Master of Science Thesis

Immunological priming in the wood tiger moth (*Parasemia plantaginis*)

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10.10.2014

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Kaukoniitty, M.: Immunological priming in the wood tiger moth (*Parasemia plantaginis*)

Master of Science Thesis: 33 p.
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October 2014

Key Words: adaptive immunity, innate immune system, insect herbivore, Lepidoptera, *Serratia marcescens*, within generational immune priming

ABSTRACT

Insects have various important roles as pests, disease vectors and beneficial species, which make insect diseases, pathogens and immune responses important study areas not only from ecological but also from economical and medical point of view. Recent studies have revealed remarkable specificity and long-lasting immunity in insect and other invertebrate immune systems. A phenomenon called 'immunological priming' produces memory-like immune response, which help the host to fight against recurrent infections. Immune functions induced by a pathogen can remain enhanced for a period of time after the immune challenge, reducing the risk of re-infection. If the reduced risk relates more clearly to a similar than a different kind of pathogen, the immune response is considered to be specific. In order to demonstrate specificity in immunological priming, it is necessary to show that the priming was induced by the targeted pathogen. The aim of this study was to find out whether or not the wood tiger moth (*Parasemia plantaginis*) larvae show specific immunological priming after two consecutive immune challenges with the same or different Gram-negative bacteria. The experimental bacteria were two *Serratia marcescens* strains, a common insect pathogen Db11 and an environmental isolate ATCC#13880. The control bacterium was a non-pathogenic *Escherichia coli* K12 strain. The larvae were challenged in oral exposure and by injection. Db11 appeared to be considerably more virulent in oral exposure than the other two bacteria. Although Db11 proved to be too virulent for the main purpose of the study, immunological priming was observed in a group of larvae that had been orally exposed and five days later injected with ATCC#13880 in comparison to a group of larvae with prior *E. coli* exposure. Since the prior *E. coli* exposure did not provide protection against ATCC#13880 injection, the priming could be regarded as specific to ATCC#13880. This study demonstrated the ability of the invertebrate immune system to discriminate between different Gram-negative bacteria in the context of immunological priming.

JYVÄSKYLÄN YLIOPISTO, Matemaattis-luonnontieteellinen tiedekunta

Bio- ja ympäristötieteiden laitos
Ekologia ja evoluutiobiologia

Kaukoniitty, M.:	Immuunipohjustus täpläsiilikkäällä (*Parasemia plantaginis*)
Pro gradu -tutkielma:	33 s.
Työn ohjaajat:	FM Lauri Mikonranta, Akatemiaprofessori Johanna Mappes
Tarkastajat:	Dosentti Lotta-Riina Sundberg, Dosentti Anssi Karvonen
Lokakuu 2014	

Hakusanat: kasvinsyöjähyönteinen, luontainen immuniteetti, Lepidoptera, *Serratia marcescens*, sopeuttava immuniteetti, sukupolven sisäinen immuunipohjustus

TIIVISTELMÄ

Hyönteisiin lukeutuu monia merkittäviä tuholaisia, taudinvälittäjiä ja hyödyllisiä lajeja, minkä takia hyönteisten taudit, taudinaiheuttajat ja immuunipuolustus ovat ekologisesta, ekonomisesta ja lääketieteellisestä näkökulmasta tärkeitä tutkimuskohteita. Viimeaikaiset tutkimukset paljastavat hyönteisten ja muiden selkärangattomien immuunijärjestelmien pystyvän erikoistumaan ja luomaan pitkäkestoista immuniteettia. Jälkimmäinen ilmiö tunnetaan tieteellisissä artikkeleissa nimellä ”immunological priming”, jonka voisi kääntää suomeksi esimerkiksi ”immuunipohjustukseksi”. Immuunipohjustus on muistinkaltaista vastustuskykyä, joka auttaa elimistöä puolustautumaan tehokkaammin toistuvia tartuntoja vastaan. Taudinaiheuttajan laukaisemat immuunitoiminnot voivat altistuksen jälkeen pysyä jonkin aikaa aktiivisina, ja pienentää näin myöhemmän tartunnan riskiä. Mikäli tartuntariski on tällöin pienempi samanlaista kuin erilaista taudinaiheuttajaa kohtaan, on immuunipohjustus erikoistunutta. Erikoistuneisuuden havainnollistamiseksi on osoitettava, että immuunipohjustuksen on aktivoinut sen kohteena oleva taudinaiheuttaja. Tässä tutkimuksessa selvitettiin erikoistuneen immuunipohjustuksen mahdollista ilmenemistä täpläsiilikkään (*Parasemia plantaginis*) toukkien kahdessa peräkkäisessä altistuksessa samalle tai erilaisille Gram-negatiivisille bakteereille. Koebakteereina käytettiin kahta *Serratia marcescens* -kantaa, joista Db11-kanta on tunnettu hyönteisten taudinaiheuttaja, ja ATCC#13880 ympäristöstä eristetty kanta. Kontrollibakteerina käytettiin taudinaiheuttamiskyvytöntä *Escherichia coli* K12 -kantaa. Toukat altistettiin bakteereille ensin oraalisesti ravinnon välityksellä, ja viisi päivää myöhemmin injektiolla. Oraalisessa altistuksessa Db11-kannan havaittiin olevan kahta muuta bakteeria huomattavasti virulentimpi. Vaikka Db11 osoittautui tutkimuksen tarkoituksen kannalta liian virulentiksi, immuunipohjustusta havaittiin toukilla, jotka oli oraalisesti altistettu ja injektoitu ATCC#13880-kannalla, kun niiden selviytymistä verrattiin toukkiin, jotka oli altistettu *E. coli*:lla. Koska *E. coli* -altistus ei suojannut toukkia ATCC#13880-injektiota vastaan, voitiin immuunipohjustuksen päätellä olevan ATCC#13880-kannalle erikoistunutta. Tutkimuksemme osoitti selkärangattomien immuunijärjestelmän kyvyn erottaa erilaisia Gram-negatiivisia bakteereja toisistaan immuunipohjustuksen yhteydessä.

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1. INTRODUCTION

1.1. Insect immunology and applications

All animals in the world – both vertebrates and invertebrates – are susceptible to pathogens and parasites. Diseases have acted as a major selection force in the evolution of species. Today, vertebrates have a highly specific immune system capable to develop immunity to pathogens in order to avoid recurrent infections (Rowley & Powell 2007). Increasing study of insect immunology indicates that invertebrate immune systems are more complex and multiform than previously presumed. The invertebrate immune systems have also been observed to become more effective and enhanced in resistance in recurrent exposures to a pathogen (Little & Kraaijeveld 2004). This phenomenon called 'immunological priming' has been suggested to have a considerable effect on ecology and population dynamics of insects (Tidbury et al. 2010).

Insects, the biggest class of the invertebrates in the number of species, include many important pests, disease vectors and beneficial species (Hill 1997). Some insect species defoliate forests, destroy crops or spread dangerous diseases to humans and domestic animals causing serious economic losses and nutritional and healthcare problems (Morris 2004). On the other hand, the long and intensive study of insects and other invertebrates has provided valuable scientific knowledge and answers to several ecological, economical and medical problems (Hill 1997, Morris 2004). Thanks to their huge diversity, wide distribution and diverse interaction with pathogens, insects are good models in the study of evolution, speciation and the immunological defence mechanisms (Boddum 2008).

Insects have to defend themselves against various pathogens such as bacteria, viruses, nematodes and fungi (Vallet-Gely et al. 2008). The host–parasite relationships between insects and their pathogens, called the entomopathogens, have been utilized for long in biological pest control of agriculture and forestry as a replacement for harmful chemical insecticides (Hill 1997). Insecticidal properties of the entomopathogenic *Bacillus thuringiensis* bacterium were first discovered in the Mediterranean flour moth (*Ephestia kuehniella*), a common pest of dry plant produce (Sanahuja et al. 2011). The discovery led to the isolation of the bacterium strain and eventually to the development of a commercial Bt insecticide, which has been used for many decades since. Similarly, a naturally occurring entomophagous virus has been used in the control of an important defoliator of hardwood forests, the Gypsy moth larvae (*Lymantria dispar* L.) (Cook et al. 2003). Yet another entomopathogen, a fungus, has shown potential as a biological control agent of the

Anopheles gambiae mosquito, an important vector of the malaria-causing *Plasmodium* parasite (Scholte et al. 2005). Not only is it possible to control insect populations with their pathogens, but it is also possible to control pathogens by utilizing immunological defence mechanisms evolved in the arms race between the insect immune systems and pathogens. For example, maggot therapy has proved to be effective in cleansing wounds infected by the methicillin-resistant *Staphylococcus aureus* (MRSA) (Bexfield et al. 2004).

In order to defend themselves against pathogens, invertebrates have highly developed defence systems and lots of antimicrobial chemicals. The first antimicrobial peptide (AMP), called 'cecropin', was isolated from the haemolymph of *Hyalophora cecropia* moth larvae in the 1980s (Boman et al. 1991). AMPs have varied abilities to kill bacterial cells or to inhibit their growth, which makes them potential sterilization agents. AMPs could help to control problematic bacteria such as MRSA, a common causing agent of hospital-derived infections (Jenssen et al. 2006). In addition to AMPs, insects have several other widely studied immune molecules and defence mechanisms, which properties are not yet fully resolved. Some of these immune factors could play an important role in improving resistance of insect immune system through multiple exposures or infections, i.e. immune priming. Immune priming is a part of the innate immune system found both in vertebrates and invertebrates (Little et al. 2005).

1.2. Defence mechanisms of the invertebrate innate immune system

Invertebrate innate immune systems are effective in fighting different kinds of pathogens. Insects among other invertebrates have several cell-mediated and humoral defence mechanisms against entomopathogens that try to persist and to proliferate in their intestinal track or to colonize their inner tissues through the gut or the body cover called cuticula (Vallet-Gely et al. 2008). Wounding of the cuticula or assisted transport by an entomophagous nematode can provide microorganisms, such as bacteria, direct access to the insect body cavity called haemocoel. Even some relatively harmless bacteria, which commonly inhabit the insect gut, such as certain species of *Serratia*, can cause lethal septicaemia if they manage to access the haemocoel (Boucias & Pendland 1998). In fact, the main route of natural infection seems to be the intestinal track (Vallet-Gely et al. 2008).

In insect midgut ingested microorganisms face multiple challenges including low pH, digestive enzymes and cell-damaging reactive oxygen species (ROS) released by the innate immune system of gut epithelium (Rowley & Powell 2007). If the ingested pathogens survive the unfavourable conditions and oxidative burst they may persist and trigger local

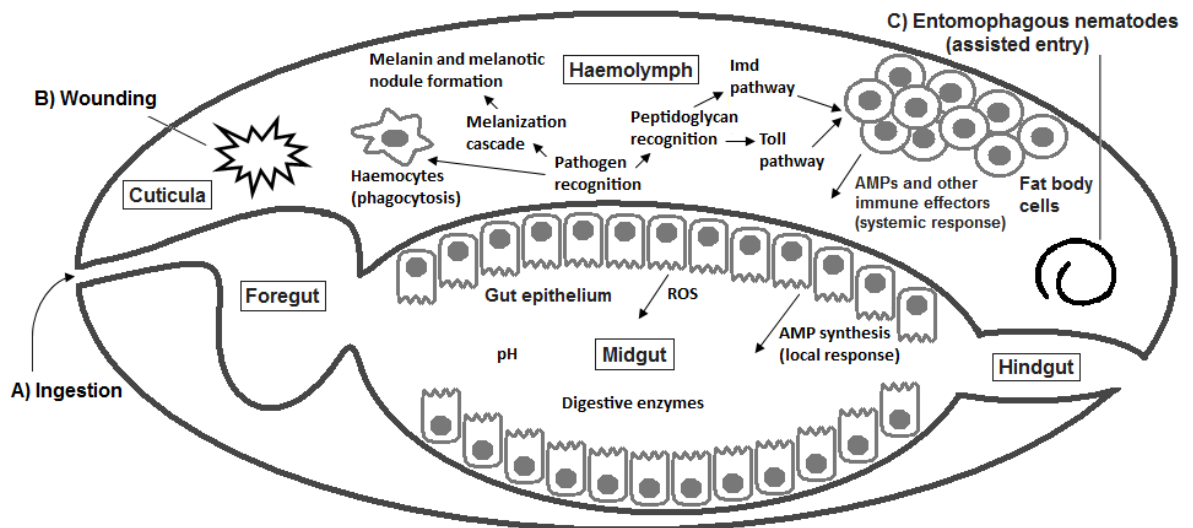

Figure 1. A simplified model of the insect alimentary canal and body cavity. Main routes of bacterial infection (A–C) and the major defence mechanisms in midgut and haemolymph. Figure modified from Vallet-Gely et al. (2008).

and systemic production of AMPs and other immune effectors (Figure 1). ROS production and local AMP response have been proposed to be the first and the second lines of inducible defence against ingested bacteria (Vallet-Gely et al. 2008).

Induction of systemic immune response requires recognition of pathogens that manage to enter insect inner tissues through the gut or due to wounding of the cuticula by an injury, invasive pathogens, parasites, parasitoids, predators or by a direct injection in a laboratory (Vallet-Gely et al. 2008). Production of AMPs is mediated by two signalling pathways called Imd and Toll. The Imd pathway is usually activated through Gram-negative bacteria recognition whereas the Toll pathway is activated through Gram-positive bacteria recognition (Rowley & Powell 2007). Gram-negative bacteria differ from Gram-positive bacteria by having a thinner peptidoglycan layer and an extra lipopolysaccharide (LPS) envelope around them (Madigan et al. 2009). Structural peptidoglycans of these outermost layers are specifically recognized by peptidoglycan recognition proteins in blood-like haemolymph of the host. Recognition may require translocation of peptidoglycan fragments from the gut into haemolymph (Vallet-Gely et al. 2008). Functionally liver-like fat body of insects produces many different AMPs, such as cecropins, diptericins, drosocins and attacins, which main purpose seems to be the suppression of microbial growth (Boman et al. 1991, Hoffmann 2003).

Two major defence mechanisms apart from AMP production are cellular response and melanisation reaction (Vallet-Gely et al. 2008). Mobile or stationary invertebrate blood cells called haemocytes are responsible for many important immune functions. They

initiate damage repair and coagulation of haemolymph in order to prevent pathogens from entering the haemocoel. Haemocytes are capable of phagocytosis, i.e. they can eat and digest small intruders like bacteria, fungi and viruses (Rowley & Powell 2007). They can also encapsulate bigger parasites by accumulating and forming multiple layers around them. The parasite isolation process involves interaction and cooperation between numerous haemocytes and melanisation reaction (Vallet-Gely et al. 2008). In melanisation reaction black brown melanin pigment and melanotic nodules are formed (Christensen et al. 2005, Vallet-Gely et al. 2008). Encapsulated microbes are removed from the haemocoel through nodulation and isolated from the rest of the host within melanised haemocytes (Rowley & Powell 2007). Melanisation reaction is induced by melanisation cascade, which ultimately relies on the activation of prophenoloxidase cascade by prophenoloxidase enzyme (Vallet-Gely et al. 2008).

Prophenoloxidase cascade is a part of invertebrate humoral immune defence based on humours, or body fluids (Rowley & Powell 2007, Vallet-Gely et al. 2008). Humoral immune defence is a key factor in the activation of many important immune functions such as haemolymph coagulation, localized melanisation, production of AMPs and other cytotoxic substances, and synthesis of proteolytic, hydrolytic and antimicrobial enzymes, e.g., lysozyme (Nappi & Ottaviani 2000). Lysozyme is one of the major antibacterial factors with an ability to lyse cell walls of Gram-positive bacteria (Hultmark 1996). The LPS envelope makes Gram-negative bacteria resistant to lysozyme (Costerton et al. 1974).

Some of the previously introduced immune reactions, molecules and phagocytes may remain active in haemolymph a period of time after an immune challenge (McTaggart et al. 2012). During this kind of immunological loitering the risk of infection is generally lower. Depending on the insect and the immune factor, immunological loitering can last several days or weeks (Haine et al. 2008). What is essential, is that immunological loitering is temporary and does not provide resistance for the rest of the life of the host, or across generations. However, recent studies indicate that invertebrate innate immune system is capable to respond faster and more effectively to a subsequent immune challenge even after longer time and apart from immunological loitering (Sadd & Schmid-Hempel 2006, Tidbury et al. 2010). This means that the immune defence of invertebrates does not seem to depend on directly induced immune response nor persistent immune reactions alone. The phenomenon of increased resistance through prior immune challenges is called ‘immunological priming’, or ‘immune priming’ in short (Little & Kraaijeveld 2004).

1.3. Non-specific and specific immunological priming

Discussion about the capability of invertebrates to acquired resistance extends at least as far as Wagner (1961). His review was based upon realization that invertebrates such as insects could be 'immunized' against pathogens through vaccination with pathogens or with haemolymph of an infected individual. The enhanced resistance was reported to last a couple of days or weeks at maximum. No counterpart was found at mechanistic level to the antibody-based adaptive immune system that produces specific and long-lasting immunity to vertebrates (Wagner 1961). Therefore the enhanced resistance of invertebrates was supposed to result from simple defence mechanisms sustaining their activity a period of time after induction, i.e. immunological loitering (Wagner 1961, McTaggart et al. 2012). Immunological loitering is gradually declining non-inducible resistance. For some time innate immune system was thought to provide only temporary, non-specific and constant protection against infection. As study continued the belief that invertebrates are incapable of lifelong or specific immune responses was challenged with new evidence of an adaptive response that functionally resembles the acquired immune response in vertebrates: immunological priming (Moret & Siva-Jothy 2003, Little & Kraaijeveld 2004).

Fundamentally immune priming can be seen as acquired element that adapts the host to environment depending on past experience with pathogens (Sadd & Schmid-Hempel 2009). Immune priming can be told apart from immunological loitering by the rapid and powerful induction of immune defence against re-infection. Immune response involved in immune priming does not have to be constant nor based simply on specific host–pathogen relationships or genotypes. Immune priming in invertebrates can be non-specific but long-lasting as demonstrated by McTaggart et al. (2012) with two clones of a crustacean *Daphnia magna* and its natural bacterial pathogen *Pasteuria ramosa*. The risk of infection was reduced for both clones irrespective of the type of primary exposure or the timing of secondary exposure. It was shown that immune priming can provide robust protection even after the loitering defence mechanisms and immune molecules induced by prior immune challenge are expected to be ceased or decreased (McTaggart et al. 2012).

Immunological loitering could explain immune priming, yet it does not suffice alone to represent 'true memory' in the same sense as acquired immunity in vertebrates (Kurtz 2005). So far the closest counterpart to specific acquired immunity of vertebrates seems to be the recently discovered specific immune priming. Specific immune priming not only protects the host from subsequent infections but offers pathogen-specific protection against previously encountered pathogens. In the case of immune priming the risk of subsequent

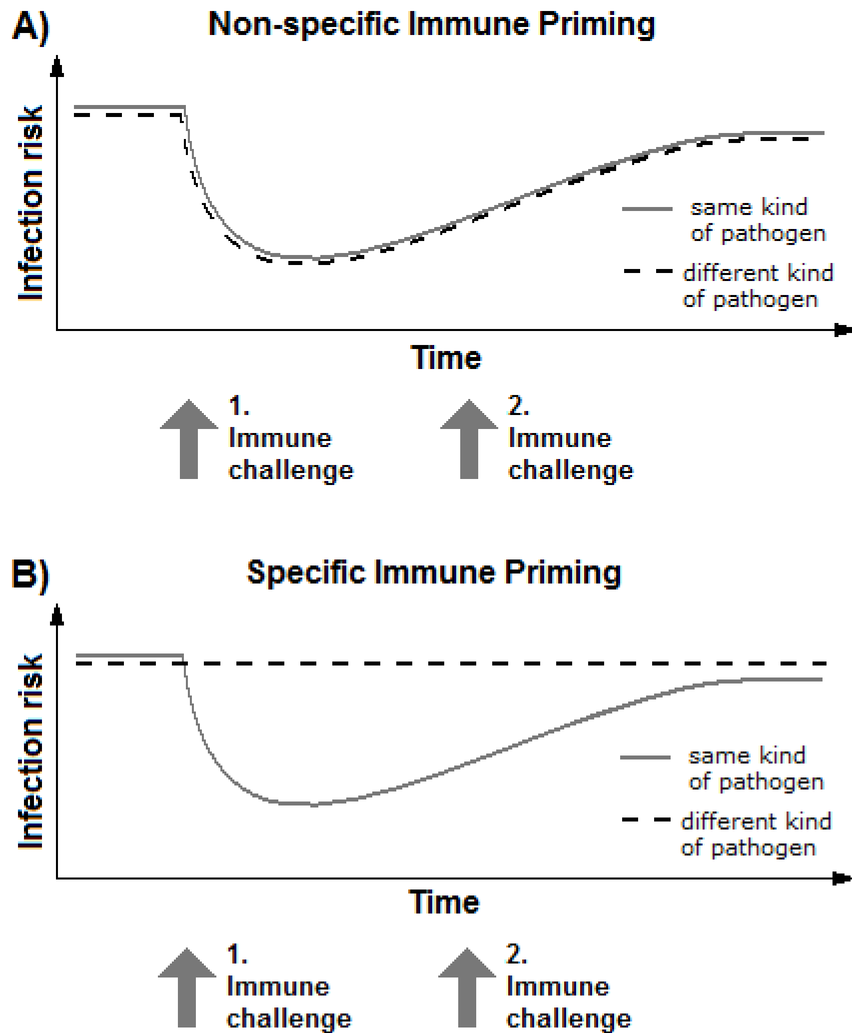

Figure 2. The effects of activated immune response and specific immune response on infection risk. A) Activated but non-specific immune reactions reduce infection risk (continuous line) also for different kinds of pathogens (dash line). B) When activated immune response is specific, infection risk is even more reduced for the same kind of pathogen (continuous line) than for a different kind of pathogen (dash line). Figure modified from Kurtz (2005).

infection is generally lower whereas in specific immune priming infection risk is even lower for the same kind of pathogen than a different kind of pathogen (Figure 2) (Kurtz 2005, Roth et al. 2009).

Problems in detecting specificity in innate immune system were assessed already in Kurtz (2005). Kurtz brought out some ambiguity in results concerning specific immune response of invertebrates and stressed the need for more accurate ways of study. In order to demonstrate specificity in immune priming it is necessary to show that an activated immune response does not act equally strongly against the third party, i.e. a pathogen unknown to the host (Kurtz 2005). Kurtz also sought to clarify the multiple and often

confusing immunological terminology. Since the meaning of some terms still seems to be misleading throughout articles, further definition might be in order. In this paper I will not take a stand on which terms are valid and how they should be defined in general. Yet from here on I will relate the term 'acquired immunity' to the specific antibody-based immune mechanism peculiar to vertebrates, and the term 'specific immune priming' to a different mechanism with equivalent function in the innate immune system of invertebrates.

As any novel scientific discovery should, specific immune priming has raised criticism mostly due to the lack of accurate evidence of its mechanisms (Hauton & Smith 2007). Another difficult question has concerned the ecological significance of specific immune priming to relatively short-lived invertebrates, since the trait is expected to cause an additional cost to the host (Schmid-Hempel 2005).

1.4. Ecological significance and occurrence of specific immunological priming

It has been questioned what ecological factors could justify evolution of specific immune priming in invertebrates. While specificity might increase the host's resistance against infections, better immunity could require resources or even trade-offs with other life-history traits such as survival and reproduction (Rigby & Jokela 2000). In order to create sufficiently strong selection pressure, there should be a real need for specific immune priming for an invertebrate with a life span of a few weeks or months at most (Kurtz 2005). Specific immune priming should benefit an animal with higher probability to be exposed multiple times to the same kind of pathogens. Rapid replication and clumped distribution of many pathogens supports the chance of short-lived animals to encounter similar types of pathogens several times during their lifetime, especially if these animals reproduce clonally or live in colonies (Kurtz 2005). Specific immune priming would be even more advantageous to short-lived animals if immunity is transferred from one individual to another, e.g., between members of a colony or from a mother to offspring (Moret & Schmid-Hempel 2001, Little et al. 2003, Kurtz 2005). Trans-generational specific immune priming would be the most beneficial if the mother and its offspring live in a similar environment (Sadd & Schmid-Hempel 2009).

Selection for immunological specificity might work, for example, in evolutionary arms race between pathogens and their hosts (Kurtz 2005). Many pathogens have evolved ways to evade host immune defence by rapidly varying their surface antigens. Recognition of such pathogens could demand diversification of the host antigen receptors (Kurtz 2005, Schmid-Hempel 2005). A hypervariable immunoglobulin domain-encoding gene, *Dscam*,

which transcripts into different phagocytosis mediating receptors depending on infecting pathogens, has already been found in *Anopheles gambiae* mosquito (Dong et al. 2006).

Past experiences with pathogens coupled with specific immune priming could make a host population increasingly resistant and the population size more robust to pathogen- or parasite-mediated impacts (Little & Kraaijeveld 2004). For estimation of the protective effect of immune priming on population-level, a new mathematical infection model, SPI (susceptible-primed-infectious) model, was developed by Tidbury et al. (2012). The SPI model differs from the traditional SIR (susceptible-infectious-recovered) model by highlighting the possibility that in immune priming the host can become ‘primed’ and immune in pathogen exposure without having to become infected. A great proportion of quickly primed individuals may significantly alter the likelihood of persistence of a pathogen in the population (Tidbury et al. 2012).

According to Little & Kraaijeveld (2004), consequences of specific immune priming could be even more striking in systems that incorporate genetic specificity. Without priming effect involved the most successful pathogens and parasites should infect common host genotypes, and through sufficient proliferation lead to their demise. However, this process could be slowed down if a past experience with pathogens or parasites provided the hosts with common genotypes and their offspring enhanced immune responses (Little & Kraaijeveld 2004). Thus specific immune priming could have an important role in host-pathogen interactions, population dynamics and evolution (Tidbury et al. 2010).

Specific immune priming has been reported to occur both within generation and between generations. Tidbury et al. (2010) observed priming effect to extend to the next generation in the Indian meal moth (*Plodia interpunctella*), which was exposed either repeatedly or once to its natural DNA virus. Offspring of the parents that had been exposed to the virus was less susceptible to the same virus compared to offspring of the parents without exposure. Pham et al. (2007) demonstrated specific immune priming by exposing the common fruit fly (*Drosophila melanogaster*) to a pathogenic fungus and a Gram-positive bacterium. Gram-negative bacteria as priming agents were studied by Roth et al. (2009) who reported that specific immune response of the red flour beetle (*Tribolium castaneum*) was strongest against its natural pathogen. They assumed that specific priming effect could vary depending on the host and pathogen combination.

While the number of articles concerning specific immune priming is steadily increasing, so far the evidence has been mostly phenomenal and the exact mechanisms behind immune priming are yet to be resolved (Kurtz 2005, Tidbury et al. 2010).

1.5. Possible mechanisms of specific immunological priming

Development of B cells and T cells into memory cells during an infection provides acquired immunity to vertebrates (Rowley & Powell 2007). These lymphocytes have a unique antibody receptor in order to recognize a specific antigen and to proliferate in its presence extremely rapidly especially in the case of re-infection, thus expressing memory and specificity. This kind of an elaborate adaptive immune system based on lymphocytes and antibodies requires more cells than the body plan of most invertebrates could maintain (Kurtz 2005). Therefore specific and adaptive immune response should base on a different mechanism within the invertebrate innate immune system (Rowley & Powell 2007). Alternative transcription of pathogen-recognition related genes, such as the previously mentioned *Dscam* of *Anopheles* mosquitoes, could create sufficient amount of receptor diversity (Watson et al. 2005, Dong et al. 2006, Kurtz & Armitage 2006). Some other essential and well-studied immune mechanisms of invertebrates were introduced earlier in this paper. Next I will review some of those mechanisms as possible contributors of specific pathogen recognition in the invertebrate immune system.

The Imd and Toll signalling pathways show a certain degree of specificity through different induction patterns. The Imd pathway activates in the presence of Gram-negative bacteria whereas the Toll pathway is induced mainly by Gram-positive bacteria and fungi (Vallet-Gely et al. 2008). Indeed, the study of Pham et al. (2007) showed that in the case of *Drosophila melanogaster* and a Gram-positive bacterium *Streptococcus pneumoniae* the Toll pathway is necessary (yet not sufficient alone) for immune priming whereas the Imd pathway is not required. Rapidly degrading AMPs were ruled out as protective agents. Phagocytosis was proposed to play an important role in the specific recognition and killing of pathogens (Pham et al. 2007). Increased phagocytosis against previously encountered bacteria was also demonstrated by Roth & Kurtz (2009) in an arthropod species *Porcellio scaber*. Here, phagocytic activity was largely decreased when the host encountered a Gram-negative bacterium *Escherichia coli*. This led to an assumption that the surface of Gram-negative bacteria might lack the cell wall components recognized by phagocytes.

Regardless of several attempts to explain the phenomenon, there is still a lot of study to be done in order to resolve the mechanisms of specific immune priming. Conducting studies in as natural way as possible could help to justify the existence of the phenomenon. In this respect the most obvious weaknesses in the previous experiments have been the use of dead pathogens and/or the less likely route of infection through inoculation or scratching. Since feeding exposes an insect to pathogens far more often than wounding,

specific immune priming should be expected to benefit the host the most in its intestinal track. Based on this assumption we conducted a specific immune priming study where living pathogens were first introduced to the host *Parasemia plantaginis* (the wood tiger moth) in the most potential infection site, the gut.

We investigated whether *P. plantaginis* larvae show specific immune priming when orally exposed to and subsequently injected with the same or different Gram-negative bacterium. We assumed specific immune priming to manifest itself in differences of resistance, which could be further measured as differences in survival (Roth et al. 2009). According to our hypotheses, immune priming was expected to occur through better survival of the larvae with an earlier exposure to the same bacterium. Immune priming could be further regarded as specific if the larvae survived better from the previously encountered bacterium compared to an unknown bacterium. Our null hypothesis would predict no differences in survival depending on the prior exposure.

2. MATERIALS AND METHODS

2.1. Study species

2.1.1. *Parasemia plantaginis*

Parasemia plantaginis (the wood tiger moth) is a Lepidopteran species without known economic importance. It belongs to the same diverse family of moths (Arctiidae) (Marttila et al. 1996) as *Hyphantria cunea*, a severe invasive pest on a variety of hardwood trees and shrubs (Yang et al. 2006). *P. plantaginis* larva feeds on many herbaceous plants (Marttila et al. 1996) and is very mobile in its search of food (Ojala et al. 2005). In Finland the active food search, growth and reproduction period of *P. plantaginis* is approximately two months. Larvae usually moult their skin 5–7 times before pupation (Ojala et al. 2005). A phase of eating and growing before each moulting is called an ‘instar’. From the third instar on *P. plantaginis* larvae have an orange patch on the back of their black and hairy body (Lindstedt et al. 2009). Larger patches function as better warning signals to predators, but the larger the signal is, the less melanin there is in cuticula (Lindstedt et al. 2008). In the Finnish cold climate *P. plantaginis* reproduces only once per summer and overwinters as an immature larva (Marttila et al. 1996). In laboratory conditions several *P. plantaginis* generations can be reared per year (Ojala ym. 2005).

Thanks to its small size as an insect species, relatively short generation time and easy maintaining *Parasemia plantaginis* is a highly potential model for immunological studies.

Since *P. plantaginis* is not a known pest species, it is also a safe model for immunological study of its relatives. For the invertebrates like *P. plantaginis*, which can encounter many similar kind of pathogens several times during its lifetime, immune priming should be expected to be a useful part of immune defense (Kurtz 2005).

P. plantaginis larvae were collected from the wild of southern Finland and reared for twelve generations in the greenhouse of University of Jyväskylä (see methods for rearing from Lindstedt et al. 2009). Normal laboratory diet was a mixture of dandelion (*Taraxacum* sp.) supplemented with plantain (*Plantago major*). Plantain supplement in the diet coupled with a smaller warning signal, i.e. higher melanin content, has been observed to improve survival of *P. plantaginis* larvae from an oral exposure to a bacterium *Serratia marcescens* strain ATCC#13880 (Zhang et al. 2012). According to the same study, genotype can also have a significant impact on survival of *P. plantaginis*.

The experiment larvae were picked from as many different families as possible in order to ensure sufficient genetic variation. Heterogeneity of the population was assumed to decrease the effect of genotype on resistance of the larvae and to minimize the effect of coincidence on survival from bacterial exposure. Unnecessary stress and selection pressure was further decreased by introducing the bacteria to the larvae in oral exposure. Melanin content of the larvae was assessed by measuring the size of the orange patch, i.e. signal, in segments similarly to Ojala et al. (2007).

2.1.2. *Serratia marcescens*

Serratia marcescens is a Gram-negative bacterium from the family Enterobacteriaceae (Mahlen 2011). It is a widespread saprophytic bacterium that grows well on different culture media. *S. marcescens* is an opportunistic pathogen of humans, animals and insects. It is an important causative agent of many kinds of nosocomial infections (Mahlen 2011, Falkiner & Hejazi 1997). Several strains of *S. marcescens* have been isolated from tens of different insects (Grimont & Grimont 1978).

Two strains of *S. marcescens* were used in the experiment: Db11 and ATCC#13880. The bacteria originated from the same stock as the bacteria used in the study of Zhang et al. (2012). The Db11 strain is a spontaneous mutant of *S. marcescens* Db10 strain, which has been isolated from a fly (*Drosophila* sp.). Like Db10 strain, Db11 strain can be lethal when fed or injected to *Drosophila*. Db11 is able to extract toxins with enzymatic and proteolytic activity in order to cleave proteins and to inactivate antibacterial activity in haemolymph of *Cecropia* (Flyg et al. 1980). The ATCC#13880 strain is an environmental

isolate originating from pond water (Martinec & Kocur 1961). Unlike the colourless strain Db11, the ATCC#13880 strain synthesizes red pigment called prodigiosin (Grimont & Grimont 1978). Non-diffusible prodigiosin is toxic to protozoa (Groscop & Brent 1964). Thus it may offer ecological advantage to *S. marcescens* living in water and soil (Grimont & Grimont 1978). Zhang et al. (2012) observed that the Db11 strain infected *P. plantaginis* larvae faster than the ATCC#13880 strain in oral exposure, although eventually both strains caused almost equal mortality. Perhaps due to the lack of close evolutionary history with insects, ATCC#13880 seems to be less virulent to *P. plantaginis* than Db11. The two strains might differ from each other enough by their origins, genotypes and phenotypes in order to be distinguished by the host immune system (Flyg et al. 1980, Kurtz 2005).

The purpose of our experiment was to determine the level of specificity of immune priming by observing and comparing survival of the larvae that were exposed orally and through injection to one or both of the *S. marcescens* strains.

2.1.3. *Escherichia coli*

Escherichia coli is a well-studied Gram-negative bacterium that belongs to the same family of Enterobacteriaceae as *S. marcescens*. It is ubiquitous in nature and occurs as several strains. Most of the *E. coli* strains are harmless or even beneficial part of the normal intestinal flora of warm-blooded organisms, but some strains are pathogenic and can cause diseases such as gastroenteritis and urinary track infections (Madigan 2009). *E. coli* has been used as a susceptible target to different antibacterial peptides and other defence responses in immunological study of moth species such as *Hyalophora cecropia* (Carlsson et al. 1991) and *Helicoverpa armigera* (Mackintosh et al. 1998).

A common, laboratory-adapted strain *E. coli* K-12 was used in the experiment as a non-lethal control species in order to stimulate immune system without causing serious harm to the host.

2.2. Study setting

Throughout the rest of this section and the following results the bacteria used in the experiment will be abbreviated as follows: ‘SmA’ stands for *S. marcescens* ATCC#13880, ‘SmB’ stands for *S. marcescens* Db11, and ‘EcC’ stands for *E. coli* K12 control.

The experiment included an oral and a septic exposure. Three larvae groups of equal size, the sample groups S1 and S2 and the control group C, were orally exposed to SmA, SmB or EcC, after which they were divided into three subgroups (Figure 3). Five days later the subgroups were injected with the same or different bacterium. The idea was to compare

survival between the subgroups in the following way: S1a could be compared with Ca, and S2a with Cb, in order to detect non-specific immune priming. S1a could be compared with S2b, and S2a with S1b, in order to detect strain-specific immune priming. Survival of the subgroups was compared between the triplets rather than within the triplets in order to minimize the effect of different virulence of SmA and SmB on the results.

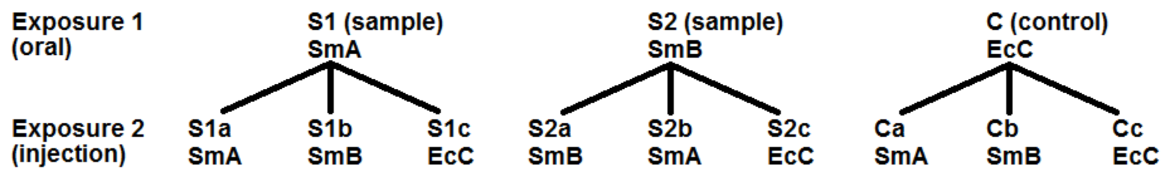

Figure 3. Division of the first exposure groups into the second exposure subgroups and the bacterial exposures.

2.2.1. Study preparations

A total of 900 c.a. three weeks old *Parasemia plantaginis* larvae were separated into their own ø 9 cm Petri plates. The larvae were picked from a weight range of 50–130 mg to ensure sufficient development of their immune system. The larvae represented a total of 107 families. Since the composition of the larvae was based on desired weight rather than family, the number or larvae representing each family could differ. However, all family representatives were distributed as equally as possible into two sample groups S1 and S2 and a control group C (300 larvae each).

The plates were piled up on tables in a laboratory with windows on one side of the room. All individual data of the larvae such as initial weight, the size of the orange patch in segments, and daily survival was recorded on the plates. Position of the plates in the piles was changed during the daily checks. Position of the sample groups closest and farthest to the windows was changed with each other every other day in order to exclude possible effect of extra sunlight. Room temperature was about 21 °C in the daytime and lower at night. The larvae were fed *ad libitum* with non-sterilized mixture of dandelion (*Taraxacum* sp.) picked from several locations close to the university.

The bacteria were grown for 24 hours in 25 ºC on bacterial growing medium (LB) consisting of 10 g NaCl, 10 g tryptone and 5 g yeast extract in 1 L dH_2O, which had been solidified to 1.5 per cent agar gel on Petri plates. After incubation some bacterial lawn was scraped off the plates with a sterile loop (VWR) and diluted with dH_2O to obtain 0.50 optical density (OD) for the first exposure and 0.16 OD for the second exposure at 600 nm

wavelength measured with Bioscreen C^{TM} spectrophotometer (manufactured by Growth Curves Ltd, Helsinki, Finland).

2.2.2. First exposure

The sample group S1 was orally exposed to SmA, the sample group S2 to SmB, and the control group C to EcC by offering each larva a piece of dandelion leaf dipped into 0.5 OD solution of living bacteria. An additional 200 µl droplet of the same solution was added on the leaf piece for extra exposure and moisture. All three groups were treated at the same time. The mean size of a leaf piece was 54.45 mg (SD 18.78 mg, N = 29).

After 24 hours the condition of each larva was checked and the leaf pieces were observed for signs of consumption. Moulted skins were removed and recorded. The larvae that had not yet eaten were placed on their leaf pieces to ensure exposure. The larvae that had consumed the entire leaf piece were given normal food in order to avoid causing them stress by starvation. After another 24 hours the larvae and the leaf pieces were checked again. The larvae were fed *ad libitum* with untreated dandelion on three consecutive days.

Before the second exposure 15 larvae, which had evidently consumed some amount of contaminated leaf, were picked from each treatment group and dissected in order to collect haemolymph and gut samples for measurement of lytic activity and further analyses of gene expression by Dr. Dalial Freitak. Lytic activity was determined from the haemolymph samples with a lytic zone assay similarly to Freitak et al. (2009): 2 ml of each haemolymph sample was pipetted into a 2 mm well of 1.5 per cent agar gel plate containing 35 ml of autoclaved Sørensen's buffer for correct pH, 2.1 mg of streptomycin sulphate antibiotic against Gram-negative bacteria, and 21 mg of freeze-dried Gram-positive *Micrococcus luteus* cells. *M. luteus* was used instead of either of the *S. marcescens* strains because lysozyme has been reported to be bactericidal only to Gram-positive bacteria (Boman et al. 1991). A control curve, which acted as a calibration curve, was created by adding a chicken egg-white lysozyme dilution series of 2, 1, 0.750, 0.500, 0.250, 0.125, 0.62 and 0.31 mg ml^{-1} on each gel plate. After 24 hours incubation in 37ºC the plates were observed for clear circles indicating dead bacteria around the samples. Radius of these clear circles should have determined lytic activity. However, any circles indicating lytic activity were not detected. The dissected and pupating larvae were excluded from the experiment and most of the statistical analyses.

2.2.3. Second exposure

The second exposure was carried out by injection in order to ensure infection. Three days after the transition to untreated diet both samples and the control group were divided into three subgroups as described above, creating a total of nine subgroups: S1a, S1b, S1c, S2a, S2b, S2c, Ca, Cb and Cc (Figure 3). The subgroups of samples S1 and S2 were injected in the following way: the larvae in the subgroups ‘a’ were injected with the previously introduced SmA/B strain, the subgroups ‘b’ were injected with the unfamiliar SmA/B strain, and the subgroups ‘c’ were injected with EcC. From the three control subgroups Ca was infected with SmA, Cb with SmB, and Cc with EcC. Infection was inflicted by injecting 2 μl of 0.16 OD solution of living bacteria between the sixth and seventh abdominal segment of the larvae into the haemocoel. The treatment was carried out in three pairs, where one person measured the injection doses while the other handled and injected the larvae. Each pair treated one subgroup from all of the three groups by injecting each of the different bacteria once. After the treatment survival of the larvae was observed at three-hour intervals. The experiment was terminated after 51 hours when most of the larvae had died and their mortality rate had dropped close to zero.

Haemolymph samples were collected from the larvae that had been orally exposed to either EcC or SmA and injected with SmA or EcC. Lytic activity was determined with the same lytic zone assay as summarized before, but it was not detected in any of the samples. Therefore the lytic activity analyses have been excluded from our results.

2.3. Statistical analyses

Analyses were performed using statistical analysis software SPSS v. 17.0 manufactured by SPSS Inc., Chicago, IL. Survival data of the larvae in injected subgroups was analysed using Cox regression analysis. The factors analysed were the main effects of the first and the second exposure, larval weight and signal, and all interactions between these four factors. After this the statistically significant factors were sorted out and analysed together. Kaplan-Meier survival analysis was used to make pairwise comparisons between the survivals of the subgroups. Since the multiple comparisons were made to test predefined hypotheses, the Bonferroni correction was not used. Difference in larval weight between the three oral exposure groups was analysed with ANOVA. Correlations were analyzed with Spearman’s rho test. Significance of the observed differences were confirmed with Chi-squared test.

3. RESULTS

Here, as in the previous section, 'SmA' is used as an abbreviation for *S. marcescens* ATCC#13880, 'SmB' for *S. marcescens* Db11, and 'EcC' for *E. coli* K12. The first and the second exposures will be expressed as abbreviations of the bacteria used in these two exposures separated with a dash. For example, an abbreviation 'SmA–SmB' would stand for the subgroup of larvae that was orally exposed to *S. marcescens* ATCC#13880 and later injected with *S. marcescens* Db11.

3.1. Contaminated leaf consumption

Roughly 75.8 per cent of the 900 larvae consumed contaminated leaf during the oral exposure (Figure 4A). That is, at least three quarters of the larvae were evidently orally exposed irrespective of the bacterium, but probably even larger proportion was exposed, e.g., by drinking the bacterial solution or due to general contamination. Proportion of the larvae that consumed leaf in the SmB group was 14.5 per cent lower compared to the SmA and the EcC group (Chi-squared test: $X^2_2 = 14.513$, P = <0.001). It seems that the larvae may have somehow detected and avoided Db11, yet despite of this mortality was still high in the SmB group (Figure 5A). At least 68.4 per cent of the larvae in all subgroups had consumed contaminated leaf before the second exposure (Figure 4B), but presumably all larvae were exposed to some extent as explained above.

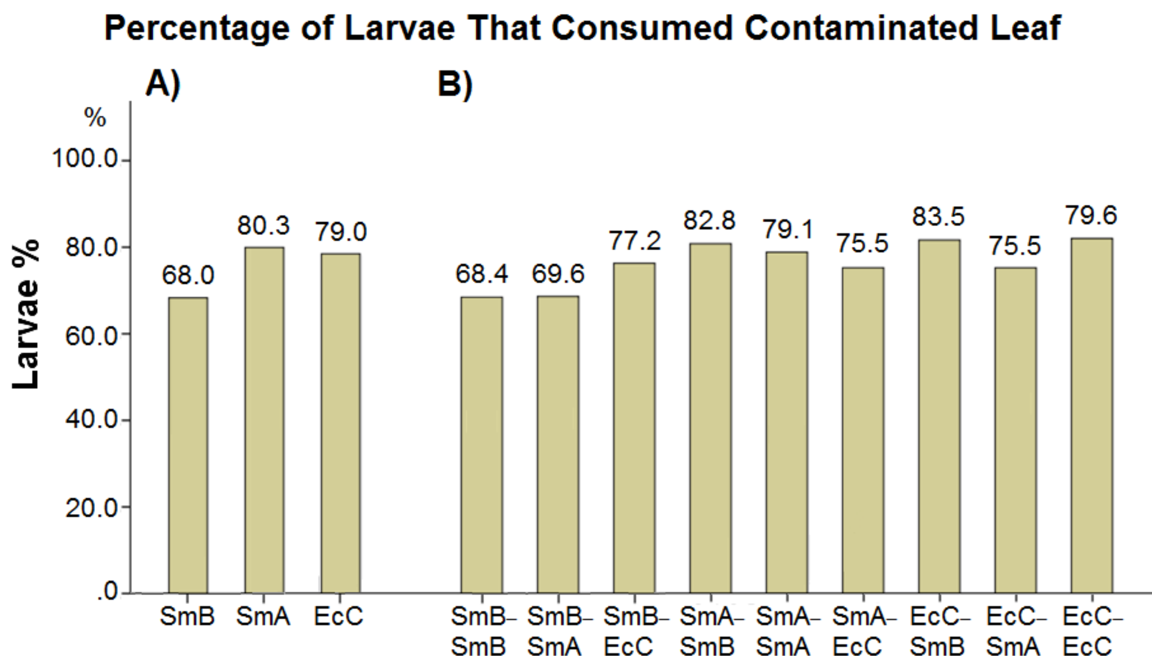

Figure 4. Percentage of the larvae that were observed to consume contaminated leaf during oral exposure A) within the oral exposure groups and B) within the injected subgroups.

3.2. Survival after the first exposure

During the two-day oral exposure and the following three days before the second exposure about 40.0 per cent (114 out of 284) of the larvae died in the SmB exposed group, ~2.5 per cent (7 out of 285) in the SmA exposed group and ~3.9 per cent (11 out of 284) in the EcC exposed control group (Figure 5A). One pupating larva was excluded from both the SmB group and the EcC group, leaving a total of 284 larvae in the samples. Mortality was higher in the SmB group compared to both the SmA group (ANOVA: $F_{2,131}$ = 37.987, P = <0.001) and the EcC group (ANOVA: $F_{2,131}$ = 37.987, P = <0.001). SmB seemed to be significantly more virulent, i.e. faster to infect the larvae, compared to the other two bacteria. There was a positive correlation between weight and survival of the larvae in the SmB group (Spearman rank correlation: $r_s = 0.154$, $N = 284$, $P < 0.01$), i.e. larger larvae survived better. There were no significant difference in the number of larvae that died in the SmA group compared to the EcC group (ANOVA: $F_{2,131}$ = 37.987, P = 0.522). At this point SmA seemed to be less virulent than SmB, or even non-virulent since it caused approximately the same amount of mortality as the presumably non-virulent EcC.

3.2. Survival after the second exposure

Over 90.0 per cent of the larvae died in the subgroups with SmB oral exposure or injection including the SmB–EcC subgroup (Figure 5B). This confirmed the high virulence of SmB

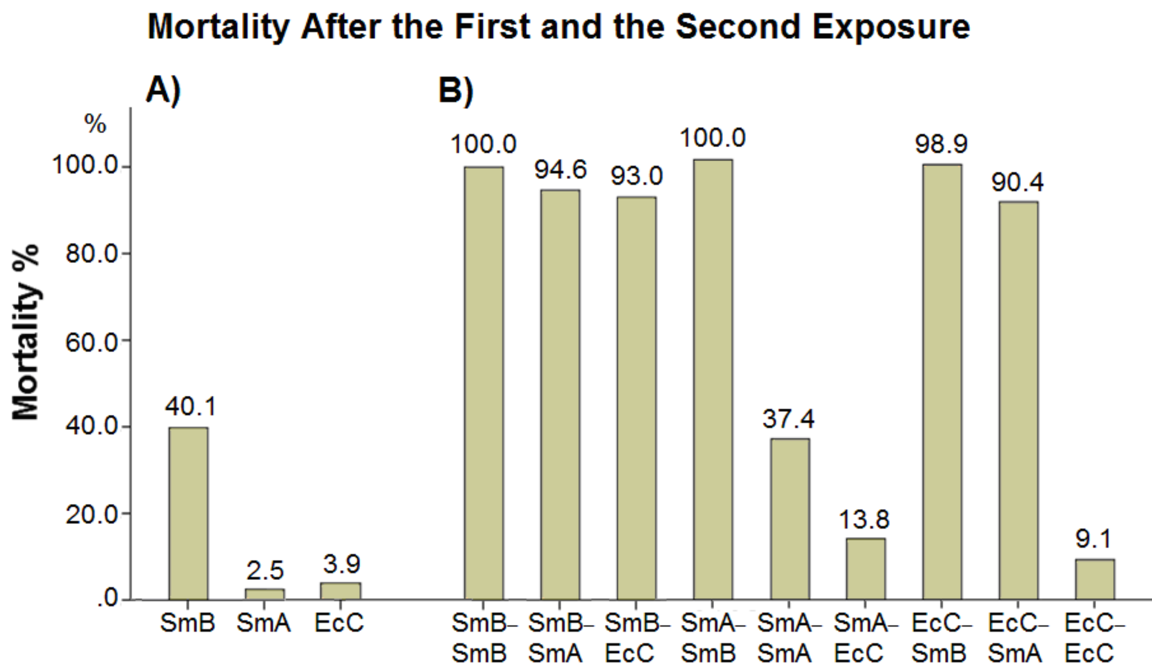

Figure 5. Mortality of the larvae A) within each exposure group after the first (oral) exposure and B) within the nine subgroups after the second exposure (septic injection).

in oral exposure. Mortality was high also in the subgroups with SmA injection except for the SmA–SmA subgroup in which mortality was unexpectedly low. Mortality was the lowest in the SmA–EcC and EcC–EcC subgroups, which supports the previous observation of the non-virulence of SmA in oral exposure and the non-virulence of EcC in general.

Survival of the larvae depended considerably on the bacteria combination of the first and second exposure (Cox Regression, χ^2=594,210, P <0.001) (Table 1). According to the full survival analysis including all factors, weight of the larvae in combination with the bacterium of the first exposure had a significant effect on survival of the larvae after the injection (Table 1A). Therefore weight was included along with other significant factors in the final survival analysis, in which it indicated significant effect on survival after the injection (Table 1B). There were no difference in mean weight of the larvae between the oral exposure groups (ANOVA: $F_{2.797} = 0.413$, $P = <0.662$).

Table 1. Results of the Cox regression survival analysis: A) the full model and B) the final model with significant covariates. The main effect of weight was included to the final analysis since it was a part of the significant weight*1st exposure interaction in the full model.

A)

	Wald	df	p
1st exp	17.974	2	**<0.001**
2nd exp	17.533	2	**<0.001**
weight	2.195	1	0.138
signal	2.122	1	0.145
1st exp*2nd exp	100.654	4	**<0.001**
weight*1st exp	8.174	2	**0.017**
weight*2nd exp	1.573	2	0.455
weight*signal	1.517	1	0.218
signal*1st exp	1.168	2	0.558
signal*2nd exp	4.674	2	**<0.001**

B)

	Wald	df	p
1st exp	25.528	2	**<0.001**
2nd exp	113.772	2	**<0.001**
weight	4.869	1	**0.027**
1st exp*2nd exp	101.661	4	**<0.001**
weight*1st exp	7.526	2	**0.017**

In pairwise comparisons the subgroups differed significantly or highly significantly in survival except for the following three pairs of subgroups: the SmA–EcC and EcC–EcC pair of subgroups, the SmB–SmA and EcC–SmA pair of subgroups, and the SmB–EcC and EcC–SmA pair of subgroups (Table 2). The first exception indicates that there were no difference in virulence between SmA and EcC in oral exposure. The second and the third exception indicate that SmA injection was as lethal to the larvae as SmB oral exposure except in the case of the subgroup with prior oral exposure to SmA (Figure 6A).

Table 2. Pairwise comparisons of survival in the subgroups with different exposure combinations.

exp1-exp2	SmB-SmB		SmB-SmA		SmB-EcC		SmA-SmB		SmA-SmA		SmA-EcC		EcC-SmB		EcC-SmA		EcC-EcC	
	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p
SmB-SmB			51.30	***	77.82	***	4.97	**0.026**	134.10	***	151.64	***	11.36	**0.001**	91.92	***	168.24	***
SmB-SmA	51.30	***			9.69	**0.002**	29.15	***	68.73	***	113.97	***	8.39	**0.004**	3.07	0.080	129.94	***
SmB-EcC	77.82	***	9.69	**0.002**			67.15	***	48.29	***	99.41	***	38.47	***	2.90	0.089	121.17	***
SmA-SmB	4.97	**0.026**	29.15	***	67.15	***			144.36	***	178.51	***	4.37	**0.037**	64.97	***	193.48	***
SmA-SmA	134.10	***	68.73	***	48.29	***	144.36	***			12.64	***	119.86	***	64.92	***	19.72	***
SmA-EcC	151.64	***	113.97	***	99.41	***	178.51	***	12.64	***			163.20	***	115.37	***	1.07	0.300
EcC-SmB	11.36	**0.001**	8.39	**0.004**	38.47	***	4.37	**0.037**	119.86	***	163.20	***			27.51	***	179.90	***
EcC-SmA	91.92	***	3.07	0.080	2.90	0.089	64.97	***	64.92	***	115.37	***	27.51	***			130.57	***
EcC-EcC	168.24	***	129.94	***	121.17	***	193.48	***	19.72	***	1.07	0.300	179.90	***	130.57	***		

***** < 0.001**

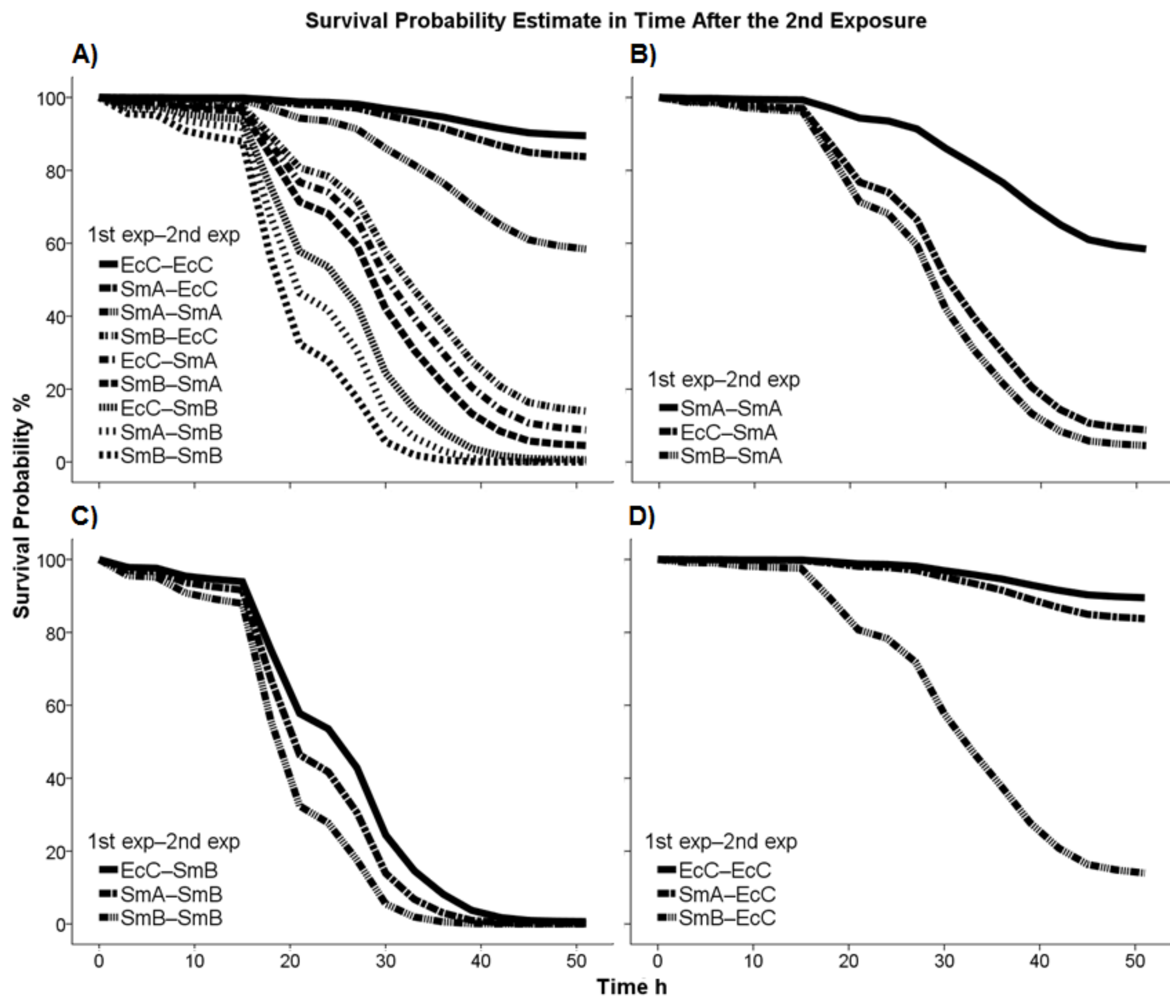

Figure 6. Survival probability estimate in time for the larvae in the subgroups after the second exposure. The bacteria used in the first and the second exposures have been abbreviated and separated by a dash, i.e. 1st exposure–2nd exposure. The order of the lines in the charts corresponds to the order of the subgroups on the lists. Survival probability estimate A) for all subgroups of larvae, B) for the subgroups with SmA in the second exposure, C) for the subgroups with SmB in the second exposure, and D) for the subgroups with EcC in the second exposure.

From the three subgroups that were injected with SmA the SmA–SmA subgroup survived highly significantly the best (Figure 6B). It seems that prior SmA oral exposure offered the larvae such protection against SmA injection that EcC exposure could not offer.

Difference in survival between the three subgroups that were injected with SmB was statistically significant (Table 2); from these subgroups the EcC–SmB survived the best, the SmA–SmB survived the second best and the SmB–SmB survived the worst (Figure 6C). In this case the prior SmA oral exposure could not protect the larvae against the SmB injection. In comparison with the prior EcC exposure, the prior SmA exposure seemed to be rather harmful than beneficial.

From the three subgroups that were injected with EcC only the SmB–EcC suffered from high mortality (Figure 6D). Again, it seems that SmB was highly virulent in oral exposure whereas SmA was not. EcC was generally non-virulent.

4. DISCUSSION

4.1. Conclusion of the results

The aim of this study was to find out whether or not *Parasemia plantaginis* show specific immunological priming when orally exposed to and subsequently injected with the same or different Gram-negative bacterium. Immune priming was expected to present itself in better survival of the larvae that were exposed twice to the same bacterium. Specificity in the immune priming could be confirmed if the larvae survived better from the previously encountered bacterium compared to an unknown bacterium.

In the oral exposure of *P. plantaginis* larvae the SmA (*S. marcescens* ATCC#13880) strain seemed to be as benign as the EcC (*E. coli* K12) control bacterium whereas the SmB (*S. marcescens* Db11) strain, a known insect pathogen, was considerably more lethal than the other two bacteria. In the second exposure through injection SmA caused high mortality similarly to SmB. This suggests that the SmA strain, an environment isolate, could be a relatively harmless inhabitant or visitor in the insect gut, but can cause lethal septicaemia if it manages to enter the haemocoel (Boucias & Pendland 1998). Unlike the pathogens, EcC was non-virulent in the second exposure. High mortality in the SmB–EcC subgroup of larvae could be explained by prolonged effect of the SmB oral exposure. Unfortunately, SmB turned out to be too virulent in order to be analyzed directly alongside SmA or EcC in the context of non-specific or specific immune priming. However, EcC became to have an important role in the detection of both of these phenomena.

The first and the second exposures and their interaction turned out to have a highly significant effect on survival of the larvae (Table 1). The larvae that had been orally exposed to SmA survived SmA injection considerably better than the larvae with prior EcC

exposure (Figure 6B). In other words, the preceding exposure to SmA seemed to activate the immune system in a way that a significant amount of larvae could withstand SmA injection. This kind of an effect can be considered as immune priming (Kurtz 2005). On the other hand, the larvae with prior SmA oral exposure survived significantly worse from SmB injection compared to the larvae with prior EcC exposure, which might be explained by mixed infections. Protection provided by SmA oral exposure seemed to be limited to the SmA strain since the same immune priming turned out to be useless or even harmless against the other strain of *S. marcescens* (SmB). Sometimes priming against one pathogen species can lower protection against other pathogen species as demonstrated in the trans-generational immune priming study with bumblebees (Sadd & Schmid-Hempel 2009). Considering SmA's higher virulence and closer genotypic relation to SmB coupled with its potential to act as a stronger immune activation agent, the SmA exposed larvae could have been expected to survive better from SmB injection compared to the EcC exposed larvae in the case of general and non-specific immune priming. Since SmA oral exposure improved survival of the larvae against otherwise lethal SmA injection while being ineffective or even impairing against SmB injection the immune response induced by SmA (*S. marcescens* strain ATCC#13880) could be regarded as specific.

It is likely that SmB was so highly virulent that once it was injected into haemocoel it was not the potential protection provided by prior EcC or SmA oral exposure but rather the possible prior stress caused by SmA oral exposure that affected survival of the SmB injected larvae. There might also have occurred mixed infections, where two species of bacteria were infecting the host simultaneously. Although this was probably true in the case of the larvae with prior SmB oral exposure, it would be more difficult to explain why the larvae with prior SmA exposure survived better from subsequent SmA injection. The protective priming may have been acquired without infection (Tidbury et al. 2012).

Another possible explanation is that the induced immune response could last from the first exposure to the second as immunological loitering (McTaggart 2012). Based on the data we cannot tell whether the observed priming resulted from immunological loitering or fast readjustment of stronger immune response. A small amount of the ingested bacteria might have survived in the alimentary canal and maintained the immune response. However, maintaining of constant immune response for several days is likely to impair the host's essential life-history traits such as growth and reproduction (Rigby & Jokela 2000). Presuming that immunological resources are also limited the induced immune response should decay over time. Thus, if the immune response was constantly induced and the

amount of immune molecules was limited, the more stressed larvae, which had spent more of their immune capacity during the oral exposure, should have died faster after the injection. Yet this kind of ‘immunological exhaustion’ was not observed in the subgroup with SmA oral exposure and injection. An opposite argument would be that the exposures were two completely separate events and the immune responses were not linked to each other. Clearly, this possibility seems unlikely considering the remarkable survival advance offered by prior SmA oral exposure against lethal SmA injection.

Weight of the larvae in interaction with the first oral exposure indicated significant effect on survival from the subsequent injections (Table 1A). Weight alone also indicated significant effect when analysed together with the both exposures and their interaction (Table 1B). Mortality during the oral exposure was significantly high only in the SmB group, in which the larger larvae survived better. Better survival of the larger larvae might have resulted from smaller relative doses or even from higher development stage and more developed immune defence. Although the larvae were of the same age, their instar could vary. The difference of only one instar has been observed to account for better immunity (Kirkpatrick 1998).

Difference in the amounts of melanin indicated by the orange patch of the larvae, i.e. the size of the warning signal, did not seem to have significant effect on survival. Yet even more surprisingly no lytic activity was detected in any of the haemolymph samples collected during the experiment. Although lysozyme has been suggested to be bactericidal only to Gram-positive bacteria (Boman et al. 1991), it is normally present in haemolymph of most insects, including moths, and lytic activity is often strongly induced during infection (Hultmark 1996). It is difficult to explain why lytic activity was not detected or why the amount of melanin did not seem to affect survival since both lysozyme and melanin are important parts of the invertebrate immune defence. The analysis methods were tested and proved to be valid before (Ojala et al. 2007, Freitak et al. 2009).

Minor errors may have occurred during the treatments and the measurements, although both were carried out by skilled individuals with utmost care. Possible unknown bacterial exposure originating from the non-sterilised dandelion leaves could not be excluded, but the risk was natural and uniform to all larvae. Although the exposure dose was not constant, i.e. the amount of leaf that the larvae consumed varied, contamination of the plates was prominent. Since SmB was too virulent for the purpose, the study setting could be improved in certain ways, which will be discussed in the last section.

4.2. Comparison of the results to other similar studies

Our study setting differed from previous immune priming studies in some respects. Firstly, delay between the first and the second exposure was three to four days longer than in the study of McTaggard et al. (2012) to minimize the effect of short-term immunological loitering. Secondly, instead of priming the hosts with dead bacteria by scratching as in the study of Roth et al. (2009) or by inoculation as in the study of Pham et al. (2007) we used living bacteria in oral exposure. Willingness of the *P. plantaginis* larvae to consume highly contaminated food enabled us to carry out the exposure in a way that most natural infections are presumed to occur (Vallet-Gely et al. 2008).

According to Vallet-Gely et al. (2008), majority of ingested bacteria are killed by the unfavourable conditions or by ROS in the insect midgut before they can induce systematic immune response. However, in order to vaccinate immunity from one moth to another as reviewed by Wagner (1961), the priming effect should take place in haemolymph. Both *S. marcescens* and *E. coli* produce catalase enzyme that offers resistance against ROS (Campbell & Dimmick 1966, Loewen 1984). Even the non-pathogenic *E. coli* has been observed to stimulate the insect immune system, e.g., by inducing the expression of antimicrobial peptides (Freitak et al. 2007, Pham et al. 2007). Our study setting enabled us to see whether or not naturally infectious bacteria are able to overcome the midgut defence barriers and to induce immune priming in haemocoel, where the subsequent infection through an injection was targeted.

Eleftherianos et al. (2006) have observed that prior infection with non-pathogenic *E. coli* K-12 strain protects the moth species *Manduca sexta* against pathogen infection, but according to Roth et al. (2009) insects cannot be primed against *E. coli*. In the case of *P. plantaginis* there were no significant difference in survival of the EcC injected larvae having either prior EcC or SmA exposure. Since *E. coli* was not lethal to the larvae in either of the two exposure methods, it seemed well-suited as a control bacterium. Using a non-pathogenic bacterium in the control treatment instead of a more neutral substance was a considered decision and seemed to work as well if not better than the water treatment in the previous study by Zhang et al. (2012).

Similarly to Zhang et al. (2012), we observed that *S. marcescens* strain Db11 is higher in virulence and kills *P. plantaginis* larvae faster than *S. marcescens* ATCC#13880 strain. Larger larvae survived significantly better in both studies. Zhang et al. (2012) reported that the amount of melanin had a significant effect on survival of the larvae whereas our results were the opposite. Contrary to the observation of the previous study

ATCC#13880 seemed to be similarly non-virulent with the control bacterium *E. coli* in oral exposure. Although the density of bacteria was only half of the density used by Zhang et al. (2012), the dose was over hundredfold. Potentially stressful measures such as diet change and food-deprivation were not involved. It is noteworthy that Zhang et al. (2012) observed the effect of oral exposure at least three times longer, but during this time mortality in the water control group was unusually high. All things considered, the result obtained in our experiment, albeit short in duration, demands further analysis of the virulence of *S. marcescens* ATCC#13880 in oral exposure.

4.3. Ecological and evolutionary implications

The ability of *P. plantaginis* to detect and to avoid Db11 indicates that this pathogen might be a natural enemy of the host. Immune priming against such a natural pathogen would be justified as emphasized in the results of Roth et al. (2009). In our study immune priming was observed to be induced by the less virulent *S. marcescens* strain ATCC#13880, which also could share evolutionary history with *P. plantaginis*. Recognition of the pathogen could be based simply on the red prodigiosin pigment that ATCC#13880 along with many other *S. marcescens* strains extract. Another explanation could be the high sensitivity and ability of invertebrate immune system to detect inconspicuous bacteria such as *E. coli* (Eleftherianos et al. 2006, Freitak et al. 2009), which can persist even in large numbers several hours in gut without inducing systemic AMP production (Vallet-Gely et al. 2008).

Immune system is presumably more sensitive to more virulent pathogens. Smaller amounts of highly virulent pathogens such as the *S. marcescens* strains should be enough to induce immune priming. Yet too large amount of pathogens can lead to infection and demise of the host before priming is formed. The immune priming process differs from that of the acquired immunity in a way that the host can gain immunity following exposure without having to become infected first. However, immune priming does not necessarily provide full immunity, i.e. primed individuals can become infected as well as non-primed individuals (Tidbury 2012). Most of the previous phenomena took place in our experiment, in which the bacteria were fed to the larvae in large amounts and the subsequent injection was expected to cause almost certain infection and death. While the amount of Db11 contamination was fatal to the larvae in oral exposure, ingested ATCC#13880 seemed to induce priming without infecting the host.

Immune priming has implications for the survival of individuals as well as whole populations. As the proportion of primed individuals increases, the ability of the pathogen

to infect the susceptible individuals reduces (Tidbury et al. 2012). Thus immune priming can alter the likelihood of persistence of a pathogen in a host population. Immune priming can act to destabilize host–pathogen population dynamics especially when populations experience high levels of immune priming together with fitness-related trade-offs. Epidemics could have surprising outcomes on population depending on the amount, quality and reproductive costs of priming. Anticipation of such outcomes requires mathematical models that can indicate the effects of immune priming on population dynamics (Tidbury et al. 2012). This kind of information might be essential for predictions of the severity and persistence of epidemics (Little & Kraaijeveld 2004), e.g., in biological control.

4.4. Applications and future prospects

Immune priming may have a significant impact on economy and public health of society by increasing resistance of pest insects and disease vectors against biological control and insecticides. It may have complicated and unexpected effects on host–pathogen population dynamics in general. Better understanding together with new mathematical models, such as the SPI model by Tidbury et al. (2012), may help to predict the effects of immune priming on pest control and epidemics. Closer study of immune responses related to priming should increase knowledge of specific and adaptive abilities of innate immune system. This kind of information could benefit especially immunocompromised individuals such as the AIDS patients who cannot rely on acquired immune system (Pham et al. 2007). Functional similarity between the vertebrate and invertebrate innate immune systems may justify new ways of using invertebrates as model species (Little & Kraaijeveld 2004).

Our results contribute to the study of insect immunology and invertebrate innate immune system in at least three major ways: Firstly, we demonstrated that the immune system of a moth species *Parasemia plantaginis* is capable to differentiate a pathogenic Gram-negative bacterium from a non-virulent Gram-negative bacterium and to induce protective priming against it. Specificity of this priming could reach as high as strain level, but this needs more study. Secondly, we observed the ability of a moth larva to detect and to avoid highly lethal substances such as Db11. Thirdly, we showed that immune priming is inducible in oral exposure to living pathogens, which is the most likely way for natural infections to occur (Vallet-Gely et al. 2008). In other words, immune priming should be a real phenomenon in the wild as well as in laboratory.

Now that specifically induced immune priming has been observed within generation and between two Gram-negative bacteria, the next step could be a trans-generational study

between two strains of the same pathogen species. Alternatively, a study could be focused within generation with longer time lag between the exposures in order to study the duration of protection provided by priming. Natural infection routes should be preferred when possible. Yet in case of highly virulent bacteria the priming should be executed with dead bacteria or very low doses. Naïve larvae could be included in order to study if there is an effect of using a non-pathogenic bacterium in a control treatment. More attention should be paid on priming inducers, physiological changes and mechanisms related to priming. It would be intriguing to explore prodigiosin pigment as a possible induction agent. Also, the gut samples collected from our experiment for further RNA studies by Dr. Dalial Freitak may still reveal more about immunological priming factors and products in *P. plantaginis*.

The phenomenon and concept of immune priming and specific immune priming alike has become more and more confirmed and accepted in science, but many questions still remain unanswered especially at mechanistic level. Instead of one simple answer there might be a variety of solutions to the problem of immune priming. Studying invertebrate immune system in as natural way as possible should help to see the big picture of the functions of innate immune system. Realistic approach to the problem is essential not only in discussion of the ecological aspects but also in study of this newly acknowledged and exciting phenomenon.

ACKNOWLEDGEMENTS

I want to thank my supervisors Lauri Mikonranta and Johanna Mappes for their excellent advice and enormous support and patience during this exciting and sometimes exhausting journey to the world of insect immunology. Special thanks go to Kaisa Suisto and Dalial Freitak for their great expertise and huge help in the experiment. I also owe thanks to all the wonderful people in the Plantaginis research group who helped and supported me during the seminars and the study. The warm and friendly atmosphere of the group had big positive impact on me. Last but not least I want to thank dear Joonas Hovi, lovely Mana and all my dear friends who helped me to cope with the hard times.

The study was funded by the Finnish Centre of Excellence in Evolutionary Ecology and Evolutionary Research funded by the Academy of Finland. Personal costs were funded by grants from the Finnish Centre of Excellence in Evolutionary Ecology and Evolutionary Research, Societas Biologica Fennica Vanamo and Betty Väänänen Foundation.

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