Ecological Immunology and Host Plant Adaptation in Lepidopteran Herbivores

Dissertation

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1 General Introduction

One of the most fascinating aspects of nature is the complexity of ecosystems, wherein herbivorous insects and plants represent about one half of the world's macroscopic biodiversity (Strong et al. 1984). Since herbivorous insects cannot exist in the absence of plants, insects and plants have developed a reciprocal co-evolutionary relationship (Ehrlich and Raven 1964). Just as herbivorous insects exploit various plant habitats, microorganisms have evolved to attack these insects. This has created the situation where insects require a range of efficient defense mechanisms to cope with all potential pathogens as specifically and immediately as possible. Since environments differ in their threats they pose, immune system variation is high between individual herbivores (Sadd and Schmid-Hempel 2009). Ecological immunology attempts to explain these variations by invoking evolutionary costs and trade-offs between immunity and other life history traits (Sadd and Schmid-Hempel 2009). Studies on insect ecology and the driving forces for immune system variation are essential to better understand immunity in the context of natural environments.

1.1 Innate immunity in insects

1.1.1 Classical immune system

Immediately upon hatching an insect faces many challenges from opportunistic bacteria, parasites, viruses as well as potentially pathogenic organisms. Obviously, protection against these challenges requires an efficient immune system that can cope with all potential pathogens as specifically and immediately as possible. Insects represent the most diverse group of all known animal species on earth and have successfully conquered almost all environments (May 1988). To cope with certain aspects of this environmental variety, insects have evolved a multilayered innate immune system consisting of various defense mechanisms that are similar to many aspects of the vertebrate innate immune system (Ratcliffe et al. 1985; Hoffmann 1995). In contrast to vertebrates, insects lack adaptive immunity, including the formation of antibodies and an immunological memory.

The first line of defense in insects against microorganisms from external threats involves the epithelial barrier, as it is in direct contact with environmental microorganisms

and thus prevents the penetration of these microorganisms (Davis and Engstrom 2012). These barriers include the digestive, reproductive and respiratory organs as well as the cuticle of insects, which provide an impenetrable physical and chemical barrier by producing local reactive oxygen species (ROS) and antimicrobial peptides (AMPs) (Davis and Engstrom 2012). Although entomopathogenic fungi are known to invade insects by direct penetration of the cuticle, this strategy has not been reported for bacteria (Clarkson and Charnley 1996; Vallet-Gely et al. 2008). However, bacteria from the external environment can enter the hemocoel through bites or wounds of insects or more likely through the ingestion from soil or diet, such as leaf surfaces. If a microorganism crosses an epithelial barrier it is immediately recognized by a broad range of pattern recognition proteins (PRPs) that can bind pathogen associated molecular pattern (PAMP) molecules produced by these microorganisms (Jiang et al. 2004; Kanost et al. 2004; Hultmark and Borge-Renberg 2007). Although pattern recognition receptors of insects lack the binding specificity of antibodies, they instead bind to classes of characteristic surface molecules of microorganisms, including, ß-1,3-glucan, peptidoglycan, lipopolysaccharides and lipoteichoic acid (Yu and Kanost 2002; Kurata et al. 2006). These PAMPs are not present in insects and thus ensure a distinction of self from nonself motifs. The recognition of these nonself motifs results in the rapid and broad induction of highly efficient humoral and cellular responses.

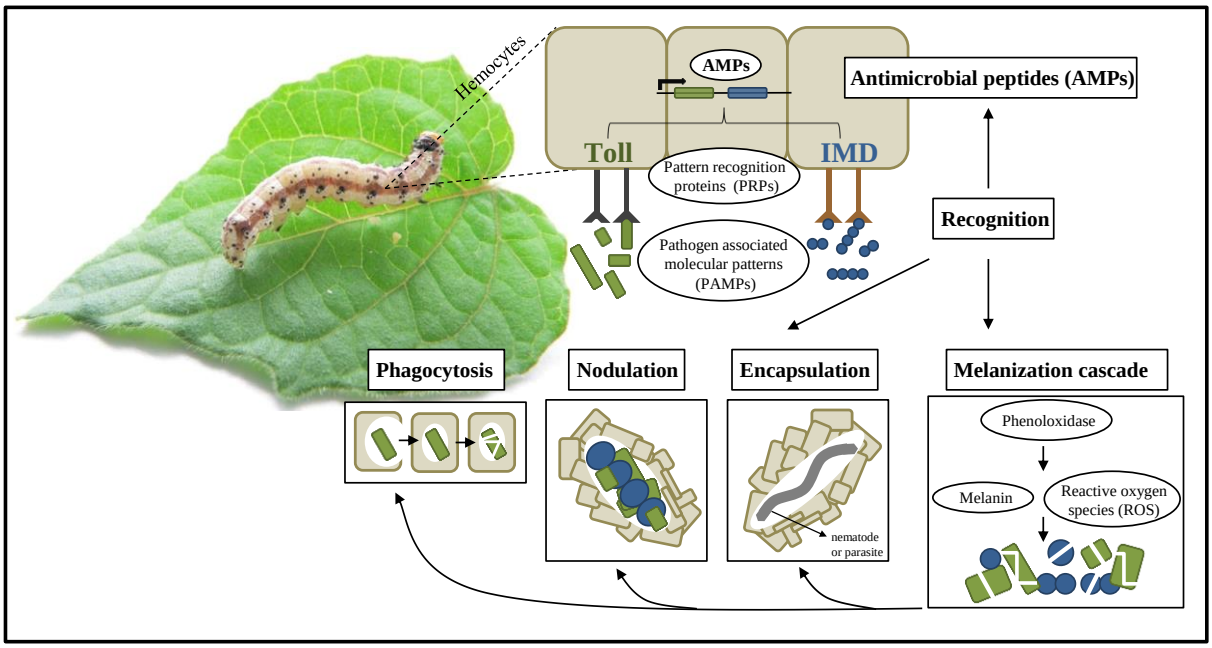

Figure 1.1: A schematic overview of the insect innate immune system. Bacteria gain entry by ingestion or wounding and consequently activate the immune system of insects. The pathogen associated molecular pattern (PAMP) molecules of bacteria serve as elicitors of the IMD (Immune deficiency) and TOLL pathway upon recognition by pattern recognition proteins (PRPs), which lead to the production of antimicrobial peptides (AMPs). In addition, the recognition of microbial compounds can also induce phagocytosis, nodulation, encapsulation and melanization which limit the spread of infecting microorganisms.

Humoral responses are characterized by various enzymes and antimicrobial peptides (AMPs), which are synthesized in the fat body, hemocytes and epithelial tissues, and are released into the hemolymph (Hoffmann 1995; Gillespie et al. 1997; Nappi and Vass 2001). More than 150 AMPs have been identified in insects (Tsakas and Marmaras 2010). Although usually cationic, the primary structures of insect AMPs vary markedly. These peptides are small, consist of 12-50 (sometimes >50) amino acids, and exhibit a broad range of activities against bacteria and/or fungi (Broderick et al. 2009b; Tsakas and Marmaras 2010). The Toll and IMD pathways have been shown to regulate these AMP genes (Lemaitre et al. 1995; Lemaitre et al. 1996). Gram-positive bacteria and fungi principally stimulate the Toll pathway while Gram-negative bacteria mainly stimulate the IMD pathway (Lemaitre et al. 1997). Despite the separate nature of these two pathways, some AMP genes, like defensin, depend on both pathways (Rowley and Powell 2007).

The cellular arm of the innate immunity is mediated by hemocytes and includes many defensive responses (Gillespie et al. 1997). Several types of hemocytes are present in the hemolymph and originate from mesodermally derived stem cells that differentiate into specific lineages (Hoffmann et al. 1979; Ratcliffe et al. 1985; Marmaras and Lampropoulou 2009). The most common hemocyte types are prohemocytes, granular cells, plasmatocytes, spherule cells and oenocytoids (Lavine and Strand 2002). Like blood cells in mammals, the various types of hemocytes in insects have specific functions. In lepidopteran species, granular cells and plasmatocytes are the only cell types capable of adhering to foreign surfaces, and account for more than 50 % of circulating hemocytes, whereas all other hemocyte types, including spherule cells, oenocytoids and prohemocytes, are non-adhesive (Ratcliffe 1993; Lavine and Strand 2002). However, all these types of circulating hemocytes are essential for cellular immunity.

An important component of hemocyte mediated immune defense is phagocytosis, a complex process that is based on recognition of invading pathogens, followed by engulfment and intracellular destruction (Gillespie et al. 1997). Phagocytosis is initiated by recognition and binding of a foreign particle to the phagocytic cell, followed by ingestion through cytoskeleton modification and intracellular vesicular transport to the phagosome where the engulfed particle is destroyed (Jiravanichpaisal et al. 2006). Phagocytic immune reactions necessarily involve migration to guide hemocytes toward foreign material (Tsakas and Marmaras 2010). The active hemocytic migration is induced and regulated by soluble molecules (chemotaxins), which are released either by foreign particles, like fMLP from

Escherichia coli, or by the host cells, like eicosanoids (Jiravanichpaisal et al. 2006; Merchant et al. 2008). Besides phagocytosis, hemocytes can form multicellular aggregates, called nodules, to entrap larger numbers of bacteria (Gillespie et al. 1997). In case of infections with parasites or nematodes, hemocytes bind to the target in multiple layers until they form a capsule around the invader (Rosales 2011). Inside these nodules or capsules the foreign organisms are killed by reactive cytotoxic products (Rosales 2011).

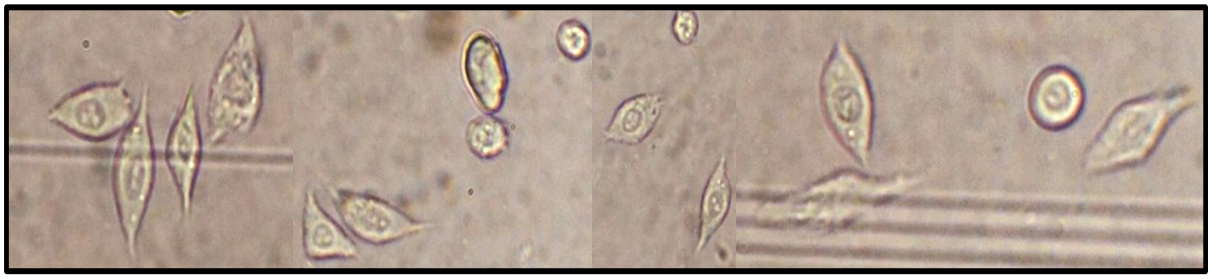

Figure 1.2: Different cell types of hemocytes of *Heliothis virescens* observed *in vitro*. Hemocytes (200x) show a varying cellular morphology from spindle-shaped to more circular cells. The cell types of hemocytes were not identified.

An important component of both humoral and cellular responses is the melanization cascade, which involves the rapid formation and deposition of melanin (a brown to black pigment) at the site of infection or injury (Eleftherianos et al. 2010). Melanin as well as other by-products of this cascade possesses cytotoxic activity towards microorganisms (Nappi et al. 2004; Eleftherianos et al. 2010). Phenoloxidase (PO) is the key enzyme responsible for melanization reactions, in which it catalyzes the oxidation of phenols to quinones leading to the formation of insoluble melanin (Soderhall and Cerenius 1998; Cerenius and Soderhall 2004; Nappi et al. 2004; Eleftherianos et al. 2010). PO exists as an inactive precursor called prophenoloxidase (proPO), which is activated through the action of serine proteases (Kanost et al. 2004). The activation of the melanization cascade can be triggered by pathogen associated molecules and is involved in encapsulation, phagocytosis and wound healing.

Besides melanization, the coagulation system is another important part of innate immunity which overlaps the humoral/cellular boundary and involves a combination of soluble and cell-derived factors (Johansson et al. 1999; Theopold et al. 2002; Jiravanichpaisal et al. 2006). A general difference between vertebrates and invertebrates is the fact that body fluids in vertebrates are mostly confined to blood and lymphatic vessels, whereas invertebrates have an open circulatory system (Theopold et al. 2004; Jiravanichpaisal et al. 2006). Therefore, after injury, the formation of stable clots quickly prevents the loss of hemolymph but also traps microorganisms to preclude spreading into the hemocoel (Theopold

et al. 2004). Clot formation is a remodeling of tissue integrity at the wounded site and is achieved by granular cells and plasmatocytes in lepidopteran species (Theopold et al. 2004).

1.1.2 Counteracting the insect immunity: Bacillus thuringiensis and Serratia entomophila

Two major strategies are utilized by pathogenic bacteria to overcome the immune response in insects: they can either avoid detection by the host because they lack (or hide) immune elicitors on their surface, or they are able to suppress or modulate the immune response to ensure their survival (Vallet-Gely et al. 2008). In this respect, *Bacillus thuringiensis* and *Serratia entomophila* are potent pathogenic bacteria, because they have evolved various virulence factors and strategies that can impair both cellular and humoral immune responses in insects. In nature, these bacteria are taken up by the larva by ingestion or accidently by wounding through the cuticle (Nielsen-LeRoux et al. 2012)

Bacillus thuringiensis (Bt) is a gram-positive, sporulating entomopathogen that is found in many ecological niches, including soil, plant surfaces and insects. This entomopathogenic bacterium can infect diverse insect species and is widely used for biocontrol of crop pests (Macintosh et al. 1990; Shelton et al. 2002; Zhao et al. 2003). The main insecticidal factor of Bt is a crystalline inclusion, containing a δ-endotoxins (Cry toxins), which is produced with spore formation (Hofte and Whiteley 1989; Schnepf et al.1998). When ingested by a susceptible insect, these crystal inclusions are solubilized in the alkaline gut and protoxins are activated by digestive enzymes (Nielsen-LeRoux et al. 2012). The activated toxins are able to cross the peritrophic barrier and bind to midgut receptors resulting in the formation of pores in the epithelial midgut cells, thus providing bacterial cells access to the hemocoel (Hofte and Whiteley 1989; Schnepf et al.1998). The impact of Cry toxins on larvae is highly variable among insects and was shown to be dependent on the type of Cry toxin, combination and concentration of Cry toxins, the insect species and the presence of microorganisms in the gut (Hansen and Salamitou 2000; Broderick et al. 2009a; Raymond et al. 2010).

Bacillus thuringiensis can also kill insects in other ways, i.e. not mediated by Cry toxins. Bt is known to produce various putative virulence factors, including phospholipase C, hemolysins, bacteriocins, modified cell surface proteins, chitinases, and metalloproteases, which are involved in establishing infections (Fedhila et al. 2004; Raymond et al. 2010; Nielsen-LeRoux et al. 2012). For instance, the production of metalloproteases, such as InhA1 and InhA3, by Bt induces the cleavage of AMPs (Dalhammar and Steiner 1984; Guillemet et

al. 2010). Bt also produces a hemolysin, HlyII, which is a pore forming toxin able to induce lysis of hemocytes by apoptosis (Tran et al. 2011). All these virulence factors of Bt counteract the humoral and cellular immune response of insects.

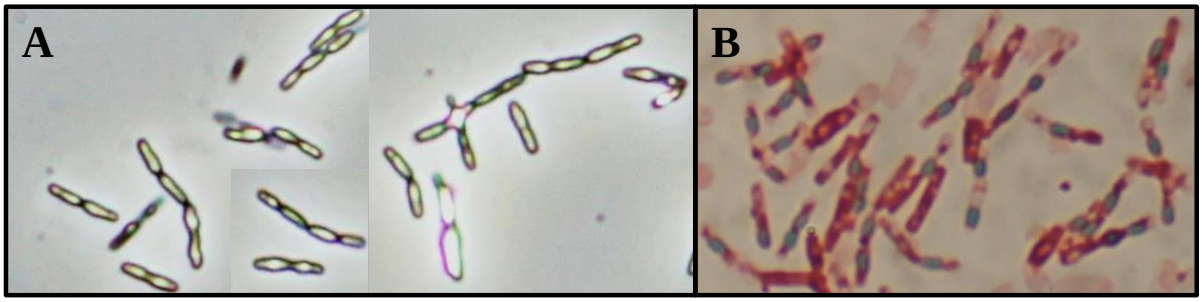

Figure 1.3: Vegetative cells (A) and endospores (B) of *Bacillus thuringiensis.* Endospores are stained by Wirtz-Conklin method showing endospores as green and vegetative cells as red.

Serratia entomophila is a gram-negative entomopathogen that is commonly isolated from grassland soils. This bacterium has been mostly studied in relation to the scarab beetle *Costelytra zealandica* where it causes the amber disease (Grimont et al. 1988). However, *S. entomophila* was also found to be lethal to lepidopteran larvae (Núñez-Valdez et al. 2008). S*erratia entomophila* contains a specific prophage consisting of genes which imparts its pathogenicity, including a lysis cassette and an antifeeding cluster (Núñez-Valdez et al. 2008). Further, the products of sep*ABC* genes from *S. entomophila*, which are virulence-associated genes located on a large plasmid, are considered homologues of the insecticidal toxin complex of *Photorhabdus luminescens* (Hurst et al. 2000; Núñez-Valdez et al. 2008). This insecticidal toxin has been shown to cause damage to the gut epithelium, inhibit hemocytic phagocytosis and to modify actins (Lang et al. 2010; Nielsen-LeRoux et al. 2012). Besides toxin production, resistance to insect immunity might be an additional virulence factor of *S. entomophila*. For instance, *Serratia* species are known to produce proteases that are able to destroy AMPs (Flyg and Xanthopoulos 1983).

1.1.3 Ecological immunology

Traditional studies on insect immunity determining physiological and molecular mechanisms suffer from a lack of knowledge of the ecology of their model organism. Since insect immunity is an evolved trait, the field of ecological immunology seeks to understand the evolutionary and ecological processe which shaped and maintained variation in insect immunity (Rolff and Siva-Jothy 2003; Schmid-Hempel 2005).This field of research relies on two main theoretical approaches (Siva-Jothy et al. 2005). The first principle is based on the

theory of the evolution of life history traits and suggests that the evolution and the use of immune functions are costly (Roff 1992; Stearns 1992; Sheldon and Verhulst 1996; Siva-Jothy et al. 2005) (see Section 1.2). The second approach relies on arms-race models of coevolution, which suggests that parasites and pathogens become rapidly adapted to frequent host genotypes, which in turn favor rare host genotypes (Van Valen 1973; Hamilton 1980; Peters and Lively 1999; Siva-Jothy et al. 2005). However, only a few studies have focused on invertebrates and even fewer studies have tested the predictions arising from these theoretical approaches in an experimental context.

A basic idea of ecological immunology is that immune defense varies in relation to the surrounding environment (Sadd and Schmid-Hempel 2009). Since insects inhabit diverse niches and interact with various microorganisms, insects are an attractive research subject to gain insights in possible evolutionary arms race between herbivores, evolving novel defense strategies and pathogens, evolving novel coping strategies. The optimal immune response of individual insects should be most efficient at the lowest possible cost (Schmid-Hempel 2005). However, the optimal immune response can be expected to vary based on environmental conditions (Lazzaro and Little 2009). Environments differ in the threats they pose, including abiotic factors, exposure to pathogens and nutrient availability and composition. These heterogeneous environments can shape selective pressures acting on the immune system (Sadd and Schmid-Hempel 2009). Natural selection on immune function in individual insects will favor different optimal strategies to counteract these threats in different environments. This variation in immune system response in turn might thus be important for adaptation to new environmental conditions.

1.1.3.1 Environmental factor: Pathogen exposure

A principal aim of ecological immunology is to understand variation in host-pathogen outcomes. Every organism will be infected by pathogens at some point in its life. Thus the ubiquitous nature and diversity of pathogens cause a strong selection pressure on the evolution of insect immunity. Since herbivorous insects inhabit diverse niches and ecosystems while feeding on host plants, they encounter a broad variety of microorganisms, including entomopathogenic bacteria. Consequently, to survive on these plants herbivores must adapt to plant-associated microorganisms and are in need of the ability to combat these pathogens as efficient as possible. Among herbivores, host plant generalists are expected to possess a more efficient and diverse immune response to deal with the high pathogen diversity in their

various habitats, compared to specialists which live in a restricted, more constant habitat. The frequency of immune challenges might also affect the selective advantage of a particular defense strategy, with a high frequency of attacks favoring greater immunocompetence. Immune defense under the selection pressure of high pathogen diversity should select for specific and immediate immune functions. Benefits of an efficient immune response are obvious, as it will alleviate or prevent the fitness loss caused by pathogens that pose a significant threat to the integrity of self. However, efficient immune responses might be costly in terms of needed resources, so that other life history traits are expected to trade off due to limits set by the range of possible resource allocation strategies by the organism. Consequently, pathogen pressure on herbivorous insects is likely to influence immune defense strategies and taken together with data from life history traits would provide a more detailed understanding of immunological variation, than life history traits alone.

1.1.3.2 Environmental factor: Diet breadth

The ability of insects to combat microbial infections depends largely on their nutritional state (Chandra 1996; Lochmiller and Deerenberg 2000). There are few studies on the association between feeding behavior and immunity. For example, in *Rhodnius prolixus* starvation compromises the effectiveness of its immune response against microorganisms (Feder et al. 1997). Similarly, starvation conditions lead to a downregulation of immune function in *Tenebrio molitor* (Siva-Jothy and Thompson 2002) and to an increased susceptibility in the genus *Glossina* (Welburn et al. 1989).

In nature, insects do not only encounter the two extremes of unlimited food or starvation. In herbivorous insects a diversity and variation of phytochemicals and nutrients between and within host plants affect the physiological state of insects. For example, in larvae of the arctiid moth, *Parasemia plantaginis*, the encapsulation rate varied depending on the host-plant species they consumed, corresponding to the amount of antioxidants in leaves of the host plants (Ojala et al. 2005; Klemola et al. 2007). Interestingly, antioxidants acquired from the diet can prevent cell damage and thus might reduce the cost of immune response (Johnson and Felton 2001; Ojala et al. 2005). Phytochemicals can also decrease the immunocompetence and increase parasitism rates in insects (Zvereva and Rank 2003; Haviola et al. 2007; Smilanich et al. 2009). Interestingly, phytochemicals consumed by herbivores can also directly affect entomopathogens infecting them (Keating et al. 1988; Cory and Hoover 2006).

The general nutritional content of a plant has effects on insect immunity as well. For instance, infected larvae of *Spodoptera littoralis* had an increased survival rate when fed on a protein-rich diet, compared to control diet (Lee et al. 2006a). Also, *Plodia interpunctella* reared on poor diet had poorer body conditions with a lower immune response than those reared on high-quality diet, and *Rhodnius prolixus* larvae kept on blood plasma had a reduced immunocompetence compared to larvae reared on their preferred diet (Feder et al. 1997; Triggs and Knell 2012). These studies show that the effect of plant nutrition on the immune response and pathogen resistance is complex and can be quite different from that of phytochemicals alone.

The interaction of different plant features that affect the immune response of herbivorous insects might be an important ecological and evolutionary force driving immunological variation. Among herbivorous insects, diet breadth may vary greatly, with generalists consuming a wide variety of plant families and specialists being restricted to a few species. Generalists may thus be exposed to a wider range of phytochemicals and nutrient quality exerting stronger selection on the innate immune system. Thus it is likely that a wide diet breadth may elicit generalists to evolve diverse immune defense strategies based on the impact of their host plant. In addition, diet breadth can also have indirect effects on the immunity of herbivores. It has been shown that predation pressure on herbivorous insects varies depending on their host plant species (Fox et al. 1990; Farrar and Kennedy 1993; Lill et al. 2002; Ojala et al. 2005). Furthermore, phytochemicals in herbivores can make them either inedible or more attractive for predators or parasitoids (Rothschild 1973; Dobler and Rowellrahier 1994; Nieminen et al. 2003).

In summary, the variability that we observe between immune responses of different individuals and populations can be the consequence of a response to the prevailing variability in the environment of herbivorous insects.

1.2 Trade-offs in life-history: Consequences of the innate immune response in insects

Insect immunity and its relationship to the evolution of other components of life history is a major topic in many studies (Roff 1992; Stearns 1992). The basic idea of life history is the competitive allocation of resources, referred to as "trade-offs" in life history theory (Reznick 1992; Zuk and Stoehr 2002). Trade-offs between immunity and life-history traits are diverse. Evidence for energetic trade-offs has been demonstrated in bumblebees and birds (Konig and Schmid-Hempel 1995; Hasselquist et al. 2001). Further costs have been shown for survival, growth, learning and antipredator defense (Moret and Schmid-Hempel 2000; Mallon et al. 2003; Brommer 2004). Costs associated with immunity are usually divided into two categories, evolutionary costs and physiological costs. Evolutionary costs rely on a negative genetic covariance between traits, assuming the existence of an antagonistic pleiotropy, where a gene with positive effects on one component of fitness has a negative effect on another (Stearns 1992; Siva-Jothy et al. 2005). This suggests that evolving more potent immunity may correlate with a loss in another fitness-relevant trait (Schmid-Hempel 2005). These genetic relationships between traits cannot be modified by the individual during its lifetime (Schmid-Hempel 2005; Siva-Jothy et al. 2005). Physiological costs, on the other hand, result from resource-based trade-offs between immunity and other fitness traits, whereby these costs are not fixed during a lifetime (Siva-Jothy et al. 2005). The assumption of physiological costs is that different functions of an organism compete for the same pool of resources and that allocation of resources to immune functions is expected to limit other traits and vice versa (Schmid-Hempel 2005; Siva-Jothy et al. 2005). Resources for immune functions are needed to keep immune functions at a given level of readiness and to actually mount an immune response upon infection.

In addition to physiological costs, inducing an immune response upon infection can cause self-damage. The hypothesis of "cost of immunity" proposes that an optimal immune response is not necessarily the maximal immune response (Sheldon and Verhulst 1996; Westneat and Birkhead 1998; Zuk and Stoehr 2002). In vertebrates, the result of a maximal immune response is the risk of immunopathology, including autoimmunity, hypersensitivity reactions and tissue damage (Wegmann et al. 1993; Hillgarth et al. 1997). In insects, the open circulation system, transporting defense molecules to all body parts, and the production of reactive oxygen species, generating cell damage, are features which might promote

immunopathology (Zuk and Stoehr 2002; Rolff and Siva-Jothy 2003; Schmid-Hempel 2005). For instance, it was shown in *Drosophila* that the production of melanotic tumors in response to infection is lethal to host tissues (Watson et al. 1991).

Some life-history traits, such as egg and sperm production, are obviously sex limited, while others, such as developmental time, have different consequences for both sexes. At first appearance, the cost of immunity should be similar for both sexes, since it is expected that females and males benefit from a proper immune response and thus both sexes should expend the same resources. However, females and males balance the cost of immune defense against requirements of other life history traits differently. Sex-specific immunity might be a consequence of sex-differential patterns of resource allocation, which are in turn caused by differences in requirements for sex-specific reproduction, such as egg and sperm production. In vertebrates it has been found that females display a higher immunocompetence than males (Wilson et al. 2002). This observation has been attributed to the difference in life histories of both sexes or the immunosuppressive effect of testosterone, the major male sex pheromone in vertebrates (Alexander and Stimson 1988; Zuk 1990; Folstad and Karter 1992; Zuk and Stoehr 2002). In insects, males have been found to invest fewer resources in immune response as well (Rheins and Karp 1985; Gray 1998; Rolff 2001). These sex-specific differences in immunity have been related to Bateman's principle, which posits that males maximize their fitness by increasing mating frequency, while female fitness is positively correlated with longevity (Bateman 1948; Trivers and Willard 1973; Zuk 1990; Roth et al.2011). Assuming that a longer lifespan requires a more efficient immune system in females and that a maximized mating frequency relies on immediately available resources in males, Bateman's principle could thus explain the sexual dimorphism in immunity (Bateman 1948; Zuk 1990; Rolff 2002; Roth et al.2011). How complex and intimate the immunity is associated with life history traits, such as reproduction, remains to be discovered and thus awaits further investigations.

1.3 Insects, plants and their complex interactions

The complexity of environments, wherein herbivorous insects and pathogens interact, is also impacted by plants. However, the relationship between plants and herbivores cannot be fully understood without incorporating the impact of herbivorous pathogens. Many laboratory studies on host plant adaptation neglect the impact of environmental threats in the natural habitat of herbivores. Thus, combined studies are necessary in order to elucidate the impact of host plants on the ecology of herbivores and their natural pathogens

1.3.1 Specialist - generalist paradigm

In nature, plants and insect herbivores are continuously interacting. Therefore, the evolution of diet breadth of herbivores is a popular topic in ecology and evolutionary biology (Futuyma and Moreno 1988; Berenbaum and Zangerl 1994). Coevolution between plants and herbivores is the generally accepted theory that has been put forward in detail by Ehrlich and Raven (1964). Presumably, a narrow diet breadth arises because of a long coevolutionary history between herbivores and their host plants as an outcome of reciprocal interactions (Ehrlich and Raven 1964; Feeny 1976). Insect herbivores can be grouped into two categories based on their degree of diet breadth (Ali and Agrawal 2012). When limited to only one or a few closely related plant genera, these herbivores are considered as monophagous or specialists (Ali and Agrawal 2012). Insect herbivores that feed on plants from more than one family are designated as polyphagous or generalists (Ali and Agrawal 2012). A general pattern that has been observed across all herbivorous insects is a predominance of specialist herbivores over generalist herbivores, as > 95 % of all herbivores are specialists (Fox 1981; Bernays and Graham 1988; Jaenike 1990). One might expect that unlimited resource availability is a benefit occurring in generalists (Bernays and Minkenberg 1997). However, with the loss of ability to use many host plants, specialists are predicted to gain physiological advantages when consuming their preferred host plant (Krieger et al. 1971; Whittake and Feeny 1971). There have been several hypotheses posed about the specialist-generalist paradigm (Ali and Agrawal 2012). First, specialists should be less harmed by defenses mediated by their preferred host plants as compared to generalists, overcoming these defenses via specialized mechanisms such as sequestration or detoxification and excretion (Whittake and Feeny 1971; Adler et al. 1995; Schoonhoven et al. 1998; Ali and Agrawal 2012). Secondly, generalists should possess more general mechanisms to tolerate or detoxify a wide array of plant defenses

than specialists do (Krieger et al. 1971; Whittake and Feeny 1971). The third prediction is that specialists and generalists will elicit different plant responses upon damage (Ali and Agrawal 2012).

Since both broad and restricted feeding patterns exist, both types of herbivores are likely to possess specific adaptations for different life styles, but the much larger number of specialists imply that these specialists have broad physiological advantages over their generalist counterparts (Bernays and Graham 1988). Presumable, a generalist is a 'jack of all trades, but a master of none', with respect to food utilization (Bernays and Graham 1988; Ali and Agrawal 2012). In line with this, although generalists are able to grow and develop on various host plant species, their level of performance differs. For instance, when fed on different hosts, commonly observed effects in generalists include reduced growth and larval survival, abnormal development in the pupal stage, delayed emergence of adults, decreased adult weight and a reduced number of generations (Hunter and McNeil 1997; Lazarevic et al. 1998; Tikkanen et al. 2000; Mello and Silva-Filho 2002). Dietary specialists are hypothesized to have more efficient forms of adaptation to reduce costs and enhance excretion of plant secondary metabolites, either involving the production of specific enzymes to detoxify their food, or storage mechanisms (Bernays and Graham 1988; Jongsma and Bolter 1997; Price 1997; Ali and Agrawal 2012). Further, specialist herbivores have developed mechanisms to either use phytochemicals as oviposition and feeding cues or as protection against parasitoids and predators (Giamoustaris and Mithen 1995; Agrawal and Sherriffs 2001). It has also been shown that specialists experience fewer signs of toxicity on their preferred host plant compared to generalists (Rhoades 1979; Berenbaum and Zangerl 1998). Together, these data indicate that specialists have a significant advantage over generalists when consuming their preferred host plant species. However, some specialists may also be negatively impacted by plant defensive traits of their own host plant (Malcolm et al. 1989; Agrawal and Kurashige 2003; Harvey et al. 2007). Physiological specialization results in a decreased capacity to eliminate or detoxify non-host phytochemicals. A lower capacity to process novel phytochemicals restricts specialists to habitats where their preferred plant species is abundant and makes specialists less resistant to changes in plant availability (Futuyma and Moreno 1988). Therefore, it is not surprising that narrow physiological tolerances are sometimes found in specialists that inhabit relatively constant environments (Futuyma and Moreno 1988), whereas generalists might experience trade-offs between adaptation to various

environments and costs of a broad chemical tolerance (Futuyma and Moreno 1988; Berenbaum and Zangerl 1994).

1.3.2 Plant-herbivore interactions in a tritrophic context

A central goal in the study of plant-herbivore interactions has been to identify driving forces leading to dietary specialization. The toxicity of many plant secondary compounds to insects has led to the hypothesis that feeding specialization is a consequence of biochemical coevolution of plants and their herbivorous insects (Ehrlich and Raven 1964; Feeny 1976; Futuyma 1976). This theory of insect-plant coevolution, however, considers primarily a two trophic level system (Gilbert 1979; Price et al. 1980). In nature, ecological communities are complex and compose of at least three interacting trophic levels: plants, herbivores, and their natural enemies (Price et al. 1980; Dicke et al. 1990; Agrawal 2000b; Singer and Stireman 2005). Natural enemies of herbivorous insects, such as microorganisms, predators and parasitoids, can either be indirectly or directly affected by properties of individual plants (Hare 2002). Indirect effects include plant traits which modify the interactions between herbivores and their enemies by operating directly on the herbivore (Price et al. 1980; Agrawal 2000b). These traits include physiological modification of herbivores by phytochemicals and nutrients, which might affect the susceptibility of herbivores to infection or predation (Ali et al. 1998; Cory and Hoover 2006; Kessler and Halitschke 2007). Effects that operate directly on natural enemies of herbivores are various and can either have positive or negative impacts on the herbivore (Price et al. 1980). For instance, herbivore-induced plant damage can result in the attraction of natural enemies by specific plant signals (Takabayashi et al. 2006; Kessler and Halitschke 2007). Further, herbivorous insects can be concealed from their enemies by specific plant structures, which provide a barrier to parasitoid penetration and thus a so-called “enemy-free space” (Price et al. 1980; Oppenheim and Gould 2002). The consumption and sequestration of phytochemicals by herbivores for their own defense can make them less attractive for predators and parasites (Price et al. 1980; Dicke et al. 1990; Agrawal 2000b; Singer and Stireman 2005). Additionally, plant-produced chemicals can directly influence the outcome of herbivore-pathogen interactions, because several classes of secondary metabolites possess antimicrobial and antiviral activity and thus might alter the infection dynamics of pathogens within herbivores (Hoover et al. 1998a; Cory and Hoover 2006). All these effects of plant properties on natural enemies can have important evolutionary consequences for herbivorous insects, because host plant use or shifts that

reduce predation or infection are advantageous (Price et al. 1980; Bernays and Graham). Therefore, to understand plant-herbivore interactions in any system the third trophic level should be considered as an important and even dominant selective force driving host plant preferences (Price et al. 1980).

1.3.2.1 Insect microbial pathogens in a tritrophic context

Plants can influence the interactions between herbivorous insects and their microbial pathogens, including viruses, bacteria and fungi, in numerous ways (Schultz et al. 1992; Cory and Hoover 2006). The extent to which plants can affect insect-pathogen interactions depends on the life history of both, and whether plants can directly or indirectly alter key processes, such as infection or environmental persistence of these pathogens (Cory and Hoover 2006). Plant phytochemicals can directly affect entomopathogens by either reducing or enhancing their pathogenicity (Price et al. 1980; Hoover et al. 1998a; Cory and Hoover 2006). For instance, caffeic acid and other plant phenolics in the gut of *Bombyx mori* were converted to active compounds against *Streptocoocus faecalis* (Iizuka et al. 1974; Koike et al. 1979; Ludlum et al. 1991). Nicotine was found to inhibit colony formation by *Bacillus thuringiensis* and reduced its pathogenicity to *Manduca sexta* (Krischik et al. 1988). In contrast, chlorogenic acid and polyphenol oxidase increased the susceptibility of *Heliothis zea* toward *B. thuringiensis* (Ludlum et al. 1991). These plant-mediated effects will impact herbivore-entomopathogen dynamics in nature. Only through consideration of these interactions will it be possible to understand the impact of entomopathogenic bacteria in the complex web of plant-herbivore-pathogen relationships (Cory and Hoover 2006).

1.3.3 Study organisms: Heliothis subflexa and Heliothis virescens

Heliothinae are a cosmopolitan subfamily of moths in the family Noctuidae and include some major worldwide crop pests. Within the Heliothinae, *Heliothis virescens* and *Heliothis subflexa* are two closely related representatives of the *virescens* group with non-overlapping host ranges, but overlapping geographical distribution in both North and South America (Figure 1.4) (Fitt 1989; Mitter et al. 1993; Sheck and Gould 1993). It is assumed that both species evolved from a shared *H. virescens*-like, generalist ancestor (Matthews 1991; Mitter et al. 1993; Cho et al. 1995; Fang et al. 1997; Oppenheim and Gould 2002; Cho et al. 2008).

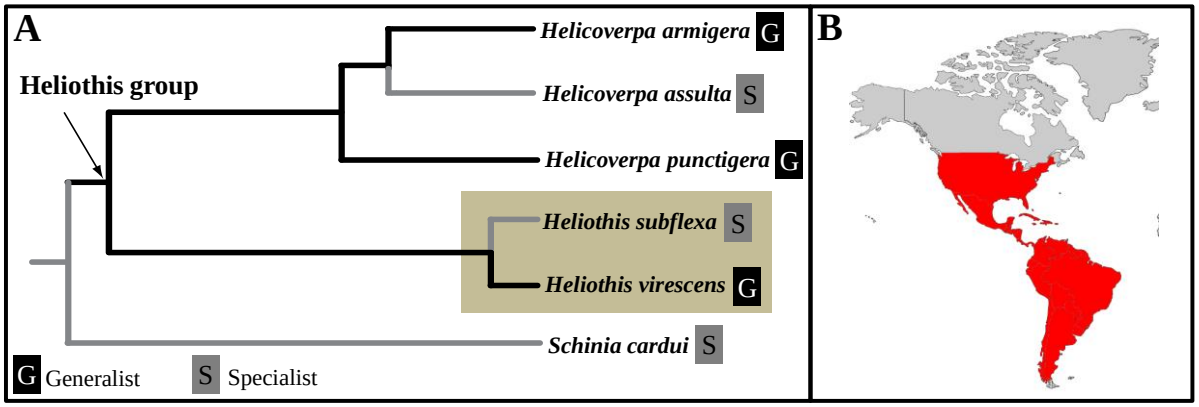

Figure 1.4: Phylogenetic relationship (A) and geographical distribution (red) (B) of *H. virescens* and *H. subflexa.* Source: (A) adapted from Choe *et al.* 2008 and (B) adapted from http://www.freeusandworldmaps.com/images/WorldPrintable/WorldMercator6NoLinesPrint.jpg (date 12.06.2014)

The tobacco budworm (*H. virescens*) is an exemplary polyphagous herbivorous species whose diet breadth is estimated at more than 37 species of plants in at least 14 families, including tobacco, cotton, soybean and other crop plants (Fitt 1989; Sheck and Gould 1993; Oppenheim and Gould 2002). It is regarded as one of the major pests of tobacco (*Nicotiana tobaccum* L.) and cotton (*Gossypium hirsutum* L.) in the United States (Johnson 1979). Contributing to its pest status in these crop plants is a long history of insecticide resistance in *H. virescens* (Lingren and Bryan 1965; Sparks et al. 1998). Like most generalist feeders, *H. virescens* has developed an efficient general detoxification system, however, this pest species displays preferences for particular host plants (Fitt 1989). In the last decades, transgenic crop plants expressing *Bacillus thuringiensis* endotoxin (Bt) have revolutionized the pest control of *H. virecsens* across the United States (Abney et al. 2004). Besides pressure associated with transgenic plants, the broad habitat of *H. virescens* possesses many other abiotic and biotic environmental challenges. This generalist would be expected to possess efficient defense strategies to combat these environmental threats resulting from its broad host range to survive within its variable habitat.

In contrast to *H. virescens* which is one of the major pests within the Heliothinae family, its closely related counterpart *Heliothis subflexa* exclusively feeds on fruits in the genus *Physalis*, it is considered as a minor pest species in the United States due to its restricted host range (Laster 1972). However, in Mexico, *Physalis* plants are severely infested by *H. subflexa* that it is now considered to be a major pest of the agricultural crop *Physalis philadelphica* in this region (Mitter et al. 1993; Lee et al. 2006c). Interestingly, *H. virescens* cannot develop on *Physalis* plants (Oppenheim and Gould 2002; De Moraes and Mescher

2004). *Physalis* fruits are enclosed by a thin-walled and inflated calyx called a "lantern", thus they provide a so-called enemy-free space for fruit-feeding larvae of *H. subflexa* (Oppenheim and Gould 2002; Puente et al. 2008). The importance of the structural refuge of *Physalis* plants in shielding *H. subflexa* from parasitoids was demonstrated by Sisterson (Sisterson and Gould 1999), showing an eight times higher parasitism rate for larvae fed on fruits with cut lanterns, even though the volatiles released from cut lanterns did not make *Physalis* plants more attractive to searching parasitoids. These results suggest that the specialization of *H. subflexa* on *Physalis* plants was under strong selection towards avoidance of natural enemies (Sisterson and Gould 1999; Oppenheim and Gould 2002). Oppenheim and Gould (2002) demonstrated that *H. virescens* larvae, which were experimentally induced to feed on *Physalis*, show a ten times higher parasitism rate than that of *H. subflexa* on *Physalis*, supporting their hypothesis that specialized behaviors of *H. subflexa* have a substantial impact on its dietary specialization (Oppenheim and Gould 2002). The study by De Moraes *et al.* (2004) suggested the lack of linolenic acid and *H. subflexa's* ability to apparently synthesize this essential fatty acid as a factor for specialization, however other studies found linolenic acid in *Physalis* plants making this theory debatable (De Moraes and Mescher 2004; Puente et al. 2011; Ramadan 2011). Based on the antifeedant and immunosuppressive activity to various insects, fruits of *Physalis* plants are well protected against herbivory by high concentrations of withanolides in the calyx and leaves of the plant (Glotter 1991; Waiss et al. 1993; Elliger et al. 1994; Lan et al. 2009; Ramadan 2011) and specialized *H. subflexa* must have developed strategies to circumvent these defenses in the course of specialization. However, the impact of withanolides on specialized *H. subflexa* had not been evaluated prior to our own studies. Consequently, *H. subflexa* appears to possess behavioral and physiological properties which are highly adapted to feeding on *Physalis* plants, which are likely to have resulted from a history of co-evolution between the herbivore and its host (Sisterson and Gould 1999; Oppenheim and Gould 2002; De Moraes and Mescher 2004; Puente et al. 2008).

Figure 1.5: Adult moths and larvae of *Heliothis virescens* (A) and *Heliothis subflexa* (B).

1.4 Aim of this thesis

As outlined above, herbivorous insects live in complex environments in which they intimately interact with their host plants and natural pathogens. As a consequence, plants and pathogens are an important selective force on their hosts. The major aim of this thesis was to examine the importance of insect ecology on immune system variation and host plant adaptation in lepidopteran herbivores. More specifically, I was interested in the role of environmental factors in shaping immune defense strategies and thus the outcome of insect-pathogen interactions in a generalist and specialist herbivore. Herbivorous insects have been used extensively to study the invertebrate immune system responses. However, many immunological studies do not take into account whether herbivore insects are specialists or generalists and thus do not link measures of immune system response to the lifestyle of the herbivore. In this thesis I attempted to compare the immune defense strategy of generalist and specialist herbivores that have different probabilities of exposure to pathogens. Furthermore, I try to answer how herbivores trade off the cost of immune defenses with life-history traits such as sexual traits. My thesis also attempted to demonstrate the important role of secondary metabolites in herbivore-plant interactions by incorporating the impact of herbivorous pathogens in this interaction.

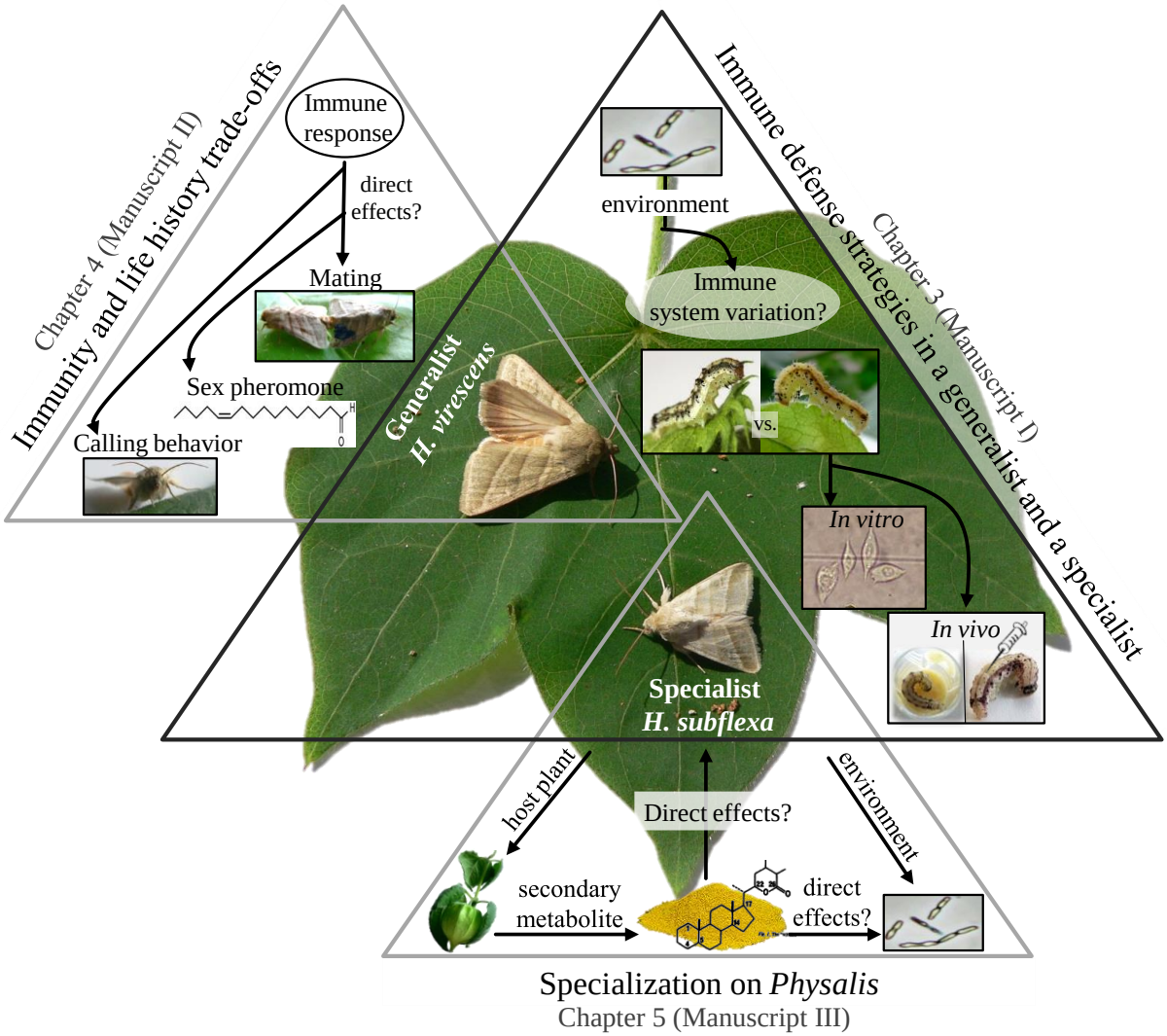

Figure 1.6: Scheme of the interactions studied in this thesis.

Chapter 3 (Manuscript I) deals with the question whether diet breadth and pathogen exposure have an effect on the evolution of immune system variation in herbivores. More specifically, we compared immune defense responses of the generalist herbivore *H. virescens* and the specialist *H. subflexa* challenged by entomopathogenic and nonpathogenic bacteria. Immune defense responses were examined on different levels. Using primary hemocyte cultures of both species and *in vivo* experiments, we were able to specifically test whether generalist herbivores have evolved different immune defense strategies than specialists to overcome infections.

In Chapter 4 (Manuscript II), the consequence of an induced immune response on reproductive life history traits were investigated. As investments into immunity have been

shown to be costly, I studied physiological and behavioral changes associated with entomopathogenic infections in females and males of *H. virescens*. In addition, I compared the immune response of females and males to assess possible sex-specific immune defense strategies.

Chapter 5 (Manuscript III) addresses the question whether specialized herbivore-host plant interactions are guided by plant chemistry. To evaluate the impact of withanolides, the secondary metabolites of *Physalis* plants, to specialized *H. subflexa* larvae, we analyzed life-history traits, such as development and immunity of larvae reared on diet containing withanolides. Although the antibacterial activity of withanolides is well known, we were particularly interested in their activity against entomopathogenic bacteria. Moreover, the question of possible tritrophic interactions was raised by studying the interaction among withanolides, *H. subflexa* and entomopathogenic bacteria.

2 Overview of Manuscripts

Manuscript I (Chapter 3)

Immune defense strategies of generalist and specialist insect herbivores

Andrea Barthel, Isabell Kopka, Heiko Vogel, Peter Zipfel, David G. Heckel & Astrid T. Groot

Proceedings of the Royal Society B: Biological Sciences 281(1788), 2014

In Manuscript I, we compared the immune defense strategy of the generalist *Heliothis virescens* and the specialist *Heliothis subflexa*, two closely related heliothine moths with non-overlapping host plant preference but geographically overlapping territories in the Americas. We used *in vitro* and *in vivo* experiments to examine the effect of entomopathogenic and non-pathogenic bacteria on hemocytes and larvae of *Heliothis virescens* and *Heliothis subflexa*. We found that the specialist *Heliothis subflexa* showed a higher mortality, a higher number of recoverable bacteria, a stronger induced hemocytic proliferation and a lower phagocytic activity compared to the generalist *Heliothis virescens*. We suggest that diet breadth and related environmental factors are involved in the evolution of immune defense strategies in these moth species.

Andrea Barthel planned, performed and established the *in vitro* and *in vivo* experiments, analyzed the data, prepared all figures and wrote the manuscript. Isabell Kopka helped with the implementation of all in vitro assays. Dr. Heiko Vogel, Dr. Astrid T. Groot and Prof. David G. Heckel participated in the design and coordination of this study and revised the manuscript. Prof. Peter Zipfel provided analysis tools for all *in vitro* assays.

Manuscript II (Chapter 4)

Consequences of an induced immune response on sexual traits in *Heliothis virescens*

Andrea Barthel[1], Heike Staudacher[1], Antje Schmalz, David G. Heckel & Astrid T. Groot

[1]These authors contributed equally

Manuscript in preparation, to be submitted to *Evolution*

In Manuscript II, we analyzed the costs caused by an activated immune defense response to pathogenic bacteria in males and females of *Heliothis virescens*. We studied the expression level of immune related genes in the context of mating behavior in both sexes. Furthermore, sex pheromone production and calling behavior of females were analyzed for changes due to immune challenge. Our data demonstrate that an immune challenge in *H. virescens* provokes a sex-specific immune response, which was associated with a reduced mating success and a modified sex pheromone composition in immune challenged females. We conclude that female moths invest more in an efficient immune defense response against pathogens at the cost of reproductive effort, compared to male moths.

Andrea Barthel planned and performed the experiments, analyzed the data, prepared all figures and wrote the manuscript. Heike Staudacher helped planning the experiments, performed all statistical analysis and wrote the manuscript. Antje Schmalz helped with GC analysis and behavioral assays. Dr. Astrid Groot and Prof. D. G. Heckel participated in the design and coordination of this study and revised the manuscript.

Manuscript III (Chapter 5)

What a big impact a small berry can make:
Herbivorous specialization on *Physalis* plants

Andrea Barthel, Heiko Vogel, Yannick Pauchet, Gerhard Pauls, Astrid T. Groot, Wilhelm Boland, David G. Heckel & Hanna M. Heidel-Fischer

Manuscript in preparation, to be submitted to *Proceedings of the National Academy of Sciences of the United States of America*

In Manuscript III, we investigated the cause and consequences of host plant specialization of the heliothine moth *Heliothis subflexa*. We used a withanolide extract isolated from *Physalis peruviana* to determine the impact of these secondary plant metabolites on larvae of *H. subflexa*. We found an improved larval growth rate, an induced phenoloxidase activity and an up-regulated immune gene expression of larvae which were fed on diet containing withanolides. Further, we found that withanolides possess antimicrobial activity against vegetative cells and spores of the bacterium *Bacillus thuringiensis*, and that withanolides improve the survival rate of larvae infected with this bacterium. This work demonstrates that withanolides, as secondary plant metabolites, indirectly favor the host plant specialization of *H. subflexa* by their effects on the immune system and pathogen defense.

Andrea Barthel planned, performed and established the experiments, analyzed the data, prepared all figures and wrote the manuscript. Hanna M. Heidel-Fischer planned and helped with the experiments and wrote the manuscript. Dr. Heiko Vogel and Dr. Yannick Pauchet participated in the design and implementation of all experiments and the discussion of the results. Gerhard Pauls performed the HPLC analysis and extracted withanolides. Dr. Astrid Groot and Prof. D. G. Heckel participated in the design and coordination of this study and revised the manuscript. Prof. Wilhelm Boland provided analysis tools to extract withanolides.

3 Manuscript I

Immune defense strategies of generalist and specialist insect herbivores

Proceedings of the Royal Society B: Biological Sciences 281(1788), 2014

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3.1 Abstract

Ecological immunology examines the adaptive responses of animals to pathogens in relation to other environmental factors, and explores the consequences of trade-offs between investment in immune function and other life-history traits. Among species of herbivorous insects, diet breadth may vary greatly, with generalists consuming a wide variety of plant families and specialists restricted to a few species. Generalists may thus be exposed to a wider range of pathogens exerting stronger selection on the innate immune system. To examine whether this produces an increase in the robustness of the immune response, we compared larvae of the generalist herbivore *Heliothis virescens* and the specialist *H. subflexa* challenged by entomopathogenic and nonpathogenic bacteria. *H. virescens* larvae showed lower mortality, a lower number of recoverable bacteria, lower proliferation of hemocytes and higher phagocytic activity. These results indicate a higher tolerance to entomopathogenic bacteria by the generalist, which is associated with a more efficient cell-mediated immune response by mechanisms that differ between these closely related species. Our findings provide novel insights into the consequences of diet breadth and related environmental factors, which may be significant in further studies to understand the ecological forces and investment trade-offs that shape the evolution of innate immunity.

3.2 Introduction

Ecological immunology is an emerging field that seeks to understand the causes and consequences of variation in immune system responses in relation to evolution and ecology (Rolff and Siva-Jothy 2003; Schulenburg et al. 2009). The general importance of the influence of environmental factors, e.g. interactions with pathogens and diet breadth, on host immune system evolution has been highlighted in several theoretical articles (Schulenburg et al. 2009). Interactions with pathogens will influence the evolution of immune system responses to protect the host from tissue damage by pathogenic infections. The unpredictable assembly of pathogens in the environment of herbivorous insects requires a high diversity and flexibility in host recognition and immune effector mechanisms (Schulenburg et al. 2009). As an example of the influence of environmental factors on the evolution of the immune system, it has been shown that invasive species can display variation in their immune defenses, such as expansion of the antimicrobial peptide repertoire, and are thus better defended against potential pathogens compared to closely-related non-invasive species (Lee and Klasing 2004; Vilcinskas et al. 2013). Immune defense strategies among species with different diet breadth might vary due to fact that these species compensate costs and benefits differently depending on environmental factors related to their life history (Moret 2003; Lee et al. 2006a). Here, we posed the question of whether generalist herbivores have a more effective immune system than closely related specialist herbivores. To address this question, we selected the generalist *Heliothis virescens* (Hv) and the specialist *Heliothis subflexa* (Hs), two closely related heliothine moths co-occurring in North and South America. Hv is a major pest in many crops and can feed on at least 14 different plant families (Sheck and Gould 1993). The broad host plant range provides great resource availability, but also leads to an elevated level of exposure to stress factors (e.g. diverse plant defensive compounds) and a large variety of potential pathogens encountered in this variable environment. In contrast to the broad generalist Hv, Hs has a narrow nutritional niche, it is specialized on *Physalis* plants, and cannot develop on plants outside this genus (Laster et al. 1982). Larvae of Hs could theoretically profit in two ways from the *Physalis* fruits they are feeding on: first, the *Physalis* fruit is covered by a calyx that creates a so-called enemy-free space (Oppenheim and Gould 2002; Puente et al. 2008); second, withanolides, secondary metabolites of *Physalis* species, display antibacterial activity (Pietro et al. 2000; Januário et al. 2002; Nathiya and Dorcus 2012), which may protect the feeding larvae from environmental pathogens.

The diversity of potential pathogens on a single plant species is likely to be much lower than on the high number of plant species that Hv utilizes. Although the large majority of bacteria in the environment of herbivores may be harmless or sometimes even beneficial, a number of pathogenic bacteria can cause insect-specific infectious diseases. For instance, the gram-positive bacterium *Bacillus thuringiensis* (Bt) and the gram-negative bacterium *Serratia entomophila* are pathogenic to insects (Macintosh et al. 1990; Jackson et al. 1991; Núñez-Valdez et al. 2008). Although in *B. thuringiensis* the endotoxin that is produced during the process of spore formation is the major virulence factor (Schnepf et al. 1998), vegetative cells of this bacterium can kill insects *in vitro* and *in vivo* (Zhang et al. 1995; Estruch et al. 1996). Since *B. thuringiensis* generates toxins against various pests, it is not only frequently used for insect pest management, but also a common entomopathogenic bacterium in the environment of herbivorous insects (Betz et al. 2000). *Serratia entomophila* has been mostly studied in relation to the scarab beetle *Costelytra zealandica* and only a few studies focused their attention on their pathogenicity to lepidopteran larvae (Núñez-Valdez et al. 2008). Since both entomopathogenic bacteria occur on plants, caterpillars may acquire these bacteria either by ingestion, with bacteria colonizing the digestive tract, or through wounding, such as a bite or cut.

In general, ingestion of pathogens is probably the main route of infection in insects. However, pathogens may also access the haemocoel directly, either through a breaching of the cuticle, or by assisted transport by entomophagous parasites (Vallet-Gely et al. 2008). Once entomopathogenic bacteria reach the hemocoel of insects, they must have the ability to attack or manipulate host immunity to colonize the host. Many bacteria are mediators of apoptosis to host cells during their pathogenesis and killing host hemocytes might be an important tool to successfully multiply within the host organism (Chen and Zychlinsky 1994; Zychlinsky and Sansonetti 1997a; Zychlinsky and Sansonetti 1997b). Subsequently, pathogenic bacteria are able to grow unrestricted by the host immune system (Daborn et al. 2002) and by producing toxins they are able to kill the host (Ffrench-Constant et al. 2003). In addition, non-pathogenic or opportunistic bacteria, like the gram positive *Bacillus subtilis*, have the potential to cause damage in insects with impaired immunity but not in healthy insects (Pirofski and Casadevall 2012).

The very different probabilities of exposure to pathogens or at least pathogen diversity of these closely related species inspired us to study differences in their immune defense responses. Since the immune response results in a competition of limited metabolic resources,

the final outcome of the competition between host and pathogen depends largely on the effectiveness of co-occurring physiological, biochemical and behavioral mechanisms of the host. One would expect highly polyphagous insects to differ from specialist herbivores in requiring a more efficient and diverse immune defense response to deal with the high variety of microorganisms. However, so far no studies have been conducted in herbivorous insects to address the influence of environmental factors (i.e. likelihood of pathogen exposure) on immunity. We chose to expose both Hv and Hs to the non-pathogenic bacterium *Bacillus subtilis* and the entomopathogenic bacteria *B. thuringiensis* and *Serratia entomophila* to compare the immune defense responses between a generalist and a specialist. Our data suggest that the specialist Hs is more sensitive to pathogenic bacteria and possesses a weaker cell-mediated immune response when compared to the closely related generalist Hv.

3.3 Material and Methods

3.3.1 Insects

Heliothis subflexa (Hs) and *Heliothis virescens* (Hv) were provided from North Carolina State University (NCSU) laboratory colonies. Hv originates from larvae collected in Clayton, North Carolina in 1988 (JEN2 strain), while Hs larvae were collected in 1985 near Gainesville, Florida and reared at the USDA Insect Attractants, Behavior and Basic Biology Research Laboratory until 1989, after which the rearing was continued at NCSU (Sheck and Gould 1995) (see Supplementary Material section 3.6.1 for rearing details).

3.3.2 Bacterial strains

Bacterial strains used in this study were the non-pathogenic *Bacillus subtilis,* and two entomopathogenic species *Bacillus thuringiensis* subsp. *kurstaki* strain HD73 and *Serratia entomophila.* The preparation of vegetative cells of these bacteria is described in the Supplementary Material section 3.6.2. Vegetative cells of *S. entomophila*, *B. thuringiensis* and *B. subtilis* were used for larval survival analysis (Figure 3.2), bacterial survival analysis (Figure 3.3), phagocytosis assay (Figure 3.4), migration assay (Figure S3.3), apoptosis assay (Figure 3.5), lytic zone assay and phenoloxidase assay (Figure S3.4).

3.3.3 Effect of Bt spores and Cry1Ac on larval growth and survivorship

Feeding experiments using *B. thuringiensis* HD73 spores were conducted to investigate effects on Hv and Hs larval growth and survival. Early 3rd instar larvae were exposed to diets containing $5x10^4$, $7.5x10^4$, 10^5 and $5x10^5$ Bt spores per ml diet, whereby the respective specific larval diet for Hv and Hs was used. For each treatment, the spore solution of *B. thuringiensis* HD73 was applied to 1 ml diet and larval growth and survival of third instar larvae was recorded daily for 7 days. In the control treatment, larvae were reared on pure artificial diet. The larval growth rate is given as average larval growth rate over 7 days. Further, to investigate the possibility that Hv and Hs differ in their susceptibility to Cry1Ac toxin, we determined the efficacy of Cry1Ac apart from spore effects in a feeding experiment with both species (see Supplementary Material section 3.6.3).

3.3.4 Effect of vegetative bacterial cells on larval survival and bacterial recovery in vivo

Direct injection of bacterial cells into the body cavity mimic that bacteria directly breach the cuticle, and has been utilized to decipher molecules that are involved in the immune response (Shelby and Popham 2008). Using injection, we achieved a defined, equal and straightforward immune response in hemocytes of both larvae. For all *in vivo* experiments, fourth instar larvae of Hs and Hv were injected with 2 µl PBS containing vegetative cells of *B. subtilis*, *B. thuringiensis* or *S. entomophila* into the larval haemocoel using a 10 µl Hamilton syringe. See Supplementary Material section 3.6.4 for details about larval survival and bacterial recovery analysis.

3.3.5 In vitro assays to evaluate cell-mediated immunity

To determine whether Hv and Hs caterpillars showed a differential response to vegetative cells of entomopathogenic or non-pathogenic bacteria *in vitro*, we assessed the rate of phagocytosis, hemocyte migration and cell apoptosis by hemocytes of both species. For these assays, we needed to prepare a primary hemocyte culture (see Supplementary Material section 3.6.5 for more details). See Supplementary Material for details about phagocytosis (3.6.6), migration (3.6.7) and apoptosis assay (3.6.8).

3.3.6 Enzyme activity in the hemolymph in vivo

To estimate the hemocytic lysozyme-like and phenoloxidase (PO) activity in response to injection with PBS and vegetative cells of different bacterial strains in both species,

hemolymph was directly collected from larvae. Twenty larvae of each strain were sampled from each treatment, 60 and 120 minutes post injection. Hemolymph was collected by puncturing the larvae with a sterile hypodermic needle. The freely flowing hemolymph was colected on a Parafilm®. See Supplementary Material for details on the lysozyme-like (3.6.9) and phenoloxidase assay (3.6.10).

3.3.7 Data analysis

All statistical tests were run with the computer program SPSS 17.0 and are described in the Supplementary Material section 3.6.11.

3.4 Results

3.4.1Effect of Bt spores and Cry1Ac toxin on larval growth and survivorship

In general, growth rate of untreated Hv larvae was higher compared to Hs on their respective artificial diets (Figure 3.1 A). A gradual decrease in larval growth rate was correlated with an increase in Bt spore concentration in both larvae. Already the lowest concentration of $5x10^4$ Bt spores had a significant negative impact on the larval growth rate of both species. With the highest concentration of $5x10^5$ Bt spores, larval growth rate turned negative due to a high weight loss and mortality of both larvae.

In the 7-day growth experiment, larval mortality was similar in both species (Figure S3.1) except in treatments with concentrations of $7.5x10^4$ Bt spores, where Hs larvae showed a significantly higher mortality than Hv larvae (Figure 3.1 B). In addition, there was an obvious lower susceptibility in Hv larvae when exposed to $7.5x10^4$ Bt spores: Growth rate of Hv larvae was not significantly different between concentrations of $5x10^4$ and $7.5x10^4$ spores, whereas Hs larvae were significantly more affected by $7.5x10^4$ Bt spores compared to $5x10^4$ Bt spores (Figure 3.1 A).

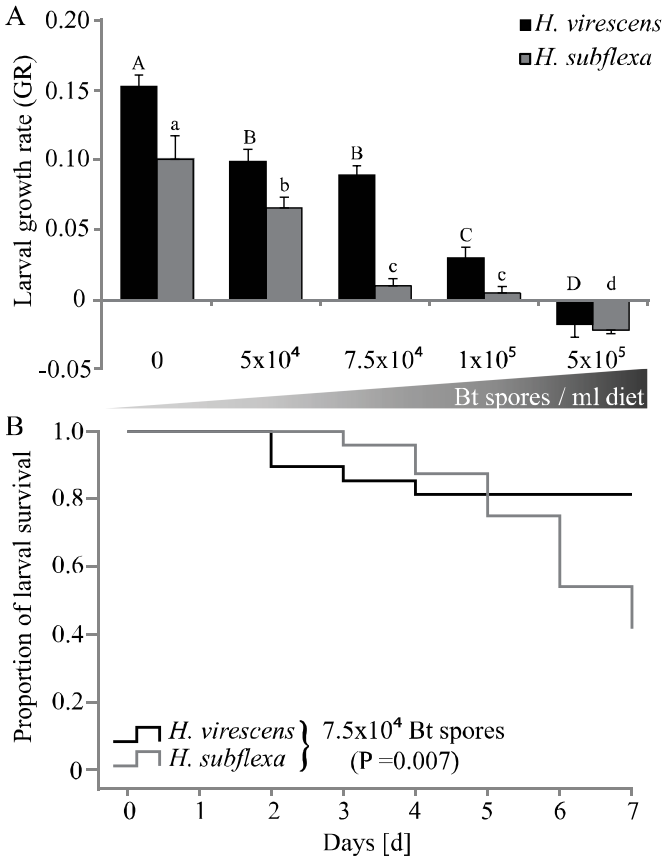

Figure 3.1: Larval growth rate and survival for *H. virescens* and *H. subflexa* on different concentration of Bt spores. (**A**) Average growth rates of *H. virescens* (black) and *H. subflexa* (grey) larvae on different Bt spore concentrations after 7 days. Bars indicate means and standard errors (n=24). Mann-Whitney U-test was used for the paired comparisons between the treatments within each species. Different letters above the bars represent significant differences. (**B**) Kaplan-Meier survival plot of *H. virescens* (black) and *H. subflexa* (grey) larvae fed on $7.5x10^4$ Bt spores (n=24). Statistical significance was determined using Cox regression survival analysis.

When we tested the susceptibility of both species to Cry1Ac (MVP) apart from spores, we found the opposite result. Hs appeared to have a lower susceptibility than Hv when exposed to Cry1Ac (Figure S3.2). This was evident in a significantly higher larval growth rate of Hs compared to Hv, when larvae fed on diet containing 0.1 µg Cry1Ac (Figure S3.2 A). With the highest concentration of 1 and 10 µg Cry1Ac we did not find any significant differences in the larval growth rate between both species. Furthermore, Hv larvae showed a significantly higher mortality than Hs larvae at all Cry1Ac concentrations tested (Figure S3.2 B-D).

Finding a lower susceptibility of Hv larvae compared to Hs larvae to Bt spores in a specific concentration range, we assessed whether there are additional, immune-related differences between both species. To directly compare defense strategies of both species, we continued with *in vivo* (injections) and *in vitro* (primary cell cultures) experiments to target hemocyte mediated immune responses using vegetative cells of all three bacterial strains.

3.4.2 Effect of vegetative bacterial cells on larval survival and bacterial recovery in vivo

We found that vegetative cells of both entomopathogenic bacteria, *S. entomophila* (Figure 3.2 A) and *B. thuringiensis* (Figure 3.2 B) were indeed pathogenic to Hv and Hs larvae. Overall, 10^5 cells of either bacterial species significantly increased larval mortality of both insect species compared to 10^4 cells of these bacteria, thus the toxicity of these bacteria is dose-dependent in both cases (Table S3.1). There was no significant overall difference in mortality between the two insect species when 10^4 or 10^5 cells of *S. entomophila* (Figure 3.2 A) were injected. Also, in both insect species larval mortality started sooner and was higher in response to injection with *B. thuringiensis* compared to injections with *S. entomophila* (compare Figure 3.2 A to B). However, a concentration of 10^5 cells of *B. thuringiensis* (Figure 3.2 B) resulted in a significantly higher mortality in the specialist Hs than in the generalist Hv. In addition, all dosages of both pathogenic bacteria induced larval death four to six hours earlier in Hs than Hv larvae.

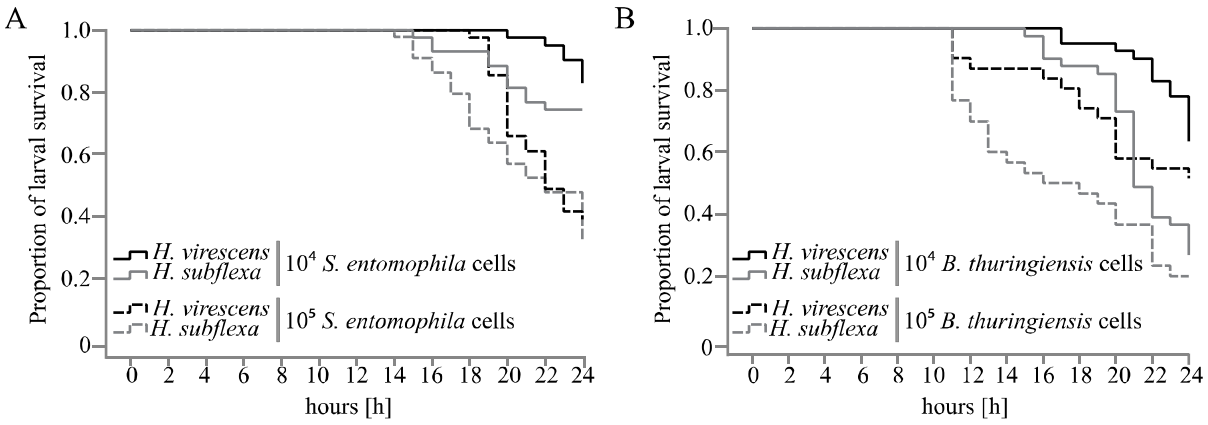

Figure 3.2: Larval survival of infected *H. virescens* and *H. subflexa* upon injection with different entomopathogenic bacterial doses. Kaplan-Meier survival plot of *H. virescens* (black) and *H. subflexa* (grey) larvae injected with (**A**) 10^4 (Hv: n=41; Hs: n=43) or 10^5 (Hv: n=44; Hs: n=41) cells of *S. entomophila* and (**B**) 10^4 (Hv: n=41; Hs: n=31) or 10^5 (Hv: n=41; Hs: n=30) cells of *B. thuringiensis.*

Given the high mortality rate of both Hs and Hv larvae in response to the pathogenic bacteria, we investigated the survival rate of these bacteria within infected larvae. Following injection of $5x10^4$ vegetative cells of *S. entomophila* (Figure 3.3 A) or *B. thuringiensis* (Figure 3.3 B), the number of recoverable bacteria increased over time. With *S. entomophila,* the increase in CFU was significantly higher at every time point in Hs than in Hv (Figure 3.3 A). With *B. thuringiensis*, CFU was only significantly higher in the specialist Hs than in the generalist Hv larvae at 24 hour post-injection (Figure 3.3 B). Interestingly, the time frame in which the highest number of recoverable pathogenic cells occurred correlated with the highest

mortality rate of both larvae (compare Figure 3.3 to Figure 3.2). In contrast, the non-pathogenic *B. subtilis* was cleared from the hemolymph of both species with no live bacteria being recoverable after 30 hours (Figure 3.3 C).

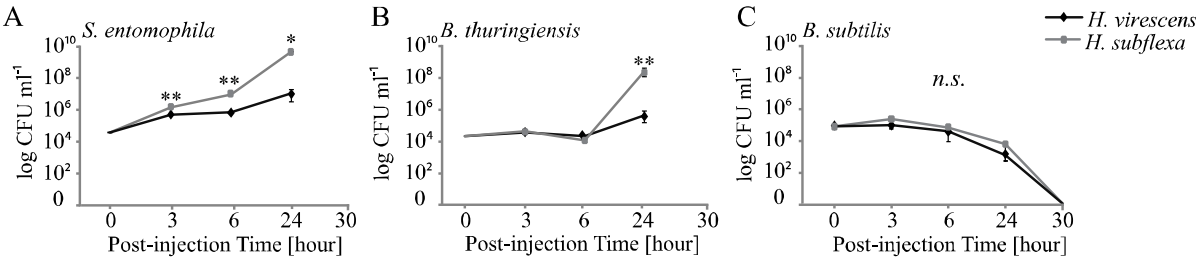

Figure 3.3: CFU of *S. entomophila*, *B. thuringiensis* and *B. subtilis* in infected *H. virescens* and *H. subflexa* larvae. Number of bacteria over specific time points after injection of Hv and Hs with (**A**) $1x10^4$ cells of *S. entomophila*, (**B**) $1x10^4$ cells of *B. thuringiensis* and (**C**) $4x10^4$ cells of *B. subtilis.* Each time point represents the mean of six insects and their associated standard error. Differences between the two species for each time point within one treatment were assessed with Student's t-test after normalization by log-transformation (Kolmogorov-Smirnov Test: P>0.05). Significant differences are indicated by $^{**}P<0.01$; $^{*}P<0.05$; *n.s.* = not significant.

3.4.3 Hemocyte phagocytosis in vitro

In their ability to phagocytize either vegetative cells of *S. entomophila* or *B. thuringiensis in vitro*, Hv and Hs did not differ within the first 30 minutes after incubation with *S. entomophila* (Figure 3.4 A): Hv and Hs hemocytes showed the same phagocytic activity against this entomopathogenic bacterium. However, after an incubation of 60 and 120 min there was a significant difference in phagocytosis between both hemocytes: Hv hemocytes showed a higher phagocytic activity against *S. entomophila* compared to Hs hemocytes. Similarly, the rate of phagocytosis of *B. thuringiensis* by Hv hemocytes was greater at all time-points than that of Hs hemocytes and significantly greater after 7.5, 30 and 60 minutes (Figure 3.4 B). When the non-pathogenic bacterium *B. subtilis* was incubated with either hemocytes, we found no significant difference in phagocytosis between Hv and Hs hemocytes (Figure 3.4 C). Thus, the higher number of recoverable pathogenic bacteria in the specialist Hs larvae is based on a lower Hs hemocyte phagocytosis of pathogenic bacteria compared to the generalist Hv hemocytes. However, we found the overall phagocytosis rate of bacterial cells by hemocytes never to reach 100 %, which is because the assay was *in vitro*, where bacterial cells only have to deal with hemocytes and are in perfect buffered solution which allows multiplication of bacterial cells. Due to the limited lifetime of hemocytes *in vitro*, we only observed phagocytosis for up to 120 minutes.

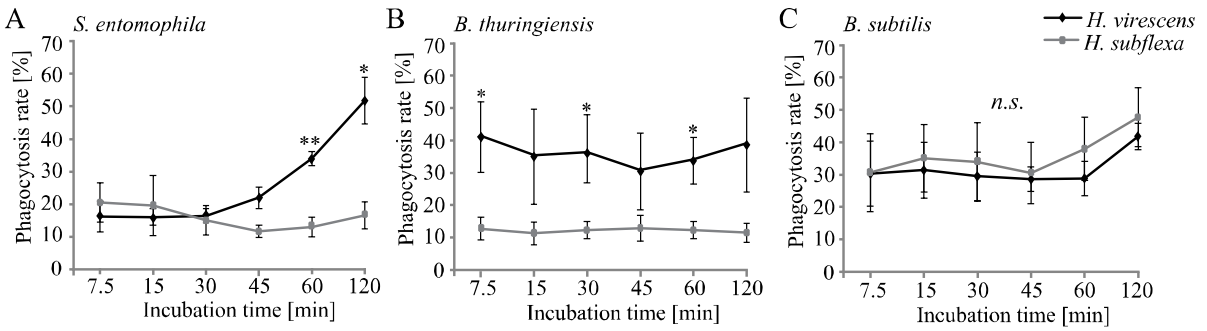

Figure 3.4: Time kinetics of phagocytosis of *S. entomophila, B. thuringiensis* and *B. subtilis* by *H. virescens* and *H. subflexa* hemocytes *in vitro.* Hemocytes were stimulated with (**A**) *S. entomophila*, (**B**) *B. thuringiensis* and (**C**) *B. subtilis*. Error bars represent the SEM of at least three independent experiments. Differences between the two species for each time point within one treatment were assessed with Student's t-test after normalization by arcsine-square root transformation (Kolmogorov-Smirnov Test: P>0.05). Significant differences are indicated by ***P*<0.01; **P*<0.05; *n.s.* = not significant.

3.4.4 Hemocyte migration in vitro

We found no significant difference between Hv and Hs hemocyte migration rates towards the pathogenic *B. thuringiensis* and the non-pathogenic *B. subtilis* and these migration rates were not different compared to untreated hemocytes either. However, Hv and Hs hemocyte migration was significantly higher towards *S. entomophila* when compared to untreated hemocytes (Figure S3.3). Thus, the higher phagocytosis rate in response to pathogenic bacteria in Hv larvae was not based on a higher Hv hemocyte migration towards pathogenic bacteria compared to Hs hemocytes.

3.4.5 Hemocyte apoptosis in vitro

When Hv hemocytes were incubated with vegetative cells of *S. entomophila,* a time dependent increase in annexin-positive cells was evident, with a significant increase in apoptotic cells after 60 and 120 minutes compared to the base line (Figure 3.5 A). In contrast, Hs larvae showed a significantly induced proliferation of hemocytes (negative percentage of annexin-positive cells) in response to *S. entomophila* after 30 minutes, but after 120 minutes of incubation annexin-positive hemocytes significantly increased compared to the base line (Figure 3.5 A). While there was a significant difference between Hv and Hs hemocytes in apoptosis rate after 60 minutes incubation with *S. entomophila,* after 120 minutes *S. entomophila* induced apoptosis of hemocytes in both species.

Vegetative cells of *Bacillus thuringiensis* and *B. subtilis* had no significant effect on Hv hemocytes, neither on apoptosis nor on proliferation of hemocytes (Figure 3.5 B and Figure 3.5 C). In contrast, there was a significant proliferation of Hs hemocytes when incubated with

B. subtilis at all time points and with *B. thuringiensis* after 60 and 120 minutes of incubation (Figure 3.5 B and Figure 3.5 C). Overall, Hs hemocytes showed an induced proliferation in response to all bacterial cells, however at different time points, than Hv hemocytes (Figure 3.5).

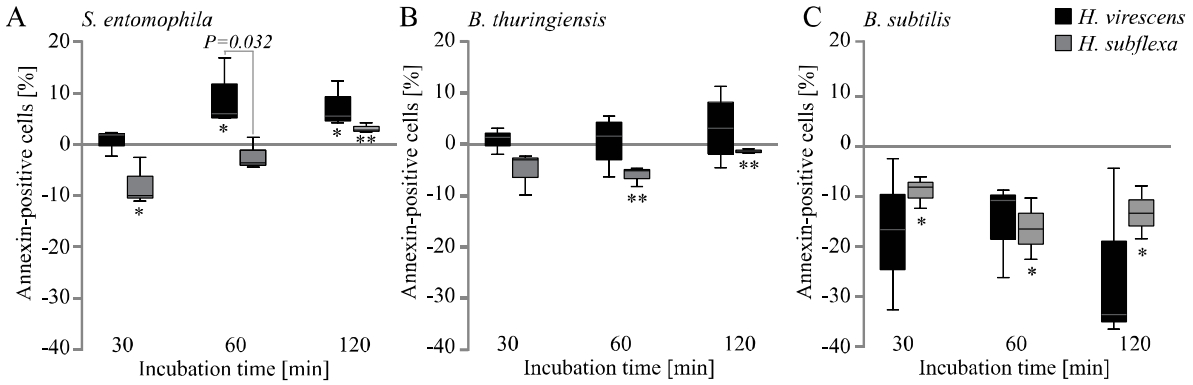

Figure 3.5: Percentage of Annexin-positive hemocytes exposed to *S. entomophila*, *B. thuringiensis* and *B. subtilis*. Hemocytes were incubated with (**A**) *S. entomophila*, (**B**) *B. thuringiensis* and (**C**) *B. subtilis* for 30, 60 and 120 minutes. Values on the y-axis represent percentages of Annexin-positive hemocytes, as proportions of total hemocytes relative to the control (base line 0 % apoptosis). Each time point represents the mean of three to four biological replicates and their corresponding SEM. Significant differences were determined between the two species for each time point within one treatment (*P*-value above the bars) and within one species compared to their corresponding control (Asterisks below the bars) using Student t test of arcsine-square root transformed data. Significant differences are indicated by **P<0.01; *P<0.05.

3.4.6 Enzyme activity in the hemolymph in vivo

When Hv larvae were injected with vegetative cells of the entomopathogenic bacteria *S. entomophila* or *B. thuringiensis*, lysozyme activity significantly increased after 60 minutes compared to the control, but this increase in activity was not significantly different from wounded larvae. However, 120 minutes post injection of *S. entomophila*, lysozyme activity significantly increased in Hv larvae compared to untreated and wounded larvae (Figure S3.4 A). In contrast, injection of *S. entomophila* and *B. thuringiensis* in Hs larvae led to a significant decrease in lysozyme activity compared to uninfected and wounded control larvae at both time points (Figure S3.4 B).

No upregulation was found for PO activity in Hv larvae following 60 minutes post injection of *S. entomophila* or *B. thuringiensis* compared to untreated control larvae (Figure S3.4 C). However, after 120 minutes the PO response against *B. thuringiensis* was enhanced in Hv larvae, although this was not significantly different from wounded Hv larvae, whereas no changes in PO activity was observed in Hs larvae after 60 and 120 minutes (Figure S3.4 D). Following injection with the non-pathogenic bacterium, *B. subtilis*, Hv larvae showed no significant response in their lysozyme and PO activity, while Hs larvae had a significantly

lower lysozyme after 120 minutes compared with wounded and untreated larvae. Overall, Hv larvae showed an increase in both lysozyme and PO activity, while Hs larvae showed a decrease in lysozyme activity and no change in PO activity in response to both pathogenic and non-pathogenic bacterial cells.

3.5 Discussion

The data presented here demonstrates the fundamental role of diet breadth and pathogen exposure on the evolution of immune defense strategies in two very closely related species, the generalist *H. virescens* and the specialist *H. subflexa.* Our results show that the closely related Hv and Hs, with overlapping geographic ranges but non-overlapping host plant ranges, combat bacterial infections in remarkably different ways.

3.5.1 Lower susceptibility of Hv larvae fed on Bt spores

Our findings that the generalist Hv can withstand higher Bt spore concentrations than the specialist Hs may reflect a higher likelihood of Hv encountering Bt spores on diverse host plant surfaces. Our results are consistent with previous studies, showing that Bt spores are acutely toxic in high amounts and growth-inhibiting at low concentrations (Gould et al. 1991; Johnson and McGaughey 1996). One important virulence factor is the parasporal crystalline inclusion of the pore-forming Cry-type protein toxin, which lyses the insect's midgut epithelial cells and allows vegetative bacterial cells to enter the body cavity and proliferate (Aronson 1993). Spore toxicity is greatly reduced or absent in Bt strains that do not produce Cry toxins (Raymond et al. 2012). However, a toxin-spore mixture causes higher mortality than toxin alone (Li et al. 1987). The JEN2 strain of Hv we studied does not possess toxin-resistance mechanisms found in other Hv strains, such as altered proteolytic activity affecting protoxin processing and maturation (Oppert 1999) or modification of toxin receptors (Gahan et al. 2001; Gahan et al. 2010). In fact, we found that the JEN2 strain of Hv was more sensitive to Cry1Ac toxin than was Hs, thus the lower sensitivity of Hv to spores must be due to factors other than the direct response to toxin. Previously it has been shown that an elevated immune status of *Ephestia kuehniella* was associated with a higher tolerance against a mixture of endotoxins and spores of *B. thuringiensis* (Rahman et al. 2004). In our studies, the lower susceptibility of Hv to Bt spores suggests the presence of efficient immune defense

mechanisms which combat newly germinated vegetative bacteria resulting in a retarded or reduced sepsis in the hemocoel. Further, the higher efficacy of Bt spores in the specialist Hs might indicate that the bacteria can cope better with cellular and humoral immune effectors of Hs, by modifications of their surface proteins or secreted proteases that degrade antimicrobial proteins. Because of the importance of Bt toxins in pest control, most studies have focused their attention on purified Bt endotoxins or transgenic Bt plants (Shelton et al. 2002; Zhao et al. 2003) and only a few have tested Bt spores in feeding experiments (McGaughey 1978; Gould et al. 1991; Johnson and McGaughey 1996). Therefore little is known about the infection strategy of entire Bt spores and how this could be combated by insect innate immunity.

3.5.2 Higher larval mortality and CFU in Hs than in Hv larvae

To date only a few studies exist where insecticidal activities of vegetative cells of *B. thuringiensis* to lepidopteran larvae were tested (Lövgren et al. 1990; Estruch et al. 1996; Salamitou et al. 2000), and even fewer studies focused their attention on the toxicity of *S. entomophila* to lepidopteran larvae (Núñez-Valdez et al. 2008). Similar to what has been documented for pathogenic bacteria in *M. sexta* larvae (Au et al. 2004), the high mortality rate in response to entomopathogenic bacteria correlated with high bacterial titers in the hemolymph of Hv and Hs larvae. The higher pathogenicity and the unhindered growth of both pathogenic bacteria in larvae of the specialist Hs compared to Hv may be due to a relative resistance of those bacteria to phagocytosis by Hs hemocytes, resulting in multiplication of bacterial cells, while a significantly lower bacterial multiplication in Hv larvae may reflect efficient phagocytosis by Hv hemocytes. This suggests that Hv both recognizes and can resist these bacteria up to a certain number, while Hs does not. The fact that we did not find a significant difference in mortality between Hs and Hv in response to *S. entomophila* may be due to a lower distribution frequency of *S. entomophila* in nature compared to *B. thuringiensis.*

3.5.3 Hv hemocytes showed a higher phagocytic activity against pathogenic bacteria than Hs

Our results suggest a more efficient capability of the generalist Hv to kill pathogenic bacteria, compared to the specialist Hs. The low phagocytic activity of Hs hemocytes might indicate that both pathogenic bacteria resist phagocytosis by Hs hemocytes due to immune suppressive

factors. For instance, pathogenic bacteria, such as *Photorhabdus*, are able to inhibit their own phagocytosis due to immune suppressive factors (Van Sambeek and Wiesner 1999; Ffrench-Constant et al. 2003). In addition, it is important to note that distinct hemocyte cell types appear to be involved in phagocytosis in different insect species (Lavine and Strand 2002; Ribeiro and Brehelin 2006). For instance, in *Helicoverpa armigera*, granular cells and plasmatocytes appear to be the only phagocytic cells (Mazet et al. 1994; Lavine and Strand 2002). It may thus be possible that Hv and Hs vary in the amount of hemocyte cell types, which ultimately results in different phagocytic capabilities.

3.5.4 Hemocyte migration rate of both species is similar

Our results reveal that (i) the higher phagocytosis rate of Hv hemocytes is not based on a higher migration capability of hemocytes and (ii) hemocytes of both Hv and Hs are attracted only to the gram-negative bacterium *S. entomophila*. Similarly, Schneeweiß *et al*. (1993) (Schneeweiß and Renwrantz 1993) reported that *Serratia marcescens* stimulates the migration of mussel hemocytes. Interestingly, *B. thuringiensis* was not attractive to Hv and Hs hemocytes, while previous studies showed that invertebrate cells are attracted to gram-positive bacterial cells (Cheng and Howland 1979). The lack of *Heliothis* hemocyte migration towards the gram-positive *B. thuringiensis* suggests a pathogenic strategy to avoid (or delay) detection by not displaying immune elicitors on their surfaces (Vallet-Gely et al. 2008).

3.5.5 Hs hemocytes induced proliferation instead of apoptosis in response to bacterial challenge

Studies in *Bombyx mori* have shown that the gram-negative *S. marcescens* induces apoptosis via a lipopolysaccharide (LPS) dependent mechanism (Ishii et al. 2012). Such an LPS-induced apoptosis may also have occurred in Hv and Hs hemocytes in our study, because we measured apoptosis in response to the gram-negative *S. entomophila* in both hemocytes. *In vitro* studies showed that entomopathogenic *B. thuringiensis* (Bt13) can kill insect cells (Zhang et al. 1995). However, our present study does not confirm that another strain *B. thuringiensis* (HD 73) is able to kill Hv and Hs hemocytes within 120 minutes of incubation. Interestingly, we found exclusively in the specialist Hs an induced proliferation of hemocytes in response to *B. thuringiensis* and *S. entomophila.* The induction of hemocyte proliferation in Hs likely consumes energetic resources. Based on the observed low larval survival rate in Hs, in combination with high growth rate and low phagocytosis of bacterial

cells, the immune defense strategy of Hs appears to be disadvantageous, because an optimal immune defense response should be most efficient at the lowest energetic cost. In addition, pathogenic bacteria might be able to kill Hs hemocytes by using mechanisms that suppresses key cellular immune functions such as phagocytosis, and therefore Hs has to induce hemocyte proliferation to try to overcome the infection (Eleftherianos et al. 2010).

3.5.6 Hv larvae display a different immune-related enzymatic response than Hs larvae

Different immune response strategies in both species might account for the fact that the immune system is a coherent system within an organism, where energy expenditure in one component of immunity may lead to trade-offs in other components. In this case, increased phagocytic activity in the generalist Hv may lead to the down regulation of further components of immunity, reflected in lower PO or lysozyme levels. However, we found that Hv significantly induced lysozyme activity in response to *S. entomophila* after 120 minutes of incubation, where we also detected a significantly induced phagocytosis activity. However, there was a reduced lysozyme activity in the specialist Hs after incubation with both pathogenic bacteria. Thus, a reduced lysozyme and phagocytosis activity during infections in Hs larvae might be due to a certain level of defenselessness against pathogenic bacteria, which might have evolved in an enemy-free environment within the calyx of *Physalis* fruits over time.

3.5.7 Differences in the defense strategy against non-pathogenic bacteria between both species

Although *B. subtilis* is a non-pathogenic bacterium, it was recognized by both Hv and Hs as foreign, resulting in phagocytosis, the most effective mechanism for eliminating foreign material from the hemolymph (Anderson et al. 2005). However, the time period of clearance in Hs larvae was associated with a significantly induced hemocyte proliferation *in vitro*, suggesting that exposure to non-pathogenic bacteria leads to the formation of more phagocytes in Hs larvae, whereas Hv does not require induction of proliferation in response to non-pathogenic bacteria. A response to non-pathogenic bacteria may be dependent on the immune status of the organism. Hs larvae may have to invest in protection against any kind of potentially infectious microbes, whereas Hv larvae may differentiate more specifically between pathogenic and non-pathogenic bacteria, and thus invest significant resources only in case of "dangerous" infections.

3.5.8 Impact of environmental factors on the innate immunity in herbivorous insects

Dietary specialization of *H. subflexa* may seem to be a suboptimal evolutionary strategy, because our laboratory study found no apparent benefit that outweighs the increased susceptibility to pathogens due to the reduced efficiency of immune defense. However, a narrow diet breadth may yield benefits seen only under natural conditions, taking the effects of nutrients and secondary metabolites of the host plant additionally into account. Studies on the relationship between insect immunity and diet breadth have revealed that variation in host plant quality can alter the immune defense response (Klemola et al. 2007), and that secondary metabolites can decrease the immunocompetence and increase parasitism rates in insects (Zvereva and Rank 2003; Haviola et al. 2007; Smilanich et al. 2009). In contrast, a recent study demonstrated that the immune system response of a generalist did not vary when fed on three different plant species, suggesting that feeding on various host plants of different nutritional qualities and secondary metabolite content has no detrimental effect on immune system response in this case (Smilanich et al. 2011). Interestingly, phytochemicals consumed by herbivores can also affect entomopathogens infecting them (Keating et al. 1988; Cory and Hoover 2006). Considering the impact of host plants on the immune status and pathogen susceptibility of insects, our results might have been different in a natural setting. However, it has been predicted that organisms in good environmental conditions have optimal resource amounts to invest in immunity (Triggs and Knell 2012). Due to the high complexity of secondary metabolites and the various nutritional conditions experienced by generalists in nature, measurements of the immune status of a generalist on a single host plant are unlikely to reveal its overall immune defense ability. Therefore, we used artificial diet to provide both species with their optimal nutritional requirements, to obtain their immune system response under controlled conditions.

Besides diet breadth, pathogen exposure is likely to influence immune defense strategies in insects and might provide explanations for immune system variations. Herbivorous insects with a broad host range are more likely to invest more into developing an efficient and specific immune system, because of a high frequency of challenges by pathogens throughout life. Therefore, a higher likelihood of pathogen exposure might affect the selective advantage of specific immune defense strategies by structuring an enhanced immune response sensitivity and specificity to broad environmental factors in generalist herbivores. An alternate strategy to investing in a highly efficient immune system is to minimize exposure to pathogens by specialization on host plants which provides an enemy-free space. For instance,

host plants could possess chemicals on leaf surfaces or an architecture which affects pathogen load or persistence (Hoover et al. 1998b; Oppenheim and Gould 2002; Nathiya and Dorcus 2012). Larvae of Hs spend most of their time surrounded by the large calyx enclosing the fruit on which they feed. Even if specialists gain such a benefit from host plant specialization, they may sacrifice some degree of resilience to environmental changes as they become increasingly dependent on a restricted host plant. On the other hand, there might be a trade-off in evolving a more powerful immune response and other fitness-relevant traits (Schmid-Hempel 2005), however, this might be required when exploiting a broad host range. Whether these traits coevolved in generalist herbivores, and whether the evolution of less effective immune defense strategies in specialist herbivores is a general pattern, can be judged when additional comparisons of generalists and specialists are carried out in the future.

3.5.9 Conclusion

This study confirms that the specialist employs a different strategy to overcome infections with non-pathogenic and entomopathogenic bacteria by investing in hemocyte proliferation-based defense, but sacrifices phagocytosis efficiency and larval survival, compared to the closely related generalist species. Our data are thus consistent with the hypothesis that generalist herbivores have a more efficient immune defense strategy than specialist herbivores. This study is one of the few that has considered the ecological and evolutionary impact of differences in diet breadth on the herbivorous innate immune response in an experimental context. Further immunological studies should take the fundamental role of diet breadth and pathogen exposure on the evolution of innate immunity into account, to enhance our understanding of the ecological and evolutionary forces structuring variation in the immune system of invertebrates.

3.6 Supplementary Material

3.6.1 Insects

In Jena, both strains were maintained by single-pair matings of non-siblings to avoid inbreeding, and fertility and genetic variation (AFLPs) remained high and constant throughout the experiment. Larvae of Hs were reared on a corn-soy blend diet (provided by NCSU), whereas larvae of Hv were reared on artificial diet described in Burton *et al*. (1970) (Burton

1970). Larvae of both species were held in a climate chamber (Snijders, Tilburg, The Netherlands) at 26 °C, 60 +/- 10 % relative humidity and 14:10 light cycle. Third to fourth instar Hv and Hs larvae were used in all experiments.

3.6.2 Preparation of vegetative bacterial cells

Bacillus subtilis and *Serratia entomophila* were obtained from the Department of Bioorganic Chemistry (MPICE, Jena, Germany), except *B. thuringiensis*, which was provided by Dr. Yannick Pauchet (Dept. Entomology, MPICE, Jena, Germany). Bacterial strains were cultured at 30°C and 250 rpm in lysogeny broth (LB) broth or on LB agar (Bertani 1951), except for *S. entomophila* which was grown in CASO medium (Trustees 1995). Vegetative bacterial cells were obtained from overnight cultures and cell counts were estimated by optical density at 600 nm (BioPhotometer, Eppendorf, Hamburg, Germany). Beforehand, known numbers of colony forming units (CFU) were plotted against their corresponding optical density at 600nm to obtain a standard curve for bacterial cell concentration of each strain.

3.6.3 Preparation of B. thuringiensis HD73 spore solution and application of MVP

During sporulation, the strain *B. thuringiensis* HD73 produces the Cry1Ac protein that is toxic to certain lepidopteran insects (Liu et al. 2013). The spore solution was newly prepared beforehand using the following protocol. Bacterial cells from a glycerol stock were plated on LB agar and kept at 30 °C overnight. Subsequently, one bacterial colony was harvested and resuspended in 5 ml LB medium and allowed to grow at 30 °C overnight on a bacterial shaker at 200 rpm. The following day, 100 µl of this bacterial culture was added to 50 ml HCO medium (containing per liter: 7 g casein hydrolysate; 6.8 g KH_2PO_4; 0.12 g $MgSO_4$ $7H_2O$; 0.0022g $MnSO_4$ $4H_2O$; 0.014 g $ZnSO_4$ $7H_2O$; 0.02 g $Fe_2(SO_4)_3$; 0.018 g $CaCl_2$ $4H_2O$; 3 g glucose; the pH was adjusted to 7.2) (Lecadet et al. 1980). After seven days at 30 °C and 250 rpm, spores were harvested. To estimate their concentration, serial dilutions of this suspension were plated onto LB agar. The agar plates were incubated at 30 °C for 48 hours and germinated bacterial colonies were counted (CFU/ml; colony forming units). The spore suspension was stored at 4 °C and before each application the spore concentration was newly determined. Spore solutions of *B. thuringiensis* HD73 were only used for feeding experiments with Hs and Hv to investigate differences in their larval growth and survival (Figure 3.1 and Figure S3.1).

In addition, we tested the efficacy of Cry1Ac toxin against both species apart from the effect of Bt spores. The Cry1Ac toxin used in this study was the formulated product MVP® (Mycogen Corporation, USA). MVP contains only the Cry1Ac toxin of *Bacillus thuringiensis* Berliner var. *kurstaki*, encapsulated within dead cells of *Pseudomonas fluorescens* (Soares and Quick 1992). Aliquots of MVP were added to artificial diet to obtain concentrations of 0.1, 1.0 and 10 µg Cry1Ac/ml diet, which were provided to early 3rd instar larvae of both species. Larval growth and survival was recorded daily for 7 days. In the control treatment, larvae were reared on pure artificial diet. The larval growth rate is given as average larval growth rate over 7 days relative to the corresponding control growth rate.

3.6.4 Larval survival and bacterial recovery analysis upon injection experiments

To document differences in the toxicity of entomopathogenic bacteria (*B. thuringiensis* and *S. entomophila*) to Hs and Hv larvae, each larva was injected with 10^4 or 10^5 vegetative cells of the corresponding bacterial strain. Infected larvae were kept at room temperature and the number of survivors was counted from 10 until 24 hours after injection. The sample size for each treatment varied from 30 to 44 larvae.

To investigate whether entomopathogenic bacteria (*B. thuringiensis* or *S. entomophila*) and non-pathogenic bacteria (*B. subtilis*) survive within Hs and Hv larvae, $5x10^4$ vegetative bacterial cells were injected into the haemocoel. Treated larvae were kept at room temperature and hemolymph samples were obtained after fixed time-points over a period of 24 hours after injection using a hypodermic needle. Serial dilutions of the hemolymph samples were plated onto LB agar. The agar plates were incubated at 30 °C for 48 hours and bacterial colonies were counted and given in CFU/ml (colony forming units). The bacterial colonies were calculated on average of six hemolymph samples, i.e. from six larvae, per time point.

3.6.5 Preparation of primary hemocyte culture

Hemolymph was collected by bleeding uninfected 4^{th} instar larvae of Hv and Hs using a hypodemic needle. Freely flowing hemolymph of approximately 30 larvae was collected into 15 ml FALCON tubes (VWR International GmbH, Darmsadt, Germany) containing 10 ml of cold and sterile anticoagulant buffer (62 mM NaCl, 100 mM glucose, 10 mM EDTA, 30 mM trisodium citrate, 26 mM citric acid; all chemicals were obtained from Carl Roth, Karlsruhe, Germany) (Mead et al. 1986). The solution was centrifuged (2500 pm for 15 sec) and the resultant hemocyte pellet was resuspended in anticoagulant buffer. The number of hemocytes

was determined using a CASY cell counter (Roche Innovatis AG, Bielefeld, Germany) and cell concentrations were adjusted depending on the *in vitro* assay (see below). These hemocyte solutions were directly used in all *in vitro* experiments and referred to as primary cell cultures.

3.6.6 Phagocytosis assay

To determine whether Hv and Hs differ in the rates of phagocytosis of vegetative cells of *B. subtilis*, *B. thuringiensis* and *S. entomophila*, phagocytosis by Hv and Hs hemocytes was determined. Hemocytes of primary cell cultures from Hv and Hs were stained with Vybrant7DiO cell labeling solution (green-fluorescent carbocyanines, Invitrogen, Darmstadt, Germany) in a 1:200 dilution in anticoagulation buffer for 20 min at room temperature. After staining, 10^6 insect cells were added to 10^7 bacterial cells, which had been dyed with Vybrant7DiD cell labeling solution (red-fluorescent carbocyanines; Invitrogen) in a 1:200 dilution for 20 minutes at 37 °C and washed afterwards two times with DPBS (Lonza) supplemented with 1 % BSA (AppliChem).

The rate of phagocytosis was observed in 400 µl DPBS / 1 % BSA and by analyzing samples after 7.5, 15, 30, 45, 60 and 120 minutes. To remove excess bacteria after incubation, hemocytes were washed two times with DPBS / 1 % BSA and centrifuged at low speed. The samples were assayed with a BD LSR II flow cytometer and the DIO^+ / DID^+ positive hemocytes, i.e. DIO^+ positive hemocytes which incorporated DID^+ positive bacterial cells, were analyzed with the BD FACSDiva™ software. Only hemocytes with a larger size and a higher granularity than bacteria were calculated. Additionally, labeled-hemocytes and labeled-bacteria separately served as positive controls, whereas unlabeled hemocytes and unlabeled bacteria are used as negative staining controls to verify a successful staining. The assay was performed at least five times.

3.6.7 Migration assay

The migration activity of hemocytes is a measure of bacteria recognition by these hemocytes. To compare the migration activity of Hv and Hs hemocytes to vegetative cells of *B. subtilis*, *B. thuringiensis* and *S. entomophila*, a Boyden chamber assay was performed. A Boyden chamber is an *in vitro* method to measure the chemotactic activity of solutes on free moving cells e.g. hemocytes (Boyden 1962). For this assay, we used BD Falcon™ 24-well multiwell plates and BD Falcon™ cell culture inserts with a translucent PET (polyethylene

terephthalate; VWR International GmbH, Darmsadt, Germany) membrane containing 8.0 µm pores. The bacteria were washed once with DPBS (Lonza, Basel, Switzerland) supplemented with 1 % BSA (AppliChem, Darmstadt, Germany). The bacteria were used as a source of chemoattractant and $5x10^7$ cells were diluted in 500 µl DPBS / 1 % BSA and placed in the lower compartment of a Boyden chamber. Cells of primary Hv and Hs hemocyte cultures were harvested and the cell count was calculated with a CASY7 cell counter in a 1:100 dilution with physiological saline solution (CASYTON, Innovatis AG) to discriminate between dead and living cells. Living Hv and Hs cells have a size of at least 6.5 µm. To reseed the hemocytes, in the upper compartment of the Boyden chamber $5x10^5$ hemocytes were suspended in 300 µl anticoagulation buffer. After incubation for 1 hour at room temperature, the cells on the upper surface of the filter were completely removed by a sterile applicator (Böttger, Bodenmais, Germany) before 500 µl of anticoagulation buffer was added at high pressure. The pushed-through buffer was analyzed with a CASY7 cell counter and the living cells were calculated. Due to the fact that hemocytes are highly motile cells, buffer without bacteria served as the base line, i.e. 100 % migration rate. To quantify the percentage of hemocyte migration towards the bacterial cells, we calculated the percentage of migrated hemocytes relative to the control. The assay was performed at least four times.

3.6.8 Apoptosis assay

To examine the influence of vegetative cells of *B. subtilis*, *B. thuringiensis* and *S. entomophila* on the viability of Hv and Hs hemocytes, apoptosis assays were conducted. Hv and Hs cells of primary hemocyte cultures were harvested and washed once with anticoagulation buffer. Bacteria were washed once with DPBS (Lonza) supplemented with 1 % BSA (AppliChem). 10^6 hemocytes were incubated with 10^7 bacteria in 50 µl anticoagulation buffer for 30 minutes, 1 hour and 2 hours at room temperature. Hemocytes without bacteria served as a control at every time point. To remove bacteria, after incubation the insect cells were washed three times with TC-Buffer (140 mM NaCl, 10 mM Tris, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) and centrifuged at low rpm. In the process of apoptosis, annexin (a calcium-dependent phospholipid-binding protein) binds phosphatidylserine which is translocated to the extracellular membrane. Therefore, the apoptosis marker Rh Annexin V-APC (eBioscience) was added to the hemocytes in 100 µl TC-Buffer for 15 minutes at room temperature. Stained hemocytes were analyzed with a LSR II flow cytometer (BD Bioscience, Heidelberg, Germany). The percentage of apoptotic hemocytes (annexin-positive) was

calculated as a proportion of total hemocytes. Due to the fact that apoptosis also occurs in untreated hemocytes, the amount of annexin-positive cells in untreated hemocytes served as the base line, i.e. 0 % apoptosis, thus the percentage of untreated annexin-positive cells was subtracted from treated annexin-positive cells. Negative percentages have to be viewed as proliferation of hemocytes. To rule out false positive signals, only hemocytes with a higher size and a higher granularity than bacteria were counted (BD FACSDiva™ software), thus potential annexin-positive bacteria can be excluded. Necrotic hemocytes produced by heating living insect cells at 65 °C for 30 minutes served as positive control for the annexin staining. The assay was performed at least four times.

3.6.9 Lytic zone Assay

Petri dishes were filled with 10 ml Sörensen buffer (0.066 M KH_2PO_4, 0.066 M $Na_2HPO_4*2H_2O$, pH 6.4) containing 0.6 mg ml^{-1} of lyophilized *Micrococcus lysodeikticus* ATCC no. 4698 (Sigma-Aldrich), 0.06 mg ml^{-1} Streptomycin sulfate (Calbiochem), PTU (Phenylthiourea; to inhibit melanization) with a final concentration of 1.5 % agar. Holes within the petri dish were made by puncturing the agar with a plastic pipette (Eppendorf Research 5000) and removing the agar plug by suction. Hemolymph samples (2 µl) were placed in each well and the plates were incubated for 24 h at 37 °C. Different dilutions of chicken egg white lysozyme (2, 1, 0.75, 0.5, 0.25, 0.125, 0.0625, 0.03 mg ml^{-1}) (Sigma) were used as a positive control and a calibration curve was created based on these standards. Lytic activity in the hemolymph was determined as the radius of the clear zone around a sample well.

3.6.10 Phenoloxidase (PO) activity assay

Hemolymph phenoloxidase activity was estimated using 10 µl of hemolymph sample diluted in 500 µl of ice-cold sodium cacodylate buffer (0.01 M Na-cacodylate, 0.005 M $CaCl_2$) and directly frozen in liquid N_2 and stored at -80 °C. Samples for PO activity measurements were prepared by thawing frozen hemolymph samples at room temperature then centrifuged at 4 °C and 2800 x g for 15 minutes. The supernatant was removed and used for measurements where 100 µl of supernatant was added to 200 µl of 3 mM L-Dopa (Sigma). Absorbance was measured for 45 minutes at 490 nm and 30 °C, taking absorbance measurements once every minute. (Multiskan Spectrum multiplate reader; Thermo-Electron). As the enzymatic reactions is linear from 5 - 45 min after adding the substrate (personal observation), in our

analyses the fastest change in absorbance from 15 - 26 minutes (v_{max}) of the reaction was used. Data was acquired with SkanIt Software for Multiskan Spectrum version 2.1 (Thermo-Electron).

3.6.11 Data analysis

To test the effect of the treatment on larval survival post exposure, larval survival experiments were analyzed using the Cox proportional harzard model. To illustrate the effect of treatment on survival of both species, we used the Kaplan-Meier survivorship function.

Bacterial recovery data was log-transformed and normal distribution was estimated using Kolmogorov-Smirnov test. Log-transformed data were assessed for significant differences between the species within one treatment using the Student *t* test ($P < 0.05$).

All data presented as proportions (phagocytosis and apoptosis, not migration) were arcsine-square root transformed and normal distributions were estimated using Kolmogorov-Smirnov test. After transformation, significant treatment/species effects were assessed by Student *t* test ($P < 0.05$). Since migration data were higher than 100 %, migration data was log-transformed and normal distribution was estimated using Kolmogorov-Smirnov test. Significant differences between the species within one treatment were determined using the Student *t* test ($P < 0.05$).

Larval growth rate on Bt spores, phenoloxidase activity and lysozyme activity were not normally distributed, even after transformation, and non-parametric statistics were conducted, using Kruskal-Wallis test followed by pairwise comparisons with Mann-Whitney *U* test ($P < 0.05$). Larval growth rate data upon MVP feeding were normally distributed and a 1-way ANOVA and Tukey's post hoc test was carried out.

3.6.12 Supplementary Tables

Table S3.1. Cox Regression Model for larval survival of *H. virescens* and *H. subflexa* upon injection with different concentrations of vegetative cells of *S. entomophila* and *B. thuringiensis.*

Bacterial challenge		b[1]	SE[2]	Wald[3]	P[4]
S. entomophila	*H. virescens* 10^4 vs. *H. subflexa* 10^4	-0.061	0.422	2.2028	0.154
	H. virescens 10^5 vs. *H. subflexa* 10^5	-0.266	0.272	0.960	0.327
	H. virescens 10^4 vs. *H. virescens* 10^5	-1.425	0.392	13.243	**0.000**
	H. subflexa 10^4 vs. *H. subflexa* 10^5	0.978	0.318	9.465	**0.002**
B. thuringiensis	*H. virescens* 10^4 vs. *H. subflexa* 10^4	-0.558	0.366	2.322	0.128
	H. virescens 10^5 vs. *H. subflexa* 10^5	-0.559	0.276	4.105	**0.043**
	H. virescens 10^4 vs. *H. virescens* 10^5	-1.104	0.319	11.956	**0.001**
	H. subflexa 10^4 vs. *H. subflexa* 10^5	0.804	0.332	6.437	**0.011**

[1] = b, regression coefficient of overall survival function
[2] = SE, Standard error of b
[3] = Wald statistic
[4] = *P*, Significance value for Wald statistic; Significant differences in bold

3.6.13 Supplementary Figures

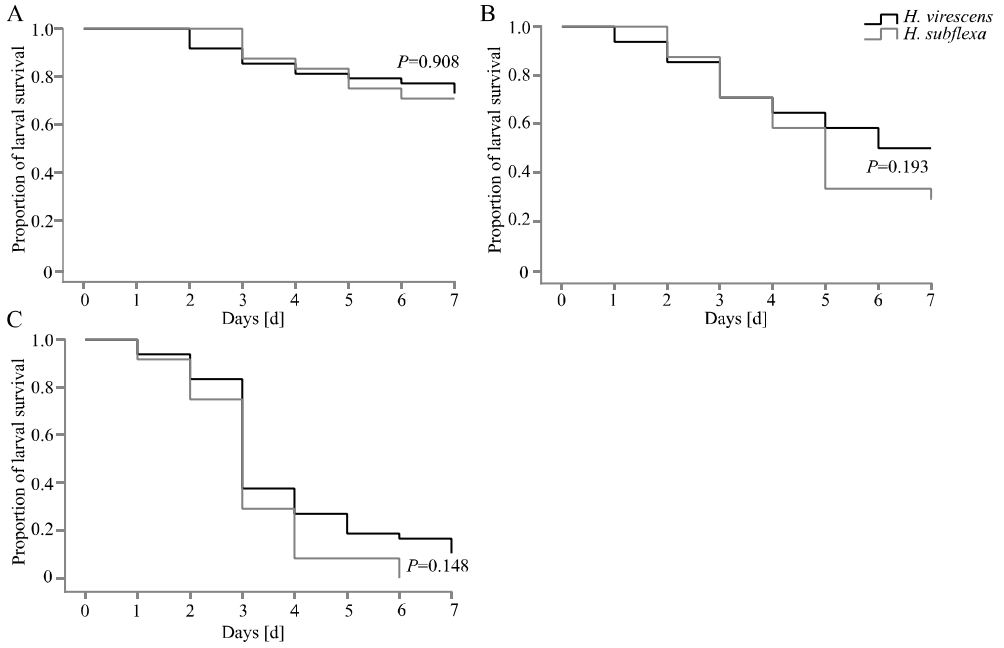

Figure S3.1. Larval survival of *H. virescens* and *H. subflexa* on different concentrations of Bt spores. Kaplan-Meier survival plot of *H. virescens* (black) and *H. subflexa* (grey) larvae fed on (**A**) $5x10^4$, (**B**) 10^5 and (**C**) $5x10^5$ Bt spores (n=24). Statistical significance was determined using Cox regression survival analysis.

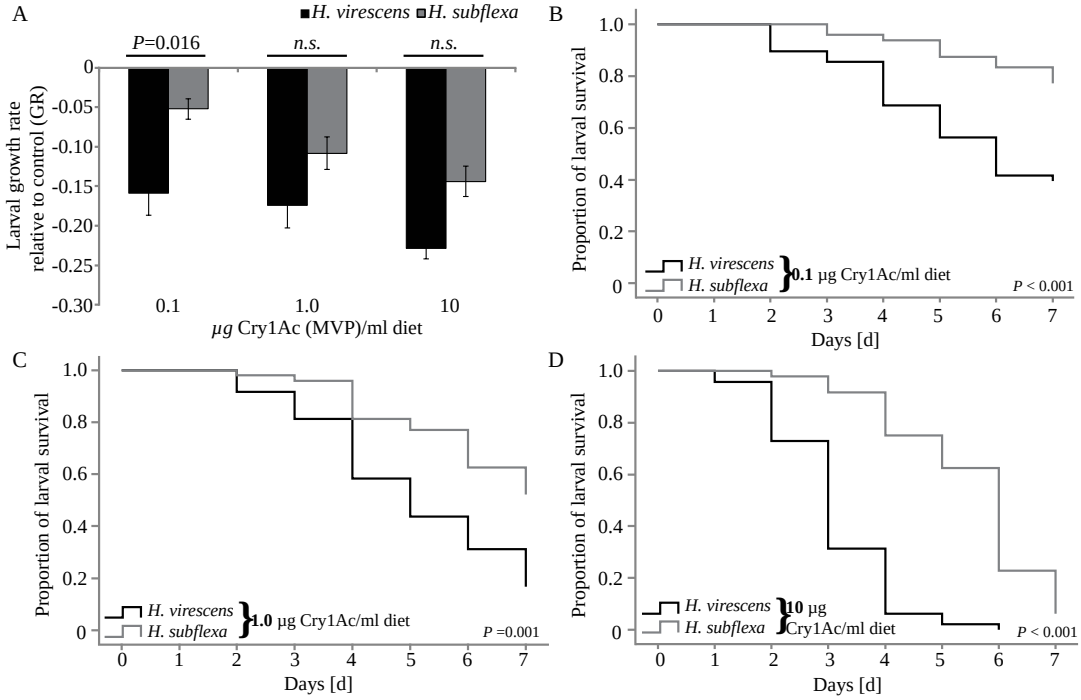

Figure S3.2: Larval growth rate and survival of *H. virescens* and *H. subflexa* on different concentrations of Cry1Ac toxin (MVP). (**A**) Average growth rates of *H. virescens* (black) and *H. subflexa* (grey) larvae on different MVP concentrations after 7 days. Values on the y-axis represent the growth rates for all treatments relative to their corresponding control, i.e. artificial diet. Bars indicate means and standard errors (n=48). Statistical significance between the species was calculated using one-way ANOVA and Tukey's post hoc test (*n.s.* = not significant). (**B-D**) Kaplan-Meier survival plot of *H. virescens* (black) and *H. subflexa* (grey) larvae on different MVP concentrations (n=48). Statistical significance was determined using Cox regression survival analysis.

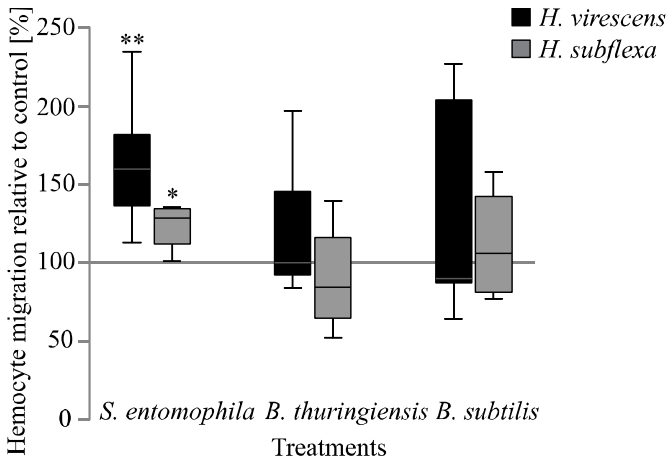

Figure S3.3: The percentage of *H. virescens* and *H. subflexa* hemocytes migrated to *S. entomophila*, *B. thuringiensis* and *B. subtilis*. Hemocytes were incubated with vegetative cells of *S. entomophila*, *B. thuringiensis* and *B. subtilis* for 1 hour. Values on the y-axis represent percentages of hemocytes, as proportions of total hemocytes relative to the control (base line 100 % migration). Each bar represents the mean of three or four biological replicates and their associated SEM. Significant differences were determined between the two species for each time point within one treatment (not significant) and within one species compared to their corresponding control (Asterisks above the bars) using Student's *t* test of log-transformed data. Significant differences are indicated by $^{**}P<0.01$; $^{*}P<0.05$.

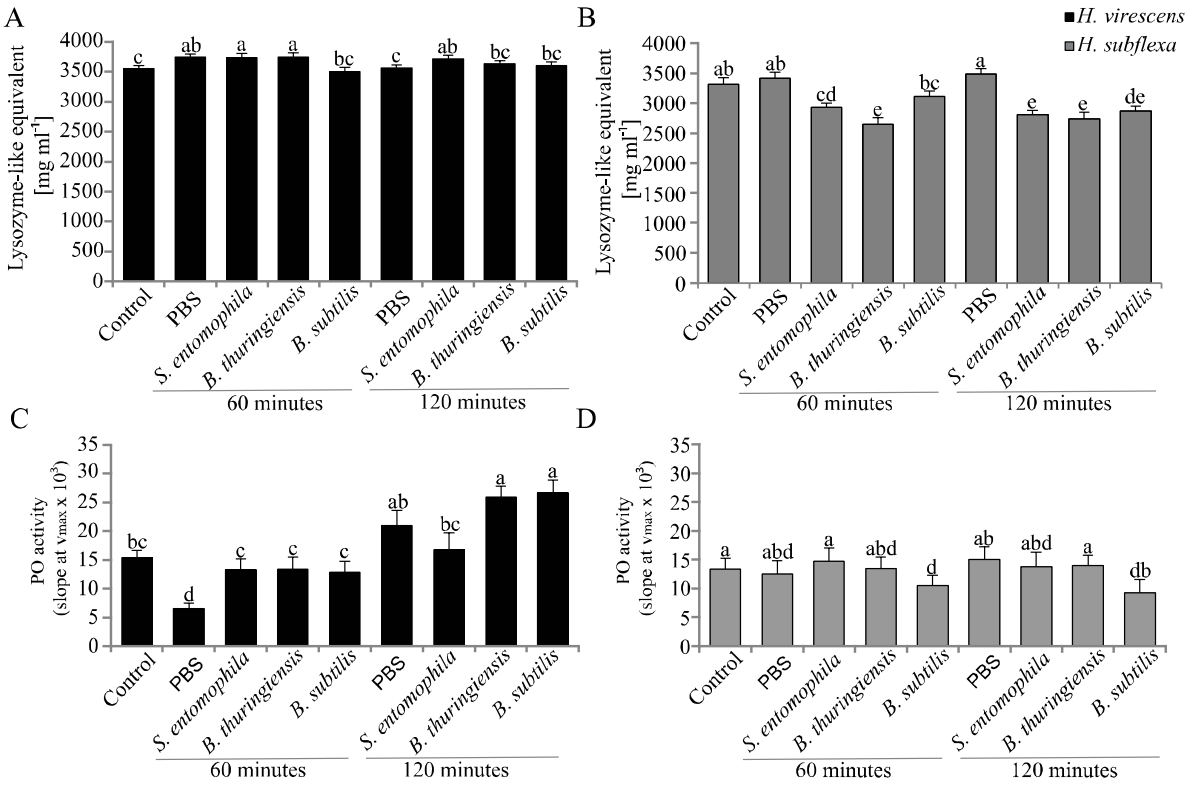

Figure S3.4: Enzyme activity in the hemolymph of *H. virescens* and *H. subflexa* larvae injected with vegetative cells of *S. entomophila*, *B. thuringiensis* and *B. subtilis*. Lysozyme-like activity in the hemolymph of (**A**) *H. virescens* (black) and (**B**) *H. subflexa* (grey) after 60 and 120 minutes. Phenoloxidase activity in (**C**) *H. virescens* (black) and (**D**) *H. subflexa* (grey) after 60 and 120 minutes. Bars represent the mean of 20 larvae ± S.E.M. Influence of wounding (PBS) and different bacterial strains on the enzyme activity was contrasted using a non-parametric Kruskal-Wallis test (df=17, $P < 0.001$). Mann-Whitney U-test was used for the pair comparisons between the treatments within one species. Different letters above the bars represent significant differences.

4 Manusript II

Consequences of an induced immune response on sexual traits in *Heliothis virescens*

Manuscript in preparation, to be submitted to Evolution

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4.1 Abstract

Immune response induction may benefit insects by preventing infectious diseases. However, as resources are limited, trade-offs between immune defense response and other life-history traits can be expected. According to Bateman's principle, life-histories often differ between the sexes. Sex specific differences in traits, such as disease susceptibility and immunocompetence are common among vertebrates and invertebrates. We hypothesized that immune response induction by pathogenic infections will provoke sex-specific changes in life-history traits, such as reproduction related traits. We tested this hypothesis by measuring physiological and behavioral changes associated with infection of *Serratia entomophila* in the heliothine moth *Heliothis virescens*. We found that injections of *S. entomophila* induced sex-specific differences in the expression level of immune-related genes. Females appeared to invest more in the activation of an immune response than males. However, males were found to maintain a higher baseline expression of immune-related genes compared to females. The physiological changes in infected females were associated with reduced mating success and modified sex pheromone composition, indicating a trade-off. Overall, we conclude that female moths appeared to invest in immune response against pathogens at the cost of reproductive effort.

4.2 Introduction

The concept of trade-offs is a central component of life history theory (Stearns 1989; Roff and 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. A fitness increase due to resource investment in one trait may cause a lack of resources available for another trait that decreases fitness (Stearns 1989; Roff and Fairbairn 2007).

Immunity and reproduction are both costly (Sheldon and Verhulst 1996; Moret and Schmid-Hempel 2000) and various studies have shown a negative correlation between these two traits (McKean and Nunney 2001; Rolff and 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 and Karter 1992; Rolff and Siva-Jothy 2002; Zuk and Stoehr 2002). It is well known in vertebrates that females display higher immunocompetence than males (Wilson et al. 2002). This phenomenon has been attributed to the difference in life histories of both sexes or the immunosuppressive effect of testosterone, the major male sex hormone in vertebrates (Alexander and Stimson 1988; Zuk 1990). In insects, males have been found to invest fewer resources in their immune response as well (Rheins and Karp 1985; Gray 1998; Rolff 2001; Rolff and Siva-Jothy 2002). These sex-specific differences in immunity have been related to Bateman's principle (Zuk 1990; Rolff and Siva-Jothy 2002; Sadd and Siva-Jothy 2006), 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. Males gain fitness by increasing their mating frequency, and should thus invest in increasing their mating rates instead of in immunity (Bateman 1948; Trivers and Willard 1973; Zuk 1990; Rolff 2002; Roth et al. 2011).

Like in other Lepidoptera, both females and males of the tobacco budworm (*Heliothis virescens*) invest substantially into their offspring (Fye and 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 and McAda 1972; Proshold et al. 1982; Blanco et al. 2009). Other than in many animal species where males can mate numerous times every day, *H. virescens* males and females only mate once per night (Raina and Stadelbacher 1990; Heath et al. 1991; Blanco et al. 2009). Consequently, being able to discriminate the condition

of a potential mate would greatly increase the reproductive success of both sexes by avoiding unhealthy and thus less fecund partners (Lockhart et al. 1996; Penn and Potts 1998; De Roode and Lefevre 2012).

Possibly, the trade-off between reproductive success and immune response is not only related to the investment of each sex into mating effort, but also to sexual signaling. Parasite-mediated sexual selection suggests that sexual signals honestly reflect the quality of the signaler to the receiver (Hamilton and Zuk 1982). Honest signals have to be costly and may be condition dependent, so that animals in good condition are better able to afford signal production, whereas animals in poor condition are less able to invest into sexual signaling (Zahavi 1975; Sheldon and Verhulst 1996; Rantala et al. 2003a). Most studies that investigated trade-offs 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). Interestingly, parasitic infection or food limitation of *Tenebrio molitor* was shown to reduce the attractiveness of male pheromones to females (Worden et al. 2000; Rantala et al. 2003a). Studies by Clark and coworkers (1997) revealed that the male sex pheromone in cockroaches (*Nauphotea cinerea*) is also condition dependent (Clark et al. 1997).

In moths, females produce a sex pheromone to attract males from a long 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 and Baker 1983). In *H. virescens*, differences in qualitative but also quantitative composition of the female pheromone blend have been shown to affect female attractiveness to males (Vetter and Baker 1983; Groot et al. 2009; Groot et al. 2014). Interestingly, in natural populations there is variation in the ratio of hexadecanal (16:Ald) and (Z)-11-hexadecenal (Z11-16:Ald) in the female sex pheromone composition (Groot et al. 2014). This variation was shown to affect female attractiveness to males in the field. Females with low ratios of 16:Ald to Z11-16:Ald were more attractive than females with high ratios (Groot et al. 2014).

Only few studies consider the female sex pheromone a sexual selection signal that is costly to produce and plastic depending on the condition of the females (Harari et al. 2011). A negative change in sex pheromone production following infection might provide 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 trade-offs

between immune system activation and sexual traits in males and females *H. virescens* on both a physiological as well as a behavioral level. Specifically, we tested the hypotheses (H) that (1) immune system activation is higher in females than in males; (2) an induced immune response evokes a reduced mating success in females, but not in males; (3) infected females trade off immunity against sexual attraction, apparent in (a) a modified sex pheromone profile and (b) an altered calling behavior.

4.3 Material and Methods

4.3.1 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 h 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.

The bacterial strain used in this study was the entomopathogenic species *Serratia entomophila*. This bacterium was obtained from the Department of Bioorganic Chemistry (MPICE, Jena, Germany). *S. entomophila* was grown at 30 °C and 250 rpm in Caso medium (Trustees 1995). 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. Lyophilized cells of *S. entomophila* were stored at -20 °C.

4.3.2 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 mimic that bacterial cells directly break through the cuticle, and has been commonly used to measure molecules that are involved in the immune response (Shelby and 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), to which we refer to as infected. 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.

4.3.3 H1: Immune system activation is higher in females than in 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). To quantify the immunocompetence of female and male moths, we evaluated expression differences of immune-related 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). 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 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 the gene expression, a ribosomal protein S18 (RpS 18), based on a sequence from an In-house database, was used as a reference. The gene expression is given as copy number per

1000 molecules RpS18. All primers used in this study are shown in Table S4.1 (Supplementary Material).

4.3.4 H2: An induced immune response evokes a reduced mating success in females, but not in males

To investigate the consequence of an induced immune response on mating success in females and males of *H. virescens*, we conducted mate choice experiments. Climate and light conditions in the experimental room were the same as for the rearing, i.e. at 26 °C, 60 % relative humidity, and a reverse 16:8 h light-dark cycle (lights off at 8.00). 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 (Potential mate 1 and 2).

Table 4.1. Experimental Setup for mate choice experiments

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

[1]Referred to as control indivduals; [2]Referred to as infected individuals; [3]Referred to as wounded individuals

Mate choice experiments were conducted in six assays, three male and three female choice assays (Table 4.1). 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 behavior, we conducted assays 2, 3, 5 and 6 as controls. In these assays, choosers were never injected and the choice was between infected and wounded potential partners (assay 2 and 5) or between wounded and control potential partners (assay 3 and 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. 50 cages were observed in one night. To distinguish between the two potential mates in one cage, one was marked with a water proof 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 in scotophase (Proshold et al. 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 behavior was observed with the use of a red LED light (Sigma LED safety light). Once a mating was recorded in a cage, the cage was discarded.

To check whether mating behavior was associated with the longevity of adult moths, 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 cl) and were provided with a 10 % honey-water solution.

4.3.5 H3a: Infected females trade off immunity against sexual attraction, apparent in a modified sex pheromone profile

To assess a possible trade-off between an induced immunity and the sex pheromone blend, we analyzed the sex pheromone profile of virgin *H. virescens* females that 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.

4.3.6 H3b: Infected females trade off immunity against sexual attraction, apparent in altered calling behavior

To test whether the frequency and temporal pattern of calling in females was affected by the induction of the immune response, we recorded the calling behavior of female moths. 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 behavior experiments were conducted on three consecutive days. Each experimental day, females of all three treatments were tested to avoid block effects. Each female was observed at only one day and in total 50 females were observed per day. Experiments started at the onset of the scotophase, the experimental room was kept dark during the experimental period. Calling behavior (yes or no) was recorded every five minutes until 450 minutes into scotophase. Females were considered to be calling when the ovipositor was clearly extruded from the female abdomen.

4.3.7 Statistical analysis

Expression levels of immune-related genes were analyzed with one-way ANOVAs and consecutive Least-Squares means (LS-means) pairwise comparisons with Tukey adjustment. Data were log-transformed to obtain normaility 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 (Figure S4.1). 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 for male attraction 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 = $P < 0.001$), 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 essential for male attraction (Vetter and Baker 1983; Blanco et al. 2009), and the ratio 16:Ald / Z11-16:Ald was shown to have biological relevance in the attraction of males as well (Groot et al. 2014). To meet the assumption of normally distributed residuals, we also log10-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 behavior of females was tested with a generalized linear mixed model using Poisson distribution in the glmmADMB package (Fournier et al. 2011; Skaug et al. 2011) 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 females 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 4.4). 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 the individual females with time as a slope as a random effect to the model. 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. All statistical analyses were conducted in the program R, version 3.0.2 (R Development Core Team, 2013).

4.4 Results

4.4.1 H1: Immune system activation is higher in females than in males

Males had higher baseline expression levels of immune-related genes than females (Figure 4.1 A). Hsp 70, lysozyme and gloverin were significantly higher expressed 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 4.1 B 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 4.1 B). In males, only lysozyme was significantly up-regulated in males after injection with *S. entomophila* compared to wounded and control males (Figure 4.1 C). Levels of gloverin and hemolin, which were significantly up-regulated upon *S. entomophila* injection, were also up-regulated 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 immune related genes of males were similarly induced after wounding and bacterial injections (See Table S4.2 for significance values).

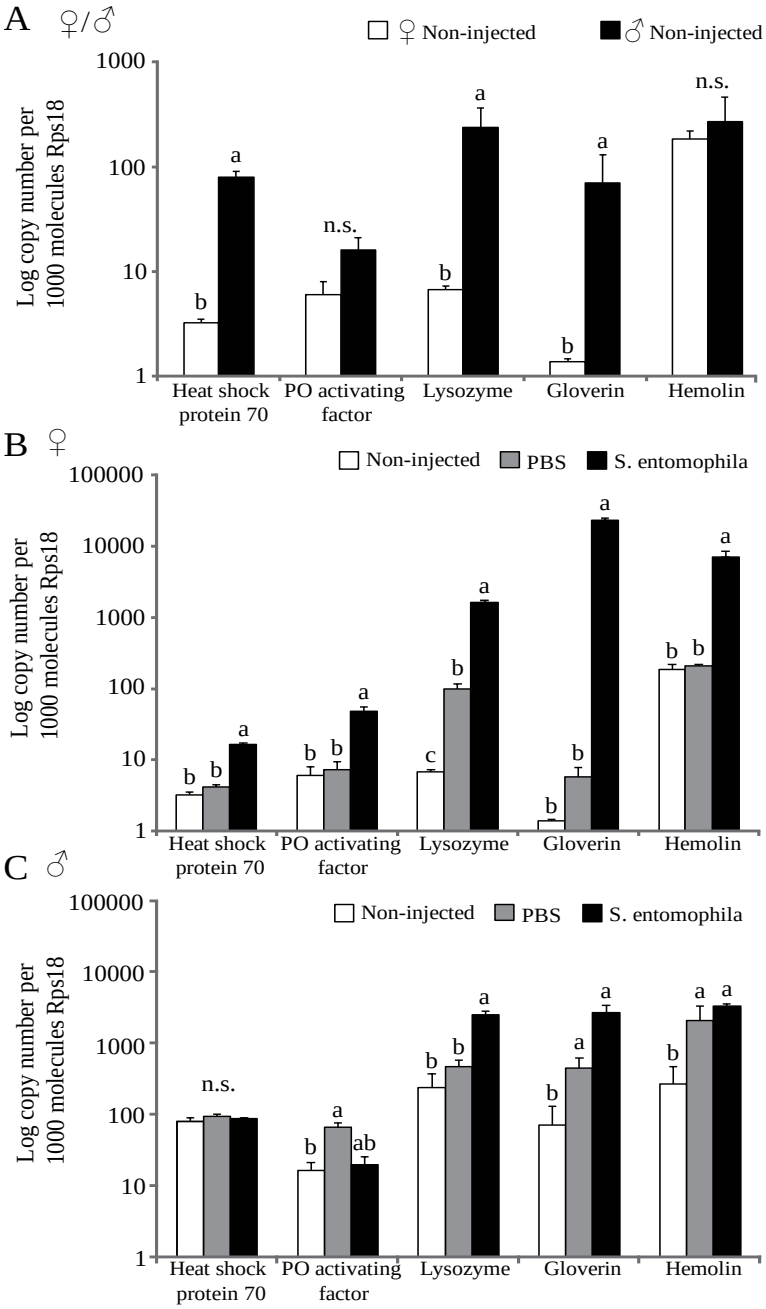

Figure 4.1: Expression level of immune-related genes in Non-injected, *S. entomophila* or PBS injected *Heliothis virescens* females and males. Expression levels were compared between Non-injected females and males (**A**), among all treatments in females (**B**), and males (**C**). Values are given 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 differences based on ANOVA and LS-means pairwise comparisons with Tukey adjustment (Table S4.2).

4.4.2 H2: An induced immune response evokes a reduced mating success in females, but not in males

Males that had a choice between *S. entomophila* injected and control females mated significantly more with control females than with *S. entomophila* injected females (P = 0.005) (Figure 4.2 A). Males that had a choice between *S. entomophila* and wounded females or between control and wounded females did not mate significantly more with one type of female (Figure 4.2 A). In the female choice experiments, females mated similarly often with all types of males offered (Figure 4.2 B). We did not observe any adult mortality within three days after the mating experiment among all treatments.

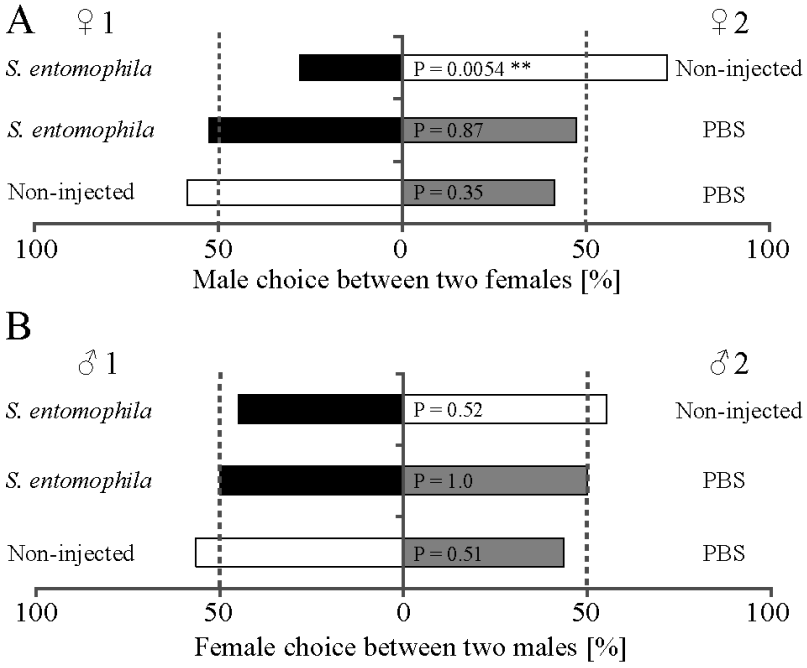

Figure 4.2: Effect of immune system activation on mating behavior in *Heliothis virescens*. (**A**) Male choice in three independent two-choice mating experiments. (**B**) Female choice in three independent two-choice mating assays. Black colors correspond to *S. entomophila* injected adults, grey colors correspond to PBS injected adults and white colors to Non-injected adults. Dashed lines indicate 50 percent of the total mated females (see Table 4.1 for sample sizes). Significant differences are indicated by ***P<0.01*, as tested with two-sided binomial tests.

4.4.3 H3a: Infected females trade off immunity against sexual attraction, apparent in a modified sex pheromone profile

In female moths, the sex pheromone profile was affected by the immune challenge. The ratio between 16:Ald and Z11-16:Ald was significantly higher in *S. entomophila* injected females compared to wounded and control females (Figure 4.3 B). Moreover, females injected with *S. entomophila* produced significantly lower amounts of Z11-16:OH than control females, but not less than wounded females (Figure 4.3 A). The relative amounts of 14:Ald, Z9-14:Ald, 16:Ald, Z11-16:Ald were not significantly different between the treatments (see Supplementary Table S4.3 A-C for significance values).

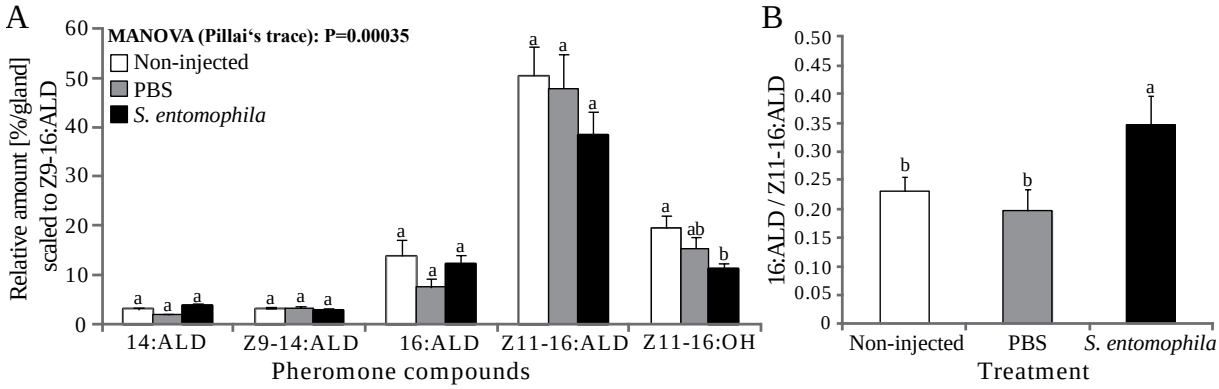

Figure 4.3: Effect of immune system activation on sex pheromone composition of *Heliothis virescens* females. (**A**) Relative amounts of five compounds scaled to Z9-16:Ald, (**B**) Ratio between 16:Ald and Z11-16:Ald. Non-injected n=38; PBS n=25*; S. entomophila* n=38. Different letters above the bars indicate significant differences between treatments at a level of alpha < 0.05 based on LS means pairwise comparisons with Tukey adjustment for multiple comparisons (Table S4.3 A-C).

4.4.4 H3b: Infected females trade off immunity against sexual attraction, apparent in an altered calling behavior

The number of females calling was not affected by the treatment (P=0.085). We did not found an interaction effect of treatment and time, suggesting that calling timing was not affected by the immune challenge (Figure 4.4). The calling behavior of all females followed similar temporal and frequency patterns with an 'on and off' calling behavior 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.

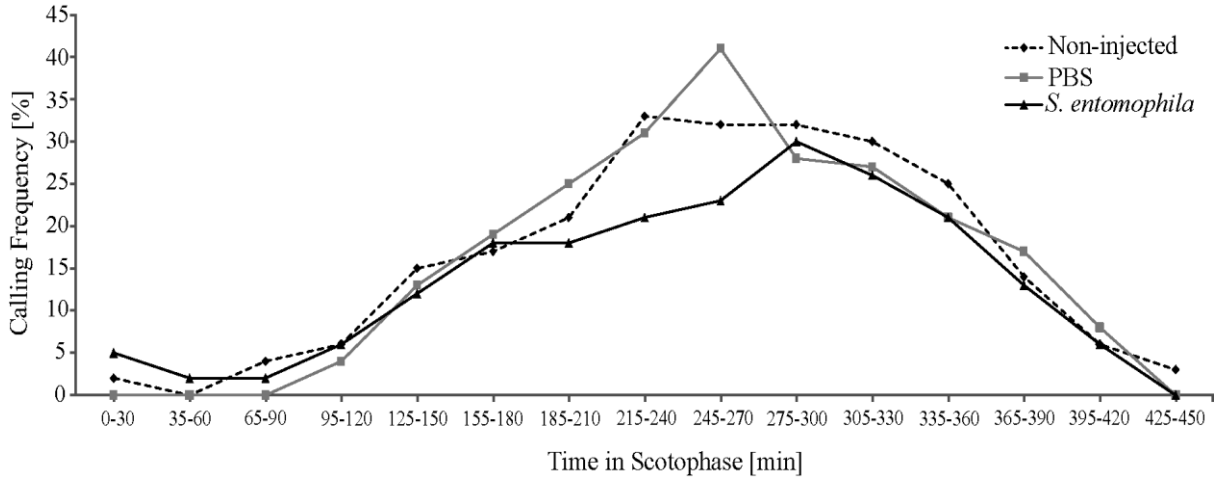

Figure 4.4: Calling activity of virgin *Heliothis virescens* females during scotophase. Individual calling behavior was registered every 5 minutes, calling events of 30 minute periods were grouped together. Calling behavior is depicted as the percentage of females that called per time interval (Non-injected n=58; PBS n=60; *S. entomophila* n=60). The overall treatment effect was tested with a generalized linear mixed model (glmmADMB in R).

4.5 Discussion

Our study revealed trade-offs between immune system activation and sexual traits in males and females *H. virescens* on both physiological as well as behavioral levels. We confirmed our hypotheses (H) that (1) immune system activation is indeed higher in females than in males; (2) an induced immune response evokes a reduced mating success only in females; (3) infected females do trade off immunity against sexual attraction, which was apparent in a (a) modified sex pheromone profile but not in (b) an altered calling behavior.

4.5.1 H1: Immune system activation is higher in females than in males

When assessing the immune status of individuals it is important to distinguish between the maintenance of immune function, to keep the immune system at a certain level of readiness, and the activation of immune system to combat pathogens (immunocompetence). We tested the latter by measuring the expression level of immune-related genes in adults upon challenge with *S. entomophila*, and found that the immunocompetence was higher in females than in males of *H. virescens*. This is in line with reports from other insects where females were found to be more immunocompetent than males. For example, in the cricket *Acheta domesticus*, females were found to be more resistant than males to infection with *Serratia liquefaciens* (Gray 1998). In the grasshopper *Melanoplus sanguinipes*, adult females possess higher PO activities than males (Gillespie and Khachatourians 1992). As suggested by various authors, a higher immunocompetence is more beneficial for females than for males, because it can prolong a female's life span and thus give the female more time to oviposit (Zuk 1990; Rolff 2002; Zuk and Stoehr 2002). Moreover, a high immunocompetence might be more advantageous to females than to males, because females have internal fertilization and have a higher risk of obtaining a sexually transmitted disease (Sheldon 1993; Adamo et al. 2001).

The results of our immune gene study further indicate that females differentiate between wounding and bacterial challenge and males do not. We found a stronger response towards *S. entomophila* in females compared to the controls, while males showed nearly the same response to wounding as to *S. entomophila* injections and had high baseline expression of immune genes. We therefore conclude that in *H. virescens* the activation of an immune response in males is less specific than in females, and might thus be less efficient in males. Since the activation of an immune system response can negatively affect other life-history traits, it is important to distinguish between wounding and bacterial challenge (Stearns 1992;

Zuk and Stoehr 2002; Schmid-Hempel 2005). The fact that males do not seem to distinguish well between wounding and bacterial injection could mean that the male immune system in *H. virescens* has not been under as strong a selection pressure as the female immune system. Assuming that a longer lifespan requires a more efficient immune system in females and that a maximized mating frequency relies on immediately available resources in males, Bateman's principle could thus explain the sex-specific differences found in *H. virescens* immunity (Bateman 1948; Zuk 1990; Rolff 2002; Roth et al. 2011).

4.5.2 H2: An induced immune response evokes a reduced mating success in females, but not in males

Since behavior is intimately linked to the physiology of insects, we predicted that changes in the physiology upon immune challenge will provoke a modified behavior in reproductive life traits in *H. virescens*. Interestingly, in the mate choice experiments we found male choice but not female choice. Specifically, *H. virescens* males mated less with *S. entomophila* injected females and chose more for control females in a two-choice test. Males thus seem to choose the healthiest female, as would be expected by parasite-mediated sexual selection theory (Hamilton and Zuk 1982). By avoiding infected females, choosy males could reduce their risk of passing on contagious diseases. Furthermore, the reduced mating success of infected females indicates a trade-off between immune system activation and sexual traits in females of *H. virescens*. A possible mechanism underlying this trade-off in females might be energy expenditure upon immune challenge resulting in an altered allocation of resources. For example, immune-challenged cabbage butterflies, *Pieris brassicae*, increase their metabolic rate about 8 % compared to control insects (Freitak et al. 2003). Therefore, the energy used for immune system activation upon infection in females might be taking away from sexual traits in females of *H. virescens.*

Apparently, it is the male who chooses the mating partner in *H. virescens*, at least at close range (less than 33 cm). An explanation for male choice, which is much less common than female choice in animals, might lie in the life history of *H. virescens*. Males also invest in their offspring by producing a spermatophore that 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 and Mason 1980; Blanco et al. 2009). This investment may limit male mating rate to only one mating per night (Proshold et al. 1982; Raina and Stadelbacher 1990), which gives an indication for why we did not see female choice: if there

is a 50:50 ratio of males and females in a population, male competition, as one of the arguments for the existence of female choice, might play a minor role in *H. virescens* (Bateman 1948; Trivers and Willard 1973; Clutton-Brock 1991).

Surprisingly, we found in the male mate choice experiments that males did not distinguish between wounded females and *S. entomophila* injected females or wounded females and control females. Based on the expression levels of immune-related genes, 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* infected females, respectively, were not as big as between control and *S. entomophila* injected females (Figure 4.1 B). 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 a shortened life time of infected females, because females of all three treatments had the same longevity.

In the female mate choice experiments, all types of males were chosen similarly often, indicating that females do not choose the mate partner in *H. virescens*, at least when confined in cages of 33 x 33 x 33 cm. As males reacted less physiologically to the bacterial challenge than females (in terms of significant upregulation of immune-related genes), it is likely that all males appeared similar to the females in the female mate-choice experiments. Furthermore, it seems that infected males allocate fewer resources to immunity than females as we found a lesser immune response in males upon immune challenge. These results fit the Bateman's principle, which states that males gain fitness through increased mating success, whereas females trade off immunity, and thus longevity, against mating success.

4.5.3 H3: Infected females trade off immunity against sexual attraction, apparent in a modified sex pheromone profile, but not in an altered calling behavior

We found the immune system activation in *H. virescens* females to be associated with an altered sex pheromone profile. The ratio between 16:Ald and 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 and Baker 1983; Groot et al. 2014). 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). Our results therefore indicate that infected females would attract fewer males than healthy

females under field conditions. Additionally, relative amounts of Z11-16:OH were lowest in *S. entomophila* injected and highest in control animals. The major component, Z11-16:Ald by itself was not significantly different between the treatments, but showed a trend to contain higher relative amounts of the major component in control and PBS-injected females compared to *S. entomophila* injected animals. Possibly, infection causes a decrease in desaturated compounds of the blend. However, relative amounts of the second critical sex pheromone component of *H. virescens*, Z9-14:Ald remained unaffected by our treatments.

Contrary to our expectations, immune system activation did not significantly modify calling behavior of infected females of *H. virescens*. However, we did see a slight trend for infected females to call less than control and wounded females. The changes in the sex pheromone towards a less attractive profile together with the slight reduction in calling activity after bacterial challenge, suggests that these life history traits are linked. The sex pheromone is likely to be an honest signal that indicates the quality of a female to the male, which fits the framework of parasite mediated sexual selection theory (Hamilton and Zuk 1982). However, the differences in sex pheromone profile and calling behavior between wounded and infected females were not coherent with female mating success in our mate choice experiments, as the pheromone profile differed between wounded and infected females while males did not mate more with wounded than with infected females and thus can not explain our mate choice results. We therefore conclude that other traits than the female sex pheromone and calling behavior must have been important for the mating success in a close range situation as our mating experiments.

One possible explanation is that the cuticular hydrocarbon profile (CHCs) play 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 and Simmons 2009). CHCs have hardly been investigated in moths, with a few exceptions (Espelie et al. 1990; Jurenka and Subchev 2000), and their role in moth sexual communication is still unknown. Another possible explanation is that infected females might simply smell differently than control females because of the immune reactions inside the individual. For instance, in vertebrates the major histocompatibility complex (MHC) is involved in mate choice through olfactory cues in house mice, humans and non-mammalian vertebrates (Penn and Potts 1998; Milinski 2006). Although it is not exactly known how MHC-specific odors are recognized, it is believed that individuals use an odor-based selection strategy to select for mates with an optimal level of MHC diversity (Penn and Potts 1998; Milinski 2006). For

example, in sticklebacks (*Gasterosteus aculeatus*), individuals release MHC-related odors that provide information about the composition of MHC alleles and thus about the individuals fitness (Milinski 2006). Although insects are believed to lack a MHC, it might be that they have similar immune gene clusters that might influence mate preference. Therefore, the reason for our results that *H. virescens* males choose a healthy mate partner over an infected one might lie in the fact that male moths used an odor-based (CHC profile or other odors) strategy to select for mates with an optimal fitness i.e. good genes.

4.5.4 Conclusion

Immune system activation was found to affect several sexual traits in *H. virescens*. As expected, we found sex-specific immunity, with females having higher immunocompetence upon immune challenge than males. Furthermore, immune system activation by *S. entomophila* was associated with a lower mating success of infected females but not of infected males. Furthermore, infected females had an altered sex pheromone composition and a non-significant reduction of calling behavior, suggesting a trade-off between immunity and the sex pheromone production in these females. This supports Bateman's principle, as our findings suggest that *H. virescens* females gain fitness by an increased investment in immunity and thus longevity.

4.6 Supplementary Material

4.6.1 Supplementary Tables

Table S4.1. Primer sequence 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 S4.2. LS-means pairwise comparisons with Tukey adjustment of expression levels 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 S4.3A. 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 S4.3B. Effect of treatment on individua)l compounds of *Heliothis virescens* female sex pheromone tested with ANOVAs and LS-means pairwise comparisons (Tukey adjusted). 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 S4.3C. 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

4.6.2 Supplementary Figures

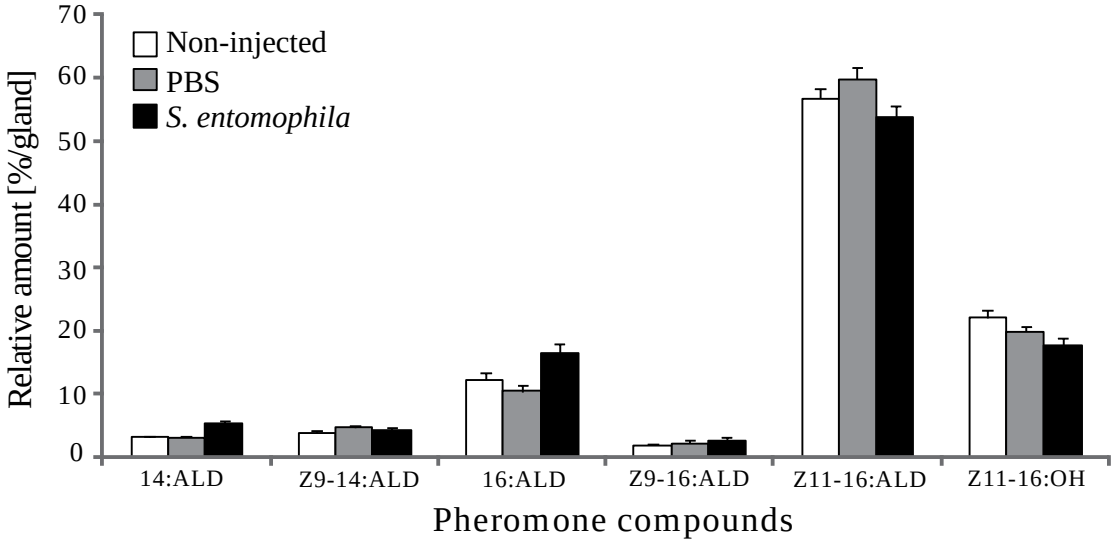

Figure S4.1: Pheromone composition of *Heliothis virescens* Non-injected (n=38), *S. entomophila* (n=38) or PBS injected (n=25) females. Z7-16:Ald was excluded.

5 Manuscript III

What a big impact a small berry can make:

Herbivorous specialization on *Physalis* plants

Manuscript in preparation, to be submitted to Proceedings of the National Academy of Sciences of the United States of America

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5.1 Abstract

Specialist herbivorous insects are more commonly observed in nature than generalists, although adaptations to a specific host plant appear to harbor strong drawbacks as host range and thereby food availability is constrained. We are interested in the causes and consequences of host plant specialization of the heliothine moth *Heliothis subflexa*, which feeds exclusively on plants in the genus *Physalis*. Specialization on *Physalis* plants necessitates the ability to tolerate withanolides, the secondary metabolites of *Physalis* species that are known to have antifeeding, immune-inhibiting and antibacterial properties on other insects. However, the antibacterial activity of withanolides has not been investigated in an ecologically relevant context before. We found that *H. subflexa* does not display any negative effects of withanolide exposure, neither at the level of larval performance nor the immune status. Instead, withanolides stimulate the immune system of the specialist *H. subflexa* and also increase the survival of the larvae when infected with *Bacillus thuringiensis* spores. We also found withanolides to have antibacterial and growth inhibiting activity against *B. thuringiensis*. We argue that the specialization in the *H. subflexa-Physalis* system has been guided to a large extent by plant chemistry. Furthermore, the specialization of *H. subflexa* on *Physalis* plants has likely led to an adaptation of its immune system to the immune-altering properties of withanolides.

5.2 Introduction

A major theme in evolutionary biology is the co-adaptation of interacting organisms. One well known example of reciprocal adaptation is the co-evolutionary arms race between plants and their insect herbivores, as first described by Ehrlich and Raven (Ehrlich and Raven 1964). The ecology of such arms races has been intensively studied and a general pattern that has been observed is a predominance of specialized herbivores, consuming a few closely related species of host plants, over generalized herbivores that utilize plants from several genera or families (Fox 1981; Jaenike 1990). A specialized host range might evolve due to several factors, including host-plant chemistry and usage of enemy-free space (Fox 1981; Schultz 1988; Bernays 1989). While specialization limits host plant range and therefore food sources, it is proposed that specialist herbivores benefit from less expensive detoxification mechanisms and elimination or even usage of plant secondary metabolites in a more efficient manner than generalists. Specialist may also evolve strategies to reduce predation and parasitism (Krieger et al. 1971; Whittake and Feeny 1971; Ali and Agrawal 2012). Understanding the causes of wide spread specialization in herbivores is crucial, when aiming to comprehend one of the driving forces of adaptation.

Here we study the cause of specialized adaptation of *Heliothis subflexa* to the plant genus *Physalis* (Laster 1972), specifically to withanolides, its secondary plant metabolites. The genus *Physalis* belongs to the Family Solanaceae and includes 120 species, which are distributed throughout the tropical parts of North and South America (Fang et al. 2012). In contrast to its close relative *Heliothis virescens,* a highly polyphagous species feeding on 37 species of plants of at least 14 different families but usually not on *Physalis* (Sheck and Gould 1993; Cho et al. 2008; Petzold et al. 2009), the larvae of *H. subflexa* exclusively feed on the *Physalis* fruits. These fruits are surrounded by an inflated calyx or "lantern" (see Figure S5.1). To reach the enclosed fruit, larvae chew a hole through the thin membrane of the inflated calyx. To complete development a caterpillar must consume several fruits (Petzold et al. 2009). It has been hypothesized that the lantern surrounding *Physalis* fruits offers *H. subflexa* an enemy-free space, and confirming this, larvae fed on fruits with cut lanterns suffered parasitism rates eight times higher than larvae fed on intact lanterns (Laster 1972; Oppenheim and Gould 2002; De Moraes and Mescher 2004). These results imply that the avoidance of natural enemies was a factor that could have promoted specialization of *H. subflexa* on *Physalis* plants. Another previous study suggested the lack of linolenic acid in *Physalis* fruits,

and *H. subflexa´s* ability to apparently synthesize this essential fatty acid as a factor for specialization (De Moraes and Mescher 2004); but other studies found linolenic acid in *Physalis* plants, making this theory debatable (Puente et al. 2011; Ramadan 2011). To our knowledge, no other insect has specialized on *Physalis* plants, indicating that the *Physalis* environment harbors challenges besides low linolenic acid concentrations that need to be overcome to successfully feed and develop on the plant.

The major secondary plant compounds of *Physalis* are withanolides present in leaves and in fruits of the plants. Withanolides belong to the group of triterpenoids and share ergostan-type steroids as core structures (Misico et al. 2011). More than 650 different withanolides are known so far (Ebada 2011). In the present study we focus on *Physalis peruviana*, also known as the cape gooseberry, native to high altitude tropical South America. Withanolides possess a broad spectrum of biological properties and withanolide isolates from *P. peruviana* were shown to exhibit insect-antifeedant activity against lepidopteran and coleopteran larvae, as well as activity against some but not all tested human pathogenic bacterial strains (Ascher et al. 1980; Ascher et al. 1981; Elliger and Waiss 1988; Silva et al. 2005; Ebada 2011). In addition, withanolides have been shown to be a potent immunosuppressant by inhibiting antibacterial activity, phagocytosis and proliferation of hemocytes in insects (Castro et al. 2008; Castro et al. 2009). Thus, withanolides form a powerful protection of the plant against insect herbivory (Ascher et al. 1980; Ascher et al. 1981; Elliger and Waiss 1988; Glotter 1991; Baumann and Meier 1993; Silva et al. 2005; Castro et al. 2008; Castro et al. 2009; Lan et al. 2009; Ebada 2011; Ramadan 2011).

Besides secondary metabolites, herbivorous insects face various other environmental factors, such as pathogens, in their variable host plant habitat. The ubiquitous entomopathogenic bacterium, *Bacillus thuringiensis* (Bt) displays a major environmental and ecological impact on herbivores by killing a wide range of insect species (Macintosh et al. 1990; Estruch et al. 1996; Schnepf et al. 1998). The toxicity of this endospore-forming bacterium is characterized by the presence of crystal proteins, also called endotoxins, during sporulation (Macintosh et al. 1990; Schnepf et al. 1998). While *Physalis* fruits provide a protected environment for *H. subflexa*, the fruit calyx is not completely closed during its development and will also contain small ‘drilling holes’ after caterpillars penetrate the calyx. Hence, while *H. subflexa* is physically protected by the fruit calyx, they do not live in a sterile environment and are exposed to pathogens either on the fruit or the plant surface when moving from a consumed to a new fruit on the plant surface. In tritrophic interactions between

herbivorous insects, microorganism and host plants, secondary compounds can either reduce toxicity of pathogens by reducing consumption rates of insects, or increase toxicity of pathogens by adding an extra stress effect to the insect metabolism (Berenbaum 1988; Navon et al. 1993). While withanolides are known to negatively affect insect immunity and have antimicrobial activities against human pathogens (Silva et al. 2005; Castro et al. 2008; Ebada 2011), we hypothesized that withanolides have a protective function against insect pathogens and thus a positive effect on the specialist *H. subflexa*.

To examine the possibility that host plant chemistry played an important part in *H. subflexa* specialization, we tested the effects of withanolides on larval growth rate, survival rate and immune status. Our data reveal a remarkable and complex relationship between the host plant and the specialist herbivore, identifying the antimicrobial activity of withanolides as an important factor that can explain its host plant specialization.

5.3 Material and Methods

5.3.1 Insects

Heliothis subflexa and *Heliothis virescens* were provided from North Carolina State University (NCSU) laboratory colonies using larvae collected in Clayton (North Carolina) and in 1985 near Gainesville (Florida) and reared at the USDA Insect Attractants, Behavior and Basic Biology Research Laboratory until 1989, after which the rearing was continued at NCSU (Sheck and Gould 1995). Larvae of *H. subflexa* were reared on a corn-soy blend diet (offered by NCSU), whereas larvae of *H. virescens* were reared on artificial diet described in Burton (1970) (Burton 1970). Larvae of both species were held in a climate chamber (Schnijder chambers) at 26 °C, 55 +/- 10 % relative humidity and 16 h / 8 h light-dark cycle. Third instar larvae were used in all experiments. All larval experiments were performed in 24-well polystyrene plates (VWR International, Darmstadt, Germany), whereby each well contained one larvae on 1 ml artificial diet.

5.3.2 Extraction, cleanup and analysis of Physalis peruviana extracts

The aerial parts (leaves and fruits) of *P. peruviana* were collected from green house plants and freeze dried. The dry plant material (5 g) was extracted three times with MeOH (2 L) at room temperature to obtain a crude extract. The volume of the crude extract was reduced to

approx. 400 ml, 100 ml H_2O was added and subjected to PS/DVB SPE cartridges (Chromabond HR-X, Macherey & Nagel) according to standard protocol. The effluent of the sample application step was collected, dried, and diluted before use.

Analyses were carried out using a Finnigan LTQ (Thermo Electron Corp., Im Steingrund 4-6, 63303 Dreieich, Germany) in the APCI positive mode (vaporizer temperature: 450 °C, capillary temperature 275 °C) connected to an Agilent HP1100 HPLC system equipped with an RP18 column, LiChroCART (250 × 4 mm, 5 µm; Merck KGaA, 64271, Darmstadt, Germany). Samples were analyzed by using gradient elution at 1 ml min^{-1} (solvent A: H_2O + 0.5 % CH_3COOH; solvent B: MeCN + 0.1 % CH_3COOH) according to the following protocol: starting with 20 % B, holding for 5 min, going to 100 % B in 30 min, with subsequent washing.

5.3.3 Production of Bt spores

The strain HD 73 carries the Cry1Ac toxin that is toxic against various lepidopteran insects (Liu et al. 2013). The spore solution was newly prepared beforehand using the following protocol. Bacterial cells from a glycerol stock were plated on LB agar and kept at 30 °C overnight. The following day, one bacterial colony was picked and resuspended in 5 ml LB medium and allowed to grow at 30 °C overnight on a bacterial shaker at 250 rpm. Subsequently, 100 µl of this bacterial culture was added to 50 ml HCO medium (HCO contained(l^{-1}): 7 g casein hydrolysate; 6.8 g KH_2PO_4; 0.12 g $MgSO_4$ $7H_2O$; 0.0022 g $MnSO_4$ $4H_2O$; 0.014 g $ZnSO_4$ $7H_2O$; 0.02 g $Fe_2(SO_4)_3$; 0.018 g $CaCl_2$ $4H_2O$; 3 g glucose; the pH was adjusted to 7.2) (Lecadet et al. 1980). After seven days at 30 °C and 250 rpm, serial dilutions of this suspension were plated onto LB agar. The agar plates were incubated at 30 °C for 48 hours and germinated bacterial spores were counted (CFU/ml; colony forming units). To verify the formation of endospores, a modified spore-staining method by Wirtz-Conklin was used (Hamouda et al. 2002). The spore suspension was stored at 4 °C and before each application the spore concentration was newly determined.

5.3.4 Feeding assay

To measure the impact of withanolides on *Heliothis subflexa* larvae, withanolide extracts were added to 1 ml artificial diet within 24-well polystyrene plates (see Figure S5.2). All applied withanolide concentrations correspond to naturally occurring withanolide concentration in the berry of *Physalis peruviana* (Baumann and Meier 1993). To incorporate

these compounds into the artificial diet, withanolide powder was dissolved in 40 % methanol and applied to the diet. Afterwards the 24-well plates were placed under a fume cupboard to allow the solvent to evaporate. Early 3rd instar larvae were placed separately in a well containing 1 ml diet incorporated with 10, 25, 50, 100, 150 or 200 µg withanolide, reflecting natural concentrations in *Physalis peruviana* (Baumann and Meier 1993). For each treatment 24 larvae were tested for 7 days and on every second day larvae were provided with freshly prepared diet containing withanolides to ensure the activity of the compounds. Larval weight was recorded daily for seven days. The growth rate was estimated as mean of the daily growth rate. In the control treatment, larvae were reared on pure artificial diet. Previous studies showed that the evaporated pure solvent (40 % methanol) had no effect on larval performance, thus as a control treatment, larvae were reared on pure artificial diet.

To investigate the effect of *B. thuringiensis* HD73 spores on *H. subflexa* larvae, Bt spores were added to 1 ml artificial diet within 24-well polystyrene plates. Early 3rd instar larvae were exposed to diets containing 10^3, 10^4, $5x10^4$, $7.5x10^4$, 10^5, $5x10^5$ and 10^6 Bt spores per ml artificial diet. For each treatment, the spore solution was applied to 1 ml diet. As soon as the spore layer had been absorbed, larval growth and survival of 24 *H. subflexa* larvae per treatment was recorded daily for 7 days. For the control treatment, larvae were reared on pure artificial diet.

To determine the impact of withanolides on infected *H. subflexa* larvae, a combined feeding experiment of Bt spores and withanolides was performed. First of all, 100 µg or 150 µg withanolide diluted in 40 % methanol was applied on artificial diet. The 24-well plates were placed under a fume cupboard to allow the solvent to evaporate. After the solvent was evaporated, the lethal Bt spore concentration of $5x10^5$ / ml diet was added in each well containing artificial diet incorporated with withanolide. As soon as the Bt spore solution was soaked in the diet, early 3rd instar larvae were allocated separately in each well. For each treatment, 24 larvae were used and for each larva in all three feeding experiments the growth rate (GR) was calculated per day over 7 days using the following equation:

Growth rate (GR) = ((log larval weight d+1) - (log larval weight d-1))/1

5.3.5 Phenoloxidase (PO) activity and antibacterial activity of the larval hemolymph

To compare PO activity of hemolymph between larvae on artificial diet and larvae exposed to artificial diet containing 100 µg withanolides, a PO activity assay was performed. Forty-two larvae of each treatment were assessed for their PO activity. Hemolymph was extracted after 7

days of treatment by puncturing the larvae with a sterile hypodermic needle. 10 µl hemolymph of each larva was collected separately in 500 µl of ice-cold sodium cacodylate buffer (0.01 M Na-cacodylate, 0.005 M $CaCl_2$) and directly frozen in liquid N_2 and stored at -80 °C. To measure PO activity, frozen samples were thawed at room temperature and centrifuged at 4 °C and 2800 x g for 15 minutes. Subsequently, 100 µl of the resulting supernatant was transferred to a 96-well polystyrene plate (VWR International, Darmstadt, Germany) containing 200 µl of 3 mM L-Dopa (Sigma) per single well and absorbance was measured for 45 minutes at 490 nm and 30 °C, taking absorbance measurements once every minute. (Multiskan Spectrum multiplate reader; Thermo-Electron). Since the enzymatic reactions are linear from 5 - 45 min after adding the substrate (personal observation), in our analyses the fastest change in absorbance from 15 - 26 minutes (v_{max}) of the reaction was used. Data was obtained with SkanIt Software for Multiskan Spectrum version 2.1 (Thermo-Electron). To measure antibacterial activity of larval hemolymph in response to exposure with 100 µg withanolide, an inhibition zone assay was performed. Hemolymph was extracted from larvae after 7 days of treatment as described above. An aliquot (500 µl) of an overnight culture of *Escherichia coli* was added to 500 ml LB medium. Petri dishes were filled with 10 ml LB medium containing N-phenylthiourea (PTU) to inhibit the melanization of the hemolymph. Holes within the petri dish were made by puncturing the agar with a plastic pipette (Eppendorf Research 5000) and removing the agar plug by suction. 2 µl of undiluted hemolymph samples of 42 individual larvae were placed into the holes of the agar plate. As a positive control, chicken egg white lysozyme at concentration between 2 and 0.03 mg ml^{-1} was used and a calibration curve was created based on these standards. After 24 hours of incubation at 37 °C, the antibacterial activity was determined as the radius of the clear zone around a sample well.

5.3.6 Antibacterial activity and growth inhibition of withanolides

The antibacterial activity of plant extracts from *P. peruviana* was tested against selected microorganisms. To further examine the antibacterial activity of withanolide in an ecological important context for *H. subfl*exa, inhibition zones on LB agar inoculated with spores of *Bacillus thuringiensis* subsp. *kurstaki* strain HD 73 were determined. An aliquot (25 µl; $4x10^9$ CFU ml^{-1}) from overnight cultures or spores of *B. thuringiensis* were added to 500 ml LB medium. Petri dishes were filled with 10 ml LB medium. Holes within the petri dish were made by puncturing the agar with a plastic pipette (Eppendorf Research 5000) and removing

the agar plug by suction. Two µl methanol extracts containing 20 µg or 40 µg withanolides were placed into the holes of the agar plates. As controls, crude withanolide extracts containing flavonoids, plant material, methanol and gentamycin were tested for their antibacterial activity. After 24 hours of incubation at 30 °C, the antibacterial activity was determined as the radius of the clear zone around a sample well.

To reproduce the conditions on *Physalis* plants, we inoculated 10 ml LB agar with 1000 µg withanolide. As a negative control, agar plates were inoculated with 40 % methanol. After the agar plates were dried, an aliquot (10 µl; $4x10^3$ CFU ml^{-1}) from vegetative cells and spores of *B. thuringiensis* were spread on the agar plates. After 48 hours of incubation at 30 °C, colony forming units (CFU) were counted.

5.3.7 Data analysis

Larval growth rate in response to withanolide treatments was normally distributed and the mean of each treatment was compared to the control using an ANOVA analysis according to Dunnett's test.

Phenoloxidase activity and antibacterial activity in response to withanolide treatments and larval growth rate in response to Bt spore treatment were not normally distributed and non-parametric statistics were conducted, using Kruskal-Wallis test followed by pairwise comparisons with Mann-Whitney U test ($P<0.05$).

Larval survival experiments were analyzed using the Cox proportional harzard model to test the effect of the treatment on larval survival post exposure. To illustrate the effect of treatment on survival of both species, we used the Kaplan-Meier survivorship function.
All statistical tests were run with the computer program SPSS 17.0.

5.3.8 Feeding assay for transcriptome analysis

H. virescens and *H. subflexa* larvae were fed on artificial diet and artificial diet containing in a final concentration of 100 µg / ml withanolides. To incorporate withanolides into the artificial diet, withanolide powder was dissolved in 40 % methanol and mixed into the cooled diet (37 °C). Afterwards diet was placed under a fume cupboard to allow the solvent to evaporate. Early 3rd instar larvae were placed separately in a cup containing either diet or diet with withanolides. For each treatment 16 larvae were tested for 3 days and then gut and rest body were extracted pooling always 4 larvae, resulting in 4 pools per treatment.

5.3.9 Transcriptome sequencing, assembly and annotation

Total RNA was extracted from *H. virescens* and *H. subflexa* larval material (reared on artificial diet only, artificial diet including plant secondary metabolites) using the innuPREP RNA Mini isolation kit (Analytik Jena, Jena, Germany) according to the instructions of the manufacturer. RNA integrity was verified using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA), and RNA quantity was determined using a Nanodrop ND-1000 spectrophotometer. For each species and treatment group 5 µg of total RNA was used for poly(A)+ mRNA purification and library preparation and subjected to NextGen sequencing. Sequencing was carried out by the Max Planck genome Center Cologne (MPGCC) using the Illumina HiSeq-2500 platform, and utilizing the paired-end (2 x 100 bp) read technology. This yielded a total of 96 million reads for *H. virescens* and 80 million reads for *H. subflexa*. Quality control, read trimming and *de novo* assembly was carried out using CLC Genomics Workbench software v6.0.1 (http://www.clcbio.com) according to published details (Vogel et al. 2014). Any conflicts among the individual bases were resolved by voting for the base with highest frequency. Contigs shorter than 250 bp were removed from the final analysis. The resulting final *de novo* reference assembly for *H. virescens* consisted of 37,614 contigs with a N50 contig size of 848 bp, an average contig length of 730 bp and a maximum contig length of 16,476 bp. The *de novo* reference assembly for *H. subflexa* consisted of 33,628 contigs with a N50 contig size of 1,329 bp, an average contig length of 896 bp and a maximum contig length of 15,361 bp. Annotation of both transcriptomes through BLAST, gene ontology and InterProScan searches was performed using BLAST2GO PRO software suite v2.6.1 (www.blast2go.de).

5.3.10 Remapping and digital expression analysis

Digital gene expression analysis was carried out by using QSeq Software (DNAStar Inc.) to remap the Illumina reads onto the reference transcriptomes and then counting the sequences to estimate expression levels. For read mapping, we used the following parameters: *n*-mer length = 25; read assignment quality options required at least 25 bases (the amount of mappable sequence as a criterion for inclusion) and at least 90 % of bases matching (minimum similarity fraction, defining the degree of preciseness requires) within each read to be assigned to a specific contig; maximum number of hits for a read (reads matching a greater number of distinct places than this number are excluded) = 10; *n*-mer repeat settings were automatically determined and other settings were not changed. Biases in the sequence datasets

and different transcript sizes was corrected using the RPKM algorithm (reads per kilobase of transcript per million mapped reads) to obtain correct estimates for relative expression levels.

5.4 Results

5.4.1 Withanolides increase the growth rate and improve the immune system response of H. subflexa larvae

Characterization of the impact of withanolides on *H. subflexa* revealed that larval growth rate was dependent on the withanolide concentration (Figure 5.1 A). Individuals reared on high withanolide concentrations, such as 100 µg and 150 µg (concentrations typically found in *Physalis* plants), had a significantly higher larval growth rate compared to individuals reared on artificial diet only. Larvae fed on both lower concentrations (< 100 µg) or higher concentrations (> 150 µg) of withanolide showed no significant difference in their growth rate compared to control treatments. None of the naturally occurring concentrations had a negative impact on larval growth. Based on these results, we standardized the dose of 100 and / or 150 µg ml^{-1} withanolide in our subsequent experiments.

When evaluating the immune status of larvae that had been previously fed with diet containing 100 µg of withanolide, we found that the immune-related enzyme activity of hemolymph samples taken from these larvae showed an enhanced immune response of *H. subflexa*, compared to control treatments (Figures 5.1 B, C): The hemolymph from withanolide-treated larvae showed a significantly higher phenoloxidase activity than hemolymph from untreated larvae (Figure 5.1 B). The induction of antibacterial response obtained for treated larvae was also higher than in control larvae but this was not significant (Figure 5.1 C).

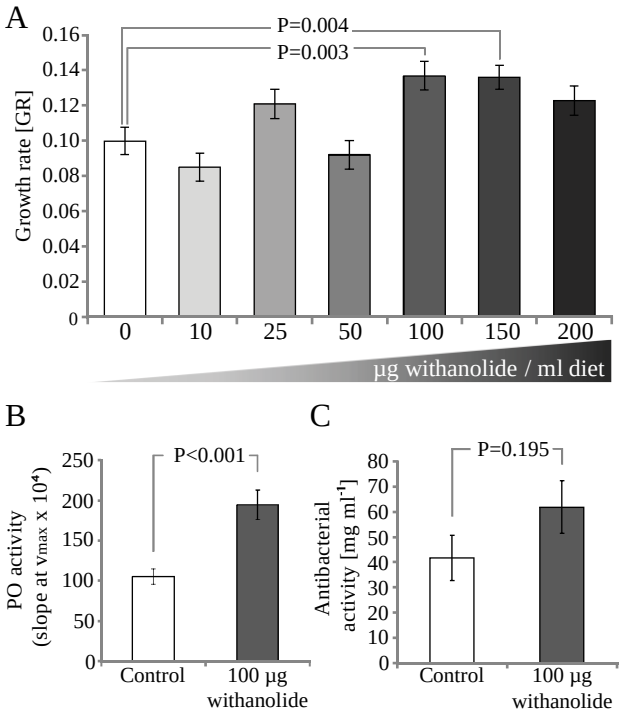

Figure 5.1: Impact of withanolides on *H. subflexa* larvae. (**A**) Average of larval growth rate of *H. subflexa* larvae after 7-day exposure to diet containing 0 - 200 µg withanolides. Increasing withanolide concentrations are indicated by a grey gradient. Bars represent the mean of 24 larvae and their corresponding standard error (P<0.05; ANOVA was conducted according to Dunnett's test). (**B**) Phenoloxidase activity after 7-day exposure to 0 µg (control; white) and 100 µg withanolide (grey) containing diet. Bars represent the mean of 42 larvae and their corresponding standard error. Mann-Whitney U-test was used for the paired comparison between the control and the treatment. (**C**) Antibacterial activity after 7-day exposure to 0 µg (control; white) and 100 µg withanolide (grey) containing diet. Bars represent the mean of 42 larvae and their corresponding standard error. Mann-Whitney U-test was used for the paired comparison between the control and the treatment.

5.4.2 Withanolides stimulate the immune system of H. subflexa but inhibit the immune system of H. virescens on the transcript level

Relative expression analysis of immune related genes collected in a RNA Seq approach revealed a contrary influence of withanolides on the immune system of *H. virescens* and *H. subflexa* (Figure 5.2). Fed on control diet and diet containing withanolides, the majority of identified immune genes in *H. virescens* were down-regulated or unaffected by the withanolide treatment. In total 19 out of 26 genes were down-regulated after withanolide treatment, with 13 genes having a relative transcript level change of more than two fold. Seven out of the 26 genes were up-regulated after withanolide treatment, with two of them having a relative transcript level change of more than two fold. Both transcripts encode lysozymes. The identified immune genes of *H. subflexa* were mostly up-regulated or unaffected by the withanolide treatment. Fourteen out of the 21 genes were up-regulated after withanolide treatment, with four having a relative transcript level change of more than two fold. Seven out of the 21 genes were down-regulated after withanolide treatment with one

having a relative transcript level change of more than two fold. See Supplementary Material Table S5.1 for relative fold change values.

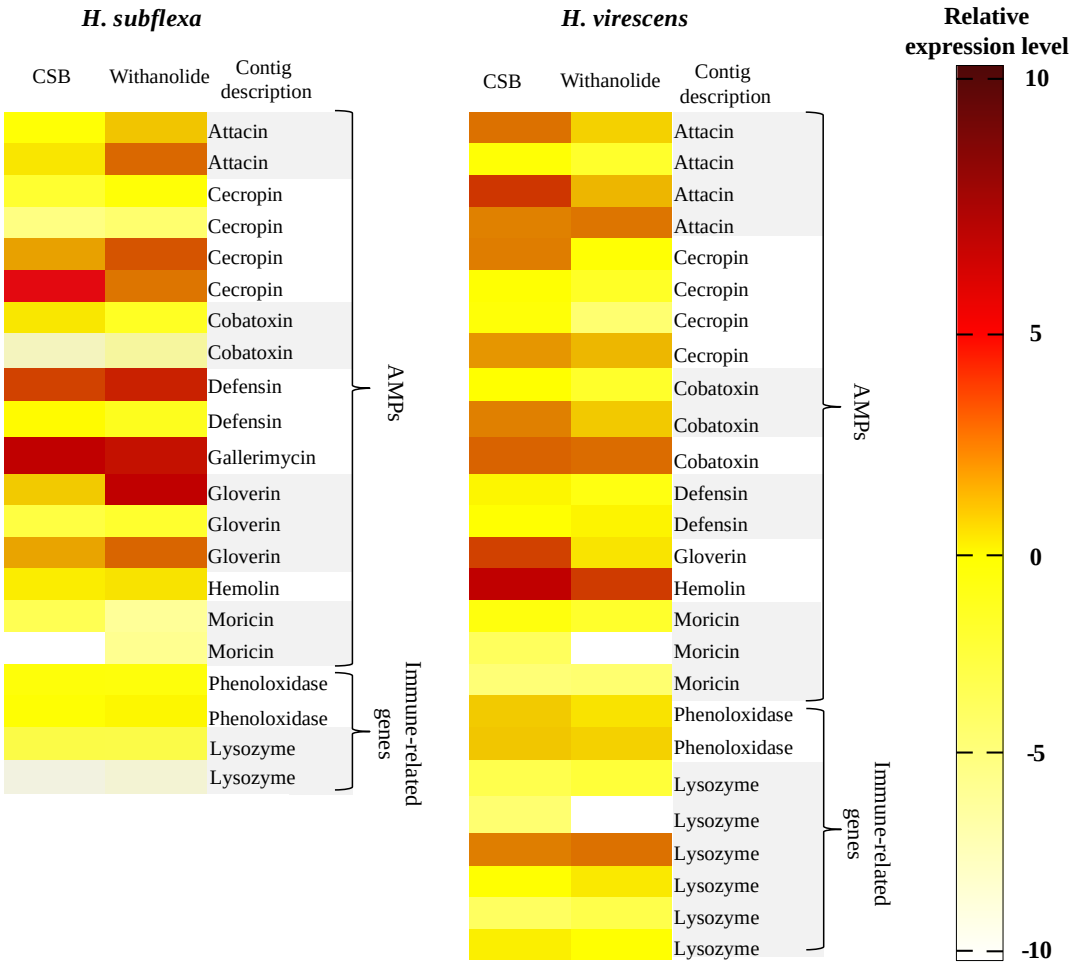

Figure 5.2: Heat maps illustrating the expression patterns of immune-related genes in *H. subflexa* and *H. virescens*. Heat map showing the expression of immune genes in *H. subflexa* and *H. virescens* after feeding on control diet (CSB) and diet containing withanolides. Low relative expression levels are depicted in white, and high relative axpression levelss in dark red.

5.4.3 Withanolides have antibacterial and growth inhibition activity against specific bacteria

Crushed leaves from *P. peruviana* displayed antibacterial activity against vegetative cells of *B. thuringiensis* (Bt), *B. subtilis* and *Pseudomonas putida*, whereas the extracts were inactive against *Escherichia coli*, *Serratia entomophila* and *Lactobacillus casei* (Figure S5.3). We confirmed that this antibacterial activity is restricted to the *Physalis* genus and not a general feature of plants, by showing that extracts of tomato (Solanacaea) and rape plants (Brassicaceae) do not possess such antimicrobial activity (Figure S5.3).

To test whether the observed antimicrobial activity can be related to withanolides or additional compounds found in extracts of *P. peruviana*, we investigated the inhibitory potential of isolated withanolides, crude leave extracts and crushed leaves of *P. peruviana* against Bt spores. Bt spores were found to be susceptible to 20 µg and 40 µg of withanolide from *P. peruviana*, while crude extracts from *P. peruviana* containing withanolide and flavonoids did not show any antimicrobial activity (Figure 5.3 A, 3 and 4). Juice from crushed leaves of fresh *P. peruviana* plants displayed a lower inhibitory activity compared to pure withanolide extracts (Figure 5.3 A, compare 5 and 1, 2). As a positive control, germination of *B. thuringiensis* spores was inhibited by 2 µg - 0.5 µg ml^{-1} gentamycin (Figure 5.3 B, 8-10). As negative control, methanol (used as a solvent for extracts) did not show any antimicrobial activity (Figure 5.3 B, 5).

To test whether withanolides also inhibit Bt growth, 1000 µg withanolides were mixed into 10 ml bacterial growth medium and poured into agar plates. When Bt spores were added to the plates, we found an inhibition of Bt spore germination (compare Figure 5.3 C and D) and a growth inhibition of vegetative cells of Bt by withanolides in the agar (Figure S5.4). Bacterial colonies found on agar plates inoculated with withanolides were lower in numbers and smaller compared to colonies on control agar plates.

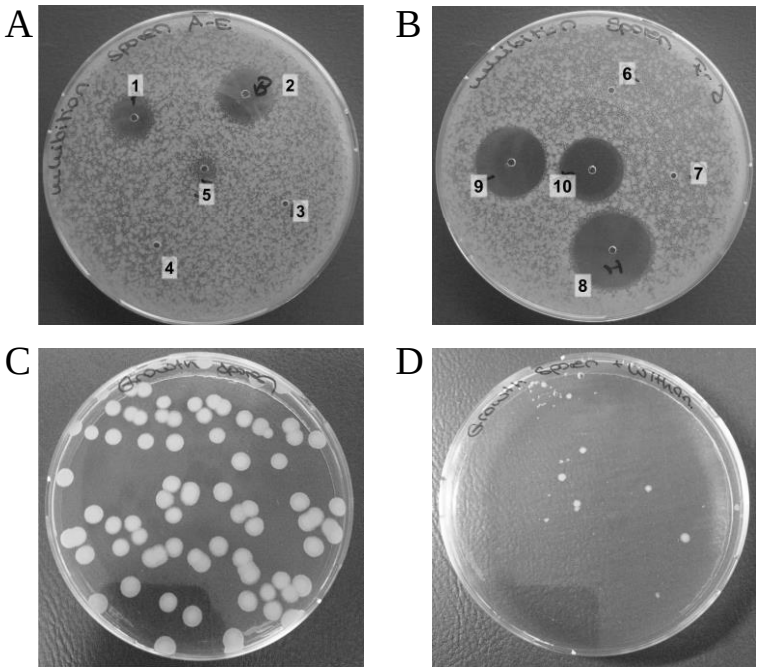

Figure 5.3: Antibacterial activity of withanolides against Bt spores. (**A-B**) Inhibition zone assay showing the bacteriostatic activity of 20 µg withanolide (1), 40 µg withanolide (2), 20 µg crude withanolide extract containing flavonoids (3), 40 µg crude withanolide extract containing flavonoids (4), *P. peruviana* leaves (5), 40 % methanol (6), DMSO (7), 2 µg gentamycin (8), 1 µg gentamycin (9), 0.5 µg gentamycin (10) against Bt spores. (**C-D**) Growth inhibition assay showing that 1000 µg withanolide ml^{-1} was able to inhibit germination of Bt spores.

5.4.4 Negative impact of Bt spores on H. subflexa larvae

When analyzing the growth rate of *H. subflexa* at Bt doses ranging from 0 to 10^6 CFU ml^{-1}, we found that larval growth rate of *H. subflexa* was significantly negatively affected by concentrations greater than or equal to 10^4 Bt spores per ml artificial diet (Figure 5.4 A). Increasing Bt spore concentrations up to 10^6 Bt spores caused a decrease in larval growth rate in a dose-dependent manner. With the highest concentration of $5x10^5$ and 10^6 Bt spores, larval growth rate turned negative due to a very high weight loss and mortality of treated larvae.

When analyzing the larval mortality in response to Bt spores, we found that concentrations greater or equal to $7.5x10^5$ Bt spores per ml artificial diet induced a significantly higher mortality in infected larvae compared to control treatments (Figure 5.4 B and Table S5.2). Larvae fed on the highest concentrations ($5x10^5$ and 10^6) of Bt spores showed the highest mortality rate with no or one surviving larvae after 7 days of treatment. Therefore, we used the lethal dose of $5x10^5$ Bt spores for all further experiments.

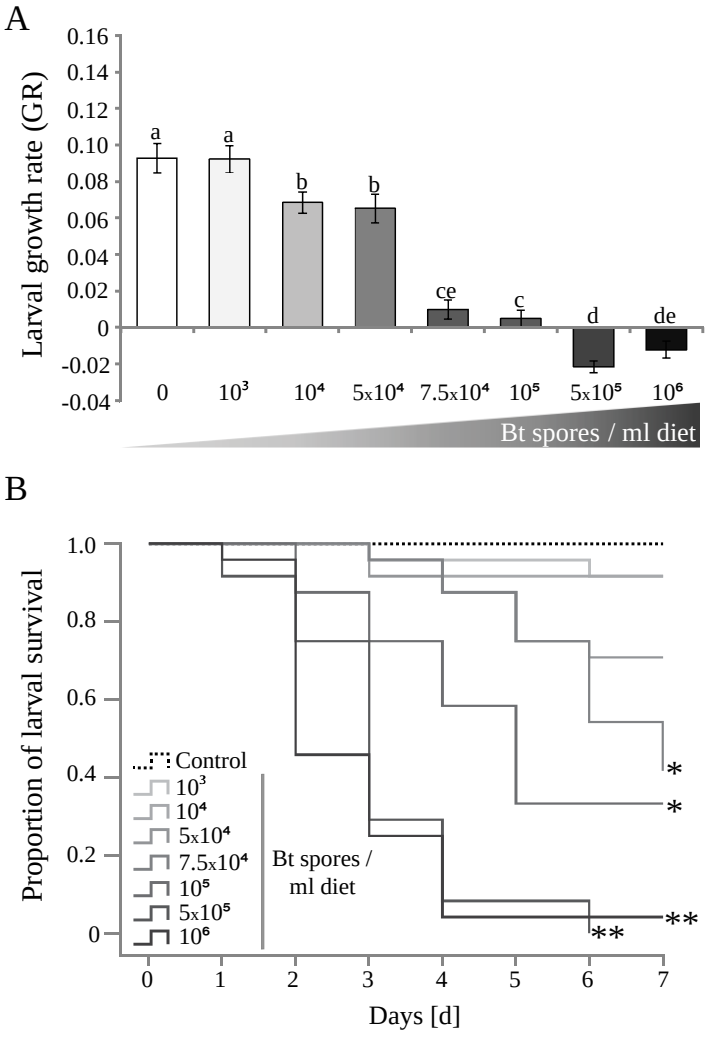

Figure 5.4: Impact of Bt spores on *H. subflexa* larvae. (**A**) Average of larval growth rate of *H. subflexa* larvae after 7-day exposure to 0 - 10^6 / ml Bt spores applied on artificial diet. Increasing Bt spore concentrations are indicated by a grey gradient. Bars represent the mean of 24 larvae and their corresponding standard error. Mann-Whitney U-test was used for the paired comparison between the treatments. Different letters above the bars represent significant differences. (**B**) Kaplan-Meier survival plot of larvae fed on 0 - 10^6 / ml Bt spores (n=24). Increasing Bt spore concentrations are indicated by a grey gradient. Significant differences are indicated by $^{**}P<0.01$; $^{*}P<0.05$.

5.4.5 Withanolides increase survival rate of Bt infected H. subflexa larvae

In contrast to the negative effects of Bt spores on *H. subflexa* larvae on pure artificial diet, larvae fed with 100 µg or 150 µg withanolide in combination with Bt spores showed no difference in growth rate compared to controls (Figure 5.5 A). The survival rate of larvae treated with 100 µg or 150 µg withanolide in addition to Bt infections was significantly higher than survival rate of control larvae (Figure 5.5 B and Table S5.3).

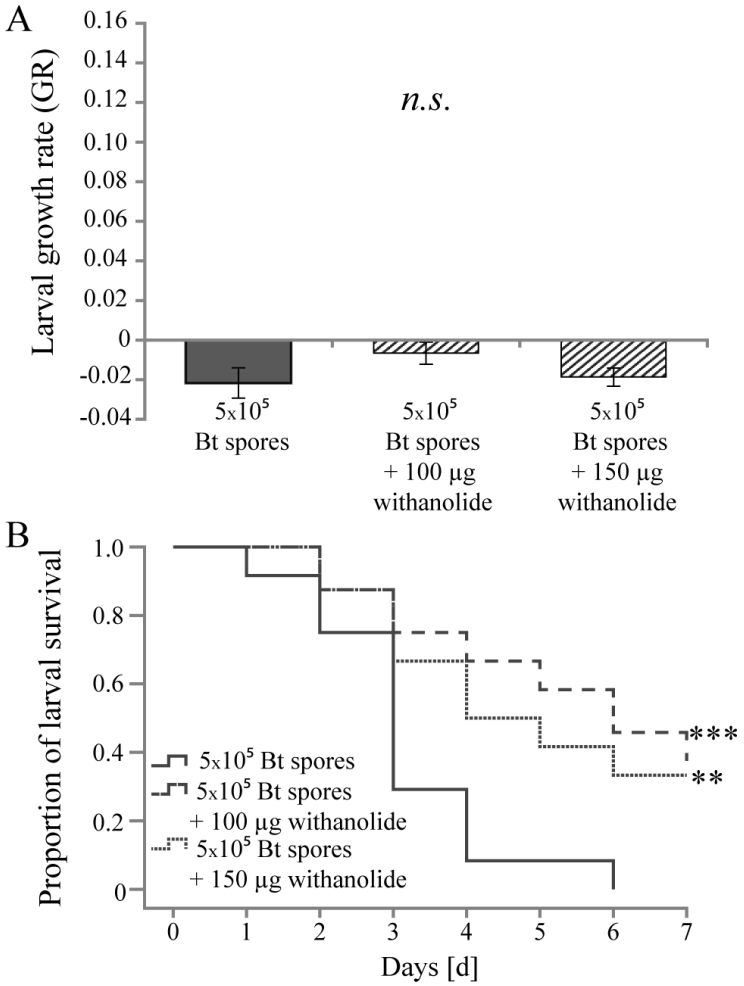

Figure 5.5: Impact of withanolide on infected *H. subflexa* larvae. (**A**) Average of larval growth rate of larvae after 7-day exposure to (i) $5x10^5$ Bt spores/ml diet, (ii) $5x10^5$ Bt spores and 100 µg withanolide /ml diet and (iii) $5x10^5$ Bt spores and 150 µg withanolide /ml diet. Bars represent the mean of 24 larvae and their corresponding standard error. Mann-Whitney U-test was used for the paired comparison between the treatments (*n.s.* = not significant). (**B**) Kaplan-Meier survival plot of larvae fed on (i) $5x10^5$ Bt spores/ml diet, (ii) $5x10^5$ Bt spores and 100 µg withanolide /ml diet and (iii) $5x10^5$ Bt spores and 150 µg withanolide /ml diet. Significant differences are indicated by ***P<0.001; **P<0.01.

5.5 Discussion

Withanolides, the major secondary plant compounds in the studied *Physalis* family, have antifeedant and immunosuppressive activities in several insect species and possess antimicrobial activity against human pathogens (Silva et al. 2005; Castro et al. 2008; Castro et al. 2009). Our study demonstrates for the first time that withanolides actually have a positive impact on the specialist *H. subflexa* larvae that feed exclusively on *Physalis* plants, by increasing growth and immune system activity of the larvae. Moreover, withanolides improved the survival rate of *H. subflexa* larvae infected with Bt, indicating that withanolides have played an important part in host plant adaptation of *H. subflexa*, which is in accordance with the coevolutionary arms-race theory.

5.5.1 Withanolides and their impact on the specialist H. subflexa

Withanolides belong to the phytoecdysteroids, which are plant-produced compounds that have been shown to possess ecdysteroid activity in insects (Dinan 2001). The ecdysteroid metabolism is known to control insect development and disruption of this metabolism compromises insect development. Furthermore, ecdysteroids were shown to modulate the cellular and humoral immunity in *Drosophila melanogaster* by increasing the phagocytic activity, pathogen encapsulation and AMP gene expression (Meister and Richards 1996; Lanot et al. 2001; Sorrentino et al. 2002). Withanolides have been shown to possess antagonistic ecdysteroid activities in *Drosophila melanogaster* cell lines (Gilbert et al. 1980; Dinan et al. 1996). The antifeedant and growth inhibitory effect of withanolides on most insects might therefore arise from a disruptive impact of withanolides on insect development and immunity (Ascher et al. 1981; Ascher et al. 1987; Elliger and Waiss 1988). In contrast, we found a significant positive impact of withanolides on the growth rate of *H. subflexa* larvae when the caterpillars were fed with relevant concentrations of withanolides. The larvae instead use the phytochemical as a feeding cue and feeding stimulant (Metcalf et al. 1980). Thus, *H. subflexa* might have evolved mechanisms to circumvent binding of withanolides to their ecdysteroid receptors and by this circumvent its negative effects on insect development and immunity.

The gut microbiota in herbivorous insects can play an important part in nutrition and digestion. In general, it is thought that food-derived phytochemicals might change the composition of the bacterial gut microbiota (Berebaum 1980; Felton et al. 1992; Priya et al.

2012). Since withanolides possess antimicrobial properties (Silva et al. 2005; Ebada 2011), they potentially affect the composition of the gut microbiota. For example, in the true bug *Rhodnius prolixus*, a blood-sucking bug and an important vector of the Chagas disease, withanolide treatment resulted in higher numbers of bacterial microbiota but also in a lower antimicrobial activity (Castro et al. 2008; Castro et al. 2012b). The fact that we found an initial elevated growth rate of *H. subflexa* larvae feeding on withanolides could be indicative of a nutritional or digestive microbial-driven effect that reached its full potential in our experiments at 150 µg of withanolides (Figure 5.1 A). Possibly, withanolides favor the abundance of specific bacterial strains that promote nutrition and digestion in the specialist *H. subflexa*, or *H. subflexa* might be able to overcome limitations in the composition of the gut microbiota with the help of withanolides.

Previous studies showed that withanolides have a potent immunosuppressive activity in insects by inhibiting antibacterial activity, phagocytosis and hemocytic proliferation, and in human cells by suppressing T- and B-cell activation and proliferation (Castro et al. 2008; Castro et al. 2009; Huang et al. 2009; Yu et al. 2010). This might be an effect of the ecysteroid modulating properties of withanolides as discussed earlier. Such an immune inhibitory effect of withanolides was not evident in the specialized *H. subflexa* larvae. Instead, we observed a stimulation of the immune system by a significant induction of the phenoloxidase activity (Figure 5.1 B). Phenoloxidase catalyzes early steps in melanin formation and is a key factor of the immune response in invertebrates, as it is involved in cellular defense, hemolytic clotting and wound repair (Cerenius and Soderhall 2004; Cerenius et al. 2008). We also observed a non-significant increase in general antibacterial activity in *H. subflexa* hemolymph after ingestion of withanolides (Figure 5.1 C). The stimulatiory effect of withanolides on the *H. subflexa* immune system is further supported by our transcript data (Figure 5.2), as we found the majority of the identified immune genes in *H. subflexa* to be up-regulated after withanolide ingestion.

5.5.2 Withanolides and their role in defense against entomopathogenic bacteria

The evolution of plant defense is not only driven by herbivorous insects but to a large extent also by microorganisms (Walker and Stahmann 1955; Thompson 1994; Cornell and Hawkins 2003). Many antibacterial agents in nature are secondary metabolites (Krischik et al. 1988; Chun et al. 2005; Igbinosa et al. 2009) and some have been shown to prevent insect infection by bacteria or viruses (Felton et al. 1987; Reichelderfer 1991). Withanolides are known to be

active against a broad range of gram-negative and gram-positive bacteria relevant in human pathology (Silva et al. 2005; Ebada 2011). However, the antibacterial activity of withanolides has not been investigated in an ecologically relevant context before. We found that vegetative cells and even spores of the microorganism *B. thuringiensis*, which are toxic to many insects (Macintosh et al. 1990; Estruch et al. 1996; Schnepf et al. 1998), were susceptible to *P. peruviana* withanolide extracts. The antibacterial activity of *Physalis* plants may therefore be a plant defense mechanism against plant pathogens, and the antimicrobial activity against the insect pathogen *B. thuringiensis* might only be a side-effect of withanolides.

The mode of action of withanolides in bacteria is not clear yet. The microorganism's susceptibility to withanolides and plant extracts of *P. peruvivana* was not correlated to a particular class of bacterial cells, e.g. gram-positive or gram-negative bacteria. For example, *P. peruviana* extracts have been found to be non-active against the entomopathogenic gram-negative bacterium *Serratia entomophila*, suggesting a more specific mode of action against the gram-positive *B. thuringiensis*. A triterpenoid from the plant *Maytenus blepharodes*, structurally related to withanolides, was found to inhibit cell wall and macromolecule synthesis of *B. subtilis*, and withanolides might act in a similar manner (De Leon et al. 2005). Since withanolides are active against a broad class of bacterial cells, it will be interesting to study the mode of action of this compound in bacteria.

5.5.3 Tritrophic interactions among B. thuringiensis, withanolides and H. subflexa

Ingestion of the Bt toxins that are produced by Bt spores mostly leads to the death of insects (Macintosh et al. 1990; Schnepf et al. 1998). We found that withanolides reduce larval mortality of *H. subflexa* caused by Bt spores. We did not observe an improvement in larval growth, likely due to a strong larval antifeedant behavior caused by Bt (Gould et al. 1991). Our results suggest that *H. subflexa*, which are able to tolerate withanolides in high amounts, gain protection from entomopathogenic bacteria such as Bt while feeding on *Physalis*.

As the toxin produced by the Bt spores, rather than the Bt bacteria or spores themselves, is the main cause of insect death (Macintosh et al. 1990; Schnepf et al. 1998), the antibacterial activity of withanolides against Bt cannot *per se* explain the observed increased survival rate of *H. subflexa*. Withanolides would inhibit further multiplication of Bt bacteria, but the toxin in ingested spores would still be effective. However, the larvae might profit from the immune stimulating effect of withanolides when challenged by Bt, similarly to *Ephestia kuehniella*, the flour moth, which has an increased survival rate on low Bt concentration,

when immune stimulated (Rahman et al. 2004). Withanolides might for example enhance the regenerative capacity of the gut cells after Bt toxins are released in the larval gut lumen and induce cell lysis. In some insects, septicemia caused by other midgut bacteria invading the hemolymph after ingestion of Bt toxins has been suggested to be the major cause of mortality (Broderick et al. 2006; Broderick et al. 2009a). The antibacterial activity of withanolides might reduce invasion of bacteria into the hemocoel and thereby suppress septicemia. Thus, while the precise mode of action by which withanolides increase survival of *H. subflexa* feeding on Bt spores remains unknown, our results confirm previous work showing that the host plant cannot be ignored in assessing Bt toxicity in insects (Raymond et al. 2011).

5.5.4 Is H. subflexa now dependent on Physalis for optimal immune system function?

In a previous study we found that *H. subflexa*, reared on artificial diet, had a lower tolerance to Bt bacteria and a less efficient cell-mediated immune response compared to *H. virescens* (Barthel et al. 2014). In the evolutionary divergence of these closely related species from a common ancestor about half a million years ago (Cho et al. 2008), immune competence must therefore have changed in one or both lineages. Since the host range of the common ancestor is unknown, we cannot rule out the possibility that *H. virescens* evolved a more efficient immune response as its host range increased, accounting for some of the present-day differences between the two species. However, it is likely that the immune system in the *H. subflexa* lineage evolved as well: it not only overcame the immune-inhibition effect of withanolides but also now experiences an immune stimulation by withanolides, possibly by circumventing ecdysteroid antagonistic activities. Thus adaptation to withanolides by *H. subflexa* has most likely resulted in a partial dependence on them for optimal functioning of the immune system. This may be an unexpected corollary of the dictum that evolutionary specialization is a dead end.

5.5.5 Conclusion

Withanolides derived from the host plant directly and indirectly protect *H. subflexa* from bacterial pathogens. Withanolides actively inhibit cell growth and germination of Bt and thereby reduce pathogen loads for *H. subflexa*. Withanolides also induce the immune response of *H. subflexa*, thereby priming the larvae for pathogen attacks in general and increasing survival on Bt diet. Hence, a consequence of *H. subflexa* specialization on *Physalis* plants is the adaptation of its immune system to the impact of withanolides, such that *H. subflexa* is

now able to exploit the positive antibacterial properties of withanolides. Beyond simply countering the adaptation of the plant producing an anti-herbivore compound, the insect has used this weapon to its own advantage, demonstrating a benefit to specialization.

5.6 Supplementary Material

5.6.1 Vegetative bacterial strains

Vegetative cells of *Bacillus thuringiensis* subsp. *kurstaki* strain HD 73, *Bacillus subtilis*, *Pseudomonas putida*, *Escherichia coli*, *Serratia entomophila* and *Lactobacillus casei*, which were obtained from the Department of Bioorganic Chemistry (MPICE, Jena, Germany), were used in this study to determine the antibacterial activity of plant extracts and withanolide extracts from *P. peruviana*. All bacterial strains were cultured at 30 °C and 250 rpm in lysogeny broth (LB) broth or on LB agar (Bertani 1951), except for *S. entomophila* which was grown in CASO medium (Trustees 1995) and *E. coli* which was grown in LB medium at 37 °C. Bacterial cells were obtained from overnight cultures and cell counts were estimated by optical density at 600 nm (BioPhotometer, Eppendorf, Hamburg, Germany). Beforehand, known numbers of colony forming units (CFU) were plotted against their corresponding optical density at 600 nm to obtain a standard curve for bacterial cell concentration of each strain. For all antibacterial activity assays, approximately $4x10^9$ CFU ml^{-1} bacterial cells were used on one agar plate.

5.6.2 Supplementary Tables

Table S5.1. RPKM values and relative fold change between treatments from RNA seq data.

H. subflexa				*H. virescens*			
CSB	**Withanolide**	**Fold change**	**Gene**	**CSB**	**Withanolide**	**Fold change**	**Gene**
5.08998	5.77649	**1.609 up**	**Attacin**	6.05157	4.71721	*2.521 down*	*Attacin*
5.48345	6.61419	**2.189 up**	**Attacin**	3.83383	1.81865	*4.042 down*	*Attacin*
3.62983	5.08249	**2.737 up**	**Cecropin**	6.86127	5.10119	*3.387 down*	*Attacin*
0.87425	1.54611	**1.593 up**	**Cecropin**	5.8282	5.98995	**1.118 up**	**Attacin**
6.09975	6.79706	**1.621 up**	**Cecropin**	5.88451	3.89569	*3.969 down*	*Cecropin*
6.22232	6.21322	*1.006 down*	*Cecropin*	3.99413	2.11515	*3.678 down*	*Cecropin*
5.47665	4.09134	*0.382 down*	*Cobatoxin*	3.72634	-1.7591	*44.80 down*	*Cecropin*
-1.83312	-0.8753	**1.942 up**	**Cobatoxin**	5.53234	5.08626	*1.362 down*	*Cecropin*
7.11005	7.6904	**1.495 up**	**Defensin**	4.09222	1.8528	*4.722 down*	*Cobatoxin*
3.86051	2.91066	*1.931 down*	*Defensin*	5.84076	4.83795	*2.003 down*	*Cobatoxin*
8.26423	7.98183	*1.216 down*	*Gallerimycin*	6.23535	6.11495	*1.087 down*	*Cobatoxin*
5.73729	7.5451	**3.501 up**	**Gloverin**	4.20811	3.20381	*2.005 down*	*Defensin*
3.01963	3.71883	**1.623 up**	**Gloverin**	4.05908	4.2441	**1.136 up**	**Defensin**
6.07575	6.64155	**1.480 up**	**Gloverin**	6.70216	4.46659	*4.709 down*	*Gloverin*
5.41919	5.50971	**1.064 up**	**Hemolin**	7.59761	6.79523	*1.743 down*	*Hemolin*
2.44105	0.11177	*0.198 down*	*Moricin*	3.43781	1.80457	*3.102 down*	*Moricin*
-3.39976	0.39356	**13.864 up**	**Moricin**	-0.77266	-9.3212	*374.4 down*	*Moricin*
3.52261	3.46662	*1.039 down*	*Phenoloxidase*	-2.09157	-1.7299	**1.284 up**	**Moricin**
3.71299	3.93917	**1.169 up**	**Phenoloxidase**	4.82259	4.46971	*1.277 down*	*Phenoloxidase*
1.68436	1.66461	*1.013 down*	*Lysozyme*	4.86751	4.72244	*1.105 down*	*Phenoloxidase*
-2.86463	-2.45736	**1.326 up**	**Lysozyme**	0.05499	1.09467	**2.055 up**	**Lysozyme**
				-1.77454	-9.3212	*186.9 down*	*Lysozyme*
				5.87001	6.04918	**1.132 up**	**Lysozyme**
				4.04176	4.39565	**1.277 up**	**Lysozyme**
				-0.92165	0.17692	**2.141 up**	**Lysozyme**
				4.3108	4.0706	*1.181 down*	*Lysozyme*

Table S5.2. Cox Regression Model for larval survival of *H. subflexa* treated with Bt spores.

Control vs. Bt spores/ml	b[1]	SE[2]	Wald[3]	df	*P*[4]
10^3	4.201	5.802	0.524	1	0.469
10^4	2.100	2.901	0.524	1	0.469
$5x10^4$	1.433	1.037	1.911	1	0.167
$7.5x10^4$	1.127	0.558	4.070	1	**0.044**
10^5	0.909	0.420	4.691	1	**0.030**
$5x10^5$	0.870	0.297	8.608	1	**0.003**
10^6	0.708	0.260	7.417	1	**0.006**

[1] = b, regression coefficient of overall survival function
[2] = SE, Standard error of b
[3] = Wald statistic
[4] = *P*, Significance value for Wald statistic; Significant differences in bold

Table S5.3. Cox Regression Model for larval survival of infected *H. subflexa* treated with withanolides.

$5x10^5$ Bt spores vs.	b[1]	SE[2]	Wald[3]	df	*P*[4]
$5x10^5$ Bt spores + 100 µg withanolide	-1.293	0.367	12.375	1	**0.000**
$5x10^5$ Bt spores + 150 µg withanolide	-0.467	0.168	7.760	1	**0.005**

[1] = b, regression coefficient of overall survival function
[2] = SE, Standard error of b
[3] = Wald statistic
[4] = *P*, Significance value for Wald statistic; Significant differences in bold

5.6.3 Supplementary Figures

Figure S5.1: Structural shelter of *Physalis peruviana*. (**A**) The calyx and surrounding leaves of *P. peruviana* fruits. (**B**) The calyx was partially removed to show different stages of fruit development. Larvae of *Heliothis subflexa* feed on all developmental stages of the fruit and to do so they have to penetrate the calyx.

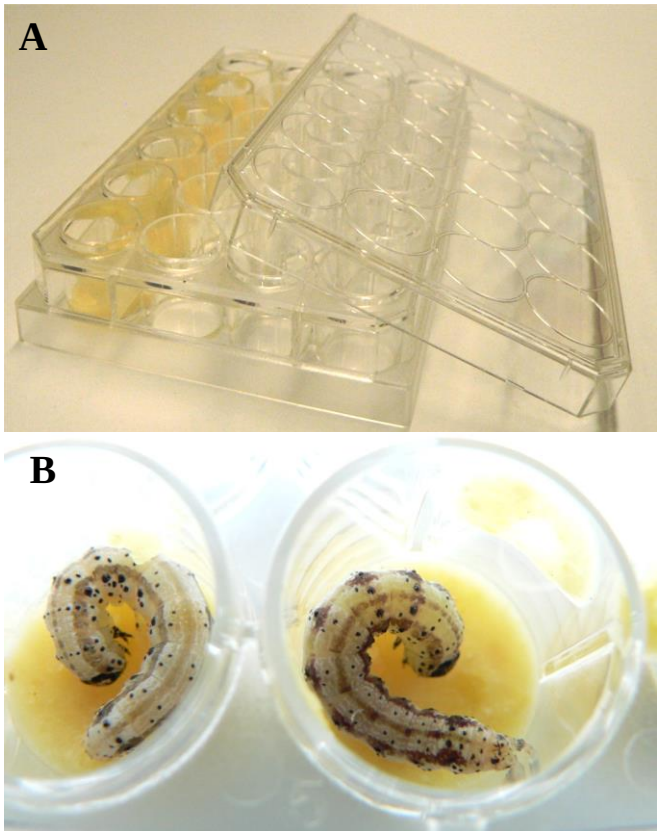

Figure S5.2: Experimental setup for feeding experiments. (**A**) 24-well plates used for all feeding assays. (**B**) 4th instar larvae of *Heliothis subflexa* feeding on 1 ml artificial diet within 24-well plates.

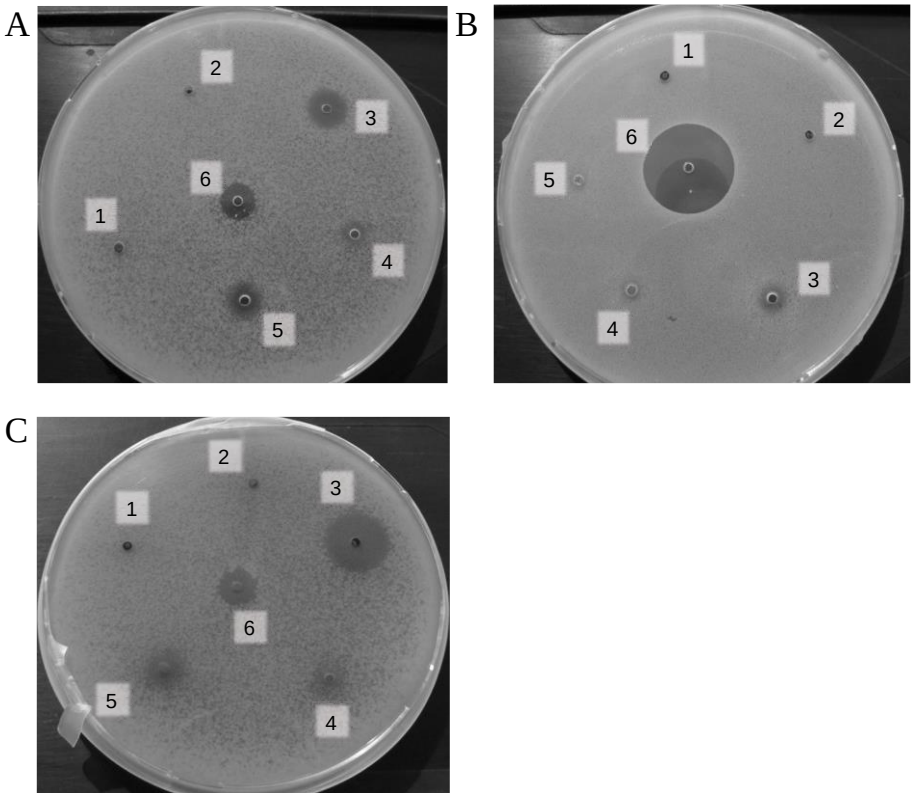

Figure S5.3: Antibacterial activity of *Physalis peruviana* extracts against bacterial strains. (**A**) Vegetative cells of *B. thuringiensis*, (**B**) *B. subtilis* and (**C**) *Pseudomonas putida* were tested against their susceptibility to tomato leave extracts (1), rape leave extracts (2), *P. peruviana* leave extracts (3), *P. peruviana* calyx extracts (4), stale *P. peruviana* leave extracts (5), 1 mg ml^{-1} ampicillin (6).

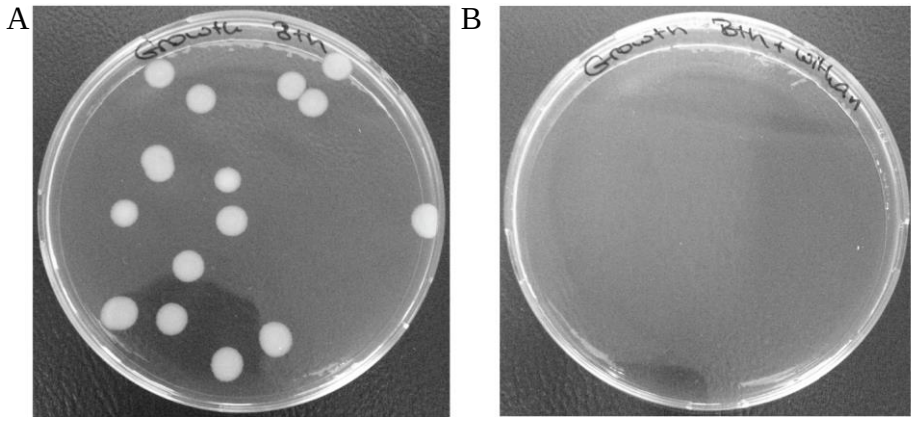

Figure S5.4: Growth inhibition assay of vegetative cells of *B. thuringiensis*. (**A**) Vegetative cells of *B. thuringiensis* grown on a normal LB agar. (**B**) Vegetative cells of *B. thuringiensis* grown on a LB agar inoculated with 1000 µg withanolide.

6 General Discussion

All herbivorous insects share their biotic environment with plants and pathogens. As a consequence, plants and pathogens are an important selective force on their hosts. In this thesis, I studied the role of environmental factors in immune system variation of two herbivorous insects, a generalist and specialist (Chapter 3) and the impact of immune system activation on reproductive life history traits in a generalist herbivore (Chapter 4). Moreover, I investigated the impact of secondary plant metabolites on plant - specialist herbivore interactions (Chapter 5). Based on immune system variation found in these generalist and specialist herbivores, the effect of environmental factors as driving forces will be discussed and I will propose how host plants, non-immunological defenses and the sex might affect immune system function in herbivores (Section 6.1). Furthermore, I will outline how herbivores trade off the cost of immune system functions with life-history traits such as reproduction and I will speculate on the role of these trade-offs in shaping immune system variations in herbivorous insects (Section 6.2). Since this study revealed the crucial role of withanolides in the interaction between the specialist and its host plant, the impact of secondary metabolites as a driving force will be reviewed and their important role in plant - herbivore - pathogen interactions will be discussed (Section 6.3). In this thesis I link the impact of plants and pathogens to the ecology and immunity of herbivorous insects to identify and further understand the selective forces for their immune system variation and evolution of diet breadth.

6.1 Network of interactions affecting immune system functions in herbivores

The environment in which herbivorous insects and pathogens interact is complex. Most immunological studies do not reflect the conditions that insect populations experience in natural settings. In the following section, I will discuss the network of interactions affecting immune system functions of herbivores in general and for the generalist *H. virescens* as well as the specialist *H. subflexa* in particular.

6.1.1 Environmental impact on the immune system of generalist and specialist herbivores

Environments differ in the threats they pose: the risk of exposure to pathogens varies, as well as the pathogenesis of those microorganisms. Thus, this landscape shapes selective pressures acting on immune system functions (Sadd and Schmid-Hempel 2009). The local optimization of immunity will lead to differences in immune system responses of different species that have experienced diverse environments (Sadd and Schmid-Hempel 2009). For instance, generalist herbivores have a greater capacity to exploit host plants and thereby withstand various environmental challenges. The heterogeneous environments of generalists thus generate a complex natural selective pressure on their immune system. In contrast, specialist herbivores evolve in environments that are relatively homogenous and might therefore experience a lower selective pressure on immune system functions than generalist herbivores. To examine whether the heterogeneous environment of generalists produces an increase in the robustness of immune system functions, we compared larvae of the generalist herbivore *H. virescens* and the specialist *H. subflexa* challenged by entomopathogenic and nonpathogenic bacteria (Chapter 3).

The most commonly method to quantify immune system functions in herbivores is to measure their ability to resist infections by pathogens (Fuxa and Richter 1989; Huang et al. 1999). This method can tell us a great deal about variation in pathogen susceptibility, and thus about the efficiency of immune system functions to combat these pathogens. Herbivorous insects vary tremendously in their susceptibility to pathogens, suggesting that the immune system has adapted to local environmental conditions (Kraaijeveld and Godfray 1997; Lazzaro et al. 2004). Considerable variation in pathogen susceptibility exists within and between populations of the model arthropods *Drosophila melanogaster*, *Daphnia magna*, *Acyrthosiphon pisum* and *Anopheles gambiae* (Collins et al. 1986; Kraaijeveld and Godfray 1997; Ebert et al. 1998; Ferrari et al. 2001; Lazzaro et al. 2004). Our results, presented in Chapter 3, indicate that the generalist *H. virescens* has a higher tolerance to entomopathogenic bacteria than the specialist *H. subflexa*. The variation between both types of herbivores in pathogen susceptibility could either be correlated with variation in genes conventionally associated with the immune system, or reflect variation in traits influencing resources available to mount an immune response or tolerate pathogens. Whatever their nature, a weakness with the measure of pathogen susceptibility is that the immune response is being measured only indirectly and thus behavioral and other non-immunological compounds may confound this result. Differences in immune system-based defense strategies might be

the key factor influencing the different outcome of pathogen - herbivore interaction observed in these generalist and specialist herbivores.

To quantify immune defense functions directly we established primary cell cultures of hemocytes to assay various components of the immune system of both species. Generally, this will reveal the immune defense strategy pursued by the individual herbivore. As an example of the influence of environmental factors on immune system functions, it has been shown that invasive species can display variation in their immune defense functions, such as expansion of the antimicrobial peptide repertoire, and are thus better defended against potential pathogens compared to closely-related non-invasive species (Lee and Klasing 2004; Vilcinskas et al. 2013). Since we found that specialized *H. subflexa* showed a higher susceptibility to vegetative cells and spores of pathogenic bacteria than the generalized *H. virescens* (Chapter 3), we suggested that immune defense mechanisms differ between these closely related species. The outcome of our experiments revealed that generalized *H. virescens* possesses a higher phagocytosis efficiency than specialized *H. subflexa* (Chapter 3). To internalize pathogens more efficiently it might be necessary to induce proliferation of phagocytes (Gilbert 2012). Therefore, it seems likely that the generalist *H. virescens* has an improved phagocytic capacity by raising the number of phagocytically active hemocytes in their hemolymph. However, our results show that the efficient phagocytic activity in the generalist was not correlated with an induced hemocyte proliferation. Interestingly, we found that the specialist *H. subflexa* showed an induced hemocyte proliferation in response to pathogenic bacteria, although its phagocytosis activity was lower compared to the generalist. Our results thus suggest that the generalist *H. virescens* might possess a higher proportion of phagocytes than the specialist to keep its system at a given level of readiness in the absence of infection. Furthermore, hemocytes are not only involved in phagocytosis but also in the production of melanin via the phenoloxidase system (Soderhall and Cerenius 1998). Possibly, the specialist *H. subflexa* might induce hemocyte proliferation to induce their phenoloxidase activity to combat pathogens. Again, our results can not confirm this hypothesis, because we found no induction of phenoloxidase activity in response to pathogens in *H. subflexa*. Based on the observed low phagocytosis and phenoloxidase activity in combination with hemocyte proliferation in *H. subflexa*, the immune defense strategy of the specialist appears to be disadvantageous, because an optimal immune defense response should be most efficient at the lowest energetic cost.

An alternative explanation for our results might be based on the fact that pathogens are able to hide from or even modulate the host immune response to ensure their survival (Ernst 2000; Espinosa and Alfano 2004; Portnoy 2005). The higher pathogen susceptibility and the lower phagocytosis and phenoloxidase activities against pathogenic bacteria which were found in the specialist when compared to the generalist (Chapter 3) suggest a specific pathogenic strategy to avoid (or delay) detection by not displaying immune elicitors on their surfaces or by killing hemocytes in specialists (Vallet-Gely et al. 2008). Generalist herbivores, however, might have evolved efficient strategies to detect these pathogens due to a greater selection pressure caused by pathogens in their heterogeneous environment.

6.1.2 Impact of host plants on the immune system of specialist herbivores

So far I have considered and discussed interactions between pathogens and herbivores as possible driving forces in immune system variations between generalist and specialist herbivores. However, it is well established that the impact of infection is often dependent on the quality and quantity of the host's diet (Duffey et al. 1995; Wilson 2005). Herbivorous insects might be in a position to influence their susceptibility to pathogens by specialization on host plants which might increase their physiological immunocompetence (Ponton et al. 2011; De Roode and Lefevre 2012). For example, *Anopheles stephensi* requires both a blood meal and a sugar source to develop an effective melanization immune response (Koella and Sorensen 2002). Similarly, larvae of *Spodoptera littoralis* choose to incorporate a high-protein and low-carbohydrate diet to increase their survival upon immune challenge (Lee et al. 2002; Lee et al. 2006b; Povey et al. 2009). Furthermore, ingestion of plant chemicals can increase host fitness due to a positive impact on host physiology or detrimental effects on the pathogen (Christe et al. 2003; Castella et al. 2008b; Povey et al. 2009). For example, *Manduca sexta* infected with *Bacillus thuringiensis* showed an improved fitness and a decreased bacterial growth associated with a lower susceptibility when larvae ingested nicotine from tobacco leaves (Krischik et al. 1988). Therefore, one possibility to explain the apparently less efficient immune system found in the specialist *H. subflexa* (Chapter 3) is that our results might be different when the specialist feeds on secondary metabolites of its host plant.

This thesis provides evidence consistent with the previously mentioned self-medication in *Manduca sexta*, as withanolides, the secondary metabolites of *Physalis* plants, were found to improve larval immunocompetence and decrease pathogen susceptibility in the specialist

H. subflexa (Chapter 5). Withanolides might be a form of medication for *H. subflexa* that is displayed by both infected and uninfected *H. subflexa*. Evidence for this kind of medication has also been documented in wood ants, *Formica paralugubris*, which utilize conifer resin to increase their survival when infected with entomopathogenic bacteria or fungi (Castella et al. 2008a). Our results suggest that the incorporation of withanolides seem to be a host adaptation that evolved to combat pathogens and improve the immunocompetence in *H. subflexa*. Therefore, specialized *H. subflexa* might not require an efficient and sophisticated immune system due to positive effects of withanolides on its immune system and pathogen defense. It might be a common strategy of specialist herbivores to choose host plants that offer chemicals which are able to increase their physiological immunocompetence. Such patterns may reveal how the evolution of immune system variation is associated with adaptation to specific host plants in herbivores.

6.1.3 Impact of non-immunological defenses on the immune system of herbivores

Over evolutionary time, herbivores have evolved a cascade of defense mechanisms to combat pathogens. I have mainly discussed immune system-based functions in herbivorous insects, but given the various environmental threats, hosts might employ alternative defense mechanisms, including behavioral adaptation (Moore 2002; De Roode and Lefevre 2012).

Behavioral adaptation of herbivores might reduce the probability of infections in the first place by avoiding contact to pathogens and thus avoiding infections. Jeffries and Lawton (1984) hypothesized that herbivorous insects use particular host plants as a refuge from their natural enemies to enhance their survival (Jeffries and Lawton 1984). Several studies have demonstrated the presence of so-called “enemy-free space” in several host plants of herbivorous insects (Denno et al. 1990; Feder 1995; Gratton and Welter 1999; Ballabeni et al. 2001; Oppenheim and Gould 2002). Studies of Andow (1990) and Lukianchuk (1997) showed that host finding success in parasitoids is higher on simply structured plants than on plants with complex structures, suggesting the importance of structural complexity for host-parasitoid interactions (Andow and Prokrym 1990; Lukianchuk and Smith 1997; Obermaier et al. 2008). Similarly, the importance of the structural refuge of *Physalis* plants in shielding *H. subflexa* from parasitoids was demonstrated by Sisterson (1999), showing an eight times higher parasitism rate for larvae fed on fruits with cut lanterns (Sisterson and Gould 1999). The results of this might imply that *H. suflexa* is not as dependent as the generalist *H. virescens* upon effective immune system-based defenses (Chapter 3), since larval feeding

behavior of *H. subflexa* is effective in reducing parasite pressure. This hypothesis is supported by studies showing that not only do non-immunological defenses reduce the likelihood of infections or predations; they also decrease the use of inducible immune system-based defenses (Castella et al. 2008b; Simone et al. 2009; Parker et al. 2011). Therefore, it might be a common strategy of specialist herbivores to use non-immunological defenses which reduce their likelihood of infection, to compensate for a possible inefficient immune system-based defense.

Behavioral adaptations to reduce infections in adult herbivores might also include sexual behavior. Sexually transmitted infections are common in natural populations and thus individuals should prefer uninfected mates to avoid infection (Loehle 1997; De Roode and Lefevre 2012). Fitness benefits of mate choice for uninfected partners have been well documented in vertebrates (Lockhart et al. 1996; Penn and Potts 1998; Nunn 2003; Knell and Webberley 2004; De Roode and Lefevre 2012). In contrast, studies on insects provide no support for the theory that adults can avoid mating with infected individuals (Abbot and Dill 2001; Webberley et al. 2002; Knell and Webberley 2004; De Roode and Lefevre 2012). However, it can be predicted that if individuals choose an uninfected mate partner over an infected one, the individual is obviously getting a selective advantage by avoiding infection from a sick mate. Indeed, our results presented in Chapter 4 showed that males of *H. virescens* prefer uninfected females over infected females, suggesting an avoidance of infections in male moths. However, a general avoidance of infected females is not supported in our study, because males showed no mating preference when they had to choose between infected and wounded females, suggesting that males recognize immune system activation in females, but cannot discriminate if this is caused by bacteria or wounding. Further, the fact that females did not discriminate between infected and uninfected males fails to support a general avoidance of infected mate partners in these moths.

One potential explanation might be the so-called "handicap principle" for sexual signals which proposes that healthy individuals are able to produce more elaborate sex characteristics than infected individuals because these signals are costly to produce (Hamilton and Zuk 1982; Andersson 1994; Wedekind 1994; Worden et al. 2000). Individuals that selectively mate with uninfected mates may benefit if infected mates provide fewer resources or lower fertility (Hoelzer 1989; Rosenqvist and Johansson 1995). Therefore, sexual signals might act as honest indicators of mate quality if there were a trade-off between sexual advertisement and immunity (Andersson 1994; Worden et al. 2000; López and Martín 2005). Sex pheromones

have been proposed to be good indicators of an individual's condition (Worden et al. 2000). For instance, males of *Tenebrio molitor* are less attracted by odour extracts from infected females than from uninfected females (Hurd and Fogo 1991). Therefore, differences in the attractiveness of infected females to males found in *H. virescens* could be the result of a qualitative or quantitative change in pheromone production caused as a direct cost of bacterial infection in these females. *H. virescens* females produce a volatile pheromone blend that attract males, with (Z)-11-hexadecanal (Z11-16:Ald) as the major component essential to attract males (Tumlinson et al. 1975; Klun et al. 1980; Teal et al. 1981; Vetter and Baker 1983). Our result that the ratio between 16:Ald and Z11-16:Ald was significantly higher in injected females than in control females might be a possible explanation for the male preference reported in this thesis, because it was shown 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). Similarly, Rantala (2003) found that pheromone production in *T. molitor* is condition dependent. Studies by Clark and coworkers (1997) revealed that sex pheromones in cockroaches (*Nauphotea cinerea*) are also condition dependent (Clark et al. 1997). Together with our finding of condition dependence of pheromone production in females of *H. virescens*, it seems likely that sexual attractiveness is negatively correlated with infection or immune system activation in females and that males of *H. virescens* might assess the quantity of sex pheromones for their mate choice (Rantala et al. 2003a).

6.1.4 Impact of sex on the immune system of herbivores

In vertebrates, sexual dimorphism in immune functions is a common pattern, whereby females are more immunocompetent, meaning that both humoral and cellular mediated immune responses are more efficient, than in males (Zuk 1990; Zuk and McKean 1996). The underlying causes are explained by either the role of testosterone as immunosuppressive substance, or by fundamental differences in male and female life history (Nunn et al. 2009). As in vertebrates, studies of crickets, scorpion flies, fairy shrimp and damselflies showed a stronger immune response in females than males (Radhika et al. 1998; Kurtz et al. 2000; Adamo et al. 2001; Rolff 2001; Zuk et al. 2004). We tested the hypothesis that males are less immunompetent than females by measuring the expression level of immune related genes of both males and females of *H. virescens* upon immune challenge (Chapter 4). Such a sex-specific difference in the expected direction was indeed found: infected females of

H. virescens had a stronger induced immune response, compared to infected males. This mirrors results from other insect species showing that females tend to invest more in the immune system upon challenge than do males (Rolff 2002).

Although immune defense mechanisms are inducible, their constitutive (baseline) expression may be costly for both males and females (Harvell 1990; Frost 1999). In insects it was shown that many individuals prophylactically increase their investment in immunity to have their immune system in a state of readiness when the risks of parasitism are high (Wilson and Cotter 2013). For instance, various studies have shown that immune functions are constitutively expressed in species where population density is high (Reeson et al. 1998; Barnes and Siva-Jothy 2000). Therefore, we predicted that both females and males of *H. virescens* would have similar expression patterns of immune-related genes under normal conditions. However, unlike the sex differences found upon immune challenge in *H. virescens*, we observed that uninfected males had a higher constitutive expression of immune-related genes than females under normal conditions (Chapter 4). Since our study only determined one aspect of the immune system in *H. virescens* adults, we cannot claim that males possess a higher constitutive and lower induced expression of all immune traits compared to females. Our results, however, suggest at least that it is very likely that sex-specific differences in immune system functions are not restricted to vertebrates (Kurtz et al. 2000). Finally, whether the observed sex-specific differences in immunity of *H. virescens* might be caused by trade-offs between immunity and reproduction will be discussed in section 6.2.3, because it has been suggested that males trade off immunity for reproduction (Sheldon and Verhulst 1996; Zuk and McKean 1996).

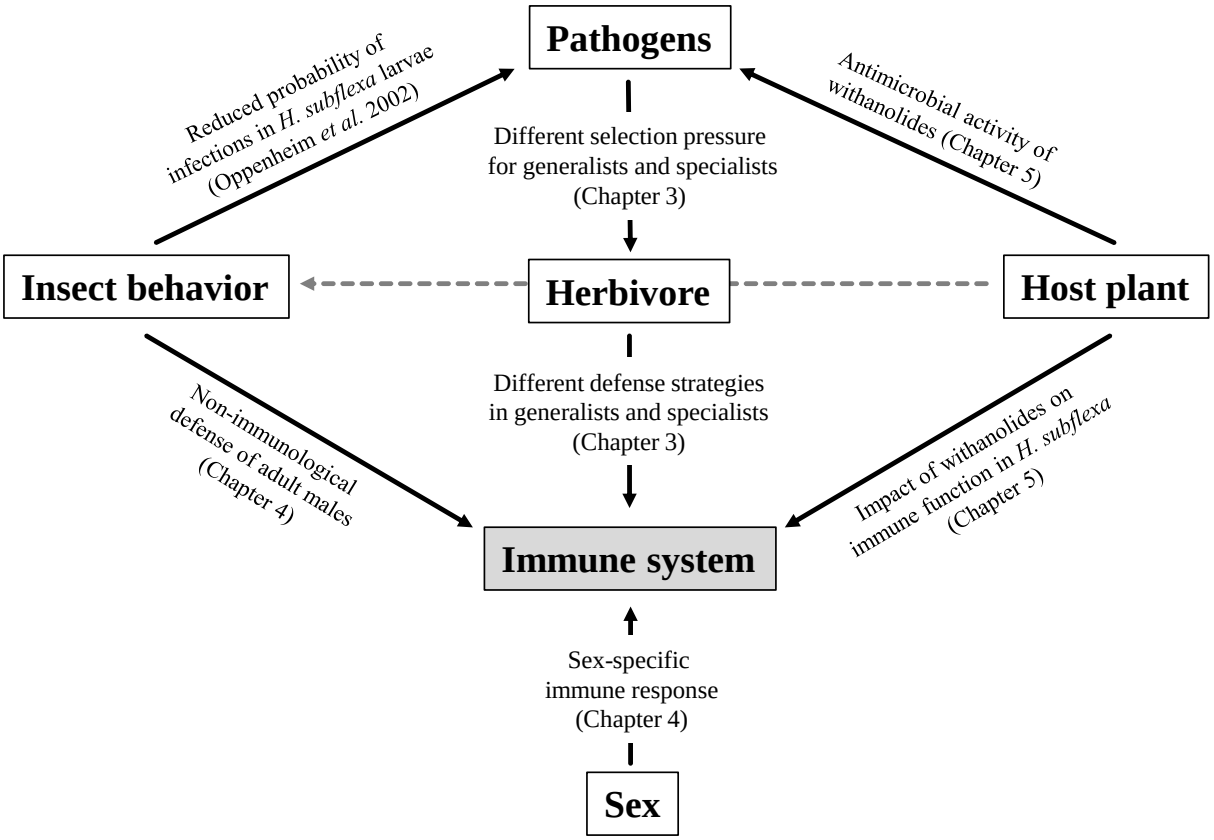

Figure 6.1. Network of interactions affecting immune system functions in the generalist *H. virescens* and specialist *H. subflexa*. Host plant usage affects insect behavior and environmental pathogens. All three in turn affect variation in the immune system of generalist and specialist herbivores. Furthermore, immune system functions of herbivorous insects vary between males and females.

In conclusion, for a complete understanding of the evolutionary dynamics of immune system functions in any system, studies must include the complexity that environmental and life history variations possess. In this light, the results of this thesis provide a rare example of the complex network of interactions which might affect immune system function in herbivorous insects in general, and in the generalist *H. virescens* and specialist *H. subflexa* in particular.

6.2 Trade-offs associated with immunity in herbivores

As described and discussed in the previous section, it is important to study immune system functions in the context of environmental factors and life history strategies. Fundamental to this approach is the assumption of ecological immunology that immunity is costly, such that it may alter the expression of another life-history component in the host (Stearns 1989; Roff 2002; Wilson 2005). This section will address possible trade-offs in immunity of *H. virescens* and *H. subflexa*, and reveal how such costs might have affected the immune system variation observed between a generalist and a specialist.

6.2.1 Trade-offs within the immune system of generalist and specialist herbivores

It is conceivable that there are trade-offs even within the immune system itself, with high levels of one component resulting in low levels of another (Cotter et al. 2004). A number of studies in vertebrates have examined this correlation and have identified potential trade-offs within the immune system, such as the upregulation of the humoral response at the expense of cellular responses (Gross et al. 1980; Siegel and Gross 1980; Grencis 1997; Gehad et al. 1999; Ibanez et al. 1999; Gill et al. 2000; Cotter et al. 2004). However, only few studies on insects provide evidence for a trade-off within the immune system of insects. For example, a study in larvae of *Spodoptera littoralis* found that antibacterial activity exhibits a negative correlation to hemocyte density and phenoloxidase activity (Cotter et al. 2004). Furthermore, several studies on insect immune functions have identified a negative correlation between phenoloxidase activity and antibacterial activity suggesting that these trade-offs may occur across several insect taxa (Moret and Schmid-Hempel 2001; Moret and Siva-Jothy 2003; Rantala et al. 2003a; Cotter et al. 2004; Freitak et al. 2007).

Based on these studies it can be predicted that trade-offs within the immune system might occur in both the generalist *H. virescens* and the specialist *H. subflexa*. As expected, we found that *H. subflexa* appeared to invest available resources into hemocyte proliferation, at the expense of phenoloxidase and lysozyme activity, suggesting a possible physiological trade-off between the cellular and humoral immune system of this specialist (Chapter 3). However, our results did not reveal a negative trade-off within the immune system of the generalist *H. virescens*. The mechanism driving the trade-off in specialists is unclear; however, one possibility is that generalists have a greater ability for resource acquisition and thus generate positive correlations between all components of the immune system, compared

to specialists. Further experiments are required to test whether trade-offs within the immune system occur in general in these species and whether these trade-offs differ between generalists and specialists.

6.2.2 Trade-offs associated with diet breadth in specialist herbivores

Maintaining the immune system and mounting an immune defense response are expected to be costly in terms of specific nutrients and energy that have to be allocated from other life history traits. Costs of immunity may therefore be positively impacted by the intake of specific nutrients to compensate for the loss. In insects, nitrogen is often considered to be the essential limiting resource (McNeill and Southwood 1978). For life history traits such as reproduction and growth it was shown that salt is a fundamental factor (Trumper and Simpson 1993). However, the essential nutritional requirements for an effective immune response are not yet identified (Lee et al. 2002; Wilson 2005). Given that the production and maintenance of the phenoloxidase system is dependent on phenylalanine, an essential amino acid, and more generally on nitrogen, it can be suggested that these dietary compounds might support immune functions (Johnson et al. 2003; Wilson 2005). Moreover, studies indicate that the phenoloxidase system is a costly trait, whose production and maintenance have fitness costs for hosts (Schwarzenbach and Ward 2006; Vermeulen and Bijlsma 2006; Cotter et al. 2008). If phenoloxidase is diet-dependent, then individuals fed on their optimal diet should have higher levels of phenoloxidase activity.

An interesting finding of this thesis was that *H. subflexa* larvae fed on withanolides had higher levels of both phenoloxidase and antibacterial activity than larvae deprived of withanolides (Chapter 5). Increased phenoloxidase levels are associated with increased dopamine levels in *Drosophila*, and this biochemical pathway provides the main source of reactive oxygen species, which are thought to contribute to early senescence (Vermeulen and Bijlsma 2006; Cotter et al. 2008). In the yellow dung fly, *Scathophaga stercoraria*, it has been shown that individuals with high phenoloxidase suffer from reduced longevity, possibly due to a coincident increase in dopamine as phenoloxidase substrate (Schwarzenbach and Ward 2006; Cotter et al. 2008). These studies strongly indicate that the costs of increased phenoloxidase activity might not justify its benefits. Nutritional composition of *Physalis* fruits might compensate for possible negative effects of withanolides. Lee and coworkers (2006) showed that high-protein diets allowed caterpillars to maintain higher phenoloxidase activity

than low-protein diets. Further studies on potential costs associated with diet breadth in other host plant specialists would be informative.

6.2.3 Trade-offs between immunity and sexual traits in H. virescens females

Possibly, the trade-off most critically important to insect life history is that between immunity and sexual traits. In insects, males have been found to invest fewer resources in immune response than females (Rheins and Karp 1985; Gray 1998; Rolff 2001). These sex-specific differences in immunity have been related to Bateman's principle, which posits that males maximize their fitness by increasing mating frequency, while female fitness is positively correlated with longevity (Bateman 1948; Trivers and Willard 1973; Zuk 1990; Roth et al.). Assuming that a longer lifespan requires a more efficient immune system in females and that a maximized mating frequency relies on immediately available resources in males, Bateman's principle could thus explain that males of *H. virescens* were found to be less immunocompetent upon immune challenge than females (Chapter 4). Further, our results revealed that the strong induced immune response in infected females was associated with a reduced mating success and a modified sex pheromone composition. By contrast, infected males had the same mating success than uninfected males, suggesting that males of *H. virescens* trade off immunity for reproduction, whereas females invest in immunity at the cost of reproductive effort. Since the sexual dimorphism and altered mating behavior was already discussed in previous sections, this section will focus on possible trade-offs between pheromone quality and immunity in females of *H. virescens*.

A possible explanation for the trade-off in females of *H. virescens* might be the impact of juvenile hormone (JH), which is involved in reproduction and immunity traits of insects (Rantala et al. 2003b). Juvenile hormone is secreted by the corpora allata and plays crucial roles in the development and reproduction of all insects (Wyatt and Davey 1996). Studies have shown that JH is associated with sex pheromone production and that titers of this hormone are elevated after mating (Couche et al. 1985; Duportets et al. 1998; Sréng et al. 1999). There is evidence that JH also affects immune function in insects (Rantala et al. 2003b). For instance, high titers of JH have been implicated in humoral immunosuppression in insects (Hiruma and Riddiford 1993; Rolff and Siva-Jothy 2002; Rantala et al. 2003b). In addition, a study in *T. molitor* showed that JH increased the attractiveness of male pheromones but suppressed immune functions, like phenoloxidase activity (Rantala et al. 2003b). Therefore, JH might control the trade-off between immunity and reproduction

necessary to maintain pheromone production (Rantala et al. 2003a). If JH is involved in the trade-off between pheromone production and phenoloxidase (PO) activity in females of *H. virescens*, we predict an increased phenoloxidase activity in infected females since the production of the major pheromone component was decreased in these females (Chapter 4). Indeed, infected females of *H. virescens* showed an increased transcription level of a phenoloxidase activating factor compared to uninfected females, suggesting an elevated PO activity in infected females. Thus, low titers of JH may positively affect PO levels but negatively affect pheromone production in infected females of *H. virescens*. Studies in *Danaus plexippus* have shown that high titers of JH reduce longevity (Herman and Tatar 2001). Females of *H. virescens* may thus reduce their JH level which in turn might increase their longevity and simultaneously elevate their immune response functions to combat infections at the expense of sexual traits.

Another possible explanation for the trade-off between pheromone quality and immunity in females of *H. virescens* might be a genetic trade-off between sexual traits and immunity. Sexual traits in moths display an extensive regulation by circadian clock genes, including female calling behavior and pheromone release (Rosén 2002; Silvegren et al. 2005). Female pheromone production and male response usually occurs synchronously at a specific time of day, e.g. in most noctuid moths during the mid-scotophase (Rosén 2002). Therefore, moth pheromone production should be under a strict and precise circadian regulation to obtain the maintenance of reproduction in both sexes. Insect immune genes are also under circadian control (Shirasu-Hiza et al. 2007; Lee and Edery 2008). Shirasu-Hiza and coworkers (2007) demonstrated that *Drosophila melanogaster* infected with *Streptococcus pneumonia* exhibit disrupted circadian rhythm, by showing that infected flies slept for shorter periods compared to healthy ones. These data might lead to the hypothesis that an altered circadian rhythm caused by infection in females of *H. virescens* might have affected the sex pheromone production resulting in a reduced mating success of these infect females. It can be speculated that infected females are trapped in a vicious cycle: infection causes loss of circadian rhythm and the loss of circadian rhythm alters pheromone production, leading to a trade-off between immunity and sexual traits.

6.3 Interactions among *Physalis* plants, the specialist *H. subflexa* and *B. thuringiensis*

Life history theory explains how natural selection influences patterns of survivorship and reproduction in order to optimize fitness in the face of ecological challenges posed by the environment (Roff 1992; Stearns 1992; Fabian and Flatt 2012). Moreover, the study of life history evolution is also about the understanding of adaptation, the most fundamental aspect in evolutionary biology (Fabian and Flatt 2012). A general pattern that has been observed is a predominance of specialist herbivores over generalist herbivores (Fox 1981; Jaenike 1990). However, only a limited understanding of the ecological and evolutionary processes that drive host specialization exists (Gould 1988; Futuyma 1991; Thompson and Pellmyr 1991). This section will illustrate the impact of secondary plant metabolites and natural pathogens on the evolution of *Physalis* specialization in *H. subflexa*.

6.3.1 Adaptation of H. subflexa on Physalis plants

A fundamental implication of Ehrlich and Raven's work on co-evolution is that the structure of herbivore-plant communities reflects their evolutionary history (Ehrlich and Raven 1964; Mitter and Farrell 1991). In the relationship between plants and herbivores, plants evolve chemicals which are repellent or harmful to herbivores, and in turn herbivores evolve abilities to overcome these defenses, and vice versa (Ehrlich and Raven 1964; Mitter and Farrell 1991). The impact of plant defenses on herbivorous insects depends on whether the herbivore is a specialist or generalist on a particular host plant (Traw and Dawson 2002). In general, specialist herbivores tend to be unaffected or even benefited by chemical defenses, many of which are repellent or lethal to generalist herbivores (Agrawal et al. 1999; Agrawal 2000b; Traw and Dawson 2002; Becklin 2008).

Withanolides are common chemical compounds in *Physalis* plants and were shown to possess antifeedant and immunosuppressive activities against several lepidopteran larvae, such as *Helicoverpa zea* (Ascher et al. 1980; Ascher et al. 1981; Elliger and Waiss 1988). The overall effect of withanolides is presumably to reduce the level of herbivory on *Physlalis* plants by making it more difficult for herbivores to process the plant material. In fact, to our knowledge no other herbivorous insect, except *H. subflexa*, has specialized on *Physalis* plants, suggesting that withanolides are an efficient defense to protect the plant against herbivory. Interestingly, even the closely related counterpart of *H. subflexa*, *H. virescens*, which has

shown to be an extreme generalist, cannot develop on *Physalis* plants (Oppenheim and Gould 2002; De Moraes and Mescher 2004). As previously mentioned, specialists are more likely to display adaptations to plant defenses of a particular host plant than generalists, therefore we predicted that the specialist *H. subflexa* evolved efficient mechanisms to overcome plant defenses of *Physalis* and thus gained the ability to successfully feed and develop on these plants. In fact, previous research showed that the specialist *H. subflexa* adapted to morphological, nutritional and biochemical features of *Physalis* plants, whereas the generalist *H. virescens* did not show any behavioral and physiological adaptation towards *Physalis* plants (Sisterson and Gould 1999; Oppenheim and Gould 2002; De Moraes and Mescher 2004). In this thesis, an attempt has been made to investigate the possible role of withanolides in *Physali*s specialization of *H. subflexa* (Chapter 5).

Our results revealed that withanolides improve larval development, induce phenoloxidase activity and immune gene expression in *H. subflexa*. Interestingly, the immunosuppressive effect of withanolides was evident in the generalist *H. virescens*, because immune genes were found to be down-regulated after withanolide ingestion, suggesting that the generalist *H. virescens* can not avoid detrimental effects of plant defenses by *Physalis*. Previous studies have shown that withanolides possess ecdysteroid antagonistic activities, suggesting that the antifeedant effect arise from a disruptive effect of withanolide on development of non-adapted insects (Dinan et al. 1996; Savchenko et al. 2000). Furthermore, ecdysteroids, including ecdysone and 20-hydroxy-ecdysone (20E), were shown to modulate the cellular and humoral immunity in *Drosophila melongaster* by increasing the phagocytic activity, pathogen encapsulation and AMP gene expression (Meister and Richards 1996; Lanot et al. 2001; Sorrentino et al. 2002). A disturbed ecdysteroid release in *Rhodnius prolixus* was shown to induce immune system depression (Garcia et al. 1990). Therefore, the ecdysteroid antagonistic activity of withanolides might account for the detrimental effects observed in non-adapted herbvirores fed on withanolides (Ascher et al. 1981; Elliger and Waiss 1988) (Chapter 5). The specialist *H. subflexa*, however, might have evolved mechanisms to circumvent plant defenses by *Physalis* plants e.g. by evading binding of withanolides to their ecdysteroid receptors, increasing their own ecdysteroid production or increasing their ecdysteroid receptor repertoire. Although *H. subflexa* larvae accept a restricted host range by the specialization on *Physalis* plants, they may benefit from a near-exclusive access to a food source that is incompatible to many herbivorous competitors.

Based on our findings, I suggest that the positive impact of withanolides on *H. subflexa* is likely to have been a strong selection force during the speciation by *H. subflexa* from a *H. virescens*-like generalist. Adaptation to *Physalis* chemistry by *H. subflexa* may constitute a further adaptation equal in importance to morphological and nutrional aspects of specialization shown by other researchers (Sisterson and Gould 1999; Oppenheim and Gould 2002; De Moraes and Mescher 2004).

6.3.2 Interactions among Physalis plants, H. subflexa and B. thuringiensis

In nature, plants can influence the interactions between herbivores and their microbial pathogens (Schultz et al. 1992; Cory and Hoover 2006). One well-documented example is the plant-mediated effect on the efficacy of *B. thuringiensis* on herbivorous insects (Meade and Hare 1993; Appel and Schultz 1994; Kleiner et al. 1998; Kouassi et al. 2001). Many studies have shown that host plants can either alter the severity of insect's disease induced by *B. thuringiensis* or inhibit the growth of this entomopathogenic bacterium *in vitro* (Smirnoff and Hutchison 1965; Morris 1972; Meade and Hare 1993; Appel and Schultz 1994). However, plant chemicals that change the toxicity of *B. thuringiensis* often do so by either affecting the insect or the entomopathogenic bacterium itself (Felton and Dahlman 1984; Krischik et al. 1988; Ludlum et al. 1991; Berenbaum and Zangerl 1998).

This thesis demonstrates that withanolides from *Physalis* plants can alter the pathogenicity of *B. thuringiensis* by both acting directly on the bacterium and indirectly on the specialist *H. subflexa* (Chapter 5). In our study, withanolides were found to be active against vegetative cells and spores of *B. thuringiensis* (Chapter 5). Feeding by *H. subflexa* caterpillars on withanolide-containing diet decreased the susceptibility of caterpillars to spores of *B. thuringiensis* (Chapter 5). However, the mode of action by which withanolides increase survival of *H. subflexa* when infected with *B. thuringiensis* is unknown. Our results suggest that *H. subflexa* might profit from both an immune system stimulating effect and a spore germination inhibiting effect of withanolides.

One theory on the mode of action of *B. thuringiensis* postulates that some lepidopteran larvae die of septicemia from gut bacteria invading the hemocoel after ingestion of *B. thuringiensis* (Broderick et al. 2006; Broderick et al. 2009a). In *Rhodnius prolixus*, Castro *et al.* (2012) found that consumption of withanolides resulted in higher numbers of bacterial microbiota, which was associated with a lower antimicrobial activity and higher levels of nitrite and nitrate in the gut of insects infected with *Trypanosoma cruzi*, compared to control

insects. For insects it is important to maintain gut homeostasis which provides resistance to invading microorganisms (Garcia et al. 2010; Castro et al. 2012a). It may thus be that the withanolide-based induced antimicrobial activity in specialized *H. subflexa* affects the microbiota population and the physiology of the larval gut and thus decreases the pathogenicity of *B. thuringiensis*.

From a plant-centered viewpoint, plants are expected to enhance the effectiveness of entomopathogen populations to reduce herbivory, rather than suppress their pathogenicity. If a plant instead provides the benefit of pathogen protection, this plant might be 'attractive' for many herbivores. However, only a few herbivorous insects are known to feed on *Physalis* plants, suggesting the presence of other drawbacks for herbivores. It remains to be seen whether *Physalis* plants prevent entomopathogenic infections in field populations of *H. subflexa*.

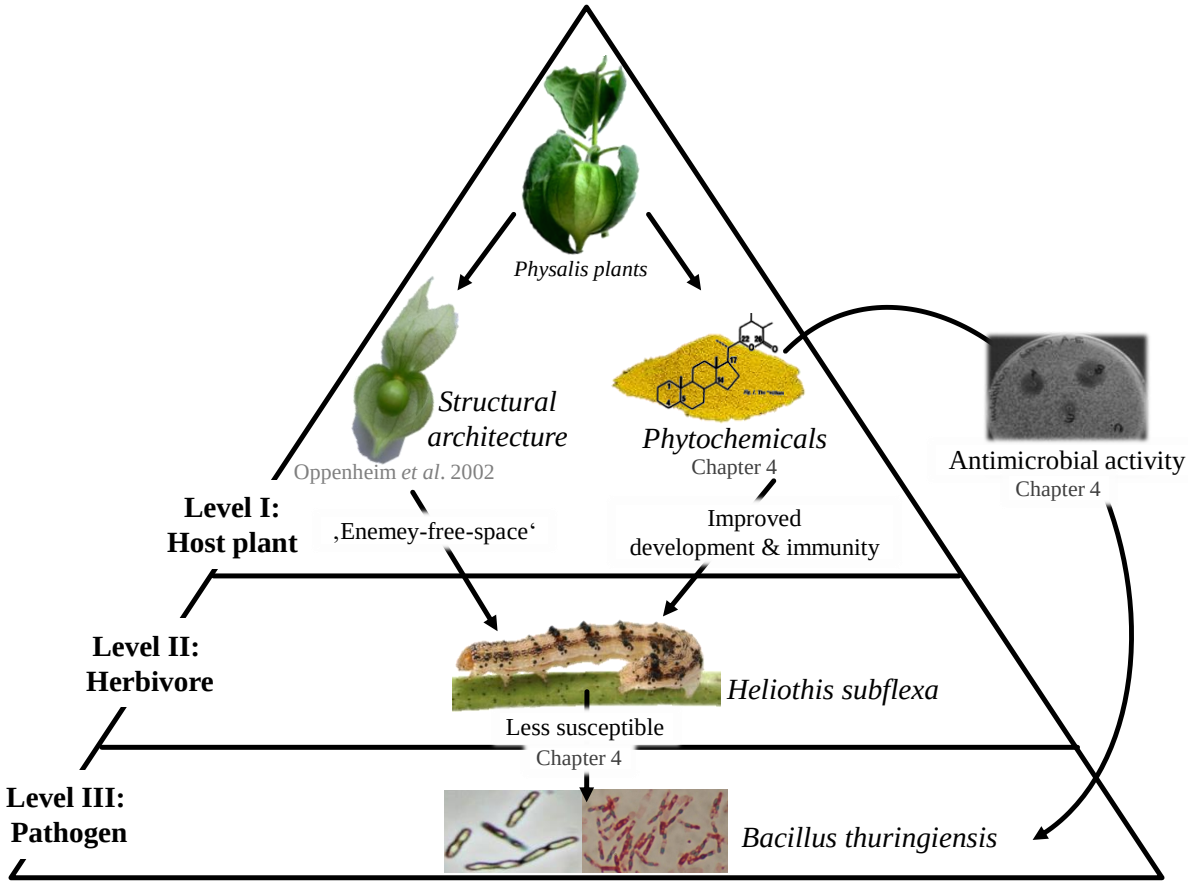

Figure 6.2. Impact of *Physalis* on *H. subflexa* and its interactions with *B. thuringiensis*.

6.3.3 Interactions among tritrophic levels: Prospects for application in pest control

Tritrophic interactions among insect pests, their host plant and their microbial pathogens have become increasingly recognized as an important factor in insect pest management (Duffey et al. 1995). Several strains of *B. thuringiensis* have been used as biocontrol agents for larval stages of economically important lepidopteran species (Kleiner et al. 1998). Therefore, the knowledge of plant-mediated effects on the pathogenicity of *B. thuringiensis* is essential for improvements of commercial formulations of this bacterial insecticide and thus will minimize the development of resistance in the targeted species (Appel and Schultz 1994; Kouassi et al. 2001). Several studies demonstrated an inhibitory effect of plant extracts on *B. thuringiensis* activity or toxicity towards its target insects (Smirnoff and Hutchison 1965; Krischik et al. 1988; Schultz et al. 1992). For instance, oak tannins were shown to reduce the effectiveness of *B. thuringiensis* towards the gypsy moth *Lymantria dispar* (Appel and Schultz 1994). Nicotine was found to inhibit colony formation by *B. thuringiensis* and reduced its pathogenicity to *Manduca sexta* (Krischik et al. 1988).

In line with this, our results show that withanolides from *Physalis* plants possess antimicrobial activity against *B. thuringiensis* and reduce its pathogenicity towards specialized *H. subflexa* (Chapter 5). Researchers that might plan to develop transgenic *Physalis* plants that produce their own insecticide, like the toxin of *B. thuringiensis*, have to take the antimicrobial properties of this plant into account in order to successfully manage the control of *H. subflexa*. Although *H. subflexa* is not yet considered as a serious agricultural pest in the United States (Laster 1972), the successful biological control of this species might be of major concern in the near future. For instance in Mexico, *Physalis* plants are severely infested by *H. subflexa* which is a major pest of the agricultural crop *Physalis philadelphica* in this region (Mitter et al. 1993; Lee et al. 2006c). Since *P. philadelphica*, i.e. the tomatillo, is a fundamental diet component of Mexican people, the cultivation of this plant is shifting from small farms to larger monocultures (Gould et al. 2005). Therefore it will become important to develop pest management methods that farmers can use to sustainably manage *H. subflexa* (Gould et al. 2005).

Given the decline in the efficacy of available insecticides and the growing concern over their impact on ecological and human health, it is important to understand the bases for plant-mediated effects on the efficacy of *B. thuringiensis*. Manipulation of plant chemistry to improve biological control agents will clearly be an important component in the further research of integrated pest management.

7 Summary

Herbivorous insects live in complex environments in which they intimately interact with a broad range of pathogens. Therefore, herbivores require a range of efficient immune defense mechanisms that can cope with all potential pathogens as specifically and immediately as possible. Environments differ in the threats they pose: the risk of exposure to pathogens varies, as well as the pathogenesis of those microorganisms. Therefore, heterogeneous environments of herbivores will generate a complex natural selective pressure on their immune system, possibly causing variations in immune system functions among herbivorous insects. Variation in immune system functions will also impose different costs of defense upon herbivores, leading to complex patterns of trade-offs against other life-history traits, such as sexual traits.

The complexity of environments, wherein herbivorous insects and pathogens interact, is also impacted by plants. The evolutionary arms race between plants and their herbivores provides plants with a highly sophisticated defense system. However, the relationship between plants and herbivores cannot be fully understood without incorporating the impact of herbivorous pathogens. We are only beginning to understand the complexity of herbivore - pathogen and herbivore - plant interactions that occur in complex environments. General explanations for both immune system variation and host plant adaptation in herbivorous insects are rare. Most immunological studies on herbivorous insects have almost exclusively focused on physiological and molecular mechanisms of the innate immune system, generally neglecting the fact that herbivores interact with their environment. The aim of this thesis was to examine the importance of insect ecology on immune system variation and host plant adaptation in lepidopteran herbivores.

7.1 Environmental factors have an impact on immune defense strategies of the generalist Heliothis virescens and the specialist Heliothis subflexa

Immune system responses vary across invertebrate species as consequence of environmental heterogeneity. Among herbivorous insects, specialists evolve in environments that are relatively homogeneous due to their restricted host range, whereas herbivorous generalists evolve in environments that are heterogeneous due to their broad host range. Generalists may thus be exposed to a wider range of environmental conditions than specialists, exerting stronger selection on the innate immune system. To investigate whether generalist herbivores

possess a more robust immunity than specialists, pathogen susceptibility and immune system-based function were analyzed and compared between larvae of the generalist *H. virescens* and the specialist *H. subflexa* (Lepidoptera, Noctuidae). Pathogenic bacteria ingested with diet or injected into the body cavity were found to affect the generalist and specialist differently. The generalist *H. virescens* showed a higher tolerance towards pathogens, apparent by a lower mortality and higher larval growth rate, than the specialist *H. subflexa*. It has also been shown that the difference in pathogen susceptibility was based on differences in immune system-based defense strategies among both herbivores. *In vitro* and *in vivo* experiments demonstrated that the generalist *H. virescens* possess a lower number of recoverable bacteria, lower proliferation of hemocytes, and higher phagocytic and antimicrobial activities compared to the specialist *H. subflexa*. These results indicate that generalist herbivores have a more efficient immune defense strategy than specialist herbivores and thus may be the base for further studies to understand the ecological forces that shape the evolution of innate immunity in herbivorous insects.

7.2 Immune system activation induces changes in sexual traits of H. virescens females

Immune system responses may vary not only among generalist and specialist herbivores, but also between males and females as a result of different life histories of both. In this study, I examined the hypothesis that females trade off reproduction for immunity. The quantitative gene expression study conducted on females and males of *H. virescens* injected with pathogenic bacteria revealed that an immune challenge in these adults provoked a sex-specific expression pattern of immune-related genes. Males were found to be less immunocompetent than females upon immune challenge, suggesting that males invest less in immunity than in reproduction. The increase in immunocompetence by females upon immune challenge was found to be negatively correlated with a lower attractiveness and altered sex pheromone profile of infected females, suggesting that infected females invest in immunity instead of attractiveness. These results indicate that future studies on trade-offs between immunity and reproduction have to consider sex-specific strategies to activate an immune defense response which in turn affects sexual traits differently among sexes.

7.3 Tritrophic interactions play an important role in the adaptation of the specialist H. subflexa on Physalis plants

The relationship of herbivores with their host plants is influenced to a large extent by plant chemistry and counter adaptations of the herbivore host. The study system for herbivore-plant interactions in this thesis involves the specialist *H. subflexa* and its host plant, *Physalis* (Solanaceae). To develop and survive on its host plant, *H. subflexa* has adapted to morphological, nutritional and biochemical features of *Physalis* plants. Here the impact of withanolides, the secondary metabolites of *Physalis* plants, on *H. subflexa* was investigated. Withanolides are known to possess antifeedant and immunosuppressive activities against several lepidopteran larvae, such as the generalist *Helicoverpa zea*. Interestingly, we found that *H. subflexa* fed on artificial diet containing withanolides display an improved larval performance and immunocompetence, apparent by an induced phenoloxidase activity and up-regulated immune gene expression, compared to control individuals. The immunosuppressive effect of withanolide, however, was evident in the generalist *H. virescens*, because immune genes were found to be down-regulated after withanolide ingestion. This indicates that the specialist *H. subflexa* has evolved mechanisms to circumvent immunosuppressive effects of withanolide and instead experiences an immune stimulation by withanolides. Although the antibacterial activity of withanolides against human pathogens is well known, its activity against entomopathogenic bacteria has been little studied. Here, a novel finding was that withanolides possess antimicrobial activity against spores and vegetative cells of *Bacillus thuringiensis*. Moreover, we could demonstrate that withanolide treatments increases survival of *H. subflexa* larvae infected with spores of *B. thuringiensis*, implying that adapting its immune system to immune altering properties of withanolides and thus utilizing their antibacterial activities was a crucial step for *H. subflexa* during host plant specialization. However, the mode of action by which withanolides increases survival of infected *H. sublfexa* remains unknown and will be an exciting challenge for future research.

In conclusion, my thesis has shown that: (1) generalist and specialist herbivores possess different immune defense strategies due to their very different life styles; (2) herbivores exhibit sex-specific trade-offs in life-history traits, such as immunity and reproduction; and (3) plant secondary metabolites have a significant impact on the physiology and immunity of specialized herbivores and affect the interaction between herbivores and their natural pathogens. This study is one of the few that has linked the impact of plants and pathogens to

the ecology and immunity of herbivorous insects, and thereby identified the fundamental role of environmental factors in shaping immune system variation and host plant adaptation in two lepidopteran herbivores. The results of my thesis show that there is still much to learn on ecological immunology and diet breadth evolution in herbivorous insects.

8 Zusammenfassung

Herbivore Insekten leben in komplexen Habitaten in denen sie mit vielen verschiedenen Pathogenen interagieren müssen. Um dieser Herausforderung gerecht zu werden, benötigen sie entsprechende Abwehrmechanismen, welche gezielt und schnell Mikroorganismen erkennen und bekämpfen. Die Bedrohungen innerhalb der Umwelt, in der herbivore Insekten leben, unterscheiden sich je nach Art des Lebensraumes, sowohl hinsichtlich des Risikos einer Konfrontation mit Pathogenen, als auch der Pathogenität dieser Mikroorganismen an sich. Dies kann zu einem komplexen Selektionsdruck auf das Immunsystem führen, infolgedessen sich die Immunsysteme zwischen verschiedenen Arten von herbivoren Insekten in ihrer Funktionsweise unterscheiden. Variationen in der Funktionsweise des Immunsystems implizieren auch unterschiedliche Kosten für herbivore Insekten. Diese können zu einem komplexen Muster von *trade-offs* und anderen *life-history* Merkmalen führen, und beispielsweise Auswirkungen auf die Reproduktion von Insekten haben.

Es ist offensichtlich, dass herbivore Insekten in ihren komplexen Lebensräumen neben Pathogenen, auch von ihren Wirtspflanzen beeinflusst werden. Das evolutionäre Wettrüsten zwischen Wirtspflanzen und ihren Fraßfeinden brachte Pflanzen mit hochgradig angepassten Resistenzmechanismen hervor. Jedoch kann die Interaktion zwischen Wirtspflanzen und ihren herbivoren Insekten nicht vollkommen verstanden werden, ohne den Einfluss von Pathogenen zu berücksichtigen. Wir beginnen erst zu verstehen, wie komplex Herbivor-Pathogen- und Herbivor-Wirtspflanzen-Interaktionen sind. Bisher gibt es kaum allgemeine Erklärungen für die Variationen in der Funktionsweise des Immunsystems und die Wirtspflanzen Adaption von herbivoren Insekten. Vorausgegangene immunologische Studien an Insekten konzentrierten sich eher auf physiologische und molekulare Mechanismen des Immunsystems, jedoch vernachlässigten diese Studien die Tatsache, dass herbivore Insekten mit ihrer Umwelt interagieren. Das Ziel dieser Dissertation war es, den Einfluss der Interaktion von Herbivoren mit ihrer Umwelt auf die Funktionsweise des Immunsystems und Wirtspflanzen Adaption in der Ordnung der Lepidoptera zu untersuchen.

8.1 Umweltfaktoren haben einen Einfluss auf die Funktionsweise des Immunsystems im Generalisten Heliothis virescens und im Spezialisten Heliothis subflexa

Die Funktionsweise des Immunsystems innerhalb der Klasse der herbivoren Insekten variiert aufgrund einer heterogenen Umwelt, die je nach Nische andere Anforderungen stellt.

Spezialisierte Herbivoren leben in homogenen Lebensräumen, aufgrund ihres eingeschränkten Spektrums an Wirtspflanzen. Im Gegensatz dazu, leben generalistische Herbivoren in heterogenen Lebensräumen, da sie sich von einer großen Vielfalt verschiedener Wirtspflanzenarten ernähren können. Um unsere Hypothese zu untersuchen, dass Generalisten effizientere Mechanismen der Immunabwehr besitzen als Spezialisten, wurden verschiedene Komponenten des Immunsystems sowie die Empfindlichkeit gegenüber Pathogenen zwischen einem Generalisten, *H. virescens*, und einem Spezialisten, *H. subflexa* (Lepidoptera, Noctuidae), verglichen. In der vorliegenden Arbeit konnte gezeigt werden, dass pathogene Bakterien, die mit der Diät aufgenommen oder injiziert wurden, einen unterschiedlichen Effekt auf den Generalisten und Spezialisten haben. Hierbei zeigte *H. virescens* eine höhere Toleranz gegenüber Pathogenen, das heißt eine geringe Sterblichkeit und erhöhte Wachstumsrate, im Vergleich zu *H. subflexa*. Weiterhin konnte gezeigt werden, dass der Unterschied in der Anfälligkeit gegenüber Pathogenen auf Unterschieden in den Mechanismen der Immunabwehr der beiden Arten beruht. *In vitro* und *in vivo* Versuche zeigten eine geringere Anzahl an lebenden Bakterien, eine reduzierte Hämozyten-Teilungsrate, eine erhöhte Phagozytose und antimikrobielle Aktivität in der Hämolymphe von infizierten *H. virescens* verglichen mit *H. subflexa*. Diese Ergebnisse weisen darauf hin, dass der Generalist, im Vergleich zum Spezialist, eine effizientere Immunabwehr-Strategie besitzt. Diese Arbeit bildet somit die Grundlage für weiterführende Studien zum Einfluss von Umweltfaktoren auf die Evolution des Immunsystems.

8.2 Die Aktivierung des Immunsystems induziert Veränderungen in der Reproduktion von weiblichen H. virescens

Die Mechanismen der Immunabwehr können nicht nur zwischen Generalisten und Spezialisten variieren, sondern, aufgrund ihrer verschieden *life-history* Merkmale, auch zwischen Männchen und Weibchen derselben Spezies. In dieser Arbeit wurde die Hypothese untersucht, ob weibliche Falter ihre Ressourcen eher in die Immunabwehr investieren als in die Reproduktion Quantitative Genexpressionsanalysen an weiblichen und männlichen *H. virescens*, welche mit pathogenen Bakterien injiziert waren, resultierten in einem geschlechtsspezifischen Expressionsmuster von Genen des Immunsystems. Hierbei konnte gezeigt werden, dass infizierte Männchen weniger immunkompetent waren als Weibchen. Die höhere Immunkompetenz der Weibchen wirkte sich negativ auf deren sexuelle Attraktivität (Paarungshäufigkeit) aus und veränderte zusätzlich ihr Pheromon-Profil. Das Ergebnis lässt

schlussfolgern, dass infizierte Weibchen eher in die Immunabwehr investieren als in die Reproduktion. Zukünftige Studien über *trade-offs* zwischen Immunabwehr und Reproduktion sollten daher geschlechtsspezifische Immunantworten berücksichtigen.

8.3 Tritrophische Interaktionen spielen eine wichtige Rolle in der Adaption des Spezialisten H. subflexa an Physalis-Pflanzen

Die Herbivor-Wirtspflanzen-Interaktion ist stark beeinflusst durch pflanzliche Abwehrmechanismen und die darauf abgestimmten Adaptionen von herbivoren Insekten. Unser Versuchsmodell um diese Interaktion zu studieren, umfasst den Spezialisten *H. subflexa* und seine Wirtspflanze *Physalis* (Solanaceae). Um auf seiner Wirtspflanze zu überleben, konnten frühere Studien zeigen, dass *H. subflexa* sich an die Morphologie, den Nährstoffhaushalt sowie biochemische Eigenschaften von *Physalis*-Pflanzen angepasst hat. In dieser Arbeit wird der Einfluss von Withanoliden, den sekundären Metaboliten der *Physalis*-Pflanze, auf *H. subflexa* untersucht. Withanolide sind dafür bekannt fraßhemmend und immunsuppressiv auf viele Lepidoptera Larven zu wirken, wie zum Beispiel auf den Generalisten *Helicoverpa zea*. Interessanterweise wirkten Withanolide, die mit der Diät aufgenommen wurden, positiv auf das Wachstum von *H. subflexa* Larven. Weiterhin konnte eine erhöhte Immunkompetenz, das heißt eine erhöhte Phenoloxidaseaktivität und hochregulierte Expression von Immungenen, gemessen werden, wenn Larven auf einer Withanolid-Diät gefressen haben. Die immunsuppressiven Eigenschaften von Withanoliden konnten jedoch im Generalisten *H. virescens* nachgewiesen werden, da mehrere Immungene stark herunter reguliert waren, wenn Withanolide vom Generalisten mit der der Diät aufgenommen wurden. Diese Ergebnisse weisen darauf hin, dass der Spezialist *H. subflexa* Mechanismen entwickelt hat, um die immunsuppressiven Eigenschaften von Withanoliden zu umgehen und stattdessen sein Immunsystem stimuliert wird. Auch wenn die antibakterielle Aktivität von Withanoliden gegen humanpathogene Bakterien seit langem bekannt ist, wurde deren Aktivität gegen entomopathogene Bakterien bisher nicht untersucht. Die Tatsache, dass Withanolide eine antibakterielle Aktivität gegen Sporen und vegetative Zellen von *Bacillus thuringiensis* besitzen, ist daher eine neuartige Entdeckung dieser Arbeit. Larven von *H. subflexa* welche mit *B. thuringiensis* infiziert wurden, zeigen ein höheres Überleben, wenn diese auf einer Withanolid-Diät gefressen haben. Diese Ergebnisse implizieren, dass die Adaption des Immunsystems von *H. subflexa* an die immunverändernden Eigenschaften von Withanoliden und die Nutzung der antibakteriellen Aktivität von Withanoliden einen

wichtigen Schritt in der Wirtspflanzen-Spezialisierung von *H. subflexa* darstellen. Die Wirkungsweise durch welche Withanolide das Überleben von infizierten *H. subflexa* Larven verbessert ist unbekannt und daher ein herausforderndes Thema für zukünftige Studien.

Zusammenfassend konnte meine Doktorarbeit zeigen, dass (1) generalistische und spezialisierte Herbivoren unterschiedliche Immunabwehr-Strategien, aufgrund von unterschiedlichen Umwelteinflüssen, besitzen; (2) dass herbivore Insekten geschlechtsspezifische *trade-offs* in *life-history* Merkmalen, wie Immunabwehr und Reproduktion, besitzen; und (3), dass sekundäre Pflanzen-Metabolite einen signifikanten Einfluss auf die Physiologie und das Immunsystem von spezialisierten Herbivoren haben und weiterhin die Interaktion zwischen Herbivoren und seinen Pathogenen beeinflussen können. Die vorliegende Arbeit stellt damit eine der wenigen Studien dar, welche den Einfluss von Wirtspflanzen und Pathogenen auf die Ökologie und das Immunsystem von herbivoren Insekten verknüpft und dabei die fundamentale Rolle von Umweltfaktoren auf die Evolution von Immunsystemvariationen und Wirtspflanzen Adaption in zwei Lepidoptera Arten identifizieren konnte. Die Ergebnisse meiner Dissertation zeigen, dass es noch viel zu erforschen gibt, hinsichtlich ökologischer Immunologie und Wirtspflanzen Adaption in herbivoren Insekten.

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11 Curriculum vitae

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Since 03/2010	PhD student at the Max Planck Institute for Chemical Ecology, Jena Title: *"Ecological Immunology and Host Plant Adaptation in Lepidopteran Herbivores"*
02/2009-02/2010	Diploma thesis at the Max Planck Institute for Chemical Ecology, Jena Title: *"Protein analysis and tissue culture of the sex pheromone gland of Lepidoptera"*
10/2006-02/2010	Diploma Study: Biology, Friedrich-Schiller-University Jena (Main subjects: microbiology, immunology and neurobiology)
10/2004-10/2006	Intermediate Diploma in Biology, University of Kassel
06/2004	Secondary school: Abitur Tilesius-Gymnasium, Mühlhausen, Thüringen

Practical and Laboratory Experience

Since 03/2010	Member of the International Max Planck Research School (IMPRS) on *"The Exploration of Ecological Interactions with Molecular and Chemical Techniques"*
10/2008-12/2008	Research assistant (HIWI) at the Max Planck Institute for Chemical Ecology (Department Entomology), Jena
07/2008-09/2008	Research assistant (HIWI) at the Leibniz Institute for Natural Product Research and Infection Biology (Department Infection Biology), Jena
06/2008-07/2008	Practical course at the Leibniz Institute for Natural Product Research and Infection Biology (Department Infection Biology), Jena
07/2007-09/2007	Practical course at the Friedrich Schiller University (Department of Applied and Environmental Microbiology), Jena
03/2005-04/2005	Practical course at the Max Planck Institute for Biophysical Chemistry (Department Molecular Cell Biology), Göttingen

Certificates

06/2013	Gene Safety Project Leader

Advanced training

06/2014	Leadership Skills for research and economy (Graduate Academy, Jena)
01/2014	Compact course: Business administration (Graduate Academy, Jena)
12/2013	Project Management for Postdocs (Graduate Academy, Jena)
02/2013	Compact course: Academic education for scientists (LehreLernen, Jena)
02/2013	PhD course: New Frontiers in Microbial Ecology (The Netherlands)
12/2012	Introduction to the GxPs – with special focus on GMP and GLP (Graduate Academy, Jena)

Teaching and Public relation activities

2013	Long Night of Science; Jena
2012	Supervision Bachelor thesis *"Hemocyte-mediated immune response in Heliothis virescens and Heliothis subflexa"*
2011	Supervision practical course on *"AFLP-based analysis to study differences in the immune system response of Heliothis virescens and Heliothis subflexa"*
2011	Long Night of Science, Jena

Publication and Presentations

Publications

- **Barthel A,** Kopka I, Zipfel P, Vogel H, Heckel DG and Groot AT (**2014**) Immune defense strategies of generalist and specialist insect herbivores. *Proceedings of the Royal Society B: Biological Sciences 281(1788)*

- **Barthel A**, Vogel H, Pauchet Y, Pauls G, Groot AT, Boland W, Heckel DG and Heidel-Fischer HM (**2014**): What a big impact a small berry can make: Herbivorous specialization on *Physalis* plants. (in prep. for *Proceedings of the National Academy of Sciences of the United States of America*)

- **Barthel A**, Staudacher H, Schmalz A, Groot AT (**2014**): Consequences of an induced immune response on sexual traits in *Heliothis virescens*. (in prep. for *Evolution*)

- Groot AT, Staudacher H, **Barthel A**, Inglis O, Schöfl G, Santangelo RG, Gebauer-Jung S, Vogel H, Emerson J, Schal C, Heckel DG, Gould F (**2013**): One quantitative trait locus for intra- and interspecific variation in a sex pheromone. Molecular Ecology 22(4), 1065-1080

- Terenius O, Papanicolaou A, Garbutt JS, Eleftherianos I, Huvenne H, Kanginakudru S, Albrechtsen M, An C, Aymeric JL, **Barthel A**, Bebas P, Bitram K, Bravo A, Chevalier F, Collinge DP, Crava CM, de Maagd RA, Duvic B, Erlandson M, Faye I, Felfoldi G, Fujiwara H, Futahashi R, Gandhe AS, Gatehouse HS, Gatehouse LN, Giebultowicz JM, Gomez I, Grimmelikhuijzen CJP, Groot AT, Hauser F, Heckel DG, Hegedus DD, Hrycaj S, Huang L, Hull J, Iatrou K, Iga M, Kanost MR, Kotwica J, Li C, Li J, Liu J, Lundmark M, Matsumoto S, Meyering-Vos M, Millichap PJ, Monteiro A, Mrinal N, Niimi T, Nowara D, Ohnishi A, Oostra V, Ozaki K, Papakonstantinou M, Popadic A, Rajam MV, Saenko S, Simpson RM, Sobero M, Strand MR, Tomita S, Toprak U, Wang P, Wee CW, Whyard S, Zhang W, Nagaraju J, ffrench-Constant RH, Herrero S, Gordon K, Swevers L, Smagghe G (**2011**): RNA interference in Lepidoptera: An overview of successful and unsuccessful studies and implications for experimental design. *Journal of Insect Physiology* 57 (2), 231-245

Presentations

- **Barthel A** (2013): Generalist vs specialist: consequence of a specialized lifestyle. *ESF-EMBO Symposium: Integrated Insect Immunology: From Basic Biology to Environmental Applications,* Pultusk, Poland

- **Barthel A** (2013): Hemocyte-mediated immune response: Consequences of a specialized lifestyle. *12th IMPRS Symposium* / MPI for Chemical Ecology, Jena, Germany

Poster presentations

- **Barthel A** and Heidel-Fischer HM (2014) Just a matter of taste? Impact of phytochemicals on two closely related moth species. *SAB Meeting 2014* / MPI for Chemical Ecology, Jena, Germany

- **Barthel A** (2014): What a big impact a small berry can make. *Keystone Symposium: Mechanisms and Consequences of Invertebrate-Microbe Interactions,* Tahoe City, USA

- **Barthel A** (2013) Hemocyte-mediated immune response in heliothine moths - is a generalist better defended? *SAB Meeting 2012* / MPI for Chemical Ecology, Jena, Germany

- **Barthel A** (2012) A multilayered response against bacteria in Heliothine moths. *11th IMPRS Symposium* / MPI for Chemical Ecology, Jena, Germany

- **Barthel A** (2011) Immune defense response in a generalist and specialist heliothine moth. *Sixth International Symposium on Molecular Insect Science*, Amsterdam, The Netherlands

- **Barthel A** (2011) Immune defense response in a generalist and specialist heliothine moth. *ICE Symposium* / MPI for Chemical Ecology, Jena, Germany

- **Barthel A** (2011) Immune defense response in a generalist and specialist heliothine moth. *8th 'Horizons in Molecular Biology' Int. PhD Student Symposium*, Göttingen, Germany

12 Selbstständigkeitserklärung

Die zurzeit geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena ist mir bekannt. Die vorliegende Dissertation mit dem Titel „Ecological Immunology and Host Plant Adaptation in Lepidopteran Herbivores“ wurde von mir eigenständig angefertigt und keine anderen als die von mir angegebenen Hilfsmittel und Quellen benutzt. Personen, die an der Gewinnung der Daten, Auswertung des Datenmaterials sowie bei der Erstellung der Manuskripte hilfreich waren, sind benannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorliegende Dissertation wurde bisher weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

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Andrea Barthel

Jena, den 25. Juni, 2014