Immunity and physiology of Lepidoptera is influenced by midgut mediated environmental signals

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Gutachter: Prof. David G. Heckel, Max-Planck-Institute für chemische Ökologie, Jena

Prof. Jonathan Gershenzon, Max-Planck-Institute für chemische Ökologie, Jena

Prof. Andreas Vilcinskas, Justus-Liebig-Universität, Gießen

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1 General introduction

Carl Zimmer describes the existence of parasites in a very colorful way - “Forget lions, tigers, and sharks. The billions of tiny parasites that make a living castrating and brainwashing their hosts may be the new kings of the food web” (Zimmer, 2002). All free-living organisms are attacked by parasites, most free-living species have at least one unique parasite and many parasites have parasites themselves (superparasitism). We can safely say that there are more parasites than free-living species. It shows that the parasitic lifestyle has proved to be extremely beneficial and wide spread. Parasites can be both prokaryotic and eukaryotic organisms, from unicellular to multicellular body plans. Parasitism is defined as “an intimate association between organisms of two or more kinds; especially: one in which a parasite obtains benefits from a host which it usually injures” (Merriam-Webster online dictionary). It has been shown in many cases that depending on parasite virulence, parasites and pathogens can reduce host fitness tremendously (Schmid-Hempel, 2005a). This leads to the conclusion that parasites can be a major source of selection pressure on their hosts by decreasing their life expectancy or their reproductive success. This creates a situation where hosts require a range of physiological counter-adaptations to increase the resistance or tolerance in the case of infections. Studies on the factors influencing development of defenses against parasitism are essential to a better understanding of mechanisms leading to higher resistance against parasites and pathogens.

1.1 Eco-immunology – costs of immune defense

In 1973 the Red Queen hypothesis was postulated by American evolutionary biologist Leigh M. Van Valen. This hypothesis states that interactions between species (such as host and parasites) lead to constant natural selection for adaptation and counter-adaptation. This hypothesis has become a paradigm in studies of evolution of defense mechanisms against pathogens and parasites (Strand & Pech, 1995; Schmid-Hempel, 2005a).

Ecological immunology examines how and why micro-evolutionary processes generate, and maintain, variation in immune effector systems and the coordinated host response to pathogens and parasites (Schmid-Hempel, 2005a and 2005b). The reasoning behind these processes is based on two main theoretical approaches. The first one relies on the theory of the evolution of life-history traits and assumes that the evolution and the use of immune defenses are costly (Sheldon & Verhulst, 1996). The second approach is based on

arms-race models of coevolution (Van Halen, 1973), which assume that coevolution between hosts and parasites can lead to sustained oscillations in host genotype frequencies through negative density-dependent selection, in favor of the rare host genotypes (Peters & Lively, 1999, Ebert, 2005).

Two types of costs associated with immune defenses can be distinguished (Siva-Jothy et al., 2005):

1. *Evolutionary cost of immune defense* – this is assumed to rely on the existence of "antagonistic pleiotropy", where a gene that has a positive effect on one fitness component, has a negative effect on another fitness component. This type of genetic relationship between traits is considered to be fixed during the lifetime of the organism.
2. *Physiological cost of immune defense* – these costs result from resource-based trade-offs between components of the immune system and other essential functions of the organism. A prerequisite for physiological costs associated with immunity is that the same pool of resource and allocation of resources to the immune function will have constrains on other bodily functions that are sustained simultaneously and *vice versa*. Schmid-Hempel (2003) divides these costs into two parts – the cost of immune system maintenance and actual mounting of immune responses upon infection.

We can further divide the physiological costs, according to the magnitude and pattern of investment into immune defense that an individual makes during its lifetime. In a single individual we can divide these immunity associated costs into four parts:

1. *Development of immune system components* - e. g. certain populations of hemocytes or antimicrobial proteins constantly present in the hemolymph and enzymes participating in rapid immune responses. Here we can also list the quantitative and qualitative presence of pattern recognition molecules in various tissues and hemocoel.
2. *Maintenance of immune status* - the population of hemocytes, but also other components of the immune system, have to be renewed from time to time and this requires additional investments.
3. *Mounting costs of immunity* - after recognition of potential intruders, the organism has to initiate an immune response, and this requires additional synthesis of various enzymes and proteins, but also hemocytes.
4. *Dealing with autoimmune effects* - since insects possess only innate immunity, the vertebrate-type of autoimmunity resulting from the generation of antibodies recognizing self- instead of non-self molecules does not apply. Rather it is assumed that autoimmune

costs result from the direct negative effects of innate immune responses, such as the release of free radicals and non-specific activity of immune response related enzymes. This requires a certain investment into antioxidative enzymes and tissue repair mechanisms.

1.2 Insect immunity

Insects have proved to be one of the most successful animal groups on earth, as they are present in all the possible habitats in the world. Many of these are heavily loaded with parasites and pathogens, and present many opportunities for infection. This has imposed enormous selection pressures on defense systems, among them immune responses. Comparatively recent studies of the molecular mechanisms of immune response have emphasized that insects have evolved a very complex immune system, with many different components. The complexity of immune defenses is related to life style; for example the honey bee (*Apis mellifera*) immune system seems to be lacking many components present in other insects, and relatively fewer immunity related genes have been identified after genome information became available (Honeybee Genome Sequencing Consortium, 2006; Evans et al., 2006). At the same time in many Lepidopteran larvae a huge variety of different antibacterial proteins have been identified, and the same holds true for some Diptera and Coleoptera (Altincicek, Knorr & Vilcinskas, 2008; Altincicek & Vilcinskas, 2007; Altincicek & Vilcinskas, 2008).

1.2.1 Recognition

The insects' immune system is very similar to vertebrate innate immunity, but lacks the vertebrate mechanisms of acquired immunity, in particular the formation of antibodies resulting in a systemic "memory" of previous infections. Insects differentiate non-self from self by relying largely on a range of pattern recognition proteins that can recognize pathogen associated molecular patterns (PAMPs) (Hultmark & Borge-Renberg, 2006; Wang et al., 2006; Kanost et al., 2004; Jiang et al., 2004) (Figure 1). The insect immune system recognizes a range of non-self motifs, characteristic of microbe cell surface molecules, like peptidoglycans, β-1,3 glucans, lipopolysaccharides and other sugar moieties (Theopold et al., 1999). Pattern recognition proteins can be located intracellularly, be exposed on the cell

surface or secreted into the hemocoel (Steiner, 2004), and several of the pattern recognition proteins are known to be inducible upon exposure to bacteria (Dziarski & Gupta, 2006; Steiner, 2004; Dziarski, 2004).

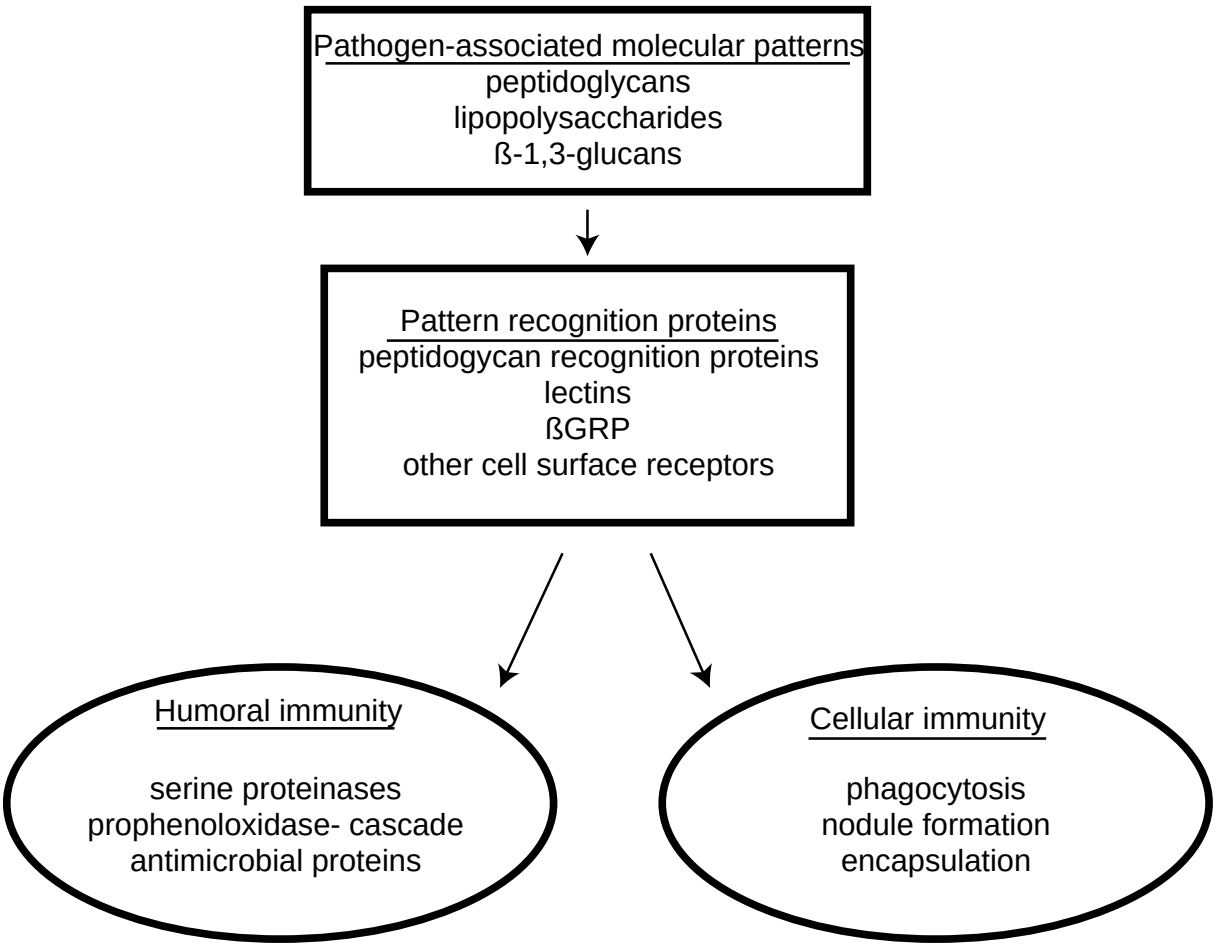


Figure 1. A schematic overview of the immune system in the Lepidopteran larval hemolymph (adapted from Jiang, 2008).

1.2.2 Cellular immunity

Cellular immune responses in insects mediated by hemocytes have been known for about 100 years (Gillespie et al., 1997). Hemocytes are “blood cells” circulating freely in the hemolymph. For a long time the most common way to classify hemocytes was on the basis of their morphology, which, however led to oversimplification. A more precise method to characterize hemocytes is based on molecular markers on their cell surface, like different

lectins. A number of monoclonal antibodies have been developed to identify insect hemocytes and analyze their function (Willott et al., 1994).

Phagocytosis is the primary response of hemocytes after encountering small foreign particles, such as bacteria. Predominant phagocytotic cells have been reported to be granolocytes and plasmatocytes (Gillespie et al., 1997). The mode of action of these cells can be described as receptor mediated endocytosis. Phagocytosis involves several steps – attachment, recognition, signal transduction, activation of pseudopodia formation, ingestion and assembly of phagosomes (Bayne, 1990). Binding to foreign particles does not always lead to endocytosis. Sometimes hemocytes form multicellular aggregates, called nodules, to entrap a large number of bacteria in extracellular material. These kinds of nodule formations may adhere to tissues and larger nodules may also be encapsulated (Gillespie et al, 1997 and references therein). Encapsulation takes place in cases where the invading organism is too big to be phagocytosed. The encapsulation process involves covering the parasite with multiple layers of hemocytes and/or a melanin coat. Two types of encapsulation processes have been described – humoral and cellular. Cellular encapsulation is characteristic for Lepidoptera and involves the generation of a hemocytic capsule around the parasite, whereas humoral encapsulation (also known as melanotic) is typical for Diptera and hemocytes do not take part in it (Gillespie et al., 1997). Melanotic encapsulation always involves phenoloxidase activity and the formation of melanin, whereas cellular encapsulation can take place also without any melanization process (Gillespie, et al., 1997). Cellular immune responses relying on encapsulation are targeted usually against protozoans, metazoan parasites and eggs or larvae of parasitoids (Lavine & Beckage, 1995; Strand & Pech, 1995).

The precise biochemistry underlying the encapsulation process is not well understood. There is some evidence that phenoloxidase can enhance encapsulation processes. Also some antimicrobial peptides have been thought to stimulate formation of hemocyte capsules around parasites (Ling & Yu, 2005; Jiang, 2008).

1.2.3 Humoral immunity

Humoral immune responses involve various enzymes and antibacterial proteins, produced in the fat body or hemocytes, or in epithelial tissues. Humoral responses are thought to be targeted primarily against unicellular parasites, like bacteria and some fungi. Another class of pathogens are viruses, but antiviral defenses are not very well understood, although

the involvement of apoptotic processes, RNA interference and some antibacterial proteins has been proposed (Wilkins et al., 2005; Terenius, 2008; Wang & Zhang, 2008).

The majority of the studies on immune inducible proteins in the hemolymph are based on examining the hemolymph protein profile after injection of bacteria into the hemocoel (Gillespie et al., 1997). Although these means of induction of immunity are artificial and not always suitable for making ecologically valid interpretations, they have proved very useful in studies of the mechanistics of immune responses. The fat body is of central importance in the synthesis of antimicrobial proteins (AMPs); smaller contributions are made by hemocytes and other tissues (e. g. pericardial cells, Malphigian tubules, midgut, cuticle) (Tzou et al., 2000; Boulanger et al., 2002; Siden-Kiamos & Louis, 2004). Insects possess a large variety of AMPs with different substrate specificity and inducibility, many of which are found in multiple insect orders (Table 1).

Insect lysozymes are relatively small enzymes around 14kDa with sequence similarity to chicken egg-white lysozyme, and hence are called c-type lysozymes (Yu et al., 2002). Lysozymes are present in the hemolymph constitutively, but are often induced dramatically upon immune insult (Yu et al., 2002; Jarosz, 1993; Morishima et al., 1995). Lysozymes hydrolyze β-1,4 linkages between *N*-acetylglucosamine and *N*-acetylmuramic acid in the peptidoglycans of bacterial cell walls (Gillespie et al., 1997; Yu et al., 2002). There is still some disagreement in the literature about the specificity and targets of lysozymes from different insect species. However, activities against both Gram-positive and Gram-negative bacteria have been reported (Yu et al., 2002: Boman & Hultmark, 1987).

Phenoloxidase is an enzyme responsible for melanization reactions, which is a common response to parasite entry in invertebrates. Phenoloxidase is an oxidoreductase and is part of the complex system of proteinases, pattern recognition proteins and proteinase inhibitors forming the so-called prophenoloxidase-activating system (Söderhäll & Cerenius, 1998; Cerenius and Söderhäll, 2004; Cerenius et al., 2008). Recent studies indicate that this system is also tightly involved in the appearance of factors in the hemolymph stimulating cellular response and phagocytosis (Cerenius et al., 2008). Activation of the prophenoloxidase cascade can be triggered by minute concentrations of pathogen associated molecules, like lipopolysaccharides, peptidoglycans and β-1,3-glucans (Söderhäll & Cerenius, 1998). After detection of an infection in the hemolymph, inactive prophenoloxidase is converted into active phenoloxidase by serine proteases. Phenoloxidase catalyses the oxidation of phenols to quinones, which will then nonenzymatically polymerize to melanin. Melanin is then deposited on the surface of the pathogens and bacteria, thus isolating them from insect tissues and

hemocoel. This will create a highly toxic microenvironment for intruders, where they are exposed to high amounts of cytotoxic compounds and their own waste products (Nappi & Christensen 2005).

Table 1. Major inducible antibacterial peptides in insects

AMP family	Activity spectra & Mode of action	Size	Present in	References
Cecropins A, B, C, D - type	Gram-positive and Gram-negative bacteria; disintegration of bacterial cell wall	4 kDa	Lepidoptera Diptera	Gillespie et al., 1997; Lee et al., 2007; Abdel-latief & Hilker, 2008
Attacins	Gram-negative bacteria; prevent cell division by inhibiting synthesis of outer membrane proteins	20 – 28 kDa	Lepidoptera Diptera	Kwon et al., 2008; Gillespie et al., 1997; Carlsson et al., 1991
Defensins	Gram-positive bacteria; forming channels in the cell wall	29 – 34 kDa	All studied insects species	Gillespie et al, 1997; Bulet & Stöcklin, 2005; Cociancich et al., 1993
Proline-rich AMPs – apidaecins and abaecins, drosocins and metchnikowins, metalnikowins and pyrrhocoricins, lebocin	Gram-positive and Gram-negative bacteria and Fungi; increase the permeability of the bacterial cell membrane	18 – 34 kDa	Hymoneptera Diptera Hemiptera Lepidoptera	Bulet & Stöcklin, 2005; Gillespie et al., 1997; Hara & Yamakawa, 1995

Despite the conceptual division of immune responses into cellular and humoral, events and elements described under both systems act synergistically in the course of the immune insult. Immune signaling cascades have been best described in *Drosophila*, where Toll (triggered by Gram-positive bacteria and fungi), imd (triggered by Gram-negative bacteria) and JAK/STAT (some indication to involvement in cellular immunity) immune pathways are characterized (Hultmark, 2003; Brennan & Anderson, 2004). How similar the immune signaling cascades are among other insect species remains to be discovered after more genomic data becomes available.

1.3 Midgut as an interaction stage with the environment

An understanding of insect ecology and evolution goes hand in hand with knowledge of insect nutritional physiology. The effects of food quality and quantity on the physiology and behavior of various insects has been in the focus of insect ecophysiologists for a long time. Lepidopteran insects are mostly herbivorous during the larval stages. Plants pose a complex and often heavily herbivore-defended dietary source (Cohen et al., 1992; Berenbaum&

Zangerl, 1992; Kliebenstein et al., 2005; Mumm & Hilker, 2006; Steppuhn & Baldwin, 2007), that requires multiple adaptations on the insect side (Ratzka et al., 2002; Wittstock et al., 2004). Adaptations to plant defenses include different detoxification, resistance, tolerance or avoidance mechanisms. These accommodations to plant primary and secondary defenses are different among specialist and generalist insects (Daborn et al., 2002; Li et al., 2004; Li et al., 2002). Most of the studies on insect-plant interactions focus on the effects of plant secondary metabolites on insects. Another potentially important part of the dietary challenge encountered due to consumption of large amounts of different plant tissues has been neglected, namely the microbiota present on all the plant surfaces (Meyling & Eilenberg, 2006). A major route of acquisition of bacterial and viral infection is by oral ingestion. Thus the gut epithelium is an essential interface between insects and the various pathogens that infect them (Boulanger, et al., 2002).

In vertebrates, epithelial intestinal innate immunity plays a major role in the control of infectious diseases (Ayabe et al., 2000). In invertebrates the role of the midgut in shaping the innate immune responses and immunocompetence of an organism is still largely unknown. In host-parasite interactions, concerning insect-vectored diseases of vertebrates such as malaria, a little more is known about the relations of induction of gut epithelial immune responses by parasites (Boulanger, et al., 2002; Siden-Kiamos & Louis, 2004; Gupta et al., 2005; Xi et al., 2008). It has been shown that an important part of the gut-based immune defenses is the release of large amounts of reactive oxygen species (Gupta et al., 2005; Ha et al., 2005). This is similar to innate immune responses of gut epithelial tissues to infections in vertebrates (Janeway, 2005). In the studies made with oral infection of *Drosophila* with entomopathogenic bacteria, it has been shown that expression of several antibacterial proteins and parts of the immune signaling cascades can be induced in midgut tissue (Tzou, et al., 2000; Ha et al., 2005; Liehl et al., 2006). In the case of vertebrates it is known that the presence of commensal microflora is essential in the formation of effective immune responses against pathogens (Iweala & Nagler, 2006). Little is known about how the immune reactivity of epithelial tissues is shaped in invertebrates, and what roles are played by orally-acquired non-pathogenic bacteria that may present some of the same molecular markers as pathogenic microbes.

1.4 Trans-generational immune priming

The different aspects of innate responses can be stimulated or primed to fight certain types of infections, both in vertebrates (He et al., 2007; Weir et al., 2004) and in invertebrates (Roth et al., 2009, Moret & Siva-Jothy, 2003; Kurtz, 2005). Of special interest when considering priming of innate immune responses are trans-generational effects of enhanced resistance and their possible fitness-related consequences. Trans-generational immune priming accompanied with maternal effects is a phenomenon which so far has been mostly associated with vertebrate immunology. It is known that mammals are capable of priming the immune system of their offspring via lactation, and even earlier in the fetal stage via transmission of gut microflora (Grindstaff et al., 2003; Blümer et al., 2007; Janeway, 2005). It is beneficial to prepare offspring against possible infections and maternally transferred immunity could have an essential role in infant survival (Holt & Jones, 2000).

In insects evidence for trans-generational immune priming has been reported in only a few cases, namely for the mealworm beetle (*Tenebrio molitor*) (Moret, 2006), bumblebee (*Bombus terrestris*) (Moret & Schmid-Hempel, 2001; Sadd & Schmid-Hempel, 2006; Sadd et al., 2005), mosquito (*Anopheles stephensi*) (Grech et al., 2007), the Mediterranean flour moth (*Ephestia kuehniella*) (Rahman, et al, 2004), and in a crustacean (*Daphnia magna*) (Little et al., 2007). In contrast, a study on the peach-potato aphid *Myzus persicae* did not provide evidence for trans-generational priming of resistance (Vorburger et al., 2008). The heritability of immune function, specifically the encapsulation response, has also been studied, but to a somewhat lesser extent (Cotter et al., 2008; Fellowes et. al., 1998).

1.5 Aim of this thesis

The major aim of this thesis was to analyze if and how environmental factors influence insect immunity. More specifically I was interested in the impact of essentially non-pathogenic bacteria, which are consumed along with food, on the immunocompetence of Lepidopteran larvae. Insects have been extensively utilized in describing different immune recognition and signaling pathways, but the methods used in these studies usually involve injections of large amounts of bacteria or PAMPs directly into the hemocoel, which may only rarely occur under natural conditions. In this thesis I attempted to induce the immune system in ways that could

be more related to how insects encounter microorganisms in the natural environment and depict more realistic challenges for the immune system in nature.

The first part of this thesis focuses on the role of the Lepidopteran midgut on the immune status and related fitness components of insects. The larval midgut is a large central organ and one of the interfaces between environment and organism. To understand the role of the midgut in shaping the immune status, I fed *Trichoplusia ni* larvae with a constant supply of non-pathogenic bacteria and measured the effects on hemolymph and midgut based immunity. As investments into the immune system have been known to be in trade-off with other life-history traits, essential fitness traits were also measured.

Another focus of this thesis is on the overall differential gene expression in relation to variable dietary conditions in *T. ni.* Transcripts of two and seven day old larvae grown on plant, bacterial and bacteria-free diet were assessed. In addition, the question of possible trans-generational effects of parental diet was raised, by studying the gene expression differences among the eggs laid by parents grown on bacterial and bacteria-free diet.

The third part of this thesis carries this one step further, by addressing the possibility of influencing the immune status of the offspring depending on immune defense relevant signals coming from the environment to the parental generation. Reciprocal and non-reciprocal crosses were made to investigate the importance of parental diet on the immune status of the next generation. Immune factors were examined on different levels and multiple life-history traits were assessed in parallel.

The last part of the thesis focussed on a novel 40 kDa protein discovered during a survey of the midgut lumen proteome of *Helicoverpa armigera*. This protein was suspected to play a role in the immune response due to its high similarity to previously characterized lepidopteran pattern recognition proteins, βGRPs (β-1,3-glucan recognition protein). Biochemical characterization coupled with tissue-specific expression suggested an alternative function combining aspects of both digestion and immune defense.

2 Chapter 1: Immune system responses and fitness costs associated with consumption of bacteria in larvae of *Trichoplusia ni*

Abstract

Background: Insects helped pioneer and persist as model organisms for studying specific aspects of immunity. Although they lack an adaptive immune system, insects possess an innate immune system that recognizes and destroys intruding microorganisms. Its operation under natural conditions has not been well studied, as most studies have introduced microbes to laboratory-reared insects via artificial mechanical wounding. One of the most common routes of natural exposure and infection, however, is via food and thus, the role of dietary microbial communities in herbivorous insect immune system evolution invites study. Here, we examine the immune system response and consequences of exposing a lepidopteran agricultural pest to non-infectious microorganisms via simple oral consumption.
Results: Immune system response was compared between *Trichoplusia ni* larvae reared on diets with or without non-pathogenic bacteria (*Escherichia coli* and *Micrococcus luteus*). Two major immune response-related enzymatic activities responded to diets differently – phenoloxidase activity was inhibited in the bacteria-fed larvae, whereas general antibacterial activity was enhanced. Eight proteins were highly expressed in the hemolymph of the bacteria fed larvae, among them immune response related proteins arylphorin, apolipophorin III and gloverin. Expression response among twenty five putative immune response-related genes were assayed via RT-qPCR. Seven showed more than five fold up regulation in the presence of bacterial diet, with twenty two in total being differentially expressed, among them apolipophorin III, cecropin, gallerimycin, gloverin, lysozyme, and phenoloxidase inhibiting enzyme. Finally, potential life-history trade-offs were studied, with pupation time and pupal mass being negatively affected in bacteria fed larvae.
Conclusions: The presence of bacteria in food, even if non-pathogenic, can trigger an immune response cascade with life history tradeoffs. *Trichoplusia ni* larvae are able to detect and respond to environmental microbes encountered in the diet, possibly even using midgut epithelial tissue as a sensing organ. Potential benefits of this immune system priming may outweigh the observed tradeoffs, as priming based on environmentally sensed bacterial may decrease risk of serious infection. These results show that food plant microbial communities represent a dynamic and unstudied part of the coevolutionary interactions between plants and their insect herbivores.

2.1 Introduction

Herbivorous insects are one of the most diverse and successful groups of animals on earth, having been able to invade and exploit nearly every available ecological niche (Chown & Nicolson, 2004). Having relatively short generation times and large numbers of progeny per adult allows insects to adapt quickly to various biotic and abiotic stressors in the environment, including pathogens. Invertebrate immunity studies have revealed valuable information on the induction and propagation of the immune response, focusing on the signaling cascades activated after pathogen recognition (Hultmark, 2003; Khush & Lemaitre, 2000; Hoffmann et

al., 1999; Hoffmann & Reichhart, 2002; Kim et al., 2000). Immune responses are costly and result in trade-offs with other life-history traits, such as reproduction and development (Schmid-Hempel, 2005a). In most studies, lab reared insects have been infected with bacterial strains via artificial mechanical wounding (i.e. injection), neglecting the main routes of natural exposure to bacteria, most notably via plant consumption (Vodovar et al., 2005). Thus, the role of plant microbial communities in herbivorous insect host use and performance is largely unknown.

Herbivorous lepidopteran larvae consume large quantities of plant material over the course of their development from neonate to late instar larvae, increasing as much as up to 20% of their total body weight per day (Gotthard, 2004; Esperk & Tammaru, 2004). Studies of host shifts onto novel host plants have traditionally focused on considerations of the new abiotic factors (eg. thermal, temporal) (Feder et al., 1988) and biotic conditions (e.g. competition, secondary plant metabolites) (Ehrlich & Raven, 1964; Chown & Nicolson, 2004) to which the herbivore must adapt. However, new host plants could also harbor different, possibly pathogenic microorganisms (Meyling & Eilenberg, 2006; Vodovar et al., 2005). Both the surface and the interior of the plant leaf are known to contain diverse and dense bacterial communities, which are distributed both as single cells and extensive biofilms (Monier & Lindow, 2004). Microbial communities are known to vary between the conspecific plants as well as between different leaves and parts of the same plant (Vodovar et al., 2005). Therefore, larvae are naturally exposed to microbes via consumption and this diversity adds to the list of novel niche conditions to which herbivorous insects' immune system must adapt.

One of the major foci of the evolutionary ecology of immunity is the identification and understanding of the selective forces shaping and maintaining immune defenses, focusing on both the factors that induce an immune response and the consequences of that response (Schmid-Hempel, 2005a). The immune defense system of insects consists of behavioral barriers, passive defensive barriers (cuticles), and cascades of active responses which follow after cuticular injury and exposure of the hemocoel to pathogens (Dunn, 1986; Siva-Jothy et al., 2005). Lacking an adaptive immune system, insects rely on an innate immune system, which controls most infections through inflammatory responses after pathogen recognition. This provides a potent first line immune defense resembling the innate immune system of mammals (Finlay & Hancock, 2004; Boman & Hulmark, 1987).

Antimicrobial peptides were first discovered in Lepidoptera, with the recognition, signaling, and antimicrobial peptide production of the innate immune system being subsequently determined in detail in *Drosophila melanogaster* and *Bombyx mori* (Barat-

Houari et al., 2006; Kanost et al., 2004; Hultmark, 2005). Although antigen specific antibodies are not produced by insects, an immune response to a later immune challenge can be enhanced by previous exposure (Moret & Siva-Jothy, 2003; Kurtz & Frank, 2003; Little et al., 2003; Sadd & Schmid-Hempel, 2006). In this case the initial microbial encounter serves as immunological priming with specific hemolymph synthesized proteins remaining in circulation for weeks (Jarosz, 1993). Epithelial tissues do appear able to recognize pathogens and express antibacterial protein encoding genes (Boulanger et al., 2002; Ferrandon et al., 1998; Kang et al., 2002; Gupta et al., 2005; Basset et al., 2000; Vodovar et al., 2005). Thus, midguts would be expected to have the potential to sense microbial presence which would allow a timely and relevant immune system priming. However activating the immune system can be costly, having consequences on other life-history traits (Schmid-Hempel, 2005a; Siva-Jothy et al., 2005).

Immune response costs of resistance, avoidance and tolerance towards pathogens can differ (Restif & Koella, 2004; Schmid-Hempel, 2005a). Ideally, depending on the probability and nature of microbes encountered in the environment, different host resistance mechanisms should be employed (Resitf & Koella, 2004; Boots & Bowers, 1999). Thus, for efficient allocation of resources, an organism needs to be able to differentiate among pathogenic and nonpathogenic microorganisms and to react accordingly, i.e. prime the immune system when necessary. In the case of pathogens using the digestive tract as a gateway to infect the host or posing a threat in the local environment, the midgut could act as a sensing organ for priming the immune system (Vodovar et al., 2005; Zaidman-Remy et al., 2006). A well-studied example for a specific host-parasite interaction is the work done on the immune system and immune gene repertoires in the mosquito *Anopheles gambiae*. The relationship between insect and parasite has been finely tuned, enabling the parasite to partially evade the insect immune system, develop in the insect gut epithelium and travel to the insect salivary glands (Dimopoulos, 2003; Siden-Kiamos & Louis, 2004; Meister et al., 2005). Several mosquito genes have been identified that control the immune response of *Anopheles*, directly affecting development of the malaria parasite within the insect gut (Osta et al., 2004). However, except for these very specific cases of host-parasite interactions, little is known about what kind of effect digested microbes, even naturally occurring essentially non-pathogenic microbes, have on insect immunological ecology.

Here, we examine the consequences of exposing insects to non-infectious microorganisms via simple oral consumption. The ability of an herbivorous insect, the cabbage semilooper *Trichoplusia ni* (Lepidoptera), to both detect and respond to non-

pathogenic, non-infectious bacterial communities through normal consumption, as well as the potential fitness consequences of such a response are studied. Comparisons were made between sterile artificial diet vs. plant feeding and artificial diet supplemented with both gram positive and gram negative bacteria. Immune system response was assayed with a detailed analysis of artificial diet treatments at three different levels: enzyme activity, mRNA expression, and protein levels. Fitness consequences were observed for two key indicators of fitness parameters - larval maturation rate and pupal weight - to be correlated with presence or absence of bacteria in the food, indicating a significant cost to the immune system induction. We believe this to be the first study of the ability of ingested non-pathogenic, non-infectious bacteria as inducers of invertebrate immune system responses in lepidopteran larvae. Ingested non-pathogenic bacteria can upregulate lepidopteran immune genes, with consequences, and our results suggest this effect may be important in host disease resistance.

2.2 Results

2.2.1 Enzyme activities in the hemolymph

Hemolymph samples were collected from 9 day old *T. ni* larvae grown on bacterial and bacteria-free diet. We measured enzyme activities for two commonly used immune status indicators in insect immunology studies – general antibacterial and phenoloxidase activity. For estimating the differences in general antibacterial activity we used standard lytic zone assays. Significant differences were found in the general antibacterial activity and phenoloxidase activity of the hemolymph depending on the type of diet in *T. ni* larvae.

Adding bacteria to the artificial diet lead to higher lysozyme activity in comparison to larvae grown on the diet without bacteria (Kruskall-Wallis ANOVA; $H_{1,58} = 7.77$; $P = 0.003$) (Fig. 1A). Immune induction can generally lead to an increase of the titer of antibacterial proteins and peptides in the hemolymph. These are usually lytic enzymes (e.g. lysozyme) causing bacterial cell wall degradation, and small pore forming peptides leading to the lysis and leakage of the bacterial membranes. The antibacterial “cocktail” measured by lytic zone assays may consist not only of different lysozymes but also of unknown lytic and antibacterial proteins.

In contrast, bacterial diet had a negative effect on hemolymph phenoloxidase, as animals fed on bacteria-free diet had a significantly higher steady state activity as compared to larvae fed with bacteria (Kruskall-Wallis ANOVA; $H_{1,130} = 31.39$; $P = 0.000$) (Fig. 1B). Arthropod

melanisation is controlled by a cascade of serine proteases that ultimately activates prophenoloxidase (PPO) to the enzyme phenoloxidase (PO), which, in turn, catalyzes the synthesis of melanin and is widely used as an estimate of immunocompetence. The PO level is believed to be in good correlation with insects' immunocompetence, especially against invading fungi or insect parasitoids (Rantala, et al., 2000; Barnes & Siva-Jothy, 2000; Söderhäll & Cerenius, 1998; Cerenius & Söderhäll, 2004).

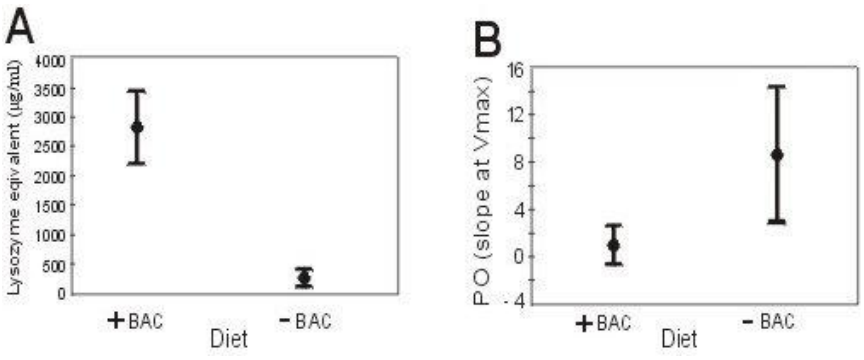


Figure 1. Enzyme activities in the hemolymph of last instar *T. ni* larva, fed on bacteria-free (-BAC) and bacteria-supplemented diet (+BAC). (A) General antibacterial activity measured as the diameter of the lytic zone on agar plates and transformed into lysozyme equivalents (µg/ml). (B) Phenoloxidase activity (slope at Vmax) measured from hemolymph samples. Results represent mean values ± SE.

2.2.2 Identification of differentially expressed proteins in the hemolymph

On one-dimensional protein gels (1D SDS-PAGE), we observed increased expression of eight proteins in the hemolymph of bacterial diet fed larvae and plant-fed (data not shown) as compared to larvae from bacteria-free diets (Fig. 2). This pattern of increased expression was very similar to that produced by hemocoel injection of bacteria into larvae fed bacteria-free diets. For the identification of induced hemolymph proteins, tryptic digests were performed, peptide mass mapping using MALDI-TOF mass spectrometry was carried out, and de novo sequencing of peptides conducted by nano LC-MS/MS. To complete the searches, tandem mass spectra were interpreted *de novo* and the obtained sequences were used for MS-BLAST database searches. Three proteins out of eight were identified: arylphorin, apolipophorin III and gloverin. Most of the unidentified gel bands are very small proteins, and failure of identification is potentially related to extraction and digestion during sample handling and

general limitations of MS in identifying very small proteins. Identified peptides and their relative position within the protein sequences are shown in Supplementary Table 1.

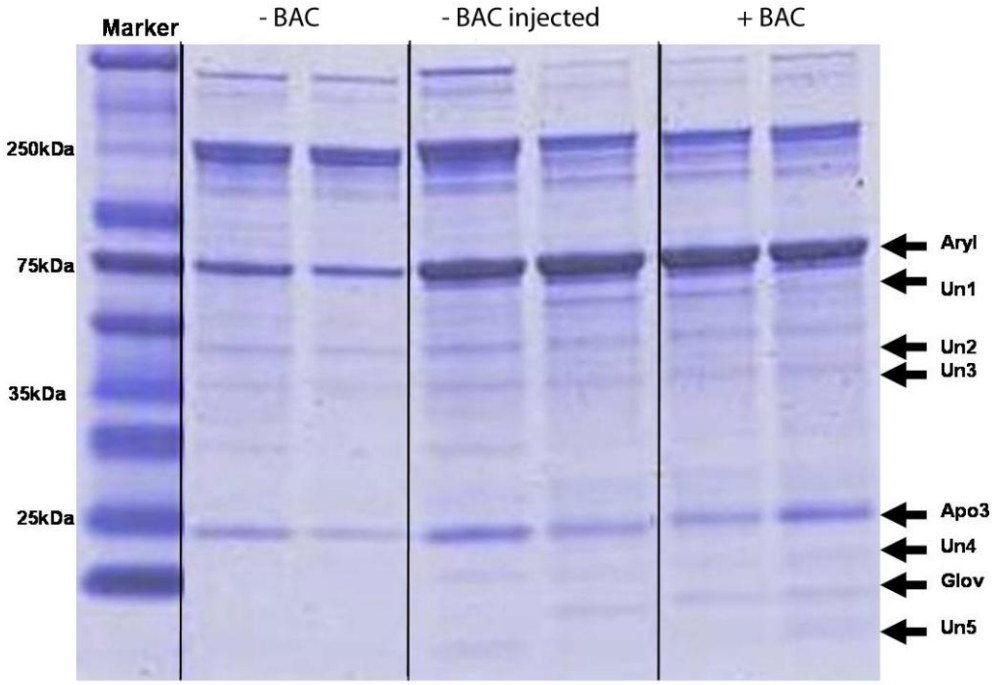


Figure 2. SDS gel electrophoresis of *T. ni* hemolymph proteins stained with Coomassie blue. Treatments are bacteria-free diet (- BAC), injection of bacteria into hemocoel of larvae fed bacteria-free diet (- BAC injected), or bacteria-supplemented diet (+ BAC). Duplicate lanes of each of the three treatments are shown. Identified differentially expressed proteins are arylphorin (Aryl), apolipophorin III (Apo3) and gloverin (Glov). Five additional unknown proteins (Un1, Un2, Un3, Un4, Un5) were observed as differentially expressed.

All three identified proteins are thought to participate in immune responses. Arylphorin, for which we observed the greatest up-regulation of protein in the hemolymph, has been proposed to play a role in humoral immune defense in response to bacterial challenge (Kunkel et al., 1990; Asgari & Schmidt, 2004; Shelby & Webb, 1994). Additional assays were performed to confirm the connection of higher arylphorin expression with larval immune challenge, ruling out the possible effect of nutritional differences between bacterial vs. bacteria-free diets. Larvae grown on bacteria-free diet were injected with the mixture of the *E. coli* and *M. luteus* and a similar up-regulation of arylphorin expression was seen as in bacterial diet fed larvae (Fig. 2). Injecting bacterial diet fed larvae with the bacterial mixture or saline did not cause any additional increase in arylphorin expression (data not shown).

Second, larvae on bacterial diet also had a higher protein expression of apolipophorin III in the hemolymph. Apolipophorin belongs to the functionally important family of apolipoproteins that play critical roles in lipid transport and lipoprotein metabolism (Weers & Ryan, 2006). The third protein identified as being differentially expressed was gloverin. Gloverin is an inducible antibacterial insect protein first isolated from the silk moth *Hyalophora* (Axen et al., 1997). It is a small, basic, heat stable protein containing a large numbers of glycine residues, but no cysteine residues as is found in many other antimicrobial peptides (e.g. defensin). Gloverin was also previously described to be expressed upon immune insult in *T. ni* and *Bombyx mori* (Lundström et al., 2002; Hwang et al., 2006).

2.2.3 EST analysis and identification of immune-related genes in T. ni

For many lepidopteran species, including *T. ni*, only a very limited number of sequences are available in public databases. To identify immune-related and general housekeeping genes in larval tissues, a cDNA library was constructed from whole *T. ni* larvae of different instars and fed a combination of dietary inducers (i.e. plant secondary metabolites). DNA sequencing from the 5' ends of clones followed by clustering produced 1675 distinct genes, 1082 represented by single reads. For putative functional assignments, the assembled sequences were compared against protein and nucleotide NCBI databases, using the locally installed BLAST search tool.

BLAST searches and annotation using Gene Ontology terms showed that several ESTs were similar to known immune-related genes from other insects, including genes involved in pathogen recognition (pattern recognition proteins), direct antimicrobial defense (antimicrobial peptides) and genes related to physiological changes upon immune challenge. Among the ESTs were several known immunity-related genes (lysozyme, gloverin, pro-phenoloxidase) and several genes with similarities to immune-induced genes (HDD1, hemolin) with a total of 25 immune candidate genes. BLAST results and putative functions for the immune-related genes identified from *T. ni* are listed in Table 1.

Table 1. Immunity-related products discovered from *Trichoplusia ni* EST projects

TC/EST name	No. of ESTs present	Best BLAST hit / Closest homologue [Species]	Description / Putative Function	Accession no.	BLAST score (E-value)
TNI-CON0233	23	Apolipophorin-3 precursor (Apolipophorin-III) [Manduca sexta]	Lipid transport; immune stimulating factor	P13276	1,00E-75
TNI-CON0998	1	Apolipophorin-3 precursor (Apolipophorin-III) [Spodoptera littoralis]	Lipid transport; immune stimulating factor	O77248	7,00E-15
TNI-CON0275	3	Large subunit arylphorin p76 [Heliothis virescens]	Storage protein; expressed after immune challenge	AAO20844	3,00E-77
TNI-CON0268	1	Chemosensory protein 11 [Bombyx mori]	GNBP-like domain; immune responsive	NP_001037068	1,00E-33
TNI-HCN384-03G20	4	Beta-1,3-glucan-binding protein 2 precursor (BGBP-2) [Manduca sexta]	Bacterial cell wall binding/recognition protein	Q8ISB6	5,00E-52
TNI-CON1186	1	Beta-1,3-glucan-binding protein precursor (BGBP) [Plodia interpunctella]	Bacterial cell wall binding/recognition protein	Q8MU95	2,00E-31
TNI-CON0313	1	Beta-1,3-glucan recognition protein [Plodia interpunctella]	Bacterial cell wall binding/recognition protein	AAM95970	4,00E-38
TNI-CON1099	2	Beta-1,3-glucan-binding protein precursor (BGBP) [Plodia interpunctella]	Bacterial cell wall binding/recognition protein	Q8MU95	1,00E-28
TNI-CON0703	3	KUN-5 [Ixodes pacificus]	Kalicludin-like; Kunitz family of serine protease inhibitors	AAT92116	6,00E-12
TNI-HCN384-03D11	2	Kunitz-like protease inhibitor precursor [Ancylostoma caninum]	Kalicludin-like; Kunitz family of serine protease inhibitors	AAN10061	2,00E-10
TNI-CON0522	1	Phenoloxidase inhibitor protein [Anopheles gambiae]	Inhibition of phenoloxidase cascade	AAX22219	7,00E-05
TNI-CON1448	1	Prophenol oxidase activating enzyme 1 [Spodoptera litura]	Activating enzyme of Pro-PO	AAW24480	7,00E-46
TNI-CON0527	3	Conotoxin scaffold VI/VII precursor [Conus arenatus]	Conotoxin-like protein; ion channel antagonist	AF215057	7,00E-05
TNI-CON0581	1	Gallerimycin [Spodoptera frugiperda]	Defensin-like antifungal peptide	AAQ18896	2,00E-20
TNI-CON1119	1	Putative hemolin [Hyphantria cunea]	Immunoglobulin domains; induced by microbial challenge	AAD09287	4,00E-51
TNI-HCN384-02J03	3	Putative hemolin [Hyphantria cunea]	Immunoglobulin domains; induced by microbial challenge	AAD09287	4,00E-85
TNI-CON0811	1	Attacin-A precursor [Trichoplusia ni]	Inducible antibacterial peptide	P50725	2,00E-22
TNI-CON0122	22	Cecropin D [Bombyx mori]	Antimicrobial peptide; Lysis of bacterial cell walls	BAA31507	9,00E-10
TNI-CON0128	9	Cecropin [Helicoverpa armigera]	Antimicrobial peptide; cecropin B	AAX51304	1,00E-16
TNI-CON0196	2	Immune-related Hdd1 [Hyphantria cunea]	Immune-related protein; induced by microbial challenge	AAD09279	4,00E-09
TNI-CON0498	2	Defensin precursor [Spodoptera frugiperda]	Antimicrobial peptide; spodoptericin-like	AAM96925	5,00E-21
TNI-CON0644	4	Cobatoxin short form A [Spodoptera frugiperda]	Scorpion toxin-like; Induced after bacterial challenge	AAQ18897	5,00E-07
TNI-CON1679	2	Gloverin precursor [Trichoplusia ni]	Antibacterial protein; binds to LPS	AF233590	8,00E-51
TNI-CON0507	3	Lysozyme [Spodoptera exigua]	Lysozyme a; destroys bacterial cell walls	AAP03061	3,00E-44
TNI-CON1157	1	Lysozyme precursor [Trichoplusia ni]	Lysozyme b; destroys bacterial cell walls	P50718	1,00E-36

2.2.4 RT-qPCR of immune-related genes in T. ni midgut tissue

Transcript profiles from *T. ni* larvae fed on bacterial and bacteria-free diet were compared using quantitative real-time PCR. Experiments were conducted each with three biological replicates and randomized between the treatments. A total of 28 gene-specific primer pairs (see Additional file 2 for primer information) were designed on the basis of sequences obtained for selected *T. ni* genes known to be involved in immune response in other insect species or were based on proteins identified as immune-responsive in our protein expression experiments. In addition, elongation initiation factor-4α (EIF4α), elongation factor-1α (EF1α) and mitochondrial ribosomal protein S18 (RPS18) were selected to serve as potential house-keeping genes. All three were tested as invariant endogenous controls in the assay to correct for sample-to-sample variation in RT-qPCR efficiency and errors in sample quantitation and sample concentration. EIF4α performed best as an endogenous control (‘normalizer’) and was used for the remaining assays.

Relative fold changes for each gene were set to 1 for the control treatment (larvae grown on bacteria-free diet). Seventeen genes were up-regulated by bacterial feeding, including apolipophorin III, arylphorin, cecropin B, cecropin D, cobatoxin, defensin, gallerimycin, gloverin, HDD1, kalcitin, lysozyme(a+b), a beta-1,3-glucan recognition protein, and phenoloxidase inhibiting enzyme. Apolipophorin III, arylphorin, a beta-glycan

recognition protein, cecropin B, HDD1 immunity related protein and lysozyme(a) were more than five times up regulated in the midguts of larvae grown on bacterial diet (Fig. 3). Notably, lysozyme(a) which is highly upregulated at the transcript level, was also identified by MS as being upregulated at the protein level. No statistically significant changes in transcript abundance by bacterial feeding could be detected for azurocidin, attacin and a Gram negative-binding protein.

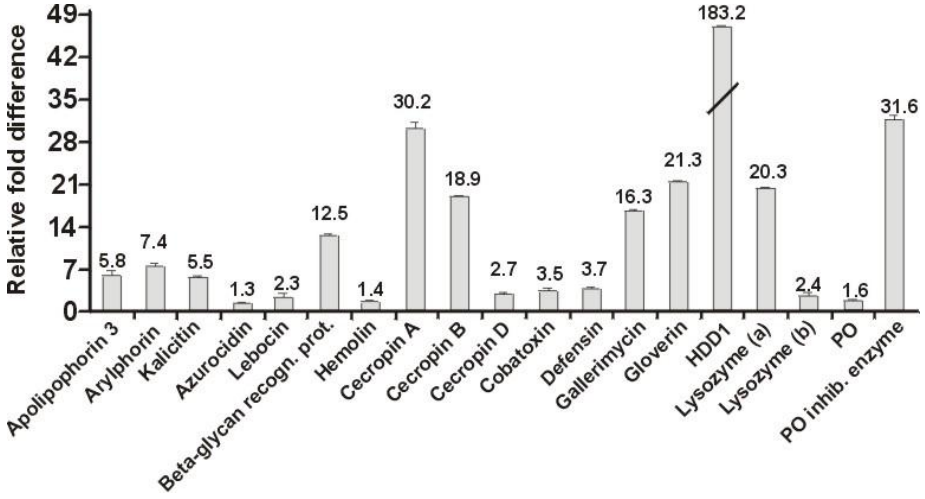


Figure 3. RT-qPCR results of differential gene expression between *T.* ni larvae grown on bacterial diet in comparison to larvae grown on bacteria-free diet. Relative fold changes for each gene were set to 1 for the control treatment. Results represent mean values of three independent biological replicates ± SD (experimental error).

2.2.5 Life-history traits

To examine whether the observed changes in protein expression and enzyme activity in the hemolymph, as well as differential gene expression in the gut, have any life history consequences, we examined the larval developmental time and pupal mass of animals grown on the different diets. Animals grown on bacterial diet had smaller pupal masses and delayed development. The complete life cycle of *T. ni* from egg to adult death under the conditions used for our experiments is approximately 4-6 weeks, with the larval stage lasting, on average, two weeks. Bacteria fed larvae reached the pupation state 1-1.5 days later than larvae grown on bacteria-free diet (ANOVA; $F_{1,\ 204} = 11.16$, $P = 0.001$) (Fig. 4B). The diet did not influence the developmental time of the two sexes differently (ANOVA; $F_{1.143} = 0.03$, $P = 0.858$). Animals grown on bacterial diet also had smaller pupal masses in comparison to animals grown on bacteria-free diet (ANOVA; $F_{1.143} = 9.77$, $P = 0.002$) (Fig. 4A). Again,

diets had no differential effect on sex, as the trend for mass loss was the same for males and females on both diets (ANOVA; $F_{1, 143} = 0.08$, $P = 0.778$). The bacterial-fed *T. ni* larvae, though having a longer developmental time, fail to reach the same pupal mass as conspecifics grown on the bacteria free diet. We could not observe any differences in mortality, failure to pupate or development into adults between the treatment groups.

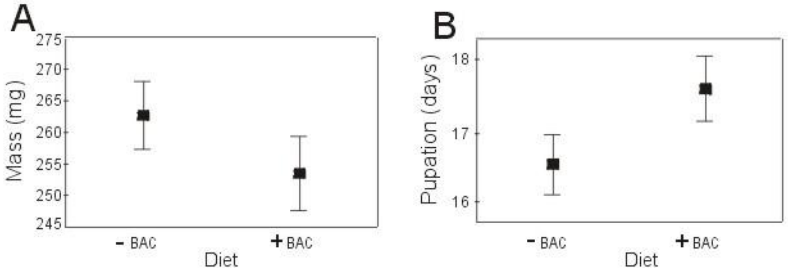


Figure 4. The effect of bacterial diet (+BAC) and bacteria-free diet (-BAC) on the pupation time and pupal masses in *T. ni*. Graphs shows least square means of the model, representing mean ± SD. (A) Pupal masses are decreased when larvae are fed bacterial diet in comparison to larvae grown on bacteria-free diet. (B) Bacterial diet leads to delayed pupation times in *T. ni* larvae.

2.3 Discussion

Insects possess a range of defense mechanisms to effectively combat invasion by microbial pathogens. Here we document for the first time that ingested non-pathogenic bacteria can induce an immune response in invertebrates with fitness related costs. These effects can be seen for both direct and indirect immune responses. A direct response was observed as bacterial diet altered two important immunity related functions of the hemolymph.

Phenoloxidase (PO) activity is widely used as an indicator of insects' immunocompetence (Wilson et al., 2001; Reeson et al., 1998; Adamo, 2004; Barnes & Siva-Jothy, 2000). In *T. ni,* bacterial diet has an inhibiting effect on PO activity in the hemolymph. However, at the same time the overall antibacterial lytic activity of the hemolymph was significantly increased. The lytic activity usually consists of a cocktail of small lytic enzymes causing bacterial cell wall degradation, leading to the lysis and leakage of the bacterial membranes. Lysozymes and other lytic proteins can be active against both Gram-positive and Gram-negative bacteria, potentially activating the Toll-related signaling pathway by releasing bacterial cell wall material (Hultmark, 2003; Dunn, 1986). Opposite responses of general lytic and PO activity could represent a trade-off between different types of immune responses,

correlated with the nature of the immune induction. Lysozyme activity is usually more related to bacterial infection and PO activity to fungal and multicellular hemolymph-invading organisms. A trade-off between PO and lysozyme activity has been reported in larvae from the related Noctuid moth *Spodoptera littoralis* (Cotter et al., 2004). It is unclear whether these apparent tradeoffs, observed both here and the previous study, arise from similar induction pathways, limited resources, or a mechanistic trade-offs due to the potentially severe harmful side effects of high PO activity on the tissues in the form of oxidative and lytic stress (Nappi & Christensen, 2005). Further study at the individual level more directly focused on this apparently general phenomena are now warranted.

Initial experiments performed with larvae fed *Brassica* plants grown in the greenhouse provided intermediate results for both the lytic zone assays and the phenoloxidase activities (data not shown). However, variations in plant secondary metabolites potentially interfere or overlap with results obtained from bacterial community variations. This is also reflected in the high overall variability in the responses of *T. ni* larvae placed on individual leaves or plants. Using plants as a food source does not facilitate clear differentiation between effects caused by leaf surface bacteria and plant secondary metabolite variations. In order to exclude effects due to secondary plant chemistry, we focused our research on artificial diet manipulation.

The observed increase in transcript abundance of a ProPO inhibiting enzyme in the midgut tissue of *T. ni* larvae fed on bacterial diet could potentially contribute to the lower overall PO activities in the hemolymph. Lower levels of PO activity in the hemolymph could also be related to up regulation of apolipophorin III expression in the plasma, as has been shown in wax moth (*Galleria mellonella*) (Halwani et al., 2000).

We observed an increase in arylphorin, apolipophorin III, and gloverin protein levels in hemolymph, as well as an increase in transcript abundance in the midgut, in larvae fed bacterial-supplemented diet. Arylphorin is usually highly abundant during the last larval instars, but its synthesis ceases during the molt, during starvation, and at the wandering stage. Arylphorin is one of the major storage proteins, has been proposed to play a role in humoral immune defense as a response to bacterial challenge but is also known to be either more abundant after parasitization by wasps (Rantala et al., 2000) or dramatically reduced relative to the levels of arylphorin detected in nonparasitized larvae (Asgari & Schmidt, 2004; Shelby & Webb, 1994; Beckage & Kanost, 1993; Shelby & Webb, 1997), however its exact function in immune response has not been clearly established.

Apolipophorin III (ApoIII) belongs to the functionally important class of lipoproteins, which are responsible for lipid transport and lipoprotein metabolism in various animal classes (Weers & Ryan, 1006). ApoIII seems to be an insect-specific protein as it has not been described in vertebrates (Hoffmann et al., 1999). The immune response related properties of ApoIII were first described in greater wax moth (*Galleria mellonella*) (Wiesner et al., 1997). Although ApoIII has mainly been described to be a storage protein, it was also shown to have general immune stimulating activity and to bind bacterial lipoteichoic acid. Arylphorin is generally able to bind to molecules characteristic to microorganisms, classifying it as a pattern recognition protein, involved in sensing the presence of bacteria, or in a more protective role by neutralizing pathogen cell wall components (Kim et al., 2004; Ma et al., 2006; Halwani et al., 2000; Weers et al., 2006). Besides having a general immune stimulating activity, injection of ApoIII as well as *E. coli* in *Hyphantria* dramatically induced the expression of antimicrobial peptides, and as previously mentioned, repressed PO activity (Cotter et al., 2004; Kim et al., 2004), which is consistent with our findings.

We have also detected high levels of the immune-related effector protein gloverin in the hemolymph of bacteria fed larvae. Gloverin is an antibacterial protein, and was shown to be synthesized after bacterial immune challenge, being active against Gram-negative bacteria and yeast in *Helicoverpa armigera* (Mackintosh et al., 1998), inhibiting the growth of *E. coli* at concentrations far below the concentration found in the hemolymph of infected pupae (Axen et al., 1997). It is also expressed in *T. ni* hemocytes following bacterial induction (Lundtsröm et al, 2002). The prime effect of gloverin seems to be binding to lipopolysaccharides on the bacterial outer membrane, inhibiting synthesis of essential outer membrane proteins, leading to increased permeability (Axen et al., 1997). Notably, a similar spectrum of the induction of immune-responsive proteins was identified in *Galleria mellonella* by using comparative proteomic analyses of hemolymph proteins and RT-qPCR analysis from larvae that were challenged with either injecting microbial metalloproteases or LPS (Altincicek et al., 2007).

The increase in transcript abundance of several immune response related genes in midgut tissues suggests that midgut cells themselves are able to recognize and respond to the presence of bacteria in the gut lumen. The innate immune response of epithelial cells has been studied rarely (Boulanger et al., 2002; Ferrandon et al., 1998). *T. ni* larvae feeding on bacteria supplemented diet show higher expression of an azurocidin-like protein in the gut (Kang et al., 2002). Experiments done with *Drosophila melanogaster* show that the expression of a drosomycin-GFP reporter gene in epithelial tissues responds to infection

(Ferrandon et al., 1998). Furthermore, some bacterial species are able to trigger a strong systemic immune response in Drosophila after oral infection, possibly mediated through a peptidoglycan receptor protein (PGRP-LB), which was suggested to being activated only in the case of severe infection and bacterial proliferation (Zaidman-Remy et al., 2006). We know of only one study showing that non-pathogenic bacteria can induce immunity-related genes after oral feeding, with honey bees showing expression differences in a single gene coding for an antimicrobial protein (abaecin) after feeding on a bacterial mix (Evans & Lopez, 2004). However, the consequence of an epithelial immune response for the systemic immune response, immunocompetence and other life-history traits is largely uninvestigated.

Two important life-history traits were affected by the consumption of bacterial diet. The increase in developmental time and decrease in pupal mass observed in the bacterial-fed treatments are both likely to have negative effects on overall fitness. We have used a nutrient-rich diet, providing optimal conditions for both larval growth and development. Costs in the wild are likely to be greater due to the likely substandard abiotic and biotic conditions. Moreover, the opportunity for additional growth during the increased larval period of approx 1.5 day was not sufficient to compensate for the reduced growth rate, as bacterial-fed individuals failed to reach the same pupal mass as conspecifics reared on nonsupplemented diet. For both the pupal mass and developmental time, the diet had no differential effect on sex, as the trend for mass loss was the same for males and females on both diets. Furthermore, we could not observe any differences in mortality, failure to pupate or development into adults between the treatment groups. This would support the idea that, although costly due to immune priming, bacterial-supplemented diet did not have any direct deleterious effects reflected in the survival of the insects.

Negative fitness related effects have been reported also for the larvae of the gypsy moth (*Lymantria dispar)* infected with the entomopathogenic microsporidium *Vairimorpha* sp (Microsporidia: Burenellidae). These larvae have prolonged development due to decreased food utilization, also resulting in a decreased body mass (Henn et al., 2000). In mosquito (*Aedes aegypti*) malarial infection reduces the fecundity, increases mass loss, and lowers metabolic rate during food digestion (Gray & Bradley, 2006). In our experiments and as an important contrast, nonpathogenic bacteria without infection (bacteria not being present in the hemolymph) resulted in comparable effects, which we interpret as a cost of "priming" the innate immune system.

Such an anticipatory up-regulation of immune defenses can have benefits as well, as illustrated in a recent study. In *Manduca sexta* larvae, prior hemolymph injection of non-

pathogenic bacteria elicited up-regulation of several genes which provided some protection against subsequent infection with pathogenic *Photorabdus*. These protective effects were weakened by experimental manipulation of transcript levels by RNA interference (Eleftherianos et al., 2006). These findings support the adaptive significance of a `priming´ of the immune system, leading to a higher level of immune responses which would enable the insects to better cope with real pathogens they may encounter. Moreover, pathogenicity may be context-dependent and 'priming' may actually be directly defensive against the entire bacterial community experienced by an insect. Broderick et al. (2006) have shown that the insecticidal activity of *Bacillus thuringiensis* is dependent on interactions with other microorganisms of the larval midgut. Eliminating most of the midgut bacteria drastically reduced larval mortality even in the presence of the insecticidal crystal protein of Bt, suggesting a complex interaction of non-pathogenic and pathogenic bacteria.

Our results suggest that the midgut may play a more active role in sensing foreign organisms and mounting protective responses than previously suspected. Moreover, immune-related properties of the hemolymph may be affected even if the foreign organisms never enter the hemocoel. The nature of the signal and the mechanisms for modulating a gut signal into a hemolymph and fat body response remains unclear and is likely to be a rewarding avenue of research. Dissected *T. ni* larvae exposed to non-pathogenic bacteria in their diet have no detectable lesions in the peritrophic matrix or epithelium. Preliminary feeding tests performed with fluorescently labeled bacteria also indicate that no marker can be detected in the hemolymph (data not shown), although we cannot rule out the possibility of bacterial fragments crossing the gut wall. Expression of both pattern recognition and antimicrobial proteins by midgut cells points to the potential of recognizing and fighting bacteria directly in the gut tissue and gut lumen. Thus, the midgut deserves attention not only as an organ of digestion and resource assimilation, but also of defense. Further studies will lead to the molecular characterization of receptor molecules and signal transduction pathways involved in guarding this vulnerable portal.

2.4 Conclusions

This work has addressed the consequences of exposing insects to non-infectious microorganisms via simple oral consumption. Here we show that larvae can sense microbes through consumption, as hemolymph specific defense mechanisms can be induced without actual exposure to and infection with microorganisms. Nonpathogenic bacteria in larval

food induce specific changes in the larval proteome, transcriptome and enzyme activity levels. Although such physiological changes negatively affect fitness related traits, like body mass and developmental time, the potential benefits of immune system priming may outweigh the observed tradeoffs, as priming based on environmentally sensed bacteria may decrease the risk of serious infections. These results strongly suggest that host plant microbial communities may represent a dynamic and unstudied part of the evolutionary interactions between plants and their insect herbivores.

2.5 Methods

2.5.1 Animals

Cabbage semilooper (*Trichoplusia ni*) eggs were obtained from Entopath Inc. (USA). Larvae of *Trichoplusia ni* were grown on artificial diet (casein 31.5g, sucrose 33.76g, wheat germ 43.76g, Wess salt 9g, potassium sorbate 1g, cellulose 6.26g, methyl paraben 1.36g, lepidopteran vitamin mix 9g, aureomycin 1g, ascorbic acid 3.5g, propyl gallate 0.2g, 40% formaldehyde 1.5ml, linseed oil 6.5ml, 45% potassium hydroxide 2.5ml, 24g agar and 750ml water) at room temperature (23^0 C) and a 16/8 h light/dark cycle, and 55% relative humidity. For initial tests, larvae were also reared on cabbage plants (*Brassica oleracea oleracea*, var. Rosella). Seeds from Brassica plants were sown on a Mini-Tray: vermiculite (3:1) soil mix (Einheitserdenwerk) and cold stratified for 7 days at 4 °C. Afterwards, plants were moved to the greenhouse and grown at 23 °C with fluorescent light banks with wide spectrum lights.

T. ni eggs were either placed in plastic cups with artificial diet or on 3 week old plant leaves and allowed to hatch. To estimate the impact of bacteria in the diet, three feeding groups were formed: larvae were fed on artificial diet with or without bacteria (later referred to as bacterial and bacteria-free diet) and for initial experiments on *Brassica* plants. Bacterial diet was soaked with overnight cultures (OD600 = 4) (2.5ml/40cm^2) of *Escherichia coli* and *Micrococcus luteus* (approximately 80 µg per 125 g of diet). Diets were changed every three days to keep the bacterial concentration in the diet at approximately same level. In the case of growth rate experiments, larvae were kept in individual cups (~30 ml) with a piece of artificial diet and pupation was estimated on a daily basis. Pupal weight was measured using an electric balance to the nearest mg on the third day after pupation. For injection control experiments, 3µl of saline (control) and *E.coli* and *M. luteus* (induction) pelleted cells in

saline were injected into 9 days old last instar larvae of both dietary groups using a FemtoJet microinjector (Eppendorf).

Enzyme activities and protein expression in the hemolymph were measured from early stage last instar larvae (9^{th} day after hatching from egg). Same age last instar larvae were also dissected and their midguts removed and stored in RNA stabilizing buffer (Qiagen) for gene expression analyses.

2.5.2 Lytic zone assay

For estimation of the total lytic activity of the hemolymph, a lytic zone assay was performed. 12 x 12cm Petri dishes were filled with 35ml of autoclaved Sörensen buffer with 21mg *Micrococcus luteus* lyophilisized cells (Sigma) and 2.1 mg streptomycin sulfate (Calbiochem) with a final concentration of 1.5 % agar. Wells within plates (2 mm diameter) were made by puncturing the agar with a plastic pipette and removing the agar plug by suction. Hemolymph samples (3 µl) were pipetted directly into the wells and the plates were incubated for 24 h at 37°C. Dilution series of chicken egg white lysozyme (Sigma) (2 mg/ml, 1 mg/ml, 0.750 mg/ml, 0.500 mg/ml, 0.250 mg/ml, 0.125 mg/ml, 0.62 mg/ml, and 0.31 mg/ml) was added to each plate as a control and a calibration curve was created based on these standards. Lytic activity was determined as the radius of the clear zone around a sample well.

2.5.3 Phenoloxidase activity assay

Hemolymph phenoloxidase activity was estimated using 10 µl of hemolymph sample diluted in 1 ml of ice-cold sodium cacodylate buffer (0.01M Na-cacodylate and 0.005M CaCl2) and directly frozen in liquid N_2. PO activity was assayed by thawing frozen hemolymph samples at 37° C for 4 minutes and then centrifuged at 4°C and 2800g for 15 minutes. The supernatant was removed and used for measurements where 100µl of supernatant was added to 200 µl of 3mM L-Dopa (Sigma). Kinetic activity of the enzyme was measured at 30°C, 490nm for 45 minutes, taking absorbance measurements once per minute. As the absorbance curve was linear from 5 – 45 min after adding the substrate (personal observation), in later analyses the slope of the curve from 15-26 minutes of the reaction was used. Measurements were made on

Multiskan Spectrum multiplate reader (Thermo-Electron) and data was acquired with SkanIt Software for Multiskan Spectrum version 2.1 (Thermo-Electron).

2.5.4 Protein gel electrophoresis and protein identification by MALDI-MS and nanoLC-MS/MS

To estimate protein expression in the hemolymph, Sodium Dodecyl Sulphate Polyacrylamide Gradient Gel Electrophoresis (SDS-PAGE) was performed in a XT-MES buffer system. 2µl of hemolymph sample was diluted into 50µl of ice-cold 4% SDS containing TrisHCl buffer with EDTA-free protease inhibitor cocktail (Pierce), directly frozen in liquid N_2 and stored at -20°C until use. For measurements, samples were allowed to melt on ice and centrifuged at 9200g for 10 minutes. Supernatant was transferred to new tubes, loading buffer was added to the supernatant, heat denatured and loaded on a 4 – 12 % Bis-Tris Criterion XT Precast Gel (BioRad). Gels were run at 80V for ~ 3.5 hours or until the dye front reached the gel end. On the gels, two different protein markers were used. Rainbow marker (Amersham) served as a running control marker and the Precision Plus Protein Unstained Standard (BioRad) for precise protein molecular weight estimation. After the run was complete, gels were washed 3 times, followed by staining with Coommassie blue (Imperial Blue, Pierce) for 2-3 hours, then destained overnight. For protein identification, spots were manually cut out from SDS-gels, transferred to 96-well microtiterplates (MTP) and processed on an automatic Ettan TA Digester (GE Healthcare). The gel plugs were rinsed with 50 mM ammonium bicarbonate/50% Acetonitrile three times for 20 min to remove the coomassie stain. The gel plugs were then air-dried and digested with trypsin overnight at 37°C. The resulting peptides were extracted from the gel plugs, collected in a MTP and vacuum-dried. Samples were submitted for MALDI-TOF mass spectrometry and denovo sequencing by Q-TOF to our in-house MassSpec service group.

For further processing, a MALDImicro MX mass spectrometer (Waters) was used for monitoring of the protein digestion. The tryptic peptides were reconstituted, mixed with α-cyano-4-hydroxy cinnamic acid, and an aliquot of the mixture was spotted on a metal 96-spot MALDI target plate. MassLynx v4.0 software served for data acquisition (Waters). Bovine serum albumin tryptic digest was used to calibrate the mass spectrometer (MPrep, Waters). The MALDI-TOF peptide signal intensities were used to estimate the volume of the sample for the nanoLC-MS/MS *de novo* sequence analysis.

Liquid chromatography-tandem mass spectrometry was performed to acquire fragmentation data from selected peptides. Aliquots of tryptic peptides were injected on a CapLC XE 2D nanoLC system (Waters). After concentration and desalting, eluted peptides were transferred to the NanoElectroSpray source of a Q-TOF Ultima tandem mass spectrometer (Waters). MS/MS spectra were collected by MassLynx v4.0 software (Waters). ProteinLynx Global Server Browser v.2.2 software (PLGS 2.2, Waters) was used for baseline subtraction and smoothing, deisotoping, *de novo* peptide sequence identification, and database searches. Obtained chromatograms were analyzed using NCBI Insecta database (http://www.ncbi.nlm.nih.gov) for MALDI – TOF samples and Swissprot database for Q-TOF samples. Amino acid sequences of peptides which did not provide conclusive results from the database searches were searched using an MS-BLAST server installed in-house or via the ButterflyBase web page (http://heliconius.org). Details of both the sample processing and instrument settings and handling have been described elsewhere (Giri et al., 2006).

2.5.5 Preparation of Trichoplusia ni cDNA libraries

For RNA isolation from larval tissue, in total 15 male and 15 female third, fourth and fifth instar larvae each were dissected in 100 mM Tris-HCl, pH 7.5. RNA and poly(A)+ mRNA was isolated with standard methods. Double-stranded, full-length enriched cDNA from dissected and whole larvae were generated by primer extension with the SMART cDNA library construction kit (Clontech) according to the manufacturer's protocol but with several modifications. 2 µg of poly(A)+ mRNA was used for each cDNA library generated. cDNA size fractionation was performed with SizeSep 400 spun columns (GE Healthcare) that resulted in a cutoff at ~300 bp. The full-length-enriched cDNAs were cut with SfiI and ligated to the SfiI-digested pDNR-Lib plasmid vector (Clontech) instead of the λTriplEx2 vector provided with the kit. Ligations were transformed into *E. coli* ELECTROMAX DH5α-E electro-competent cells (Invitrogen).

2.5.6 Generation of a Trichoplusia ni EST sequence database

Plasmid isolation from bacterial colonies grown in 96 deep-well plates was performed using the 96 robot plasmid isolation kit (Eppendorf) on a Tecan Evo Freedom 150 robotic platform (Tecan). Single-pass sequencing of the 5'-termini of a total of approximately 5600 clones of

the directionally cloned *T. ni* cDNA libraries was carried out on an ABI 3730xl automatic DNA sequencer (PE Applied Biosystems). Vector clipping, quality trimming and sequence assembly was done with the Lasergene software package (DNAStar). Of the total of 5,300 ESTs, 770 were removed during the quality trimming steps. The average readable insert length after vector clipping and quality trimming was 545 bp. Blast searches were conducted on a local server using the National Center for Biotechnology Information (NCBI) blastall program. Sequences were aligned using ClustalW software (http://sf01.bic.nus.edu.sg/clustalw/). *T. ni* sequences were submitted to Genbank under accession numbers EF605248, EU016384-EU016407.

2.5.7 RNA isolation and Quantitative real-time PCR

All larvae were 9 days old at the time of dissection. Dissected insect midguts were rinsed with PBS, ground using a motorized hand pestle and total RNA was isolated using the TRIzol Reagent (Invitrogen) according to the manufacturers´ protocol. An additional DNAse (Turbo DNAse, Ambion) treatment was included prior to the second purification step to eliminate any contaminating DNA. A second purification step was performed with RNeasy MinElute columns (Qiagen). RNA integrity was verified on an Agilent 2100 Bioanalyzer using RNA Nano chips (Agilent). RNA quantity was determined photospectrometrically using a BioPhotometer 6131 (Eppendorf).

500 ng of DNA-free total RNA was converted into single-stranded using a mix of random and oligo-dT20 primers according to the ABgene protocol (ABgene). Real-time PCR oligonucleotide primers were designed using the online Primer3 internet based interface (http://frodo.wi.mit.edu). Primers were designed by the rules of highest maximum efficiency and sensitivity rules were followed to avoid formation of self and hetero-dimers, hairpins and self-complementarity (Supplementary Table 2). Gene-specific primers were designed on the basis of sequence obtained for selected *T. ni* genes and several additional genes as potential house-keeping genes to serve as the endogenous control (normalizer). Q-RT-PCR was done in optical 96-well plates on a MX3000P Real-Time PCR Detection System (Stratagene) using the Absolute QPCR SYBR green Mix (ABgene) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye included in the PCR master mix.

A dissociation curve analysis was performed for all primer/probe pairs, and all experimental samples yielded a single sharp peak at the amplicon’s melting temperature. The

dynamic range of a given primer/probe system and its normalizer was examined by running triplicate reactions of tenfold-dilution series (five different RNA concentrations). Since target and normalizer had similar dynamic ranges, the comparative quantitation method (ΔΔCt) was used to contrast the different treatments and tissues, and transformed to absolute values with $2^{-\Delta\Delta Ct}$ for obtaining relative fold changes (Livak & Schmittgen, 2001). All of the assays were run in quadruplicate (biological replication, each representing a pooled mRNA of 4 individuals) and triplicate (technical replication) to control for overall variability. Relative fold changes for each gene were set to 1 for the control treatment (larvae grown on bacteria-free diet).

2.5.8 Statistical analysis

Statistical analyses were performed with the software package Statistica 7 (StatSoft). Normality of the data was estimated by using Shapiro-Wilkoxon and Levene's tests. In the case where assumptions for normality and homogeneity were not violated, our hypotheses were tested using an ANOVA model, otherwise a nonparametric Kruskall-Wallis ANOVA test was used.

Supplementary Table 1. Peptides from 1D SDS-PAGE gel-isolated proteins identified by MS

Q-TOF

HE_DF_TN47_1GEL8.raw **Apolipophorin III**

Submitted Mass	Submitted	Experimental Mass	mW	Delta (Da)	Delta (ppm)	Probability	Ladder Score	Start	End	Sequence	Query Tool
779.3933	2	1556.771	1556.816	0.0449	28.8558	134.634	76.7123	1	14	(-)LQAAVQNTVQETQK(-)	De Novo Sequencing
660.3101	2	1318.6046	1318.637	0.0319	24.1622	201.8095	73.7705	1	12	(-)DLASNVETTNQK(-)	De Novo Sequencing
660.3101	2	1318.6046	1318.637	0.0319	24.1622	203.7849	64.1791	1	13	(-)VEASNVETTNAGK(-)	De Novo Sequencing
660.3101	2	1318.6046	1318.637	0.0319	24.1622	204.4652	67.1642	1	13	(-)EVASNVETTNAGK(-)	De Novo Sequencing
660.3101	2	1318.6046	1318.637	0.0319	24.1622	205.6361	64.1791	1	13	(-)LDASNVETTNAGK(-)	De Novo Sequencing
660.3101	2	1318.6046	1318.637	0.0319	24.1622	206.1665	64.1791	1	13	(-)DLASNVETTNAGK(-)	De Novo Sequencing
779.3933	2	1556.771	1556.816	0.0449	28.8558	133.6574	68.3544	1	15	(-)LQAAVQNTVQETAGK(-)	De Novo Sequencing
655.3352	2	1308.6548	1308.668	0.0127	9.701	41.7448	72.1311	1	12	(-)DAPPSKVQQPDK(-)	De Novo Sequencing
779.3933	2	1556.771	1556.816	0.0449	28.8558	131.5314	68.4931	1	14	(-)LQAAVQNTVQTEQK(-)	De Novo Sequencing
779.3933	2	1556.771	1556.816	0.0449	28.8558	133.4949	68.3544	1	15	(-)LQAAVQNTVQETGAK(-)	De Novo Sequencing
779.3933	2	1556.771	1556.816	0.0449	28.8558	133.5025	79.4521	1	14	(-)QLAAVQNTVQETQK(-)	De Novo Sequencing
655.3352	2	1308.6548	1308.668	0.0127	9.701	41.6201	72.1311	1	12	(-)GEPPSKVQQPDK(-)	De Novo Sequencing

MALDI-TOF

C:\MassLynxBackUp_1\Service Measurements.PRO\Data\HAE_DF_TN13G19_1.raw **Arylphorin**

Name	Score	% Probability	Peptide Mɛ	Coverage	mW	pI	Description	Average Mas	RMS Mass	% Modified Peptides	% Peptides with Missed Cleavage Sites
5869989	7.479	1.0964493	8	10.458	84059	7.119	arylphorin subu	37.658	41.996	0	12.5

Submitted Mass	Submitted	Experimental Mass	mW	Delta (Da)	Delta (ppm)	Probability	Ladder Score	Start	End	Sequence	Modificatio	Query Tool
941.542	1	940.535	940.502	-0.033	-34.653	-0.493	0	34	40	(K)FVEYQKK(I)		Databank Search
961.508	1	960.501	960.471	-0.03	-30.883	4.007	0	97	103	(K)FSIFYER(M)		Databank Search
949.468	1	948.46	948.423	-0.037	-39.255	0.147	0	120	126	(K)DFETFYK(T)		Databank Search
915.488	1	914.48	914.439	-0.041	-44.785	0.221	0	332	338	(R)FLDTYEK(T)		Databank Search
1058.597	1	1057.589	1057.56	-0.029	-27.586	0.828	0	339	346	(K)TFFQFLQK(A)		Databank Search
1300.706	1	1299.698	1299.625	-0.073	-55.978	1.824	0	417	426	(R)DPAFYQLYQR(I)		Databank Search
2058.058	1	2057.05	2057.048	-0.002	-0.949	1.088	0	436	452	(K)QYVKPYNHNDLHFVGVK(I)		Databank Search
1374.766	1	1373.758	1373.666	-0.092	-67.177	-0.144	0	542	551	(K)FYELDWFVQK(L)		Databank Search

C:\MassLynxBackUp_1\Service Measurements.PRO\Data\HAE_DF_TN31G19_1.raw **Gloverin**

Name	Score	% Probability	Peptide Mɛ	Coverage	mW	pI	Description	Average Mas	RMS Mass	% Modified Peptides	% Peptides with Missed Cleavage Sites
AF233590_1	7.67	1.3272074	4	22.414	19186.553	9.656	gloverin precur	24.389	27.354	0	25

Submitted Mass	Submitted	Experimental Mass	mW	Delta (Da)	Delta (ppm)	Probability	Ladder Score	Start	End	Sequence	Query Tool
1214.509	1	1213.501	1213.548	0.047	38.326	2.071	0	44	53	(R)DTTWEHNVGR(G)	Databank Search
938.456	1	937.448	937.462	0.014	15.04	3.43	0	85	93	(R)LSGQAYGSR(V)	Databank Search
1208.633	1	1207.625	1207.667	0.042	34.773	0.991	0	117	127	(R)AALDVHKEIGR(G)	Databank Search
921.471	1	920.463	920.472	0.009	9.416	1.177	0	147	155	(R)FSAGGNLQK(N)	Databank Search

Supplementary Table 2. Real-time quantitative PCR primers used in this study

Gene	Forward Primer Sequence 5`-3`	Reverse Primer Sequence 5`-3`
Elongation init. factor 4 alpha (control)	GTGAGCGCGAAGTTATTATGC	AGAAACTTGCTGCACGTCAAT
Apolipophorin III	TCGTTAAGGAAGTAGCCAGCA	AGTTTCTTCTGCACCTGTTC
Arylphorin	GAACAACTCGACCTTCACTCG	AGCCTCATTCCTCATCCTTTC
Kalicitin	GATGATCGACGCTAGAGATGC	CAGAATTTCCGGTCTCACTGT
Azurocidin	GAAGGCGGTCCTTTAGTATGC	GGTAGCCGATGATGACGTAGA
Lebocin	GAGGGTTGAGAGGAGTCTTGG	TTAGGAACGAATGGAGGTGTG
BGRP	AACCAGACGGCATCGAATTAT	GAAGACCAAGGGTCACATGAA
Hemolin	TTCTGTTCAGGGCTGACAACT	GAGCAACCTCTTTCCTCCAGT
Cecropin A	TTCGCTTGTCTGGTCTTCACT	CACGAATGTTCTGTCCAACCT
Cecropin B	ATATGAATTTCTCCCGCGTGT	GCCTTGATGATACCGTCTCTG
Cecropin D	ATTTGGAAGGAATTGGTCAGC	CTTATTCCCTTTGCTGCTGCT
Cobatoxin	TTACATCCCAATCCTGACGAG	CGGATGATGGTAGATAAGGTAGG
Defensin	CAATAAGCAGTGAAGCCTTGG	GCATATGCCGTAGTTGTAGCC
Gallerimycin	TGCATTGCCAGTTGTAGACAG	ATAGCCTCAAGCTCATCACCA
Gloverin	CTTGATGTCCACAAGCAGGTT	CAAAGGTCTTGTCCAGATTGC
HDD1	GGCTATACTACCGCGAGATCC	CGCCATTGTGGTCTTCTCTTA
Lysozyme (a)	ATGCGCCAAGAAGATCTACAA	GTTTAGCATTTGCTGATGTCG
Lysozyme (b)	TGTAGCAAGACCAGCACTCCT	CCTGGAACTTGTGACGTTTGT
PO inhib. enzyme	TGGCCTTCGTCTTCAAACTTA	CGACAGAACAACACGTTTACC
ProPO activating enzyme	AAGTCGGAAGAAGAGGTCGAG	CTGGCGTGTAACATGATCCTT
Ribosomal protein S18 (control)	TGTCCTATTTGTCGGGATGAG	TGGTCCCATGCTCTTTCTATG
Attacin	GGGAACCAAACTTCGGACTTA	CAGAGTCGGTGGCTACCTATAAA
GNBP	CGCTATGGTCGTGTTGAAGTT	CTGGATGTCAAGGTCATCGTT
BGBP	GCACTGGGCTAAAGGAACTGT	ATCGGGAAATTCGTTGATACC
Apolipophorin 3 (b)	GCACAATGGCAGCTAAGTTGT	GGTTGGTTCTTCGGAGTAAGG
Arylphorin (b)	AACTAGCTTCGTGATCCGTCA	TTAACTGGGTATCCGTTGCTG
ProPO activating enzyme (b)	ATGCATCGCGAAATACAGAAC	ATGCCTTCAGGCTTTCTCTTC
Elongation factor 1 alpha (control)	CGGTCAAATCTCAAACGGATA	CGTCACCAGACTTGATGGATT

3 Chapter 2. Bacterial feeding induces changes in immune-related gene expression and has trans-generational impacts in the cabbage looper (*Trichoplusia ni*)

Abstract

Background: Poly- and oligophagous insects are able to feed on various host plants with a wide range of defense strategies. However, diverse food plants are also inhabited by microbiota differing in quality and quantity, posing a potential challenge for immune system mediated homeostasis in the herbivore. Recent studies highlight the complex interactions between environmentally encountered microorganisms and herbivorous insects, pointing to a potential adaptational alteration of the insects´ physiology. We performed a differential gene expression analysis in whole larvae and eggs laid by parents grown on different diets to identify potential novel genes related to elevated microbial content in the caterpillars' food.

Results: We used GeneFishing, a novel differential display method, to study the effects of dietary bacteria on the general gene expression in different life stages and tissues of the cabbage looper (Trichoplusia ni). We were able to visualize several hundred transcripts on agarose gels, one fifth of which were differentially expressed between treatments. The largest number of differentially expressed genes was found in defense-related processes (13) and in recognition and metabolism (16). 21 genes were picked out and further tested for differential gene expression by an independent method (qRT-PCR) in various tissues of larvae grown on bacterial and bacteria-free diet, and also in adults. We detected a number of genes indicative of an altered physiological status of the insect, depending on the diet, developmental stage and tissue**.**

Conclusions: Changes in immune status are accompanied by specific changes in the transcript levels of genes connected to metabolism and homeostasis of the organism. Our findings show that larval feeding on bacteria-rich diet leads to substantial gene expression changes, potentially resulting in a reorganization of the insects´ metabolism to maintain organismal homeostasis, not only in the larval but also in the adult stage. Furthermore, differences in gene expression levels can also be seen in the next generation, strongly influenced by parental diet.

3.1 Introduction

Most Lepidopteran larvae are herbivorous and many among them are important pests in agriculture, causing severe damage to various crop plants growing in monocultures. The level of specialization even within a Lepidopteran family can vary dramatically. Larval feeding can be restricted to a specific plant part, like leaf material only or it can be extended to allow exploiting various plants including different parts of the plant (e.g. leaves, stem, flowers, and fruits) as a food source. In addition to the enormous variation in defensive proteins and secondary metabolite production, different parts of the plant are inhabited by different microorganisms (Meyling & Eilenberg, 2006). Feeding on different plants and plant organs or even moving up and/or down on the leaves of the same plant is accompanied by potential changes in the ingested microflora, both qualitatively and quantitatively. Previously (Freitak et al., 2007) we showed that feeding on large amounts of essentially non-pathogenic bacteria

causes substantial changes in the immune status of larvae of the cabbage looper (*Trichoplusia ni*). Changes can be seen in immune response related enzyme activities and protein expression in the hemolymph, but also in transcription of immune-related genes in midgut tissue. Moreover, fitness related traits are impaired in animals due to ingestion of large amounts of bacteria in comparison to larvae feeding on sterile diet.

The mounting of immune responses is costly (Freitak et al., 2003) and can result in severe autoimmune effects in insects (Sadd & Siva-Jothy, 2006; Nappi & Christensen, 2005). However, very little is known about the accompanying changes in metabolic processes and the physiology of insects in the course of immune responses. This probably stems from the fact that researchers have mostly focused on known immune effectors and have also often restricted their analysis to direct immune repertoire cells, like hemocytes. A number of physiological changes taking place in the body during any immune insult may not be directly linked to the immune system, but to dealing with harmful side effects of the targeted immune response, allowing the organism to maintain homeostasis under stressful conditions.

An increasing amount of genomic data is accumulating for numerous invertebrates, as whole genome sequences are available now for honey bee (*Apis mellifera*) (The Honeybee Genome Sequencing Consortium, 2008), fruitfly (*Drosophila melanogaster*) (Adams et al., 2000), mosquito (*Anopheles gambie*) (Holt et al., 2002), and the flour beetle (*Tribolium castaneum*) (*Tribolium* Genome Sequencing Consortium, 2008), and this has led to the flourishing of comparative immunology as an approach to study host-parasite interactions. Although the screening of various EST libraries and comparing strictly immune induced markers has revealed much information about immunity, this approach is based on previously identified genes from other organisms. This leads to the situation where it is hard to study new factors associated with a changed immune status, not necessarily directly involved in classical comprehension of the immune response. Furthermore, most studies focus on strictly pathogenic interactions. We therefore applied a random screening approach to identify novel genes involved in immune status changes of *T. ni*. We chose the GeneFishing method, a novel differential display technique, in order to study differential gene expression in a system with very little prior DNA sequence information.

In our study we examined global gene expression level differences, dependent on the dietary conditions of an herbivorous Lepidopteran larva. Transcripts of two and seven day old larvae grown on plants, on bacteria-supplemented and on non-supplemented artificial diet were compared. In addition we wanted to address the question whether parental diet can induce changes in gene expression in the following generation. To accomplish that, we

compared the transcripts of eggs laid by parents grown on bacterial and bacteria-free diet. We show that changes in immune status are accompanied by specific changes in the transcript levels of genes connected to metabolism and homeostasis of the organism.

3.2 Results and Discussion

3.2.1 Differential gene expression analysis in dietary challenged *T.ni* larvae and eggs

We examined gene expression patterns using GeneFishing, a PCR-based differential display technique. Analysis was carried out using purified RNA from 2 and 7 day old whole larvae and eggs. After 40 PCR cycles, agarose gel electrophoresis revealed cDNA bands ranging in size from 100 bp to 2.5kb. Using a combination of 60 arbitrary ACP primers and two anchored oligo(dT) primers, a total of 323 bands were visualized on agarose gels. 60 bands were identified as differentially expressed between larvae fed on three different diets and those bands were cut out of the agarose gels, purified, cloned and sequenced. In the case of eggs, 31 differentially expressed bands were identified out of 141 total bands. Obtained sequences were compared to the National Center for Biotechnology Information databases using a blastx program (Table 1).

Like all differential display techniques, the GeneFishing method can be expected to reveal a subset of expression differences; and may be biased towards more abundant transcripts. Although relatively new, it has already been applied to studies of animal neurophysiology (Bevan et al. 2008), cancer (Choi et al. 2007) and plant allellopathy (Junaedi et al 2007) and development (Park 2006). So far a quantitative evaluation of the technique compared with others such as cDNA-AFLP, RAPD-differential display, or microarray analysis has not been carried out. Thus we selected 21 genes for further testing by an independent method (qRT-PCR) for differential gene expression in various tissues of larvae grown on bacterial and bacteria-free diet, and also in adults.

3.2.2 Physiological changes upon feeding on different diets in larvae

We were able to identify 102 differentially expressed genes among larvae grown on different diets (Table 1). Several of these genes show age dependent expression levels, being influenced by diet only at certain developmental stages. We divided the identified genes into

eight functional categories/clusters – defense and recognition, development, digestion, DNA-related, metabolism, ribosomal proteins, signaling and genes with unknown function. In addition we also listed the transcripts which gave no significant hit to any known protein or expressed sequence tag (EST) library (Table 1). In total we were unable to identify 49 of our transcripts via Blast searches. The reasons we failed to identify a number of transcripts could be partially embedded in the approach we took for studying global gene expression patterns. Due to the methodology of the GeneFishing technique, we mainly amplify regions of the mRNA close to the polyA tail and the 3´ UTR region of the transcript, which is not the most informative for identification of the gene, as it contains non-coding sequence. This method in combination with the lack of the sequence information when studying a non-model organism can lead to difficulties with gene identification.

When examining the transcripts involved in defense and recognition we found several strictly immune response related genes, but also general stress and detoxification related indicators (Table 1). The immunity related genes show a very interesting expression pattern when comparing larvae feeding on different diets. The immune inducible effector molecule gloverin (Lundström et al., 2002) is highly abundant in 2 and 7 day old larvae fed on plant and bacterial diet. The expression patterns for hemolin and HDD1, both known to be part of the immune response and up regulated upon immune challenge and bacterial feeding (Freitak et al., 2007), are more complex. We see higher expression of hemolin in 2 day old bacteria-free diet fed larvae and 7 day old bacterial and plant diet larvae. HDD1 is expressed in all the larval stages and eggs at the same level, with the exception of a higher expression in 7 day old bacterial diet fed larvae. Hemolin is an immune protein in Lepidoptera, participating in phenoloxidase mediated immune responses (Terenius, et al., 2007, Labropoulou, et al., 2008), and its silencing in *Hyalophora cecropia* pupae is lethal for the next generation (Bettencourt, et al., 2002). Hemolin is supposedly also involved in antiviral defense (Terenius, 2008), whereas the function of HDD1 in immunity is not really known so far (Shin et al., 1998). A cathepsin L-like protease was up regulated in 2 day old larvae grown on plant and bacteria-free diet in comparison to bacterial diet fed larvae, and it was highly expressed in 7 day old bacteria fed larvae. Cathepsin L-like proteases are cystein proteases, which are known to participate in tissue remodeling during insect metamorphosis (Kanost & Clarke, 2005). In the case of vertebrates, this enzyme is also known to be involved in immunological processes, and in the leech *Theromyzon tessulatum* cathepsin L is involved in direct immune responses (Lefebvre et al., 2008). We observed the down regulation of a C-type lectin receptor gene in 2 day old larvae fed on plants and in 7 days old larvae fed on bacterial diet (Table 1). These

lectin receptors are involved in antifungal immunity mediating fungal binding, uptake and killing, and are probably also contributing to initiation and/or modulation of the immune response of the whole organism (Williment & Brown, 2008). Most interestingly we also observed the down regulation of C-type lectins, possibly implying that also general immune response related genes are influenced upon bacteria-rich diet. In case that lectins are also involved in antifungal defenses of *T. ni*, then down regulation of this type of defense-related genes could be caused by trade-offs between various immune system components, e.g. antibacterial vs. antifungal defenses. This is in good correlation with our previous findings, where we see several immune inducible genes up regulated in midgut tissue upon larval exposure to bacterial diet (Freitak et al., 2007). Two different cytochrome c related genes were up regulated in 2 and 7 days old larvae fed on plant and bacterial diet (Table 1). Cytochromes c (cytC) are electron-transfer proteins, having one or more heme c groups attached to proteins. Cytochromes c possess a wide range of properties and function in a large number of different redox processes, present in bacteria and mitochondria (Ambler, 1991; Luo et al., 2008). Seven day old plant feeding larvae also show a higher expression of a glutathione S-transferase (GST) when compared to the other diets. GST proteins are known to be involved in insecticide and plant toxin detoxification. The up regulation of a GST in the larvae feeding on plants could thus be explained by a strict correlation with the detoxification of plant secondary compounds (Ranson & Hemingway, 2005) rather than a response to the bacterial load of the plant material. We can assume that our observation of the up regulation of several defense reaction-related transcripts in the larvae feeding on plant and bacterially challenged diet to be symptomatic of increased stress levels for the organism. Up regulation of immune responses is considered costly, as it involves often release of multiple cytotoxic molecules, what cause activation of other stress related defenses (Nappi & Christensen, 2005; Adamo, 2009; Altincicek et al., 2008).

Table 1. cDNAs from GeneFishing for Trichoplusia ni. Expression data, based on agarose gel banding pattern is shown for different life stages (2B – 2 day old larvae on bacterial diet, 2N – 2 day old larvae on bacteria-free diet, 2P – 2 day old larvae on cabbage plants; 7B – 7 day old larvae on bacterial diet, 7N – 7 day old larvae on bacteria-free diet, 7P – 7 day old larvae on cabbage plants; EN – eggs laid by parents grown on bacteria-free diet, EB - eggs laid by parents grown on bacterial diet). Banding pattern is shown in correlation with band visibility on agarose gel (- … band is absent, + … band is visible, ++ … strongly visible band).

Cluster	Accession No	Best BlastX match	e value	Banding pattern on agarose gel							
				2N	2P	2B	7N	7P	7B	EN	EB
Defence and recognition	GH270329	ABQ43785: mitochondrial cytochrome c oxidase subunit VIa	3.00E-06	-	+	++	-	+	++	-	-
	GH270411	ABV68856.1: gloverin [Trichoplusia ni]	2.00E-07	-	++	++	-	++	++	-	-
	GH270330	NP_001106738: ubiquinol-cytochrome c reductase [Bombyx mori]	2.00E-99	-	-	++	-	+	-	-	-
	GH270331	NP_001037299: Mn superoxide dismutase [Bombyx mori]	9.00E-95	+	+	+	+	+	+	++	+
	GH270389	NP_001040131.1: GST omega1 [Bombyx mori]	5.00E-52	+	+	+	+	++	+	+	+
	GH270405	ABV68857.1: Hdd1-like protein [Trichoplusia ni]	7.00E-14	+	+	+	+	+	++	+	+
	GH270332	P_001040251: lectin 4 C-type lectin [Bombyx mori]	1.00E-96	+	-	+	+	+	-	-	+
	GH270378	ACC91897.1: hemolin [Heliothis virescens]	5.00E-15	+	-	-	-	+	+	-	-
	GH270333	AQ75437: cathepsin L-like protease [Helicoverpa armigera]	7.00E-75	++	+	-	-	-	++	+	++
	GH270348	XP_974384: PREDICTED: similar to homologue of Sarcophaga	1.00E-33	-	-	-	-	-	++	-	-
	GH270334	NP_001106742: cytochrome c oxidase polypeptide Vb [Bombyx mori]	9.00E-47	-	-	-	-	-	-	+	++
	GH270337	AAS79891: gst1 [Spodoptera litura]	9.00E-11	-	-	-	-	-	-	-	++
	GH270339	NP_001037057: BCP inhibitor [Bombyx mori]	2.00E-04	-	-	-	-	-	-	+	-
Development	GH270340	BAG30780: muscle protein 20-like protein [Papilio xuthus]	3.00E-09	-	-	++	-	-	++	-	-
	GH270341	XP_001662353: hypothetical protein AaeL_AAEL012245 [Aedes	4.00E-39	+	-	+	++	++	+	-	++
	GH270382	XP_001988215: GH10690 [Drosophila grimshawi], contains chitin	5.00E-11	-	-	-	-	-	-	++	+
	GH270345	NP_001108405: titin1 [Bombyx mori]	4.00E-05	-	-	-	-	-	-	+	++
Digestion	GH270346	AAA29341: trypsin [Manduca sexta]	3.00E-40	-	-	++	-	-	++	+	+
	GH270365	BAD22559: lipase [Antheraea yamamai]	7.00E-50	+	+	+	+	++	+	-	-
	GH270347	AAV91434: serine protease 3 [Lonomia obliqua]	2.00E-52	+	-	+	+	-	+	-	-
	GH270394	P35045: Trypsin, alkaline A precursor	2.00E-39	-	++	-	+	++	+	-	-
	GH270343	XP_972363.2: similar to trypsin-like serine protease [Tribolium	6.00E-122	+	+	-	-	-	+	-	-
	GH270376	XP_001650916: tyrosine-protein kinase [Aedes aegypti]	2.00E-33	-	-	-	+	-	-	-	-
DNA related	GH270368	NP_001040495: C14orf124 protein [Bombyx mori]	5.00E-20	-	-	++	-	-	-	-	+
	GH270385	XP_001952287: PREDICTED: similar to chromosome 19 open	6.00E-22	++	++	++	++	++	++	+	-
	GH270400	XP_001200852: PREDICTED: similar to histone H2B (aa 1-123)	6.00E-07	++	-	-	++	++	-	-	++
Metabolism	GH270353	NP_001091831: enolase [Bombyx mori]	9E-58	+	+	++	+	+	++	+	+
	GH270417	XP_001960088: GF13192 [Drosophila ananassae], similar to short-	4.00E-23	+	+	++	+	+	+	+	++
	GH270358	XP_552371: AGAP011872-PA [Anopheles gambiae str. PEST],	2.00E-66	+	+	++	+	+	++	++	++
	GH270375	EEA92944.1: alcohol dehydrogenase (acceptor) [Pseudovibrio sp.	4.00E-11	+	+	++	+	+	+	+	++
	GH270397	NP_001040294: protease inhibitor 1 [Bombyx mori]	8.00E-18	-	-	+	-	-	-	-	-
	GH270384	NP_001037171: protein disulfide isomerase [Bombyx mori]	2.00E-100	-	-	+	-	-	+	-	-
	GH270355	XP_396707: PREDICTED: similar to Serine/threonine-protein kinase	4.00E-47	-	-	+	++	-	-	++	+
	GH270409	NP_001040233: ATP synthase [Bombyx mori]	7.00E-146	-	-	+	++	-	-	-	-
	GH270415	XP_001599579: PREDICTED: similar to ENSANGP00000017562	8.00E-28	+	+	-	-	-	+	-	-
	GH270418	NP_001040310: light-induced protein-like brain protein 44 [Bombyx	2.00E-34	++	-	-	++	-	-	-	++
	GH270388	NP_001040436: hydroxysteroid dehydrogenase [Bombyx mori]	8.00E-56	-	-	-	-	-	++	-	-
	GH270407	XP_564957: Elongase_AGAP007264-PA [Anopheles gambiae str.	2.00E-45	-	-	-	-	-	++	+	-
	GH270433	ABG77272: ubiquitin-53aa extension protein [Pieris rapae]	7.00E-05	-	-	-	-	-	-	+	++
	GH270423	NP_001037047: silk proteinase inhibitor [Bombyx mori]	4.00E-10	-	-	-	-	-	-	+	-
	GH270386	AAL60239: takeout [Aedes aegypti]	1.00E-08	-	-	-	-	-	-	++	+
Ribosomal protein	GH270406	ABS57435: ribosomal protein S25 [Heliconius melpomene]	1.00E-27	++	++	++	++	++	++	+	-
	GH270431	AAK92157: ribosomal protein L14 [Spodoptera frugiperda]	4.00E-06	+	-	++	+	+	++	-	-
	GH270381	AAN86048: ribosomal protein S2 [Spodoptera frugiperda]	4.00E-49	-	-	++	-	-	-	-	-
	GH270374	AAL26578: ribosomal protein S3 [Spodoptera frugiperda]	1.00E-51	+	+	+	+	++	+	+	+
	GH270396	AAV91399: ribosomal protein 27 [Lonomia obliqua]	4.00E-11	+	+	+	+	+	+	++	-
	GH270410	NP_001091753: ribosomal protein L36A [Bombyx mori]	2.00E-29	++	-	-	+	+	-	-	++
	GH270377	NP_001037570: ribosomal protein S15A [Bombyx mori]	2.00E-30	-	-	-	-	-	-	++	+
	GH270416	AAV91403: ribosomal protein 5 [Lonomia obliqua]	3.00E-26	-	-	-	-	-	-	+	-
Signalling	GH270352	NP_724186: Paxillin [Drosophila melanogaster]	1.00E-111	+	+	++	+	+	++	-	+
	GH270363	XP_974400: PREDICTED: similar to lots wife CG33968-PA, Nose	8.00E-12	+	+	+	+	+	-	-	+
	GH270422	XP_001599614.1: signal peptidase 12kda [Nasonia vitripennis]	4.00E-07	+	+	-	-	++	+	+	+
	GH270420	NP_001040123: electron-transfer-flavoprotein beta polypeptide	4.00E-32	-	-	-	-	-	-	-	+
Unknown	GH270366	XP_001604274.1: similar to CG12009-PA [Nasonia vitripennis]	3.00E-04	+	++	+	-	-	+	-	-
	GH270399	XP_001121582: PREDICTED: similar to CG10710-PA [Apis	1.00E-06	++	+	+	++	+	+	+	++
No significant match	GH270336	YP_001329156: glyoxalase/bleomycin resistance	e>-1	++	+	++	-	-	-	++	+
	GH270350	XP_001421381: predicted protein [Ostreococcus lucimarinus	e>-1	+	+	++	+	++	++	+	+
	GH270359	XP_001759276: predicted protein [Physcomitrella patens subsp.	e>-1	+	+	++	+	+	+	+	++
	GH270390	XP_001717683: PREDICTED: hypothetical protein [Homo sapiens]	e>-1	+	+	++	+	+	+	++	++
	GH270429	NP_001040436: hydroxysteroid dehydrogenase [Bombyx mori]	e>-1	-	+	++	-	+	++	-	-
	GH270401	P_001943585: PREDICTED: similar to zinc finger protein 624	e>-1	+	-	++	-	-	-	-	-
	GH270403	EAW95139.1: serine arginine repetitive matrix 1 [Homo sapiens]	e>-1	+	-	++	-	-	-	-	-
	GH270428	YP_001744939: hypothetical protein EcSMS35_2915 [Escherichia	e>-1	+	-	++	-	-	-	-	-
	GH270387	NP_927355: hypothetical protein glr4409 [Gloeobacter violaceus	e>-1	+	-	++	-	-	-	-	-
	GH270403	ZP_01465929: hypothetical protein STIAU_3143 [Stigmatella	e>-1	+	-	++	-	-	-	-	-
	GH270351	BAB89324: putative G-protein coupled receptor [Homo sapiens]	e>-1	+	-	+	+	+	+	-	-
	GH270356	XP_001647881: Gustatory receptor 61a, putative [Aedes aegypti]	e>-1	++	+	+	++	+	+	+	++
	GH270389	ABU41034: hypothetical protein [Lepeophtheirus salmonis]	e>-1	+	+	+	+	++	+	+	+
	GH270395	AAA26387: outer membrane protein A [Rickettsia akari]	e>-1	+	+	+	++	++	+	-	-
	GH270424	ZP_02747435: two-component sensor histidine kinase [Clostridium	e>-1	-	+	-	-	-	-	-	-
	GH270342	XP_001867382: myosin I [Culex pipiens quinquefasciatus]	e>-1	++	+	-	-	+	+	-	-
	GH270362	YP_001910237: putative secretion/efflux abc transporter, ATP-	e>-1	++	-	-	+	+	+	-	-
	GH270338	CAA43583: hydroxyproline-rich glycoprotein [Oryza sativa (indica	e>-1	-	-	-	-	-	-	++	+
	GH270335	XP_001952211: PREDICTED: similar to corneal wound healing-	e>-1	-	-	-	-	-	++	-	-
	GH270344	XP_001017307: Chitinase class I family protein [Tetrahymena	e>-1	-	-	-	-	++	-	-	-
	GH270413	NP_508266: Serpentine Receptor, class H family member (srh-19)	e>-1	-	-	-	-	-	-	+	++
	GH270391	XP_318947: Porin AGAP009833-PA [Anopheles gambiae str. PEST]	e>-1	-	-	-	++	++	-	-	-
	GH270404	XP_821286: hypothetical protein Tc00.1047053506401.350	e>-1	-	-	-	++	++	-	-	-
	GH270367	YP_812104: hypothetical protein LACR_2573 [Lactococcus lactis	e>-1	-	-	-	-	-	-	-	+
	GH270426	XP_001652430: hypothetical protein AaeL_AAEL001147 [Aedes	e>-1	-	-	-	-	-	-	+	++
	GH270432	ZP_01222271: hypothetical protein P3TCK_18689 [Photobacterium	e>-1	-	-	-	-	-	-	+	-
	GH270357	XP_976032: PREDICTED: hypothetical protein [Tribolium	e>-1	-	-	-	-	++	+	++	+
Hit only against EST	GH270392	EB827756: 1151973 KZ03 Plodia interpunctella cDNA clone 584001,	9.00E-08	++	+	++	++	+	++	-	-
	GH270360	Sf1F01923-3-1 Spodoptera frugiperda Fat Body cDNA Library	4.00E-12	+	+	++	+	+	++	+	+
	GH270373	CF258147: 90 Trichoplusia ni fifth instar digestive system cDNA	6.00E-63	+	+	+	-	+	++	-	-
	GH270371	EY265223: BF01036X1D07.f1 Normalized subtracted keck library	8.00E-07	-	-	+	-	-	-	+	+
	GH270425	AT001017: AT001017 Bombyx mandarina library (Hwang JS)	2.00E-19	-	-	+	+	+	+	-	-
	GH270354	TN-LN-384-G-03-libF_J07 Trichoplusia ni whole larvae normalized	7.00E-85	-	+	-	-	+	-	+	-
	GH270398	FF375565: TN-28-lipF_D07 Trichoplusia ni larval non-normalized	6.00E-42	+	+	-	+	++	+	+	+
	GH270412	FF377777: TN-58-pDNR-lipF_N24 Trichoplusia ni larval non-	5.00E-16	++	-	-	+	+	-	-	++
	GH270419	FF377954:TN-60-pDNR-lipF_N13 Trichoplusia ni larval non-	6.00E-72	-	-	-	+	-	-	-	-
	GH270379	EX212089: EST # 0000117 Spodoptera litura oxidative-stress	2.00E-50	-	-	-	-	-	-	+	-
No hit at all	GH270393	No hit against EST databases as well		-	+	+	+	+	+	-	-
	GH270408	No hit against EST databases as well		++	+	+	++	+	+	-	++
	GH270372	No hit against EST databases as well		-	-	+	-	-	-	-	-
	GH270427	No hit against EST databases as well		-	-	+	-	-	-	+	+
	GH270364	No hit against EST databases as well		+	+	-	+	+	-	++	++
	GH270421	No hit against EST databases as well		++	-	-	++	-	-	-	++
	GH270349	No hit against EST databases as well		++	-	-	++	-	-	-	++
	GH270369	No hit against EST databases as well		-	-	-	-	-	+	+	-
	GH270370	No hit against EST databases as well		-	-	-	-	+	+	++	+
	GH270414	No hit against EST databases as well		-	-	-	-	-	-	+	+

Among the genes related to insect development we identified a member of the chitinase gene family and two genes involved in muscle development. There is no clear diet dependent expression pattern of these genes (Table 1). Differences in the expression of genes involved in developmental processes are possibly related to the physiological costs for feeding on nutritionally (plant *vs.* artificial diet) and microbially (bacterial *vs.* bacteria-free diet) different diets. These might be associated to delayed development and reduced pupal masses, as could be seen in our previous study (Freitak et al., 2007). Such negative effects in life-history traits are often accompanied with reduced reproductive success (Schmid-Hempel, 2005a).

The differential gene expression analysis revealed altered expression levels of various digestive protein genes depending on the diet (Table 1). Major differences could be observed when comparing larvae fed on plant diet and both artificial diets. Both a trypsin and a lipase show a higher expression level in 7 day old larvae fed on plant diet and trypsin also in the 2 day old larvae fed on similar diet. These changes in expression levels could be linked to the occurrence of protease inhibitors in leaf tissue (Terra & Ferreira, 2005) or to overall differences in lipid and protein concentrations in the plant tissue in comparison to the optimal artificial diet. There is no clear presence or absence pattern in the expression of genes coding for digestive enzymes, but differences in expression levels. Bacterial diet and bacteria-free diet show no differences in the expression of digestive enzyme coding genes, with the exception of one trypsin, which is expressed only in bacteria fed larvae. Thus the costs of fitness-related traits, seen with larvae feeding on bacterial and bacteria-free larvae (Freitak et al., 2007) are probably not directly linked to a complete remodeling of the digestive processes.

Several metabolism related genes were identified as differentially expressed in our analysis. Enolase was found to be highly expressed in 2 and 7 day old larvae fed on bacterial diet. Enolase is a metalloenzyme with catalytic activity involved in glycolysis and is present in all tissues and organisms capable of glycolysis or fermentation (Gerlt et al., 2005). 2 day old larvae fed on bacterial diet had higher mRNA levels for a glucose-methanol-choline oxidoreductase (GMC oxidoreductase) and protease inhibitor 1. GMC oxidoreductases belong to a large family of diverse FAD enzymes. In insects these enzymes are often involved in the regulation of common developmental and physiological processes related to ecdysteroid metabolism (Iida et al., 2007) but in many cases their functions are still unknown. Protease inhibitors are a class of proteins involved in regulating the activity of various endogenous and exogenous proteases. However, they can also have a role in digestive processes, as well as defense or development (Terra & Ferreira, 2005). We could also identify a number of

enzymes involved in ATP binding and synthesis and several ribosomal proteins to be differentially expressed in 2 and 7 day old larvae grown on different diets (Table 1). When examining the expression pattern of genes putatively identified as proteins involved in signaling, we detected a general trend of higher expression levels in plant and bacterial diet fed larvae (Table 1). The changes in genes involved in general metabolism advocate serious constraints exerted on the organism by alteration of the quality of the diet plant vs. artificial but also bacterial vs. bacteria-free diet.

3.2.3 Tissue-specific differential gene expression

To examine the expression of a selected subset of genes more closely within the organism, we used midguts and non-midgut tissue (the rest of the body) of 9 day old *T. ni* larvae grown on bacterial and bacteria-free diet. We found a number of genes to be down regulated in the midgut tissue of bacteria fed larvae, namely GMC oxidoreductase, lectin 4, a protease inhibitor and titin1. Glutathione S-transferase 1 was the only gene we found to be down regulated in the rest of the body of bacteria-fed larvae, whereas GMC oxidoreductase, a putative G-protein coupled receptor, alcohol dehydrogenase, tyrosine-protein kinase and a serine protease were up-regulated (Table 2). The alcohol dehydrogenase belongs to the short-chain dehydrogenases/reductases (SDR), a large enzyme family, most of which are known to be NAD- or NADP-dependent oxidoreductases (Kallberg et al., 2002). These alterations are possibly connected with an elevated immune status (Freitak et al., 2007) caused by feeding on bacteria rich diet and could be directed to deal with harmful side effects of the elevated immune status and/or help in the case of possible infection.

Table 2. RT-qPCR results for 9 day old larval midguts and restbodies for genes identified by GeneFishing. Results are shown for animals grown on bacterial diet, where bacteria-free diet treatments were set to 1 (values are mean ± SD).

Gene	Midgut	ct value (Bac)	Restbody	ct value (Bac)
Defence				
cathepsin L-like protease	1.77 ± 0.018	19.99	1.17 ± 0.034	19.22
cytochrome c oxidase polypeptide Vb	1.07 ± 0.074	17.81	-1.08 ± 0.094	18.61
mitochondrial cytochrome c oxidase subunit VIa	-1.02 ± 0.115	17.93	-1.15 ± 0.004	19.3
Mn superoxide dismutase	-1.03 ± 0.026	20.18	-1.75 ± 0.030	20.38
lectin 4 C-type lectin	**-3.49 ± 0.048**	25.53	-1.96 ± 0.129	21.9
Glutathion S-transferase 1	1.80 ± 0.037	20.35	**-9.17 ± 0.058**	27.94
ubiquinol-cytochrome c reductase	-1.16 ± 0.005	19.27	-1.10 ± 0.014	19.74
Development				
trypsin-like serine protease	-1.35 ± 0.212	32.96	**2.02 ± 0.037**	
hypothetical protein AaeL_AAEL012245, contains chitin binding domain	-1.29 ± 0.210	31.98	-1.42 ± 0.163	31.89
Perithrophin A	**-78.25 ± 0.067**	29.15	-1.28 ± 0.148	28.17
myosin I	-1.27 ± 0.014	22.73	-1.72 ± 0.035	22.47
titin1	**-2.32 ± 0.018**	20.1	1.29 ± 0.028	16.64
Digestion				
tyrosine-protein kinase	-1.40 ± 0.134	25	**3.96 ± 0.007**	28.89
DNA related				
C14orf124 protein	-1.03 ± 0.023	22.48	-1.15 ± 0.030	21.59
zinc finger protein	-1.05 ± 0.065	21.13	-1.08 ± 0.267	21.97
Metabolism				
alcohol dehydrogenase (acceptor)	**-2.05 ± 0.939**	19.8	**2.25 ± 0.062**	22.76
protease inhibitor 1	**-2.45 ± 0.325**	29.81	-1.18 ± 0.131	19.04
Serine/threonine-protein kinase polo	-1.45 ± 0.097	31.06	-1.17 ± 0.048	26.2
short-chain dehydrogenase	-1.20 ± 0.018	21.09	**6.12 ± 0.117**	26.29
protein disulfide isomerase	-1.09 ± 0.078	17.63	1.25 ± 0.074	17.66
Signalling				
putative G-protein coupled receptor	-1.40 ± 0.25	19.31	**9.48 ± 0.129**	25.49

3. 2. 4 Differential gene expression in *T. ni* adults

21 selected genes were also examined for differential expression in pooled adult female and male insects, and three were found to differ in expression levels between the diet the larvae had encountered. A putative chitin binding protein was down regulated and an alcohol dehydrogenase up regulated (Table 3) if pooled mRNA from both sexes was examined. In addition, we studied the expression of some of the genes in both sexes separately, and we could find higher levels of cytochrome c oxidase and alcohol dehydrogenase transcripts in bacteria-fed males. With the exception of these three genes we were not able to see any differential expression in genes selected from our GeneFishing analysis in the adult stage of *T.ni*. The relatively small number of differentially expressed genes found in the adult stage could be due to the fact that we have pre-selected gene

candidates based on expression data in larvae, and the physiological requirements for adult moths are quite different from those of larvae.

Table 3. RT-qPCR results for adults fed on different diets for genes identified by GeneFishing. Results are shown for animals grown on bacterial diet, where bacteria-free diet treatments were set to 1 (values are mean ± SD).

Gene	Genders pooled	Females	ct value	Males	ct value
Defence					
cathepsin L-like protease	1.01 ± 0.100				
cytochrome c oxidase polypeptide Vb	1.41 ± 0.010	1.02 ± 0.046	18.23	**2.16 ± 0.158**	17.46
mitochondrial cytochrome c oxidase	1.29 ± 0.045				
Mn superoxide dismutase	1.27 ± 0.050	-1.01 ± 0.036	19.87	1.89 ± 0.097	19.57
lectin 4 C-type lectin	1.47 ± 0.005				
Glutathion S-transferase 1	1.04 ± 0.310	1.59 ± 0.258	30.63	1.54 ± 0.236	30.38
ubiquinol-cytochrome c reductase	1.01 ± 0.070				
Development					
trypsin-like serine protease	-1.19 ± 0.040				
hypothetical protein AaeL_AAEL012245, contains chitin binding domain	**-2.91 ± 0.240**	-1.43 ± 0.050		1.55 ± 0.015	
Perithrophin A	-1.08 ± 0.115				
myosin I	-1.18 ± 0.115				
titin1	-1.42 ± 0.090	-1.06 ± 0.236	19.52	1.17 ± 0.356	18.43
Digestion					
tyrosine-protein kinase	-1.31 ± 0.000				
DNA related					
C14orf124 protein	1.07 ± 0.060				
zinc finger protein	-1.18 ± 0.070				
Metabolism					
alcohol dehydrogenase (acceptor)	**2.20 ± 0.005**	1.44 ± 0.285	28.36	**2.08 ± 0.030**	30.64
protease inhibitor 1	1.52 ± 0.010	1.04 ± 0.175	19.56	1.17 ± 0.305	16.42
Serine/threonine-protein kinase polo	-1.20 ± 0.045				
short-chain dehydrogenase	1.15 ± 0.175				
protein disulfide isomerase	1.16 ± 0.020				
Signalling					
putative G-protein coupled receptor	-1.64 ± 0.110	1.05 ± 0.245	27.92	-1.41 ± 0.150	30.15

3 .2. 5 Differential gene expression in T. ni eggs laid by parents grown on different diets

Our analysis shows that a number of defense related genes are differentially expressed between eggs laid by parents grown on bacterial (EB) or bacteria-free (EN) diet. Cytochrome C oxidase and GST1 are highly expressed in EB eggs, whereas a BCP inhibitor-like gene and Mn superoxide dismutase transcripts are more abundant in EN eggs (Table 1). GST1, like cytochrome C, belongs to a large family of proteins also involved in various detoxification processes (Ranson & Hemingway, 2005). BCP inhibitor is a cysteine proteinase inhibitor, which has been isolated and characterized from *Bombyx mori* eggs and is known to be involved in degradation of yolk proteins (Yamamoto et al., 1999). We also found two developmental gene transcripts highly abundant in EB eggs, namely titin1 which is known to be important in muscle development (Tskhovrebova & Trinick, 2003) and a hypothetical chitin binding domain containing protein. In addition to developmental genes we have also

identified two genes with DNA binding function to be highly expressed in EN and EB eggs, namely ORF 29 protein and histone H2B protein. As already seen in the larvae, also in the eggs a number of ribosomal proteins showed differential expression depending on the diet (Table 1).

Taken together, these results indicate that environmental conditions experienced by larvae of the parental generation can have substantial effects on the physiology of the next generation, and that these effects can be measured at the gene expression level. If expression differences in eggs carry over into the larvae that develop from them, this would support the idea that parents are able to prime their offspring against possible environmental stressors, like increased microbial content, but probably also other factors. The phenomenon of trans-generational priming of immunity has been reported for insects in studies that show increased survivorship or tolerance of infection among offspring of pre-exposed parents; however the underlying genes have not yet been investigated (Little et al., 2003; Moret & Siva-Jothy, 2003; Rahman et al., 2004; Roth et al., 2009). In the present study we show that trans-generational priming of genes expressed in the eggs can be caused by exposure to bacteria in the parental diet. In a separate study (Freitak et al, *submitted*) we have extended these findings to genes expressed larvae of the offspring generation. These findings draw attention to the parental environment, as one of the factors influencing the phenotype of the organism. It can be one of the major sources for the phenotypic variation of several physiological features influencing fitness and reproduction. Trans-generational effects require more attention in studies of the factors shaping the ecology and physiology of organisms.

3.3 Conclusions

The ecophysiology of any organism is a complex and multifaceted set of processes involving responses to all possible changes in biotic and abiotic environmental factors. Important biotic stress related effectors of the physiology of any organism are diet and defense linked changes in habitat. Innate immunity is a central part of the insects' defense mechanism for dealing with physiological adaptations to biotic stressors in the form of parasites and pathogens. The immune regulatory pathway cascades include a huge number of different cellular and humoral components, many of which are in tight and complex relation to other physiological processes.

Here we report a comparative analysis of differentially induced transcripts for *T.ni* larvae and eggs laid by parents grown on bacterially challenged diet. We selected a differential gene expression study method that does not require previous sequence information, as we use a non-model Lepidopteran species. Comparing transcripts of whole larvae fed on three different diets shows that changes in gene expression are connected with different processes related to metabolism and homeostasis. We also detected several immunity related genes with our differential gene expression analysis. Gloverin, HDD1 and hemolin have been found to be highly abundant also in midgut tissue of bacterial feeding larvae (Freitak et al., 2007). Changes in immune status are accompanied by alterations in the expression of genes coding for diverse physiological processes. We were able to identify a number of genes showing diet dependent expression, linked to an adjusted physiological status. We observed different expression patterns for a number of classically stress-related genes among others. This is in good correspondence with studies indicating that a variety of stresses can affect immune function in insects (Brey, 1994) and that immune response and alteration of immune status are stressful for an organism (Adamo, 2009; Altincicek et al., 2008). It is also clear that these differences are not necessarily the same in different life stages and/or tissues. Furthermore, gene expression in eggs produced by individuals exposed to dietary bacteria was affected in some cases, indicating the potential for transgenerational transmission of an immune response.

3.4 Methods

3.4.1 Animals

Cabbage semilooper (*Trichoplusia ni*) eggs were obtained from Entopath Inc. (USA). Larvae of *Trichoplusia ni* were grown on three different diets at room temperature (23º C) and a 16/8 h light/dark cycle, and at 55% relative humidity.

To estimate the impact of bacteria in the diet, three feeding groups were formed: larvae were fed on artificial diet (Freitak et al., 2007) with or without bacteria (later referred to as bacterial and bacteria-free diet) and on cabbage plants (*Brassicae oleraceae*). Bacteria-free diet and plants may in fact contain low levels of environmental bacteria. Bacterial diet was specifically enriched for bacteria by soaking with overnight cultures (OD600 = 4) (2.5ml/40cm^2) of *Escherichia coli* and *Micrococcus luteus* (~80 µg per 125 g of diet). Diets were changed every three days to keep the bacterial concentration in the diet at approximately

the same level. To equalize the amount of handling stress between the plant diet and artificial diets, all the larvae feeding on cabbage plants were resettled on new plants also with the interval of three days.

To study the effect of parental diet on the gene expression in the eggs, two crosses were set up. Adults grown on bacteria-free diet were mated with each other (N♀N♂) and adults grown bacterial diet (B♀B♂) were crossed with each other. Matings were carried out in 21x13x13cm cages. Three day old eggs were collected and used for further expression analyses.

3.4.2 Differential gene expression analysis

To study differential gene expression between *T. ni* larvae grown on plant, bacterial and bacteria-free diet the DEG GeneFishing Kit (SeeGene, Seoul) was used, following the manufacturer's protocol. Briefly, 3 µg of DNA-free total RNA was converted into single-stranded DNA using annealing control primer one (dTACP1) and two (dTACP2) which prime from the polyA tail, and a mixture of different reverse transcriptases (Array Script, Ambion; Bioscript, Bioline). Second-strand cDNA synthesis and subsequent PCRs were performed essentially as described in the DEG GeneFishing protocol. We used a total of 60 different ACP (annealing control primer) pairs in the analysis. ACP primers are designed for highly specific PCR in a two-stage process. In the first stage, amplification is based on a perfect match between the short 3' end of the ACP and the cDNA template; and the second more stringent stage further amplifies the specific product based on pairing of the longer 5' end of the ACP to a additional primer; with no further nonspecific amplification from the cDNA template. Like all differential display techniques, this method identifies a fraction of the total gene expression changes, depending on the number of different primers employed.

PCR products were separated and visualized on 2% agarose gels. Bands were scored visually and differentially expressed bands were cut out from the agarose gels and PCR products were extracted using the Zymoclean Gel DNA Recovery Kit ™ (Zymo Research) according to the manufacturer's instructions (Supplementary Figure 1). DNA fragments were cloned into the TOPO TA cloning vector (Invitrogen) following the manufacturer's protocol. For identification of inserted DNA, isolated plasmids were sequenced (Applied Biosystems, ABI). BLAST searches were conducted on a local server using the National Center for Biotechnology Information (NCBI) Blastall program and best hits were recorded.

To assess the amount of internal variation within treatments, we selected two treatments, formed three different pooled samples for each, and used 20 ACP primers to search for variation among the samples from the same treatment group. No differences were found for this subset (data not shown); therefore we used a single superpool for each treatment group in subsequent analysis.

3.4.3 RNA isolation

Dissected insect midguts and the rest of the bodies (except for the head capsules) were ground using a motorized hand pestle and total RNA was isolated using the TRIzol Reagent (Invitrogen) according to the manufacturers´ protocol. To isolate RNA from whole larvae, eggs and adults, tissues were submersed in liquid nitrogen and homogenized using mortar and pestle, the powder was dissolved in TRIzol Reagent (Invitrogen) and RNA was extracted according to the standard protocol. An additional DNAse (Turbo DNAse, Ambion) treatment was included prior to the second purification step to eliminate any contaminating DNA. A second purification step was performed with RNeasy MinElute CleanUp Kit (Qiagen). RNA integrity was verified on an Agilent 2100 Bioanalyzer using RNA Nano chips (Agilent). RNA quantity was determined photospectrometrically using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific).

3.4.4 Quantitative real-time PCR

500 ng of DNA-free total RNA was converted into single-stranded cDNA using a mix of random and oligo-dT20 primers according to the ABgene protocol (ABgene). Real-time PCR oligonucleotide primers were designed using the online Primer3 internet based interface (http://frodo.wi.mit.edu). Primers were designed by the rules of highest maximum efficiency and sensitivity rules were followed to avoid formation of self and hetero-dimers, hairpins and self-complementarity. Gene-specific primers were designed on the basis of sequences obtained for selected *T. ni* genes and several additional genes as potential house-keeping genes to serve as the endogenous control (normalizer) (Suppl. Table 1.). Q-RT-PCR was done in optical 96-well plates on a MX3000P Real-Time PCR Detection System (Stratagene) using the Absolute QPCR SYBR green Mix (ABgene) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye included in the PCR master mix.

Abbreviations

GST – glutathione S-transferase

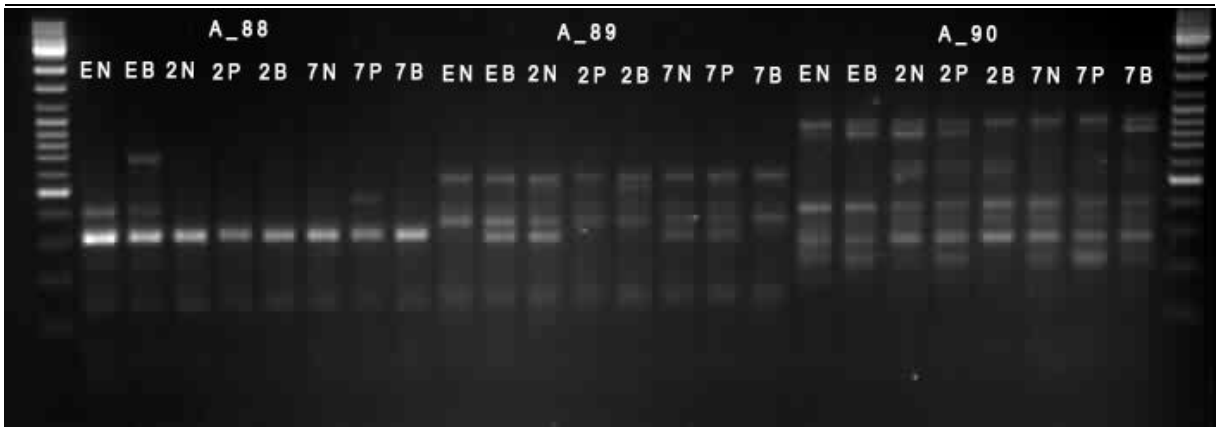
SDR – Short-chain dehydrogenase/reductase

FAD – fatty acid dehydrases

ATP – adenosine triphosphate

NAD – nicotinamide adenine dinucleotide

GMC – glucose-methanol-choline oxidoreductase



Supplementary Figure 1. Example of gel with separated PCR products from differential gene expression analysis. (2B – 2 day old larvae on bacterial diet, 2N – 2 day old larvae on bacteria-free diet, 2P – 2 day old larvae on cabbage plants; 7B – 7 day old larvae on bacterial diet, 7N – 7 day old larvae on bacteria-free diet, 7P – 7 day old larvae on cabbage plants; EN – eggs laid by parents grown on bacteria-free diet, EB - eggs laid by parents grown on bacterial diet). A_88, A_89, A_90 are primer pairs used for GeneFishing analysis.

Supplementary Table 1. Primers used in RT-qPCR, (H) – hemocyte specific sequences.

Gene	Forward primer	Reverse primer
Glutathion S-transferase 1	CCTGCATCTCGACCATTTCTA	TTACGCCTTGCTGTTCTTCTC
Cytochrome C oxidase Vb	CGAGGAACCATAGACGTTTGA	GTGCGGACACTGGTACAAACT
Titin-like protein	AACGGGCTTAAGCTGTACCTC	GAAGGAAGAAGAACCCAAGGA
Mn superoxide dismutase	GCCAGCTGGTTCTTCATATTG	TCAGCACAGTCATCAGTCTGG
Chitin binding (b)	TCACAGCTTAATAGCCGAGGA	CCGCAAATGTACCACAATGTA
Serine/threonine-protein kinase	TGCAGCATACGTTTGTCATGT	CATAGATTGAACGGCCAGGTA
NAD dependent epimerase/dehydratase	GCGAATTCTTCACAAGCTACG	GCAATGATCATGACGAAAGGT
Ubiquinol-cytochrome c reductase	CCTTCACCTACCTGATTGCTG	GCCTGATGAACAGTGGCTTAC
Serine protease inhibitor	AAAGTGACATTAGCGCCACAC	CGCATGTCCAGTAACATCTGA
Cation channel family protein	AGAGCTTGTGTGGGAGACTGA	CGAAACACTCCCATACTTCCA
Retinol dehydrogenase	AGCTCCTTCCCATGATGTCTT	TTAATAGTGCGAACCCTGGTG
Protein kinase	TTTCTTCAGCTCAGGTTCTGG	TGACGATGTTATCCAGCATGA
Protein disulfide isomerase	CTCAACGAAGCTGGTCATAGC	ACACTTCGAGAAGGACGATGA
Cathepsin	AGGTGACGAGGAGAACGTGAT	TAGTAGACGCCGTCGGAGTAG
Trypsin-like serine protease	TCCCACTACTTCCACCACTTG	TCTTCGTCTTGGTACGTGCTT
G-protein coupled receptor	CCTGAGGATACCACCACTGAA	TCGGATGAAGAGGAAGACTCA
Chitin binding (a)	AGACCAAGGATGGTTTCGAGT	ACCTCTGGGACTCCTCATTGT
Tyrosine-protein kinase	TTTGGGAGATTGCCACATTAG	CCATCAGCTGGTAGAGTCTCG
GMC oxidoreductase	GGGAACTTGCTCTATGGGTTC	ATGAAGTCCGACGCTCTTTCT
Cytochrome C oxidase Via	CGCGAAATTGCGCTACTATAC	ATGGCCAGAAGTCGCTCTT
C-type lectin	CGTGACAAGCTGTCTCCTACC	GGAAGTACCGTTGATGTCGAA
Lectin_547	CTCGTTCACGTTGCAATCTCT	TCGACGTGGAATCATGTATCA
PPOEA_355	ATGCCTTCAGGCTTTCTCTTC	ATGCATCGCGAAATACAGAAC
ProPO_565	TCCAGTCCCATGTTACTCAGG	TCTTGATAGCGTGGAACAACC
BGRP_736	CAAGTTATGCGAGGTCTCCAA	TTGTCCGGAATATAAGCGTTG
Hemolin_202	GGCACTGCAAGTTTGAGATTC	ACAAAGAAGGCAGGGTGATTT
Metalloprot	CTAGACCCGTTCACCACTCTG	GGTGTGGCGTGAAAGATATTG
C_Lys_886	GACTTTGTTGGCGCATACACT	GAAGTGAGTGGTGCAAAGAGG
IMPI_807	CGTATCCATCTTCGCAGTAGC	AGGTGCACACGAGAACCTAGA
Lectin_82	TCTTCCTCCCACCAATCTTCT	AAAGCCGTTTGGAAAGAGAAC

4 Chapter 3. Dietary-dependent trans-generational immune priming in an insect herbivore

Abstract

Trans-generational effects on immunity are well known in vertebrates and are considered in many evolutionary and ecological theories of species interaction. Maternal effects have been identified to be of special importance, and are now recognized as a mechanism for adaptive phenotypic response to environmental heterogeneity. We have previously shown that exposure to dietary nonpathogenic bacteria can induce several aspects of the immune response in the insect *Trichoplusia ni*. Here we test the effects of this exposure on the immune status of the next generation. We measured parameters reflecting immune responses on three different levels – enzyme activities, protein expression and transcript abundance. We also monitored several fitness related traits, such as body mass and developmental time, which are often negatively correlated with increased immunocompetence. We found substantial maternal effects on hemolymph based immune parameters and immune response related transcripts in the next generation. However, the physiology and immune condition of the offspring does not mirror the parental status, but is modulated in several significant respects. This scenario indicates that this transgenerational priming of the immune response is a complex and multifaceted phenomenon, potentially playing a role in short term environmental adaptation.

4.1 Introduction

The phenotype of an organism is not only determined by its genotype, but also by interactions with the environment and epigenetic factors (Ghalambor et al., 2007; Nussey et al., 2007; Gotthard & Nylin, 1995; Poulin & Thomas, 2008). Epigenetic inheritance is increasingly recognized as playing a role in shaping the phenotype and life-history of an organism (Poulin & Thomas, 2008). Maternal effects especially have been reported in many different taxa as a response to various environmental factors, including pathogens. In many cases it has been shown that these kind of maternal effects are adaptive, optimizing life-history traits in the long term (Mousseau & Fox, 1998).

Pathogens and parasites profoundly influence the fitness of their hosts. The ability to cope with infections is a life-history trait with a high degree of phenotypic plasticity, and its phenotypic costs result in well-documented trade-offs with fitness (Schmid-Hempel, 2005a). Under certain conditions such trade-offs could be ameliorated if information about the likelihood of future infection was available. Sorci and Clobert (1995) suggested that in a relatively stable environment, infection levels experienced by mothers are good indicators for

what the offspring are likely to encounter. Trans-generational priming of the immune system could therefore result in lower mortality of progeny by reducing the delay required by independent induction of progeny immune response, and possibly increasing efficiency by priming those aspects of the immune response most appropriate to dealing with the particular stress encountered by the parents.

In vertebrates it is known that immunocompetent mothers can transmit pathogen resistance to their offspring (Grindstaff et al., 2003). Mucosal immune responses in the fetal gut are primed by maternal gut flora (Blümer et al., 2007). Evidence for trans-generational immune priming has also been reported in arthropods, namely mealworm beetle (*Tenebrio molitor*) (Moert, 2006), bumblebee (*Bombus terrestris*) (Moret & Schmid-Hempel, 2001), mosquito (*Anopheles stephensi*) (Grech et al., 2007), Mediterranean flour moth (*Ephestia kuehniella*) (Rahman et al., 2004), and the crustacean *Daphnia magna* (Little et al., 2003). In contrast, studies on peach-potato aphid *Myzus persicae* did not provide evidence for trans-generational priming of resistance to its parasitoid *Diaeretiella rapae* (Vorburger et al., 2008). The heritability of immune function, specifically the encapsulation response, has also been studied (Cotter & Wilson, 2002; Fellowes et al., 1998; Collins et al., 1986).

We have previously shown that exposure to dietary nonpathogenic bacteria during the larval stage can induce several aspects of the immune response in the insect *Trichoplusia ni* (Freitak et al., 2007). Here we assess the extent to which components of an immune response can be seen in the unexposed progeny of exposed individuals. Reciprocal crosses were made with parents grown on sterile artificial diet or bacteria-laden diet to study trans-generational maternal and paternal effects. We examined different aspects of the immunity and physiology of the insect by measuring enzyme activities, hemolymph protein profiles and transcript levels of a number of immune-related genes in different tissues, as well as fitness-related parameters including larval developmental time, pupal mass and survival. Instead of the parental immune status being faithfully mirrored in the next generation, we observed a complex, mixed-pattern scenario, with strong maternal effects in many aspects. Thus the pattern of trans-generational priming affects diverse aspects of the progeny immune response in a highly selective manner.

4.2 Results

4.2.1 Immune-related changes in the hemolymph

Hemolymph samples were taken from 14 day old (early stage last instar) larvae. The effect of the absence or presence of bacteria in the diet of the parental generation on the general antibacterial activity was not significant ($F_{3,78} = 1.99$, $p = 0.124$) (Fig. 1A). Although the mean general antibacterial activity is higher in offspring of the B♀B♂ cross (129.2µg of lysozyme) in comparison to other crosses (N♀N♂ = 94.1 µg of lysozyme, N♀B♂ = 84.6 µg of lysozyme, B♀N♂ = 74,6 µg of lysozyme), the difference is not statistically significant after Bonferroni corrections on the Post-hoc analysis (B♀B♂ vs B♀N♂, $p = 0.149$; B♀B♂ vs N♀B♂, $p = 0.469$ and B♀B♂ vs N♀N♂, $p = 0.609$).

A significant effect of bacteria in the parental diet was observed on the phenoloxidase (PO) activity in the hemolymph of second generation *T. ni* larvae, but not on the additional PO activity accounted for by activation of ProPO. The activity of PO was significantly affected by parental diet (Kruskal-Wallis ANOVA, $H_{3,79} = 10.87$, $p = 0.012$) whereas that accounted for by activation of ProPO was only marginally affected (Kruskall-Wallis ANOVA, $H_{3,79} = 6.7$, $p = 0.082$) (Fig. 1B).

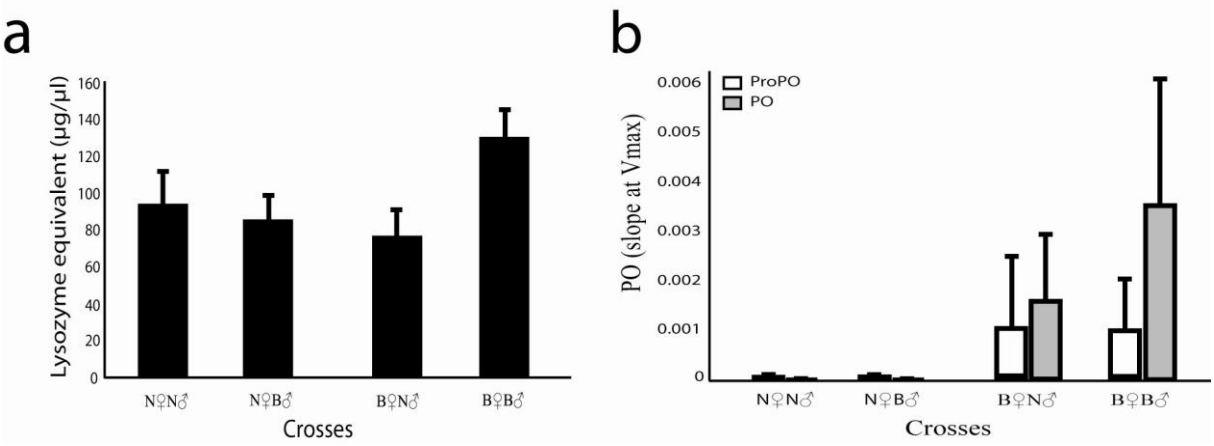


Figure 1. Enzyme activities in the hemolymph of last instar *T. ni* larvae originating from N♀N♂, B♀B♂, B♀N♂ and N♀B♂ crosses. *(A)* General antibacterial activity measured as the diameter of the lytic zone on agar plates with lyophilized *M. luteus* and transformed into lysozyme equivalents (µg/µl). Results represent mean values ± SE. *(B)* Prophenoloxidase and phenoloxidase activities measured in the hemolymph samples. V_{max} is measured as the maximum change in optical density per minute ± SE.

We found strong maternal effects on protein expression in the larval hemolymph. The analysis of the protein profiles of one-dimensional protein gel electrophoresis (1D SDS PAGE) resulted in 20 differentially expressed bands, 18 of which were more pronounced in B♀B♂ and B♀N♂ crosses, and two in N♀B♂ and N♀N♂ crosses. Based on mass-spectrometry data three proteins were identified, including storage proteins, such as arylphorin and vitellogenin, but also juvenile hormone suppressing protein involved in developmental processes, all of which were highly expressed in B♀B♂ and B♀N♂ crosses (Fig. 2) (Supplementary Table 2).

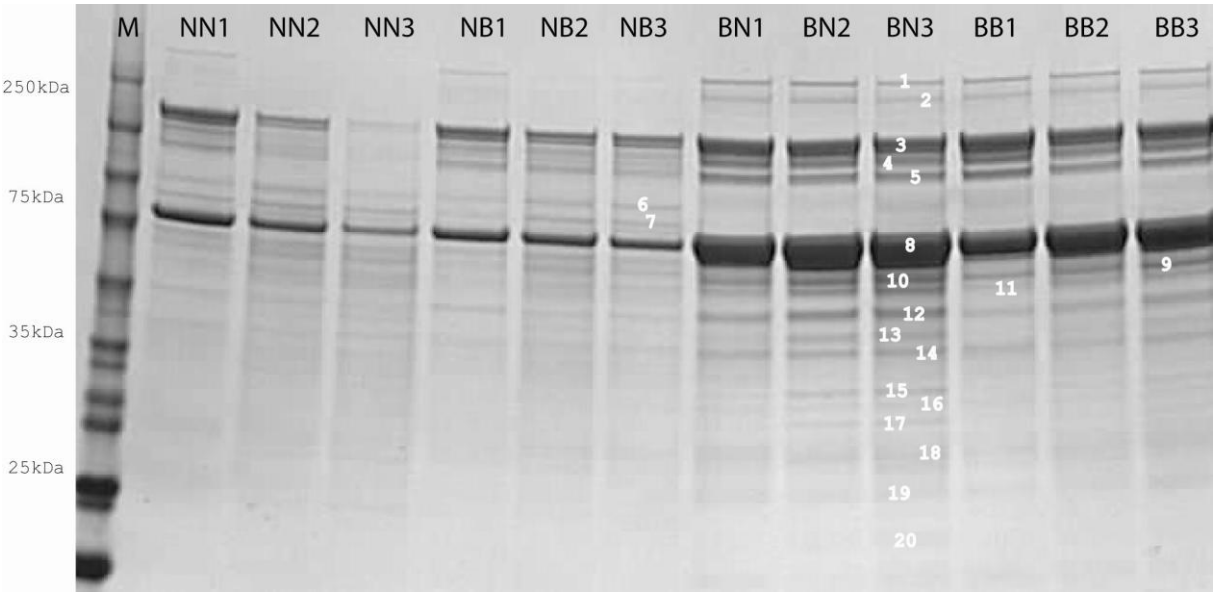


Figure 2. SDS gel electrophoresis of *T.ni* last instar larvae hemolymph proteins stained with Coomassie blue. Three individual larval hemolymph samples from each cross are presented – BB1-BB3 from B♀B♂ cross, BN1-BN3 from B♀N♂ cross, NB1-NB3 from N♀B♂ cross, NN1-NN3 from N♀N♂ cross. Protein bands identified as differentially expressed are vitellogenin (3, 4, 7), arylphorin (8, 10) and juvenile hormone suppressive protein (11, 12). 17 additional unknown proteins were observed as differentially expressed.

4.2.2 Tissue specific gene expression in second generation

Differential expression of immune response related genes was examined in the midgut, rest of the body, hemocytes and whole larvae of the crosses. A number of immune inducible genes were up regulated in the B♀B♂ cross larval midguts and rest of the body, but also in the rest of the body of B♀N♂ and N♀B♂ crosses (Fig. 3 A and B). In addition, apolipophorin III and

arylphorin were up regulated in the rest of the body of B♀N♂ cross larvae. Apolipophorin III and beta-glycan recognizing protein (BGRP) were up regulated in the hemocytes of larvae from B♀N♂ cross. In the cases where we used pooled larvae to study differential gene expression, only a metalloprotease showed higher expression in the B♀N♂ cross. We examined the expression pattern of 12 immune inducible genes in hemocytes, only two of them showed higher expression in B♀N♂ cross (Supplementary Table 3).

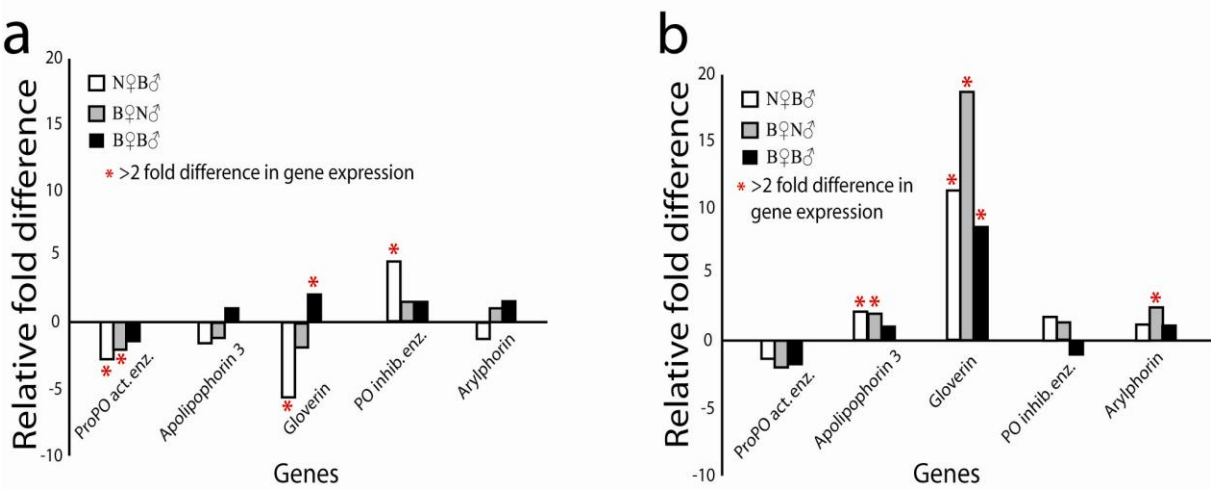


Figure 3. Differential gene expression in last instar *T.ni* larvae originating from different crosses. *(A)* The effect of parental diet on the relative gene expression in midgut tissue of offspring originating from different crosses in *T.ni*. *(B)* The effect of parental diet on the relative gene expression in rest of the body of offspring originating from different crosses in *T.ni*. In both cases relative gene expression in offspring of N♀N♂ cross is set to 1, values are mean ± SD, NE – not expressed.

4.2.3 Immunity related gene expression in parental generation

To compare the changes in immune status reflecting gene expression we also examined transcripts for immune inducible genes in parental generation, actually exposed to bacterial diet. We have previously found that the transcription of several immune inducible genes is influenced in the midgut tissue by bacterial feeding in larvae (Freitak et al., 2007). At the same time in the rest of the body of larvae grown on bacterial diet only two out of seventeen examined genes showed different expression, namely attacin was highly upregulated and cobatoxin was down regulated (Supplementary Table 4).

Expression of 24 immune response related genes was studied in the adult stage of *T. ni* moths. Six of them were up regulated in the animals grown on bacterial diet and one down

regulated. Among the up regulated genes were attacin, cecropin A, a chemosensory-like protein, cecropin D, lebocin and azurocidin (Supplementary Table 5). All of these are directly involved in immune response, having a function in recognizing bacteria and acting on pathogen cell wall integrity (Carlsson et al., 1998; Liu et al., 2000; Kang et al., 2002; Steiner et al., 1988; Lee et al., 1996; Sun et al., 1990). Hemolin, a protein with sequence homology to mammalian immunoglobulins by virtue of shared Ig-C2 domains, was at the same time significantly down regulated. Hemolin is one of the first proteins to appear in the hemolymph of the giant silkmoth *H. cecropia* upon infection, binding to the surface of bacteria (Sun et al., 1990).

For a number of genes (9) also gender specific differential gene expression patterns were examined. All of the studied genes, with exception of gallerimycin, were differentially expressed in males and females. Major up regulation of attacin, cecropin A and azurocidin was found in females fed on bacterial diet in comparison to females grown on bacteria free diet. In the case of males, both attacin and cobatoxin were significantly up regulated in the bacteria fed group. We also found that when both sexes were grown on bacterial diet, GMC oxidoreductase was highly expressed in adult *T. ni* males in comparison to females (Supplementary Table 5).

4.2.3 Effect on fitness

To estimate the effects of the parental diet on the life-history traits of the next generation, a number of fitness related traits were studied. We found no effect of the different crosses on pupal mass (ANOVA, $F_{3,323} = 0.3$, $p = 0.8$). However, survival into the adult stage was significantly lower among offspring originating from B♀B♂ (78%) and N♀B♂ (76%) in comparison to N♀N♂ (87%) and B♀N♂ (90%) crosses (Chi-square test, $\chi^2_{3,400} = 9.7$, $p = 0.021$) (Fig. 4a). No effect of sex on developmental time was discovered (all tests $p<0$). Crosses where both parents originated from different diets (N♀B♂ and B♀N♂) had prolonged developmental times (Kruskal Wallis ANOVA, $H_{3.331} = 24.5$, $p > 0.000$) (Fig. 4b).

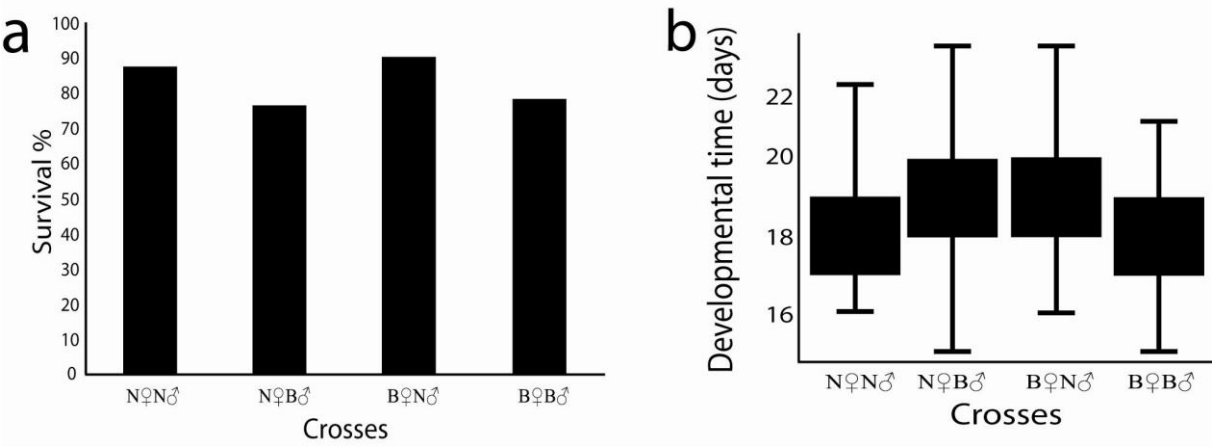


Figure 4. Effect of parental diet on the fitness-related traits in *T. ni*. *(A)* The effect of parental diet on the survival of offspring originating from different crosses in *T.ni*. Graph shows % survival in different crosses (n = 100 per each cross). Survival is reduced in B♀B♂ and N♀B♂ crosses. *(B)* The effect of parental diet on developmental time of the offspring originating from different *T.ni* crosses. Offspring of the parents forming mixed crosses B♀N♂ and N♀B♂ have prolonged developmental times. Results represent medians with upper and lower quartiles.

4.3 Discussion

In the current work we present evidence that constant ingestion of bacteria during larval development has an effect on immune-related aspects of the phenotype of the next generation. The induced changes cover many aspects of the immune response, including enzyme activities, protein expression and transcript levels of many genes. These effects are predominantly but not exclusively maternal. At the transcriptional level, comparison of parental and offspring generations does not indicate a simple transfer of the changed immune and physiological status from the parents, but rather a new immune status quality in offspring.

Strong diet related maternal effects were found on the immune system of next generation *T. ni* larvae. In B♀B♂ and B♀N♂ crosses immune response related enzyme activities were higher and a number of proteins were notably expressed. In a previous study (Freitak et al., 2007) we found that in the parental generation fed on bacterial and bacteria free diet, lysozyme is highly active in bacterial diet fed larvae and phenoloxidase in bacteria-free diet larvae. It has been proposed that these two enzyme activities exhibit a trade-off with each other and are not displayed at high levels at the same time (Cotter & Wilson, 2002), potentially due to possible mechanistic reasons and severe autoimmune effects (Cotter et al., 2004; Sadd & Siva-Jothy, 2006; Nappi & Christensen, 2005). We observed such a trade-off in

the first, directly exposed generation but not the second generation. This could be due to additional physiological changes to cope with higher enzyme activities and increased autoimmune risk. One of the candidates enabling the insects to avoid harm caused by free radicals is vitellogenin, a protein we find to be highly expressed in the hemolymph of larvae of both B♀B♂ and B♀N♂ crosses, showing both high lytic and high phenoloxidase activity. Vitellogenin is known to act as an antioxidant and to reduce oxidative stress related harmful effects to the organism (Seehuus et al., 2006). The elevated vitellogenin expression in the hemolymph of larvae whose mothers have been feeding on bacterial diet might be an adaptive induction, possibly enabling the insects to cope with high free radical stress due to elevated phenoloxidase activity. As in the first generation, in the second generation arylphorin is one of the predominant hemolymph proteins. Arylphorin is highly inducible upon immune challenge (Kunkel et al., 1990; Asgari & Schmid-Hempel, 2004) and bacterial feeding (Freitak et al., 2007), but at the same time its role in the immune response is still largely unknown, although growth promoting and mitosis enhancing effects have been reported (Hakim et al., 2007). Another highly abundant protein in B♀B and B♀N crosses was identified as juvenile hormone suppressing protein. This could be related to the high expression of vitellogenin, as a high titer of juvenile hormone leads to suppression of this protein (Pinto et al., 2000).

Strong maternal effects can also be seen at the transcript level. It is interesting to note that a number of immune response related genes are up regulated in the rest of the body of B♀B♂ and B♀N♂ crosses, among them apolipophorin III and arylphorin, also known to be involved in storage processes. It is very interesting to see, that a number of immune response related genes known to be antimicrobial effector molecules (e.g. gloverin, cecropins, hemolin) are highly expressed in the midgut tissue. Although no direct induction of the immune system elements occurred in the second generation, we can observe higher abundance of several markers, characteristic for immune challenged organism. Our findings support the idea that parental diet is able to alter the immune status in the next generation. Up regulation of antimicrobial genes in the midgut tissue could be seen as an adaptive response preparing offspring to elevated bacterial load in the environment by increasing their chance for survival in the case of infection.

Alterations in immune status can lead to certain trade-offs with other life-history traits, such as developmental time and body mass (Schmid-Hempel, 2005a). We did not see any differences in the pupal masses between different crosses. Developmental time was prolonged in mixed crosses - B♀N♂ and N♀B♂. Mortality was higher in the B♀B♂ and N♀B♂

crosses, indicating a paternally-related cost associated with an elevated immune level and possibly an imbalance in homeostasis. Our experimental design kept larval densities constant, so we could not observe effects of varying density on immune status as seen in other studies (Barnes & Siva-Jothy, 2000; Wilson et al., 2002). Nor was there any opportunity in our experimental design for larvae to vary their diet choice in response to increased immune challenge, as seen by Lee et al. (2006).

A number of papers claiming to show adaptive immunity and trans-generational immune priming in invertebrates have been strongly criticized recently, as lacking mechanistic evidence (Hauton & Smith, 2007). The critics argue that in many cases only survival has been considered as a measure of successful immune response, and usually single enzyme or protein measurements have been used as markers reflecting the immune status. Several other studies have presented additional life-history parameters (e.g. body mass, survival, egg mass etc.) as evidence of adaptive priming of the immune system, but at the same time do not show any measurable immune markers. The complexity of the response we have observed in *T. ni* provides further evidence that approaches focusing on only one or two immune parameters in assessing trans-generational effects may give an incomplete and possibly unrepresentative picture.

Encountering non-pathogenic bacteria in the food is sufficient to trigger changes in the immune status of the next generation but this effect can only be seen on some immune markers and not on others. The immune response is multifaceted and involves coordination and reorganization of many aspects of physiology, the outcome being a complex pattern of induction and repression of different factors. These immune system parameters are clearly not transmitted in a 1:1 ratio from parent to offspring, but are selectively transmitted in a complex manner with a strong but not exclusively maternal component. The mechanism of transmission of this information is completely unknown, but could involve maternal transmission of proteins and/or RNA in the egg cytoplasm, as well as epigenetic mechanisms such as DNA methylation. Determining the relative importance of these possible mechanisms remains a challenging topic for future research.

4.4 Materials and Methods

4. 4. 1 Animals

Cabbage semilooper (*Trichoplusia ni*) eggs were obtained from Entopath Inc. (USA). Eggs were placed in plastic cups with artificial diet and allowed to hatch, and were grown at room temperature (23º C) at 16/8 h light/dark cycle, at 55% relative humidity. To estimate the impact of constant presence of bacteria in the diet, two treatment groups were formed for the parental generation: larvae were fed on artificial diet (Freitak et al., 2007) with or without bacteria. Larvae in both treatments may have initially ingested some bacteria when consuming the eggshell after hatching, or from other environmental sources, Bacterial diet was prepared by soaking with overnight cultures (OD600 = 4) (2.5ml/40cm^2) of *Escherichia coli* and *Micrococcus luteus* (~80 µg per 125 g of diet). Old diet was removed and fresh diet (sterile or freshly laden with bacteria) was provided every three days to keep the difference between treatments of the bacterial concentration approximately constant in time. Larvae were not given a choice of different types of food, to avoid affects of changes in feeding behavior on immune responses as shown by Lee et al. (2006).

Adults originating from different diets were mated to estimate the effect of parental diet on the physiology and immune system of offspring. Four different crosses were made, females and males grown on bacteria-free diet (N♀N♂), females and males grown on bacterial diet (B♀B♂), females grown on bacterial diet and males on bacteria-free diet (B♀N♂) and females grown on bacteria-free diet and males on bacterial diet (N♀B♂). Mixed matings of 30 males and 30 females were carried out in 21x13x13cm cages. All the larvae from first and second generation were kept at the same densities to avoid density dependent effects such as those shown by Barnes and Siva-Jothy (2000). Eggs were collected and allowed to hatch and fed on artificial bacteria-free diet. In total there were three replicate groups from which crosses were made and offspring tested.

4. 4. 2 Lytic activity in the hemolymph

For estimation of the total lytic activity of the hemolymph, a lytic zone assay was performed. 12 x 12cm Petri dishes were filled with 35ml of autoclaved Sörensen buffer with 21mg lyophilisized *Micrococcus luteus* cells (Sigma) and 2.1 mg streptomycin sulfate (Calbiochem) with a final concentration of 1.5 % agar. Wells within plates (2 mm diameter) were made by

puncturing the agar with a plastic pipette and removing the agar plug by suction. Hemolymph samples (4 µl) were pipetted directly into the wells and the plates were incubated for 24 h at 37°C. A dilution series of chicken egg white lysozyme (Sigma) (2 mg/ml, 1 mg/ml, 0.750 mg/ml, 0.500 mg/ml, 0.250 mg/ml, 0.125 mg/ml, 0.62 mg/ml, and 0.31 mg/ml) was added to each plate as a control and a calibration curve was created based on these standards. Lytic activity was determined as the radius of the clear zone around a sample well.

4. 4. 3 Pro-phenoloxidase (proPO) quantity and phenoloxidase (PO) activity in the hemolymph

Hemolymph was collected via puncturing the dorso-lateral part of the larval abdomen and 20 µl was diluted into 200 µl ice cold PBS and vortexed immediately. Samples were centrifuged at 4 ºC at 9000rpm for 7 minutes, supernatant removed and kept for further analysis at -80 ºC. PO assays were carried out in 96well plates using the Infinite 200 microplate reader (Tecan). 100 µl of supernatant was mixed with 200µl of 0.4mM L-Dopa (Sigma) and changes in absorbance were measured at 30ºC for 60min at 490nm. To measure total activity of enzyme per volume of the hemolymph accounted for by both (already activated) PO and (unactivated) proPO, 30µl of supernatant was incubated with 70µl of chymotrypsin solution (2mg chymotrypsin (Calbiochem, Germany) in 1.5 ml PBS) to activate the proPO for 20min at 30ºC and changes in absorbance were measured at 30ºC for 60min at 490nm. To estimate the additional PO activity accounted for by activation of proPO, readings measured without chymotrypsin digestion were subtracted from the total activity seen after incubation with chymotrypsin. Enzyme activity was measured as the slope (Vmax value) of the reaction curve during the linear phase of the reaction (15-45min after adding the substrate).

4. 4. 4 Protein expression in the hemolymph

Protein expression patterns in the hemolymph were analysed with Sodium Dodecyl Sulphate Polyacrylamide Gradient Gel Electrophoresis (SDS-PAGE) with 4-12% gradient gels performed in a XT-MES buffer system. 2µl of hemolymph sample was diluted into 50µl of ice-cold 4% SDS containing TrisHCl buffer with EDTA-free protease inhibitor cocktail (Pierce), directly frozen in liquid N_2 and stored at -20°C until further use. All the samples were analysed as described in Freitak et al., (2007).

4. 4. 5 RNA isolation

Dissected insect midguts and the rest of the bodies (except for the head capsules) were ground using a motorized hand pestle and total RNA was isolated using the TRIzol Reagent (Invitrogen) according to the manufacturers´ protocol. For isolating RNA from the hemocytes, hemolymph samples from ~15 larvae, (~ 25 µl per larvae) were pooled in 1 ml phosphate buffer and centrifuged at 4ºC at 9000g for 10 min. Supernatant was removed, the cell pellet was dissolved in TRIzol and RNA was isolated according to the manufacturers´ protocol. An additional DNAse (Turbo DNAse, Ambion) treatment was included prior to the second purification step to eliminate any contaminating DNA. A second purification step was performed with RNeasy MinElute CleanUp Kit (Qiagen). RNA integrity was verified on an Agilent 2100 Bioanalyzer using RNA Nano chips (Agilent). RNA quantity was determined photospectrometrically using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific).

4. 4. 6 Quantitative real-time PCR

500 ng of DNA-free total RNA was converted into single-stranded DNA using a mix of random and oligo-dT20 primers according to the ABgene protocol (ABgene). Real-time PCR oligonucleotide primers were designed using the online Primer3 internet based interface (http://frodo.wi.mit.edu). Primers were designed by the rules of highest maximum efficiency and sensitivity rules were followed to avoid formation of self and hetero-dimers, hairpins and self-complementarity. Gene-specific primers were designed on the basis of sequence obtained for selected *T. ni* genes and several additional genes as potential house-keeping genes to serve as the endogenous control (normalizer). Q-RT-PCR was done in optical 96-well plates on a MX3000P Real-Time PCR Detection System (Stratagene) using the Absolute QPCR SYBR green Mix (ABgene) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye included in the PCR master mix.

4. 4. 7 Statistical analysis

Statistical analyses were performed with the software package SPSS. 15 (SPSS Inc.). Normality of the data was estimated by using Shapiro-Wilkoxon and Levene's tests. In the case where assumptions for normality and homogeneity were not violated, our hypotheses

were tested using an ANOVA model, otherwise a nonparametric Kruskall-Wallis ANOVA test was used. In the case qRT-PCR data, the $2^{-\Delta\Delta CT}$ method was used (Livak & Schmittgen, 2001).

Abbreviations:

PO – phenoloxidase

ProPO – prophenoloxidase

Supplementary Table 1. Primers used in RT-qPCR, (H) – hemocyte specific sequences.

Gene	Forward primer	Reverse primer
Glutathion S-transferase 1	CCTGCATCTCGACCATTTCTA	TTACGCCTTGCTGTTCTTCTC
Cytochrome C oxidase Vb	CGAGGAACCATAGACGTTTGA	GTGCGGACACTGGTACAAACT
Titin-like protein	AACGGGCTTAAGCTGTACCTC	GAAGGAAGAAGAACCCAAGGA
Mn superoxide dismutase	GCCAGCTGGTTCTTCATATTG	TCAGCACAGTCATCAGTCTGG
Chitin binding (b)	TCACAGCTTAATAGCCGAGGA	CCGCAAATGTACCACAATGTA
Serine/threonine-protein kinase	TGCAGCATACGTTTGTCATGT	CATAGATTGAACGGCCAGGTA
NAD dependent epimerase/dehydratase	GCGAATTCTTCACAAGCTACG	GCAATGATCATGACGAAAGGT
Ubiquinol-cytochrome c reductase	CCTTCACCTACCTGATTGCTG	GCCTGATGAACAGTGGCTTAC
Serine protease inhibitor	AAAGTGACATTAGCGCCACAC	CGCATGTCCAGTAACATCTGA
Cation channel family protein	AGAGCTTGTGTGGGAGACTGA	CGAAACACTCCCATACTTCCA
Retinol dehydrogenase	AGCTCCTTCCCATGATGTCTT	TTAATAGTGCGAACCCTGGTG
Protein kinase	TTTCTTCAGCTCAGGTTCTGG	TGACGATGTTATCCAGCATGA
Protein disulfide isomerase	CTCAACGAAGCTGGTCATAGC	ACACTTCGAGAAGGACGATGA
Cathepsin	AGGTGACGAGGAGAACGTGAT	TAGTAGACGCCGTCGGAGTAG
Trypsin-like serine protease	TCCCACTACTTCCACCACTTG	TCTTCGTCTTGGTACGTGCTT
G-protein coupled receptor	CCTGAGGATACCACCACTGAA	TCGGATGAAGAGGAAGACTCA
Chitin binding (a)	AGACCAAGGATGGTTTCGAGT	ACCTCTGGGACTCCTCATTGT
Tyrosine-protein kinase	TTTGGGAGATTGCCACATTAG	CCATCAGCTGGTAGAGTCTCG
GMC oxidoreductase	GGGAACTTGCTCTATGGGTTC	ATGAAGTCCGACGCTCTTTCT
Cytochrome C oxidase Via	CGCGAAATTGCGCTACTATAC	ATGGCCAGAAGTCGCTCTT
C-type lectin	CGTGACAAGCTGTCTCCTACC	GGAAGTACCGTTGATGTCGAA
Lectin_547	CTCGTTCACGTTGCAATCTCT	TCGACGTGGAATCATGTATCA
PPOEA_355	ATGCCTTCAGGCTTTCTCTTC	ATGCATCGCGAAATACAGAAC
ProPO_565	TCCAGTCCCATGTTACTCAGG	TCTTGATAGCGTGGAACAACC
BGRP_736	CAAGTTATGCGAGGTCTCCAA	TTGTCCGGAATATAAGCGTTG
Hemolin_202	GGCACTGCAAGTTTGAGATTC	ACAAAGAAGGCAGGGTGATTT
Metalloprot	CTAGACCCGTTCACCACTCTG	GGTGTGGCGTGAAAGATATTG
C_Lys_886	GACTTTGTTGGCGCATACACT	GAAGTGAGTGGTGCAAAGAGG
IMPI_807	CGTATCCATCTTCGCAGTAGC	AGGTGCACACGAGAACCTAGA
Lectin_82	TCTTCCTCCCACCAATCTTCT	AAAGCCGTTTGGAAAGAGAAC

Supplementary Table 2. Summary of the protein identification from SDS gel electrophoresis of *Trichoplusia ni* hemolymph samples.

Band	Database Searched	Hit	Species	Accession no.	De novo sequenced peptides	Peptides matched by MS BLAST	MS BLAST Score1
1	NCBI Insecta	Chitin-binding domain type 2	*Anopheles gambiea*	XP_309884	6	6	45
2	No peptides						
3	NCBI Insecta	vitellogenin	*Spodoptera litura*	ABU68426	11	11	86
4	NCBI Insecta	vitellogenin	*Samia cynthia ricini*	BAB32641	9	8	63
5	NCBI Insecta	very high density lipoprotein	*Helicoverpa zea*	ABQ23674.1	11	11	86
6	No peptides						
7	NCBI Insecta	vitellogenin	*Lymantria dispar*	AAB03336.1	9	8	63
8	NCBI Insecta	arylphorin subunit	*Spodoptera litura*	CAB55605.1	14	13	102
9	NCBI Insecta	juvenile hormone-related protein	*Trichoplusia ni*	AAA27884.1	24	24	187
10	NCBI Insecta	arylphorin subunit	*Spodoptera litura*	CAB55605.1	18	18	138
11	NCBI Insecta	Basic juvenile hormone-suppressible protein 1 precursor (BJHSP1)	*Trichoplusia ni*	Q06342.1	16	16	108
12	NCBI Insecta	Basic juvenile hormone-suppressible protein 1 precursor (BJHSP1)	*Trichoplusia ni*	Q06342.1	16	16	108
13	Not identified				4		
14	Not identified				4		
15	NCBI Insecta	similar to venom acid phosphatase	*Nasonia vitripennis*	XP_001604473.1	5	4	40
16	No peptides						
17	No peptides						
18	No peptides						
19	No peptides						
20	Not identified				1		

1 - MS BLAST scoring is detailed in the material and methods section

Supplementary Table 3. RT-qPCR results for hemocytes originating from larvae of different crosses, comparisons are made against N♀N♂ cross (=1), values are ±SD.

Gene	NB	BN	BB
Apolipophorin III	1.52 ± 0.009	**2.37 ± 0.025**	1.69 ± 0.036
Gloverin	1.11 ± 0.018	-1.93 ± 0.096	1.22 ± 0.013
Arylphorin (b)	1.19 ± 0.025	1.64 ± 0.014	-1.77 ± 0.042
Lectin (a)	-1.08 ± 0.025	1.33 ± 0.042	1.21 ± 0.105
ProPO ectivating enzyme	1.32 ± 0.037	1.15 ± 0.067	1.07 ± 0.028
ProPO subunit 2	-1.46 ± 0.036	-1.36 ± 0.021	1.01 ± 0.054
β-glucan recognition protein	-1.19 ± 0.061	**2.14 ± 0.024**	1.35 ± 0.017
Hemolin	1.02 ± 0.019	-1.02 ± 0.023	1.05 ± 0.046
Metalloprotease	1.63 ± 0.036	1.35 ± 0.018	-1.22 ± 0.080
C-type lysozyme	1.65 ± 0.053	1.50 ± 0.024	1.55 ± 0.015
Inducible metalloprotease inhibitor proetin precursor	-1.37 ± 0.009	-1.34 ± 0.066	1.08 ± 0.047
Lectin (b)	1.10 ± 0.030	1.52 ± 0.020	1.34 ± 0.023

Supplementary Table 4. RT-qPCR results for restbodies originating from parental generation larvae fed on bacterial diet in comparison to larvae fed on bacteria-free diet (=1), values are ±SD.

Gene	Bac diet
Attacin	**175.49 ± 0.037**
Hemolin	-1.08 ± 0.086
Lysozyme (a)	-1.35 ± 0.013
Lysozyme (b)	1.35 ± 0.084
PO inh. enz.	1.17 ± 0.018
Cecropin B	1.34 ± 0.036
Gloverin	-1.14 ± 0.059
Defensin	-1.12 ± 0.013
ProPO act. enz.(b)	1.61 ± 0.119
Beta-glucan bind.	-1.22 ± 0.021
Arylphorin	1.16 ± 0.011
Galleriomycin	-1.41 ± 0.036
Cecropin D	-1.60 ± 0.027
HDD1	1.04 ± 0.014
Cobatoxin	**-2.66 ± 0.037**

Supplementary Table 5. RT-qPCR results for adults fed on bacterial diet in comparison to animals fed on bacteria-free diet (=1), values are ±SD

Gene	Genders pooled	Females	Males
Attacin	**13.13 ± 0.465**	**28528 ± 0.280**	**5.33 ± 0.465**
Hemolin	**-26.54 ± 0.540**	-1.52 ± 0.185	**2.25 ± 0.515**
Lysozyme (a)	1.04 ± 0.060		
Lysozyme (b)	1.07 ± 0.045		
Arylphorin (a)	1.17 ± 0.010		
Peptidoglycan recognition protein	-1.09 ± 0.100		
Phenoloxidase activating enzyme	-1.10 ± 0.060		
Apolipophorin III	1.14 ± 0.380		
Cecropin B	1.53 ± 0.000	1.22 ± 0.065	**2.58 ± 0.040**
Gloverin	-1.78 ± 0.025	**-2.17 ± 0.260**	1.44 ± 0.115
Defensin	1.15 ± 0.060		
Phenoloxidase inhibiting enzyme	-1.13 ± 0.040		
Cathepsin	-1.29 ± 0.065		
β-glucan recognition protein	1.01 ± 0.145		
Arylphorin (b)	-1.10 ± 0.015		
Galleriomycin	-1.78 ± 0.100	-1.83 ± 0.110	1.82 ± 0.140
Cecropin A	**3.68 ± 0.035**	**6.89 ± 0.115**	1.21 ± 0.100
Chemocensory protein	**5.96 ± 0.180**	**2.32 ± 0.105**	**3.23 ± 0.065**
Cecropin D	**2.04 ± 0.050**		
HDD1	-1.68 ± 0.040		
Lebocin	**2.25 ± 0.165**	1.06 ± 0.090	5.92 ± 0.060
Cobatoxin	-1.16 ± 0.000		
Azurocidin	**2.39 ± 0.135**	**31.89 ± 0.010**	1.19 ± 0.155

5 Chapter 4. Immunity or digestion: Glucanase activity in a glucan-binding protein family from Lepidoptera

Abstract

The cell surfaces of microorganisms display distinct molecular patterns formed from lipopolysaccharides, peptidoglycans or β-1,3-glucans. Binding of these surfaces by pattern recognition proteins such as β-1,3-glucan recognition proteins (βGRPs) activates the immune response in arthropods. We identified a 40 kDa β-1,3-glucan-binding protein with sequence similarity to previously characterized lepidopteran βGRPs from hemolymph, but unlike these it is secreted into the larval gut lumen and is an active β-1,3-glucanase. This glucanase was not detected in hemolymph. Its mRNA is constitutively and predominantly expressed in the midgut and is induced there when larvae feed on diet containing bacteria. Homologs of this predominantly midgut-expressed gene from many Lepidoptera possess key residues shown to be part of the active site of other glucanases, and form a cluster that is distinct from previously-described βGRPs. In addition, this group includes proteins from insects such as the *Anopheles gambiae* GNBP subgroup B for which a catalytic role has not been previously suspected. The current domain classification does not distinguish between the catalytic and noncatalytic clades, and should be revised. The noncatalytic βGRPs may be evolutionarily derived from this newly described enzyme family which continues to function catalytically in digestion and/or pathogen defence.

5. 1 Introduction

Recognition of invading organisms as non-self is a crucial pre-requisite for an immune response. In arthropods, immune response is triggered in the hemolymph by so-called Pattern Recognition Proteins or Receptors (PRPs or PRRs) which bind to classes of bacteria- and fungi-specific polysaccharides such as peptidoglycans, β-1,3-glucans, lipopolysaccharides (LPS) and lipotechoic acid (LTA) (Kanost et al., 2004; Medzhitov & Janeway, 1997). Little detail is known for Lepidoptera but in *Drosophila* these binding interactions lead to activation of the innate immune response, largely regulated by two main pathways, the Toll and Imd pathways. The innate immune system contains of three main effector mechanisms: the cellular response, the humoral response and melanization. The activation of the signaling pathways leads to a cascade of events that result in the induction of defensive genes in hemocytes and the fat body, including antimicrobial peptide genes (Kim et al., 2000), and also to the activation of the prophenoloxidase (proPO) cascade. Phenoloxidase (PO) is an essential enzyme for the cellular immune responses (Cerenius & Söderhäll, 2004) but is also involved

in other developmental and defensive processes such as wound healing and sclerotization (Kanost et al., 2004).

Gram negative bacteria binding proteins (GNBPs) and β-1,3-glucan recognition proteins (βGRPs) have been extensively studied as pattern recognition proteins in Lepidoptera (Fabrick et al., 2003; Jiang et al., 2004; Lee et al., 1996; Ma & Kanost, 2000; Ochiai & Ashida, 2000; Shin et al., 1998). These proteins are produced in the fat body and secreted into the caterpillar's hemolymph. Some are constitutively present (Fabrick et al., 2003; Ma & Kanost, 2000) whereas others are induced upon microbial infection (Jiang et al., 2004). Recognition of β-1,3-glucans by these proteins appears to be due to two distinct β-1,3-glucan-binding domains: a strongly binding N-terminal glucan recognition domain and a weakly-binding C-terminal glucanase-like domain. Neither domain possesses β-1,3-glucanase activity (Fabrick et al., 2004).

The immune response has been extensively studied in Lepidoptera by introducing microbes into the hemocoel of laboratory-reared insects by mechanical wounding. However, since herbivorous lepidopteran larvae consume large amount of plant material during their development, infection via food is very likely. The potential role of the midgut in triggering the immune response of herbivorous larvae has been poorly studied, but there is increasing evidence that it participates in this process. Freitak et al. (2007) showed that in larvae of *Trichoplusia ni* that were fed artificial diet containing bacteria, several immune-related genes were up-regulated and immune-related proteins increased in concentration in the hemolymph.

If the midgut were to play a role in sensing potentially invasive bacteria, one would expect to find pattern recognition proteins expressed in that tissue. Bacteria present in the *Drosophila* gut are thought to release peptidoglycan fragments that bind to specific PGRP (peptidoglycan recognition protein) receptors and induce the *imd* pathway (Liehl, et al., 2006; Nehme et al., 2007; Zaidman-Remy et al., 2006). Simpson et al. (2007) described a sequence (EpGRP1) with similarity to β-1,3-glucan binding proteins from *Anopheles gambiae* and *Bombyx mori* in ESTs derived from the larval midgut of *Epiphyas postvittana*. In a proteomic study of the lumen contents of larval midgut of *Helicoverpa armigera*, we (Pauchet et al., 2008) discovered a similar protein that we named GH16BetaGRP-1 (Glycosyl Hydrolase family 16, β-glucan recognition protein 1) secreted into the lumen. The full-length cDNA showed similarities to β-1,3-glucan recognition proteins from the hemolymph of *B. mori* and *Manduca sexta*. Since β-1,3-glucanase activity had not been previously reported from lepidopteran midguts, we suggested the possibility of an immune-related function.

Here we describe the purification and characterization of GH16betaGRP-1 from the midgut lumen of *H. armigera*. The protein does indeed possess β-1,3-glucanase activity and can account for the majority of such activity in the lumen. Thus we have renamed this protein as *H. armigera* β-1,3-glucanase-1. The mRNA is predominantly expressed in midgut, and the protein product is secreted into the lumen and persists stably there. Expression is increased when the insect feeds on diet containing bacteria. We found similar sequences from a number of lepidopteran species derived from cDNAs generated from midgut tissue samples. These form a novel protein family that is characterized by a highly conserved signature sequence including two glutamate residues previously shown to be necessary for catalytic activity in other β-1,3-glucanases (Juncosa et al., 1994). This family is related to but distinct from a family of previously-described βGRP/GNBP proteins primarily found in lepidopteran hemolymph which do not possess this conserved site. Certain proteins from insects for which a catalytic role has not been suspected, including the *Anopheles gambiae* GNBP subgroup B, belong to the catalytic clade and we suggest that the domain nomenclature be revised accordingly. These features suggest that lepidopteran larvae secrete an active β-1,3-glucanase into the midgut lumen which may function in digestion of β-1,3-glucans released by commensal or invading bacteria.

5. 2 Results

5. 2. 1 A β-1,3-Glucan Binding Protein from H. armigera larval midgut.

Midgut lumen contents of unchallenged *H. armigera* larvae were screened for glucan binding proteins using curdlan, an insoluble β-1,3-glucan isolated from the Gram negative bacterium *A. faecalis*, as an affinity matrix in pull-down assays. The bound fraction was freed from the matrix by boiling and after SDS-PAGE, a single main protein band (apparent MW: 40 kDa) was detected along with trace amounts of other proteins (Fig.1A). The peptide mass fingerprint of this main protein band by MALDI-TOF/MS analysis exactly matched the cDNA sequence of *H. armigera* GH16betaGRP-1 (**ABU98621.1**, Supplementary Information) showing that this previously identified protein, which had been named on the basis of sequence similarity to other Lepidopteran proteins, was the main β-1,3-glucan binding protein in the larval midgut lumen.

Expression patterns of this gene in different tissues were further investigated by quantitative real time PCR (RT-qPCR) (Fig. 1B). The expression level was very high in larval

midgut; much lower but detectable in salivary glands, hemocytes, and the fat body; and undetectable in the rest of the larval body tissue.

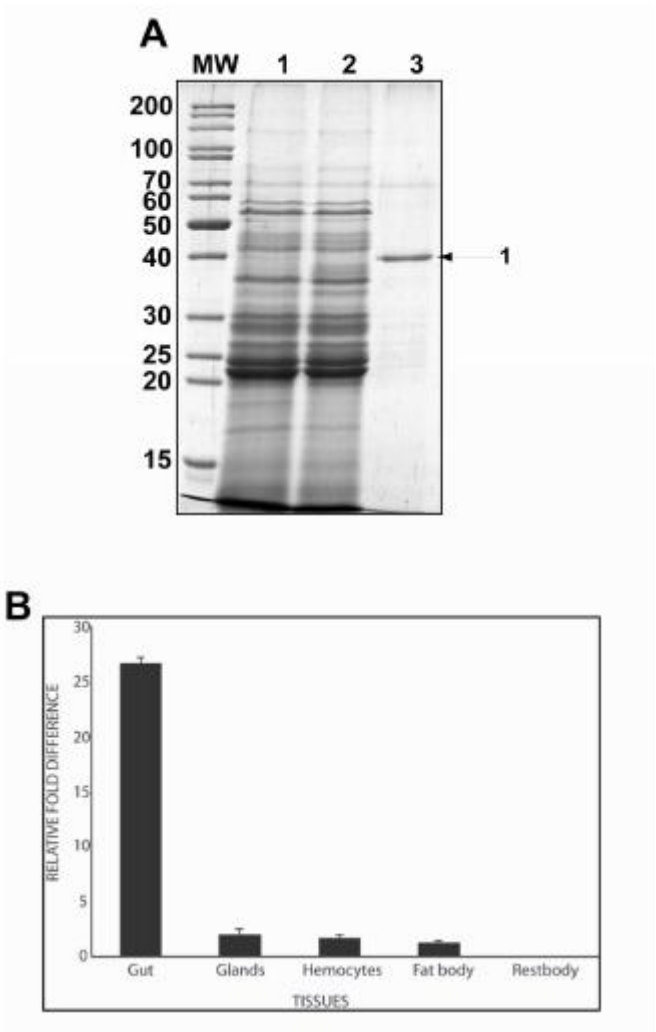


Figure 1: Purification and tissue expression of *H. armigera* glucanase-1. (A) Curdlan pull-down assay on gut larval lumen sample. Proteins were separated by SDS-PAGE using a 12% polyacrylamide resolving gel and stained with coomassie. Lane 1: 10 µg gut lumen proteins, lane 2: 10 µg gut lumen flow-through, lane 3: 15 µl elution fraction. Tryptic peptides obtained from band 1 were analyzed by MALDI-TOF/MS. (B) Tissue expression analysis of *glucanase-1* by real-time quantitative RT-PCR, the different tissue investigated were midgut, salivary glands, hemocytes, fat body and rest body. Relative fold differences were calculated in comparison to *glucanase-1* expression in a pooled sample of adults and pupae for which relative fold expression was set to 1 (whole animals).

5. 2. 2 Glucan-binding protein has β-1,3-glucanase activity.

We screened for glucanase activity in *H. armigera* larval midgut lumen contents by plate assays on carbohydrate polymers followed by staining of any remaining polymer with Congo Red (Fig. 2A). The only tested substrate that was degraded by the lumen sample was CM-curdlan which contains exclusively β-1,3-glucan linkages. Neither cellulose (exclusively β-1,4 linkages) nor lichenan and Barley β-glucan (with mixed β-1,3 and β-1,4 linkages) were degraded by the lumen sample. The latter two substrates could be partially degraded by fungal enzymes specific for β-1,3-glucans or β-1,4-glucans. However, the lumen sample was incapable of degrading the β-1,3-glucan linkages in the lichenan or barley β-glucan (Fig 2A).

To investigate the correlation between β-1,3-glucan binding and glucanase activity, an untreated gut lumen sample was compared with the flow-through from the curdlan pull-down assay by native PAGE followed by an overlay with CM-curdlan and staining with Congo Red (Fig. 2B). Although the resolution of the zymogram was poor, a broad band indicating activity could be detected only in the untreated sample whereas no activity was detected in the flow-through. The broad activity band is likely due to the major band seen in Fig. 1A after the curdlan pull-down, which is poorly resolved on the native gel; although a contribution of some of the fainter bands in Fig. 1A cannot be completely ruled out.

To further test whether the glucan-binding protein itself could be responsible for this β-1,3-glucanase activity, its coding sequence fused to a C-terminal V5/$(His)_6$ tag was transiently expressed in High Five insect cells (Fig. 2C). Using the anti-V5 antibody in a western blot, a protein with an apparent molecular weight of 44 kDa was detected in the culture medium of transfected High Five cells, and a lesser amount was associated with the crude membrane fraction. The increase in apparent molecular weight of about 4 kDa between the gut lumen form of the protein and its recombinant form expressed in insect cells is due to the V5/$(His)_6$ tag. A zymogram using CM-curdlan as substrate revealed a single activity band in the culture medium of cells transfected with the protein construct but not with empty vector (Fig. 2D). These results show that the protein that we had named GH16betaGRP-1 is an active β-1,3-glucanase and suggest that the activity detected in the gut lumen is mainly due to this protein. We thus rename this protein as β-1,3-glucanase-1 from *Helicoverpa armigera*.

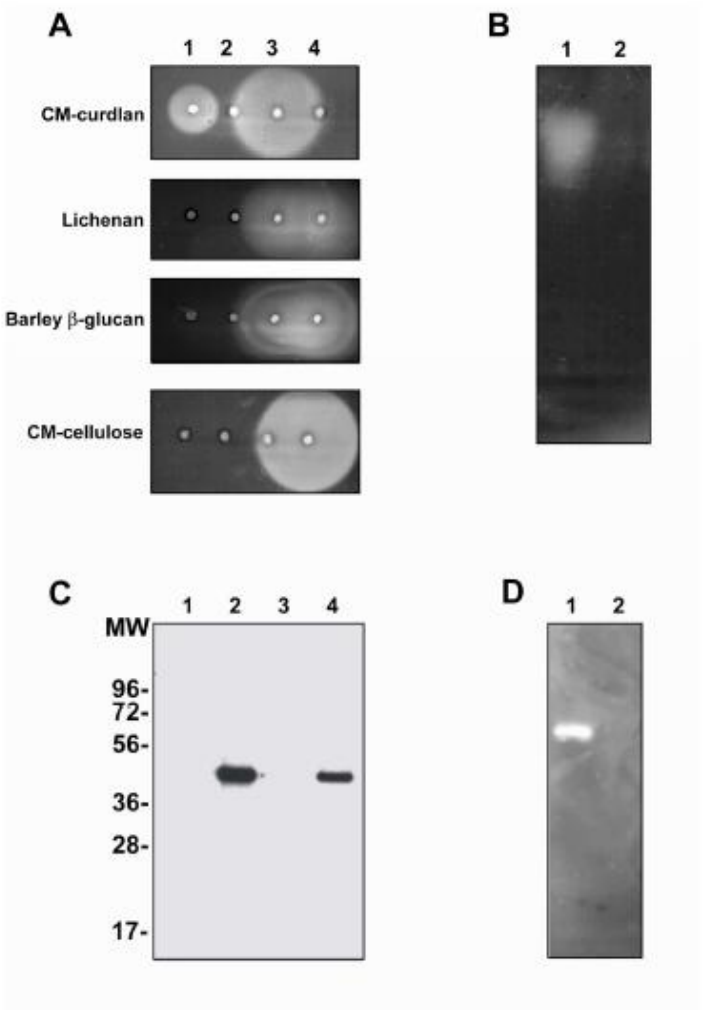


Figure 2: Glucanase activity assays on *H. armigera* larval gut lumen. (A) Assays were performed on 1.5% agar plates containing 0.25% of several types of beta-glucans: CM-curdlan (beta-1,3-glucan), lichenan and barley beta-glucan (mixed beta-1,3:1,4-glucans) and CM-cellulose (beta-1,4-glucan). Activity was revealed by Congo Red staining. Lane 1: larval gut lumen, lane 2: PBS (negative control), lane 3: endo beta-1,3-glucanase from *Trichoderma* sp. and lane 4: cellulase from *Trichoderma* sp. (both positive controls). (B) Beta-1,3-glucan zymogram of *H. armigera* larval gut lumen. Ten micrograms proteins were separated by native PAGE and the gel overlaid on a CM-curdlan-containing agar plate. Activity was revealed by Congo red staining. Lane 1: gut lumen proteins, lane 2: gut lumen proteins after curdlan-pull down assay (flow-through). (C) Analysis of the expression of *H. armigera* glucanase-1 in transfected *T. ni* High Five cells. Ten micrograms of proteins from culture medium and crude membrane fraction of both glucanase-1- and empty vector-transfected cells were resolved by SDS-PAGE using a 12% polyacrylamide resolving gel, then transferred to PVDF and revealed by western blot using the anti-V5-HRP antibody. Line 1: culture medium from control cells, Line 2: culture medium from glucanase-1-transfected cells, line 3: crude-membrane fraction from control cells, line 4: crude-membrane fraction from glucanase-1-transfected cells. (D) Beta-1,3-glucanase activity in culture medium from control and glucanase-1-transfected cells was investigated by zymogram. Ten micrograms of proteins were resolved by native PAGE and the gel overlaid on a CM-curdlan-containing agar plate. Activity was revealed by Congo red staining. Line 1: culture medium from glucanase-1-transfected cells, line 2: culture medium from control cells.

5. 2. 3 Induction of glucanase-1 after ingestion of bacteria.

H. armigera larvae were fed for 24 h on artificial diet containing one of three types of non-pathogenic microbes: Gram negative (*Escherichia coli*) and Gram positive (*Micrococcus luteus*) bacteria and yeast (*Saccharomyces cerevisiae*). Expression of *glucanase-1* was investigated by RT-qPCR (Fig. 3). A ~2.5-fold increase in mRNA levels was observed for larvae fed on Gram negative bacteria as well as a ~4.5-fold increase for larvae fed on Gram positive bacteria. No significant induction was observed for larvae fed on yeast.

Alternatively, larvae were fed on diet containing bacterial cell wall polysaccharides: LTA for Gram positive or LPS for Gram negative bacteria. A ~2-fold increase in mRNA levels was observed for larvae fed on LTA as well as a ~1.8-fold increase for larvae fed on LPS. In addition, no significant induction was observed for larvae fed on artificial diet containing one of three types of β-glucans (Curdlan, Lichenan and Barley β-glucan).

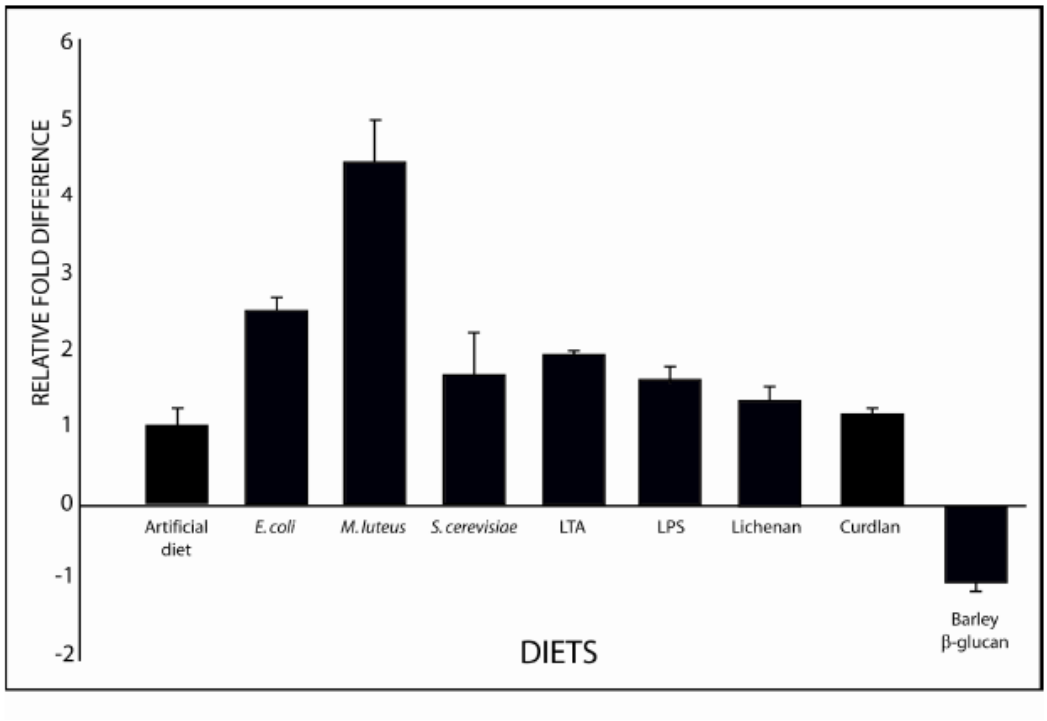


Figure 3: Variation of expression of *glucanase-1* in the midgut of *H. armigera* larvae upon feeding on microbial- or polysaccharides-contaminated diet. Larvae were fed for 24h on artificial diet contaminated with either microbes (*E. coli*, *M. luteus* and *S. cerevisiae*) or bacterial cell wall polysaccharides (LTA and LPS) or various β-glucans (Lichenan, Curdlan and Barley β-glucan). Relative fold differences were calculated in comparison to the data obtained for larvae fed on sterile artificial diet for which relative fold expression was set to 1.

5.2.4 Glucanase-1 is not detected in the hemolymph.

To further confirm the expression specificity of glucanase-1 in the midgut, affinity to curdlan was used to pull down proteins from *H. armigera* hemolymph sample of unchallenged larvae (Fig. 4). Seven protein bands were recovered and tryptic peptides were sequenced *de novo* and identified. None of the proteins matched the glucanase-1 sequence although two (bands b and c) having an apparent MW of around 56 kDa were identified as βGRPs (Table 1) by their matching ESTs from *H. armigera* fat body. Predicted protein sequences of βGRP-1 and -2a are very similar to previously characterized proteins purified from larval hemolymph of the lepidopteran species *M. sexta* and *B. mori* (8,9). In addition to βGRPs, prophenoloxidase (proPO) subunit 2 (band a), a proPO-activating factor-like protein (serine-proteinase like protein 1, band e), a C-type lectin (band f) and a serine-proteinase homolog (band g) were identified in the hemolymph curdlan pull-down fraction (Table 1). All hits obtained using MS-BLAST were of high confidence as given by the search engine except for the C-type lectin. This protein was identified with only a single 9 amino acid peptide (SVIPGNFDK) that was returned as a "borderline" hit by MS BLAST. We took this hit into consideration because the predicted MW of the full-length protein (36.8 kDa) was similar to the apparent MW observed on the gel (Fig. 4).

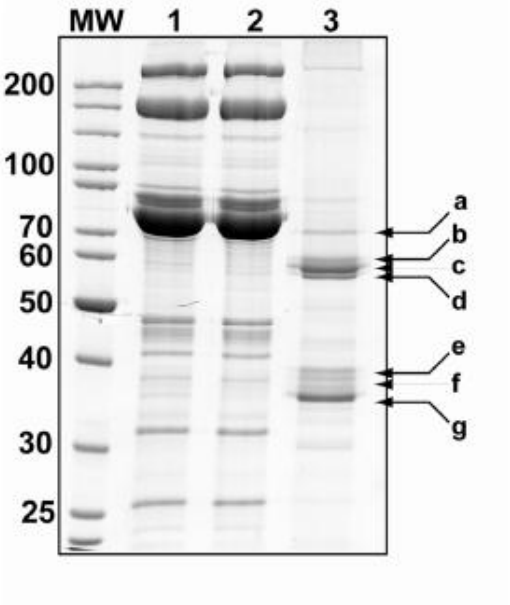


Figure 4: Curdlan pull-down assay on larval hemolymph sample collected from unchallenged larvae. Proteins were separated by SDS-PAGE using a 10% polyacrylamide resolving gel and stained with coomassie. Lane 1: 10 μg cell-free hemolymph proteins, lane 2: 10 μg cell-free hemolymph flow-through, lane 3: 15 μl elution fraction. Tryptic peptides obtained from bands a-g were analyzed by nanoLC-MS/MS and interpreted *de novo*, search results are given in Table 1.

5.2.5 Glucanase-1 is conserved among Lepidoptera and other insect orders.

The *H. armigera* glucanase-1 is 375 amino acids long and it contains a predicted signal peptide with a cleavage site between amino acid 17 and 18 suggesting secretion. Residues 40 through 375 correspond to a conserved domain identified as the glycosyl hydrolase family 16 (pfam00722 Glyco_hydro-16, E = 4e-10) and the subfamily beta-1,3-glucan recognition proteins (cd02179 GH16_beta_GRP, E = 3e-103) as indicated by a search against the Conserved Domain Database. Furthermore, a conserved GH16 active site was found between amino acids 188 and 199 including 2 glutamate residues in positions 188 and 193 that have been shown to be crucial for catalytic activity in the glucanase from *Bacillus licheniformis* (Juncosa, et al., 1994) (Fig. 5A).

Helicoverpa armigera βGRP-1 and -2a from hemolymph also possess the glycosyl hydrolase 16 domain at the carboxy terminus, however the GH16 active site with the 2 glutamate residues is not present (Fig 5A). Both proteins have an additional domain at the amino-terminus of approximately 150 residues (Fig 5B). This domain has no detectable similarity to the glycosyl hydrolase 16 family, and has not yet been assigned to a conserved

domain family, but it occurs in a variety of beta-1,3-glucan binding proteins isolated from lepidopteran hemolymph. Fabrick et al. (2004) denoted this domain as CRD (carbohydrate recognition domain) and showed that truncated proteins from *Plodia interpunctella* expressing only CRD could bind to curdlan and could activate the prophenoloxidase cascade.

Putative orthologs to *H. armigera* Glucanase-1 were found in several species including *B. mori*, *Spodoptera frugiperda*, *E. postvittana*, *Ostrinia nubilalis* and *P. interpunctella*. EST coverage was sufficient to obtain 3 full-length sequences. The *O. nubilalis* sequence was truncated missing 30 nucleotides, and the full-length sequence was kindly provided by Dr. Siu Fai Lee (University of Melbourne, CESAR centre). All EST accession numbers used to obtain the data presented here are given in Supplementary Information. An orthologous protein annotated as beta-1,3-glucanase from the sugarcane borer *Diatraea saccharalis* was found in Genbank (**ABR28479**); this protein is truncated missing a few amino acids at the amino-terminus. Finally, a *S. frugiperda* ortholog was also found in Genbank (**ABR28478**). This protein differs by 4 amino acid substitutions from the protein we obtained via ESTs. The Genbank protein is two amino acids longer than our protein due to a repetition of amino acids 165-166: DW (GATTGG) in our sequence, DWDW (GATTGGGATTGG) in the **ABR28478** sequence. Similar to the *H. armigera* sequence, the 4 orthologs are 375 amino acids long and harbor a conserved GH16 active site (Figure 5A).

Other non-lepidopteran insect species possess likely orthologs of Glucanase-1 that have previously been annotated as GNBP. They are found in several termite species (Bulmer et al., 2006), in the genome of two mosquito species *Aedes aegypti* (GNBPs, **XP_001659797.1**, **XP_001664289.1**, **XP_001659796.1**, **XP_001664288.1**, **XP_001652521.1**) and *A. gambiae* (GNBP subgroup B, **XP_312116.3**, **XP_312118.3**, **XP_312115.1**, **XP_313748.3**) and in the genome of the red flour beetle *Tribolium castaneum* (PREDICTED: similar to β-1,3-glucanase, **XP_970010.1**). All of these sequences have around 60-65% amino acid identity compared to lepidopteran Glucanase-1 and all of them possess a conserved GH16 active site (Fig. 5A). Orthologous sequences were not found in *Drosophila* sp. or in the honeybee genome.

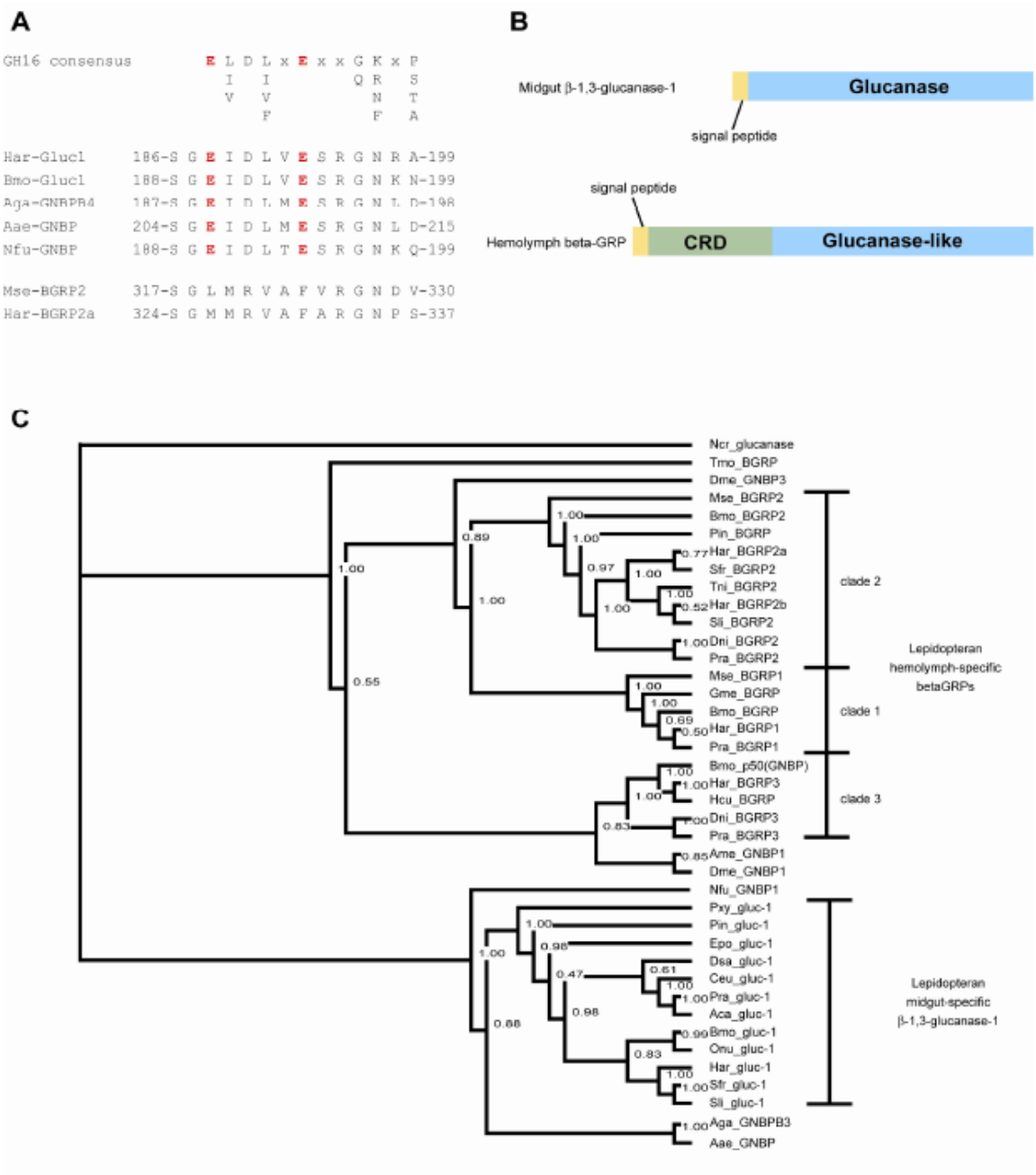


Figure 5: (A) Alignment of the predicted GH16 active site of 2 lepidopteran Glucanase-1 and GNBPs from termite and mosquitoes compared with two lepidopteran hemolymph-specific proteins. Consensus active site for GH16 family was obtained from PROSITE (http://www.expasy.ch/cgi-bin/nicedoc.pl?PDOC00794). Bmo: *B. mori*, Har: *H. armigera*, Nfu: *N. fumigatus*, Aae: *A. aegypti*, Aga: *A. gambiae* and Mse: *M. sexta*. (B) Schematic structure of lepidopteran Glucanase-1 and hemolymph-specific betaGRPs. CRD: Carbohydrate Recognition Domain. (C) Bayesian tree (rooted) of putative betaGRPs protein sequences expressed in several lepidopteran species. Sequences were obtained from Genbank, in-house EST databases and SilkDB. Posterior probability for each branch point is given.

5.2.6 Identification of 4 distinct betaGRP clades in Lepidoptera.

To examine the relationships among βGRP proteins in Lepidoptera, a total of 32 sequences from 17 species (for details refer to Supplementary information S3) were collected and used to construct a Bayesian phylogeny (Fig. 5C). The analysis revealed that these sequences clustered in two distinct clades. One of them falls into three subclades (clades 1, 2 and 3) containing many proteins that had previously been found in insect hemolymph: Mse_BGRP1 and 2 (6,8), Bmo_BGRP and p50_GNBP (7,9), Pin_BGRP (5), and Ha_BGRP1 and 2a (the present study). The second cluster (clade 4) contains the *H. armigera* Glucanase-1 protein isolated from midgut, and sequences from cDNA libraries made from midgut tissue of different Lepidoptera species. This cluster is clearly separated from the other clades by a posterior probability of one and a large branch length. The topology of the tree is unchanged when the comparison is restricted to the glycosyl hydrolase 16 domains of the proteins, and is similar when the neighbor-joining method is used (data not shown). This phylogeny suggests an ancient duplication event leading to paralogues that have different tissue specificity as well as function.

5.2.7 Orthologous glucanase genes in H. armigera and B. mori.

To obtain the intron/exon organization of the *Glucanase-1* gene, a probe from the *H. armigera* cDNA sequence was used to screen a BAC library, yielding a 120 kb clone. When sequenced, it was found to contain the entire *Glucanase-1* genomic region (3407 bp) consisting of 9 exons, with the initiator methionine codon at the start of exon 2 (Fig 6A). For an interspecific comparison, the *B. mori* cDNA sequence was used in a blastn search of the whole genome shotgun (wgs) sequences at NCBI, identifying a total of 5 hits (**AADK01004415**, **BAAB01063034**, **BAAB01133393**, **BAAB01043260**, **BAAB01083370**) that clustered together in one unique contig of 18,022 bp which covers the entire *Glucanase-1* gene between base pair numbers 9395 and 15120 for a total of 5725 bp. Similar to the *H. armigera* one, the *B. mori* gene consists of 9 exons with the transcription initiation methionine codon also at the beginning of exon 2 (Fig. 6A).

We further identified the flanking genes of *Glucanase-1* in both species (Fig. 6B). To do so, we recovered a scaffold of the *B. mori* genome assembly from SilkDB (**scaffold000559,** http://silkworm.genomics.org.cn/) that covered the entire *B. mori Glucanase-1* gene as well as flanking genes. For *H. armigera*, a part of the 120 kb BAC clone

described above was used for the comparative analysis. All of the sequences were blasted via the NCBI webpage against the non-redundant database using BLASTX. A total of 10 and 7 genes were found on the *H. armigera* and *B. mori* sequences respectively (Fig. 6B). The genomic regions of the 2 species contain four genes in common: *Transcription factor IIE*, *Tyrosine receptor kinase*, *GTPase* and *Glucanase-1*, with similar but not identical orientations. No additional or duplicated *BGRP* gene was identified within the respective genomic regions. These data strongly suggest that the *B. mori* and *H. armigera Glucanase-1* genes are orthologous and that a larger genomic region is highly syntenic between these two lepidopteran species.

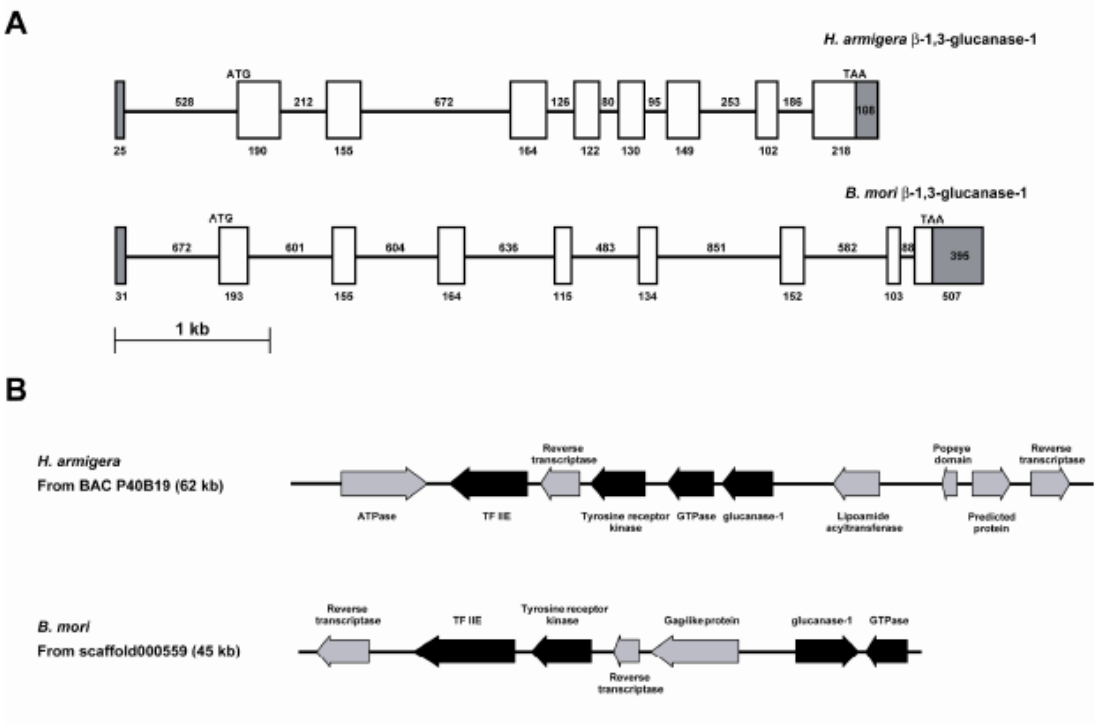


Figure 6: (A) Schematic representation of the intron/exon structure of *H. armigera* and *B. mori Glucanase-1* genes. Boxes represent exons: white boxes correspond to the gene CDS and gray boxes represent the 5'- and 3'-UTR found on the transcripts. Size (in nucleotides) is shown below each exon. Lines represent intron insertions (drawn to scale), and the size of each intron is indicated above the line. Initiator methionine codon (ATG) and stop codon (TAA) are also indicated for both species. (B) Schematic representation of the genomic region around the *Glucanase-1* gene deduced from a *B. mori* scaffold obtained from NCBI and sequencing of a *H. armigera* BAC clone. Black arrows: putative genes common between the 2 species, grey arrows: other putative genes.

5.3 Discussion

Here we present the molecular characterization of a member of a new class of β-1,3-glucan recognition protein in Lepidoptera that binds strongly to curdlan, is predominantly expressed in the midgut, is located in the gut lumen thus in direct contact with the food bolus, and exhibits β-1,3-glucanase activity.

Glucanase-1 orthologs are present in many insect orders and thus this βGRP/GNBP gene family is not mosquito-specific as suggested by Warr et al. (2008). Within the Lepidoptera, we can find a clear distinction between two classes of βGRPs, one possessing the signature of a glucanase active site and expressed primarily in midgut, and the other lacking this signature but possessing an additional C-terminal domain and expressed in other tissues including hemolymph. The difference in function however is not clear yet. We found that the expression of the gene coding for this protein is induced in the midgut of larvae fed on Gram negative or Gram positive bacteria in the diet, suggesting a role in immune response.

The result of our curdlan pull-down assay with the hemolymph sample was complex and several unexpected proteins were identified apart from the βGRPs 1 and 2a. Finding a C-type lectin associated with curdlan was not surprising because lectins are well known polysaccharide-binding proteins and a previously characterized protein from *H. armigera* has been shown to have some affinity for curdlan (Chai et al., 2008). The presence of proPO subunit 2, a proPO-activating factor protein (serine proteinase-like protein) and a serine-proteinase homolog was surprising and is unlikely due to direct binding to curdlan. ProPO is responsible for melanization and has no known affinity for polysaccharides (Hall et al., 1995) similar to proPO-activating factors (Kanost et al., 2004). PRRs (in our case βGRPs and C-type lectins), proPO and proPO-activating factors all participate in the proPO activation cascade (Kanost et al., 2004) and consequently might at some point interact together. Hence, our data suggest that we have precipitated a complex of these proteins. This is consistent with the suggestion of Yu et al. (2003) that, in *M. sexta*, Immunolectin-2 (a C-type lectin), after binding to surface polysaccharides of microbes, forms a complex together with serine-proteinase homologs and proPO-activating factors.

Our results on the dietary induction of *Glucanase-1* by bacteria as well as by LTA and LPS correlate well with observations of one of its ortholog in the mosquito *A. gambiae*, *GNBP-B1*, for which the expression is induced upon bacterial challenge with both Gram negative and Gram positive bacteria with a stronger effect observed with the latter (Dimopoulos et al., 1997). No induction of GNBP-B1 is observed upon challenge with *S.*

cerevisiae (Dimopoulos et al., 1997). Furthermore, Warr et al. (2008) showed that knocking down the expression of GNBP subgroup B genes in adult female mosquitoes by RNAi increased their susceptibility to immune challenge and subsequently their death rate. Our findings suggest that these results should now be considered in light of a possible enzymatic role of GNBP-B proteins. A similar knock-down approach with lepidopteran *Glucanase-1* genes combined with bioassays testing larvae behavior feeding on highly microbial-contaminated diet would be a valuable tool in understanding the potential role in immune defense of Glucanase-1 in caterpillars. Development of gene manipulation techniques for Lepidoptera is a growing research field, but their availability is still limited although some progress has been made recently especially with *H. armigera* (Mao et al., 2007).

In Lepidoptera and other insect orders, pattern recognition proteins including βGRPs/GNBPs trigger an innate immune response after recognition of PAMPs by activating the prophenoloxidase cascade (Kanost et al., 2004; Kim et al., 2000) and the Toll receptor pathway in hemocytes (Ao et al., 2008). These events take place in the hemolymph and thus it is unlikely that Glucanase-1 could activate this cascade from its site of activity in the midgut lumen. Furthermore, the presence of two distinct groups of βGRPs in Lepidoptera suggests an ancient duplication event leading to paralogous genes. Subfunctionalization and neofunctionalization could have led to different functions of both these paralogs. Hence, hemolymph specific βGRPs might trigger innate immune response, whereas midgut specific βGRPs (Glucanase-1) possibly fulfill a different function within the organism.

The recognition that glucanases and non-catalytic glucan-binding proteins are similar but distinct clades is hampered by current nomenclature, including the misleading definition of a conserved domain in the NCBI CDD database. Version 2.15 lists a Conserved Domain, cd02179: G16_beta_GRP, described as follows: "Beta-GRP (beta-1,3-glucan recognition protein) is one of several pattern recognition receptors (PRRs), also referred to as biosensor proteins, that complexes with pathogen-associated beta-1,3-glucans and then transduces signals necessary for activation of an appropriate immune response ... ". This description is supported by citations (Ma & Kanost, 2000; Fabrick et al., 2004) describing studies on the non-catalytic hemolymph bGRP proteins from Lepidoptera. However, none of these lepidopteran proteins lacking the GH16 active site with two glutamates are among the 14 sequences used to construct the consensus sequence for this cd02179 domain. Instead, all 14 sequences possess the active site, including 7 from fungi, 2 from earthworms, 4 from crustaceans, and 2 mosquito sequences (one is a GNBP-B from *A. gambiae*, which as far as we know has not been tested for glucanase activity). Thus biological information from those

sequences has been associated with the definition of a domain possessing the catalytic site, yet sequences from Lepidoptera have not been used to represent a separate domain lacking the catalytic site. Recognition of an additional subfamily containing bona-fide GRPs lacking the active site would better reflect the existence of similar but distinct catalytic and non-catalytic clades that we have found.

Our data show that glucanase-1 hydrolyzes specifically β-1,3-glucans. The major source for this type of polysaccharides is the cell wall of bacteria, yeast and fungi. One of its ortholog in *A. gambiae*, GNBP-B4, binds to the surface of both Gram negative and Gram positive bacteria (Warr et al, 2008), but its potential beta-1,3-glucanase activity has not been tested yet. Genta et al. (2003) purified a 46 kDa endo-β-1,3-glucanase from salivary glands of the cockroach *Periplaneta americana.* Their purified protein was able to lyse *S. cerevisiae* cells in hypotonic medium lacking nutrients, making their content available as a nutritive source, but no lysis was observed in isotonic nutritive medium. The authors also pointed out that although it is conceivable that this enzyme may protect midgut cells from microbial invasion, its presence in saliva and the large amounts of fungi usually found in detritus, the major food source of *P. americana*, points more strongly to a digestive role. We could not observe any lytic activity by plate assay on any microbe tested using partially purified Glucanase-1 from culture medium of High Five transfected cells (data not shown) although the recombinant protein exhibits glucanase activity. We cannot exclude that this lack of activity is due to a too low concentration of recombinant Glucanase-1. A more thorough investigation of the potential anti-microbial activity of Glucanase-1 should be undertaken.

Similarly, it has been proposed that β-1,3-glucanase from plants may act synergistically with other hydrolases, such as chitinases and proteinases, to disrupt the structural integrity of fungal cell wall (Mauch et al., 1988). Alternatively they may release oligosaccharides from β-1,3-glucan substrates that may serve as chemical signals leading to the activation of other defense responses (Lamb et al., 1989).

In addition, the midgut of herbivorous caterpillars is composed of a highly diverse microbial community that might contribute to its physiological function (Ping et al., 2007; Spiteller et al., 2000; Xiang et al., 2006) and it is unclear how Glucanase-1 could differentiate between them, as the molecular motifs activating the innate immune response are present in both beneficial and intruding microorganisms. One hypothesis is that Glucanase-1 has a similar function as PGRP-LB in *Drosophila* in preventing local immune activation by commensal gut bacteria (Zaidman-Remy et al., 2006). PGRP-LB, in contrast to PGRP-LC, is an active amidase able to digest peptidoglycan molecules released by dividing gram-negative

bacteria. PGRP-LB regulates the level of immune response by scavenging peptidoglycan present in the *Drosophila* larval gut. If this scavenging effect is overwhelmed, by bacterial infection for example, the excess peptidoglycan is recognized by the nonenzymatic PGRP-LC which activates the immune response through the Imd pathway. In the case of peptidoglycan released by commensal gut flora, the scavenging effect of PGRP-LB is sufficient to prevent activation of the Imd pathway and thus to inhibit any immune response.

Our results raise the following questions: (i) Can Glucanase-1 be considered as a true pattern recognition protein? (ii) What is the role of Glucanase-1 in the midgut: Immune defence protein or digestive enzyme? The present study is a first step in understanding the physiological role of this new lepidopteran midgut-specific βGRP protein family. More investigations have to be conducted in order to fully address these questions.

5.4 Experimental procedures

5.4.1 Insects.

The TWB strain of *H. armigera* Hübner (Lepidoptera: Noctuidae) was collected from the vicinity of Toowoomba, Queensland, Australia. Neonates were reared on a chemically defined diet containing only casein as the protein source and no plant-derived material, as described by Vanderzant (1968) at 26 °C with a 16:8 (L:D) photoperiod.

5.4.2 Sample preparation.

Gut lumen sample was prepared essentially as described by Pauchet et al. (2008). Briefly, midguts were dissected from actively feeding second day fifth-instar larvae in ice-cold phosphate-buffered saline (PBS). Peritrophic matrix containing the food bolus was pulled out of the midgut with forceps and gently homogenized by 10 strokes in a Potter-Elvehjem homogenizer in PBS pH 7.5 containing a cocktail of protease inhibitors (Complete EDTA-free, Roche Applied Science) in order to release soluble proteins. After centrifugation (30,000xg, 30 min, 4 °C), the supernatant containing the gut lumen soluble proteins was kept and protein concentration was determined using the Protein Dye reagent (BioRad) and bovine serum albumin (BSA) as standard. Hemolymph was collected via lateral punctuation of abdominal part of larvae with micropipette. Collected hemolymph was immediately diluted with ice-cold N-Phenylthiourea-saturated PBS containing protease inhibitors and hemocytes were removed by centrifugation (3,000xg, 15 min, 4 °C).

5.4.3 Curdlan pull-down assay.

Gut lumen and hemolymph samples (1 ml each) were incubated 30 min at 22 °C with 2.5 mg curdlan (an insoluble β-1,3-glucan isolated from *Alcaligenes faecalis*, Sigma). After centrifugation of the sample/curdlan mixture (16,000xg, 5 min, 4 °C), the supernatant corresponding to the unbound fraction was saved. The curdlan pellet was washed 5 times with PBS followed by 2 washes with PBS containing 1 M NaCl and finally 3 more times with PBS. Bound proteins were eluted from curdlan by boiling 10 min in SDS-PAGE sample buffer. After centrifugation (16,000xg, 5 min, 4 °C), the supernatant was analyzed by SDS-PAGE.

5.4.4 Protein analysis by mass spectrometry.

This was done essentially as described by Pauchet et al. (2008). Protein bands were excised manually from the SDS-PAGE gels and were destained, trypsinized, and extracted using an Ettan TA Digester running the Digester Version 1.10 software (GE Healthcare Bio-Sciences AB). Trypsin digestion was carried out overnight with 50 ng of porcine trypsin (Promega) at 37 °C. First step analysis of the tryptic peptides was done using a MALDImicro MX mass spectrometer (Waters) used in reflectron mode and was calibrated using a tryptic digest of bovine serum albumin (MPrep, Waters). The MALDI-TOF spectra searches were performed with the Protein Lynx Global Server software, version 2.2 (PLGS 2.2, Waters) against the NCBI_insecta database (downloaded on 15 March 2008 from http://www.ncbi.nlm.nih.gov/database, 372,057 entries). The search parameters were as follows: peptide tolerance of 80 ppm, one missed cleavage, carbamidomethyl modification of cysteines, and possible Met oxidation. An estimated calibration error of 0.05 D and a minimum of four peptide matches were the criteria for obtaining positive database hits.

The MALDI-TOF peptide signal intensities were used to estimate the volume of the remaining sample to be used for the subsequent nanoLC-MS/MS *de novo* sequence analysis. Liquid chromatography-tandem mass spectrometry was performed to acquire fragmentation data from selected peptides. Aliquots of tryptic peptides were injected on a CapLC XE 2D nanoLC system (Waters). After concentration and desalting, eluted peptides were transferred to the NanoElectroSpray source of a Q-TOF Ultima tandem mass spectrometer (Waters). MS/MS spectra were collected by MassLynx v4.0 software (Waters). ProteinLynx Global

Server Browser v.2.2 software (PLGS 2.2, Waters) was used for baseline subtraction and smoothing, deisotoping, and *de novo* peptide sequence identification.

Using PLGS 2.2, CID spectra were interpreted *de novo* to yield peptide sequences. Sequences with a ladder score (percentage of expected y- and b-ions) exceeding 30 were then used in a homology-based search strategy using the MS BLAST program (19). MS BLAST was developed to utilize redundant, degenerate and partly inaccurate peptide sequences in similarity searches of protein databases that may be derived from organisms phylogenetically distant from the study species. The WU-BLAST2 BLASTP search engine (W. Gish 1996, http://blast.wustl.edu) is employed with parameter values that disallow gaps within a peptide, and that score only the most significant match in the case of several peptide candidates covering the same region in the target sequence. In addition, the PAM30MS matrix which accounts for the inability to distinguish I and L residues, and allows for unknown residues X, is used in the blastp similarity search (Shevchenko et al., 2001). Scoring of the significance of peptide matches is not based on E- or p-values of the individual HSPs (high-scoring segment pairs) but instead on pre-computed threshold scores conditional on the number of query peptides and HSP hits. The color-coded output produced by the MS BLAST script identifies in red those target sequences with scores exceeding 99% of queries utilizing randomized peptide sequences by chance. Computational studies have estimated a false positive rate of <3% (Habermann et al., 2004). We installed an in-house MS BLAST server for searching NCBI_insecta and a locally generated EST database from *H. armigera* midgut and fat body cDNA libraries (5,685 protein sequences). Additional information on MS BLAST statistics and scoring can be found in Shevchenko et al. (2001).

5.4.5 Beta-glucanase activity assays.

Lichenan, Barley β-glucan and carboxymethyl (CM)-cellulose were from Sigma. CM-curdlan, endo-β-1,3-glucanase from *Trichoderma* sp. And cellulase from *Trichoderma* sp. were both from Megazyme.

Plate assays were performed on 1.5% agar Petri dishes containing 0.25 % of each substrate in 50 mM Citrate-Phosphate buffer pH 6.0. Wells within plates were made by puncturing the agar with a plastic pipette and removing the agar plug by suction. Two and a half micrograms gut lumen proteins in 2.5 µl PBS were tested together with 2.5 µl PBS as negative control, and 1 µl of a 100 times diluted solution of each endo-β-1,3-glucanase and

cellulase as positive controls. After 16 hours incubation at 37 °C, activity was revealed by staining with Congo Red (0.1 % (w/v) in water) for 30 min and destaining with 1M NaCl also for 30 min. Plates were scanned using a GS800 densitometer (BioRad).

For zymograms, samples were resolved by native PAGE using 4-15 % polyacrylamide gels (Ready Gels, BioRad) and Tris-Glycine pH 8.3 as running buffer. Electrophoresis was performed at 150 V for 60 min at 4 °C. After the run, gels were washed 3 times 15 min in 50 mM citrate-phosphate buffer pH 6.0 before being overlayed on a CM-curdlan plate prepared as described above. Incubation and Congo Red staining/destaining were performed as described above.

5.4.6 Cell culture and transfection.

The *Ha_GH16betaGRP-1* cDNA was amplified from a *H. armigera* larval midgut cDNA library clone using primers GRP1-F (5'-GCCACCATGTGGTCGGTGTTAGCGGGCGTG-3') and GRP1-R (5'-CAAAGCCCAAATGCGAACGTAGTC-3') and was inserted in pIB/V5-His TOPO (Invitrogen) by TA cloning according to the supplier's instructions. Positive clones were selected and correct insertion was confirmed by sequencing.

Trichoplusia ni High Five cells (Invitrogen) were cultured at 27 °C in Express Five serum-free medium (Gibco) supplemented with 2 mM L-Glutamine and 10 µg/ml Gentamycin. Cells were transfected in 90 mm-diameter Petri dishes with 12 µg plasmid DNA using Insect Gene Juice (Novagen) as transfection reagent. Forty-eight hours post-transfection, culture medium was saved and the cells were detached from the plate and pelleted by centrifugation. After two washes with PBS, cell pellets were resuspended in PBS supplemented with Complete protease inhibitor mixture (Roche Applied Science). Six cycles of freezing and thawing were performed to lyse the cells. Crude-membrane fraction was recovered by centrifugation (13,000xg, 20 min, 4 °C) and resuspended in cold PBS/Complete. Expression was analyzed by western blot using the anti-V5-HRP antibody (Invitrogen).

5.4.7 Preparation of cDNA libraries.

TRIzol Reagent (Invitrogen) was used to isolate total RNA from whole larvae or dissected tissues according to the manufacturer's protocol. After DNase treatment, total RNA were further purified by using the RNeasy MinElute Clean up Kit (Qiagen) following the

manufacturer's protocol. Poly(A)+ mRNA were purified by binding to an oligo d(T) column (RNA Purist, Ambion). The generation of *T. ni* whole-larvae cDNA library has been described in Freitak et al. (2007). Generation of *Plutella xylostella*, *Pieris rapae*, *Colias eurytheme*, *Anthoracharis cardamines* and *Delias nigrina* has been described in Fischer et al. (2008). For *H. armigera* midgut and fat body and *Spodoptera littoralis* whole-larvae, normalized, full-length enriched cDNA libraries were generated using a combination of the SMART cDNA library construction kit (Clontech) and the Trimmer-Direct cDNA normalization kit (Evrogen) generally following the manufacturer's protocol but with several important modifications. In brief, 2 µg of poly(A)+ mRNA was used for each cDNA library generated. Reverse transcription was performed with a mixture of several reverse transcription enzymes for 1h at 42 °C and 90 minutes at 50 °C. cDNA size fractionation was performed with SizeSep 400 spun columns (GE Healthcare) that resulted in a cutoff at ~200 bp. The full-length-enriched cDNAs were cut with SfiI and ligated to pDNR-Lib plasmid (Clontech). Ligations were transformed into *E. coli* ELECTROMAX DH5α-E electro-competent cells (Invitrogen).

5.4.8 Generation of EST Databases.

Plasmid minipreparation from bacterial colonies grown in 96 deep-well plates was performed using the 96 robot plasmid isolation kit (Eppendorf) on a Tecan Evo Freedom 150 robotic platform (Tecan). Single-pass sequencing of the 5' termini of cDNA libraries was carried out on an ABI 3730 xl automatic DNA sequencer (PE Applied Biosystems). Vector clipping, quality trimming and sequence assembly was done with the Lasergene software package (DNAStar Inc.). Blast searches were conducted on a local server using the National Center for Biotechnology Information (NCBI) blastall program. Protein domains were determined by searching the NCBI Conserved Domain Database (CDD) at http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml. Sequences were aligned using ClustalW software (Thompson et al., 1997).

5.4.9 BAC library screening and sequencing.

H. armigera BAC library nylon filters were washed, blocked and hybridized with horseradish peroxidase (HRP)-labelled DNA fragments containing part of the *GH16betaGRP-1* gene. Labelling, hybridization and probe detection were done according to specifications in the

ECL DNA labeling and detection kit (GE Healthcare). Positive clones were isolated from glycerol stocks, grown in Terrific Broth and BAC DNA was isolated with the Nucleobond Xtra Midi Kit according to the manufacturers´ instructions (Macherey-Nagel). BAC Genomic DNA quantity was measured photospectrometrically on a Nanodrop ND1000 and all of the positive clones were digested with EcoRI and HindIII to identify clone diversity and redundant inserts, blotted and re-hybridized to verify positive inserts. The BAC DNA was sheared into two different size ranges (1-1,5 kb and 4-5 kb) with a Hydroshear device (Molecular Devices), blunted with the Quick Blunting Kit (NEB), isolated from an agarose gel, column purified and ligated into the pUC19-SmaI vector (Fermentas). Ligations were transformed into *E. coli* ELECTROMAX DH5α-E electro-competent cells (Invitrogen). Plasmid preparation, sequencing and assembly were performed as mentioned above.

5.4.10 Feeding assays.

Fifteen early fifth-instar *H. armigera* larvae were fed for 24h on artificial pinto-bean based diet soaked with overnight liquid culture of either *S. cerevisiae* or *M. luteus* or *E. coli*. Alternatively, larvae were fed on artificial diet soaked in solutions containing either β-glucans (0.25 % w/v) or bacterial cell wall polysaccharides (LTA or LPS, 0.05 % w/v). Larvae fed on sterile artificial diet were used as a control group. Three times 5 larvae per diet were used for detecting diet-induced changes in *GH16betaGRP-1* expression in midgut tissue. Experiments were repeated 3 times with different patches of eggs.

5.4.11 Quantitative real-time PCR.

Total RNA was prepared from dissected insect midguts, salivary glands, fat bodies and rest of bodies (except head capsules) according to the method described above. Five hundred nanograms of DNA-free total RNA was converted into single-stranded cDNA using a mix of random and oligo-dT20 primers according to the ABgene protocol (ABgene). As an endogenous control gene, large ribosomal protein 1 (*Lrp1*) was used (forward primer: 5'-CACATCAGCAAACACATCACC and Reverse primer: 5'-AGAAGTGAGAGCCGTGTGAAA). Gene-specific primers were designed on the basis of sequence obtained for *GH16betaGRP-1* gene (forward primer: 5'-CTGGATCAGAAGGAACACTGC and reverse primer: 5'-

TACACGTCCCAGTTCCAAGTC). Q-RT-PCR was done in optical 96-well plates on a MX3000P Real-Time PCR Detection System (Stratagene) using the Absolute QPCR SYBR green Mix (ABgene) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye included in the PCR master mix.

5.4.12 Phylogenetic analysis.

Genes were aligned by their amino acid sequences using MAFFT multiple sequence alignment program (Katoh et al., 2002). The amino acid alignment was then used for phylogenetic analysis.

The phylogenetic reconstruction was done by Bayesian inference using Mr. Bayes 3.1 (Mao et al., 2007). The prior was set for the amino acid models to mix, thereby allowing model jumping between fixed-rate amino acid models. Markov Chain Monte Carlo runs were carried out for 1,000,000 generations after which log likelihood values showed that equilibrium had been reached after the first 400 generations in all cases, and those data were discarded from each run and considered as ‘burnin’. Two runs were conducted for the dataset showing agreement in topology and likelihood scores.

Phylogenetic analysis was furthermore performed using the Neighbour-Joining (NJ) method (TREECON) based on the MAFFT alignments. Distance calculations were performed after Tajima & Nei and bootstrap analysis, running 1000 bootstrap samples. The Neighbour-joining and the Bayesian tree topologies including their general subfamily relationships and node supports were in agreement.

Table 1. Protein identification from the curdlan pull-down assay on *H. armigera* larval hemolymph

band	database searched	hit1	species	accession2	*de novo* sequencd peptides		peptides matched by MS BLAST		MS BLAST score3	
a	ncbi_insecta	porPO subunit 2	*H. armigera*	AAZ52554	5		5		336	
b	HA_EST_db	beta-1,3-glucan recognition protein 2a	*H. armigera*	EU770382	4		1		78	
c	ncbi_insecta	beta-1,3-glucan recognition protein	*B. mori*	NP_001036840	5	5	3	1	182	101
	HA_EST_db	beta-1,3-glucan recognition protein 1	*H. armigera*	EU770381*						
d	not identified				6					
e	HA_EST_db	Serine-proteinase like protein 1	*H. armigera*	EU7770389*	3		2		145	
f	HA_EST_db	C-type lectin	*H. armigera*	EU770390*	3		1		64	
g	ncbi_insecta	masquerade-like serine protease	*B. mori*	NP_001037053	6	6	2	4	113	
	HA_EST_db	Serine-protease homolog-1	*H. armigera*	EU770391*					192	

1For hits obtained by searching ncbi_insecta, the annotation is the one given by the database. For hits obtained by searching our *H. armigera* EST database, the annotation has been given according to the *M. sexta* homologs (Kanost et al., 2004). 2Genebank accession numbers (*from this study). 3MS BLAST scoring is detailed in the Material and Methods section.

6 General discussion

6. 1 Midgut mediated immune responses

Insects defend themselves against pathogens by a highly evolved set of reactions involving various cellular and humoral reactions. These immune defenses show several similarities to innate immunity of mammals, and can be divided into systemic and local responses. In mammalian studies the importance of gut epithelial tissues in the immune responses has been recognized for some time (Janeway, 2005). In the case of the invertebrates, this aspect of immune defense is not well studied, although information on specific parasite-host interactions taking place in the insect midgut is accumulating. The best studied examples are the *Anopheles-Plasmodium* model (Siden-Kiamos & Louis, 2004; Gupta et al., 2005) and lepidopteran host-*Bacillus thuringiensis* (Bt) relations (Rahman et al., 2007). The dipteran model species *Drosophila* has been used to study defenses related to ingested entomopathogens and consequent immune responses in the midgut tissue itself (Tzou et al., 2000; Ha et al., 2005; Liehl et al., 2006) and in systemic immune response (Tzou et al, 2000; Vodovar et al., 2005). All these studies used actual pathogens which employ specialized adaptations to enter the host and establish the infection.

One of the most intensively studied cases of gut mediated infection in insects is the medically important mosquito - malaria (*Anopheles gambiae* – *Plasmodium*) system. The long evolutionary relationship between *A. gambiae* and *Plasmodium* has produced a good model system to study host-parasite interactions taking place in the gut. To infect its intermediate host mosquito, *Plasmodium* has to overcome both local and systemic immune defenses (Richman et al., 1997; Dimopoulos et al., 1997). The immune response against *Plasmodium* involves different steps, starting from recognition, signaling amplification cascades, immune signaling pathways and finishing with expression of downstream effector molecules (Gupta et al., 2005; Garver et al., 2008). Knowledge of these steps forms a useful basis for comparison of other gut-pathogen interactions in insects.

In the case of another Dipteran model species - *Drosophila*, it is known that against gut borne parasites, the formation of free radicals is essential to fight the infection (Ha et al., 2005). It is also proposed that communal microflora in the gut have a function in defense against entomopathogens (Xi et al., 2008; Liehl et al., 2006). In the case studies involving *B. thuringiensis* effect on the host, Cry-toxins in the bacterial spore disrupt the host's gut epithelia and enable Bt and other bacteria to proliferate (Broderick et al., 2006). Rahman and

his co-authors (2006) propose the involvement of lipophorin particles in defense against Bt by initiating a coagulation reaction that protects surrounding tissues against pathogens.

This thesis poses the question, could the midgut function as a sensing organ, and depending on the environmental signal also shape the immunocompetence of the organism? I have chosen to use essentially non-pathogenic bacteria as a stimulus, as the majority of bacteria encountered via the food are not entomopathogens. The role of nonpathogenic microorganisms in activating an immune response via the midgut has not been addressed previously, and the effect of dietary bacteria on the physiology and accompanying fitness consequences are unknown.

My results show that midgut tissue can react to the increased concentration of bacteria in the food by up regulating expression of various genes involved in immune defense (Chapter I). The response is systemic and just feeding on bacteria-rich diet leads to changes in immune status for several hemolymph based markers, including enzyme activities and protein expression (Chapter I). Two important immune response related enzymatic activities show a response in the hemolymph – phenoloxidase and general lytic activity. Phenoloxidase activity is down regulated in the hemolymph of bacteria-fed larvae and general antibacterial activity is up regulated in comparison to enzyme activities of larvae fed on bacteria-free diet. Both enzymes are widely investigated in studies of insect immunity and their high activity levels are considered to be an indication of good immunocompetence (Rantala et al., 2000; Armitage & Siva-Jothy, 2005; Wilson et al., 2001; Adamo, 2004). It has also been noted that often these two enzymes are not highly active at the same time and it has been proposed that a trade-off exists between their activities, due to high autoimmune costs (Wilson et al., 2001). Defense reactions involving phenoloxidase have been shown to be costly to the organism, having detrimental effects on host tissues due to release of free radicals, which are active against self as well as invaders (Sadd & Siva-Jothy, 2006, Nappi & Christensen, 2005). This is probably one reason why the prophenoloxidase-cascade is under very tight control by various enzymes and has usually only a limited activation window (Nappi & Christensen, 2005; Cerenius et al., 2008). A diet related trade-off between general antibacterial activity and phenoloxidase activity can be seen also in *T. ni* larvae. The general antibacterial activity has been more related to bacterial infections and phenoloxidase activity to fungal and multicellular parasite infections (Gillespie et al., 1997), current results are in good correlation with this hypothesis. General antibacterial activity is higher in larvae fed bacterial diet and phenoloxidase is higher in larvae fed bacteria-free diet. This could also mean that a certain

steady state activity for phenoloxidase is one of the sustained levels of immune system and it will be changed according to the signals coming from the environment.

A number of proteins are highly expressed in the hemolymph of bacterial diet fed larvae, namely arylphorin, apolipophorin III and gloverin (Chapter I). The first two are also known to be storage proteins and gloverin is a strictly immune inducible effector molecule actively attacking bacterial cell walls (Axen et al., 1997; Lundström et al., 2002). The precise function of arylphorin in immunity is not known to date, but apolipophorin III is known to be able to bind to bacterial surface sugar moieties and act as a pattern recognition protein and has also been proposed to function as an opsonin (Kim et al., 2004).

Costs of a changed immune status are reflected in life-history traits, like body mass and developmental time, and in both cases the negative effects of bacterial feeding are observed (Chapter I). It can be seen that feeding on bacterial diet and altering organismal immune status has fitness costs, that could result in reduced reproductive success and fecundity. As this alteration is caused by nonpathogenic bacteria, it may imply that dietary bacteria-induced signaling is not very specific and insects can not differentiate between pathogenic and non-pathogenic ones. As plant material always contains a lot of bacteria and most of them are probably non-pathogenic, one could argue that it would be beneficial for the insect to recognize only the really pathogenic bacteria and not react to others. This would reduce the threat of altering immune status without real necessity and allow the resources to be allocated into other essential life-history traits. The fact that more specific recognition mechanisms have not been evolved could be due to the so called “rare predator” phenomenon (Dawkins, 1999). As more specific recognition mechanisms would be even more costly to develop and maintain, and given that the chance to meet a very specific pathogen is not very high, one could be protected enough by developing a wider recognition margin. We might have a situation occurring where the type of alteration of steady state immune responses could be sufficient for taking care of most of the moderate infections. And in the case of infection with actual entomopathogens, a further upregulation of immune system or some of its components could follow. This could then rely on other indicators of infection, like damaged tissues, bacterial waste products or bacterial metalloproteases (Altincicek & Vilcinksas, 2008; Clermont et al., 2004).

The experiments performed in this thesis cannot answer how the signaling from midgut to hemocoel is happening. One possibility is that the midgut cells themselves sense the bacteria patterns and use an unknown cell signalling pathway to activate neighboring cells. Alternatively, it could be that small particles of the bacterial cell wall are somehow

passed through the midgut tissue and secreted/transported to the hemocoel, where they are recognized by pattern recognition molecules. This would be an example of the phenomenon of transcytosis, for which there is some experimental support in Lepidoptera (Casartelli et al. 2007).

Pattern recognition proteins are molecules able to recognize pathogen specific markers. A plethora of them are circulating in the hemolymph, but are also present on hemocyte cell membranes (Steiner, 2004). A screen of the proteome of the midgut lumen of *Helicoverpa armigera* identified a novel 40 kDa protein with β-1,3-glucan-binding capacities (Chapter IV). β-1,3-glucans are polysaccharides which are, together with peptidoglycans, lipopolysaccharides and lipotechoic acids, characteristic molecules on the surface of microorganisms. They are also binding sites for pattern recognition proteins and receptors, that will after binding to the antigens trigger an immune response (Janeway, 2005). The protein discovered (named glucanase-1), shows similarity to previously described lepidopteran βGRPs from hemolymph but in contrast to them, is an active β-1,3-glucanase. However, glucanase-1 is exclusively expressed in the midgut tissue and not highly inducible upon feeding different immune elicitors (Chapter IV). Upregulation of mRNA levels of glucanase-1 upon feeding on diet containing high amounts of *Escherichia coli* and especially *Micrococcus luteus* are seen. It is not clear from these results whether the identified protein is only involved in defense reactions or also in digestion (Chapter IV).

It can be concluded that the digestive system functions as a radar for possible infections that can be detected via food. Furthermore, signals coming from food are sufficient to alter steady state immune status in caterpillars, having at the same time fitness-related costs (Chapter I).

6. 2 Homeostasis of the organism under changed immune status

A reflection of the cost of mounting an immune response is that changes in the immune status are accompanied with other changes in physiology. Many alterations in the organism taking place at the same time with the up regulation of immune system may relate to the general stress related adaptations of the body (Altincicek & Vilcinskas, 2007; Altincicek & Vilcinskas, 2008).

Indeed, as seen in comparing the transcripts of larvae grown on different diets, the expression pattern for a number of genes with functions probably not directly related to the immune

system is changed (Chapter II). Genes were clustered into 7 different functional classes: defense and recognition, development, digestion, DNA-related, metabolism, ribosomal proteins, signaling and unknown. The patterns are very complex and not always easily explainable. Diet-dependent changes in the transcripts were monitored during different life stages (2 day old, 7 day old and adult stage) and different tissues (midguts and rest of the bodies) (Chapter II). For some genes, similar diet-linked up or down regulation can be seen in different life stages, and for others this is not the case. Some of the changes in general gene expression can be also seen in the next generation, as the gene expression was studied in the eggs laid by parents grown on bacterial and bacteria-free diet. This suggests that the physiology of the offspring is influenced by the parental environment in a complex manner.

6. 3 Trans-generational immune priming

Trans-generational immune priming is the physiological phenomenon reflecting the fact that parents can influence their offsprings' immunocompetence. In the case of mammals the passing of antibodies via maternal milk to the next generation is a well established mode of promoting the immune defenses against infections (Grindstaff et al., 2003; Hanson, 2007). Since insects lack antibody-based immunity this precise mechanism of immune priming can be excluded, however fortifying the egg with other immune-active molecules is a possibility. Even without knowledge of the mechanism, it has been shown in a number of studies that invertebrates are able to enhance the survival rate of their offspring after immune challenge.

The possibility of nonspecific immune memory in insects has received increasing attention recently. Using the term "memory" in the case of invertebrates has received criticism by those who claim that it applies only in the case of antibody-based immunity characteristic of vertebrates. However, an increasing number of studies show that invertebrate immune systems possess a certain amount of nonspecific memory. Two important steps are now being recognized: (1) once the immune defense is induced, it can last for several days; (2) it is possible to prime insects by injecting immune elicitors against infections and increase survival in the case of subsequent pathogen attack (see Pham & Schneider, 2008 for review). There is also some evidence for more specific immune memory in invertebrates. In one study, Sadd & Schmid-Hempel (2006) showed that it is possible to prime bumble bees against a specific strain of bacteria, by previously exposing them to a sublethal dose after which the animals are able to survive an otherwise lethal dose of the same type of bacteria used for

priming, but fail to survive if any other bacterial type is used for the second challenge. Similar results have been shown in the case of the copepod *Macrocyclops albidus* against its natural parasite tapeworm *Schistocephalus solidus*, where previous exposure causes increased resistance to that specific strain of parasite only (Kurtz & Franz, 2003). While both examples provide evidence for some specificity in immune memory, they do not reveal which specific component of the immune response is involved.

Most studies of trans-generational immune priming focus on changes in a single component correlated with survival of artificially-induced infection. For example Moret (2006) showed that by injecting the parental generation of *Tenebrio molitor* with LPS (lipopolysaccharides) the antimicrobial activity of the hemolymph is enhanced in the next generation. Studies on bumblebee (*Bombus terrestris*) have shown that offspring of immune challenged mothers have higher antibacterial activity (Sadd & Schmid-Hempel, 2007). For the crustacean *Daphnia magna* it has been shown that exposing the mothers to a specific strain of pathogens will increase offspring resistance to the same strain of parasites (Little et al., 2003). Another study on *Daphnia magna* underlined the importance of the maternal environment in shaping disease resistance in offspring. Mitchell & Read (2005) showed that poor growth condition of mothers increased the immunocompetence of their offspring. Selection experiments performed on the flour moth *Ephestia kuehniella* showed that by exposing the parental generation to sublethal doses of Bt toxin, the resistance of the next generation can be increased, and by selecting further the concentration of the median lethal dose can be increased. The basis for this resistance seems to be mediated by the phenoloxidase cascade (Rahman et al., 2004).

A number of these papers claiming to show adaptive immunity and trans-generational immune priming in invertebrates have been strongly criticized recently, as lacking sufficient mechanistic evidence (Hauton & Smith, 2007). In this thesis, an attempt has been made to avoid this problem and a multilevel approach has been taken to investigate trans-generational immune priming in insects. After establishing the diet-induced effects upon the steady state immune status in *T. ni*, it was interesting to determine whether the signal about bacterial presence in the environment can be passed on to the offspring. Reciprocal and non-reciprocal crosses were made with animals grown on bacterial and bacteria-free diet and their progeny were grown under similar, bacteria-free conditions. Strong parental diet-linked effects on various aspects of immune status, like enzyme activities and protein expression in the hemolymph, and also on gene expression in different tissues were observed (Chapter II and III). A number of induced changes indicate strong maternal effects on priming of immunity in

T. ni (Figure 1). At the same time no simple 1:1 transfer of immunity from one generation to the next seems to be taking place, as a number of immune factors are expressed differently in the next generation.

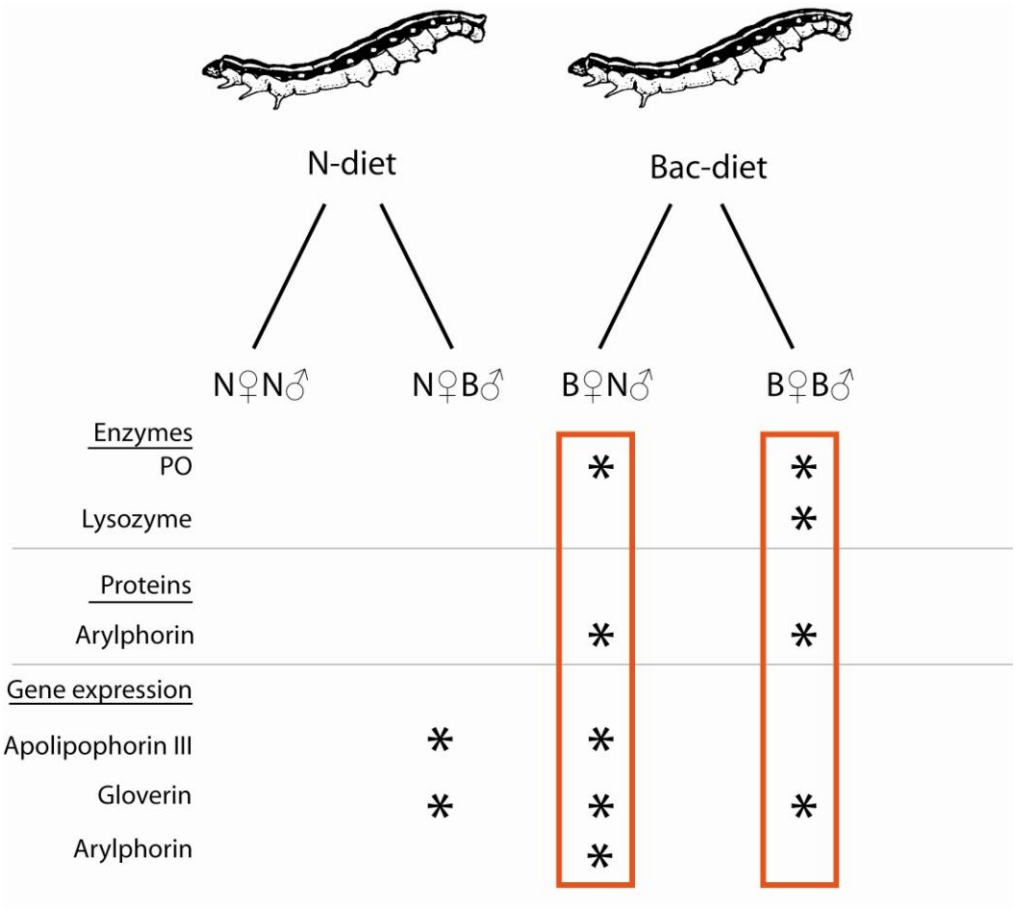


Figure 1. Parental effects on trans-generational priming of immune status reflected on different levels.

Studies focused on transgenerational priming that measure only a single element of the immune response would have missed the complex interactions that we observed among different elements. Most of these have also failed to discriminate between maternal and paternal effects. For example, different immune system properties of the hemolymph show different patterns of transgenerational priming (Fig 1), as well as different responses in bacterial-fed parents and their offspring. Some progeny classes showed an elevation of both lysozyme and phenoloxidase activity, in contrast to the trade-off observed in the parental generation (Chapter I). Moreover, the expected fitness-related costs when both systems are up-regulated were not observed. One factor reducing such costs could be the presence of high amounts of vitellogenin in the hemolymph of larvae originating from B♀B♂ and B♀N♂ crosses (Chapter III). Vitellogenin is known to act as an antioxidant and reduce oxidative stress and related harmful effects on the organism (Seehuus et al., 2006). This could represent

an adaptational maternal priming of vitellogenin expression to defend offspring against free radical associated oxidative stress. No diet-linked induction of vitellogenin was observed in the first generation (Chapter I). The other two highly abundant and maternally primed proteins in the hemolymph were arylphorin and juvenile hormone (JH) suppressive protein. The first has been discussed, and JH suppressive hormone could be linked to high abundance of vitellogenin, as JH is known to have a suppressive effect on vitellogenin expression (Corona et al., 2007).

Strong maternal effects can also be seen on the transcript level. A number of immune response related genes are up regulated in the rest of the body of B♀B♂ and B♀N♂ larvae, among them apolipophorin III and arylphorin (Chapter III). Both storage proteins are highly abundant in the hemolymph of first generation larvae fed on bacterial diet (Chapter I) and their transcripts are up-regulated in the midgut tissue of same larvae (Chapter I). Immune effector molecules that have been shown to be induced by direct immune challenge (e.g. gloverin, cecropins) are also highly expressed in the midgut tissue of offspring of bacterial-fed mothers. Up regulation of antimicrobial genes in the midgut tissue could be seen as an adaptive response preparing offspring to elevated bacterial load in the environment by increasing their chance for survival in the case of infection.

The fitness traits examined here do not suggest any clear immune status connected costs in the second generation. No differences are seen in the pupal masses between different crosses; significant differences in the developmental time and survival are present, but these are not necessarily correlated with primed immune status and do not show a simple pattern (Chapter III).

It can be concluded that encountering non-pathogenic bacteria in the food is sufficient to trigger changes in the immune status of the next generation although this effect can only be seen on some immune markers and not on others. Immune system parameters studied here that are activated in the parental generation are not transmitted in a 1:1 ratio to offspring, but are selectively transmitted in a complex manner with a strong but not exclusive maternal component. The mechanism of transmission of this information is completely unknown, but could involve maternal transmission of proteins and/or RNA in the egg cytoplasm, as well as epigenetic mechanisms such as DNA methylation. Determining the relative importance of these possible mechanisms remains a challenging topic for future research.

7 Summary

Most organisms on Earth are threatened by pathogens. For defense against pathogen-caused infections, organisms have developed a special feature, the immune system, consisting of a plethora of various cellular and humoral components. Different components of the immune repertoire are active against different pathogens and impose different costs of defense upon the host, leading to a complex pattern of trade-offs against other life-history traits, such as longevity and reproduction. Differential allocation of resources among immunity and other functions thus affects overall fitness. Investing in immunity produces a fitness benefit only if there is a real risk of actually encountering the pathogen in the environment. This leads to the question, "Is it possible to sense the "pathogenicity" of the environment and change the steady state immune status accordingly?".

One of the main sites of interaction between environment and organism for Lepidopteran larvae is the digestive system. Many bacterial infections such as *Bacillus thuringiensis* start by ingestion via the gut, thus the midgut epithelium could function as an interface for immune signaling about possible infections and for priming systemic immunity. Increasing amounts of information have become available about immune signaling pathways and molecules participating in the immune response in recent years. At the same time not much is known about what kind of environmental factors influence development of immune responses. No research has been done to date on the role of non-pathogenic bacteria digested with the food on the immunity of an insect. As bacteria are extremely abundant in the natural environment they are ingested by herbivorous insects in potentially large amounts. Do they play any role in influencing the defense reactions of insects? The aim of this thesis was to examine the importance of digested non-pathogenic bacteria on the immune status and physiology of Lepidopteran larvae.

7. 1 The midgut has a role in the shaping of immune status in *T. ni*

Non-pathogenic bacteria ingested with the diet were found to affect the organismal immune status. As a result of feeding on diet containing a constant supply of bacteria, a number of defense-related molecules showed changes in their expression on multiple levels. Changes were evident in enzyme activities and protein expression in the hemolymph. Several transcripts of immunity related genes were up regulated in midgut tissue. This indicates that

larval midgut tissue is able to react to the increased bacterial content of the diet and express various antibacterial defenses upon induction. At the same time the signal about the bacterial content of the environment is passed on to the hemocoel and the immune status is changed accordingly. Animals grown on bacterial diet had smaller pupal masses and delayed development. This indicates that feeding on bacterial diet has a measurable effect on essential fitness-related traits.

7. 2 Bacterial feeding induces changes in general physiology related gene expression in *T. ni*

The differential gene expression study conducted on animals feeding on bacterially challenged and non-challenged diet and cabbage plants found that bacterial exposure has a significant effect on a number of genes. The genes identified as differentially expressed between larvae feeding on different diets can be clustered into eight separate physiological functions: defense and recognition, development, digestion, DNA-related, metabolism, protein synthesis, signaling and unknown. It was also shown that some of the identified genes show dissimilar expression patterns during the different developmental stages of the animal and between various tissues. Some genes are also differentially expressed in the eggs coming from parents grown on diet with or without bacteria, suggesting a diet-mediated effect on gene expression in the next generation.

7. 3 Changes in the immune status can be transmitted to the next generation in *T. ni*

Another novel finding of this thesis is that dietary bacteria are also able to shape the immune status of the next generation via trans-generational priming. Strong maternal effects can be seen on a number of different immune factors. Hemolymph based immunity related enzyme activities are more pronounced in larvae whose mothers fed on bacterial diet. Changes in enzyme activities are accompanied by higher expression of several proteins in the hemolymph. The transcripts for several immune response related genes are more abundant in various tissues and life-stages in the descendants of bacteria-fed animals. However, these immune system parameters are clearly not transmitted in a 1:1 ratio from parent to offspring, but are selectively passed on in a complex manner.

7. 4 A novel protein identified from the midgut lumen of *Helicoverpa armigera*

Screening the proteome of the midgut lumen of *H. armigera* revealed the existence of a novel 40 kDa protein, with sequence similarity to lepidopteran βGRPs (β1,3-glucan recognition proteins). βGRPs are pattern recognition proteins, typically occurring in hemolymph, and thus the presence in the midgut lumen of an immune-protein was novel. Biochemical assays demonstrated that the protein was a catalytically active β-1,3-glucanase, in contrast to the hemolymph proteins. Feeding with bacteria is inducing the expression of β-1,3-glucanase. The β-1,3-glucanase may play a role in regulating the level of immune response by digesting spontaneously released glucan from commensal midgut bacteria, thus preventing them from triggering an inappropriate immune response.

In the current thesis, approaches on several different levels have been employed to answer ecologically and evolutionary pertinent questions in the immunology of insects. This is one of the first attempts to explore the molecular details of trans-generational immune priming in invertebrates by following the same participants of immunocompetence from one generation to the next. It is also evident from the current study that changes in immune status caused by dietary bacteria are accompanied by other physiological changes. The shaping of innate immune responses and accompanying physiological rearrangements in the organism are very complex, but constitute a very exciting and challenging area of research.

8 Zusammenfassung

Die meisten Organismen auf der Erde werden von Pathogenen bedroht. Um sich gegen Pathogene zu schützen, haben Organismen ein oftmals komplexes Immunsystem entwickelt. Das Immunsystem hat viele unterschiedene Zellulare und Humorale Komponenten. Verschiedene Komponenten des Immunrepertoires sind aktiv gegen unterschiedliche Pathogene und haben daher unterschiedliche Kosten für den Organismus. Das führt zu einen komplexen Muster von *trade-offs* und anderen *life-history* Eigenschaften wie zum Beispiel Auswirkungen auf Lebenslänge und Reproduktion. Die Verteilung von Rohstoffen zwischen Immunität und anderen lebenswichtigen Funktionen hat einen substantiellen Einfluss auf die generelle Fitness des Organismus. Eine Investition in Immunität ist nur dann positiv für die Fitness, wenn ein reales Risiko für einen Pathogenbefall besteht. Die Frage besteht also, ob es möglich ist, die Pathogenität der Umwelt wahrzunehmen und den eigenen Immunstatus entsprechend zu ändern.

Eine der primären Interaktionsflächen zwischen der Umwelt und der Lepidopteren-Raupe ist das Verdauungssystem. Viele bakterielle Infektionen, wie zum Beispiel *Bacillus thuringiensis*, fangen im Larvendarm bei der Verdauung an. Das heisst, dass der Darm die Grenzfläche für potentielle Immunsignale und somit den Informationsaustausch über mögliche Infektionen und die Vorbereitung des Immunsystems darstellt. Das Wissen über Immunsignalwege und Moleküle, die in der Immunantwort eine Rolle spielen, ist in den letzten Jahren erheblich gewachsen. Gleichzeitig ist nicht sehr viel über die Umweltfaktoren, die die Entwicklung des Immunsystems beeinflussen können, bekannt. Bis zum heutigen Tag gibt es keine einzige Studie über die Wirkung von nicht-pathogenen Bakterien, die von Insekten mit dem Futter aufgenommen werden, auf das Immunsystem. Da Bakterien in der natürlichen Umgebung überreichlich vorhanden sind, werden sie in großen Mengen von pflanzenfressenden Insekten konsumiert. Beeinflussen diese Bakterien die Immunabwehr von Insekten? Das Ziel dieser Dissertation war es, die Rolle von nicht-pathogenen Bakterien auf den Immunstatus und die Physiologie von *Trichoplusia ni* (Noctuidae: Lepidoptera) zu untersuchen.

8. 1 Das Mitteldarm hat eine Rolle in der Gestaltung des Immunstatus in *T. ni*

In der hier vorliegenden Arbeit wurde gezeigt, dass nicht-pathogene Bakterien, die mit der Diät aufgenommen werden, einen Effekt auf den Immunstatus von Organismen haben. Eine Anzahl von Molekülen, die an der Abwehr beteiligt sind, zeigen Expressionsunterschiede auf mehrerer Ebene, wenn Raupen auf einer Bakterien-kontaminierten Diät fressen. Diese Änderungen waren primär in der Aktivität von Enzymen und der Expression von Proteinen in der Hämolymphe zu sehen. Mehrere Immunitätsgene waren im Mitteldarmgewebe stark hochreguliert. Dies zeigt, dass der Mitteldarm von Raupen fähig ist, auf eine erhöhte bakterielle Konzentration in der Diät zu reagieren und diverse antibakterielle Abwehrstoffe dagegen zu produzieren. Zur gleichen Zeit wird das Signal über den Bakteriengehalt der Umgebung zum Haemocoel übermittelt und der Immunstatus auch in anderen Geweben entsprechend geändert. Die Tiere die auf die Diät mit hoher Bakterienkonzentration aufgewachsen sind, haben kleinere Puppenmassen und eine verspätete Entwicklung. Diese Ergebnisse deuten an, dass das Fressen auf bakteriell kontaminierter Diät einen messbarer Effekt auf grundlegende *life-history* Eigenschaften hat.

8. 2 Genexpressionsänderungen von Nicht-Immungenen werden von Bakterien in der Diät beeinflusst

Untersuchungen zur differentiellen Genexpression von Raupen, die auf bakterieller und nichtbakterieller Diät und auf Kohlpflanzen fressen, hat gezeigt, dass die Diät einen starken Effekt auf eine grosse Zahl von sexprimierten Genen hat. Die Gene, die als differentiell exprimiert in Raupen, die auf unterschiedlichen Diäten fressen, identifiziert wurden, können in acht verschiedene physiologische Kategorien eingeteilt werden: Schutz und Erkennung, Entwicklung, Verdauung, DNA-assoziiert, Stoffwechsel, Proteinsynthese, Signalwege und Gene mit unbekannter Funktion. Es wurde auch gezeigt, dass einige dieser als differentiell exprimiert identifizierten Gene unterschiedliche Expressionsmuster in verschiedene Entwicklungsphasen und in unterschiedlichen Geweben haben. Manche Gene zeigten auch Expressionsunterschiede in Eiern, die von Eltern abstammen, die auf unterschiedlichen Diäten aufgewachsen sind. Das deutet die Möglichkeit an, dass transgenerationelle Effekt auch die Genexpression beeinflussen.

8. 3 Unterschiede in der Gen- und Proteinexpression können auch an die nächste Generation weitergegeben werden

Eine weiteres Ergebnis dieser Arbeit ist die Tatsache, das Bakterien in der Diät auch die Genexpression der nächsten Generation durch sog. *transgenerational priming* beeinflussen können. Zusätzlich zu diesen generationenübergreifenden Effekten, konnten auch starke maternale Effekte an mehreren Immunfaktoren festgestellt werden. Hämolymph-basierende Aktivitäten von Immun-Enzymen sind höher in Raupen, deren Mütter auf einer Bakterien-reichen Diät aufgewachsen sind. Diese Änderungen in Enzymaktivitäten werden begleitet durch höherer Expression von mehreren Proteinen in de Hemolymphe. In dem Nachwuchs von Tieren, die auf bakterieller Diät aufgewachsen sind, sind viele der Immunantwort zugehörigen Gene hoch exprimiert - auch in unterschiedlichen Geweben und Lebensphasen. Allerdings werden diese Immunsystem-Parameter nicht in einem 1:1 Verhältnis von den Eltern zum Nachwuchs weitergegeben, sondern auf eine selektive und komplexe Art.

8. 4 Verdauung oder Abwehr? Eine neues Protein aus dem Mitteldarm von *Helicoverpa armigera*

Während der Untersuchung des Proteoms des Mitteldarmlumens von *H. armigera* ist eine neues 40kDa Protein mit Sequenzähnlichkeit zu β-GRP von Lepidopteren identifiziert worden. β-GRPs sind sog. *pattern recognition* Proteine, welche typischerweise in der Hämolymphe gefunden werden. Deshalb war die Tatsache, dass ein solches Protein auch im Mitteldarmlumen vorkommen kann, eine Neuigkeit. Die biochemische Analyse hat gezeigt, dass dieses Protein eine katalytische Aktivität gegen β-1,3-glucosidische Bindungen hat, und zwarim Gegensatz zu den Proteinen der Hämolymphe. Das Fressen von Bakterien induziert die Expression dieser β-1,3-glukanase. Somit könnte diese im Mitteldarmlumen lokalisierte β-1,3-glukanase eine Rolle in der Regulation der Immunantwort spielen.

In dieser Dissertation konnte klar der Einfluss von spezifischen (hier: Bakterien) Umweltfaktoren auf die Physiologie und die Immunantwort von Lepidopteren gezeigt werden. In der hier vorgelegten Arbeit wird auch offensichtlich, dass Änderungen im Immunstatus auch Änderungen in der Physiologie der Insekten nach sich ziehen. Besonders hervorzuheben sind die hier gezeigten generationenübergreifenden Effekte, welche auf mehreren funktionellen Ebenen erfolgen. Dies ist einer der ersten erfolgreichen Versuche, die

molekularen Hinweise auf die Existenz von *transgenerational priming* in wirbellosen Tieren zu untersuchen.. Die Existenz als auch die hier gezeigte Komplexizität von *transgenerational priming*, die Vielfalt der potentiell einwirkenden Faktoren und das Ausmaß ihrer gegenseitigen Interdependenzen zur Formung der angeboren Immunantworten und bedingten Änderungen in der Physiologie des Organismus sind deshalb ein sehr interessantes und herausforderndes Feld zukünftiger Forschung.

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10 Selbständigkeitserklärung

Die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich Schiller-Universität ist mir bekannt. Die vorliegende Dissertation habe ich selbständig verfasst und keine anderen als die von mir angegebenen Quellen, persönliche Mitteilungen und Hilfsmittel benutzt. Es wurden keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen. Alle Personen, die an der Gewinnung von Daten beteiligt, bei der Erstellung des Manuskripts hilfreich waren oder sonstige Hilfestellungen gaben, sind benannt.

Es wurde weder bezahlte noch unbezahlte Hilfe eines Promotionsberaters in Anspruch genommen.

Ich habe die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere Wissenschaftliche Prüfung eingereicht.

Jena, den 2009 ……..

Dalial Freitak

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12 Curriculum vitae

Personal data

Name: Dalial Freitak
Date of birth: 01.10. 1978
Place of birth: Valga, Estonia
Email: dfreitak@ice.mpg.de

Scientific career:

Since 01/2005	PhD student in Max Planck Institute for Chemical Ecology in Jena and Friedrich-Schiller-University Jena
09/2003-12/04	University of Tartu, PhD student in Department of Biology and Geography
09/2001 – 06/03	University of Tartu, master student in Department of Biology and Geography, defended *Master of Science* in animal ecology
09/1997 – 06/01	University of Tartu, Department of Biology and Geography, *Bachelor of Science* in zoology

Education:

09/1985 – 06/1997	Valga Gymnasium

Publications

(1) Freitak D, Heckel DG, Vogel H. **Bacterial feeding induces changes in immune-related gene expression and has trans-generational impacts in the cabbage looper (*Trichoplusia ni*).** Frontiers in Zoology **6**:7.
(2) Freitak D, Heckel DG, Vogel H. **Dietary-dependent trans-generational imprinting in herbivorous Lepidopteran *Trichoplusia ni.*** Proceedings of the Royal Society London: B (doi: 10.1098/rspb.2009.0323)

(3) Dabrowska P, Freitak D, Vogel H, Heckel DG, Boland W. **The phytohormone precursor OPDA is isomerized in the insect gut by a single, specific Glutathione S-transferase** (*in prep*)

(4) Heidel-Fisher H, Freitak D, Janz N, Söderlind L, Vogel H, Nylin S. **Analysis of gene expression differences of Comma Butterfly (Polygonia c-album Lepidoptera: Nymphalidae) upon feeding on different host plants** (*submitted – under review*)

(5) Pauchet Y, Freitak D, Heidel-Fischer HM, Heckel DG, Vogel H. (2009) **IMMUNITY OR DIGESTION: Glucanase activity in a glucan-binding protein family from Lepidoptera.** The Journal of Biological Chemistry, 284 (4), 2214-2224.

(6) Freitak D, Wheat CW, Heckel DG, Vogel H. (2007) **Immune system responses and fitness costs associated with consumption of bacteria in larvae of *Trichoplusia ni*.** BMC Biology, 5:56.

(7) Meylaers K, Freitak D, Schoofs L. (2007) **Immunocompetence of *Galleria mellonella*: Sex- and stage-specific differences and the physiological cost of mounting an immune response during metamorphosis.** Journal of Insect Physiology, 53 (2), 146-156.

(8) Freitak D, Vanatoa A, Ots I, Rantala MJ. (2005). **Formation of melanin-based wing patterns is influenced by condition and immune challenge in *Pieris brassicae*.** Entomologia Experimentalis et Applicata, 116(3), 237 - 243.

(9) Ots I, Freitak D, Vanatoa A. (2005). **Expression of immunity and general condition in the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera : Chrysomelidae), in relation to origin and gender.** Entomological Science, 8(2), 173 - 178.

(10) Freitak D, Ots I, Vanatoa A, Hõrak P. (2003). **Immune response is energetically costly in white cabbage butterfly pupae.** Proceedings of the Royal Society of London B, 270, S220 - S222.

Presentations

Oral presentations

(1) National Meeting of Developmental and Comparative Immunology, 8 – 10 October 2008, Gießen, Germany. Dalial Freitak, David G. Heckel, Heiko Vogel – **"Trans-generational imprinting in herbivorous Lepidopteran *Trichoplusia ni* is dependent on diet and shows strong maternal effects".**

(2) International Symposium on Insect Midgut Biology, 7 – 11 April 2008, Guangzhou, China. Dalial Freitak, Christopher C. Wheat, David G. Heckel, Heiko Vogel – **"The role of**

the midgut in the immune response of an insect herbivore (*Trichoplusia ni,* Lepidoptera)".

(3) 7th International Workshop on the Molecular Biology and Genetics of the Lepidoptera, 20 – 26 August, 2006, Kolympari, Crete, Greece. Dalial Freitak, David G. Heckel, Heiko Vogel – **"The effect of microorganisms in the food on the growth rate and immune system of cabbage looper (*Trichoplusia ni*)"**.

(4) 10th Jubilee Congress International Society of Behavioral Ecology, 10 -15 July 2004, Jyväskylä, Finland. Dalial Freitak, Alo Vanatoa, Inderk Ots - **"Previously encountered pathogens affect immune responses in Colorado potato beetle: synergetic adaptive immunity in insects?"**.

(5) VIIth Europan Congress of Entomology, 07 – 13 October 2002, Thessaloniki, Greece. Dalial Freitak, Alo Vanatoa, Indrek Ots, Peeter Hõrak - **"Immune challenge and standard metabolic rate in white cabbage butterfly (*Pieris brassicae* L.) diapausing pupae"**.

(6) Workshop on Evolutionary Ecology of Resistance and Defence, 12 - 14 December 2001 Konnevesi, Finland. Dalial Freitak, Alo Vanatoa, Indrek Ots - **"Immune challenge and development in White Cabbage Butterfly"**.

Oral presentations on in house symposia

(1) 3rd Biannual IMPRS Symposium / MPI for Chemical Ecology, November 4-5, 2005 Jena, Germany. **"Induced immune responses in lepidopteran larvae – food makes the difference?"**.

(2) ICE Symposium / MPI for Chemical Ecology, June 29-30, 2006, Jena, Germany. **"Food makes the difference...at least for cabbage looper…"**.

(3) 5th Biannual IMPRS Symposium / MPI for Chemical Ecology, November 10-11, 2006, Jena, Germany. **"The effect of orally ingested microorganisms on the immunity and physiology of Trichoplusia ni larvae"**.

(4) ICE Symposium / MPI for Chemical Ecology, September 25-26, 2008, Jena, Germany. **"The role of the larval midgut in triggering the insect immune response"**.

Poster presentations

(1) 11th Congress of European Society for Evolutionary Biology, 20 – 25 August 2007, Uppsala, Sweden. Dalial Freitak, Christopher C. Wheat, David G. Heckel, Heiko Vogel - **"Immune system induction and fitness costs of bacterial consumption in an insect herbivore (Trichoplusia ni, Lepidoptera)"**.

(2) Workshop on Innate immunity: bridging the gap between ecology and molecules, 06 – 09 May 2004, Plön, Germany. Dalial Freitak, Alo Vanatoa, Inderk Ots - **"Previously encountered pathogens affect immune responses in Colorado potato beetle: synergetic adaptive immunity in insects?"**.

(3) 9th International Behavioral Ecology Congress, 07 – 12 July 2002, Montreal, Canada. Indrek Ots, Dalial Freitak, Alo Vanatoa, Peeter Hõrak - **„Immune challenge and resting metabolic rate in White Cabbage Butterfly pupae"**.

(4) European Science Foundation Workshop on Ecological Immunity of Arthropods, 06 – 09 December 2001 Castelton, Great Britain. Dalial Freitak, Indrek Ots, Alo Vanatoa, Peeter Hõrak - **"Does the pupal immune challenge affect adult phenotype in White Cabbage Butterfly (*Pieris brassicae*)?"**.

(5) VIIIth Congress of the European Society for Evolutionary Biology, 20 – 25 August 2001, Aarhus, Denmark. Dalial Freitak, Alo Vanatoa, Indrek Ots, Markus Rantala, Jukka Suhonen - **"Sources of individual variation in immunocompetence of White Cabbage Butterfly (*Pieris brassicae* L.) pupae"**.

Jena, den 2009 ……..

Dalial Freitak