Trade-offs between signalling pathways in immune defence, survival and sexual selection in a pest insect (*Galleria melonella*)

2.1. OBJECTIVES

The main focus of eco-immunology, an emerging field of ecology, has been to describe and explain natural variation in immune functions, specifically why and how biotic/abiotic factors contribute to variation in immunity in free-living organisms. This approach can explain the persistence of parasitism as the most common mode of life on the Earth. This project will address general questions in the areas that are of central importance for development of ecological immunology. The main objective of this project is to test the hypotheses that there are phenotypic and genetic tradeoffs between immune defence, life-history traits, sexual signalling and trade-offs between signalling pathways to fight fungal and bacterial infections in the greater wax moth (*Galleria melonella*). Furthermore, this project will evaluate the ecological and evolutionary factors/costs (including metabolic ones) responsible for the variation in immune defence. The project will contribute to understanding of natural defences and improve methods of pest-control.

2.2. STATE-OF-THE-ART AND PROGRESS BEYOND

Parasites and pathogens are an ubiquitous threat to organisms, reducing their fitness by decreasing survival or reproductive success (Schmid-Hempel 2011). As a consequence of such attacks on fitness, organisms have developed various defence mechanisms against many parasites and pathogens. An important evolutionary question is, "how much should an organism invest in its ability to mount an immune response against parasites?" If immune defence is limited by the availability of resources, investing them in immune defence will reduce the resources available to other traits associated with the organism's reproductive success, in particular to important life-history traits such as fecundity, age at maturity, body size, sexual signalling, longevity (e.g. Zuk & Stoehr 2002; Daukste *et al.* 2012; Otti *et al.* 2012). Thus, evolutionary pressure will favour those individuals that allocate their resources to life-history traits and immune defence in a way that maximizes lifetime reproductive success. In other words, evolutionary pressure will lead to an investment of resources that balances the benefits and costs of immunity.

In insects, immune protection relies on both humoral and cellular responses that are mediated via certain recognizing receptors and activation of several signalling pathways (Lemaitre & Hoffmann 2007). Fat body and hemocytes are the origins for the production and secretion of antimicrobial agents and activators/regulators of cellular response (Aggarwal & Silverman 2008; Feldhaar & Gross 2008), while cell mediated immunity in insects is performed by hemocytes (Schmid-Hempel 2011). In the last years, research has focused on the mechanisms of microbial recognition and activation of intracellular signalling molecules in response to invaders. However, less is known about trade-offs and competition among different signalling pathways for common resources of the individual insect. The mechanisms concerning the innate immunity, three major responses can be summarized: the production of antimicrobial peptides due to specific receptors, either soluble or membrane, the internalization-phagocytosis, which follows the attachment of bacteria on the cell membrane and the role of RNA interference in the antiviral immunity. Two recognition and signalling cascades regulate expression of these antimicrobial peptide genes. The Toll pathway is activated by fungal and many Gram-positive bacterial infections, whereas the immune deficiency (IMD) pathway responds to Gram-negative bacteria. Phagocytosis is triggered by certain trans-membrane proteins on the hemocyte

surface. Further, insect immunity is characterized by the inducible expression of a large array of antimicrobial peptides and by the constitutive melanization–encapsulation response. Encapsulation is a non-specific, constitutive, cellular response through which insects defend themselves against multicellular pathogens such as fungi, nematodes and parasitoids (Gillespie *et al.* 1997), but it also plays a role in defence against viruses (Washburn *et al.* 1996). However, no studies have tested for a possible competition among the above signaling pathways, melanization-encapsulation response and other needs of the organisms for a common resource.

As fitness-related traits are often energetically costly, it has been hypothesized that selection should act directly on the energetics of individuals (Lovegrove 2003; Rezende *et al.* 2005). However, efforts to examine the relationship between fitness and components of the energy budget are surprisingly scarce. The existing studies have focused primarily on resting metabolic rate (RMR) of arthropods, reflecting the minimum energy required to keep an individual alive. It is still largely uncertain whether natural selection might influence RMR, and how consistent over time such influences might be (Boratynski & Koteja 2009).

Insects are excellent models to study trade-offs between immune function and immune defence, life-history traits, sexual signals and trade-offs between different arms of immunity because the immune defence system in insects is far less complex than the vertebrate immune system (Schmid-Hempel 2011). Insects are especially good to study metabolic basis of immunity, energetic costs of life-history traits and functions of organisms. In insects it is easy to perform infections by fungal and bacterial infections, and to measure the magnitude of the encapsulation response to a novel and standardized antigen such as a nylon monofilament (e.g. Köning & Schmid-Hempel 1995; Rantala *et al.* 2000; Rantala *et al.* 2002; Rantala *et al.* 2003a; Rantala *et al.* 2003b; Koskimaki *et al.* 2004; Rantala *et al.* 2004). It has been shown that the ability to encapsulate abiotic material is strongly related to the ability to encapsulate a parasite (Paskewitz & Riehle 1994; Gorman *et al.* 1996; Rantala & Roff 2005).

Model organism

The greater wax moth, *Galleria mellonella* L., is an international pest in bee-hives, larvae of which tunnel through the combs feeding on pollen, wax and honey. The greater wax moth combines acoustic and pheromone signalling in its sexual communication (Spangler 1987). Male greater wax moths have a small tymbal located on each tegula, with which they produce short pulses of 75 kHz sound when the wings are fluttered. Male pheromone is a strong aromatic scent (Leyrer & Monroe 1973) and females go to males in response to the pheromone (Finn & Payne 1977; Flint & Merkle 1983). Males can be stimulated to elevate their pheromone release rate not only in response to female wing produced sound, but also in response to artificially produced low- frequency sound or substrate vibration (Spangler 1987). Since both the amount of pheromone emitted and sonic signalling can easily be recorded and analysed (see Spangler 1987), the greater wax moth is an excellent object for the study of multiple signalling and immune defence. The greater wax moth is also easy to breed in laboratory and developmental time from egg to adult is very short (about 1 month, Dutky *et al.* 1962). Pilot experiments that we ran during the winter of 2010/2011 have shown that the proposed immunological methods (see Methods) work well in this species. Moreover, the use of *G.melonella* in bio-medical research will provide a novel insight into the infection biology of insect and also human pathogenic bacteria, fungi, viruses, and protozoa since this species has rich anti-microbial peptides present in the hemolymph (e.g. Harding *et al.* 2012; McLaughlin *et al.* 2012) providing important advantages in studying septic infection by several human pathogens; the large size of the larvae provides the possibility to inject desired microbial inoculums or anti-microbial therapeutics directly into the hemolymph as usually done

in mammalian models; apart from septic infections studies *G.melonella* can be used to mimic oral infections by food-born pathogens like *Bacillus cereus* (Fedhila *et al.* 2010) and many other reasons will make this species as an outstanding study object (e.g. Fedhila *et al.* 2006; Peleg *et al.* 2009; Harding *et al.* 2012; McLaughlin *et al.* 2012; Vilcinskas 2012). Hence, results obtained in this project may be important not only for eco-immunology but also to the study of human diseases.

Previous research of the topic

My research interests concentrate on the ecological immunology, and in particularly the role of immune defence on survival, sexual selection, the role of immune defence as a life history trait, personality traits and metabolism in insects and genetics of immunity. During last five years I carried out several studies on immunocompetence of mealworm beetles (*Tenebrio molitor*), water-striders (*Aquarius najas*), damselflies (*Calopteryx spp.*) and xylophagous beetles. I conducted my foreign post-doctoral research at Turku University, Finland, and carried out some research at the University of Tartu in Estonia, where I have some ongoing projects in ecological immunology of birds. Currently, in Latvia I am working as a senior researcher at the University of Daugavpils. The proposed research project is a direct continuation of my previous work. I have several papers accepted or in press and also some manuscripts.

2.3. RESEARCH METHODOLOGY AND DETAILED WORK DESCRIPTION

This project will address general questions in the areas that are of central importance for the development of ecological immunology. The main objective of this study is to test the hypotheses that there are phenotypic and genetic tradeoffs between immune defence, life-history traits, sexual signaling and trade-offs between signaling pathways to fight against fungal and bacterial infections. Furthermore, the ecological/evolutionary factors/costs responsible for the variation in immune defence will be evaluated.

I. Immune defence and multiple sexual signalling

It has been hypothesized that secondary sexual traits provide reliable signals of a male's ability to resist parasites and pathogens (Hamilton & Zuk 1982). The immunocompetence handicap hypothesis provides a potential mechanism for reliable signalling in the form of trade-off between expenditure on trait expression *versus* expenditure on immunity (Folstad & Karter 1992; Rantala *et al.* 2012). It was found that juvenile hormone of insects has a dualistic effect on sexual signalling and immunosupression, suggesting that it may be the hormonal analogue to testosterone that maintains honesty in insect sexual signalling (Rantala *et al.* 2003a). Recent studies in insects and spiders have found that immune function shows a positive correlation with the expression of sexual ornaments (e.g. Rantala *et al.* 2000; Ryder 2000; Siva-Jothy 2000; Rantala *et al.* 2002; Rantala & Kortet 2003; Rantala *et al.* 2003a; Rantala *et al.* 2003b; Ahtiainen *et al.* 2004; Rantala & Kortet 2004), but studies on insects testing whether expression of a secondary sexual ornament is associated with resistance against real pathogens are still rare (e.g. Siva-Jothy 2000). Thus, clearly more work using real pathogens is needed. Recent studies suggest that animals use multiple signals in sexual communication

(review in in Candolin 2003; Schmid-Hempel 2011). It is suggested that each component of a signal reflects a different aspect of the overall quality of an individual or that each ornament gives a partial indication of condition (e.g. Møller & Petrie 2002). However, there are only a few previous studies of immune function and multiple signals and they gave mixed results (e.g. Saino *et al.* 1999; Møller & Petrie 2002), with mixed results.

a) Multiple sexual signalling and immune defence in *G.mellonella*

This study will determinate to what extent different signals reflect different components of immune defence and disease resistance. In addition, the condition dependence of sonic and pheromone signalling in *G.mellonella* will be assessed. We will manipulate male condition through their nutritional condition by allocating them to poor quality and high quality diet treatments. After the males have become sexually mature their sonic and pheromone signalling will be measured, followed by measurement of different parameters of the immune system and resistance against real diseases (see Methods). We predict that males raised on a poor quality diet treatment will have lower sexual signalling and immune defence than males from the high quality diet treatment group. Further, we expect stronger correlations between sexual signalling and components of immune defence among males from the poor quality diet treatment than in males from the high quality diet treatments.

b) Does the increased investment to sexual signalling reduce immune defence or survival?

Theory predicts that for mate quality signals to be reliable they should be costly, a mechanism that is likely to drive condition-dependent expression of the signal in question (review e.g. in Kotiaho 2001; Schmid-Hempel 2011). However, the evidence behind this hypothesis is rather scarce. In this experiment we will test whether the increased investment to sexual signalling reduces immune defence or/and survival in *G.mellonella* and whether this cost of signalling is higher in males in poorer nutritional condition. To determinate the impact of genotype on the costs of sexual signalling, we will measure the costs of males within isofemale (genetic) lines (see **2.5).** Males of *G.mellonella* can be stimulated to elevate their pheromone release rate not only in response to female wing produced sound, but also in response to artificially produced low-frequency sound or substrate vibration (Spangler 1987). In this experiment we will manipulate male condition through their nutritional condition by allocating them to poor quality and high quality diet treatments as larvae. Two days after enclosion we will randomly assigned males to two different experimental treatments, either high sexual signalling treatment (exposed female wing produced sound) or low sexual signalling treatment (control). Then, we will assign males randomly to two different assays, either the immunological assays (see methods) or survival assays where we will measure the length of male life span. We predict that males stimulated to increase sexual signalling to have lower immune defence and reduced survival than control males. Furthermore, we predict that the cost of signalling will be greater in the low quality food treatment than in the high quality food treatment.

c) The state dependent fitness cost of induced immune response in *G.mellonella*. Despite its importance for the evolution of host defence, state-dependent fitness costs of immunity have received little attention (Moret & Schmid-Hempel 2000; Schmid-Hempel 2011). In this experiment we will manipulate the nutritional condition in *G.mellonella* and subsequently investigate the effect of an induced immune response

on life-history traits and sexual signalling. To determinate the impact of genotype on the costs of the activation of immune system, we will measure the costs in individuals from isofemale (genetic) lines (see Methods) we will activate the immune system with (1) lipopolysaccharides (LPS), i.e., surface molecules extracted from *Escherichia coli,* or (2) sterile micro-latex beads, dissolved in Ringer. In both cases, the immune system is activated, but the artificial pathogen is unable to generate any pathogenic effect (Moret & Siva-Jothy 2003). Controls only will receive Ringer but otherwise will be treated identically. After the treatments we will measure the sonic and pheromone signalling and survival. If immunity is costly, we predict that the costs of the activation of immune system will be higher for males in lower body condition and that costs will vary among genotypes.

II. Genetic correlations between components of immune defence, life history traits and sexual signalling

In this study we will test whether there are genetic correlations between sexual signalling and immune defence, and whether multiple sexual signals are genetically correlated. Further, we will examine the genetic variance and covariance within and between a number of immune function traits and other life history traits (see Methods), in order to identify potential genetic trade-offs constraining immune function expression. The good genes-theory of sexual selection predicts that a female may produce more viable offspring by choosing and mating with males who bear viable as well as attractiveness alleles that are inherited by both sons and daughters (Pomiankowski 1988). Since immune defence is an important component of the viability of an organism, it is possible that attractiveness alleles are linked with alleles for immune defence (Hamilton & Zuk 1982). On the other hand, if there is tradeoff between resistance and attractiveness, there would be negative genetic correlations between components of immune defence and components of sexual signalling (see Verhulst *et al.* 1999).

To obtain paternal half-sib families for the estimation of genetic parameters, each of 50 haphazardly selected males will be mated with three females. Larvae will be reared individually and for each individual, we will record development time, body mass, sexual signalling (sonic and pheromonal) and immune defence (see Methods).

III. Selective lines for increased resistance

Although it is commonly thought that costs and trade-offs evolving immunity exist (i.e. antagonistic pleiotropy), we know very little about the nature or magnitude of these costs and trade-offs. A powerful technique for identifying trade-offs between fitness components is the study of correlated responses to artificial selection (Roff 1992). The aim of this experiment is to measure the correlated responses to artificial selection for enhanced resistance against diseases. We predict that increased investment to immune defence should slow developmental rate and reduce fertility. Further, because there may be trade-offs between different components of immune system (e.g. Cotter *et al.* 2004; Rantala & Roff 2005), selection for enhanced resistance against a bacterial disease may reduce an individuals ability to encapsulate pathogens and to resist fungal disease.

The base stock will be split into twelve lines, four to be selected for increased resistance against *Bacillus thurigiensis* subspecies *gallerie,* four lines to be selected for increased resistance against entomopathogenic fungi using an isolate of *Beauveria bassiana* (see Methods), and the remaining four to serve as a control. Each new generation of the selection lines will be bred from larvae that survive LD50

doses of the disease. To minimize inbreeding, a population size of at least 100 will be maintained in all lines. After 12 generation we will test for correlated responses to selection by comparing a range of fitness and immunological parameters in control and selected lines (see Methods). Further, we will test whether lines selected for increased resistance have higher metabolic rate than control lines. In addition, we will test whether increased immune defence has effects on the sexual signalling of males. Parasite mediated sexual selection is an important explanation for the evolution of sexual ornaments (Hamilton & Zuk 1982; Kortet *et al.* 2010). However, most studies to date have used manipulations of parasite load to test the intraspecific predictions, and very few studies have attempted to manipulate resistance itself. The rationale underlying this study using selection lines is that selecting for increased resistance could result in a correlated response of attractiveness. This correlated response could be (i) negative, if a trade-off between resistance and arises, or (ii) positive, if parasite-mediated sexual selection shapes a positive genetic correlation between resistance and attractiveness.

IV. Cost and benefits of evolving under experimentally enforced polyandry or monogamy

The aim of this study is to examine whether experimentally enforced polyandry (conflict and sexual selection) or monogamy (no conflict and no sexual selection) influences sexual signals and immune defence. We predict that enforced monogamy leads to reduction in the production of pheromones and sonic signalling and improves immune defence in males. It is important to note that this study gives us also an opportunity to study sexual conflict as a by-product. Newly enclosed moths will be divided into two treatments with four replicate lines of each, and started simultaneously: four polyandry lines, each 25 males and 25 females housed together in containers and four monogamous lines, each consisting of 20 pairs, housed one pair to a vial. After 20 generations moths will be kept under relaxed selection for one generation (all lines from both treatments in containers with 50 males and 50 females). This ensures that any differences we subsequently note will not to be due to differential maternal effects or phenotypic differences due the different housing conditions of the lines (see Martin & Hosken 2003). The offspring of these moths will be used to measure the effect of different treatments on immune defence and sexual signalling (see methods below).

V. Effect of inbreeding on immune defence and sexual signalling

Inbreeding is predicted to decrease the resistance of a population to parasites and disease (e.g. Sorci *et al.* 1997). However, this issue is controversial and there is limited rigorous scientific evidence available, most of which is in vertebrates. In this experiment we will test whether inbreeding reduces immune defence, fecundity and sexual signalling in *G. mellonella.* Additionally, we will test whether females can use male pheromone signals to avoid inbreeding (for preference test see Rantala *et al.* 2002; Rantala *et al.* 2003a; Rantala *et al.* 2003b; Kivleniece *et al.* 2010; Daukste *et al.* 2012; Krams *et al.* 2012a). For colonizing organism, such as *G. mellonella,* that are likely to suffer relatively high levels of inbreeding at frequent intervals, inbreeding depression could significantly influence the evolution of traits and their genetic architecture.

To generate inbred individuals we will use full sib families that will be grouped into pairs. From each pair, we will form two inbred families by brother-sister mating and two outbred families by reciprocal matings of a male and a female from each family within the pair (for method see Roff 1998). This approach avoids the problem of different genetic backgrounds. The advantage of this particular breeding design is that, within each group, there is an equal representation of alleles, only their combinations changing (Roff 1998). When they are sexually mature we will measure the sonic and pheromone signaling of males and then

different parameters of immune system and resistance against real diseases (see Methods). Following the theory, we predict that in *G. mellonella* inbreeding will reduce immune defence. Because sexual signals are often condition dependent, we predict that inbreeding will also reduce sexual signalling. Our preliminary studies in *G. mellonella* suggest a strong inbreeding depression on life history traits. There are two major hypotheses to account for the existence of inbreeding depression. According to the dominance hypothesis, inbreeding increases the frequency of homozygous combinations of deleterious recessive alleles thereby decreasing fitness (Davenport 1908), whereas the overdominance hypothesis posits that inbreeding increases homozygosity and thus reduces the frequency of the superior heterozygotes (East 1908). In this experiment we will test these predictions using inbred lines. The basic design will follow Roff (2002) in that we will inbreed eight separate lines for 15 generations and then construct all possible crosses among lines. After outbred and inbred progeny achieve sexual maturity we will measure the levels of sexual signalling and immune defence (see methods). According to the overdominance hypothesis the mean trait value of the among-line crosses will be equal to that of the outbred population, whereas the partial dominance hypothesis predicts that mean trait value of the among-line crosses will exceed that of the outbred population (Roff 2002). The inbred lines established for this experiment will be used later for the experiments **3.1**(b & c).

VI. Trade-off between costs of running the Toll pathway, the IMD pathway and PO cascades

Fungal or Gram-positive bacterial infections may occur simultaneously with infections caused by Gram-negative bacteria. Infections that require different signalling pathway or the same pathway may occur in short intervals one by one. It would be important to study whether insects can easier resist co-infections of two different pathogens, which activate the same of different signalling pathways. These trade-offs will be studied also between the Toll pathway, the IMD pathway and encapsulation response, which is based on phenoloxydase (PO) cascades. To the best of my knowledge this is never done in insect studies of ecoimmunology.

Laboratory stock and methods

Insects

The greater wax moths that will be used in these experiments will be taken from a laboratory stock population originating from approximately 600 individuals and maintained on artificial diet at the University of Daugavpils by the applicant since September 2012. To reduce the likelihood of genetic drift or inbreeding, the stock population will be maintained at 2000-4000 breeding individuals.

Sexual signalling

To record sound from individual moths we will use a digital recording system, microphones sensitive to sonic and/or ultrasonic frequencies, and speakers capable of emitting sonic and ultrasonic sound (see Takács *et al.* 2003). The basic apparatus for detecting relative pheromone release rates from a single male is shown by Spangler (1987). The male pheromone will be detected with a Calectro 54-807 hydrocarbon sensor which is sensitive to both the n-nonanal and n- undecanal components of the pheromone released by the wing glands

of male greater wax moths (Spangler 1987). To test female preference on pheromones of different males we will use the traditional filter paper experiment (Rantala *et al.* 2002; Krams *et al*. 2012b *in press*).

Assays of innate immune function

Encapsulation rate will be measured by inserting a nylon implant into the hemocoel. After three hours the implant will be removed and photographed under a light microscope with a digital camera. These pictures will be analyzed using the Image J software (e.g. Kivleniece *et al.* 2010). Once implants have been removed, we will collect haemolymph samples. Phenoloxidae (PO) -activity will be measured by using L-DOPA as a substrate (see Rantala *et al.* 2002; Rantala *et al.* 2003a; Rantala *et al.* 2003b). Protein contents will be determined with BioRad Protein Assay method based on the method of Bradford on a Multilabel counter. PO-activities will be expressed as PO units per mg protein. The lysozyme activity of the haemolymph will be assayed turbidometrically using lyophilised cells of *Micrococcus lysodeicticus* bacterium (see Rantala & Kortet 2003, 2004).

Assays of resistance against real parasites and pathogens

To test resistance against bacterial disease, we will use a *Bacillus thurigiensis* subspecies *gallerie* (Gram positive) (Novosibirsk, Russia). This is a specific strait of bacteria for *G. mellonella* and it has been used as a tool for the control of greater wax moths (see Dubovskiy et al. 2005). LD50 doses will be determined by giving a separate group of larvae five increasing doses of bacteria and selecting the dose that comes closest to killing 50% of the animals. Animals will receive 3 µl injections using a Hamilton syringe. After the injection, individuals will be housed individually in plastic bins with food ad lib and mortality will be recorded daily. *Serratia marcescens* (Gram negative) also will be used for activation of the immune system (Tan *et al.* 2006).

We will assess resistance to entomopathogenic fungi using *Beauveria bassiana.* This entomo-pathogenic fungy are widely used to control pest insects and it is known to be effective against *G.mellonella* (Vilcinskas & Matha 1997). We have a large culture of this disease which I have maintained at the University of Daugavpils. LD50 doses will be determined by giving a separate group of larvae five increasing doses of conodians and selecting the dose that will come closest to killing 50% of the animals. Larvae will be dipped into conidian suspension (or control). Mortality will be recorded daily and the cause of mortality will be verified.

We will combine infections of *B.bassiana* and *B.thurigiensis* to perform subsequent activations of the Toll pathway, and *B.bassiana* or *B.thurigiensis* and *Serratia* strains to activate the both signalling pathways in the context of sexual signaling and survival.

Time schedule for the proposed research

Although the applied project is scheduled to start in January 2013, we have already established laboratory stocks for *G. mellonella.* Further, we have done all required preliminary experiments. Laboratory work for the studies I and V will be conducted during years 2013 and 2014, and study II will be conducted during the year 2015. Selective lines for increased resistance (III) will be started during the spring of 2013 and lines will be maintained until the end of 2016. Experiment IV will be started in the end of 2013 and lines will be

maintained until the summer of 2016. Experiment VI will be carried out throughout the project. Analyses of results and writing of articles will be accomplished by the autumn of 2016.