05$, based on ANOVA and LS-means pairwise comparisons with Tukey adjustment.

males after injection with *S. entomophila* compared to wounded and control males (Figure 3.1c). Levels of gloverin and hemolin, which were significantly upregulated in males upon *S. entomophila* injection, were also upregulated in wounded males compared to control males. The transcript level of PO activating factor was significantly higher in wounded males compared to control males but not compared to *S. entomophila*-injected individuals. Overall,

immune-related genes in females were induced only in response to bacterial injections, whereas in males immune-related genes were similarly induced after wounding and bacterial injections (see Additional file 3, Table S3 for significance values).

(2) Effects of immune challenge on the mating success of females and males

Males that had a choice between *S. entomophila*-injected and control females mated significantly more often with control females than with *S. entomophila*-injected females (Figure 3.2a). Males that had a choice between *S. entomophila*-injected and wounded females or between control and wounded females did not mate significantly more with one type of female (Figure 3.2a). In the female choice experiments, females mated similarly often with all types of males offered (Figure 3.2b). We did not observe any adult mortality within three days after the mating experiment among all treatments.

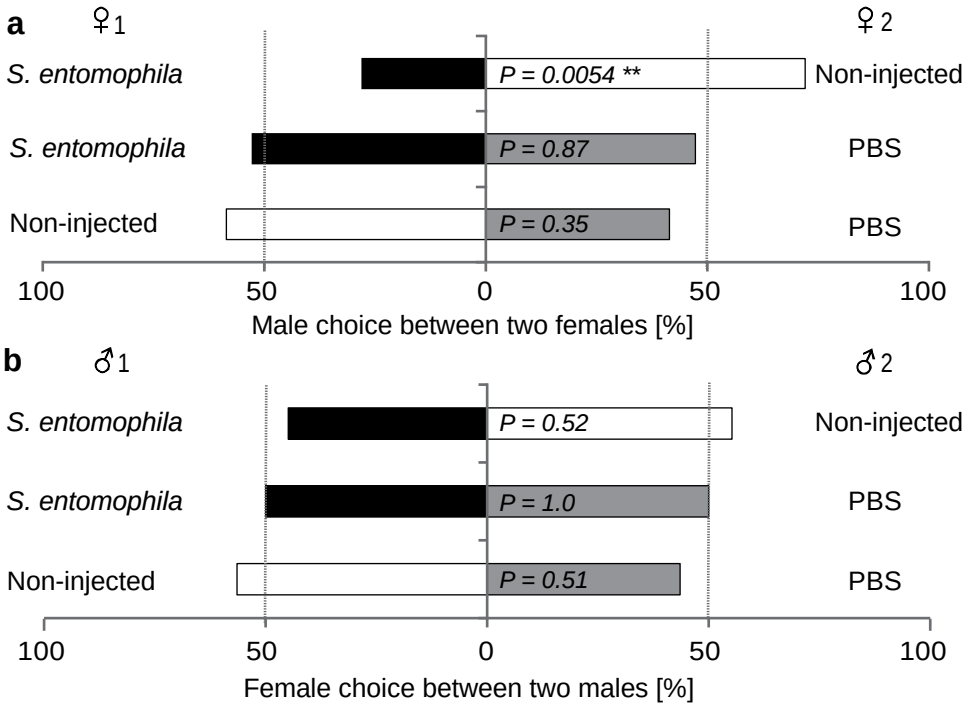

Figure 3.2. Effect of immune system activation on mating behaviour in *Heliothis virescens*. a) Male choice in three independent two-choice mating experiments. b) Female choice in three independent two-choice mating assays. Black colours correspond to *S. entomophila*-injected adults, grey colours correspond to PBS-injected adults and white colours to non-injected adults. Dashed lines indicate 50 percent of the total mated adults (see Additional file 2, Table S2 for sample sizes). Significant differences are indicated by $^{**}P < 0.01$, as tested with two-sided binomial tests.

(3a) Effect of immune challenge on the female sexual signal

In female moths, the sex pheromone profile was affected by the immune challenge. Females injected with *S. entomophila* produced significantly lower amounts of Z11-16:OH than

control females, but not less than wounded females (Figure 3.3a). The relative amounts of 14:Ald, Z9-14:Ald, 16:Ald, Z11-16:Ald were not significantly different between the treatments (Figure 3.3a and Table S3.4a-c for significance values). The ratio of 16:Ald to Z11-16:Ald was significantly higher in *S. entomophila*-injected females compared to wounded and control females (Figure 3.3b).

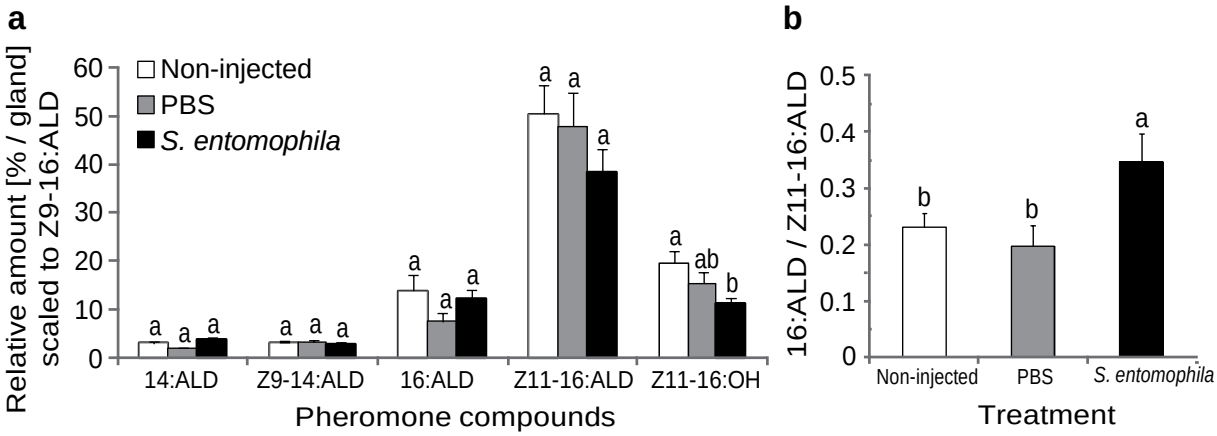

Figure 3.3. Influence of immune defense activation on sex pheromone composition of *Heliothis virescens* females. a) Relative amounts of five compounds scaled to Z9-16:Ald. b) Ratio between 16:Ald and Z11-16:Ald. Non-injected n = 38; PBS-injected n = 25*; S. entomophila*-injected n = 38. Different letters above the bars indicate significant differences between treatments at a level of $P < 0.05$ based on LS-means pairwise comparisons with Tukey adjustment for multiple comparisons.

(3b) Effect of immune-challenge on female signalling behaviour

The number of females calling was not affected by the different treatments ($\chi^2 = 4.85$, df = 2, $P = 0.088$). We did not find an interaction effect of treatment and time, suggesting that calling timing was not affected by the immune challenge (Figure 3.4). The calling behaviour of all females followed similar temporal and frequency patterns with an 'on and off' calling behaviour throughout scotophase. The majority of all females started calling 120 minutes into scotophase and stopped calling after 420 minutes, with a peak of calling activity between 240 and 360 minutes.

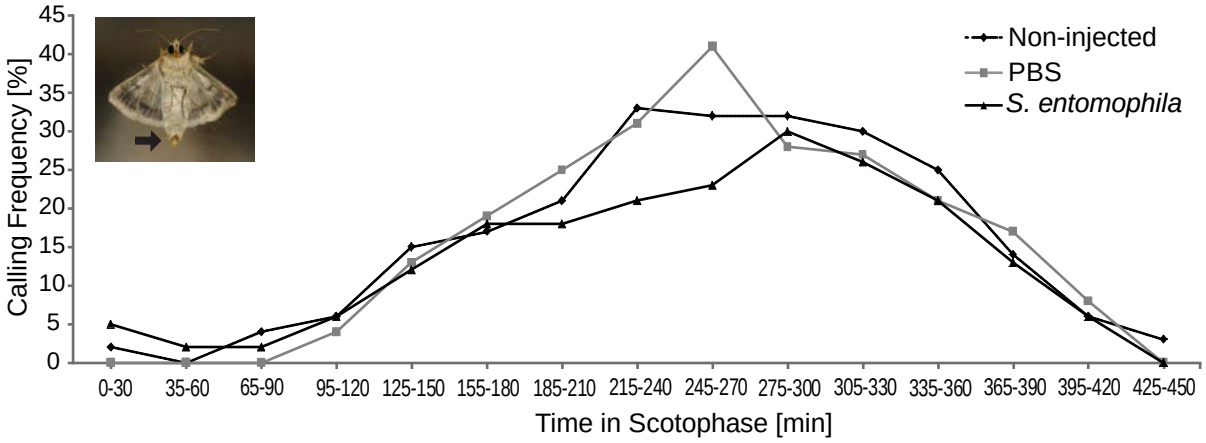

Figure 3.4. Calling activity of virgin *Heliothis virescens* females during scotophase. Individual calling behaviour was registered every 5 minutes, calling events of 30 minute periods were grouped together. Calling behaviour is depicted as the percentage of females that called per time interval (non-injected n = 58; PBS-injected n = 60; *S. entomophila*-injected n = 60). The overall treatment effect was tested with a generalized linear mixed model (glmmADMB in R). Photo: calling *H. virescens* female, arrow: extruded ovipositor with sex pheromone gland.

Discussion

Our study revealed trade-offs between immune system activation and reproduction in the moth *H. virescens* on a physiological as well as a behavioural level. However, in contrast to our hypothesis, we found that females and males invest differently in immunity: males had higher baseline immunity than females, indicating immune maintenance, while immune system activation was higher in females than in males in response to a bacterial challenge, indicating immune deployment. Our hypothesis that sexual attractiveness and immunity compete for the same resource pool and thus trade off with each other was partly confirmed: mating success was reduced in *S. entomophila*-injected females, but not in *S. entomophila*-injected males. In addition, *S. entomophila*-injected females traded off immunity against sexual attractiveness through a modified sex pheromone profile.

(1) Effect of immune challenge on immune gene expression in females and males

When assessing the immune status of organisms, it is important to distinguish between immune maintenance, which implies physiological costs to keep the immune system at a certain level of readiness, and immune deployment, which implies physiological costs of immune system activation to combat pathogens (Schmid-Hempel, 2011). We tested this by measuring the expression level of immune-related genes in adults upon challenge with *S. entomophila,* and found that immune deployment was higher in *H. virescens* females than in males. This is in line with reports from other insects. For example, in the scorpionfly

Panorpa vulgaris, females had a higher lysozyme-like activity and a higher number of (phagocytically active) hemocytes than males (Kurtz *et al.*, 2000). In the grasshopper *Melanoplus sanguinipes*, adult females showed higher PO activities than males in response to a fungus infection (Gillespie & Khachatourians, 1992).

Interestingly, our immune gene study showed that immune maintenance was higher in males than in females. If such an investment increases their longevity, male fitness is likely to be increased: *Heliothis virescens* males only mate at most once per night, so that every additional night of being alive means another mating opportunity (Proshold *et al.*, 1982; Raina & Stadelbacher, 1990). Additionally, *H. virescens* males invest in their offspring by transferring resources for a spermatophore to the female, which can make up to 5% of their body weight and contains not only sperm, but can also contain one third of the male's zinc supplies as well as sugars and proteins (Engebretson & Mason, 1980; Blanco *et al.*, 2009). Male investment into offspring has been shown to affect their investment in immunity in various species. For instance, in the pipefish *Syngnathus typhle*, a sex role reversed species where males invest more in offspring than females, males showed higher innate and adaptive immunity than females (Roth *et al.*, 2011). In another species with sex role reversal, the spider *Allocosa brasiliensis*, males showed a higher encapsulation response than females in response to a nylon filament implant (Aisenberg & Peretti, 2011). Interestingly, in *H. virescens*, which neither shows conventional sex roles nor sex role reversal but an investment of both sexes into offspring, we found that both sexes invested into immunity in different ways: males had higher immune maintenance, while females showed higher immune deployment.

The immune system of *H. virescens* females also seemed more specific than that of males, because immune gene expression levels differed more between wounding and bacterial challenge in females than in males (Figure 3.1). To save resources, it is likely important for animals to distinguish between wounding and bacterial challenge, because the activation of an immune response is costly and can negatively affect other life-history traits (Stearns, 1992; Zuk & Stoehr, 2002; Schmid-Hempel, 2005). This trade-off may affect female fitness more than male fitness in *H. virescens*, because females are the sexual signalers, whereas males have been shown to be choosy (Vetter & Baker, 1983; Groot *et al.*, 2014). Therefore, access to males is important for female fitness, and females likely need to invest resources in their attractiveness (Groot *et al.*, 2010; Harari *et al.*, 2011). Consequently, females may have evolved a more specific immune response than males, which is only activated in response to cues of pathogens, whereas males may afford constitutively high levels of immune defense and react stronger to minor cues of disease, like wounding.

(2) Effect of immune challenge on mating success of females and males

Since behaviour is intimately linked to the physiology of insects, we hypothesized that sexual attractiveness and immunity compete for the same resource pool and thus trade off with each other. In the mate choice experiments, we found male choice but not female choice. Specifically, we found in the male choice experiment that *H. virescens* males mated less with *S. entomophila*-injected females and chose more control females in a two-choice test. Males thus seem to choose the healthier female, as would be expected by parasite-mediated sexual selection theory (Hamilton & Zuk, 1982). However, we also found that males did not distinguish between wounded females and *S. entomophila*-injected females or wounded females and control females. This may be explained by comparing expression levels in our immune gene expression assays, where we found that wounding also induced an immune response in females of *H. virescens*, e.g. in the expression of lysozyme. Additionally, the differences in immune response induction between control and wounded, or wounded and *S. entomophila*-injected females were not as big as between control and *S. entomophila*-injected females (Figure 3.1b). These smaller differences might not have been enough to be recognized by the males. We can exclude the possibility that male choice was due to an acute risk of *S. entomophila*-injected females to die (in the course of three days), because in all three treatments females survived for more than three days.

In the female mate choice experiments, all types of males were chosen similarly often, indicating that females did not distinguish between differentially treated males. As males reacted less, physiologically, to the bacterial challenge than females, in terms of up-regulation of immune-related genes, it is likely that all males appeared similar to the females in the female mate-choice experiments.

(3) Effect of immune challenge on sexual signal and calling behaviour in females

We found the immune system activation in *H. virescens* females to be associated with an altered sex pheromone profile. The ratio of 16:Ald to Z11-16:Ald was significantly higher in *S. entomophila*-injected females than in wounded or control females. Z11-16:Ald is the major sex pheromone component in the *H. virescens* sex pheromone blend and is essential to attract males (Vetter & Baker, 1983; Groot *et al.*, 2014). As Groot *et al.* (2014) showed that females with higher ratios of 16:Ald / Z11-16:Ald (and thus less of the major component) were less attractive to males in the field than females with lower ratios (Groot *et al.*, 2014), these results indicate that infected females likely attract fewer males than healthy females under field conditions.

Interestingly, the changes in the sexual signal towards a less attractive profile did not coincide with a change in calling behaviour. These results indicate that female immunity and the quality of the female sexual signal are linked, and suggest that the female sex pheromone in moths is an honest signal that indicates the quality of a female to the male, which also fits the framework of parasite mediated sexual selection theory (Hamilton & Zuk, 1982).

Since the pheromone profile differed between wounded and *S. entomophila*-injected females, while males did not mate more with wounded than with *S. entomophila*-injected females in the mate choice experiments, the altered pheromone profile cannot fully explain our mate choice results. Most likely, at close range in mating cages, other traits than the female long-range sex pheromone are important for male choice and female mating success. In moths, males produce and emit a courtship pheromone that is likely important for female choice (Birch *et al.*, 1990; Hillier & Vickers, 2004). However, a male courtship pheromone cannot explain male choice for specific females. Perhaps cuticular hydrocarbon profile (CHCs) plays a role in mate choice at close range in *H. virescens*, as has been found in many insect species, e.g. *Drosophila*, crickets and beetles (Howard *et al.*, 2003; Peterson *et al.*, 2007; Thomas & Simmons, 2009). CHCs have hardly been investigated in moths, with a few exceptions (Jurenka & Subchev, 2000; Piskorski *et al.*, 2010)and their role in moth sexual communication is still unknown.

Conclusions

In the noctuid moth *H. virescens*, we found that investment in immunity is linked to the life history trajectories of males and females, and can trade off with sexual attractiveness and reproduction. Immune response is important for both sexes in *H. virescens*, because both females and males mate only once per night and invest substantially into their offspring. Accordingly, in our immune gene expression assays, we found that both males and females invested in immunity. However, the sexes differed in their investment strategies: females invested in immune defense after a bacterial challenge, whereas males had higher baseline immunity than females. Interestingly, these differences in investment were reflected in the mate choice assays. Males chose more for non-injected females than for *S. entomophila*-injected females, whereas females did not show a preference for differentially treated males. Furthermore, *S. entomophila*-injected females had an altered sexual signal compared to non-injected females. Thus, there is likely a trade-off between immune system activation and reproduction in females. We did not find this trade-off in males, probably because *H. virescens* males invest in immunity maintenance. It will be interesting to determine

whether these sex-specific differences in the type of immunity, i.e. immune deployment in females and immune maintenance in males are a general phenomenon.

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Supporting information

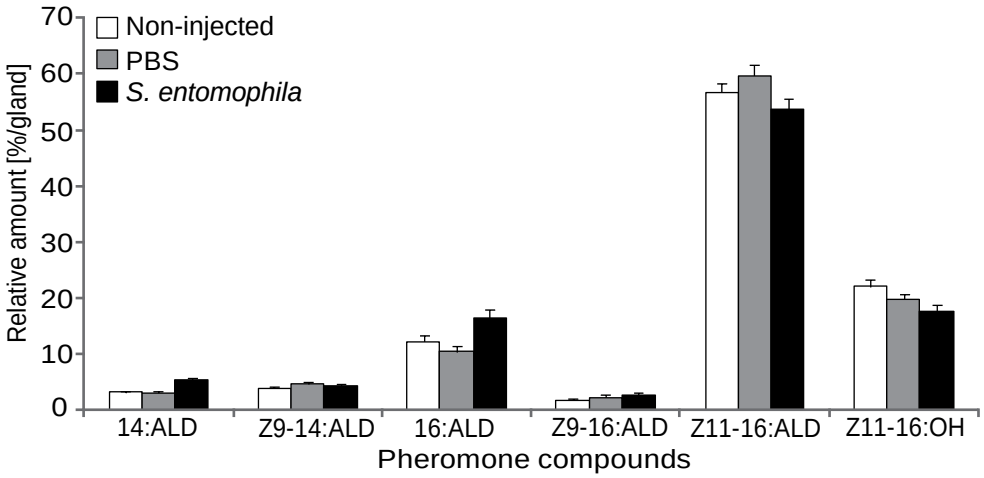

Figure S3.1. Pheromone composition of *Heliothis virescens* females that were non-injected (n=38), injected with *S. entomophila* (n=38) or with PBS (n=25). Z7-16:Ald was excluded from the analysis, see main text for explanation.

Table S3.1. Primer sequences used in qRT-PCR analysis

Gene name	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')
Heat shock protein 70	ACV32640	GTGCTCAGGATCATCAACGA	AGGTCGAAGATGAGCACGTT
PO activating factor	ACI32835	TGATCAGCCACAGCGTAAAG	CTCCGAATTTGGTCTCTCCA
Lysozyme	AAD00078	CGCTAGAAAGACGGACAAGG	CATTTCAGCGCAAGTGACAT
Gloverin	ACR78446	AGCAGCTTCTTGGGAGGAC	TCCTTATGGACATCAAGAGCAG
Hemolin	ACC91897	TTCCTGAGCCGAAGAATCAC	AATGTTCAGCCAACACCACA
Ribosomal protein S18 (RpS18)	In-house database	GCGTGCTGGAGAATGTACTG	GCCTGTTGAGGAACCAGTCT

Table S3.2. Experimental setup for mate choice experiments.

Assay		Chooser	Potential mate 1	Potential mate 2	Sample size [n]
Male choice	1	♂ Non-injected[1]	♀ *S. entomophila*	♀ Non-injected[1]	43
	2	♂ Non-injected[1]	♀ *S. entomophila*	♀ PBS[2]	38
	3	♂ Non-injected[1]	♀ Non-injected[1]	♀ PBS[2]	41
Female choice	4	♀ Non-injected[1]	♂ *S. entomophila*	♂Non-injected[1]	58
	5	♀ Non-injected[1]	♂ *S. entomophila*	♂ PBS[2]	48
	6	♀ Non-injected[1]	♂ Non-injected[1]	♂ PBS[2]	39

[1]Referred to as control individuals; [2]Referred to as wounded individuals

Table S3.3. Pairwise comparisons (LS-means with Tukey adjustment) of the expression level of immune-related genes between all treatments. Male and female moths were injected with *Serratia entomophila* (SER), PBS (PBS) or non-injected (NON); n = 3 for all groups. The overall treatment effect was tested by one-way-ANOVA with $P < 0.001$ for all genes.

Gene	Sex	Comparison	df	t-ratio	*P*-Value
Heat shock protein 70	♀	PBS – NON	12	-2.125	0.33
		SER – PBS	12	-11.837	**<0.001*** **
		SER – NON	12	-13.963	**<0.001*** **
	♂	PBS – NON	12	-1.310	0.77
		SER – PBS	12	0.477	1.00
		SER – NON	12	-0.833	0.96
	♀ vs ♂	NON-NON	12	-27.368	**<0.001*** **
Gloverin	♀	PBS – NON	12	-1.693	0.56
		SER – PBS	12	-11.711	**<0.001*** **
		SER – NON	12	-13.404	**<0.001*** **
	♂	PBS – NON	12	-3.601	**0.03***
		SER – PBS	12	-2.652	0.16
		SER – NON	12	-6.253	**<0.001*** **
	♀ vs ♂	NON-NON	12	-4.060	**0.02***
Hemolin	♀	PBS – NON	12	-0.297	1.00
		SER – PBS	12	-6.106	**<0.001*** **
		SER – NON	12	-6.403	**<0.001*** **
	♂	PBS – NON	12	-3.988	**0.02***
		SER – PBS	12	-1.371	0.74
		SER – NON	12	-5.359	**0.002****
	♀ vs ♂	NON-NON	12	0.245	1.00
Lysozyme	♀	PBS – NON	12	-7.291	**<0.001 *** **
		SER – PBS	12	-7.858	**<0.001 *** **
		SER – NON	12	-15.149	**<0.001 *** **
	♂	PBS – NON	12	-2.523	0.19
		SER – PBS	12	-4.752	**0.005****
		SER – NON	12	-7.275	**<0.001 *** **
	♀ vs ♂	NON-NON	12	-9.007	**<0.001 *** **
Phenoloxidase activating factor	♀	PBS – NON	12	-0.686	0.98
		SER – PBS	12	-4.609	**0.006****
		SER – NON	12	-5.294	**0.002 ****
	♂	PBS – NON	12	-3.505	**0.04***
		SER – PBS	12	3.107	0.08
		SER – NON	12	-0.398	1.00
	♀ vs ♂	NON-NON	12	-2.547	0.19

Table S3.4A. Overall treatment effect for differences in *Heliothis virescens* female sex pheromone between *Serratia entomophila*-injected (n=38), non-injected (n=38) and PBS-injected (n=25) females.

MANOVA	df (degree of freedom)	Pillai's trace	Approximate F	Pr(>F)
Treatment	2	0.31	3.45	0.00035
Residuals	98			

Table S3.4B. Effect of treatment on individual compounds of *Heliothis virescens* female sex pheromone tested with ANOVAs and LS-means pairwise comparisons (with Tukey adjustment) for compounds with treatment effect of P < 0.05. SER: *Serratia entomophila*-injected (n=38), NON: non-injected (n=38), PBS: PBS-injected (N=25)

ANOVAs, individual compounds				LS means pairwise comparisons (Tukey adjusted)			
Compound	df	F value	Pr(>F)	Comparison	Df	t-ratio	*P*-value
14:ALD	2	2.08	0.13	SER-NON	98	-1.40	0.35
				SER-PBS		-1.95	0.13
				PBS-NON		0.71	0.76
Z9:14:ALD	2	0.61	0.55	SER-NON	98	0.97	0.60
				SER-PBS		0.91	0.64
				PBS-NON		-0.05	0.64
16:ALD	2	1.05	0.36	SER-NON	98	-0.14	0.99
				SER-PBS		-1.36	0.37
				PBS-NON		1.23	0.44
Z11-16:ALD	2	1.47	0.24	SER-NON	98	1.67	0.22
				SER-PBS		1.08	0.53
				PBS-NON		0.40	0.91
Z11-16:OH	2	4.0	**0.021***	SER-NON	98	2.82	**0.016***
				SER-PBS		1.47	0.31
				PBS-NON		1.04	0.55

Table S3.4C. 16:ALD /Z11-16:ALD ratio in *Heliothis virescens* female sex pheromone. ANOVA for overall treatment effect and LS-means pairwise comparisons with Tukey adjustment between females of different treatment groups, SER: *Serratia entomophila*-injected (n=38), NON: non-injected (n=38), PBS: PBS-injected (n=25).

				LS means pairwise comparisons (Tukey adjusted)			
Tested Ratio	**df**	**F-value**	**Pr(>F)**	**Comparison**	**Df**	**t-ratio**	***P*-value**
16:ALD / Z11-16:ALD	2	6.74	**0.0018****	SER-NON	98	2.45	**0.042***
				SER-PBS		3.54	**0.018****
				PBS-NON		1.36	0.37

4

Effects of immune challenge on the oviposition strategy of a noctuid moth

Heike Staudacher, Steph B.J. Menken and Astrid T. Groot

Abstract

Infections can have detrimental effects on the fitness of an animal. Reproducing females may therefore be sensitive to cues of infection and be able to adaptively change their oviposition strategy in the face of infection. As one possibility, females could make a terminal investment and shift reproductive effort from future to current reproduction as life expectancy decreases. We hypothesized that females of the noctuid moth *Heliothis virescens* make a terminal investment and adapt their oviposition timing as well as their oviposition site selectivity in response to an immune challenge. We indeed found that females that were challenged with the bacterial entomopathogen *Serratia entomophila* laid more eggs than control females one night after the challenge. Additionally, bacteria-challenged females were less discriminating between oviposition sites than control females. Whereas control females preferred undamaged over damaged plants, immune-challenged females did not differentiate between the two. These results indicate that terminal investment is part of the life history of *H. virescens* females. Moreover, our results suggest that a strategy of terminal investment in *H. virescens* oviposition represents a fitness trade-off for females: in the face of infection, an increase in oviposition rate enhances female fitness, whereas low oviposition site selectivity reduces female fitness.

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Introduction

Pathogens are virtually always present in the environment of organisms and can have detrimental effects on their fitness when infection occurs (Grenfell & Dobson, 1995; Poulin, 2007; Schmid-Hempel, 2011). Under pathogen pressure, natural selection should favour adaptive changes in response to an immune challenge to minimize the costs of infection and maximize lifetime reproductive success (e.g. Adamo, 1999; Agnew *et al.*, 2000; Bonneaud *et al.*, 2004; Javoiš & Tammaru, 2004).

One possibility for organisms to adapt their life history strategy is terminal investment, that is the increase of current reproductive effort as life expectancy decreases (Clutton-Brock, 1984). Current reproductive output is expected to trade off with future reproductive output and is therefore in most cases not maximized (Williams, 1966). However, if life expectancy decreases, no resources need to be saved for future reproduction and investment in current reproduction is predicted to increase (Part *et al.*, 1992; Polak & Starmer, 1998; Velando *et al.*, 2006). Terminal investment may be apparent in increased courtship activity, a temporary increase in numbers of offspring and/or investment in offspring survival (Part *et al.*, 1992; Polak & Starmer, 1998; Adamo, 1999; Bonneaud *et al.*, 2004; Creighton *et al.*, 2009). For example, a temporary increase in numbers of eggs in response to an immune challenge has been found in crickets and freshwater snails (Minchella & Loverde, 1981; Adamo, 1999). Studies of several bird species showed an increase in parental care in response to an immune challenge and/or because of ageing (Part *et al.*, 1992; Hanssen, 2006; Velando *et al.*, 2006).

Nonsocial herbivorous insects do not generally provide parental care to their offspring (Janz, 2002). However, in many species, females show oviposition site choice that can be viewed as an investment in offspring survival, as well as a form of parental care (Wiklund & Persson, 1983; Janz, 2002; Lefèvre *et al.*, 2010). Oviposition site choice includes finding and selecting host plants on which offspring can obtain realized fitness and avoiding nonhost or herbivore-infested plants on which offspring cannot (Rothschild & Schoonhoven, 1977; Renwick, 1989; Nishida *et al.*, 1990; Tingle & Mitchell, 1991; De Moraes *et al.*, 2001; Kessler & Baldwin, 2001). Avoiding herbivore-infested plants may be especially important for offspring survival, and thus female fitness, because of enhanced intra- and interspecific competition on such plants (Denno *et al.*, 1995; Kaplan & Denno, 2007), indirect plant defences, such as the emission of volatiles that attract natural enemies of herbivores (Turlings *et al.*, 1990; McCall *et al.*, 1993; De Moraes *et al.*, 1998; Thaler, 1999) or the induced synthesis and enhanced accumulation of secondary plant metabolites that may render the plant unpalatable (e.g. Schoonhoven *et al.* 2005).

It has long been suggested that when available oviposition time is limited, ovipositing females become less selective and more likely to accept lower-ranked hosts or even non-hosts (Jaenike, 1978; Courtney & Courtney, 1982). As infection can be a cue for shortened life expectancy, oviposition site selectivity and thus investment in offspring survival can be expected to decrease with infection. Moreover, if oviposition rate actually increases in response to infection, ovipositing females become more time-limited. There is evidence that terminal investment behaviour in herbivorous insects in response to cues for shortened life expectancy indeed results in increased plant acceptance (Javoiš & Tammaru, 2004). However, to our knowledge, cues of shortened life expectancy have not yet been shown to lower oviposition site selectivity.

In this study, we investigated oviposition timing and oviposition site preference in response to an immune challenge in the generalist herbivore *Heliothis virescens* (Lepidoptera, Noctuidae). Females of this moth species can lay up to about 1500 eggs in their lifetime of about 30 days in the laboratory at 25 °C (Proshold *et al.*, 1982; Willers *et al.*, 1987; Fitt, 1989). Females oviposit throughout the night and lay their eggs singly on plants (Fitt, 1989; Ramaswamy, 1990). *Heliothis virescens* larvae are cannibalistic from their 3rd instar onwards (Gould *et al.*, 1980), so that avoiding to oviposit on plants that are infested with conspecific larvae is likely adaptive for *H. virescens* females. Accordingly, *H. virescens* females have been shown to avoid *Nicotiana tabacum* plants on which conspecific larvae had fed (De Moraes *et al.*, 2001).

Even though *H. virescens* can be regarded an r-strategist, oviposition site choice can be considered an investment into offspring survival, because females need to invest time and resources into finding and selecting an optimal site (Pianka, 1970; Wiklund & Persson, 1983; Willers *et al.*, 1987; Janz, 2002). As infection may limit the time that is available for oviposition, we hypothesized that *H. virescens* females would increase their oviposition rate after a bacterial challenge (H1). Secondly, we predicted that terminal investment would be apparent in oviposition site selectivity, such that bacteria-challenged females are less discriminating in their choice of oviposition site than control females (H2).

Material and methods

Insects and bacterial culture

Heliothis virescens was collected in July 2011 in North Carolina, USA, and reared in climate chambers at 25 °C, 60% relative humidity and a light-dark cycle of 10L:14D with lights on at

11 am. Larvae were grown on artificial pinto bean diet (Burton, 1970). Adults were provided with a 10% (wt/vol) sugar-water solution.

To induce an immune response in *H. virescens* females, we used the entomopathogenic bacterium *Serratia entomophila,* which was obtained from the Department of Bioorganic Chemistry (MPICE, Jena, Germany). *Serratia entomophila* was grown overnight in CASO medium at 30 °C on a shaker set at 250 r.p.m. After one night, cultures were centrifuged and the supernatant was discarded. To investigate effects of immune challenge without the confounding effects of the dynamics of a living pathogen, it is common to use immune elicitors like lipopolysaccharides (LPS) (Moret & Schmid-Hempel, 2000; Korner & Schmid-Hempel, 2004) or dead bacterial cells (Haine *et al.*, 2008, Cotter, 2010 #445). We therefore killed the bacteria by freezing and drying the samples in a lyophilisator at -80 °C for 5 days. To confirm that bacteria were dead, we streaked them out on Luria-Bertani (LB) agar plates. Lyophilized cells of *S. entomophila* were stored at -20 °C until used in the experiments.

Immune activation via bacterial challenge in mated females

In all experiments, two groups of females were tested; one group was injected with lyophilized cells of *S. entomophila* to induce an immune response [4 µl of a 1µg/µl solution of bacteria diluted in 1x phosphate buffered saline (PBS)]. This entomopathogenic bacterium was shown to be lethal for *H. virescens* larvae and to induce hemocyte apoptosis in *H. virescens* larvae (Barthel *et al.*, 2014). The other group of females was the control, which was injected with 4 µl of 1x PBS solution. All females were mated in single pair matings one night before they were used in the oviposition assay (night zero). Matings were observed to ensure that females were mated. At the onset of the photophase and between 15 and 17 h before the start of the experiments, the mated females were injected into their abdominal cavity with a 10-µl Hamilton syringe. Previously, we found that injecting lyophilized *S. entomophila* cells in this way elicits an immune response in *H. virescens* females (A. Barthel, H. Staudacher, A. Schmaltz, D.G. Heckel, A.T. Groot, unpublished data). Injecting bacteria has been commonly used to investigate the effects of an immune challenge and as such mimics the process of bacterial cells breaking through the cuticle (Shelby & Popham, 2008). Furthermore, injection ensures that equal amounts of bacterial and PBS solution are used for each female and in all experiments.

H1 Females increase their oviposition rate after a bacterial challenge

To test the hypothesis that females would make a terminal investment and increase their oviposition rate (that we define here as mean number of eggs/ female/ night) after a bacterial challenge, we conducted oviposition assays with two groups of 2- to 9-day-old *H. virescens* females. One group was injected with *S. entomophila* (n = 27), the other group of females served as the control and was PBS-injected (n = 25), as described above. Experimental females of different ages were distributed evenly between the two treatment groups. The mated and injected females were placed in paper cups (200 ml) at the beginning of the first night after mating (night one), and provided with one dental stick that was soaked in 10% sugar water, which was renewed every night. Cups were closed with transparent gauze. During this experiment, the females were kept without plants. For each female, eggs (all eggs in a cup) were counted at the end of each consecutive night until she died. In the course of the experiment females died, which was recorded daily. Females that did not lay eggs during the experiment were excluded. Also with these exclusions, the age distribution of females remained similar between the two treatment groups (W = 284.5, $P = 0.33$, Wilcoxon rank sum test with continuity correction) (see Figure S4.1 for age distribution of females in the two treatment groups).

Statistical analysis. Differences in number of oviposited eggs between *S. entomophila*-injected and control females for night one were tested with a linear model. Number of eggs at night one was used as response variable and treatment and female age at the start of the experiment as explanatory variables. Additionally, to test whether bacterial challenge had an influence on oviposition rate over time, we analysed the data from the first 13 nights. We chose the period of 13 nights, because after that period there were fewer than 10 surviving females in the *S. entomophila*-injected group left. We used a generalized linear mixed model with negative binomial distribution in the R package glmmADMB (version 0.8.0) (Fournier *et al.*, 2012; Skaug *et al.*, 2014). Number of eggs per female per night was used as response variable. Night and treatment as well as the interaction effect between night and treatment were used as explanatory variables. Female age at the start of the experiment was included as fixed effect in the model. To account for repeated measurements, we added individual females as random effect to the model, because the eggs of each female were counted every night. To account for temporal autocorrelation, we added night as a random effect slope to the model (night | individual female). To improve the model fit, we included experimental night square and zero inflation in the model which lowered the AIC (Akaike information criterion).

We used a linear model to test for differences in total number of eggs (number of eggs that females laid after injection until they died) between *S. entomophila*-injected and PBS-injected females. Total number of eggs was used as response variable. Treatment and female age at the start of the experiment were used as explanatory variables. To analyse the survival of control and *S. entomophila*-injected females, we used weighted Cox regression in the package coxphw in R (Heinze *et al.*, 2014), with censoring applied to females that did not die by day 13, and using average hazard ratio (AHR) as template for the case of nonproportional hazards (Schemper, 1992; Schemper *et al.*, 2009; Heinze *et al.*, 2014). Treatment and age were used as explanatory variables. One female of the *S. entomophila* group escaped at night three and was excluded from the models of survival and number of total eggs. To test whether female age and treatment influenced the death of females two nights after injection (when many females were found dead), we constructed a generalized linear model with binomial distribution, using likelihood-ratio as test statistic. Survival [n(alive) = 41] or nonsurvival [n(dead) = 10] for longer than night two was used as response variable, female age and treatment served as explanatory variables.

To determine whether an actually shortened life span affected the number of eggs oviposited in night one after the bacterial challenge, we applied another linear model to the data of the *S. entomophila*-injected group. Number of eggs was used as response variable, and female age as well as survival [n(alive) = 16] or nonsurvival [n(dead) = 10] for longer than night two after injection were used as explanatory variables.

The response variables in the linear models were square-root-transformed when it improved the residual structure of the models. Interaction effects of treatment and female age were tested for all linear models, but were excluded when they were not significant.

H2 Bacteria-challenged females are less discriminating in their choice of oviposition site than control females.

To test the hypothesis that immune system activation influences female oviposition preference, we conducted dual-choice oviposition assays with a different group of mated females, that were *S. entomophila*-injected or PBS-injected (control) as described above. The light-dark cycle of the moth rearing for this experiment was L16:D8 with lights on at 6 am. Mated females were given a choice between a damaged and an undamaged plant. We used *Nicotiana attenuata* plants, which is one of the natural host plants of *H. virescens.* To generate damaged plants, five *H. virescens* 3rd instar larvae were placed on the plants for 48 h and removed from the plants right before the start of the experiment. Undamaged plants were left

untreated. All plants were eight to nine weeks old and in their flowering stage, as females mainly lay their eggs on tobacco buds and flowers. Experiments were conducted in cages (2.0 x 0.83 x 1.0 m), which contained one damaged plant, one undamaged plant and one mated female which was 2-8 days old at the night the oviposition experiment started. Age did not differ significantly between the treatments (W = 269, P = 0.70, Wilcoxon rank sum test with continuity correction; for age structure see Figure S4.2). Damaged and undamaged plants were placed at a distance of 1.5 m from each other. Each plant was only used once for testing one female. Positions of damaged and undamaged plants in the cages were switched every night to avoid directional effects. The experiment was conducted under natural light conditions during eight nights in May 2014 (sunrise ~5:35 and sunset ~21:40 hours) and two nights in June 2014 (sunrise 5:18 and sunset 22:06 hours) at 25 °C.

Each night, 2-8 females were tested and each female was tested only once, that is for one night. Each night we tested the same number of control and *S. entomophila*-injected females. Females were released in the middle of the cages one hour before dusk and were removed from the cages on the next day. Eggs were counted on the undamaged and the damaged plant as well as on the sides, bottom and top of the cages, on the day after the experimental night. We will refer to eggs that were not found on plants but anywhere else in the cage as off-plant eggs.

Statistical analysis. To test differences in preference between control and *S. entomophila*-injected females for oviposition site (damaged plant, undamaged plant or off-plant), we performed mixed-design ANOVAs with one between variable (female age) and one within variable (oviposition site) to account for the paired character of oviposition site. The analysis was performed separately for control and *S. entomophila*-injected females. The interaction effect between female age and treatment was not significant for *S. entomophila*-injected females and thus excluded from the model for this group. To compare number of eggs between the three oviposition sites, we performed LS-means pairwise comparisons with Tukey correction based on the above-described ANOVA models. Differences in total number of eggs between control and *S. entomophila*-injected females were tested with a linear model, using number of eggs as response variable and treatment and female age as explanatory variables. All analyses were conducted with R version 3.0.2 (R Core Team, 2013).

Results

H1 Females increase their oviposition rate after a bacterial challenge

Serratia entomophila-injected females laid on average significantly more eggs than control females one night after the immune challenge ($F_{1,49}$ = 7.66, P = 0.0079, Figure 4.1a and b). Female age at the start of the experiment did not significantly affect the number of eggs one night after injection ($F_{1,49}$ = 2.48, P = 0.12, Figure S4.3).

When we analysed the effect of treatment over time, we found an overall significant interaction effect of night and treatment on the number of eggs per female per night (χ^2 = 4.56, df =1, P = 0.033, Figure 4.1b). *Serratia entomophila*-injected females laid on average more eggs per night than control females from night one to five with an exception of night three. Control females laid on average more eggs per night from night six to night 13 (Figure 4.1b).

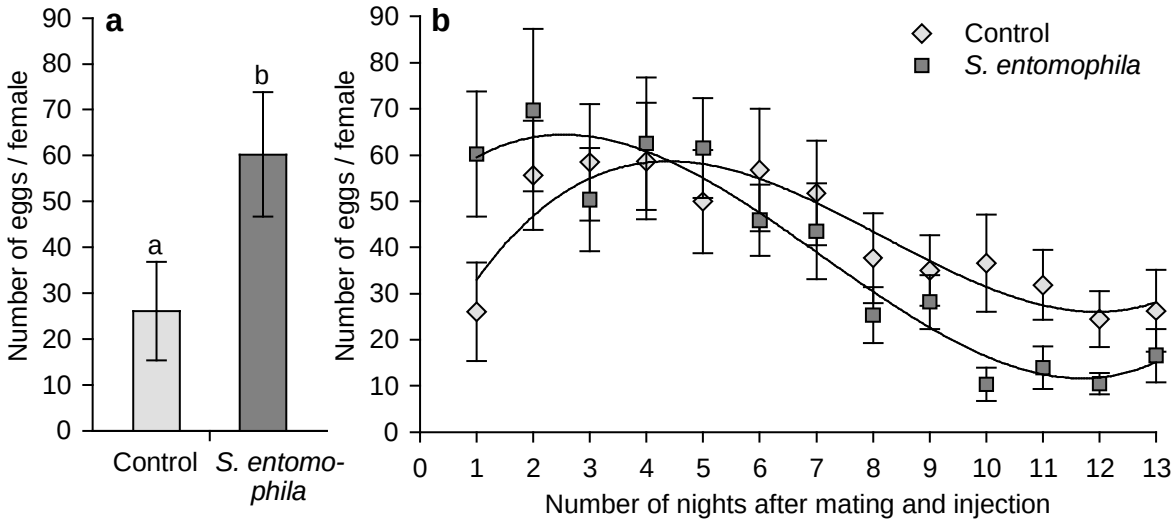

Figure 4.1. Mean (± SE) number of eggs oviposited per female in response to an immune challenge. a) Mean (± SE) number of eggs per female 24h after immune challenge; different letters above the bars indicate significant differences at a level of alpha ≤ 0.01 (linear model). b) Daily mean number of eggs (± SE) per female in the course of 13 nights with polynomial trendline (trendline is only for visualization and was not used for calculations); females were PBS-injected (n = 25 at night zero) or *S. entomophila*-injected (n = 27 at night zero).

Total number of eggs laid by control females did not differ significantly from the total number of eggs laid by *S. entomophila*-injected females ($F_{1,48}$ = 2.10, P = 0.15). Female age at the start of the experiment had a marginally significant effect on the total number of eggs of the females ($F_{1,48}$ = 3.95, P = 0.053, Figure 4.2).

Serratia entomophila-injected females died significantly earlier than control females after injection (z = -2.25, P = 0.024, Figure 4.3). Of the *S. entomophila*-injected females,

38.5% died two nights after the injection. Female age at the start of the experiment did not have a significant effect on the mortality of females (i.e. number of days from injection to death) (z = 1.07, P = 0.29). Female age at the start of the experiment also did not affect the number of females that died two nights after injection (χ^2 = 0.69, df = 1, P = 0.41). Treatment did have a significant effect on the number of females that were found dead at night two (χ^2 = 16.5, df = 1, P < 0.001, Figure 4.3). The number of eggs at night one that were laid by *S. entomophila*-injected females which survived longer than two nights after injection did not differ significantly from the number of eggs that were laid by nonsurvivors of *S. entomophila* injection ($F_{1,24}$ = 1.62, P = 0.22, Figure 4.4).

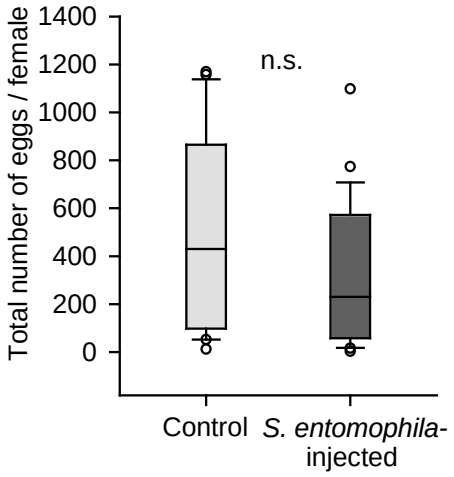

Figure 4.2. Total number of eggs laid by *S. entomophila*-injected (n = 26) and PBS-injected (n = 25) females after injection and mating. Boxes span the 25-75 percentiles, lines in the boxes represent the medians, whiskers span the 10-90 percentiles and circles represent data points outside this range; linear model, n.s. = not significant.

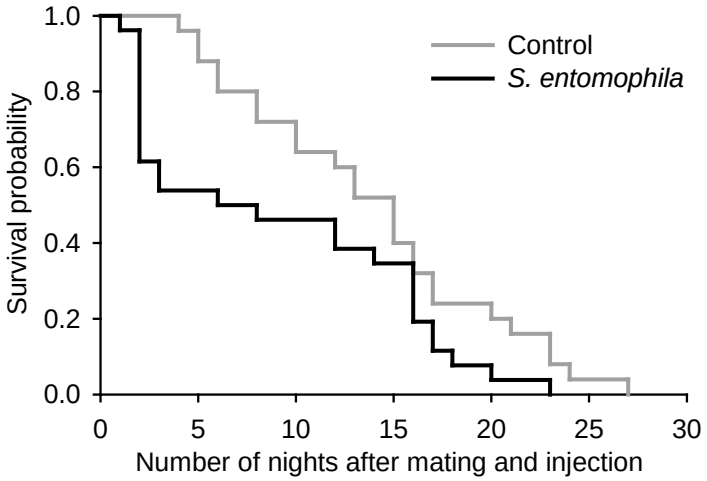

Figure 4.3. Survival probability of PBS-injected (n = 25) and *S. entomophila*-injected females (n = 26) on 27 consecutive nights after injection; weighted Cox regression.

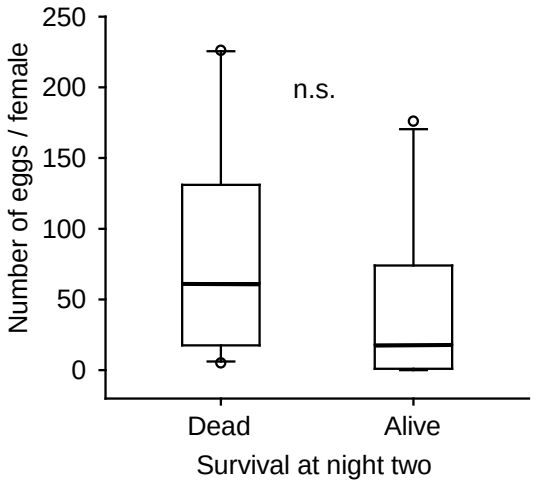

Figure 4.4. Number of eggs per *S. entomophila*-injected female one night after injection. Boxes span the 25-75 percentiles, lines in the boxes represent the medians, whiskers span the 10-90 percentiles and circles represent data points outside this range. Dead: females were dead at night two after injection (n = 10), alive: females survived longer than night two after injection (n = 16); n.s. = not significant.

H2 Bacteria-challenged females are less discriminating in their choice of oviposition site than control females.

In control females, the number of eggs differed significantly between oviposition sites (undamaged, damaged plant or off-plant) ($F_{2,46}$ = 4.24, P = 0.020, Figure 4.5a). We also detected a significant interaction effect between oviposition site and female age on the number of oviposited eggs ($F_{2,46}$ = 13.06, P < 0.001, Figure S4.4). In the pairwise comparison of oviposition sites, control females oviposited more eggs on undamaged than on damaged plants (t_{46} = 2.61, P = 0.032) or off-plants (t_{46} = 2.42, P = 0.050), and a similar number of eggs on damaged plants and off-plant (t_{46} = 0.191, P = 0.98, Figure 4.5a). In the control females, age did not have a significant effect on the number of eggs oviposited ($F_{1,23}$ = 1.38, P = 0.25). In *S. entomophila*-injected females, the number of oviposited eggs did not differ between oviposition sites ($F_{2,44}$ = 2.06, P = 0.14, Figure 4.5b), and there was no interaction effect between female age and oviposition site ($F_{2,42}$ =1.51, P = 0.23, Figure S4.5). The total number of eggs was not affected by female age when the two treatment groups were combined ($F_{1,45}$ = 0.014, P = 0.92).

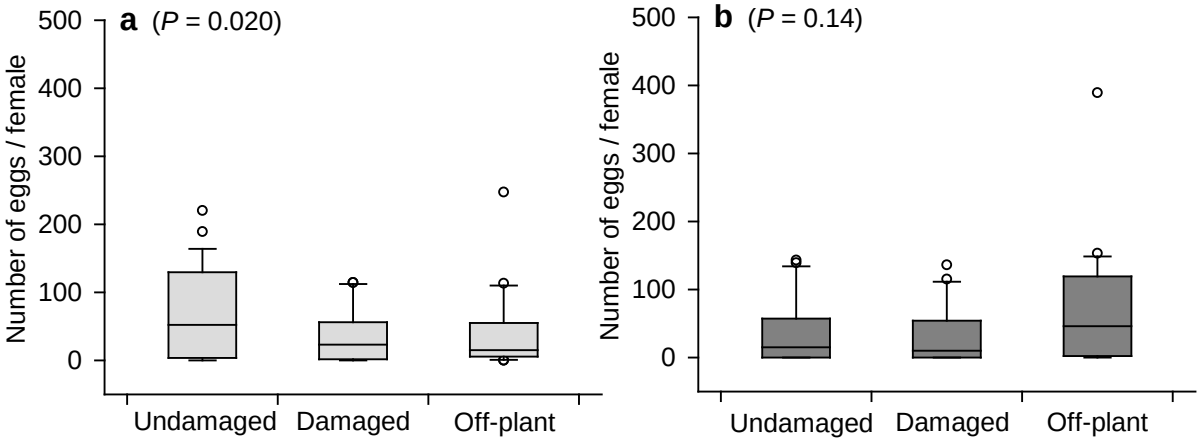

Figure 4.5. Oviposition site preference (undamaged plants, damaged plants and off-plant) in *S. entomophila*-injected and control *H. virescens* females. a) Number of eggs per female on the three oviposition sites in control females (n = 25). Undamaged vs. damaged plants: P = 0.032, undamaged plants vs. off-plant: P = 0.050. b) Number of eggs per female on the three oviposition sites in *S. entomophila*-injected females (n = 23), overall effect: not significant. Boxes span the 25-75 percentiles, lines in the boxes represent the medians, whiskers span the 10-90 percentiles and circles represent data points outside this range.

Discussion

In this study, we found evidence for terminal investment in the oviposition behaviour of *H. virescens* females in response to immune challenge with the bacterial entomopathogen *S. entomophila*. We confirmed our hypotheses that (H1) *H. virescens* females increase their

oviposition rate after bacterial challenge and (H2) bacteria-challenged *H. virescens* females are less discriminating in their choice of oviposition site than control females.

H1 Females increase their oviposition rate after a bacterial challenge

The findings that *S. entomophila*-injected females oviposited more eggs earlier and especially laid more eggs on the first night after a bacterial challenge provide further evidence for the terminal investment hypothesis. Indication for terminal investment in nonsocial herbivorous insects has been found earlier in the cricket *Acheta domesticus,* which increased its egg output in response to immune challenge (Adamo, 1999). Similarly, in the moth *Scotopteryx chenopodiata*, oviposition rate of injured females was also higher than that of females without injuries (Javoiš & Tammaru, 2004). In our study, the change in oviposition strategy in response to bacterial challenge was underlined by the fact that the total number of eggs laid by *S. entomophila*-injected and control females did not differ significantly, even though *S. entomophila*-injected females lived fewer days. This suggests that females compensate for a shortened lifetime with a shift of reproductive output from future to current reproduction in response to an immune challenge, which is in accordance with the terminal investment strategy (Williams, 1966; Clutton-Brock, 1984).

The fact that *S. entomophila* injection was deadly for many females in our study suggests a cost of immune response, which has been shown to reduce life span (Sheldon & Verhulst, 1996; Moret & Schmid-Hempel, 2000). Moreover, cytotoxic substances that are produced in the course of immune defence are possibly harmful for host tissue as well (Nappi & Vass, 1993; Zuk & Stoehr, 2002; Schmid-Hempel, 2005). Even though longevity was found to be affected by immune response in many studies (Moret & Schmid-Hempel, 2000; Armitage *et al.*, 2003; Javoiš & Tammaru, 2004), mortality is usually not as high as in our study. However, Krams *et al.* (2014) also found very high mortality in larvae of the moth *Galleria mellonella* in response to an immune challenge by nylon bead implantation: larvae which were grown on high energy food showed shorter developmental time, weaker encapsulation response and higher mortality in response to the challenge than larvae grown on low-energy food. As the shorter developmental time was associated with weak encapsulation response, the authors argue that low encapsulation response was likely responsible for high mortality in the high-energy food group (Krams *et al.*, 2014). Thus, possibly more complex relationships between life history traits and immune defence underlie the high mortality of *H. virescens* females in response to immune challenge.

H2 Bacteria-challenged females are less discriminating in their choice of oviposition site than control females.

As control females preferred undamaged over damaged plants and off-plant oviposition sites whereas *S. entomophila*-injected females did not differentiate between oviposition sites, we conclude that oviposition site selectivity can be lowered by cues of shortened life expectancy in herbivorous insects if females are given a choice between sites of different quality. Our results are in line with previous studies that investigated host plant acceptance and oviposition delay without providing a choice. For example, in the moth *S. chenopodiata*, survival of females was associated with oviposition latency on plants of different host quality (Javoiš & Tammaru, 2004): survivors of experimentally applied injuries showed a small oviposition latency on superior hosts, but a large oviposition latency on inferior hosts, whereas the reverse was found for moths that laid eggs but did not survive until the end of the experiment (Javoiš & Tammaru, 2004). We also confirmed the model prediction that oviposition site selectivity decreases when there is a cue that oviposition time is reduced (Jaenike, 1978; Courtney & Courtney, 1982).

Trade-off between early oviposition and oviposition site preference

The fact that a cue for shortened life expectancy provoked a temporary increase in oviposition rate in *H. virescens* females after a bacterial challenge indicates a change in oviposition strategy. This change likely optimizes female fitness when infected with pathogens (Minchella & Loverde, 1981; Adamo, 1999; Bonneaud *et al.*, 2004). However, a cue for shortened life expectancy also decreased female oviposition site selectivity, as we found differences in oviposition site choice between *S. entomophila*-injected and control females. A decrease in oviposition site selectivity is expected to reduce female fitness, because oviposition site selectivity has been shown to affect offspring survival in herbivorous insects (Singer, 1972; Rausher, 1982; Gripenberg *et al.*, 2010). The nonpreference for undamaged or damaged plants in *S. entomophila*-injected *H. virescens* females is possibly due to an increased pressure to oviposit early (before death) and indicates that increased egg output after an immune challenge may be linked to lowered oviposition site preference. Terminal investment in this moth may thus be characterized by a trade-off between early oviposition and oviposition site selectivity which likely translates into a fitness trade-off for the females.

The trade-off that we found in our experiments is not necessarily typical of all types of infections. In our experiments, we only used one strain of a pathogenic bacterium to induce an immune defence and one population of *H. virescens* as host. As genotype-specific host-

parasite interactions are widespread in nature, it should be stressed that our results cannot be generalized for interactions of *H. virescens* with different parasites or even different strains of the same bacterium used in this study (Schmid-Hempel & Ebert, 2003; De Roode & Altizer, 2010). We chose to conduct the experiments with *S. entomophila*, because *S. entomophila* injection has been shown to induce the immune system of *H. virescens* in a previous study (Barthel *et al.*, 2014).

The influence of female age on oviposition behaviour

The finding that female age did not affect the number of eggs laid one night after mating could be explained by the fact that in *H. virescens* mating stimulates egg maturation and oviposition (Proshold *et al.*, 1982; Ramaswamy *et al.*, 1997; Zeng *et al.*, 1997). Virgin females lay far fewer eggs than mated females and mating has a particularly stimulating effect on oviposition one day after mating (Proshold *et al.* 1982). In our experiments, the effect of female age on total number of eggs was marginally significant, which is comparable to the results of Proshold *et al.* (1982) who found that the total number of eggs laid depended on the age at which females were mated. As in our experiments the number of females that were less than three and more than six days old was very small, we could not firmly test homogeneity in the number of eggs laid across age groups.

Interestingly, when we investigated oviposition site preference, we did not find an interaction effect between female age and oviposition site in *S. entomophila*-injected moths, but we did detect such an interaction in the control females. This result indicates that older females discriminate less between oviposition sites than young females. Since lifetime expectancy generally decreases with age, this age effect fits the prediction that oviposition site selectivity decreases with less time to oviposit (Jaenike, 1978). Hence, bacterial challenge and age seem to similarly induce terminal investment behaviour: possibly, age did not affect oviposition site choice in *S. entomophila*-injected females, because terminal investment was induced by bacterial challenge in females of all ages in this group. Since our experiments were not designed to measure the effect of age, future experiments are needed to investigate the effect of age on oviposition choice in *H. virescens*.

Presence versus absence of plants in oviposition experiments

When we tested oviposition preference between damaged and undamaged plants, both *S. entomophila*-injected and control females laid about three times as many eggs compared to *S. entomophila*-injected females in the first experiment, where plants were not involved. The

presence of tobacco (*N. tabacum*) or tobacco leaf extracts is known to stimulate oviposition in mated *H. virescens* females (Jackson *et al.*, 1984; Mitchell *et al.*, 1990; Ramaswamy *et al.*, 1997): females were found to lay about three times as many eggs on cloth treated with tobacco leaf extract than on untreated cloth (Mitchell *et al.*, 1990). Conversely, Proshold *et al.* (1982) found that *H. virescens* females in mating cups without plant stimuli laid about 200 eggs per female one day after mating, which is more than three times the number that we encountered during oviposition without plant stimuli. These differences may be due to adaptation to host plant-free laboratory rearing conditions, which may inadvertently have selected for females that oviposit more readily on artificial substrate in the absence of plant stimuli: Proshold *et al*. (1982) used a *H. virescens* strain that had been reared in the laboratory for > 60 generations, whereas our oviposition timing experiment was performed with moths that were collected from the field as eggs in 2011 and reared in the laboratory for only 14 generations.

Interestingly, when we tested for an increase in the number of eggs after a bacterial challenge without plants, *S. entomophila*-injected females laid more eggs than control females one night after injection, whereas when we tested oviposition site preference, control and *S. entomophila*-injected females laid a similar total number of eggs. The absence of a suitable host plant can cause a delay in oviposition in moths (Leather & Burnand, 1987; Tammaru & Javoiš, 2000). Oviposition delay was also shown for *S. chenopodiata* on an inferior host plant for survivors of injury, but not for moths that laid eggs but did not survive until the end of the experiment (Javoiš & Tammaru, 2004). Hence, control females in our experiments possibly delayed oviposition only in the absence of a suitable host plant, whereas *S. entomophila*-injected *H. virescens* females laid high numbers of eggs one night after injection even in the absence of a suitable host. These findings are also in accordance with the terminal investment strategy.

Conclusions

We conclude that *H. virescens* females are able to adapt their oviposition strategy by shifting their egg output from future to current reproduction, when survival prospects are compromised by infection. Moreover, by this shift, immune-challenged females seem to be able to compensate for their shorter lifetime by ovipositing more eggs earlier in life, as total number of eggs was not different between immune-challenged and control females. As immune-challenged females were less selective for oviposition site than control females, we show that oviposition site selectivity is another trait that can be affected by an immune

challenge in herbivorous insects. As oviposition timing and plant selectivity are likely linked in herbivorous insects, we suggest that there is a fitness trade-off between making a terminal investment by laying more eggs early at the expense of oviposition site selectivity.

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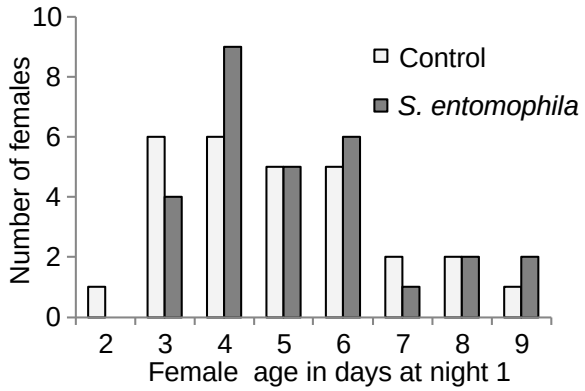
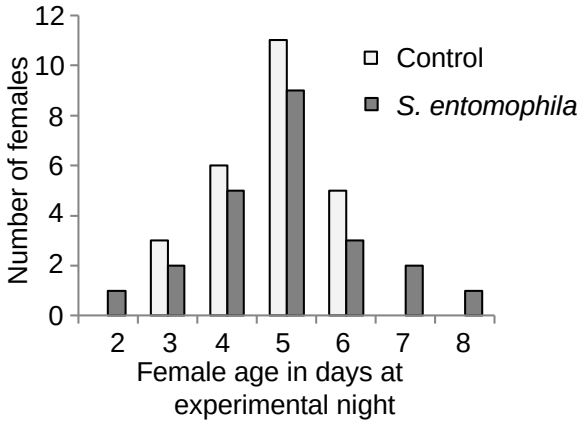
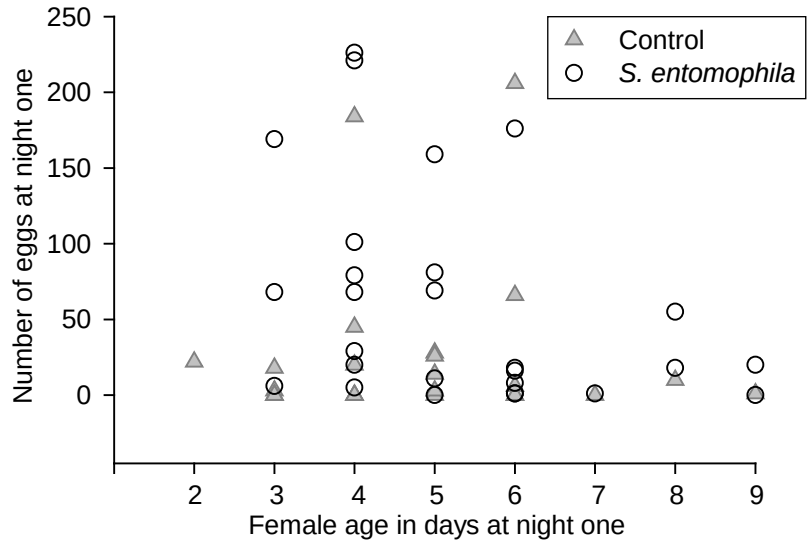
Supporting information



Suppl. Figure S4.1. Age structure of control (n = 25) and *S. entomophila*-injected females (n = 27) at night one after injection and mating in the oviposition timing experiment.



Suppl. Figure S4.2. Age structure of control (n = 25) and *S. entomophila*-injected females (n = 23) in the experiment for oviposition site selectivity.



Suppl. Figure S4.3. Effect of female age on the number of eggs at night one after injection and mating in the oviposition timing experiment in control (n = 25) and *S. entomophila*-injected (n = 27) females.

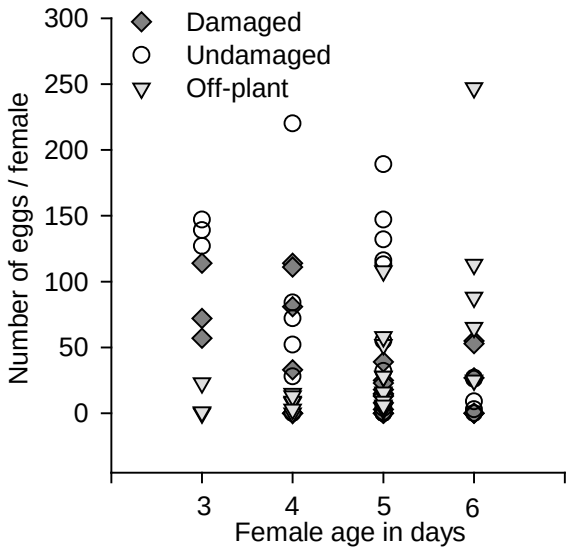


Figure S4.4. Oviposition site choice (damaged plants, undamaged plants, off-plant) in different age groups in control females (n = 25).

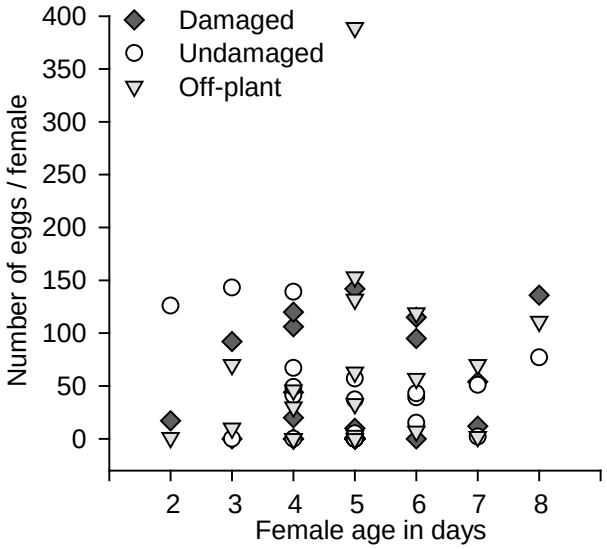


Figure S4.5. Oviposition site choice (damaged plants, undamaged plants, off-plant) in different age groups in *S. entomophila*-injected females (n = 23).

5

The bacterial symbionts *Wolbachia*, *Cardinium* and *Spiroplasma* affect gene expression and survival of their spider mite host and impact distinct induced responses in plants

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**Contributed equally to this study*

Abstract

Herbivore-associated microbial symbionts may facilitate plant utilization of their hosts directly by providing nutrients or by supporting the breakdown of food. Symbionts may also indirectly facilitate plant utilization by manipulating plant physiology to their host's benefit. Here we investigated the role of the symbiotic prokaryotes *Wolbachia* (W), "*Candidatus* Cardinium" (C) and *Spiroplasma* (S) in plant utilization by two strains of the spider mite *Tetranychus urticae*: Santpoort-2, an "inducer" strain of plant defence in tomato (*Solanum lycopersicum*), and DeLier-1, a "suppressor" strain of plant defence. We sequenced the bacterial communities of both strains and found that the inducer strain carries *Cardinium* and *Spiroplasma* (C+S+), while the suppressor strain carries *Wolbachia* and *Spiroplasma* (W+S+). After mites were treated with antibiotics to remove these symbionts, we obtained mite lines for 5 groups: W+S+, W-S+, W-S- for the suppressor and C+S+, C-S- for the inducer strain. In the suppressor strain, we found that the absence of *Wolbachia* correlated with lower mite survival on tomato, while the absence of *Spiroplasma* did not affect mite survival. In the inducer strain, food intake, survival and fecundity were increased when *Cardinium* and *Spiroplasma* were removed from the host. Using microarrays, we determined to what extent the absence of symbionts affected the mite's transcriptome. Interestingly, many of the mite genes that were differentially regulated in mites with or without bacteria encode proteins that play a role in digestion and detoxification or are predicted to be secreted, some of them from the salivary gland. Specifically, in the suppressor strain we found a reduced expression of these genes when *Wolbachia* was absent, while in the inducer strain the absence of *Cardinium* and *Spiroplasma* had the opposite effect. Finally, we investigated to what extent the antibiotics-treated mites induced or suppressed plant responses compared to the non-treated control strains. Excluding *Wolbachia* from the suppressor mites while retaining *Spiroplasma* (W-S+) resulted in a dramatically reduced accumulation of several jasmonic acid (JA)-intermediates, such as 12-oxo-phytodienoic acid (OPDA), but did not affect the end product JA-Ile nor the expression of defence marker-genes downstream of JA. We did however detect an increased salicylic acid (SA) accumulation, but without a uniform effect on the downstream marker genes. Removal of both (endo)symbionts from the suppressor strain yielded an intermediate plant phenotype. Feeding by C-S- inducer mites resulted in higher levels of abscisic acid (ABA) and lower expression of SA marker genes compared to feeding by C+S+ mites, while SA levels were unaffected. Our data suggest that *Wolbachia* is beneficial for the suppressor mites, by possibly enhancing the ability of its host to deal with toxic secondary metabolites in its food. In contrast, *Cardinium* seems to negatively affect the

performance of the inducer mites, possibly by inducing expression of (SA-responsive) pathogenesis-related genes in tomato leaflets, while simultaneously inhibiting detoxification processes in the mite. *Spiroplasma* might counteract these effects to some extent through direct interactions with *Wolbachia* and *Cardinium*. In conclusion, spider mite (endo)symbionts seem to interact directly and indirectly with their host. While *Wolbachia* has the characteristics of a mutualist, *Cardinium* has the characteristics of a parasite.

Introduction

Herbivores face the challenge to digest, absorb and utilize plant material in order to grow, develop and reproduce. In many cases, plant material is poor or unbalanced in nutrients or contains structural molecules that are hard to digest like cellulose or lignin (Schoonhoven *et al.*, 2005; Karban & Baldwin, 2007). Moreover, herbivores have to cope with plant defences ranging from mechanical barriers, such as thorns and trichomes, to the production of poisonous substances (Schoonhoven *et al.*, 2005; Karban & Baldwin, 2007; Mithöfer & Boland, 2012). Not only herbivores, but also microbes have evolved to consume plant material either as (biotrophic or necrotrophic) pathogens or as detritivores. In some cases, herbivores and microbes have established symbioses (i.e. the living together of dissimilar species; (De Bary, 1879) that benefit both partners (mutualistic symbiosis). For instance, some herbivores provide nutrients and shelter to microbes, while in return they make use of the huge metabolic capabilities of these microbes to feed on otherwise unpalatable plants or plant parts, thereby expanding their niche space (Douglas, 2009; Feldhaar, 2011; Engel & Moran, 2013; Hansen & Moran, 2014).

Plant usage by herbivores may be facilitated either by beneficial bacterial symbionts directly, or indirectly via the host plant (Barbosa *et al.*, 1991; Frago *et al.*, 2012; Casteel & Hansen, 2014). For example, direct facilitation may occur via bacteria that upgrade low quality food, by producing essential amino acids or vitamins that the host diet lacks, or by the production of enzymes which enhance the digestion of refractory food sources (Douglas, 2009; Feldhaar, 2011; Engel & Moran, 2013). In contrast, indirect facilitation may occur via interaction of symbiotic bacteria with the plant to benefit their host's fitness (Frago *et al.*, 2012; Hansen & Moran, 2014). For example, bacteria associated with oral secretions, such as that of the Colorado potato beetle (*Leptinotarsa decemlineata*), were shown to alter plant resistance and turn the host plant into better food (Chung *et al.*, 2013).

Not all bacterial symbionts are (always) beneficial for their hosts. In fact, with respect to the effect on host fitness, a symbiosis can span the entire range from beneficial to the

microbe but harmful to the host (parasitism), via a neutral relationship (commensal symbiont) to true mutualism. Moreover, symbiosis can have mixed effects and may change over (evolutionary) time, depending on environmental conditions (Werren *et al.*, 2008; Zug & Hammerstein, 2015). Among arthropods, the most prevalent microbial symbionts are so-called reproductive parasites such as *Wolbachia* (Rickettsiales), "*Candidatus* Cardinium" (Cytophagales) and *Spiroplasma* (Entomoplasmatales) (Duron *et al.*, 2008). Reproductive parasites commonly secure their prevalence in a host population by increasing the proportion of infected females through various mechanisms including cytoplasmic incompatibility, feminization, parthenogenesis or male killing (Werren, 1997; Duron *et al.*, 2008; Werren *et al.*, 2008; Engelstädter & Hurst, 2009). In most cases, these manipulations by the symbiont are not beneficial to the host, yet very effective for the persistence of the symbiont. This was demonstrated by a recent study in which approximately 40% of all terrestrial arthropod species was estimated to be infected with *Wolbachia* (Zug & Hammerstein, 2012). However, direct beneficial effects of reproductive manipulators on host fitness are thought to mediate their spread within populations as well, especially when manipulation of host reproduction is weak (Hoffmann *et al.*, 1998; Fry *et al.*, 2004). Accordingly, evidence has accumulated that reproductive manipulators, which have long been considered parasites, can benefit their hosts or vectors in various ways (Casteel & Hansen, 2014; Sugio *et al.*, 2015; Zug & Hammerstein, 2015). For instance, reproductive manipulators have been shown to protect their host against parasitoids, predators and bacterial or viral pathogens (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Moreira *et al.*, 2009; Jaenike *et al.*, 2010a; Xie *et al.*, 2010; Walker *et al.*, 2011; Hamilton *et al.*, 2014; Xie *et al.*, 2014). Beside protection, *Wolbachia* is known to function as nutritional mutualist in filarial nematodes (Foster *et al.*, 2005), while some examples also exist for arthropod hosts (Brownlie *et al.*, 2009; Hosokawa *et al.*, 2010; Unckless & Jaenike, 2012). Interestingly, infection of arthropods with *Wolbachia* has even been associated with the manipulation of plant physiology (Barr *et al.*, 2010; Kaiser *et al.*, 2010; Body *et al.*, 2013; Robert *et al.*, 2013).

The two-spotted spider mite *Tetranychus urticae* can harbour several (endo)symbiotic bacteria that are known as reproductive manipulators in mites (Breeuwer & Jacobs, 1996; Gotoh *et al.*, 2003; Enigl & Schausberger, 2007; Gotoh *et al.*, 2007). However, the infection status was shown to vary widely among and within spider mite populations (Breeuwer & Jacobs, 1996; Gotoh *et al.*, 2007). *Tetranychus urticae* is a highly polyphagous pest species found on over 1100 plant species worldwide, including economically important crops like tomato, cucumber, strawberry, bean and cotton (Bolland *et al.*, 1998; Grbic *et al.*, 2011;

Migeon *et al.*, 2011). Plants have evolved a wide array of defences, which are organized by the action of several phytohormones in which jasmonic acid (JA) and salicylic acid (SA) are the two central players (Erb *et al.*, 2012; Pieterse *et al.*, 2012). Defence against biotrophic pathogens is orchestrated by SA, while jasmonates, in particular jasmonic acid-isoleucine (JA-Ile), are crucial for defence against herbivores and pathogens with a necrotrophic lifestyle (Kessler *et al.*, 2004; Staswick & Tiryaki, 2004; Glazebrook, 2005). However, like other piercing-sucking arthropods (Walling, 2000), spider mites induce these hormones simultaneously, albeit JA-mediated defences seem to be most important for increased resistance against mites (Li *et al.*, 2002; Ament *et al.*, 2004; Kant *et al.*, 2004; Kant *et al.*, 2008; Zhurov *et al.*, 2014; Alba *et al.*, 2015). Recently, we isolated mites from natural *T. urticae* populations and demonstrated that some of them suppressed JA-mediated defences of tomato to uphold a relatively high reproductive performance on this hostile plant (Alba *et al.*, 2015). One of these suppressor strains, designated as the DeLier-1 strain, was characterized in more detail and shown to significantly reduce JA- and SA-mediated defences that were elicited by the "normal" inducer strain Santpoort-2 (Alba *et al.*, 2015).

Against the background that (endo)symbiotic bacteria can influence host fitness in various ways, we investigated the bacterial communities that are associated with the *T. urticae* plant defence suppressor strain DeLier-1 and the defence inducer strain Santpoort-2, hereafter referred to as the suppressor and the inducer strain, respectively. The suppressor strain was found to contain *Wolbachia* and *Spiroplasma*, while the inducer strain harboured "*Candidatus* Cardinium" and *Spiroplasma*. We subsequently treated both mite strains with antibiotics to kill the bacteria and tested how their presence was correlated with the performance and transcriptome of the mites. Moreover, we investigated induced plant responses in tomato (*Solanum lycopersicum*) by analyzing phytohormone profiles and expression levels of defence-related genes after infestation of the plants with antibiotics-treated and untreated control mites of both strains that differed in their bacterial community. Together, this enabled us to assess to which extent mite endosymbionts impact mites directly or indirectly via the plant.

Material and methods

Plants

Tomato (*S. lycopersicum* cv. Castlemart) and bean (*Phaseolus vulgaris* cv. Speedy) were germinated and grown in a greenhouse (25/18 °C day/night temperature, 16L/8D photoperiod, 50-60% relative humidity [RH]). Experiments involving plants were carried out in a climate

room (default settings: 25 °C, 16L/8D photoperiod, 60% RH, 300 µE m^{-2} s^{-1}), to which plants were transferred seven days in advance.

Spider mites

We used spider mites from the *Tetranychus urticae* strains Santpoort-2 ("inducer") and DeLier-1 ("suppressor"). The Santpoort-2 mites have been described before as inducers of tomato JA- and SA defences, to which they are also susceptible (Kant, 2006; Alba *et al.*, 2015), while DeLier-1 mites suppress these defences (Alba *et al.*, 2015). Spider mites from both strains were reared separately on detached bean leaflets in a climate room. For all plant infestation experiments and mite performance assays, we used age-equilibrated adult females.

I. Bacterial communities of a *Tetranychus urticae* suppressor and inducer strain, in antibiotics-treated and non-treated mite lines

Results from a preliminary assessment of the presence of bacteria in the two mite strains indicated that they harboured different endosymbiotic bacteria. Suppressor mites contained *Wolbachia* sp., while *Candidatus* Cardinium (referred to as *Cardinium*) was identified in inducer mites. In addition, *Spiroplasma* sp. was found in both mite strains (data not shown).

Ia. Antibiotics treatments and nomenclature of mite lines

We treated mites from both strains with antibiotics to remove *Wolbachia*, *Cardinium* and *Spiroplasma* bacteria. In short, offspring from randomly selected mated adult females ("founder mites") was divided over two treatments: (i) antibiotics-treated and (ii) untreated controls. For the antibiotics treatment, adult female spider mites were first kept on bean leaf discs placed on cotton wool soaked with tetracycline hydrochloride (Sigma-Aldrich, St Louis, MO, USA) for 2-3 days, after which they were transferred to new leaf discs on water-saturated cotton wool to produce eggs. Two days later, adult females were individually sampled in Eppendorf tubes and stored at -80 °C until DNA was extracted for diagnostic PCRs to establish the bacterial infection status of the mites (see below). The eggs on the leaf discs were allowed to hatch and mature in a climate room, after which the antibiotics treatment was repeated. In parallel, untreated control mites were kept on leaf discs placed on water-saturated cotton wool and after egg production mites were sampled for diagnostic PCRs as described below. Three subsequent generations of mites were treated in this way, but with increasing concentrations of tetracycline (i.e. 0.15%, 0.20% and 0.30% (v/v)) to obtain mites free of *Wolbachia*, *Cardinium* and *Spiroplasma* as assessed via diagnostic PCR. From

generation 4 onwards, all mites (antibiotics-treated or not) were reared on untreated detached bean leaflets to accommodate larger populations (from here on referred to as “lines”).

During the antibiotics treatment, we kept track of the individual mites and their offspring and only kept those lines (antibiotics-treated versus control) that both originated from the same “founder mite”, i.e. these were “sister lines”. This was done to minimize genetic variation between antibiotics-treated and untreated control lines. Following these criteria, we obtained four lines for the suppressor strain, designated as line 1, 2, 3 and 4. Each of the four lines had three sublines: W+S+ contained both *Wolbachia* and *Spiroplasma*; W-S+ was free of *Wolbachia,* but contained *Spiroplasma,* W-S- was free of *Wolbachia* and *Spiroplasma*. We did not manage to obtain W+S- sublines. For the inducer strain, we obtained four lines as well, which were designated as lines 5, 6, 7 and 8. Each of the four lines had two sublines: C+S+ contained *Cardinium* and *Spiroplasma,* and C-S- was free of *Cardinium* and *Spiroplasma*. Sublines with the same respective bacteria will be referred to as “groups”. In the suppressor strain the groups were W+S+, W-S+ and W-S-, in the inducer strain the groups were C+S+ and C-S (see Figure 5.1 and Table 5.1 for an overview of the mite lines and their bacterial communities). Mites from each strain and subline were regularly checked for their bacterial infection status by diagnostic PCR and kept on untreated detached bean leaflets for approximately 15 generations before they were used for the plant infestation assay and mite fecundity tests.

Ib. Illumina sequencing

To assess the presence of *Wolbachia, Cardinium, Spiroplasma* and other potentially present bacteria in mites from the five groups (W-S-, W-S+, W+S+, C-S- and C+S+) that were used for the plant infestation assay, we sampled five tomato-habituated mites per subline (as described above) for Illumina sequencing. DNA was extracted from single mites using a fast Chelex method modified from Breeuwer and Jacobs (1996). To isolate the DNA, a single mite was ground and homogenized in 100 µl sterile 5% (w/v) Chelex (Sigma-Aldrich) with a sterile pestle, after which 2.5 µl proteinase K (20 mg/ml, Sigma-Aldrich) was added. Samples were then incubated at 56 °C for 1 hour, followed by incubation at 95 °C for 8 min to complete the DNA extraction. DNA from the five mites from the same subline was pooled to form one sample. DNA concentration was adjusted to 25-35 ng/µl per sample. In total, 20 (pooled) samples were sent for sequencing, one for each subline. Amplification and sequencing of the 16S rRNA gene fragment was done by LGC Genomics (Berlin, Germany) using an Illumina MiSeq sequencer (2 x 250 bp paired-end reads; Illumina, San Diego, CA, USA) and the

universal primers 341F and 785R (modified from Klindworth *et al.*, 2012, see Table S5.1). Since the Chelex method does not yield highly pure DNA, the initial PCR amplification of the 16S rRNA genetic region was done on 20 times diluted DNA. Furthermore, the PCR was run with 35 instead of the usual 30 cycles.

Sequences were provided as adapter clipped FASTQ files and analysed in QIIME (quantitative insights into microbial ecology), which is a standard pipeline for microbial community analysis (Caporaso *et al.*, 2010a). First, forward and reverse reads were joined with the join_paired_ends.py algorithm. Joined sequences were quality filtered, applying a Phred threshold of 20. Subsequently, sequences were clustered into operational taxonomic units (OTUs) with the open reference OTU picking command, applying the uclust algorithm (Edgar, 2010) and 97 % similarity cut-offs. First, sequences were clustered against the reference Greengenes 16S rRNA gene database (http://greengenes.lbl.gov/; (DeSantis *et al.*, 2006)). Sequences that did not cluster with the reference sequences were clustered *de novo*. The most abundant sequence from each OTU cluster was taken as representative sequence. Representative sequences were aligned with PyNAST, using the Greengenes core set as a template (Caporaso *et al.*, 2010b). PyNAST-aligned sequences were checked for chimeras with Chimera Slayer (Haas *et al.*, 2011). Identified chimeras were removed from *de novo* clustered sequences for downstream analysis. Taxonomy was assigned to the representative sequences using the uclust consensus taxonomy classifier (Edgar, 2010). The resulting OTU table was manually edited; global singletons and sequences identified as chloroplast and mitochondrial DNA were removed from the dataset. For the graph (Figure 5.1), we show OTUs that were present at > 0.5% in at least one of the sublines.

Ic. Diagnostic PCRs on mites

To verify the presence or absence of the most common endosymbionts in mites, viz. *Wolbachia*, *Cardinium* and *Spiroplasma* (Breeuwer & Jacobs, 1996; Gotoh *et al.*, 2003; Enigl & Schausberger, 2007; Gotoh *et al.*, 2007), we performed diagnostic PCRs on DNA extracted from spider mites using genus-specific bacterial primers (Table S5.1). Adult female mites, by default not surface-sterilized, were sampled in Eppendorf tubes and their DNA was extracted using the fast Chelex method as described above. Samples were stored at 4 °C until they were used for PCR.

One µl of the mite-derived DNA solution (approximately 30-40 ng of DNA) was used as template in a 10.5 µl PCR reaction, further containing 2 µl 5x Phire Hot Start Buffer (Thermo Fisher Scientific, Waltham, MA, USA), 2 µl dNTPs (1 mM), 0.5 µl of each primer

(10 µM each) and 0.1 µl Phire Hot Start DNA Polymerase (Thermo Fisher Scientific). PCR amplification of *T. urticae actin* was used as a positive control for DNA quality. The PCRs were run on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following cycling conditions; an initial denaturation for 30 s at 98 °C, followed by 32 cycles of 5 s denaturation at 98 °C, 5 s primer annealing at 52 °C (for *Wolbachia* and *Spiroplasma*), 57 °C (for *Cardinium*), or 58 °C (for *T. urticae actin*) and 10 s elongation at 72 °C; finalized with a 1 min elongation step at 72 °C. PCR-generated amplicons were analysed by agarose-gel electrophoresis. Primer specificity was confirmed by Sanger sequencing of the amplicons.

II. Effects of *Wolbachia, Cardinium* and *Spiroplasma* on spider mites

IIa. Spider mite performance assay

To establish whether (endo)symbionts had an effect on mite performance, we assessed spider mite fecundity and survival on wild type tomato plants. For the experiment, an "egg-wave" (see Alba *et al*., 2015) was generated by allowing random adult females from each strain to produce eggs on the adaxial surface of detached bean leaflets, which had been put flat on wet cotton wool. After 48 hours of egg production, all mites were removed from the leaflets and collected in Eppendorf tubes (25-35 mites from the same subline were pooled), flash-frozen in liquid nitrogen and stored at -80 °C, until their DNA was extracted for diagnostic PCRs. The eggs were allowed to hatch and mature in a climate room for another 9 days. The bean leaflets with mites were then transferred to leaves of 21-day-old tomato plants to habituate the mites to tomato. Three days later, the then 1 ± 1-day-old adult female mites were collected from the tomato leaves and transferred to "new" 21-day-old tomato plants for the mite performance assay. Plants were infested with 5 mites per leaflet; 3 leaflets per plant; 3-6 plants per treatment. A lanolin (Sigma-Aldrich) barrier was made around the petiolule to prevent the mites from escaping. After 4 days, the number of eggs produced by the mites, as well as the number of alive, dead and missing (i.e. migrated) mites, was recorded using a stereo microscope (Leica MZ6, Leica Microsystems, Wetzlar, Germany). This experiment was repeated 2-3 times for all four lines of the suppressor strain. However, for the inducer strain, populations from the C-S- subline of line 6, as well as both sublines of line 7 went (for yet unknown reasons) extinct before we could complete the performance assays. Data from the C+S+ subline of line 6 was included for analysis.

Statistical analysis of spider mite performance assay

To test the effect of *Wolbachia*, *Cardinium* and/or *Spiroplasma* on mite oviposition, we constructed one linear mixed effect models (LMM) for each mite strain (suppressor and inducer) in the lme4 package (Bates *et al.*, 2013), using either "average number of eggs per number of females that were originally put on the leaves" (5 mites), or "the average number of eggs per number of females that survived until the end of the experiment" as response variable. To test effects of bacteria on survival of the mites, we used generalized linear mixed models in the lme4 package using a binomial distribution to analyse the proportion of mites that were dead or alive at the end of the oviposition experiment (i.e. after 4 days). In the models of oviposition and survival, we used "bacterial group" as explanatory variable and "line" was added as random effect. Additionally, since experiments were spread over different experimental days, and in total 6-9 plants were used per line (with three leaflets per plant), we added a nested random effect with "leaflet" nested in "plant", nested in "day" (1|day/plant/leaflet) to the model. Pairwise comparisons for suppressor strain were done using Tukey contrasts in the multicomp package (Hothorn *et al.*, 2008) and applying Holm adjustments to account for multiple comparisons. All analyses were performed using the statistical software R 3.0.2 (R Core Team, 2013).

IIb. Spider mite RNA isolation and microarray analysis

To determine if the presence of *Wolbachia*, *Cardinium* and/or *Spiroplasma* in the mites is associated with gene expression of their host, we isolated RNA from the same mites that were used for the plant infestation assay (see below) and analysed it by means of a microarray. After 7 days of tomato infestations, mites were sampled, as described below in the "plant infestation assay" section. All mites from the same plant (maximum 3 x 15 = 45; dead and alive) were pooled to form one biological replicate. Total RNA was isolated from the pooled spider mites using the Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA), according to the instructions of the manufacturer. RNA integrity was checked by agarose-gel electrophoresis and a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific) was used to assess RNA purity and quantity.

For the microarray hybridizations, equal amounts of RNA (400 ng), derived from the five biological replicates of each subline, were pooled to form one sample. In total there were twelve samples for the suppressor strain (4 lines x 3 sublines) and eight samples for the inducer strain (4 lines x 2 sublines), which were hybridized separately on two arrays, i.e. one with suppressor strain samples, the other with inducer strain samples.

The RNA integrity of each sample was verified with the 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA) using the RNA ScreenTape (Agilent Technologies). The amount of RNA per µl was measured on a NanoDrop spectrophotometer (ND-2000). Per sample, 100 ng of total RNA, combined with Spike A, was amplified and labeled according to the Agilent Two-Colour Microarray-Based Gene Expression Analysis guide version 6.6 (G4140-90050, Agilent Technologies) using the Low Input Quick Amp Labeling Kit (Agilent Technologies). For the common reference, an equimolar pool of all samples was made and 100 ng samples were amplified similarly as the test samples with the exception that Spike B was used. Synthesized antisense RNA (aRNA) was purified with the E.Z.N.A. MicroElute RNA Clean Up Kit (Omega Bio-Tek, Norcross, GA, USA). The NanoDrop ND-2000 was used to assess aRNA quantity and CyDye incorporation.

Each hybridization mixture was made up from 1.1 µg Test (Cy3) and 1.1 µg Reference (Cy5) sample. Samples were dried and 1.98 µl water was added. The hybridization cocktail was made according to the manufacturer's instructions (NimbleGen Arrays User's Guide - Gene Expression Arrays Version 5.0, Roche NimbleGen, Basel, Switzerland) and 7.2 µl of this mix was added to each sample. The samples were incubated for 5 min at 65 °C and 5 min at 42 °C prior to loading onto a 12x135K microarray (Roche NimbleGen), custom designed as described in the next paragraph (Kant *et al.*, 2004; Kant *et al.*, 2008; Alba *et al.*, 2015). Microarrays were hybridized for 20 hours at 42 °C with the NimbleGen Hybridization System (Roche NimbleGen). Afterwards, the slides were washed according to the NimbleGen Arrays User's Guide (Gene Expression Arrays Version 6.0, Roche NimbleGen) and scanned with an Agilent DNA microarray scanner G2565CA (Agilent Technologies). Feature extraction was performed with NimbleScan v2.6 (Roche NimbleGen).

Microarrays were designed using normalized transcriptomes of *T. urticae* strains Houten-1 ("KOP"; Kant *et al.*, 2004), Santpoort-1 ("KMT"; Kant *et al.*, 2008) and DeLier-1 (Alba *et al.*, 2015). Transcriptomes had been prepared from a normalized shotgun cDNA library via random priming (Houten-1) and three normalized 3-prime libraries cDNA (Houten-1, Santpoort-1 and DeLier-1) via 5' sequencing of poly A-selected fragments of 600-800 nts long via 454 Titanium sequencing (Eurofins, Germany) delivering 1,416,647 reads in total with an average length of 330 nts. For the microarray probe design, we assembled only the 744,649 3' fragment reads, because these will contain more cDNA-specific sequence information than the shotgun reads. These fragment reads were assembled into 16,372 isotigs (with an average length of 571 nts; N50 = 597 nts), using Newbler (using a 99% identity setting and a minimum of 40 nts overlap), from which we could extract 14,335 unique 60-mer

probes. After assembly, 167,643 3' reads remained as singleton (i.e. reads that could not be assembled), from which another 120,000 unique 60-mer probes were extracted. Together, these were printed in 12-fold on a 12 x 135k array in Nimblegen format.

Probe sequences were (re)mapped to the latest annotation of the *T. urticae* genome (December 2014) using Bowtie2-2.10 with the "very sensitive" preset option (Langmead *et al.*, 2009). Of the 134,672 probes on each array, 30,759 aligned with the *T. urticae* genome (Grbic *et al.*, 2011), representing 9,424 (51%) of the 18,414 *in silico* predicted protein-coding sequences. Prior to gene expression analysis, signal intensity data were $\log_2$-transformed and normalized (Loess and Aquantile). The biological replication within each mite strain and its cured sublines were assessed by a PCA-analysis, using the prcomp function in R. The first two principal components, which explained a cumulative proportion of the total variance of 65.6% and 32.6% for the inducer and suppressor strain, respectively, were used to explore the data and to identify outliers. Next, a linear model of a non-connected design was fitted to the processed data, using limma (Smyth, 2004; Smyth & Altman, 2013). An empirical Bayes approach (Smyth, 2004) was applied to assess relative transcript levels and the associated *P*-values between different treatments. When multiple probes aligned to the same mite gene, the expression values of the individual probes were averaged. To control for false discovery rate, obtained *P*-values were adjusted for multiple testing according to Benjamini and Hochberg's step-up procedure (FDR; Benjamini & Hochberg, 1995). A transcription heat map was constructed, using the relative gene-expression levels obtained in limma (bioproject website). All analyses, except for the genome alignment with Bowtie2-2.10 (Linux), were performed using the statistical software R 3.0.2 (R Core Team, 2013). Biological functions were ascribed to the DEG lists as described in Wybouw *et al.* (2015).

III. Effects of spider-mite associated *Wolbachia, Cardinium* and *Spiroplasma* on mite-plant interactions

IIIa. Plant infestation assay

To measure phytohormone levels and plant defence gene expression upon mite-inflicted feeding damage, tomato plants were infested with spider mites, as described before (Alba *et al.*, 2015), with an additional habituation step (of 2 days) on tomato to minimize possible effects of the previous diet (i.e. bean) on mite behaviour, -performance, and/or induced/suppressed tomato defences. Dietary effects are known to persist for at least 48 hours, after which they diminish rapidly (Storms, 1971). For the experiment, we used age-equilibrated adult female spider mites obtained from an egg-wave. The eggs were allowed to

hatch and mature in a climate room for another 12 days. The bean leaflets with mites were then taken from the cotton wool and placed upside-down on leaves of 28-day-old tomato plants to infest these (i.e. habituation step). Two days later, the 3 ± 1-day-old adult female mites were collected and transferred to 21-day-old tomato plants for the plant infestation assay, according to our standard infestation protocol (Alba *et al.*, 2015); 15 mites per leaflet, 3 leaflets per plant. To prevent the mites from escaping, a lanolin (Sigma-Aldrich) barrier was made around the petiolule, which was also applied to uninfested control leaflets. A total of 5 plants was infested per mite subline. To verify the bacterial infection status of each strain, 5 tomato-habituated mites per strain were individually collected in Eppendorf tubes, flash-frozen in liquid nitrogen and stored at -80 °C until DNA was extracted for PCR amplification and subsequent Illumina sequencing of the 16S rRNA genetic region (see "Illumina sequencing" section).

At 7 days post-infestation (dpi), mites and tomato leaflets were harvested separately. First, spider mites were removed from the leaflets and collected in Eppendorf tubes (all mites obtained from the same plant were pooled), flash-frozen in liquid nitrogen and stored at -80 °C until their RNA was extracted for microarray analysis (see "Spider mite RNA isolation and microarray analysis" section). A vacuum pump, sterile 1 ml pipet tip, and mite-proof gauze were used to quickly sample the mites without touching and hence mechanically damaging the leaflets. Subsequently, the mite-cleared leaflets were excised without the petiolule. The 3 detached leaflets obtained from the same plant, along with a scale marker, were then aligned on black paper, gently covered with a thin glass plate to flatten them out, and photographed with a Canon EOS 300D DSLR camera (Canon, Tokyo, Japan) equipped with a Canon EF-S 18-55 mm lens to enable *in silico* calculation of spider mite-inflicted feeding damage, using Adobe Photoshop CS6 Extended (Adobe Systems, San Jose, CA, USA) as described by Kant *et al*. (2004). Finally, the leaflets were flash-frozen in liquid nitrogen and stored at -80 °C until we extracted their phytohormones and RNA. The 3 leaflets obtained from the same plant were pooled to form 1 biological replicate. In total, it took less than 5 min per plant to complete these 3 steps and harvest the leaflets. Care was taken to not damage them. Except for removal of the mites, uninfested control leaflets were processed in the same way.

IIIb. Isolation of phytohormones and analysis by means of LC-MS/MS

Per sample, 200-300 mg of frozen leaf material was homogenized (Precellys 24, Bertin Technologies, Aix-en-Provence, France) in 1 ml of ethyl acetate which had been spiked with

D_6-SA and D_5-JA (C/D/N Isotopes,Pointe-Claire, Quebec, Canada) as internal standards with a final concentration of 100 ng ml^{-1}. Tubes were centrifuged at 13,000 rpm (15,493 x *g*; Sigma 3-30KS; SIGMA Laborzentrifugen GmbH, Osterode am Harz, Germany) for 10 min at 4 °C and the supernatant (the ethyl acetate phase) was transferred to new tubes. The pellet was re-extracted with 0.5 ml of ethyl acetate (without internal standards) and centrifuged again at 13,000 rpm for 10 min at 4 °C. Both supernatants were combined and then evaporated to dryness on a vacuum concentrator (CentriVap Centrifugal Concentrator, Labconco, Kansas City, MO, USA) at 30^{o}C. The residue was re-suspended in 0.1 ml of 70% methanol (v/v), centrifuged at 14,800 rpm (20,081 x *g*) for 15 min at 4 °C, and the supernatants were transferred to glass vials and then analysed using a liquid chromatography tandem mass spectrometry (LC-MS/MS) system (Varian 320-MS LC/MS, Agilent Technologies). A serial dilution of pure standards of abscisic acid (ABA), traumatic acids, 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA), jasmonic acid-isoleucine (JA-Ile) and salicylic acid (SA) was run separately. We injected 10 µl of each sample onto a Kinetix 5u C18 100A column (C18 phase, 5 µm particle size, 100Å pore size, 50 × 2.1 mm; Phenomenex, Torrance, CA, USA) equipped with a Phenex-RC guard cartridge (Phenomenex). The mobile phase comprised of solvent A (0.05% formic acid in LC-MS-grade water; Sigma-Aldrich) and solvent B (0.05% formic acid in LC-MS-grade methanol; Sigma-Aldrich). The program, with a constant flow rate of 0.2 ml min^{-1}, was set as follows: (i) 95% solvent A/5% solvent B for 1 min 30 s; (ii) followed by 6 min in which solvent B gradually increased till 98%; (iii) continuing with 98% solvent B for 5 min; (iv) then a rapid (in 1 min) but gradual decrease returning to 95% solvent A/5% solvent B until the end of the run. A negative electrospray ionization mode was used for detection. LC-MS/MS parameters, e.g. analysed compounds, their parent ions, daughter ions, and collision energies used in these analyses, are listed in Table S5.1. Figure S5.1 shows a comprehensive overview of the most important compounds and their "position" within the plant JA and SA defence response pathways induced by *T. urticae*. For all oxylipins and ABA, we used D_5-JA to estimate the recovery rate and their *in planta* concentrations were subsequently quantified using the external standard series. For SA we used D_6-SA to estimate the recovery rate and it was quantified using the external standard. Phytohormone amounts were expressed as ng per gram fresh mass leaf material (ng/g fresh weight). Compounds for which a pure standard was not available (i.e. 13(*S*)-hydroperoxyoctadecatrienoic acid (13-HPOT), 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid (OPC-8), 3-oxo-2-(2-pentenyl)-cyclopentane-1-hexanoic acid (OPC-6) and C_{12}-derivatives of the hydroperoxide lyase (HPL)

pathway) were analysed similarly, but their amounts were expressed as "Ion Count" per gram fresh mass leaf material (IC/g fresh weight).

IIIc. Gene expression analysis by quantitative reverse-transcriptase PCR (qRT-PCR)

To determine the effect of mite-associated bacteria on defence gene expression, we performed qRT-PCRs on plant defence marker genes. Therefore, total RNA was isolated from the tomato leaf tissue that was used for phytohormone isolation, using the hot phenol method (Verwoerd *et al.*, 1989). RNA integrity was checked by agarose-gel electrophoresis and a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific) was used to assess RNA purity and quantity. Per sample, 3 µg DNase (Ambion, Austin, TX, USA)-treated RNA was used as template for reverse transcription and first strand cDNA synthesis using RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific). For gene expression analysis, 1 µl of 10-times diluted cDNA (i.e. the equivalent of 7.5 ng total RNA) served as template in a 20 µl qRT-PCR using the 5x HOT FIREPol EvaGreen qPCR Mix Plus (ROX) kit (Solis Biodyne, Tartu, Estonia) and the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), according to the instructions of the manufacturers. We monitored expression of genes involved in JA biosynthesis: *Allene Oxide Synthase 1* (*AOS1*) (Howe *et al.*, 2000; Sivasankar *et al.*, 2000) and *OPDA reductase 3* (*OPR3*) (Strassner *et al.*, 2002), as well as JA-defence marker genes; *Jasmonate-inducible Protein-21* (*JIP-21*) (Lisón *et al.*, 2006), *Threonine Deaminase-2* (*TD-2*) (Gonzales-Vigil *et al.*, 2011), *Proteinase Inhibitor IIc* (*PI-IIc*) (Gadea *et al.*, 1996), SA-defence marker genes; *Pathogenesis-related protein 1a* (*PR-1a*) (Tornero *et al.*, 1997), *PR-P6* (van Kan *et al.*, 1992), and finally putative OPDA-responsive genes; *Tomato Wound-induced 1* (*TWI-1*) (Truesdale *et al.*, 1996), *Alcohol Dehydrogenase* (*ADH*) (Tieman *et al.*, 2007), an uncharacterized *Gluthathione S-transferase* ("*GST6*") (Solyc06g009020.2) and an uncharacterized *Glutaredoxin* ("*GRX*") (Solyc07g053550.1). See Figure S5.1 for a schematic overview of the plant defence response against mites, which includes the marker genes whose expression levels were analysed in this study. The amino acid sequences of established *Arabidopsis thaliana* OPDA-responsive genes (ORGs; (Taki *et al.*, 2005; Mueller *et al.*, 2008; Park *et al.*, 2013)) were used to identify their putative tomato homologs: At2g15480 (*AtUGT73B5*) for *SlTWI-1*, At1g09500 (*AtCAD*) for *SlADH*, At2g47730 (*AtGST6*) for *SlGST6*, and At1g28480 (*AtGRX480*) for *SlGRX*. *Actin* was used as a reference gene to normalize expression data and hence correct for variance in quantity of cDNA input. Standard dilution series of selected samples were included with each qRT-PCR run to calculate primer efficiency. PCR-generated amplicons were sequenced to verify primer

specificity. Gene identifiers, primer sequences and references are listed in Table S5.2. The normalized expression (NE) data were calculated by the ΔCt method as described before (Alba *et al.*, 2015): NE = $(PE_{target}{}^{Ct\_target})/(PE_{reference}{}^{Ct\_reference})$, in which PE is the primer efficiency and Ct the number of cycles to reach the cycle threshold value.

Statistical analysis of the plant infestation assay data (phytohormones, qRT-PCR, feeding damage)

To test the effect of bacteria on phytohormone levels, tomato gene expression and amount of spider mite-inflicted feeding damage, we constructed LMM using the package lme4 (Bates *et al.*, 2013). The respective amounts of phytohormones (ng/g FW or IC/g FW), normalized gene expression (NE) or total amount of feeding damage (mm^2) for three leaflets of one plant combined were used as response variable, while "presence of bacteria" (i.e. W/S/C) was used as explanatory variable. To test the level of induction or suppression as compared to uninfested control plants, in the case of phytohormones and gene expression this explanatory variable also included control plants that were not infested. Since we had four lines which were present as sublines in all mite-bacteria groups, we added "line" as a random factor to the model to account for variation between the lines. The response variables were transformed using log, sqrt or 1/sqrt if applicable for meeting the assumptions of homogeneity of variance and normality of residuals required for LMM. For pairwise comparisons, we used Tukey contrasts with Holm adjustment for multiple comparisons in the multcomp package (Hothorn *et al.*, 2008). To assess how the phytohormones SA and ODPA correlated with expression levels of putative ORGs (*TWI-1*, *GRX*, *GST-6*, *ADH* and *OPR3*), we calculated linear correlations between phytohormone amounts and the NE level of these genes using the R package Hmisc 3.15 (Harrel *et al.*, 2015). Further, we calculated linear correlations between SA amounts and NE levels of the SA marker genes *PR-P6* and *PR-1a*. *P*-values of correlations were adjusted for multiple testing, using the Holm method. All analyses were performed using the statistical software R 3.0.2 (R Core Team, 2013).

Results

I. Bacterial communities of a *Tetranychus urticae* suppressor and inducer strain, in antibiotics-treated and non-treated mite lines

Adult female mites, obtained from laboratory populations of either the *T. urticae* suppressor or *T. urticae* inducer strain (Alba *et al.*, 2015), harboured different bacterial communities, as was determined by Illumina sequencing of the 16S rRNA derived PCR products (Figure 5.1,

Table 5.1). Most evidently, in the suppressor strain a high percentage of the reads corresponded with the endosymbiotic bacterium *Wolbachia* sp. (W) (Rickettsiaceae), with an average of 30.41% (± 13.58 SD). *Wolbachia* was identified in the inducer strain as well, albeit at low relative levels with an average of 0.25% (± 0.32 SD). In contrast, in the inducer strain a high percentage of reads corresponded to the endosymbiotic bacterium "*Candidatus* Cardinium" (C) (Bacteroidaceae), with an average of 29.05% (± 8.72 SD). *Cardinium* was also found in the suppressor strain, but at low relative levels with an average of 0.0032% (± 0.0037 SD). In addition, the same *Spiroplasma* sp. (S) (Spiroplasmataceae) OTU was present in similar relative amounts in both mite strains: an average of 4.03% (± 1.06 SD) in the suppressor mites and 4.42% (± 2.67 SD) in inducer mites. Therefore, the suppressor strain was classified as W+S+ and the inducer strain as C+S+.

To assess the effect(s) of the bacteria on mite performance, transcriptome and induced plant responses, mites from both strains were treated with antibiotics (tetracycline) as described above. The antibiotics treatments successfully cleared *Wolbachia* from the suppressor mites, as only very few (0-6) reads were detected per line in the W-S+ and W-S- groups (Figure 5.1, Table 5.1). *Spiroplasma* was completely removed in the latter group, but was relatively more abundant in the W-S+ group than in the W+S+ one, i.e. on average at 7.98% (± 1.71 SD). The antibiotics treatment was also successful in the inducer mites (Figure 5.1). Only a small fraction of the reads (< 0.04%) recovered from tetracycline-treated C-S- mites corresponded to *Cardinium* or *Spiroplasma* (Table 5.1). Moreover, presence of *Wolbachia*, *Spiroplasma* or *Cardinium* was no longer detectable in the antibiotics-treated sublines by means of PCR, using bacteria-specific primers on DNA of individual mites, which was also used for Illumina sequencing (data not shown).

Besides *Wolbachia*, *Cardinium* and *Spiroplasma*, bacteria from the families Enterobacteriaceae (Enterobacteriales) and Pseudomonadaceae (Pseudomonadales) were also present in varying amounts in all groups and sublines of both mite strains (Figure 5.1). Other bacterial families reached high relative abundances in some of the sublines, e.g. two different Oxalobacteraceae (Burkholderiales), one in line 5 subline C-S-, the other line 6 subline C+S+ and line 2 subline W+S+; Sphingobacteriaceae (Sphingobacteriales) in line 2 subline W+S+, or Nocardiaceae in line 4 subline W-S+. Taken together, this demonstrates that we cleared the mites from *Wolbachia*, *Spiroplasma* or *Cardinium*, but not from all bacteria residing in or on the mites, possibly because we had not surface-sterilized mites before assaying them. Therefore, we only focused on the consistent presence/absence of *Wolbachia*, *Spiroplasma* or

Cardinium in all our analyses, to which we refer when we use the terms “bacteria” or “mite-associated bacteria”.

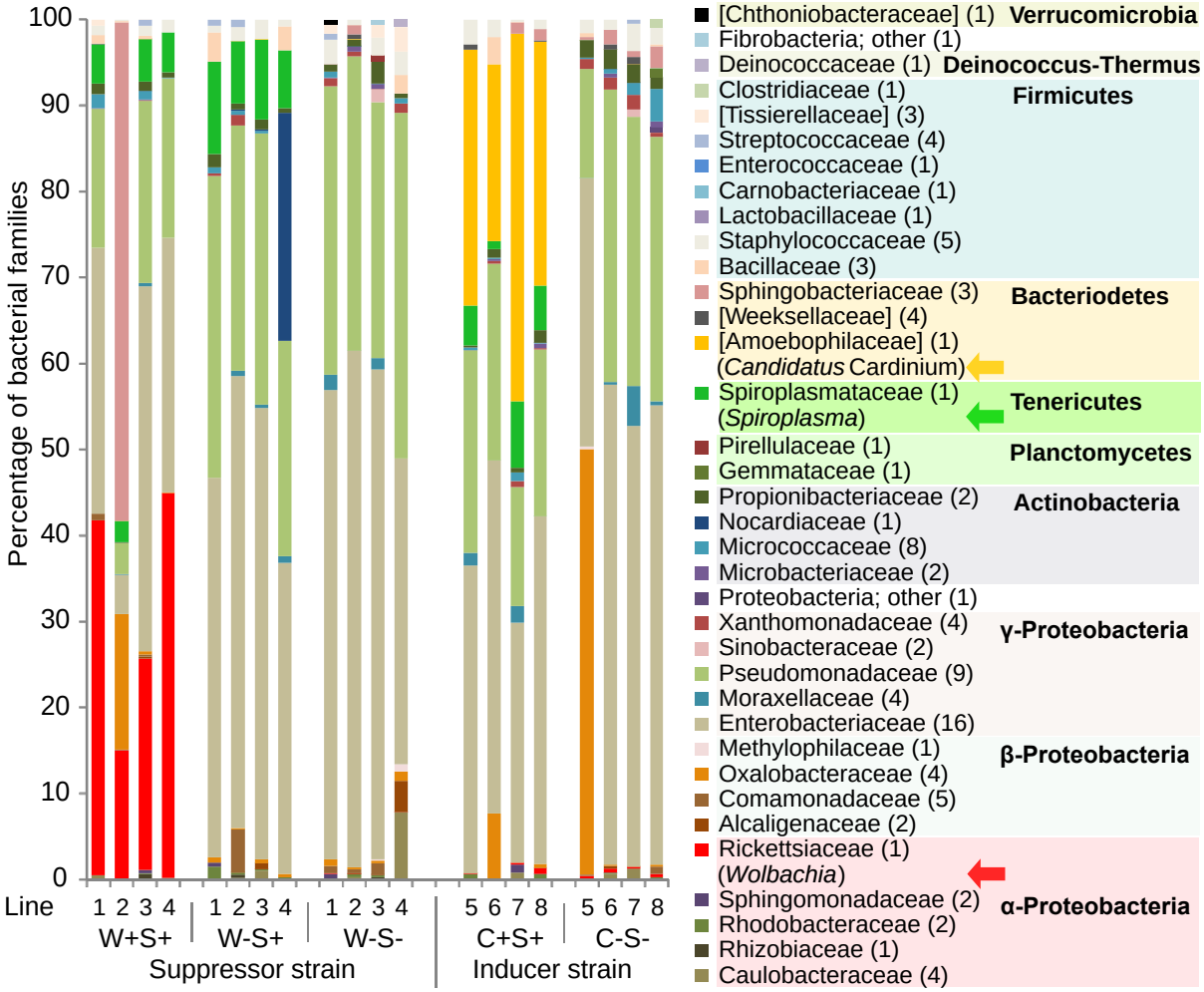


Figure 5.1. Bacterial community composition derived from Illumina 16S rRNA amplicon-sequencingin two strains of the spider mite *Tetranychus urticae* which had been treated with tetracycline and hence did (+) or did not (-) contain *Wolbachia* (W), *Spiroplasma* (S) and/or *Candidatus* Cardinium (C); Plant defence suppressor strain DeLier-1 with three mite groups: W+S+, W-S+ and W-S-; Plant defence inducer strain Santpoort-2 with two mite groups: C+S+ and C-S-; 4 lines per group. Bacterial operational taxonomic units (OTUs) were combined at the family level. Numbers behind family names indicate how many OTUs of one family were combined. Rare OTUs that were overall represented less than 0.5% are not shown; square brackets around taxa indicate that the taxon name is not fully established yet.

Table 5.1. Antibiotics treatments of two strains of the spider mite *Tetranychus urticae* resulted in the (near) complete removal of their associated bacteria *Wolbachia*, *Spiroplasma* and/or *Candidatus* Cardinium, as demonstrated by Illumina 16S rRNA amplicon-sequencing. The *T. urticae* plant defence suppressor strain DeLier-1 contained *Wolbachia* and *Spiroplasma* (W+S+) and tetracycline treatments either removed only *Wolbachia* or both bacteria, yielding the groups W-S+ and W-S-, respectively. The plant defence inducer strain Santpoort-2 harboured *Cardinium* and *Spiroplasma* (C+S+), which were both removed by the tetracycline treatments, yielding the group of C-S- mites. Each group is represented by four independent mite lines. Note that mites from lines with the same number are "sister lines" which originate from the same untreated "founder mite", hence each DeLier-1 line consists of three sublines (W+S+, W-S+, W-S-) and each Santpoort-2 line consists of two sublines (C+S+ and C-S-). Shown are the overall total number of Illumina reads obtained per subline, as well as the total number of reads corresponding to all 11 *Wolbachia* operational taxonomic units (OTUs), all 3 *Spiroplasma* OTUs and the only *Cardinium* OTU identified. The 16S rRNA amplification was done with universal 341F and 785R primers, modified from {Klindworth, 2012 #191}, see Table S5.2.

Mite strain	**Suppressor strain**												**Inducer strain**							
Group	**W+S+**				**W-S+**				**W-S-**				**C+S+**				**C-S-**			
Line	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**5**	**6**	**7**	**8**
Total	3,164	14,303	34,118	19,747	4,371	16,637	27,939	8,812	4,975	20,054	10,241	8,906	26,384	18,367	48,325	28,966	39,276	22,648	30,608	18,702
Wolbachia	1,272	2,131	7,940	8,546	1	-	6	1	3	4	2	1	7	5	108	204	100	110	66	63
Spiroplasma	141	350	1,605	890	440	1,120	2,422	567	-	-	-	-	1,159	165	3,555	1,467	1	1	-	6
Cardinium	-	1	2	-	-	1	2	-	-	1	-	-	7,443	3,643	19,744	7,904	2	1	3	-

II. Effects of *Wolbachia, Cardinium* and *Spiroplasma* on spider mites

IIa. Effects of *Wolbachia, Cardinium* and *Spiroplasma* on spider mite performance

To assess if the mite-associated bacteria were correlated with the fitness of their host, we determined the number of eggs and survival of adult female mites by performing oviposition assays on tomato. For the suppressor mites, we found an overall significant effect of the factor "bacterial group" (i.e. presence/absence of *Wolbachia* and/or *Spiroplasma*) on mite survival (Figure 5.2a). More W+S+ mites survived until the end of the experiment (4 days) compared to both W-S+ and W-S- mites, while survival did not differ between the latter two groups, with the exception of line 2 (Figure S5.2a). There was no statistical difference in number of eggs between the mites from each group when we calculated the total number of eggs produced per number of females that were initially put on each leaflet (5 mites), (Figure 5.3a). This suggests that the fewer W-S+ and W-S- mites that did survive produced more eggs. Hence, we determined the total number of eggs produced per female that survived, and here the factor "bacterial group" had an overall significant effect on number of eggs ($F_{2,207}$ = 3.14, P = 0.045) (data not shown in graph). However, *post hoc* tests with Holm correction for multiple testing indicated no significant difference between surviving W+S+ mites laying fewer eggs than W-S+ (z = -2.00, P = 0.09) and W-S- (z = -2.30, P = 0.06).

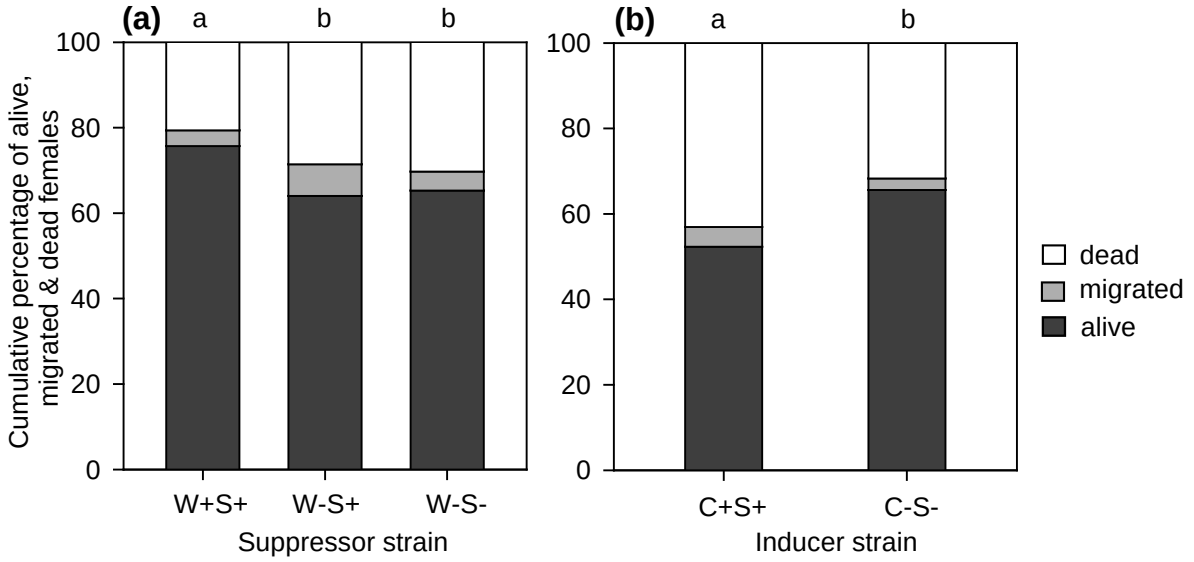


Figure 5.2. Survival, migration and mortality in two strains of the spider mite *Tetranychus urticae* which did (+) or did not (-) contain *Wolbachia* (W), *Spiroplasma* (S) and/or *Candidatus* Cardinium (C); (a) Plant defence suppressor strain Delier-1 with three mite groups: W+S+, W-S+ and W-S-. (b) Plant defence inducer strain Santpoort-2 with two mite groups: C+S+ and C-S-. Different letters above the bars indicate significant differences at a level of $P \leq 0.05$, after applying a generalized linear mixed model followed by Tukey multiple comparisons with Holm adjustment. n.s. = not significant

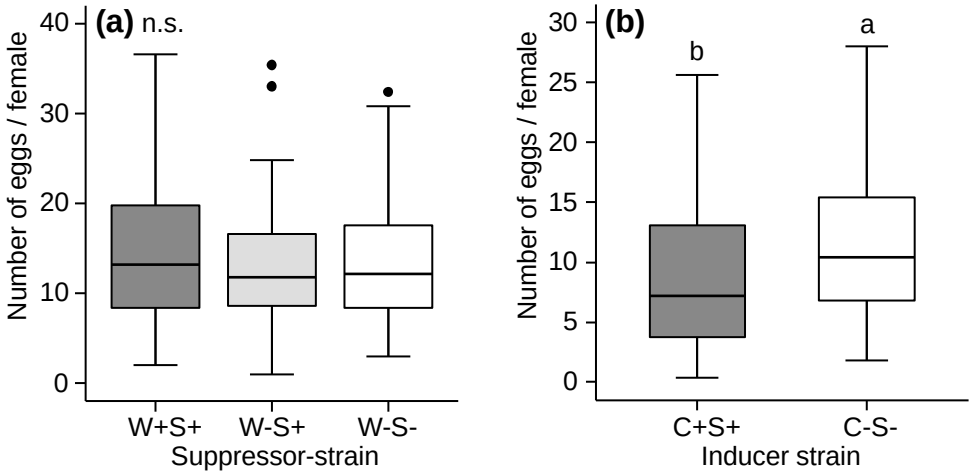


Figure 5.3. Reproductive performance (number of eggs produced per female in four days) in two strains of the spider mite *Tetranychus urticae* which did (+) or did not (-) contain *Wolbachia* (W), *Spiroplasma* (S) and/or *Candidatus* Cardinium (C); (a) plant defence suppressor strain Delier-1 with three mite groups: W+S+, W-S+ and W-S-. (b) plant defence inducer strain Santpoort-2 with two mite groups: C+S+ and C-S-. Boxes span the 25-75 percentiles, horizontal lines in the boxes represent medians, whiskers span 1.5*IQR (interquartile range), dots represent data points outside of this range. Different letters above the boxes indicate significant differences at a level of $P \leq 0.05$, after applying a linear mixed model followed by Tukey multiple comparisons with Holm adjustment. n.s. = not significant

Between the two groups of the inducer strain, significantly more mites of the C-S- group generally survived until the end of the experiment (Figure 5.2b), but this was not consistent among the lines (Figure S5.2b). The number of eggs between C+S+ and C-S- mites was also significantly different: overall, C-S- mites produced more eggs than C+S+ mites (Figure 5.3b). This difference was significant for line 5 ($F_{1,29} = 14.71$, $P < 0.001$), but not for line 8 ($F_{1,32} = 1.21$, $P = 0.27$), although both lines followed the same trend (Figure S5.3). The oviposition calculated per surviving mite did not differ between the C+S+ and C-S- groups ($F_{1,76} = 3.34$, $P = 0.07$) (data not shown in graph), possibly due to the lower survival of C+S+ mites. However, the data from the oviposition assays with the inducer mites were inconclusive, because the C-S- subline of line 6, as well as both sublines of line 7, went extinct before the performance assays were completed.

IIb. Effects of *Wolbachia, Cardinium* and *Spiroplasma* on the mite's transcriptome

In the microarray analysis, we found significant differences in expression levels of mite genes across the five groups (W+S+, W-S+, W-S-, C+S- and C-S-). Even though the microarray represented only 9,424 (51%) of the in total 18,414 *in silico* predicted protein-coding sequences (Grbic *et al.*, 2011), we identified 201 (1.1% of the total) differentially expressed

genes (DEGs; absolute fold change (FC) > 1, Benjamini-Hochberg false discovery rate (FDR)-adjusted $P \leq 0.05$) across all groups. Overall, differences were relatively small, i.e. absolute FCs ranged from +1.1 to +5.2 for upregulated genes and from -1.1 to -3.4 for downregulated genes respectively, with only 23 genes displaying an absolute FC > 2 ($P \leq 0.05$, after *P*-value adjustment). The most pronounced transcriptional differences were found between W+S+ and W-S+ mites of the suppressor strain with 152 DEGs (Figure S5.4), 30 of which were up-regulated (see Table S5.3a for the top-20) and 122 were down-regulated (see Table S5.3b for the top-20) in W-S+ mites. Comparing the transcriptome of W+S+ and W-S- mites resulted in 50 DEGs: 14 of them were up-regulated (Table S5.4a), while 36 were down-regulated (see Table S5.4b for the top-20) in W-S- mites. Out of the 50 DEGs, 29 also differed between W+S+ and W-S+ mites (Figure S5.4), hence the other 21 correlated with the presence of *Spiroplasma*. The transcript levels of only 3 genes (2 up-, 1 down-regulated in W-S-) differed significantly between W-S- and W-S+ mites (Table S5.5, Figure S5.4). Based on the principal component analysis, one of the inducer C-S- samples was identified as an outlier and therefore excluded from further analysis. Using the remaining samples, we identified 33 DEGs between the two groups of the inducer strain C+S+ and C-S-; 23 were higher expressed in C-S- mites (Table S5.6a), while 10 were lower expressed (Table S5.6b). Five of the 33 DEGs were also significantly affected by *Wolbachia* and/or *Spiroplasma* in the suppressor mites (Table S5.6a). Many of the 201 DEGs, i.e. 36 genes (17.9%), had no homology with any of the genes characterized so far, hence their function in the mite physiology remains unknown. Among the DEGs in both mite strains, those encoding proteins involved in digestion, metabolic detoxification (phase I), binding/conjugation of xenobiotics (phase II detoxification), transport of xenobiotics (phase III detoxification) or encoding putative (salivary gland-derived) secreted proteins were highly abundant (Table 5.2). Genes related to immune-responses (Grbic *et al.*, 2011), of which the expression is associated with the presence of *Wolbachia* in dipterans (Kambris *et al.*, 2009; Moreira *et al.*, 2009), were not differentially expressed among the mite groups (data not shown).

Table 5.2. Overview of differentially expressed genes (DEGs; absolute fold change > 1; $P \leq 0.05$, after *P*-value adjustment) in adult female *Tetranychus urticae* suppressor (DeLier-1) and inducer (Santpoort-2) mites after selective removal of their associated bacteria *Wolbachia* sp. (W), *Spiroplasma* sp. (S) and/or *Candidatus* Cardinium (C) and feeding from tomato (*Solanum lycopersicum*) for seven days. Shown are the DEGs encoding proteins that are involved in digestion or detoxification, or are (putatively) secreted.

▲, up-regulated in mites of the first-mentioned bacterial group; ▼, down-regulated in mites of the first-mentioned bacterial group; +, present; -, absent

		Suppressor strain			Inducer strain		
Biological process	**Gene family**	**W-S+ vs W+S+**	**W-S- vs W+S+**	**W-S- vs W-S+**	**C-S- vs C+S+**	**unique**	**TOTAL***
		▲ / ▼	▲ / ▼	▲ / ▼	▲ / ▼		
Digestion							
	Cathepsin cysteine peptidases	0 / 7	0 / 2	-	-	7	49
	Legumain cysteine peptidases	0 / 5	0 / 2	-	-	5	12
	Cystatins	-	2 / 0	1 / 0	2 / 0	2	11
Detoxification							
Phase I	Cytochrome P450 monooxygenases	1 / 4	0 / 3	-	2 / 0	9	59
	Carboxyl/choline esterases (CCEs)	0 / 7	0 / 1	-	1 / 0	8	51
	Intradiol ring-cleavage dioxygenases	0 / 2	-	-	-	2	15
Phase II	Lipocalins	0 / 6	-	-	-	6	41
	Glutathione S-transferases (GSTs)	0 / 2	0 / 1	-	-	2	26
	UDP-glycosyltransferases (UGTs)	-	-	-	1 / 0	1	51
Phase III							
	ABC-transporters	0 / 1	0 / 1	-	1 / 0	3	88
	MFS-transporters	0 / 1	-	-	1 / 1	3	97
	total	1 / 35	2 / 10	1 / 0	8 / 1	48	
Unknown (secreted)							
	Unknown (various)	2 / 9	2 / 6	1 / 1	3 / 1	16	
	total DEGs	152	50	3	33	201	

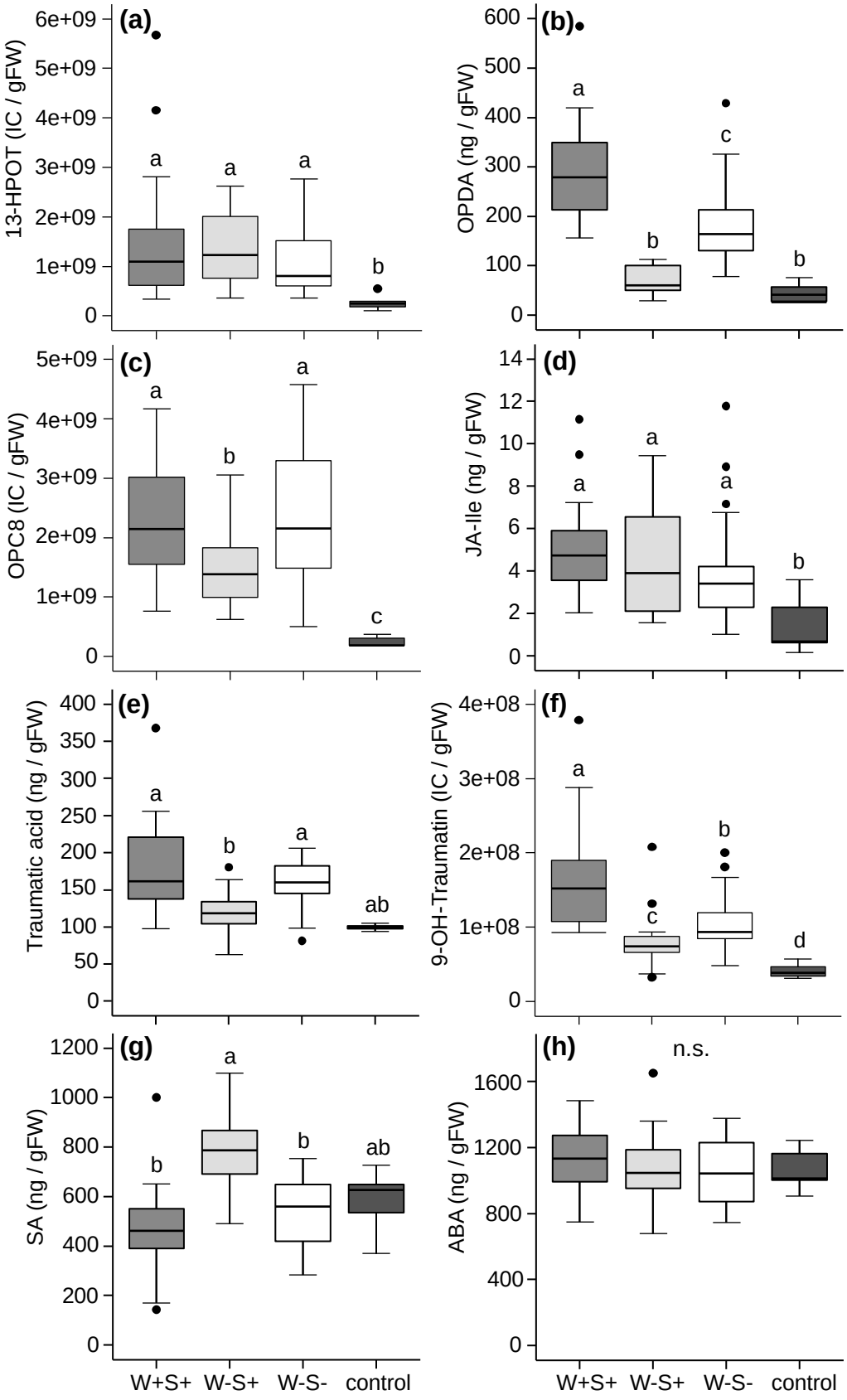
All *T. urticae* multi-gene families implicated in digestion and detoxification have been identified and/or manually annotated (Grbic *et al*., 2011; Santamaria *et al*., 2012; Dermauw *et al*., 2013; Ahn *et al*., 2014; Wybouw *et al*., 2015). *indicates the total number of genes (of the indicated gene family) that is represented on the microarray

III. Effects of spider mite-associated *Wolbachia, Cardinium* and *Spiroplasma* on mite-plant interactions

IIIa. Effects of mite-associated *Wolbachia, Cardinium* and *Spiroplasma* on tomato induced responses

For the suppressor mite infested plants, the most consistent differences in phytohormone profiles were found between leaflets infested with W-S+ mites and those infested with W-S- and/or W+S+ mites (Figure 5.4, Figure S5.5). The (combined) presence of *Wolbachia* and/or *Spiroplasma* correlated with phytohormone levels in tomato as follows. The accumulation of the JA-precursor OPDA was correlated with mite-associated bacteria (Figure 5.4b). Feeding by W+S+ mites induced the accumulation of OPDA, as did feeding by W-S- mites. However, leaflets infested with W-S- mites accumulated significantly lower amounts of OPDA than W+S+ mites. Leaflets infested with the third group of mites, W-S+, accumulated significantly less OPDA than W-S- and W+S+ infested leaflets and did not accumulate more OPDA than uninfested control leaflets. This clear pattern was detected in leaflets infested with all four suppressor mite lines (Figure S5.5a). A similar pattern was found for several other oxylipins as well, i.e. lowest hormone levels in W-S+ compared to W+S+ and W-S- infested leaflets. First, for the JA-precursor OPC8 (Figure 5.4c), which is the direct downstream product of OPDA, and to a lesser extent for OPC6 (Figure S5.5b), but not for JA itself (Figure S5.5c), nor for its biologically active conjugate JA-Ile (Figure 5.4d, Figure S5.5d). Second, we found the pattern for traumatic acid (Figure 5.4e) and 9-OH-traumatin (Figure 5.4f, Figure S5.5e), which are C_{12}-derivatives of the HPL pathway, derived from the same 13-HPOT pool as JA (Figure S5.1). The accumulation of 13-HPOT, one of the first intermediates in the biosynthesis process of oxylipin signalling molecules (Figure S5.1) (Wasternack, 2007), was not correlated with mite-associated bacteria (Figure 5.4a).

Figure 5.4. Phytohormone amounts in tomato (*Solanum lycopersicum*) leaflets after 7 days of infestation with a plant defence suppressor strain Delier-1 of the spider mite *Tetranychus urticae* which did (+) or did not (-) contain the bacteria *Wolbachia* (W) and *Spiroplasma* (S). Control plants were not infested. Phytohormones for which we assayed include (a) 13(*S*)-hydroperoxyoctadecatrienoic acid (13-HPOT), (b) 12-oxo-phytodienoic acid (OPDA), (c) 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid (OPC8), (d) jasmonic acid-isoleucine (JA-Ile), (e) traumatic acid, (f) 9-OH-traumatin, (g) free salicylic acid (SA), (h) abscisic acid (ABA); Boxes span the 25-75 percentiles, horizontal lines in the boxes represent medians, whiskers span 1.5*IQR (interquartile range), dots represent data points outside this range. Different letters above the boxes indicate significant differences at a level of $P \leq 0.05$, after applying a linear mixed model followed by Tukey multiple comparisons with Holm adjustment. Phytohormone amounts are presented as nanogram (ng) per gram fresh leaf weight (g FW). Compounds for which we did not have a pure standard are presented as ion counts (IC) /g FW. n.s. = not significant


(a)
13-HPOT (IC / gFW)
6e+09
5e+09
4e+09
3e+09
2e+09
1e+09
0
a
a
a
b
(b)
OPDA (ng / gFW)
600
500
400
300
200
100
0
a
b
c
b
(c)
OPC8 (IC / gFW)
5e+09
4e+09
3e+09
2e+09
1e+09
0
a
b
a
c
(d)
JA-Ile (ng / gFW)
14
12
10
8
6
4
2
0
a
a
a
b
(e)
Traumatic acid (ng / gFW)
400
350
300
250
200
150
100
50
0
a
b
a
ab
(f)
9-OH-Traumatin (IC / gFW)
4e+08
3e+08
2e+08
1e+08
0
a
c
b
d
(g)
SA (ng / gFW)
1200
1000
800
600
400
200
0
b
a
b
ab
(h)
ABA (ng / gFW)
n.s.
1600
1200
800
400
0
W+S+
W-S+
W-S-
control

The levels of SA in infested leaflets followed a pattern that appeared opposite of that of OPDA: amounts of SA in leaflets that were infested with W-S+ mites were significantly higher than in leaflets infested with either W-S- or W+S+ mites, but were not higher than in uninfested control leaflets (Figure 5.4g, Figure S5.5f). The accumulation of ABA was not affected by infestations with mites from any of the suppressor strain groups (Figure 5.4h).

The phytohormone profiles of inducer mite (C+S+ and C-S-) infested leaflets were as follows (see Figure 5.5, Figure S5.6). The levels of OPDA, JA-Ile and SA were induced by C+S+ and C-S- mites compared to uninfested controls, but did not differ between the two groups. However, two of the C_{12}-derivatives of the HPL pathway, i.e. traumatic acid (Figure 5.5c, Figure S5.6c) and 9,12-OH-(10*E*)-dodecenoic acid (Figure 5.5d), accumulated to higher amounts in C-S- infested than in C+S+ infested leaflets. ABA levels followed the same pattern (Figure 5.5f, Figure S5.6d).

Previously, suppression of plant defences by spider mites was shown to act downstream of phytohormones (Alba *et al.*, 2015). We therefore augmented the phytohormone data with the expression data of downstream marker genes (Figure S5.1) with qRT-PCRs. Upon infestation with suppressor mites, the amounts of OPDA in W-S+ infested leaflets differed from those in W+S+ and W-S- infested leaflets, with the latter having intermediate levels (Figure 5.4b). Transcript levels of *AOS1*, which encodes an enzyme involved in the biosynthesis of OPDA, were higher in suppressor mite-infested leaflets compared to uninfested controls, but there was no difference among the mite groups (Figure 5.6a). In contrast, expression of *OPR3*, which acts directly downstream of OPDA in the JA biosynthesis pathway, was significantly higher in W-S+ than in W+S+ or W-S- infested leaflets (Figure 5.6b, Figure S5.7a). The same pattern was found for multiple putative OPDA-responsive genes (ORGs); *TWI-1*, *ADH*, *GST6* and *GRX* (Figures 5.6c-f and Figures S5.7b-e, respectively). The expression levels of these ORGs were thus negatively correlated with OPDA amounts and positively with SA amounts, while the coefficient of determination (R^2) values were similar (Figure S5.8), except for expression levels of *ADH*, which correlated with OPDA (R^2 = 0.42, $P < 0.0001$) but not with SA (R^2 = 0.09, P = 0.22). Analogous to the overall induction of JA-Ile by W+S+, W-S+ and W-S- mites (Figure 5.4d), expression of the JA-defence marker genes *TD-2* (Figure 5.6g) and *PI-IIc* (Figure 5.6h) were induced in all three groups compared to the control, but did not differ among them. However, transcripts of *JIP-21* accumulated to significantly higher levels in W-S- infested leaflets than in those of W+S+ and W-S+ (Figure 5.6i, Figure S5.7f). As for the SA-defence marker genes, *PR-P6* transcript accumulation was higher in W-S+ leaflets than in W+S+ and W-S- leaflets (like SA

itself) (Figure 5.6j). Here it has to be noted that this *PR-P6* expression pattern was visually clear in two lines, but not in the other two (Figure S5.7g). We did not find any significant

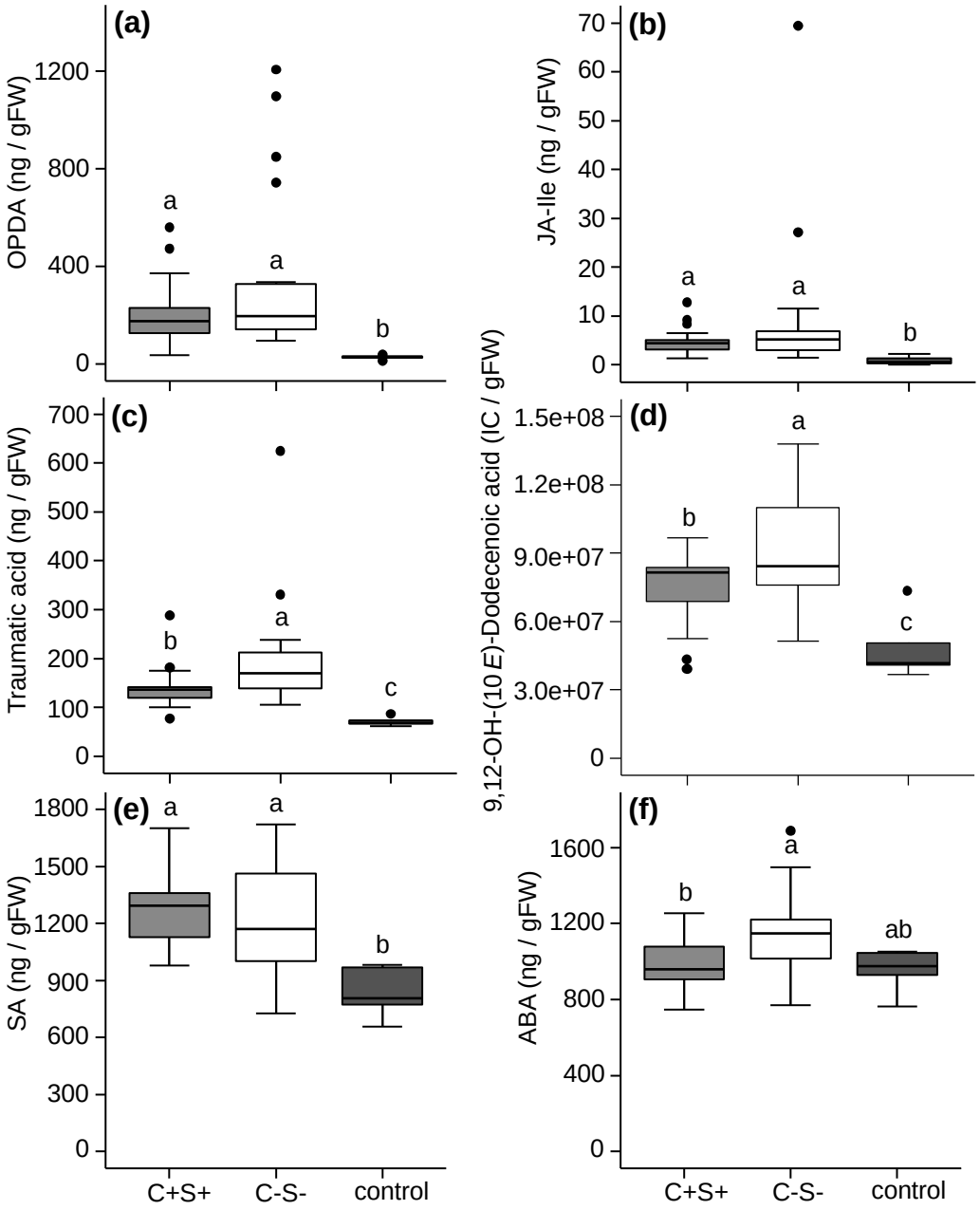


Figure 5.5. Phytohormone amounts in tomato (*Solanum lycopersicum*) leaflets after 7 days of infestation with a plant defence inducer strain Santpoort-2 of the spider mite *Tetranychus urticae* which did (+) or did not (-) contain the bacteria *Candidatus* Cardinium (C) and *Spiroplasma* (S). Control plants were not infested. Phytohormones for which we assayed include (a) 12-oxo-phytodienoic acid (OPDA), (b) jasmonic acid-isoleucine (JA-Ile), (c) traumatic acid, (d) 9,12-OH-(10*E*)- dodecenoic acid, (e) free salicylic acid (SA) and (f) abscisic acid (ABA) (f); Boxes span the 25-75 percentiles, horizontal lines in the boxes represent medians, whiskers span 1.5*IQR (interquartile range), dots represent data points outside of this range. Different letters above the boxes indicate significant differences at a level of $P \leq 0.05$, after applying a linear mixed model followed by Tukey multiple comparisons with Holm adjustment. Phytohormone amounts are presented as nanogram (ng) per gram fresh leaf weight (g FW). Compounds for which we did not have a pure standard are presented as ion counts (IC) /g FW. n.s. = not significant

differences in the expression of *PR-1a* in leaflets infested with the different groups of suppressor mites, nor between infested and uninfested leaflets (Figure 5.6k). Accordingly, SA amounts showed a weak but significant correlation with expression levels of *PR-P6* (Figure S5.9a) and did not correlate with *PR-1a* transcript levels (Figure S5.9b).

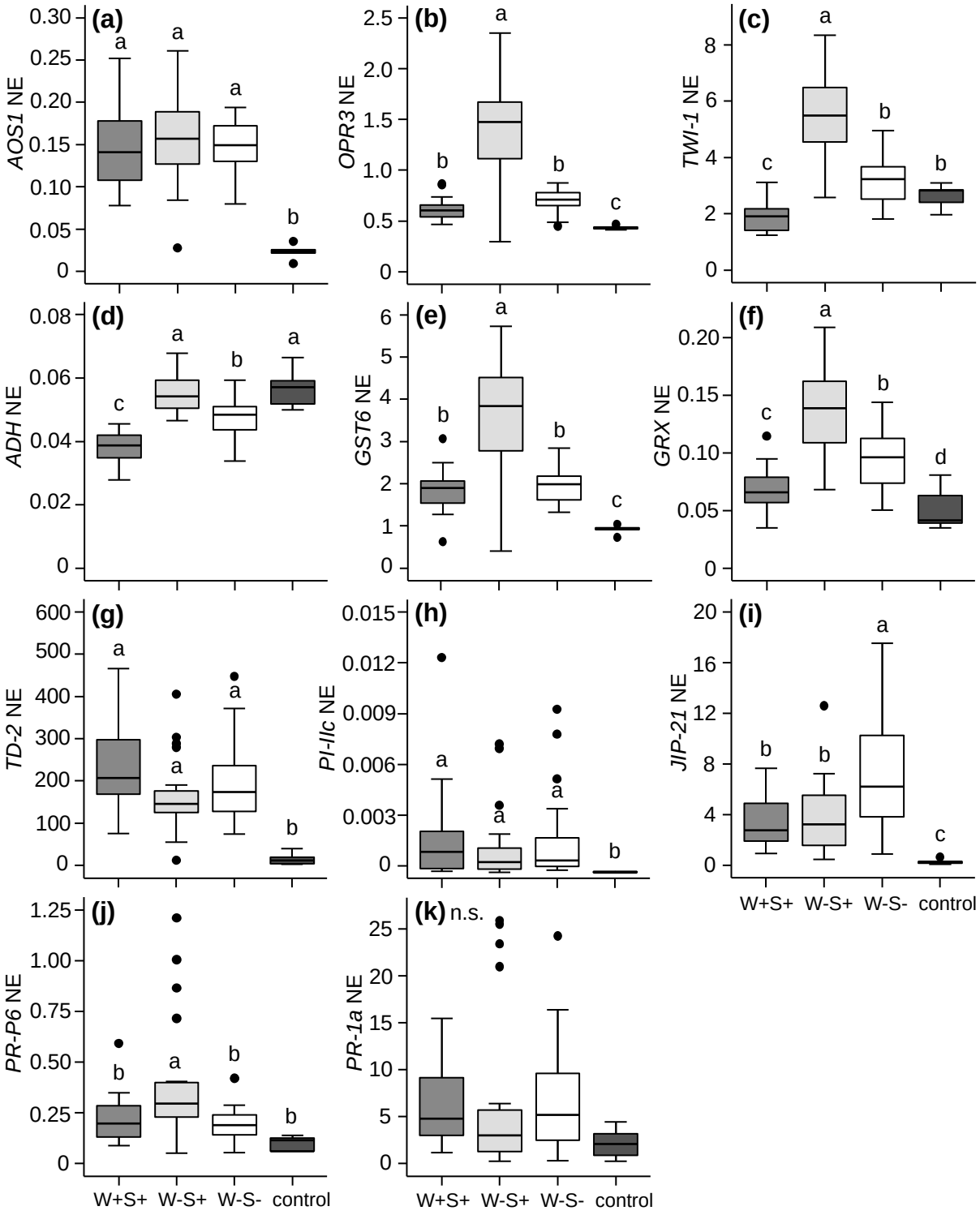


Figure 5.6. Normalized expression (NE) of plant defence related genes obtained via qRT-PCR in tomato (*Solanum lycopersicum*) leaflets after infestation (7 days) with a plant defence suppressor strain Delier-1 of the spider mite *Tetranychus urticae* which did (+) or did not (-) contain the bacteria *Wolbachia* (W) and *Spiroplasma* (S). (a*) Allene oxide synthase 1* (*AOS1*), (b) *12-oxophytodienoate Reductase 3* (*OPR3*), (c) *Tomato wound-induced 1* (*TWI-1*), (d) *Alcohol dehydrogenase* (*ADH*), (e) *Glutathione* S-*transferase 6* (*GST6*), (f*) Glutaredoxin* (*GRX*), (g) *Threonine Deaminase-2* (*TD-2*), (h) *Proteinase Inhibitor IIc* (*PI-IIc*), (i) *Jasmonate-inducible protein-21* (*JIP-21*), (j) *Pathogenesis-related protein 6* (*PR-P6*), (k) *Pathogenesis-related protein 1a* (*PR-1a*); Gene expression levels were normalized to the levels of tomato actin (Table S5.2). Boxes span the 25-75 percentiles, horizontal lines in the boxes represent medians, whiskers span 1.5*IQR (interquartile range), dots represent data points outside of this range. Different letters above the boxes indicate significant differences at a level of $P \leq 0.05$, after applying a linear mixed model followed by Tukey multiple comparisons with Holm adjustment. n.s. = not significant

In the leaflets infested with the inducer mites, none of the JA-defence marker genes (*TD-2, PI-IIc* and *JIP-21*) were differentially expressed ($P < 0.05$) between C+S+ and C-S- infested leaflets (Figure 5.7a-c). Interestingly, transcripts of both SA-defence marker genes, *PR-P6* and *PR-1a*, were more abundant in C+S+ infested leaflets than in C-S- infested ones (Figures 5.7d-e and Figures S5.10a,b).

IIIb. Effects of *Wolbachia, Cardinium* and *Spiroplasma* on the amount of feeding damage inflicted by the spider mites

A possible explanation for the observed differences in phytohormone and gene expression levels, which were correlated with the presence of mite-associated bacteria might be of behavioural nature, i.e. mites that feed more cause more damage to the plant and therefore might elicit stronger induced responses. To test if the magnitude of induced responses was correlated with the amount of feeding of the mites, we quantified the amount of spider mite-inflicted feeding damage (recognizable as chlorotic spots) on the same leaflets that were used for phytohormone extractions and tomato RNA isolation for qRT-PCRs. Overall, there was no significant difference in feeding damage between the three groups (W+S+, W-S+, W-S-) of suppressor mites (Figure 5.8a). In contrast, there was a clear difference in the amount of feeding damage caused by the two groups of inducer mites and in the type of damage these inflicted. Not only did C-S- mites feed significantly more than the C+S+ mites (Figure 5.8b), feeding by C+S+ mites resulted in rusty red/brown “scars” on the leaflets (Figure 5.8c), while those infested with C-S- mites had clear white scars (Figure 5.8d). This feeding damage phenotype was evident for three out of four lines, it was less clear for line 7 (Figure S5.11).

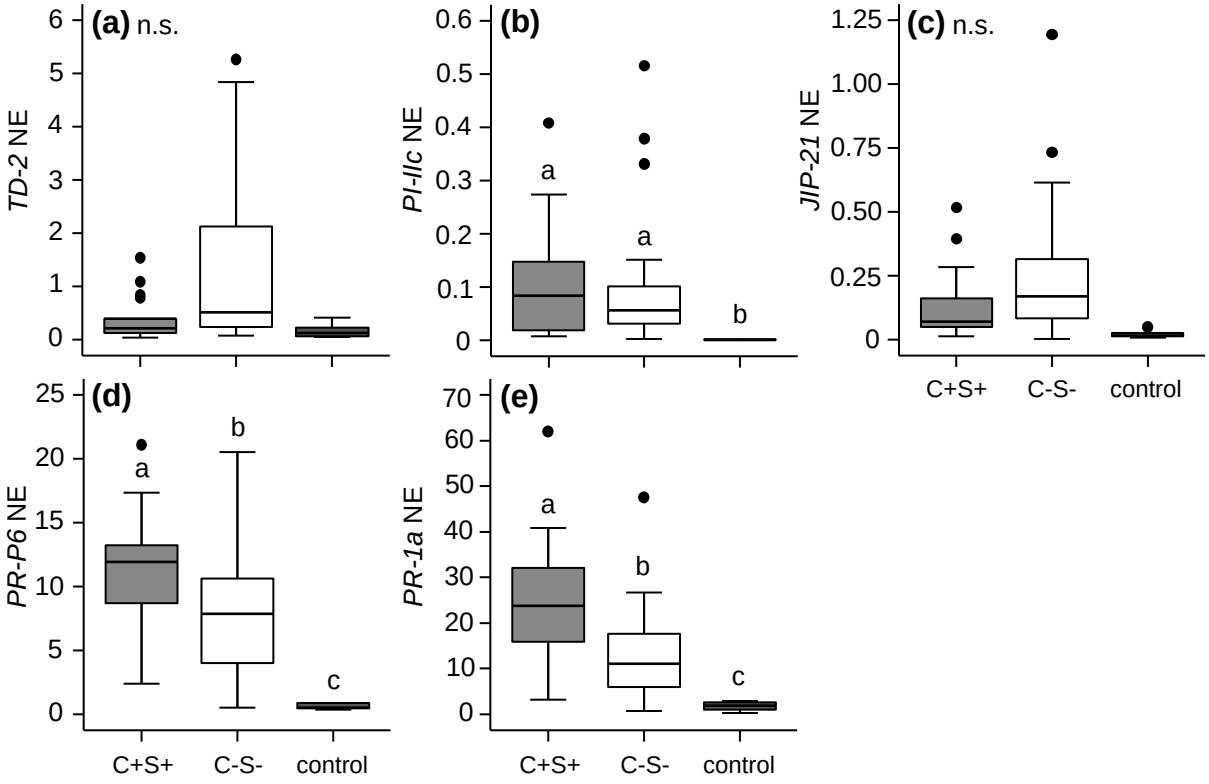


Figure 5.7. Normalized expression (NE) of plant defence related genes obtained via qRT-PCR in tomato (*Solanum lycopersicum*) leaflets after infestation (7 days) with a plant defence inducer strain Santpoort-2 of the spider mite *Tetranychus urticae* which did (+) or did not (-) contain the bacteria *Candidatus* Cardinium (C) and *Spiroplasma* (S). (a) *Threonine Deaminase-2* (*TD-2*), (b) *Proteinase Inhibitor IIc* (*PI-IIc*), (c) *Jasmonate-inducible protein-21* (*JIP-21*), (d) *Pathogenesis-related protein 6* (*PR-P6*), (e) *Pathogenesis-related protein 1a* (*PR-1a*)*;* Gene expression levels were normalized to the levels of tomato actin (Table S5.2). Boxes span the 25-75 percentiles, horizontal lines in the boxes represent medians, whiskers span 1.5*IQR (interquartile range), dots represent data points outside of this range. Different letters above the boxes indicate significant differences at a level of $P \leq 0.05$, after applying a linear mixed models followed by Tukey multiple comparisons with Holm adjustment. n.s. = not significant

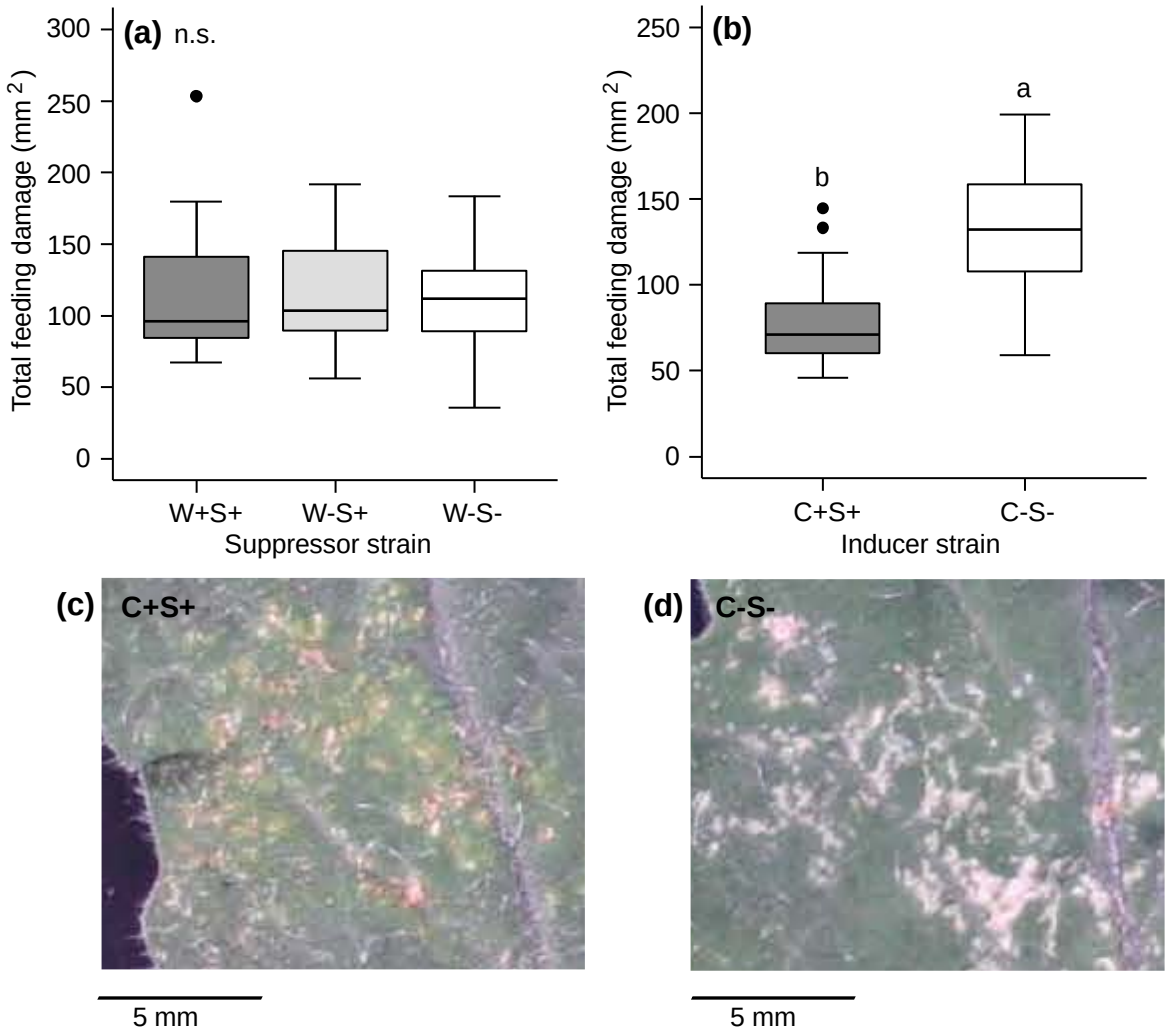


Figure 5.8. Feeding damage (mm^2) on tomato (*Solanum lycopersicum*) leaflets inflicted by two strains of the spider mite *Tetranychus urticae* which did (+) or did not (-) contain the bacteria *Wolbachia* (W), *Spiroplasma* (S) and/or *Candidatus* Cardinium (C). (a) Plant defence suppressor strain Delier-1 with three mite groups: W+S+, W-S+ and W-S-. (b) Plant defence inducer strain Santpoort-2 with two mite groups: C+S+ and C-S-. Boxes span the 25-75 percentiles, horizontal lines in the boxes represent medians, whiskers span 1.5*IQR (interquartile range), dots represent data points outside of this range. Different letters above the boxes indicate significant differences at a level of $P \leq 0.05$, n.s. = not significant after applying a linear mixed model. (c) Typical rusty red/brown scars inflicted by feeding of C+S+ mites of the inducer strain and (d) typical white scars inflicted by feeding of C-S- mites of the inducer strain.

Discussion

In this study we showed that *T. urticae* females of the plant suppressor strain DeLier-1, contained *Wolbachia* (W) and *Spiroplasma* (S) bacteria, while those from the plant defence inducer strain Santpoort-2, harboured *Candidatus* Cardinium (C) and also *Spiroplasma* (i.e. the same OTU as in suppressor mites). We determined the effects of these well-known reproductive parasites on (a) the spider mites and (b) on induced plant responses, after

removing them via antibiotic treatment. In the spider mites, we showed I) that the presence of the (endo)symbionts correlated positively with the survival, but not oviposition of the suppressor mites, while it correlated negatively with both survival and oviposition of the inducer mites. II) We showed that a subset of spider mite genes that are predicted to be predominantly involved in digestion and detoxification processes, as well as genes encoding putative secreted (salivary gland) proteins, were differentially expressed in mites, with opposite trends occurring in the suppressor versus the inducer mites. III) Plant responses to mite infestations differed between the mite groups that did or did not harbour *Wolbachia*, *Cardinium* and/or *Spiroplasma*. With the suppressor strain the most prominent result was that the amount of OPDA was lowest in W-S+ infested compared to W-S- and W+S+ leaflets, while SA followed an opposite pattern (highest amount in W-S+ infested leaflets). With the inducer strain the most interesting result was that SA-marker genes were expressed at lower levels in C-S- inducer mites, than in C+S+ infested leaflets. Moreover, the antibiotics-treated inducer mites (C-S-) consumed significantly more plant material and caused visually different feeding scars compared to the non-treated (C+S+) mites. Remarkably, well-known upstream-downstream relationships between phytohormones and expression of their marker genes were in some cases not found in our study, as discussed below. Finally, we showed IV) the combined presence of *Wolbachia* and *Spiroplasma* bacteria has consequences for mite gene expression and induced plant responses. Together, our results indicate that different (endo)symbiotic bacteria may have distinct consequences for their host, as is summarized in Figure 5.9 and discussed in the next sections.

Importantly, it has to be noted that although the antibiotic treatments resulted in the (near-) complete removal of *Wolbachia*, *Cardinium* and/or *Spiroplasma* from the mites and we found their absence/presence to correlate well with certain parameters (e.g. mite performance, mite transcriptomic responses and induced plant responses), various other bacteria were (randomly) present in/on antibiotics-treated and non-treated mites as well. These bacterial strains were not restricted to a certain group of mites and are thus not likely to be responsible for the effects that we found between the mite groups. In addition, tetracycline treatments may have had effects on the mites other than the removal of bacteria. Direct toxic effects of tetracycline, such as inhibition of mitochondrial functioning, are unlikely to play a role in our study, because we started experiments at least 15 generations after the antibiotics treatments. Selective effects, in which tetracycline treatment has selected e.g. for more toxin resistant mites, cannot be ruled out. However, subjectively we did not notice higher mortality in the tetracycline-treated lines compared to non-treated lines.

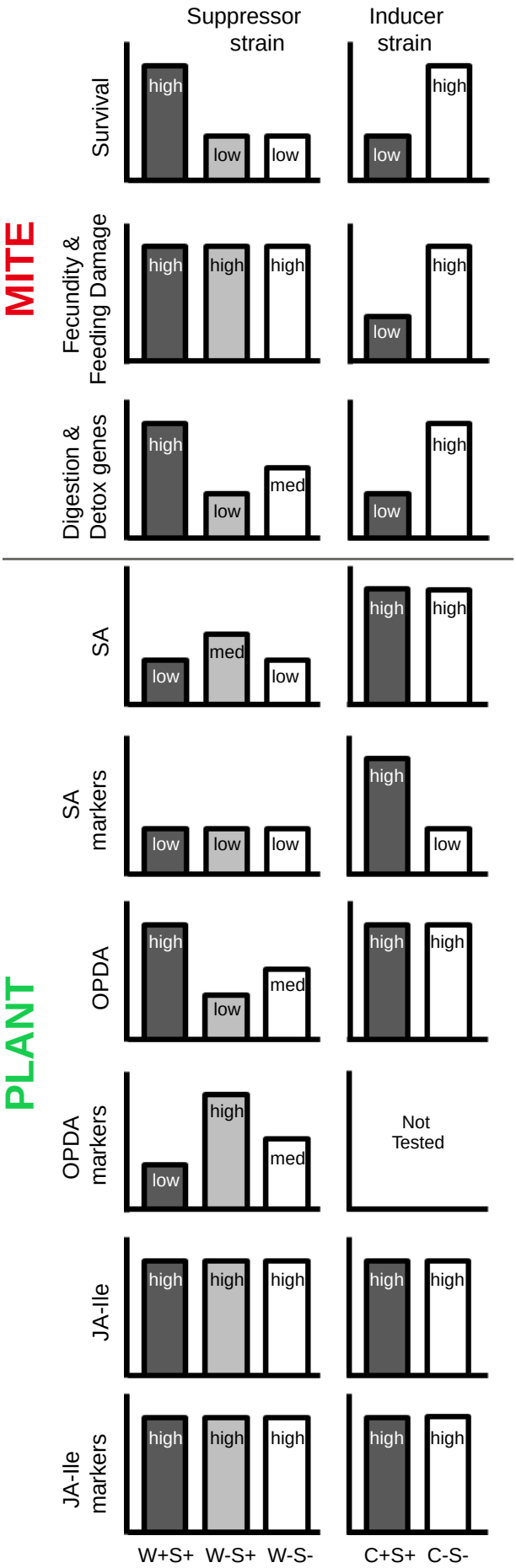


Figure 5.9. Schematic and simplified overview of the most important findings of this study. Adult female spider mites (*Tetranychus urticae*) from the plant defence suppressor strain DeLier-1 and the defence inducer strain Santpoort-2 were treated with antibiotics to remove their associated bacteria *Wolbachia* sp. (W), *Spiroplasma* sp. (S) and/or *Candidatus* Cardinium (C), after which the indicated mite and plant (tomato; *Solanum lycopersicum*) parameters were assayed. For a more detailed description we refer to the Results and Discussion sections. med, intermediate; +, bacteria present; -, bacteria absent

I) Effects of *Wolbachia, Spiroplasma* and *Cardinium* on spider mite performance

When we investigated the performance of suppressor mites in a four days trial, survival was highest in mites containing *Wolbachia* and *Spiroplasma*. The presence or absence of *Spiroplasma* did not affect mite survival. The *Wolbachia*-mediated effect on suppressor mite survival could have resulted from direct effects on host gene transcript levels, from indirect effects via induced or suppressed plant defences, or a combination hereof, as discussed below. Surprisingly, we did not find that mite fecundity was higher in the presence of *Wolbachia* than in its absence, indicating that the *Wolbachia*-containing survivors may have laid eggs at a lower frequency than mites without *Wolbachia*. Future experiments should focus on the lifetime production of eggs by mites with and without symbionts, since differences in survival are expected to have a significant effect on reproductive performance in the long run.

The performance of inducer mites was negatively correlated with the presence of *Cardinium* and *Spiroplasma*. Both mite survival and fecundity were higher for C-S- mites than for the C+S+ mites. Unfortunately, the performance assay for the inducer strain could not be completed, because the C-S- subline of line 6, as well as both sublines of line 7, went extinct before the assays started. However, the results of the plant infestation assay and mite transcriptome analysis, which were performed with all lines, were all in line with our hypothesis that *Cardinium* and/or *Spiroplasma* have a negative influence on the performance of the inducer mites: expression of SA-marker genes in C-S- infested leaflets was reduced compared to the C+S+ infested leaflets, even though C-S- mites inflicted almost twice as much damage. Furthermore, on the microarray the expression of digestion and detoxification genes was higher in C-S- mites than in C+S+ ones.

Previous studies indicate that fitness effects of reproductive parasites may strongly depend on the genotype of symbiont and host and whether symbionts optimize their prevalence in a population by reproductive manipulation and/or by positively affecting host fitness (Bordenstein & Werren, 2000; Fry *et al.*, 2004; Zug & Hammerstein, 2015). Accordingly, the reported effects of *Cardinium*, *Wolbachia* and *Spiroplasma* on host fitness are diverse. Whereas *Cardinium* and/or *Spiroplasma* negatively affected the fitness of mites in our experiments, an earlier study with a different *T. urticae* strain found that *Cardinium* did not alter survival or egg hatchability of its host, nor did it manipulate its reproduction (Gotoh *et al.*, 2007). Studies of other arthropods indicate that infection with *Cardinium* can have all possible effects on fitness parameters of its host (Weeks & Stouthamer, 2004; Ros & Breeuwer, 2009; White *et al.*, 2011; Stefanini & Duron, 2012). The same is true for *Wolbachia* infection, which seemed to positively affect *T. urticae* fitness in our study. In

T. urticae, *Wolbachia* was previously shown to have positive, negative or no effect on fitness parameters of this mite species (Vala *et al.*, 2000; Perrot-Minnot *et al.*, 2002; Xie *et al.*, 2011). Such varying effects of *Wolbachia* are also known in *Drosophila melanogaster* (Hoffmann *et al.*, 1994; Fry *et al.*, 2002). Effects of *Spiroplasma* on animal host fitness parameters were previously found to be negative or neutral (Ebbert, 1991; Fukatsu *et al.*, 2001; Montenegro *et al.*, 2006; Anbutsu & Fukatsu, 2011).

II) Effects of *Wolbachia, Spiroplasma* and *Cardinium* on the mite's transcriptome

Previous studies have established that (endo)symbiotic bacteria can affect gene expression of their host. Some data has been published on *Cardinium* (Nakamura *et al.*, 2011) and *Spiroplasma* (Hutchence *et al.*, 2011), but most research has focused on *Wolbachia* (Kambris *et al.*, 2009; Moreira *et al.*, 2009; Hussain *et al.*, 2011; Zhang *et al.*, 2013; Mayoral *et al.*, 2014).

In *T. urticae*, *Wolbachia* has been shown to impact gene expression of its host: sex-specific differences in transcript levels of up to 251 protein-coding genes (Zhang *et al.*, 2015), as well as up to 91 miRNAs (Rong *et al.*, 2014) were found when comparing *Wolbachia*-infected and uninfected female mites. In our microarray analysis with antibiotics-treated and non-treated adult female suppressor and inducer mites, we identified 201 differentially regulated spider mite genes among the mites from all groups (i.e. W+S+, W-S+, W-S- and C-S-, C+S+), i.e. roughly 1% of the 18,414 *in silico* predicted protein-coding sequences (Grbic *et al.*, 2011) of which about 50% were present on our array. Consistent with the data of Zhang *et al.* (2015), none of these mite genes putatively involved in immunity (Grbic *et al.*, 2011) were differentially regulated. This is in agreement with previous findings that the immune system is not activated in response to *Wolbachia* in arthropod host species that are naturally infected by this bacterium, but can be activated in species that are naturally *Wolbachia*-free and are experimentally infected, as was shown in *Aedes aegypti* (Rances *et al.*, 2012; Zug & Hammerstein, 2015).

Among the DEGs, there was a high abundance of genes that implicated the mite-associated bacteria in direct or indirect regulation of digestive and detoxification processes of their host. This is again similar to the findings of Zhang *et al*. (2015). However, when comparing the gene IDs of the DEGs of our study and that of Zhang *et al*. (2015) we found only five genes with an identical ID. Moreover, according to the data of Zhang *et al*. (2015), two of these five genes were differentially regulated in males but not females, while the remaining three genes were inversely regulated on our arrays. Notably, Zhang *et al.* (2015)

worked with a Chinese mite strain that was sampled from bean, whereas we worked with Dutch mite lines that were sampled from tomato, while both data sets were mapped onto the *T. urticae* London genome (Grbic *et al.*, 2011) for identification of DEGs. Possibly this is not accurate enough for identifying the homologous loci, but only for identifying homologous gene families. Additionally, *Wolbachia* strains may have differed between the two studies or *Wolbachia* may interact differently with mites on bean than with mites on tomato.

Absence of only *Wolbachia* from suppressor mites (W-S+) correlated with reduced transcript accumulation of 35 out of 36 DEGs, which belong to 9 gene families involved in digestion and in detoxification of xenobiotics. This result indicates that *Wolbachia* may have a direct effect on mites and may impact their capability to digest food and their ability to catabolize secondary metabolites. It has been shown before for various strains of *Wolbachia* that they can metabolically assist their host, in particular in filarial nematodes in which *Wolbachia* represents an obligate nutritional mutualist, for instance by providing its host with the energy source ATP (Darby *et al.*, 2012) or essential coenzymes, the cofactor heme and nucleotides, in return for amino acids (Foster *et al.*, 2005). With respect to arthropod hosts, *Wolbachia* is indispensable for the bedbug *Cimex lectularius*, because it provides essential B vitamins (Hosokawa *et al.*, 2010). Furthermore, in the parasitic wasp *Asobara tabida* and the fruitfly *D. melanogaster*, *Wolbachia* influences iron (and hence redox) homeostasis, which benefits its host in perturbed iron environments (Brownlie *et al.*, 2009; Kremer *et al.*, 2009). Hence, in suppressor mites, *Wolbachia* possibly performs early catabolic steps of (plant) nutrients or secondary metabolites and delivers intermediates which the mite can further process using the genes listed in Table 5.2. If so, this may explain why Zhang *et al.* (2015) observed similar functional processes but different loci in mites in correlation with the absence or presence of *Wolbachia*. In this scenario, *Wolbachia* may act as a nutritional mutualist when mites feed from tomato, which might explain the higher survival of suppressor mites that contained *Wolbachia*.

Strikingly, our data reveals fewer DEGs (roughly 65% less) when comparing W+S+ mites with W-S- (instead of W-S+) mites, while transcriptomic differences between W-S+ and W-S- mites were virtually absent. This suggests a direct antagonistic interaction between *Wolbachia* and *Spiroplasma*. Unfortunately we did not obtain a W+S- line to substantiate this. Possibly, part of the *Wolbachia*-associated transcriptional response of the mite is a direct consequence of the presence of *Spiroplasma*. It has been suggested before that *Spiroplasma* negatively affects the abundance of *Wolbachia* within the same host (Goto *et al.*, 2006). If so,

Wolbachia may chemically combat and/or constrain *Spiroplasma* and *vice versa*, leaving the mite to clean up "the waste" using the genes listed in Table 5.2.

The transcriptome of *Cardinium*- and *Spiroplasma*-free inducer mites also differed in the expression of genes involved in digestion, detoxification and transport of xenobiotics as compared to the mites that still harboured both bacteria. However, we found fewer DEGs and the directionality of transcriptional change was largely opposite from what we found for the suppressor strain. Expression of cysteine peptidase and lipocalin encoding genes for instance, which responded strongest to the absence of *Wolbachia*, was unaffected in C-S- inducer mites. In fact, the only overlap with suppressor mites was the increased transcript abundance of two cystatin encoding genes in C-S- versus C+S+ mites. When compared to W+S+ mites, these genes were also induced in W-S- mites, but not in W-S+ ones. Together, this suggests that these cystatin genes respond to the presence of *Spiroplasma*. Other DEGs (encoding two CYPs, a CCE, an UGT, an ABC- and two MFS-transporters) seem to respond to the presence of *Cardinium* in inducer mites, mainly because they followed a trend opposite to when *Wolbachia* was present in the suppressor strain: all but one MFS-transporter were up-regulated in C-S- mites compared to C+S+ ones. We did not manage to obtain inducer strain lines with only *Spiroplasma* or only *Cardinium*, therefore it remains difficult to pinpoint the effects of each bacterial strain more precisely. All in all, the transcriptome data suggest *Cardinium* to have a negative effect on the mite, possibly by reducing the mite's capability to detoxify and transport harmful secondary metabolites. This is consistent with our results for mite performance, in which C+S+ mites preformed worse than C-S- mites.

Among the 201 DEGs, the ones encoding (putative) secreted proteins were also highly abundant. Again this is in agreement with the data from Zhang *et al*. (2015). Four of our identified DEGs were predicted to encode proteins that are secreted from the mite salivary gland and are thus thought to indeed directly interact with the plant. Moreover, *Wolbachia* was found to be localized in the gnathosoma (mouth and feeding parts) of both male and female *T. urticae* (Zhao *et al.*, 2013), from where the proteins could also be secreted to the plant. Mite salivary gland-secreted proteins might be recognized by the plant and induce anti-herbivore defences (i.e. act as elicitors) or they may act as effectors and interfere with plant defences (Villarroel *in prep*). Pathogens (Dou & Zhou, 2012; Lo Presti *et al.*, 2015) and nematodes (Goverse & Smant, 2014) are well-known for their use of effector molecules to subvert plant immunity and while it is likely that many arthropods have evolved them as well, empirical evidence is still scarce (Kant *et al.*, 2015). In some cases defence suppression and the secretion of effectors by arthropods was reported to depend on associated microbes. For

instance, the leafhopper *Macrosteles quadrilineatu*s vectors phytoplasma bacteria, that after being transmitted to the host plant, produce and secrete molecules that suppress JA-defences, which is beneficial for the leafhopper(Sugio *et al.*, 2011). Recently, the saliva of the whitefly *Bemisia tabaci* was shown to contain a small (< 3 kDa) non-proteinaceous compound(s) responsible for the effective suppression of JA-defences via manipulation of JA-SA crosstalk (Su *et al.*, 2015). Defence suppression was only detected with saliva from whiteflies that harboured the endosymbiont *Hamiltonella defensa*, substantiating a relationship between the presence of the bacterium and the production of the effector(s) (Su *et al.*, 2015). Our microarray data indicates that mite-associated bacteria can affect the host's transcript accumulation of genes encoding secreted (salivary gland) proteins and may in this way affect plant defence responses.

Together, the transcriptional differences that we observed between mites with *Wolbachia*, *Cardinium* and/or *Spiroplasma* bacteria or without them, may have several, not mutually exclusive, causes. First of all, they may reflect processes in the host associated with suitability for bacterial colonization, as was shown in *A. aegyti* (Hussain *et al.*, 2011) and this could be a direct consequence of bacterial manipulation of host transcripts e.g. via RNA-interference (RNAi). Secondly, it may reflect responses of the host to bacterial metabolites, either plant-derived or not, eligible for further metabolization or catabolization. Such metabolites may be nutritional, may be plant toxins or be generated by multiple bacterial strains competing for space or nutrients. Thirdly, they may reflect a response of the mite to differences in plant tissue quality, e.g. due to differentially induced plant responses (discussed in the next section), directly or indirectly caused by differences in the host's bacterial composition and possibly mediated by alterations in the host's secretome.

III. Effects of *Wolbachia, Cardinium* and *Spiroplasma* on tomato induced responses

When we investigated the effect of *Wolbachia* and *Spiroplasma* infection of the suppressor strain on tomato induced responses, the most striking finding was that the JA-precursor OPDA did not significantly accumulate in W-S+ infested leaflets compared to uninfested leaflets and W+S+ infested leaflets, while OPDA levels were intermediate in leaflets infested with W-S- mites. Thus, the presence of *Wolbachia* in suppressor mites is correlated with enhanced OPDA accumulation, while the presence of *Spiroplasma* in these mites is correlated with suppression of OPDA accumulation. Note that the OPDA (or any other plant response) phenotype cannot be explained by the amount of damage inflicted due to mite feeding, as this was equal among all four groups. Expression of *OPR3* (Strassner *et al.*, 2002) was highest in

W-S+ infested leaflets, thus an increased conversion rate of OPDA might explain the reduced OPDA accumulation in the respective leaflets. However, the immediate downstream product of OPDA, OPC8 showed the same pattern as OPDA. Surprisingly, the end product of the oxylipin pathway, JA-Ile, which is considered the main biologically active molecule (Fonseca *et al.*, 2009; Wasternack & Hause, 2013), did not show any pattern that was correlated with bacterial presence, neither did JA-responsive defence marker genes. In addition, the accumulation profiles of two C_{12}-derivatives of the hydroperoxide lyase (HPL)-pathway, traumatic acid and 9-OH-traumatin, closely resembled that of OPDA across the treatments. This is notable, because both the JA and the HPL pathway represent branches of the LOX pathway that use the same substrate, i.e. 13-HPOT (Figure S5.1; Wasternack, 2007). Moreover, levels of the substrate 13-HPOT as well as transcript levels of *AOS1*, which encodes the first of two enzymes responsible for turnover of 13-HPOT into OPDA (Howe *et al.*, 2000; Sivasankar *et al.*, 2000), were not correlated with the presence of bacteria. Although gene transcript levels do not necessarily reflect protein levels and/or enzyme activity (Sullivan & Green, 1993), it remains unknown how accumulation of some wound hormones is affected by spider mite-associated bacteria, while other oxylipin pathway intermediates/products are not. One explanation for the altered OPDA amounts might be that *Wolbachia* and *Spiroplasma* differentially affect conjugation of OPDA with other cellular compounds. In Arabidopsis (*Arabidopsis thaliana*) and tobacco (*Nicotiana tabacum*) for example, OPDA is known to form conjugates with glutathione (GSH) (Davoine *et al.*, 2006; Mueller *et al.*, 2008). Although we did not detect OPDA-GSH in tomato leaflets, it is possible that other conjugates (that we did not search for) were formed.

Unexpectedly, expression levels of putative OPDA-responsive genes (ORGs) in suppressor mite infested leaflets negatively correlated with OPDA amounts. While OPDA induces expression of the ORGs *UGT73B5*, *CAD*, *GST6* and *GRX480* in Arabidopsis (Taki *et al.*, 2005; Mueller *et al.*, 2008; Park *et al.*, 2013), the expression of their respective putative tomato homologs *TWI-1*, *ADH*, *GST6* and *GRX* all followed exactly the opposite pattern in our experiments. The fact that all four tomato genes had identical expression profiles (we found this for more putative ORGs, data not shown) and that their sequences and gene ontologies were conserved between Arabidopsis and tomato (52-62% identical, 69-77% similar at the amino acid level), strongly suggests these genes are indeed functional homologs. An explanation for the inverse correlation of ORG transcript levels and OPDA amounts might be that these genes are regulated by one or more signals other than OPDA. This might for instance be attributed to SA, since we found significantly higher levels of SA in W-S+ than in

W+S+ and W-S- infested leaflets and thus SA amounts and ORG transcript levels were positively correlated. Interestingly, some Arabidopsis ORGs have been shown to be SA responsive (Uquillas *et al.*, 2004; Langlois-Meurinne *et al.*, 2005; Ndamukong *et al.*, 2007). In tomato, the ORG *TWI-1* is also responsive to SA (O'Donnell *et al.*, 1998). This suggests that SA might (co-)regulate the expression of the putative tomato ORGs.

SA amounts correlated only weakly with expression levels of *PR-P6* and did not correlate with *PR-1a* expression levels at all. Interestingly, *PR-1a* was not induced compared to the control in leaflets infested with suppressor mites, while it was induced in leaflets infested with inducer mites. This weak or lack of correlation with PR gene expression might thus be indicative of defence suppression by suppressor mites, which was demonstrated to occur downstream of phytohormone accumulation, e.g. at the *PR-1a* transcript level (Alba *et al.*, 2015).

Remarkably, plant response patterns (in which W-S+ differed from W+S+ and W-S-) were not reflected in mite performance (mite survival was lower for W-S+ and W-S- than for W+S+ mites), possibly because *Wolbachia* and *Spiroplasma* do not seem to have a significant effect on the JA defence responses to which the mites are susceptible (Alba *et al.*, 2015). However, differences in OPDA amounts might have a significant impact on the fitness of mites independently from JA, because accumulation of OPDA itself is sufficient to confer resistance to several herbivores and pathogens as has been demonstrated in various studies (Stintzi *et al.*, 2001; Stotz *et al.*, 2011; Park *et al.*, 2013; Bosch *et al.*, 2014; Scalschi *et al.*, 2015; Guo *et al.*, 2014). We can therefore not exclude, that plant responses, that were altered in correlation to bacteria, affect fitness of the suppressor strain in other traits than we have measured.

For the inducer strain, the results form a clearer picture. Leaflets infested with C-S- mites contained higher amounts of ABA, traumatic acid and 9,12-OH-(10*E*)-dodecenoic acid, while transcript levels of SA-responsive *PR-P6* and *PR-1a* were reduced. Consistent with these results, earlier studies found that feeding by arthropods with bacterial symbionts was associated with increased amounts of SA and higher expression levels of *PR* genes in host plants (Chung *et al.*, 2013; Su *et al.*, 2015). ABA appears to negatively regulate SA defences in tomato, in particular by inhibiting expression of *PR-1a* (Audenaert *et al.*, 2002). The reduced expression of *PR* genes in C-S- samples compared to the C+S+ samples might thus be explained by the negative action of ABA. Since these results were paralleled by different feeding intensities of mites with or without these bacteria, *Cardinium* and/or *Spiroplasma* may negatively affect mite fitness via induction of plant defences.

This hypothesis is complemented by different feeding scar phenotypes of C-S- and C+S+ mites. Whereas, feeding by C+S+ mites resulted in rusty red/brown scars, infestation with C-S- mites yielded white scars. Similar scar phenotypes have been reported before for the Kanzawa spider mite *T. kanzawai*. In that case, red scars were associated with increased SA amounts of bean leaves as well as increased expression of a SA defence marker gene (Matsushima *et al.*, 2006), which is in agreement with our findings. Notably, the scar colour resulting from Kanzawa mite feeding was found to be determined by the mite genotype, with dominance of the red phenotype over the white one and no maternal effect (Yano *et al.*, 2003). Thus, maternally inherited symbionts cannot be the cause of differentially coloured scars produced by *T. kanzawai* feeding. Nevertheless, since we did not select for scar phenotype and the genetic background of the mite lines used in our experiments was equal among the C+S+ and C-S- groups, we suggest that the red scars in our case did probably not have a genetic basis but were caused by the presence of a bacterium and concomitant induction of SA-responses. Most likely the presence of *Cardinium*, resulted in rusty red/brown scars, because we did not observe this scar phenotype with W-S+ suppressor mites that contained the same *Spiroplasma* OTU.

IV) The combined presence of *Wolbachia* and *Spiroplasma* bacteria has consequences for mite gene expression and induced plant responses

Within a host, the various symbionts that (can) co-occur possibly interact, which may affect host and bacterial fitness in various ways. Our results suggest that a single or double infection status of suppressor mites differentially affects host gene expression as well as induced plant responses, with *Wolbachia* demonstrating characteristics of a mutualist, while the effect of *Spiroplasma* on the mite was less clear, but its presence (partially) antagonized *Wolbachia*-associated responses. This could have resulted from competition between the two symbionts, for instance for space and/or resources. Microbial competition for space (i.e. the ovaries) within the mosquito hosts *Anopheles stephensi* and *Aedes aegypti* was reported to occur between *Wolbachia* and an unrelated *Asaia* bacterium (Hughes *et al.*, 2014; Rossi *et al.*, 2015). It is thus possible that *Wolbachia* spatially displaces *Spiroplasma* or *vice versa*, i.e. away from organs where they may affect induced plant responses or mite digestive processes, like the salivary glands or gut epithelial cells. It would therefore be interesting to determine the spatial localization of both bacteria in single and double infected mites using *in situ* hybridizations. Beside the proper localization, symbiont densities are another important parameter for fidelity of their vertical transmission as well as their effect on host fitness

(Rousset *et al.*, 1999; Goto *et al.*, 2006; Oliver *et al.*, 2006; Unckless *et al.*, 2009). Our Illumina MiSeq analysis showed that the relative abundance (as well as the total number of reads) of *Spiroplasma* in the W-S+ lines was higher than in the W+S+ lines, suggesting that *Wolbachia* may negatively affect the abundance of *Spiroplasma* in suppressor mites. However, since 16S amplicon sequencing is only a semi-quantitative method, bacterial abundance should be assessed by means of qRT-PCRs to test this hypothesis.

How co-infecting symbionts affect each other's densities depends on both the species and genotype of host and symbiont (Bordenstein & Werren, 2000; Kondo *et al.*, 2005). For instance, in double or triple infections of wasps, fruitflies or moths with different *Wolbachia* genotypes, none of the tested *Wolbachia* strains influenced densities of the other *Wolbachia* strain(s) (Rousset *et al.*, 1999; Ikeda *et al.*, 2003; Mouton *et al.*, 2003). In contrast, in the beetle *Callosobruchus chinensis* one *Wolbachia* strain suppressed a second one (Kondo *et al.*, 2005). In *D. melanogaster* infected with *Wolbachia* and *Spiroplasma*, the latter negatively affected *Wolbachia* densities, but *Wolbachia* did not affect *Spiroplasma* densities (Goto *et al.*, 2006).

In addition, fitness effects of mixed infections with different bacterial species on hosts are diverse. For instance, female *Bryobia sarothamni* mites that were doubly infected with *Wolbachia* and *Cardinium* had a higher fecundity than singly or uninfected females (Ros & Breeuwer, 2009). In contrast, pea aphids that were doubly infected with *H. defensa* and *Serratia symbiotica* had a lower fecundity than singly or uninfected aphids (Oliver *et al.*, 2006). Infection of *D. melanogaster* with *Wolbachia*, *Spiroplasma* or both did not affect fitness parameters of the flies (Montenegro *et al.*, 2006). A mutualistic interaction has been proposed for *Wolbachia* and *Spiroplasma* that co-infect the fruitfly *D. neotestaceae* (Jaenike *et al.*, 2010b). The frequency of co-infected fruitflies in sampled populations and estimated evolutionary age of the two bacteria that had co-infected the flies, were seen as a possible basis for the evolution of a mutualism between the bacteria (Jaenike *et al.*, 2010b). The fact that *Wolbachia* and *Spiroplasma* bacteria in the suppressor strain seemed to differentially influence mite survival, transcriptomic responses and induced plant responses, emphasizes the importance of considering interaction effects of multiple bacteria in/on one host.

Unfortunately, we did not obtain all bacterial combinations in *T. urticae* to disentangle the exact effects of single bacterial strains and their combined effects on mite fitness, induced plant responses and mite gene expression. For instance, we did not test mites that were infected with only *Wolbachia*, hence we can only indirectly infer the role of *Wolbachia* in our experiments from comparisons between W+S+, W-S+ and W-S- mites. Moreover, for the

inducer strain the roles of *Spiroplasma* and *Cardinium* remain difficult to interpret because we did not have C-S+ or C+S- mites. The same *Spiroplasma* OTU occurred in both the inducer and suppressor mites. If we assume that *Spiroplasma* in inducer mites had the same effect on host fitness parameters as in suppressor mites, we could attribute the negative effects of bacteria in inducer mites to *Cardinium*. However, *Spiroplasma* may interact with *Cardinium* in the mites. Furthermore, its effects on host fitness may depend on mite genotype (Schmid-Hempel, 2011).

Conclusions

We assessed the influence of the (endo)symbiotic bacteria *Wolbachia*, *Cardinium* and *Spiroplasma* on the performance and transcriptome of their respective hosts, the *T. urticae* suppressor (W+S+) and inducer (C+S+) strains. Furthermore, we tested how these spider mite-associated bacteria affect the induced responses of tomato plants triggered by feeding mites. Our data indicate that the different (endo)symbiotic bacteria may have distinct consequences for their host (see Figure 5.9):

1. The presence of *Wolbachia* correlates positively with survival of suppressor mites, possibly via manipulation of OPDA and SA-related responses. We did not observe changes in JA- and SA marker gene expression levels. Further, the presence of *Wolbachia* correlates positively with transcript levels of suppressor mite genes involved in digestion and detoxification of xenobiotics. This might reflect responses of the mite to symbiont-generated or -modified metabolites, either plant-derived (transcriptome profiles of the suppressor strain resemble the OPDA phenotype) or not, that are suitable for further processing by the mite.

2. The presence of *Cardinium* and *Spiroplasma* correlates negatively with the feeding activity, survival and fecundity of inducer mites, while it correlates positively with the induction of SA-marker-gene expression as well as with the frequently observed rusty scars of infested leaves. This indicates a distinct and clearly visible (in)direct impact of the presence of *Cardinium* and *Spiroplasma* on the mite's host plant while simultaneously their presence correlates with inhibition of digestion and detoxification processes in the mite.

3. There is no clear correlation between the absence or presence of *Spiroplasma* and survival of suppressor mites, while the induced plant responses of leaflets fed on by mites with or without *Spiroplasma* are ambiguous. The data suggests a complex interaction between *Wolbachia* and *Spiroplasma* in which the latter may suppress OPDA-related responses that are induced in the presence of *Wolbachia*. In addition, some mite digestion and detoxification genes that respond positively to the presence of *Wolbachia* seem to be

slightly suppressed when only *Spiroplasma* is present. Unfortunately, we did not obtain W+S- lines to disentangle this interaction.

4. The same *Spiroplasma* OTU was present in suppressor and inducer mites. Hence, although we did not obtain singly-infected inducer lines, a comparison of the results between the mite strains suggests that the presence of *Spiroplasma* in C+S+ inducer mites is not related with the observed negative effects on its host (e.g. induction of SA defences, impaired expression of detoxification genes and reduced feeding). However, we cannot exclude that *Spiroplasma* affects the suppressor mites differently than the inducer mites.

In conclusion, according to our data, *Wolbachia* has the characteristics of a mutualist, while *Cardinium* has the characteristics of a parasite. The role of *Spiroplasma* remains unclear, but it does seem to interact - directly or indirectly - with *Wolbachia* at the level of mite gene expression and induced plant responses (note that these two responses might be causally linked).

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Supporting information

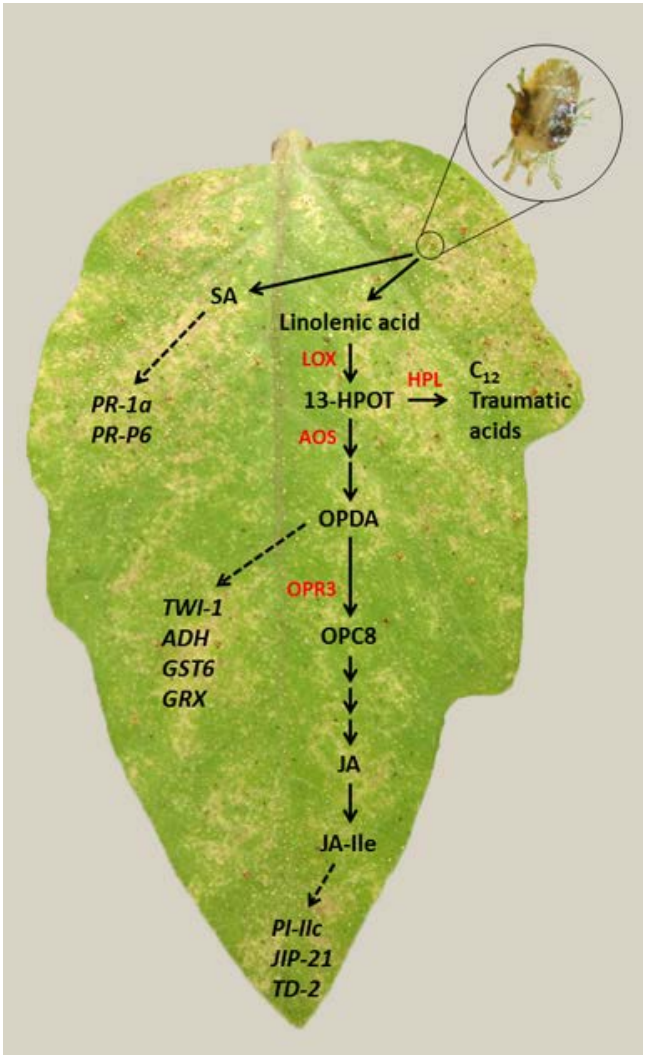

Figure S5.1. Schematic and simplified overview of tomato (*Solanum lycopersicum*) defence responses typically induced by spider mite (*Tetranychus urticae*) feeding and which were monitored in this study. Infestation with inducer *T. urticae* Santpoort-2 mites (inset) results in the simultaneous, enhanced accumulation of the phytohormones jasmonic acid (JA) and salicylic acid (SA) as well as transcript levels of their downstream marker genes. JA biosynthesis originates from linolenic acid, which is converted to 13-HPOT by LOX. This compound then serves either as substrate for the enzyme HPL to generate various C_{12} traumatic acids, or for AOS to ultimately yield JA-Ile. Arrows indicate biosynthetic steps carried out by a single enzyme, some of them are shown in red letters. Note that SA can be synthesized via different pathways and how this is done upon mite infestation is still unclear. Dashed arrows symbolize transcriptional activation of marker genes (which are in italic face). Amounts of linolenic acid and HPL transcripts were not assayed in this study.

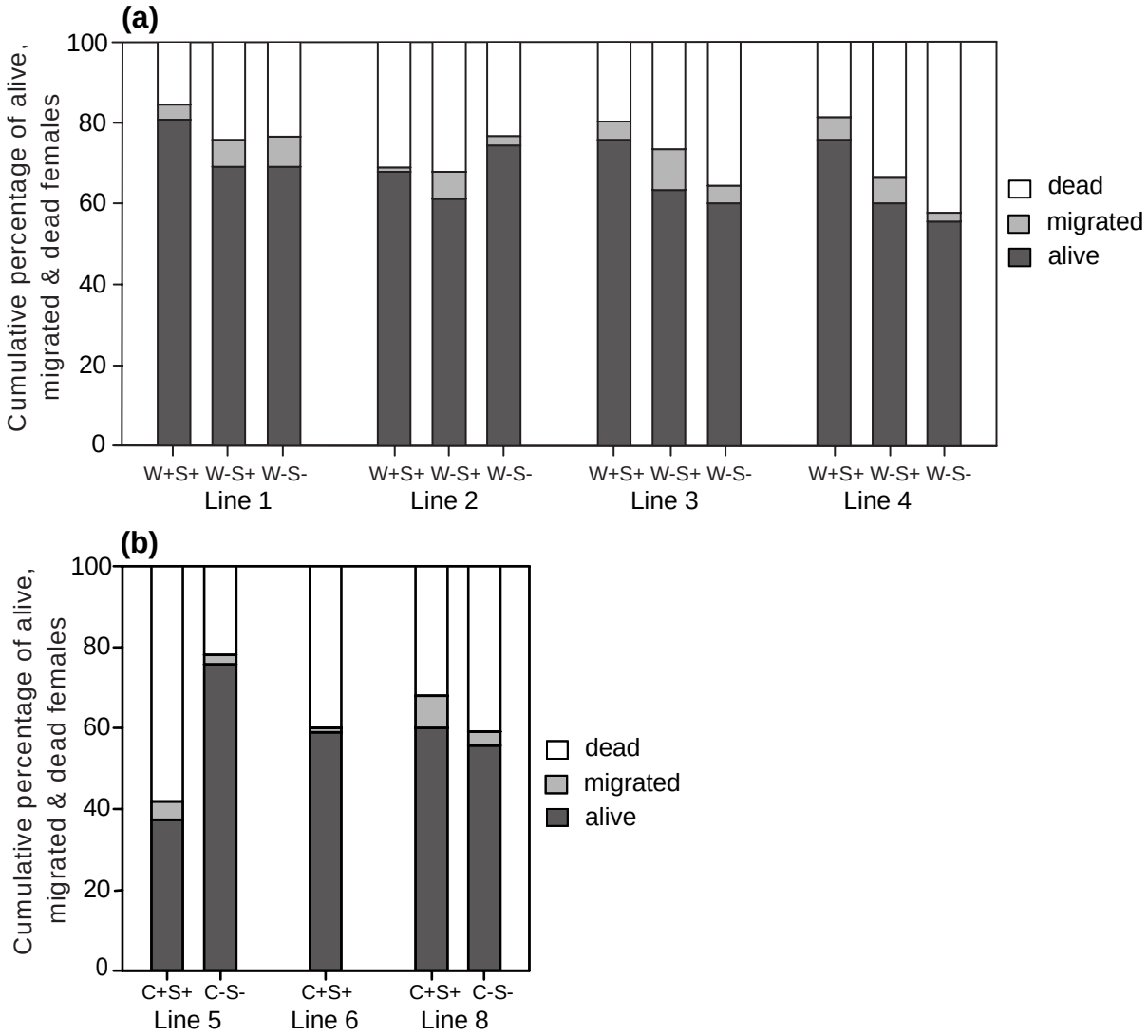

Figure S5.2. Survival, migration and mortality in two strains of the spider mite *Tetranychus urticae* which which did (+) or did not (-) contain *Wolbachia*, *Spiroplasma* and/or *Candidatus* Cardinium after feeding on tomato (*Solanum lycopersicum*) for 4 days. (a) Four lines of the plant defence suppressor strain Delier-1 were tested (1, 2, 3 and 4). Each line was subdivided into three mite groups: W+S+, W-S+ and W-S-; (b) Two lines (5 and 8) were tested for the plant defence inducer strain Santpoort-2 with two mite groups: C+S+ and C-S-. Additionally we tested C+S+ of line 6. C-S- of line 6 as well as both groups of line 7 went extinct before the experiment.

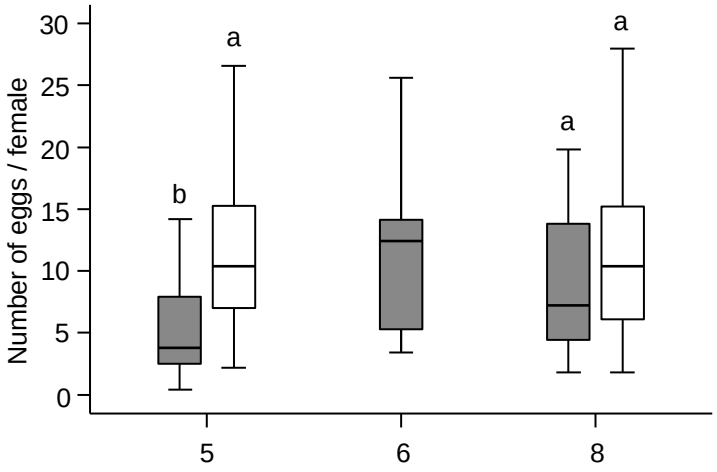

Figure S5.3. Reproductive performance (number of eggs produced per female in four days) of two lines (5, and 8) of a plant defence inducer strain Santpoort-2 of the spider mite *Tetranychus urticae*. Each of the lines was subdivided into two groups which did (+) or did not (-) contain the bacteria *Candidatus* Cardinium and *Spiroplasma*: C+S+ (grey) and C-S- (white). Additionally we tested C+S+ of line 6; the C-S- groups of line 6 as well as both groups of line 7 went extinct before the experiment. Boxes span the 25-75 percentiles, horizontal lines in the boxes represent medians, whiskers span 1.5*IQR (interquartile range), dots represent data points outside of this range. Different letters above the boxes indicate significant differences at a level of $P \leq 0.05$ (tested per line) after applying a linear mixed model.

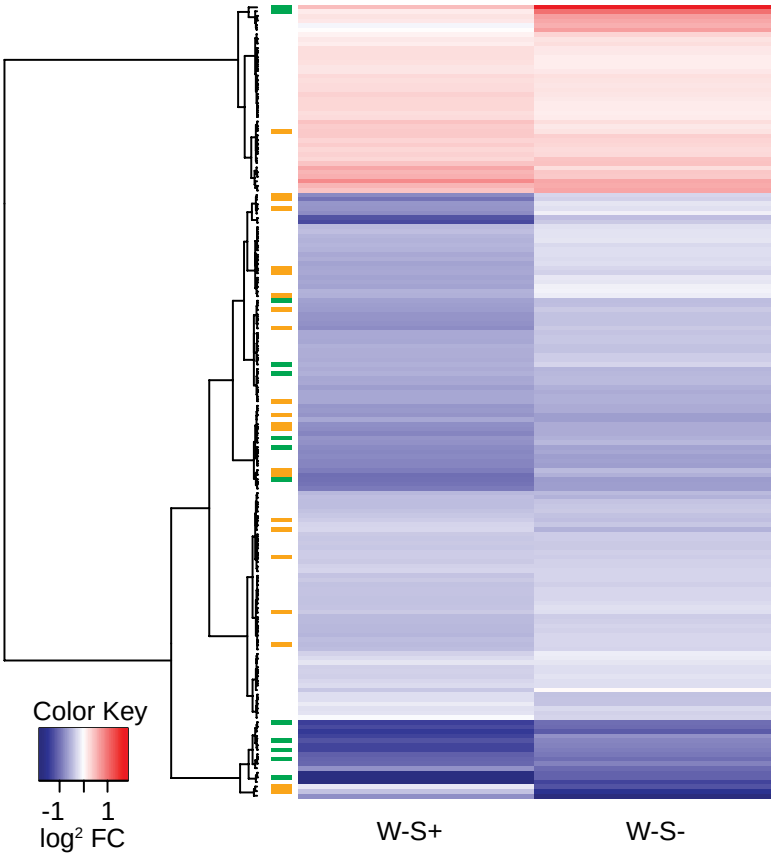

Figure S5.4. Transcription heatmap of differentially expressed genes in the *T. urticae* suppressor strain Delier-1 after removal of *Wolbachia* (W-S+) and *Wolbachia* and *Spiroplasma* (W-S-) as compared to transcription levels of the original strain (W+S+) that contained both bacteria. A total of 173 differentially expressed genes were detected after *Wolbachia* (W-S+) and *Wolbachia-Spiroplasma* removal (W-S-) and between the two treatments, mutually (FDR-corrected *P*-value ≤ 0.05). Using Euclidean distance metrics, genes were clustered based on their relative transcript levels in the W-S+ vs W+S+ and W-S- vs W+S+ comparisons. In the left sidebar, orange and green rectangles indicate genes that code for enzymes involved in the xenobiotic and digestive pathways of *T. urticae*, respectively.

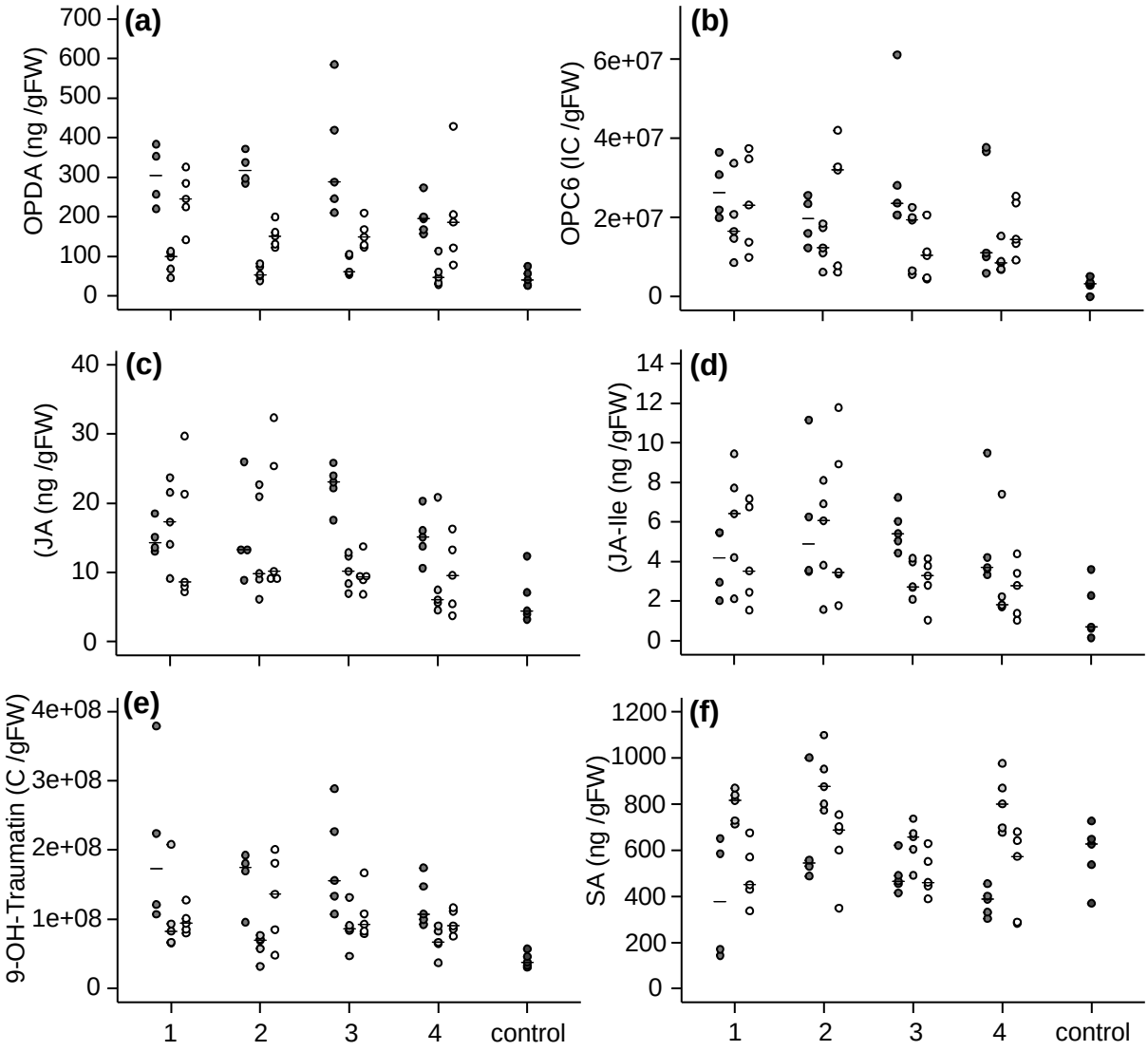

Figure S5.5. Phytohormone amounts in tomato (*Solanum lycopersicum*) leaflets after 7 days of infestation with four lines (1, 2, 3 and 4) of a plant defence suppressor strain Delier-1 of the spider mite *Tetranychus urticae.* Each of the lines was subdivided into three groups which did (+) or did not (-) contain the bacteria *Wolbachia* and *Spiroplasma*: W+S+ (dark grey), W-S+ (light grey) and W-S- (white). Control plants were not infested (darkest grey). Tested phytohormones included (a) 12-oxo-phytodienoic acid (OPDA), (b) 3-oxo-2-(2-pentenyl)-cyclopentane-1-hexanoic acid (OPC6), (c) jasmonic acid (JA) (d) jasmonic acid-isoleucine (JA-Ile), (e) 9-OH-traumatin, (f) free salicylic acid (SA); Circles represent individual data points, horizontal lines indicate the medians. Phytohormone amounts are presented as nanogram (ng) per gram fresh leaf weight (g FW). Compounds for which we did not have a pure standard are presented as ion counts (IC)/g FW.

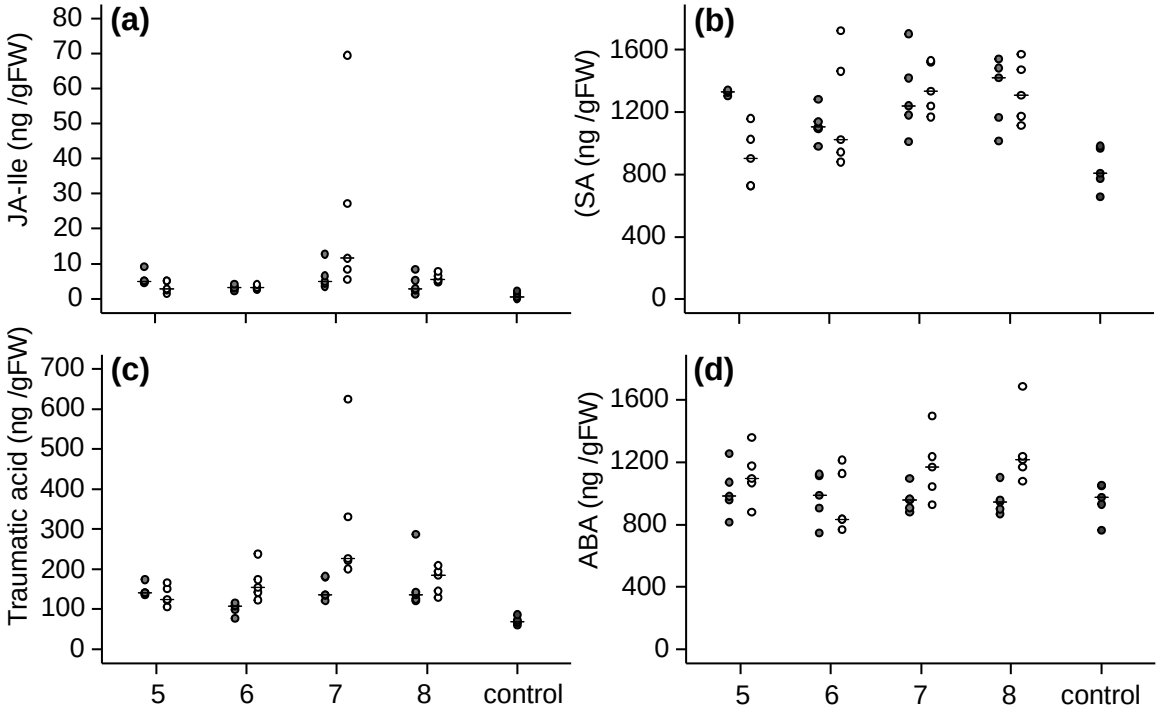

Figure S5.6. Phytohormone amounts in tomato (*Solanum lycopersicum*) leaflets after 7 days of infestation with four lines (5, 6, 7 and 8) of a plant defence inducer strain Santpoort-2 of the spider mite *Tetranychus urticae*. Each of the lines was subdivided into two groups which did (+) or did not (-) contain the bacteria *Candidatus* Cardinium and *Spiroplasma*: C+S+ (grey), C-S- (white). Control plants were not infested (dark grey). Tested phytohormones included (a) jasmonic acid-isoleucine (JA-Ile), (b) free salicylic acid (SA) (c) traumatic acid, (d) abscisic acid (ABA) (f); Circles represent individual data points, horizontal lines indicate the medians. Phytohormone amounts are presented as nanogram (ng) per gram fresh leaf weight (g FW). Compounds for which we did not have a pure standard are presented as ion counts (IC)/g FW.

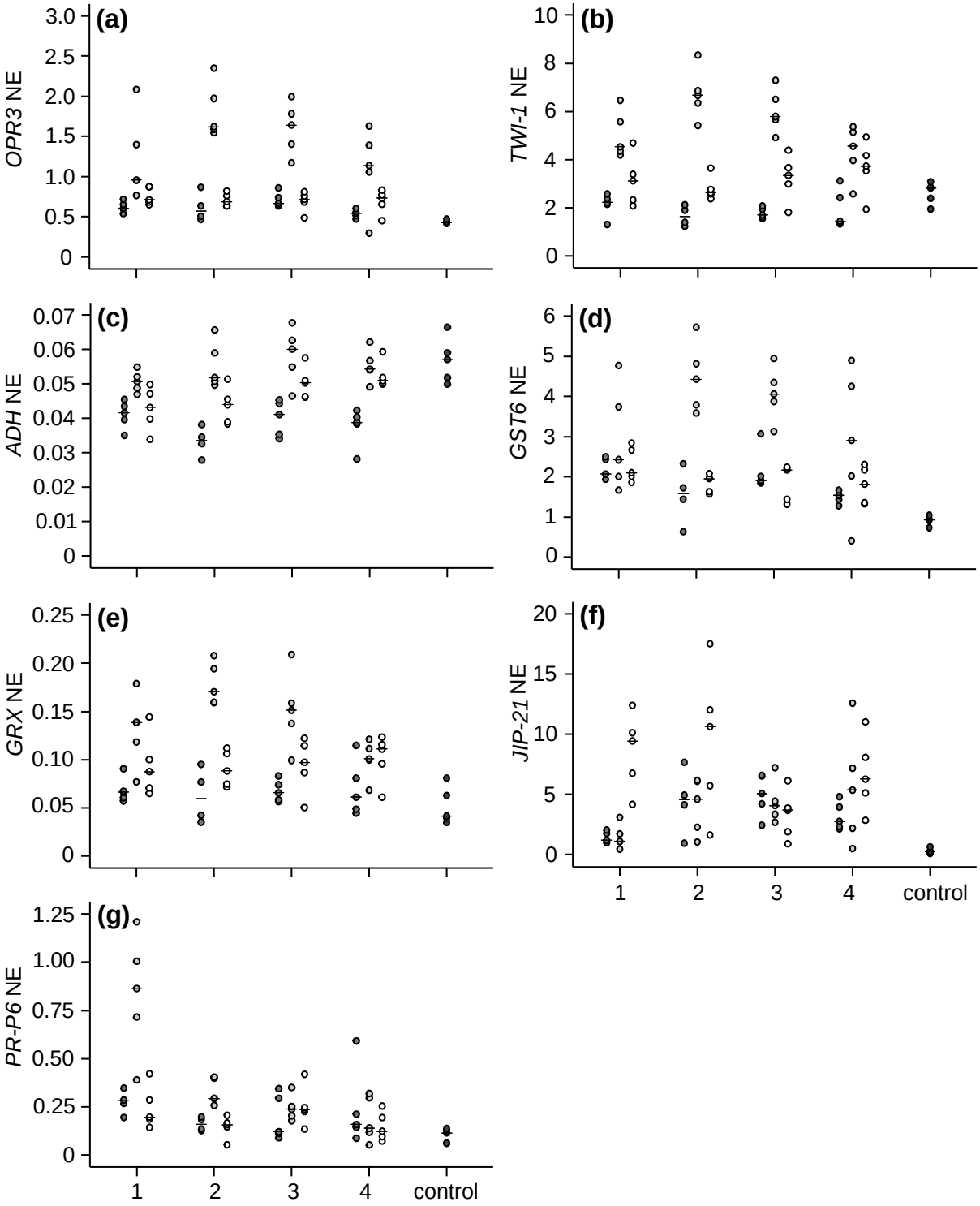

Figure S5.7. Normalized expression (NE) of plant defence related genes (qRT-PCR) in tomato (*Solanum lycopersicum*) leaflets after 7 days of infestation with four lines (1, 2, 3 and 4) of a plant defence suppressor strain Delier-1 of the spider mite *Tetranychus urticae*. Each of the lines was subdivided into three groups which did (+) or did not (-) contain the bacteria *Wolbachia* and *Spiroplasma*: W+S+ (dark grey), W-S+ (light grey) and W-S- (white). Control plants were not infested (darkest grey). (a*) 12-oxophytodienoate Reductase 3* (*OPR3*), (b) *Tomato wound-induced 1* (*TWI-1*), (c) *Alcohol dehydrogenase* (*ADH*), (d) *Glutathione* S*-transferase 6* (*GST6*), (e*) Glutaredoxin* (*GRX*), (f) *Jasmonate-inducible protein-21* (*JIP-21*), (g) *Pathogenesis-related protein 6* (*PR-P6*); Circles represent individual data points, horizontal lines indicate the medians.

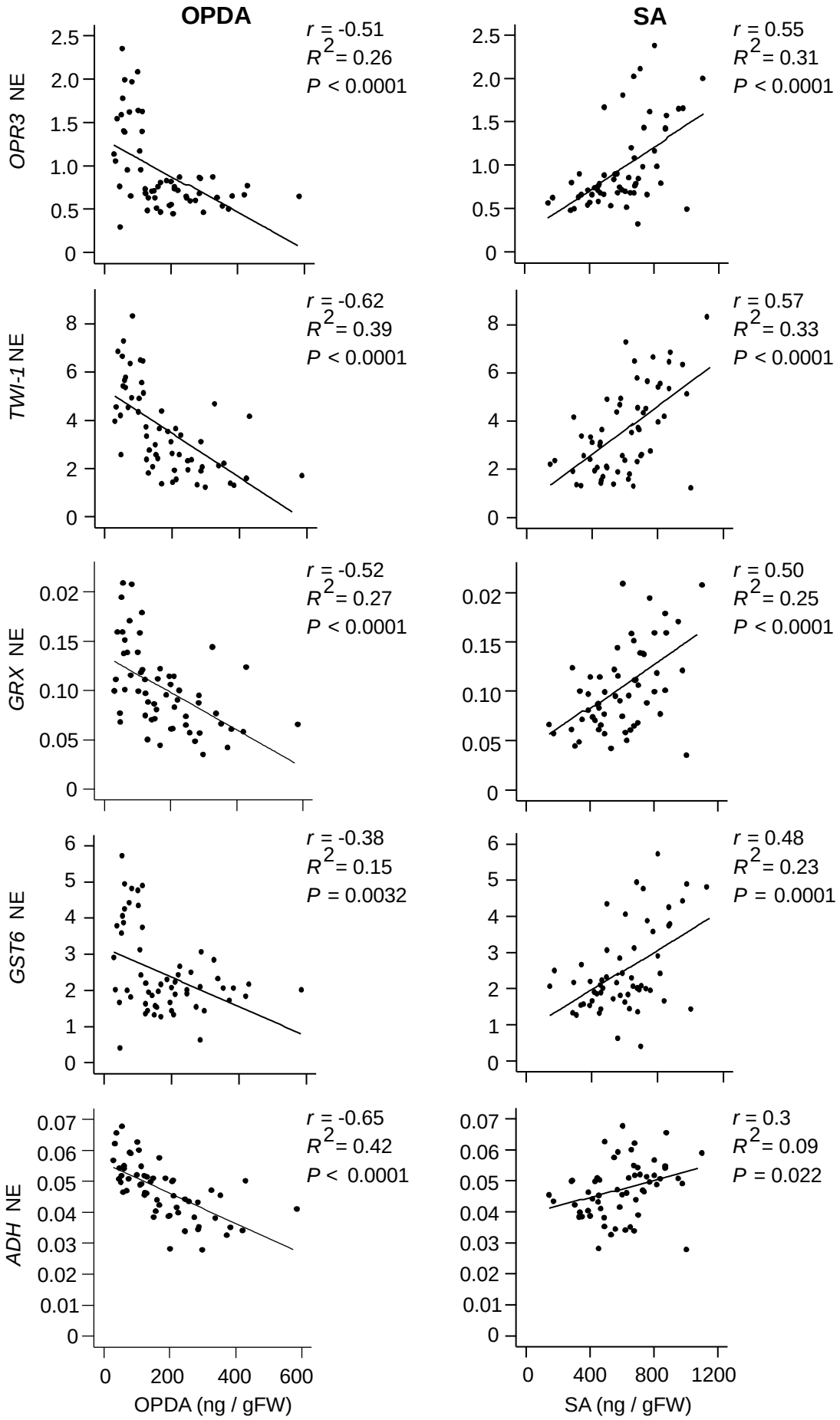
OPDA
SA
OPR3 NE
TWI-1 NE
GRX NE
GST6 NE
ADH NE
r = -0.51
R2 = 0.26
P < 0.0001
r = 0.55
R2 = 0.31
P < 0.0001
r = -0.62
R2 = 0.39
P < 0.0001
r = 0.57
R2 = 0.33
P < 0.0001
r = -0.52
R2 = 0.27
P < 0.0001
r = 0.50
R2 = 0.25
P < 0.0001
r = -0.38
R2 = 0.15
P = 0.0032
r = 0.48
R2 = 0.23
P = 0.0001
r = -0.65
R2 = 0.42
P < 0.0001
r = 0.3
R2 = 0.09
P = 0.022
OPDA (ng / gFW)
SA (ng / gFW)

Figure S5.8. Correlations between phytohormone amounts of salicylic acid (SA) and 12-oxo-phytodienoic acid (OPDA) and the normalized expression levels of putative OPDA responsive genes (*TWI-1*, *GRX*, *GST-6*, *ADH* and *OPR3*) measured in tomato leaflets after 7 days of infestations with the plant defence suppressor strain Delier-1 of the spider mite *Tetranychus urticae. TWI-1*: *Tomato wound-induced 1*, *GRX*: *Glutaredoxin*, *ADH*: *Alcohol dehydrogenase*, *GST6*: *Glutathione* S-*transferase 6*; *P*-values were adjusted for multiple comparisons with the holm method.

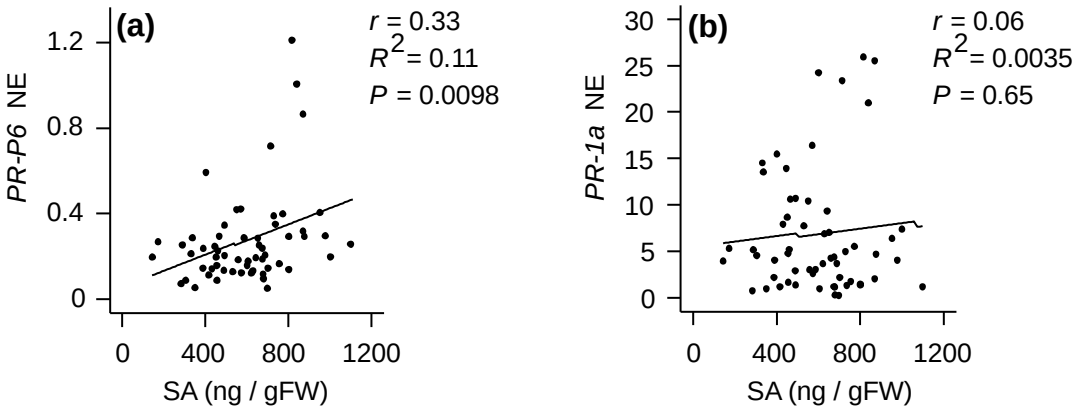

Figure S5.9. Correlations between phytohormone amounts of salicylic acid (SA) and the normalized expression levels of the two SA marker genes *PR-P6* and *PR-1a*, measured in tomato leaflets after 7 days of infestations with the plant defence suppressor strain Delier-1 of the spider mite *Tetranychus urticae. PR-P6*: *Pathogenesis-related protein 6*, *PR-1a*: *Pathogenesis-related protein 1a; P*-values were adjusted for multiple comparisons with the holm method.

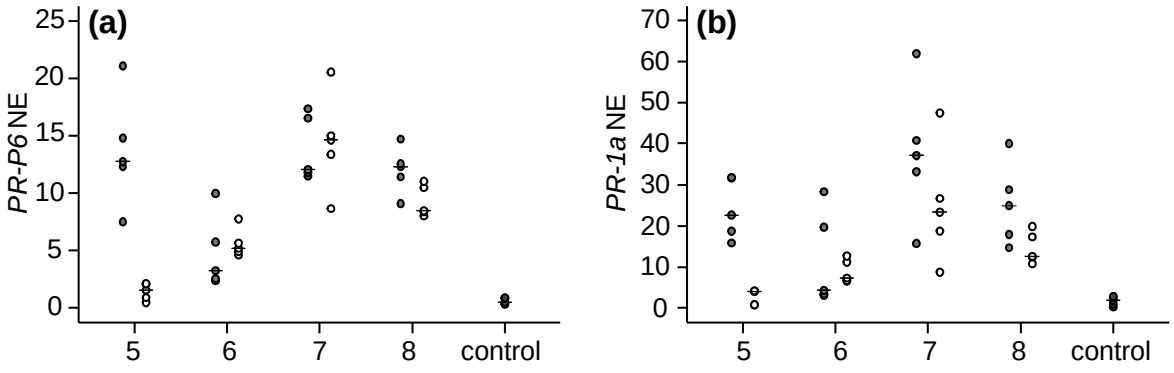

Figure S5.10. Normalized expression (NE) of plant defence related genes (qRT-PCR) in tomato (*Solanum lycopersicum*) leaflets after 7 days of infestation with four lines (5, 6, 7 and 8) of a plant defence inducer strain Santpoort-2 of the spider mite *Tetranychus urticae.* Each of the lines was subdivided into two groups which did (+) or did not (-) contain the bacteria *Candidatus* Cardinium and *Spiroplasma*: C+S+ (grey) and C-S- (white). Control plants were not infested (dark grey). (a*) Pathogenesis-related protein 6* (*PR-P6*), (b) *Pathogenesis-related protein 1a* (*PR-1a*); Circles represent individual data points, horizontal lines indicate the medians.

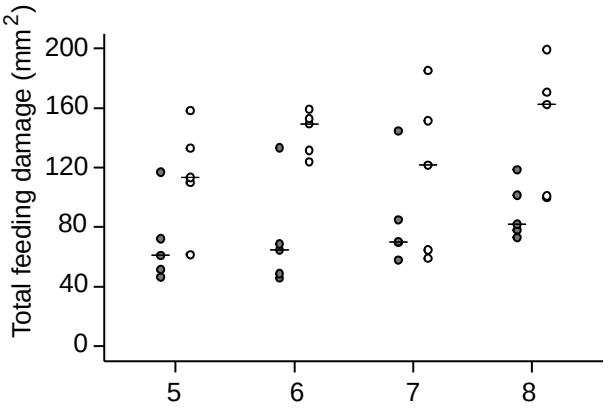

Figure S5.11. Feeding damage (mm^2) on tomato (*Solanum lycopersicum*) leaflets after 7 days of infestation with four lines (5, 6, 7 and 8) of a plant defence inducer strain Santpoort-2 of the spider mite *Tetranychus urticae.* Each of the lines was subdivided into two groups which did (+) or did not (-) contain the bacteria *Candidatus* Cardinium and *Spiroplasma*: C+S+ (grey) and C-S- (white). Circles represent individual data points, horizontal lines indicate the medians.

Table S5.1. Parameters used for detection of phytohormones and related compounds by LC-MS/MS

Compound	Q_1 *1	Q_3 *2	CID *3	CE *4	detected	Reference
SA	137	93	-35	15.0	yes	Wu *et al.*, 2007
D_6-SA (internal standard)	141	97	-35	15.0	yes	Alba *et al.*, 2015
ABA	263	153	-35	9.0	yes	Bonaventure, 2011
13-HPOT	309	291	-35	5.0	yes	Kallenbach *et al.*, 2010*5
dinor-OPDA	263	165	-35	18.0	no	Bao *et al.*, 2014
OPDA	291	165	-35	18.0	yes	Koo *et al.*, 2009
OPDA-GSH	598	306	-35	18.0	no	Dąbrowska *et al.*, 2009
OPC-8	293	275	-35	12.0	yes	Kallenbach*5; Bao *et al.* (2014)
OPC-6	265	247	-35	12.0	yes	Kallenbach*5
OPC-4	237	219	-35	12.0	no	Kallenbach*5
JA	209	59	-35	12.0	yes	Wu *et al.*, 2007
D_5-JA (internal standard)	213	61	-35	12.0	yes	Alba *et al.*, 2015
12-OH-JA	225	59	-35	19.0	no	Stitz *et al.*, 2011
12-OH-JA-Ile	338	130	-35	19.0	yes	Stitz *et al.*, 2011
12-COOH-JA-Ile	352	130	-35	19.0	no	Stitz *et al.*, 2011
12-oxo-(9*Z*)-dodecenoic acid	211	183	-35	13.5	no	Kallenbach *et al.*, 2011
(2*E*-) and (3*Z*)-dodecenedioic acid (traumatic acids)	227	183	-35	13.0	yes	Kallenbach *et al.*, 2011
12-OH-(9*Z*)-dodecenoic acid	213	183	-35	15.5	no	Kallenbach *et al.*, 2011)
9-OH-12-oxo-(10E)-dodecenoic acid (9-OH-traumatin)	227	209	-35	9.5	yes	Kallenbach *et al.*, 2011
4-OH- (2*E*)-dodecenedioic acid (4-OH-traumatic acid)	243	225	-35	9.5	yes	Kallenbach *et al.*, 2011
9,12-OH-(10E)-dodecenoic acid	229	211	-35	9.5	yes	Kallenbach *et al.*, 2011
9,12-OH-(10E)-dodecanoic acid	231	213	-35	9.5	no	Kallenbach *et al.*, 2011
9-OH-traumatin-GSH	534	306	-35	16.5	no	Kallenbach *et al.*, 2011
JA-Ala	280	88	-35	12.0	no	Koo *et al.*, 2009
JA-Gly	266	74	-35	12.0	no	Wang *et al.*, 2007
JA-Val	308	116	-35	12.0	no	Wang *et al.*, 2007
JA-Ile / JA-Leu	322	130	-35	19.0	yes	Wu *et al.*, 2007
JA-Pro	306	114	-35	12.0	no	Wang *et al.*, 2007
JA-Ser	296	104	-35	12.0	no	Wang *et al.*, 2007
JA-Thr	310	118	-35	12.0	no	Wang *et al.*, 2007
JA-Met	340	148	-35	12.0	no	Wang *et al.*, 2007
JA-His	346	154	-35	12.0	no	Wang *et al.*, 2007
JA-Phe	356	164	-35	12.0	no	Wang *et al.*, 2007
JA-Tyr	372	180	-35	12.0	no	Wang *et al.*, 2007
JA-Trp	395	203	-35	12.0	no	Wang *et al.*, 2007
JA-Gln	337	145	-35	19.0	no	Stitz *et al.*, 2011
JA-ACC	292	100	-35	12.0	yes	Wang *et al.*, 2007
JA-glucose	417	209	-35	18.0	no	VanDoorn *et al.*, 2011

*1 Q_1 mass = Molecular Weight – 1 = $[M - H]^-$ (*m/z*); *2 CID Q_3 mass (of daughter with 100% intensity) (*m/z*)
*3 Capillary Collision-Induced Dissociation (V); *4 Collision Energy (V); *5 personal communication

Table S5.2. Nucleotide sequence of primers used for PCR and (q)RT-PCR analysis

Target Organism(s)	Target Gene	Name	Gene Identifier (BOGAS/ITAG2.3)	Forward Primer 5' → 3'	Reverse Primer 5' → 3'	References
Wolbachia pipientis	*16SrRNA*	*16S ribosomal RNA*	-	TTGTAGCCTGCTATGGTATAACT	GAATAGGTATGATTTTCATGT	O'Neill *et al.*, 1992
Cardinium sp. (CLO; Cytophaga Like Organism)	*16SrRNA*	*16S ribosomal RNA*	-	GCGGTGTAAAATGAGCGTG	ACCTMTTCTTAACTCAAGCCT	Weeks *et al.*, 2003
Spiroplasma sp.	*dnaA*	*DnaA*	-	ATTCTTCAGTAAAAATGCTTGGA	ACACATTTACTTCATGCTATTGA	Fukatsu *et al.*, 2001
Bacteria (general)	*16SrRNA*	*16S ribosomal RNA*	-	TCCTACGGGNGGCWGCAG	TGACTACHVGGGTATCTAAKCC	Klindworth *et al.*, 2012
Tetranychus urticae	*Actin*	*Actin*	Tetur03g09480	CAGCCATGTATGTTGCCATC	AAATCACGACCAGCCAAATC	Feng *et al.*, 2010
Solanum lycopersicum	*AOS1*	*Allene Oxide Synthase 1*	Solyc04g079730.1	AACAGTGTGCCGGAAAAGAC	AATGGAGATGCACCGACTTC	Howe *et al.*, 2000; Sivasankar *et al.*, 2000
Solanum lycopersicum	*OPR3*	*OPDA reductase 3*	Solyc07g007870.2	GATCCAGTTGTGGGATACACAG	GCCCAACAAAATCAGGTTTC	Strassner *et al.*, 2002
Solanum lycopersicum	*TWI-1*	*Tomato Wound-induced 1*	Solyc01g107820.2	CATCTTACAATGGATGGGCTAC	CGAGATGATTGATCTTGGATTC	Truesdale *et al.*, 1996
Solanum lycopersicum	*ADH*	*Alcohol Dehydrogenase*	Solyc01g087640.2	GTCTTGAGTTGAGCGTGAAGG	CAGGCCTAGTGTATTCCGTTTC	Tieman *et al.*, 2007
Solanum lycopersicum	*GST6*	*Gluthathione S-transferase*	Solyc06g009020.2	GTGAAGAGCTTGTTCGATGC	CCTTCCTTTCAACGCGATAC	This study
Solanum lycopersicum	*GRX*	*Glutaredoxin*	Solyc07g053550.1	ATGATGCAACAAGCACTTCC	GATGATGTCGATCAACTCTTGG	This study
Solanum lycopersicum	*JIP-21*	*Jasmonate-inducible protein 21*	Solyc03g098790.1	ACTCGTCCTGTGCTTTGTCC	CCCAAGAGGATTTTCGTTGA	Lisón *et al.*, 2006
Solanum lycopersicum	*TD2*	*Threonine Deaminase-2*	Solyc09g008670.2	TGCCGTTAAAAATGTCACCA	ACTGGCGATGCCAAAATATC	Chen *et al.*, 2005
Solanum lycopersicum	*PR-1a*	*Pathogenesis-related protein 1a*	Solyc09g007010.1	TGGTGGTTCATTTCTTGCAACTAC	ATCAATCCGATCCACTTATCATTTTA	van Kan *et al.*, 1992
Solanum lycopersicum	*PR-P6*	*Pathogenesis-related protein P6*	Solyc00g174340.1	GTACTGCATCTTCTTGTTTCCA	TAGATAAGTGCTTGATGTGCC	van Kan *et al.*, 1992
Solanum lycopersicum	*PI-Iic*	*Proteinase Inhibitor Iic*	Solyc03g020050.2	CAGGATGTACGACGTGTTGC	GAGTTTGCAACCCTCTCCTG	Gadea *et al.*, 1996
Solanum lycopersicum	*Actin*	*Actin*	Solyc03g078400.2	TCAGCACATTCCAGCAGATGT	AACAGACAGGACACTCGCACT	Tomato Genome Consortium, 2012

Table S5.3a. List of top-20 genes significantly up-regulated in adult female *Tetranychus urticae* suppressor (DeLier-1) W-S+ mites as compared to W+S+ mites, after feeding from tomato (*Solanum lycopersicum*) for seven days ($P \leq 0.05$, after *P*-value adjustment).
W, *Wolbachia* sp.; S, *Spiroplasma* sp.; +, present; -, absent

#	Locus identifier	Annotation (BOGAS)	Fold change	*P*-value
1	tetur06g06585	Hypothetical protein (snosnR61, small nucleolar RNA)	1,81	0,0086
2	tetur18g02170	Hypothetical protein (no hits found)	1,54	0,0031
3	tetur24g01690	Hypothetical protein (similar to sorting nexin 13)	1,49	0,0261
4	tetur05g04420	Hypothetical protein (choline/ethanolaminephosphotransferase 1)	1,45	0,0344
5	tetur31g00280 [d]	CSPCA1: CUB domain-containing Secreted Protein Conserved in Arthropods	1,44	0,0185
6	tetur39g00780	Hypothetical protein (vitellogenin1)	1,35	0,0449
7	tetur04g06140 [d]	Hypothetical protein (IscW_ISCW012949)	1,34	0,0140
8	tetur05g02950	CYP389C2: Cytochrome P450	1,31	0,0127
9	tetur23g01800	Hypothetical protein (Rho GTPase-activating protein 29)	1,30	0,0295
10	tetur09g04980	Hypothetical protein (no hits found)	1,29	0,0069
11	tetur10g04770	Hypothetical protein (leukocyte elastase inhibitor)	1,28	0,0216
12	tetur60g00070	Hypothetical protein (TcasGA2_TC006277)	1,25	0,0184
13	tetur07g01880	SSPE2: Small Secreted Protein, Family E	1,25	0,0143
14	tetur06g03890	Hypothetical protein (trichohyalin, putative)	1,24	0,0483
15	tetur05g08770 [d]	Hypothetical protein (intracellular protein transport)	1,23	0,0014
16	tetur05g03440	Hypothetical protein (transmembrane and coiled-coil domains protein 2-like)	1,22	0,0414
17	tetur37g01020	Hypothetical protein (M-phase inducer phosphatase)	1,21	0,0086
18	tetur23g01870	Hypothetical protein (tyrosine kinase, putative)	1,21	0,0146
19	tetur02g10760	Hypothetical protein (no hits found)	1,20	0,0190
20	tetur06g01790	Hypothetical protein (mRNA splicing protein SMN)	1,20	0,0344

d, these genes were significantly up-regulated in *T. urticae* suppressor W-S- mites as compared to W+S+ mites.

Table S5.3b. List of top-20 genes significantly down-regulated in adult female *Tetranychus urticae* suppressor (DeLier-1) W-S+ mites as compared to W+S+ mites, after feeding from tomato (*Solanum lycopersicum*) for seven days ($P \leq 0.05$, after *P*-value adjustment).
W, *Wolbachia* sp.; S, *Spiroplasma* sp.; +, present; -, absent

#	Locus identifier	Annotation (BOGAS)	Fold change	*P*-value
1	tetur16g03730 [d]	Hypothetical protein (similar to WT Secreted Protein 12)	-3,44	0,0020
2	tetur16g03680 [d]	TuPap-31: Cathepsin L	-3,28	0,0014
3	tetur16g03740 [d]	WTSP12: WT Secreted Protein 12	-3,25	0,0014
4	tetur16g03620 [d]	Hypothetical protein (similar to WT Secreted Protein 12)	-2,74	0,0032
5	tetur196g00010 [d]	Hypothetical protein (Legumain)	-2,64	0,0025
6	tetur44g00311	Hypothetical protein (transposon)	-2,58	0,0121
7	tetur32g02297	SSPF13: Small Secreted Protein, Family F	-2,56	0,0223
8	tetur16g03670	TuLeg-13: Legumain	-2,52	0,0031
9	tetur16g03610	WTSP4: WT Secreted Protein, pseudogene	-2,44	0,0020
10	tetur31g00810	Hypothetical protein (similar to methyl-accepting chemotaxis sensory transducer, extracellular)	-2,43	0,0321
11	tetur16g03770	TuPap-44: Cathepsin L	-2,35	0,0086

12	tetur128g00030	Hypothetical protein (Serpin 3 inhibitory serine protease inhibitor, putative secreted salivary gland protein)	-2,32	0,0365
13	tetur31g00830	Hypothetical protein (methyl-accepting chemotaxis sensory transducer, extracellular)	-2,17	0,0310
14	tetur01g06610 [d]	TuLeg-17: Legumain	-2,11	0,0062
15	tetur39g00220	Hypothetical protein (extracellular)	-2,08	0,0143
16	tetur11g05410	Hypthetical protein (MFS-transporter)	-2,00	0,0030
17	tetur06g03540	TuLeg-7: Legumain	-1,99	0,0044
18	tetur01g00490	ID-RCD: intradiol ring-cleavage dioxygenase	-1,96	0,0101
19	tetur33g01640	Hypothetical protein (extracellular)	-1,96	0,0179
20	tetur03g02710 [d]	Hypothetical protein (phospholipid scramblase 2-like)	-1,89	0,0014

d, these genes were significantly down-regulated in *T. urticae* suppressor W-S- mites as compared to W+S+ mites.

Table S5.4a. List of all genes significantly up-regulated in adult female *Tetranychus urticae* suppressor (DeLier-1) W-S- mites as compared to W+S+ mites, after feeding from tomato (*Solanum lycopersicum*) for seven days ($P \leq 0.05$, after *P*-value adjustment).
W, *Wolbachia* sp.; S, *Spiroplasma* sp.; +, present; -, absent

#	Locus identifier	Annotation (BOGAS)	Fold change	*P*-value
1	tetur06g06650 [y]	TuPap-19: Cystatin (onchocystatin)	3,60	0,0197
2	tetur06g01060 [b y]	TuCPI-22: Cystatin (cystatin precursor)	2,03	0,0008
3	tetur02g15167	SP68: Serine Protease (Peptidase S1)	1,63	0,0443
4	tetur15g00310	CSPA1: Cell Surface Protein with GTPLASR(K,R,S) repeats	1,63	0,0247
5	tetur11g00700	Hypothetical protein (tribbles homolog 2, serine/threonine-protein kinase)	1,55	0,0151
6	tetur17g02060 [b]	Hypothetical protein (secreted salivary gland peptide, putative)	1,54	0,0002
7	tetur31g00280 [d]	CSPCA1: CUB domain-containing Secreted Protein Conserved in Arthropods	1,53	0,0176
8	tetur15g00320	CSPA2: Cell Surface Protein with GTPLASR(K,R,S) repeats	1,49	0,0443
9	tetur04g06140 [d]	Hypothetical protein (IscW_ISCW012949)	1,36	0,0197
10	tetur11g00270	Hypothetical protein (signal recognition particle 14 kDa protein)	1,33	0,0493
11	tetur11g04330	Hypothetical protein (ADP-ribosylation factor GTPase-activating protein 3)	1,24	0,0324
12	tetur05g08770 [d]	Hypothetical protein (no hits found)	1,19	0,0151
13	tetur04g05590	TuGR62: Chemosensory Receptor, Gustatory receptor family	1,17	0,0392
14	tetur14g02440	Hypothetical protein (An09g06400, *Aspergillus niger* chitinase)	1,15	0,0369

b, these genes were significantly up-regulated in *T. urticae* suppressor W-S- mites as compared to W-S+ mites. d, these genes were significantly up-regulated in suppressor W-S+ mites as compared to W+S+ mites. y, these genes were significantly up-regulated in *T. urticae* inducer (Santpoort-2) C-S- mites as compared to C+S+ mites. C, *Candidatus* Cardinium

Table S5.4b. List of top-20 genes significantly down-regulated in adult female *Tetranychus urticae* suppressor (DeLier-1) W-S- mites as compared to W+S+ mites, after feeding from tomato (*Solanum lycopersicum*) for seven days ($P \leq 0.05$, after *P*-value adjustment).
W, *Wolbachia* sp.; S, *Spiroplasma* sp.; +, present; -, absent

#	Locus identifier	Annotation (BOGAS)	Fold change	*P*-value
1	tetur03g08800 [b d]	SSPB2: Small secreted protein, family B	-3,36	0,0000
2	tetur03g05070	CYP392D8: Cytochrome P450	-2,90	0,0008
3	tetur16g03730 [d]	Hypothetical protein (no hits found)	-2,53	0,0315
4	tetur03g09961	CYP392D7: Cytochrome P450	-2,34	0,0197
5	tetur16g03620 [d]	Hypothetical protein (similar to WT Secreted Protein 12)	-2,21	0,0392
6	tetur16g03740 [d]	WTSP12: WT Secreted Protein 12	-2,13	0,0498
7	tetur16g03680 [d]	TuPap-31: Cathepsin L	-2,12	0,0443
8	tetur03g08810 [d]	SSPB1: Small Secreted Protein, Family B	-2,04	0,0151
9	tetur196g00010 [d]	Hypothetical protein (Legumain)	-2,01	0,0455
10	tetur01g06610 [d]	TuLeg-17: Legumain	-2,00	0,0199
11	tetur03g02710 [d]	Hypothetical protein (phospholipid scramblase 2-like)	-1,59	0,0197
12	tetur03g07920 [d]	TuGSTd06: Glutathione S-transferase, delta class	-1,58	0,0262
13	tetur28g01730 [d]	Hypothetical protein (3-oxoacyl-acyl-carrier-protein reductase)	-1,58	0,0315
14	tetur06g02930 [d]	TuPap-45: fibroinase precursor, cathepsin L	-1,55	0,0186
15	tetur01g03150 [d]	CCEinc-01: Carboxyl/choline esterase, incomplete	-1,50	0,0455
16	tetur22g02500 [d]	Hypothetical protein (No hits found)	-1,50	0,0151
17	tetur16g03750 [d]	Hypothetical protein (3-Pan modules Cell Surface Protein, antigen-2-like)	-1,50	0,0460
18	tetur03g05030	CYP392D6: Cytochrome P450	-1,44	0,0239
19	tetur02g10560 [d]	AGO-1G: ortholog of Argonaute-1 (*Drosophila melanogaster*)	-1,44	0,0307
20	tetur01g03410 [d]	Hypothetical protein (apical endosomal glycoprotein)	-1,40	0,0418

b, this gene was significantly down-regulated in *T. urticae* suppressor W-S- mites as compared to W-S+ mites. d, these genes were significantly down-regulated in suppressor W-S+ mites as compared to W+S+ mites.

Table S5.5. List of all genes significantly up- and down-regulated respectively, in adult female *Tetranychus urticae* suppressor (DeLier-1) W-S- mites as compared to W-S+ mites, after feeding from tomato (*Solanum lycopersicum*) for seven days ($P \leq 0.05$, after *P*-value adjustment).
W, *Wolbachia* sp.; S, *Spiroplasma* sp.; +, present; -, absent

#	Locus identifier	Annotation (BOGAS)	Fold change	*P*-value
1	tetur06g01060 [v]	TuCPI-22: Cystatin (cystatin precursor)	1,84	0,0187
2	tetur17g02060 [v]	Hypothetical protein (secreted salivary gland peptide, putative)	1,39	0,0187

#	Locus identifier	Annotation (BOGAS)	Fold change	*P*-value
1	tetur03g08800 [x]	SSPB2: Small secreted protein, family B	-1,96	0,0235

v, these genes were significantly up-regulated in *T. urticae* suppressor W-S- mites as compared to W+S+ mites and in inducer (Santpoort-2) C-S- mites as compared to C+S+ mites. x, this gene was significantly down-regulated in both suppressor W-S- and W-S+ mites as compared to W+S+ mites. C, *Candidatus* Cardinium

Table S5.6a. List of all genes significantly up-regulated in adult female *Tetranychus urticae* inducer (Santpoort-2) C-S- mites as compared to C+S+ mites, after feeding from tomato (*Solanum lycopersicum*) for seven days ($P \leq 0.05$, after *P*-value adjustment).
C, *Candidatus* Cardinium; S, *Spiroplasma* sp.; +, present; -, absent

#	Locus identifier	Annotation (BOGAS)	Fold change	*P*-value
1	tetur11g05780 [o]	Hypothetical protein (no hits found)	5,17	0,0046
2	tetur06g06650 [h]	TuPap-19: Cystatin (onchocystatin)	5,03	0,0001
3	tetur06g01060 [p]	TuCPI-22: Cystatin (cystatin precursor)	3,02	0,0001
4	tetur11g05760	TuCCE-33: Carboxyl/choline esterase (putative esterase)	2,84	0,0288
5	tetur37g00580	Hypothetical protein (4-nitrophenylphosphatase)	2,32	0,0008
6	tetur08g08060	Hypothetical protein (secreted salivary gland peptide, putative)	2,11	0,0008
7	tetur17g02060 [p]	Hypothetical protein (secreted salivary gland peptide, putative)	1,96	0,0004
8	tetur01g02310	5TM1: 5-pass Trans-membrane protein of unknown function	1,80	0,0337
9	tetur11g00550	Hypothetical protein (MFS-transporter)	1,72	0,0046
10	tetur08g07240	Hypothetical protein (secreted salivary gland peptide, putative)	1,61	0,0066
11	tetur11g05000 [o]	CYP385C2: Cytochrome P450	1,61	0,0313
12	tetur23g00470	Hypothetical protein (no hits found)	1,58	0,0108
13	tetur03g05040	Hypothetical protein (Cytochrome P450 - fragment)	1,55	0,0398
14	tetur33g01340	Hypothetical protein (ribosomal protein)	1,47	0,0017
15	tetur03g04990	CYP392D2: Cytochrome P450	1,46	0,0143
16	tetur02g09850	TuUT11: UDP-glycosyltransferase (UGT)	1,45	0,0185
17	tetur01g10390	TuABCC-02: ABC-transporter, class C	1,43	0,0064
18	tetur02g02190	Hypothetical protein (tRNA)	1,41	0,0100
19	tetur10g01570	TuCAS: β-cyanoalanine synthase	1,38	0,0100
20	tetur13g03000	Hypothetical protein (no hits found)	1,36	0,0023
21	tetur89g00030	Hypothetical protein (no hits found)	1,33	0,0307
22	tetur11g05670	Hypothetical protein (ornithine aminotransferase)	1,32	0,0145
23	tetur11g05680	Hypothetical protein (vacuolar protein sorting 13 homolog D)	1,29	0,0337

h, this gene was significantly up-regulated in *T. urticae* suppressor (Delier-1) W-S- mites as compared to W+S+ mites. o, these genes were significantly down-regulated in suppressor W-S+ mites as compared to W+S+ mites. p, these genes were significantly up-regulated in suppressor W-S- mites as compared to both W+S+ and W-S+ mites. W, *Wolbachia* sp.

Table S6b. List of all genes significantly down-regulated in adult female *Tetranychus urticae* inducer (Santpoort-2) C-S- mites as compared to C+S+ mites, after feeding from tomato (*Solanum lycopersicum*) for seven days ($P \leq 0.05$, after *P*-value adjustment).
C, *Candidatus* Cardinium; S, *Spiroplasma* sp.; +, present; -, absent

#	Locus identifier	Annotation (BOGAS)	Fold change	*P*-value
1	tetur11g05550	Hypothetical protein (MFS-transporter)	-1,59	0,0219
2	tetur18g02050	Hypothetical protein (no hits found)	-1,55	0,0369
3	tetur11g05720	PLAT10: Lipase/lipoxygenase	-1,47	0,0066
4	tetur02g13460	Hypothetical protein (very acidic salivary protein)	-1,46	0,0337
5	tetur01g13560	ZN207: Zinc Finger protein 207	-1,37	0,0436
6	tetur28g01570	Hypothetical protein (deoxyhypusine synthase)	-1,36	0,0185
7	tetur11g06310	Hypothetical protein (galactose-binding domain-like)	-1,36	0,0079
8	tetur05g03510	Hypothetical protein (ribosome production factor 2 homolog)	-1,35	0,0143
9	tetur15g00160	Hypothetical protein (no hits found)	-1,30	0,0329
10	tetur27g02556	SLCP1: secreted low complexity protein, putative	-1,29	0,0313

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6

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; Fürnkranz *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*-phytodienoic 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 persistance 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 interspecific 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 (Chapter 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 (Rolff, 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 *H. virescens* females trade off immune defence activation after bacterial challenge for mating success, while males do not.

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; Jurenka, 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 virgin females trade-off immunity with reproduction (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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Summary

Bacteria are omnipresent in nature and a multitude of associations between bacteria and higher organisms (plants, animals and fungi) have been described. The effect of bacteria can vary from beneficial to neutral to detrimental as for the fitness of the organism with which the bacteria are associated. In this thesis, I investigated the bacterial communities as well as pathogenic and non-pathogenic effects of bacteria on life history traits of two generalist herbivorous arthropods. In the noctuid moth *Heliothis virescens*, I described the variability of bacterial communities that are associated with this moth in the laboratory and the field (Chapter 2). Furthermore, I determined the effect of immune challenge on mating success, female sex pheromone composition and oviposition strategy (Chapters 3 & 4). In the two-spotted spider mite, *Tetranychus urticae*, I investigated the bacterial communities of two of its strains, as well as the effects of *Wolbachia*, *Cardinium* and *Spiroplasma* on mite survival and oviposition, mite gene expression and the induction of tomato responses after mite feeding (Chapter 5).

In **Chapter 2,** I describe the variability of bacterial communities that are associated with the moth *H. virescens* that was determined by using bacterial tag-encoded FLX pyrosequencing of 16S rRNA amplicons. Bacterial communities varied between *H. virescens* larvae that had fed on different host plants and between larvae originating from the field and those from laboratory rearing. Notably, while larvae from the laboratory contained an unidentified *Enterococcus* strain in high relative amounts, this bacterial strain was absent in field larvae. Moreover, field larvae contained a more diverse bacterial community than larvae from the laboratory. Bacterial communities also varied between biological replicates in larvae from the field and between moths of different developmental stages, i.e. eggs, larvae and female adults. Overall, the variability of bacterial communities in *H. virescens* suggests that this moth has not evolved a long-term, stable symbiosis with the detected bacteria. Whether bacteria that are transiently present in these moths have a beneficial effect on fitness and may for instance support host plant utilization remains to be investigated.

In **Chapter 3,** I show sexual dimorphism in immune system activation of the moth *H. virescens* in response to an immune challenge with dead cells of the bacterial entomopathogen *Serratia entomophila*. When measuring expression levels of immunity-related genes, females showed higher immune deployment, i.e. immune system activation in response to a pathogenic threat, whereas males had higher immune maintenance, i.e. their

immune system was at a higher basic level of immune defence than females. Furthermore, the female immune response seemed to differentiate more between wounding and bacterial challenge, suggesting a more specific and possibly more cost-efficient immune response than the male immune system. Sexual dimorphism in immune response matched the results from mating experiments: bacteria-challenged females had lower mating success than non-challenged females, while males showed no differences in mating success between bacteria-challenged, wounded or non-challenged males. This suggests that females trade off immunity for mating success, while males do not. Lastly, the sex pheromone composition of bacteria-challenged females differed from that of non-challenged and wounded females, suggesting that females may trade off immunity for sexual signalling, although the differences in female sex pheromone cannot explain the differences in mating success between females. Overall, the observed difference in the type of immunity between males and females adds a new dimension to the research of sexual dimorphism in immunity.

In **Chapter 4,** I demonstrate that immune challenge with dead cells of the bacterial entomopathogen *S. entomophila* affects the oviposition strategy of *H. virescens*. Females increased their oviposition rate in response to bacterial challenge. Notably, bacteria-challenged females had shorter life spans, but did not lay significantly fewer eggs than control females, indicating that bacteria-challenged females can compensate for a shorter life-span. However, bacteria-challenged females were less selective in terms of oviposition site than control females. Bacteria-challenged females laid equal numbers of eggs on plants that were infested with *H. virescens* larvae as on uninfested plants, whereas control females preferred uninfested plants. Since *H. virescens* larvae are cannibalistic and infested plants can be expected to have higher levels of secondary plant metabolites, non-preference of bacteria-challenged females likely has a negative effect on female fitness. The strategy of *H. virescens* females to increase their oviposition rate after an immune-challenge, which may compensate for a shortened life span, might thus come at the cost of reduced growth and survival of their offspring.

In **Chapter 5** it is shown that various (endo)symbiotic bacteria of *T. urticae*, i.e. *Wolbachia, Cardinium* and *Spiroplasma,* affect mite survival and oviposition, and mite gene expression as well as induced responses of tomato, the mite's host plant, after mite feeding. These effects were investigated in two strains of *T. urticae*, one plant defence suppressor strain (Delier-1) and one plant defence inducer strain (Santpoort-2) which contained different endosymbionts: while the suppressor strain harboured *Wolbachi*a (W) and *Spiroplasma* (S), the inducer strain harboured *Cardinium* (C) and *Spiroplasma* (S). By means of antibiotic

treatment, different mite groups were produced, containing or not containing W and S or C and S.

In the suppressor strain, the presence of *Wolbachia* was associated with higher survival of the mites, and higher expression of digestion- and detoxification-related genes, compared to mites that did not contain *Wolbachia*. Additionally, mites with *Wolbachia* reduced salicylic acid (SA) levels in tomato leaflets compared to leaflets that were infested with mites that contained only *Spiroplasma*. Further, *Spiroplasma* was associated with reduced JA-precursor [12-oxophytodienoic acid (OPDA)] levels in tomato leaflets, while *Wolbachia* had the opposite effect.

In the inducer strain, mites that contained *Cardinium* and *Spiroplasma* had lower survival, laid lower amounts of eggs and fed less than mites that did not contain the two bacterial strains. Moreover, leaflets that were infested with C+S+ mites had higher expression of SA-marker genes, and leaflets accumulated lower amounts of abscisic acid (ABA), than leaflets infested with mites without these bacteria. Further, C+S+ mites caused rusty/red feeding scars, while C-S- mites caused white feeding scars. Overall, *Wolbachia* seems to be beneficial for the mites, while *Cardinium* is likely a parasite. Even though the role of *Spiroplasma* is less clear, the results suggest that *Spiroplasma* may interact with *Wolbachia* in the suppressor strain, as *Wolbachia* seems to counteract the effects of *Spiroplasma*.

Samenvatting

Bacteriën zijn alom aanwezig in de natuur en derhalve bestaat er een grote verscheidenheid aan associaties tussen bacteriën en hogere organismen (planten, dieren en schimmels). Effecten van bacteriën kunnen variëren van gunstig tot neutraal en schadelijk voor het organisme waarmee de bacteriën geassocieerd zijn. In dit proefschrift heb ik de samenstelling van de bacteriële gemeenschappen en de pathogene en niet-pathogene effecten van bacteriën op de levensgeschiedenis kenmerken van twee generalistische geleedpotigen onderzocht. In de nachtvlinder *Heliothis virescens* heb ik de variabiliteit van de aanwezige bacteriële gemeenschappen in het veld en in het laboratorium in kaart gebracht (hoofdstuk 2). Ook heb ik het effect van een immuun uitdaging (d.w.z. infectie) op het paringssucces en de samenstelling van het seksferomoon in vrouwtjes en hun ovipositie-strategie bepaald (hoofdstuk 3 en 4). In in twee lijnen van de spintmijt *Tetranychus urticae* heb ik de bacteriële gemeenschappen onderzocht, en heb ik bepaald wat de effecten van *Wolbachia*, *Cardinium* en *Spiroplasma* bacteriën zijn op de overleving, ovipositie en gen-expressie van de gastheer en op de inductie van de reactie van tomatenplanten die door mijten zijn aangevreten (hoofdstuk 5).

In **hoofdstuk 2** beschrijf ik de variabiliteit van bacteriële gemeenschappen die geassocieerd zijn met de de nachtvlinder *H. virescens*; de bacteriën werden moleculair-genetisch getypeerd met behulp van zogenoemde bacterieële tag-gecodeerde FLX pyrosequencing van 16S rRNA amplicons. De bacteriële gemeenschappen verschilden tussen *H. virescens* rupsen die gegeten hadden op verschillende planten en tussen rupsen uit het veld vergeleken met die uit het laboratorium. Verder bleek dat rupsen uit het laboratorium voornamelijk veel van een ongeïdentificeerde *Enterococcus* soort bevatte, terwijl deze soort afwezig was in rupsen uit het veld. Rupsen uit het veld bevatten ook een meer diverse bacteriële gemeenschap dan rupsen uit het laboratorium. Aangezien de bacteriële gemeenschappen tussen biologische replicas van rupsen uit het veld en tussen verschillende ontwikkelingsstadia (eieren, rupsen en volwassen vrouwtjes) nogal varieerden, lijkt het erop dat in deze nachtvlinder geen stabiele, langdurige symbiose is ontwikkeld met de gevonden bacteria. Of de tijdelijk aanwezige bacteriën een gunstig effect hebben op de fitness van de nachtvlinders, doordat ze bijvoorbeeld betrokken zijn bij het verteren en ontgiften van voedsel zodat de rupsen op planten kunnen leven die in afwezigheid van de bacteriën niet geschikt zouden zijn, zal verder onderzocht moeten worden.

In **hoofdstuk 3** laat ik zien dat, wanneer volwassen *H. virescens* geïnjecteerd worden met dode cellen van de bacteriële entomopathogeen *Serratia entomophila*, de motten een sekse-specifieke activatie van het immuun-systeem laten zien. Bij het meten van de expressie-niveaus van immuun-gerelateerde genen vertoonden vrouwtjes een hogere activatie dan mannetjes, terwijl mannetjes een hogere handhaving van het immuunsysteem vertoonden, d.w.z. hun immuunsysteem had een hoger basis expressie-niveau dan dat van de vrouwtjes. Bovendien leek de immuunrespons in vrouwtjes meer te differentiëren tussen verwonding op zich (injectie met controle-stof) en een bacteriële injectie, hetgeen een specifiekere en mogelijk kosten-efficientere immuunrespons in vrouwtjes dan in mannetjes suggereert. Dit sekse-specifieke onderscheid in immuunrespons kwam overeen met de resultaten van de parings-exeprimenten: met bacterie geïnjecteerde vrouwtjes hadden een lager paringssucces dan niet-geinjecteerd vrouwtjes, terwijl het paringssucces van mannetjes niet verschilde tussen bacterieel-geïnjecteerde, gewonde of niet-geïnjecteerde mannetjes. Dit suggereert dat vrouwtjes een balans vertonen tussen immuniteit en seksuele aantrekking, hoewel de verschillen in paringssucces niet verklaard kunnen worden door verschuivingen in het seksferomoon van het vrouwtje. De gevonden verschillen in immuniteit tussen vrouwtjes en mannetjes voegt een nieuwe dimensie toe aan het onderzoek naar sekse-specifieke immuniteit.

In **hoofdstuk 4** laat ik zien dat activatie van het immuunsysteem met dode cellen van de bacteriële entomopathogeen *Serratia entomophila* het ovipositie-gedrag van *H. virescens* beïnvloedt. Vrouwtjes verhoogden het aantal gelegde eieren na immuun-activatie, maar leefden ook korter, zodat ze uiteindelijk dezelfde hoeveelheid eieren legden als niet-geactiveerde vrouwtjes. Dit suggereert dat bacterieel-geïnfecteerde vrouwtjes een kortere levensduur kunnen compenseren door sneller meer eieren te leggen. Echter, dergelijke vrouwtjes waren tegelijkertijd minder selectief in hun ovipositiekeuze dan controle vrouwtjes: de bacterieel-geactiveerde vrouwtjes legden dezelfde hoeveelheid eieren op planten met *H. virescens* rupsen als op planten zonder die rupsen, terwijl controle vrouwtjes bij voorkeur hun eieren legden op de niet aangevreten planten. Omdat *H. virescens* rupsen kannibalistisch zijn, en met rupsen bezette planten waarschijnlijk meer secondaire plantenstoffen bevatten (als verdedigingsreactie van de plant op de insectenvraat), heeft het ontbreken van keuzegedrag van geïnfecteerde vrouwtjes hoogstwaarschijnlijk een negatief effect op de fitness van deze vrouwtjes. De strategie van *H. virescens* vrouwtjes om meer eieren te leggen na een immuun-activatie, hetgeen kan compenseren voor een verkorte levensduur, gaat dus waarschijnlijk ten koste van een verminderde groei en overleving van hun nakomelingen.

In **hoofdstuk 5** beschrijf ik dat verschillende (endo)symbiotische bacteriën van de mijt *T. urticae*, in het bijzonder *Wolbachia*, *Cardinium* en *Spiroplasma*, de overleving en ovipositie van mijten beïnvloeden, evenals hun gen-expressie patronen en, na mijtenvraat, ook geïnduceerde responsen in tomaat, een waardplant van de mijt. Deze effecten zijn in twee mijten-lijnen onderzocht, een plantenafweer-onderdrukkende lijn (Delier-1) en een plantenafweer-inducerende lijn (Santpoort-2), lijnen die verschillende endosymbionten bevatten: terwijl de onderdrukkende lijn *Wolbachia* (W) en *Spiroplasma* (S) bevat, herbergt de inducerende lijn *Cardinium* (C) en *Spiroplasma*. Door de mijten te behandelen met antibiotica konen verschillende groepen mijten geproduceerd worden, die wel of niet W en S of C bevatten.

In de onderdrukkende lijn was de aanwezigheid van *Wolbachia* (W+) geassocieerd met hogere overleving van de mijten en een hoger expressieniveau van verterings- en detoxificatie-gerelateerde genen, vergeleken met mijten zonder *Wolbachia* (W-). Ook reduceerden W+ mijten salicylzuur (SA) niveaus in tomatenbladeren vergeleken met tomatenbladeren die met S+ mijten geinfecteerd waren. De aanwezigheid van *Spiroplasma* was geassocieerd met gereduceerde niveaus van 12-oxofytodienoische zuur,, een voorloperstof van jasmonzuur, in tomatenbladeren, terwijl *Wolbachia* juist het tegenovergestelde effect hadden.

In de inducerende mijtenlijn hadden mijten met *Cardinium* en *Spiroplasma* (C+S+) een lagere overleving, legden ze minder eieren en vraten ze minder dan mijten die deze bacteriën niet bevatten. Bovendien vertoonden bladeren die geïnfecteerd waren met C+S+ mijten een hogere expressie van SA merkergenen en accumuleerden deze bladeren lagere hoeveelheden abscisic zuur dan bladeren met C-S- mijten. Ook veroorzaakten C+S+ mijten roestige, rode vraat-littekens, terwijl C-S- mijten juist witte vraat-littekens veroorzaakten. Kortom, de aanwezigheid van *Wolbachia* lijkt gunstig te zijn voor de mijten, terwijl *Cardinium* een parasiet lijkt te zijn. Hoewel de rol van *Spiroplasma* minder duidelijk is suggereren de resultaten dat *Spiroplasma* en *Wolbachia* elkaar beïnvloeden in de onderdrukkende mijtenlijn, aangezien *Wolbachia* de effecten van *Spiroplasma* lijkt tegen te werken.

Author contributions

Variability of bacterial communities in the moth *Heliothis virescens* indicates transient association with the host

Heike Staudacher, Martin Kaltenpoth, Johannes A.J. Breeuwer, Steph B.J. Menken, David G. Heckel & Astrid T. Groot

HS, MK & ATG planned the experiments, HS conducted experiments, HS analysed the data, HS, MK, JAJB, DGH, SBJM & ATG wrote the manuscript

Sex-specific trade-offs between immune response and reproduction in a moth

Andrea Barthel*, Heike Staudacher*, Antje Schmaltz, David G. Heckel & Astrid T. Groot

AB, HS, DGH & ATG planned the experiments, AB & AS conducted the experiments, HS analysed the data, AB, HS, DGH & ATG wrote the manuscript

**Contributed equally to this study*

Effects of immune challenge on the oviposition strategy of a noctuid moth

Heike Staudacher, Steph B.J. Menken & Astrid T. Groot

HS & ATG planned the experiments, HS conducted the experiments and analysed the data, HS, SBJM & ATG wrote the article

The bacterial symbionts *Wolbachia, Cardinium* and *Spiroplasma* affect gene expression and survival of their spider mite host and impact distinct induced responses in plants

Heike Staudacher*, Bernardus C.J. Schimmel*, Mart M. Lamers, Nicky Wybouw, Astrid T. Groot & Merijn R. Kant

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Biography

Heike Staudacher was born on the 30th of January, 1982 in Schramberg, Germany. She obtained the Abitur at the Gymnasium Schramberg in 2001. After the Abitur, Heike studied media informatics for one semester and took a 1-year course in digital film directing at the SAE in Amsterdam. In 2004, she started her studies of Biology at the Free University in Berlin, with palaeontology, zoology and genetics as minors and ecology as major subject. She conducted her Diploma thesis at the Max Planck Institute for Chemical Ecology in Jena, Germany, and finished her studies in 2009. In 2010, Heike worked as a field technician on population dynamics of *Aedes aegypti* in Mexico for three months. In 2011, she started her PhD project at the Institute of Biodiversity and Ecosystem Dynamics of the University of Amsterdam with Dr. Astrid Groot as her supervisor and Prof. Dr. Steph Menken as her promotor.

Publications

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