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Katariina Kangassalo

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ABSTRACT

Phenotypic plasticity allows the same genotype to produce distinctly different morphological, physiological or behavioural characteristics depending on the environmental conditions the individual, and even its parents or more remote ancestors, have experienced. However, phenotypic change is limited by physiological, genetic and environmental constraints, which can lead to trade-offs between life-history traits. In this thesis, I investigated the intra- and trans-generational effects of larval nutrition on life-history traits of a ubiquitous pest of apiculture: the greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae). I found that a low-nutrition larval diet and periods of fasting negatively affected the moths' development rate, body size and adult longevity. However, compared with the moths that were reared on a standard diet, the moths that were subjected to fasting or a low-nutrition larval diet exhibited lower mortality from infection by an entomopathogenic fungus *Beauveria bassiana* and stronger encapsulation responses to synthetic immune challenge at the larval and adult stages. Furthermore, the activation of encapsulation response at the pupal stage reduced the strength of adult encapsulation response in males reared on a standard diet but not in males reared on a low-nutrition diet. In contrast to the general pattern, a subgroup of females subjected to a relatively short fast had distinctly high growth rates after the fast, but perhaps as a cost of this compensatory response they exhibited particularly weak encapsulation responses and short adult lifespans. Maternal, but not paternal, low-nutrition diet increased the survival time of offspring infected with *B. bassiana*. In addition, a low-nutrition parental diet had sex-specific effects on development time and body mass of the offspring. My research demonstrates that larval nutrition has diverse and long-lasting effects on immune function and other life-history traits – as well as on associations between the different life-history traits – in *G. mellonella*. The studies also add to the growing body of evidence indicating that environmental conditions experienced by the parents can contribute to variation in offspring phenotype.

KEYWORDS: *Galleria mellonella*, nutrition, immunity, phenotypic plasticity, non-genetic inheritance, insect

TURUN YLIOPISTO
Luonnontieteiden ja tekniikan tiedekunta
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KATARIINA KANGASSALO: Ravinto-olosuhteiden sukupolven sisäiset ja sukupolven yli ulottuvat vaikutukset isovahakoisan (*Galleria mellonella)* elinkiertopiirteisiin
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TIIVISTELMÄ

Ilmiasun joustavuus mahdollistaa sen, että sama genotyyppi voi tuottaa hyvin erilaisia morfologiaan, fysiologiaan tai käyttäytymiseen liittyviä piirteitä riippuen yksilön tai jopa sen vanhempien kokemista, ympäristöolosuhteista. Ilmiasun muutoksia rajoittavat kuitenkin fysiologiset, geneettiset ja ympäristöön liittyvät tekijät, mikä voi johtaa vaihtokauppatilanteisiin elinkiertopiirteiden välillä. Yksi tärkeä yksilön ilmiasua muovaava tekijä on kehitysaikainen ravinto, jonka sukupolven sisäisiä ja sukupolven yli ulottuvia vaikutuksia tutkin tässä väitöskirjassa merkittävällä mehiläispesien tuholaisella: isovahakoisa *Galleria mellonella* (Lepidoptera: Pyralidae). Havaitsin, että eripituisilla paastojaksoilla ja heikkolaatuisella ravinnolla oli pääsääntöisesti negatiivinen vaikutus isovahakoisien kehitysnopeuteen, kokoon ja aikuiseliniän pituuteen. Paastolle tai heikolle toukkaravinnolle altistetuilla yksilöillä kuolleisuus *Beauveria bassiana* -sieni-infektiosta oli kuitenkin pienempi ja enkapsulaatiovaste keinotekoista immuunihaastetta kohtaan voimakkaampi toukka- ja aikuisvaiheessa verrattuna standardiravinnolla kasvaneisiin yksilöihin. Lisäksi enkapsulaatiovasteen aktivointi kotelovaiheessa heikensi standardiravinnolla, mutta ei heikolla ravinnolla, kehittyneiden koiraiden aikuisvaiheen enkapsulaatiovastetta. Pieni osa lyhytaikaiselle paastolle altistetuista naaraista kasvoi poikkeavan nopeasti paaston seurauksena, mutta mahdollisesti tämän ns. kompensoivan kasvun seurauksena niillä havaittiin huomattavan heikko immuunivaste ja lyhyt aikuiselinikä. Äidin, mutta ei isän, heikko ravinto pidensi *B. bassiana* -infektion saaneiden jälkeläisten selviytymisaikaa vaikuttamatta kuitenkaan niiden kokonaiskuolleisuuteen. Vanhempien ravinnon laadulla oli lisäksi jälkeläisen sukupuolesta ja sen ravinto-olosuhteista riippuvia vaikutuksia kehitysaikaan ja kokoon. Tutkimukseni osoittavat, että toukka-aikana koetuilla heikoilla ravinto-olosuhteilla on moninaisia vaikutuksia isovahakoisan immuniteettikykyyn ja muihin elinkiertopiirteisiin, sekä eri elinkiertopiirteiden välisiin assosiaatioihin. Tutkimukseni tuovat myös lisänäyttöä siitä, että kehitysaikaisella ravitsemuksella voi olla jopa sukupolven yli ulottuvia vaikutuksia hyönteisten ilmiasuun.

ASIASANAT: *Galleria mellonella*, hyönteinen, ravinto, immuniteetti, epigenetiikka, ilmiasun joustavuus

Contents

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Kangassalo, K., Valtonen, T. M., Roff, D., Pölkki, M., Dubovskiy, I. M., Sorvari, J. & Rantala, M. J., 2015. Intra- and trans-generational effects of larval diet on susceptibility to an entomopathogenic fungus, *Beauveria bassiana*, in the greater wax moth, *Galleria mellonella*. *Journal of Evolutionary Biology*, 28(8), p. 1453–1464.

II Kangassalo, K., Valtonen, T. M., Sorvari, J., Kecko, S., Pölkki, M., Krams, I., Krama, T. & Rantala, M. J., 2018. Independent and interactive effects of immune activation and larval diet on adult immune function, growth and development in the greater wax moth (*Galleria mellonella*). *Journal of Evolutionary Biology*, 31(10), p. 1485–1497.

III Kecko, S., Mihailova, A., Kangassalo, K., Elferts, D., Krama, T., Krams, R., Luoto, S., Rantala, M. J. & Krams, I., 2017. Sex-specific compensatory growth in the larvae of the greater wax moth *Galleria mellonella*. *Journal of Evolutionary Biology*, 30(10), p. 1910–1918.

IV Kangassalo, K., Sorvari, J., Nousiainen, I., Pölkki, M., Valtonen, T. M., Krams, I. & Rantala, M. J. Intra- and trans-generational phenotypic responses of the greater wax moth, *Galleria mellonella*, to low-nutrition larval diet. Manuscript.

1 Introduction

1.1 Phenotypic plasticity and nutrition

Many natural environments are under constant change, with variation in such conditions as the quality and quantity of nutrition, the temperature and the abundance of pathogens or predators. This variation is reflected in organisms, which exhibit variable phenotypes in different environments: a phenomenon termed 'phenotypic plasticity'. Phenotypic plasticity is defined as the ability of a single genotype to produce alternative morphologic forms, physiological states and/or behaviours in response to environmental factors (Westeberhard, 1989) [1]. The degree of plasticity, and the developmental window when plasticity is possible vary widely among species and traits. For example, in holometabolous insects, phenotypic plasticity in external morphology must be largely initiated before metamorphosis is completed (Westeberhard, 1989, Moczek, 2010). At the level of an individual, phenotypic plasticity can have important consequences for fitness; on a larger scale, it can mediate interspecific interactions, structure ecological communities and facilitate evolutionary change (Westeberhard, 1989, Agrawal, 2001, Fordyce, 2006). Of particular interest is the extent to which the environmentally induced phenotypic changes are adaptive; that is, improve the survival and reproductive success of an individual (Via, 1993, Nijhout, 2003). A specific phenotype may be successful in one context but detrimental in another, which highlights the difficulty of determining the true adaptive value of phenotypic change. Some phenotypic responses are initiated indirectly by cues that may indicate a particular environmental state; for example, in some insect species, tactile stimulation triggers the development of a melanic, high-density phenotype (Wilson, et al., 2001).

One of the most important factors that shapes an individual's phenotype is nutrition. Nutrition has profound effects on the physiology, behaviour and

[1] Such responses can be substantial; for example, the European map butterfly (*Araschnia levana*) exhibits two such distinct morphological forms due to seasonal changes in temperatures and photoperiods, that the forms were originally classified as two different species (Nijhout, 2003, Morehouse, et al., 2013).

performance of organisms, with significant implications for communities and even ecosystems (Scriber & Slansky, 1981, Johnson, et al., 2013, Lihoreau, et al., 2015, Mischler, et al., 2016). The detrimental effects of nutritional stress, ranging from retarded growth to reduced survival and reproductive success, are well documented (Boots & Begon, 1994, Nylin & Gotthard, 1998, Suwanchaichinda & Paskewitz, 1998, McGraw, et al., 2007, Muturi, et al., 2011). An individual may mitigate the negative effects of poor nutrition through compensatory mechanisms, such as an increased growth rate, after the conditions have improved, but this may come at a cost (Metcalfe & Monaghan, 2001, De Block & Stoks, 2008). On the other hand, studies show that dietary restriction, fasting or a diet that is low in specific nutrients can also have positive effects on traits such as longevity, stress tolerance and pathogen resistance (Chippindale, et al., 1993, Koubova & Guarente, 2003, Sinclair, 2005, Wenzel 2006, Bishop & Guarente, 2007, Klemola, et al., 2007, Smith, et al., 2007, Kristan, 2008, Kelly & Tawes, 2013). Furthermore, in nature, exposure to various concurrent environmental stressors is common, and the effects of nutrition on organisms may interact with other biotic or abiotic factors such as temperature, population density and exposure to pathogens (Laing, et al., 1987, Moret & Schmid-Hempel, 2000, Triggs & Knell, 2012a, Couret, et al., 2014).

1.1.1 Life-history trade-offs

Phenotypic change is limited by physiological, genetic and environmental constraints, which can lead to trade-offs between life-history traits (Stearns, 1989, Ardia, et al., 2011). Physiological trade-offs arise when the finite resources (e.g. energy or specific nutrients) allocated to one life-history trait reduce the resources available for other needs (Roff, 1992, Stearns, 1992, Adamo, et al., 2001). Indeed, a basic principle of life-history theory predicts that an individual cannot simultaneously have maximal investment in all costly life-history traits – commonly processes related to growth, survival and reproduction – hence, negative correlations between the traits are observed (Stearns, 1989, Roff, 1992, Stearns, 1992). Genetic trade-offs, on the other hand, arise from antagonistic pleiotropic effects or the linkage disequilibrium of alleles (Stearns, 1989, Partridge & Fowler, 1993, Zwaan, et al., 1996, Sgro & Hoffmann, 2004). Interestingly, the direction or amplitude of trait associations may change as a result of environmental variation, as shown by studies in with physiological trade-offs between life-history traits only become apparent under starvation or poor nutrition (Nylin & Gotthard, 1998, Moret & Schmid-Hempel, 2000). The manifestation of genetic covariation can also be affected by environmental conditions (Sgro & Hoffmann, 2004).

1.2 Non-genetic inheritance

An individual's phenotype can be affected not just by the environmental conditions it encounters but also by influences that result from current or past environmental effects on its parent(s) or more remote ancestors. Such influences have been referred by a variety of terms, such as 'parental effects', 'trans-generational effects', 'non-Mendelian inheritance' and 'non-genetic inheritance' (Bernardo, 1996, Rossiter, 1996, Mousseau & Fox, 1998, Wolf, et al., 1998, Bossdorf, et al., 2008, Bonduriansky & Day, 2009). Non-genetic inheritance can be understood as an effect on offspring phenotype that is caused by the vertical transmission of factors other than the DNA sequence, and it can be thought of as an extension of the Mendelian model of inheritance (Bonduriansky & Day, 2009). Mechanisms of non-genetic inheritance include, for example, the epigenetic modulation of DNA (such as variations in patterns of DNA methylation and in the histone proteins that bind the DNA), the transfer of cytoplasmic factors via the gametes, nutritional provisioning (such as feeding, egg provisioning or nuptial gifts) and the transmission of behaviour through learning (Mousseau & Dingle, 1991, Bernardo, 1996, Rossiter, 1996, Mousseau & Fox, 1998, Bonduriansky & Day, 2009).

In insects and other animals, maternal effects are generally considered to be more prevalent than paternal effects, as mothers typically invest more in the production and care of the offspring (Tallamy, 1984, Mousseau & Fox, 1998, Bonduriansky & Day, 2009). For example, mothers provide the zygote with most of the essential substances such as mRNA, proteins, carbohydrates and lipids (Bonduriansky & Day, 2009). However, a growing body of research suggests that paternal non-genetic inheritance may be more prevalent than previously thought, even in species that lack conventional forms of paternal investment (Bonduriansky & Day, 2009, Friberg, et al., 2012, Valtonen, et al., 2012). Paternal effects can be mediated through variation in resource allocation when males provide resources to their offspring directly or indirectly via nuptial feeding[2]. Paternal effects may also be mediated by epigenetic mechanisms or by the transfer of paternal cytoplasmic factors, such as hormones or RNA, via the gamete (Curley, et al., 2011, Crean & Bonduriansky, 2014). It is often difficult to distinguish between the phenotypic effects of the mother and those of the father, as the paternal effects can be mediated indirectly via the female: for example, a female may show higher investment in her offspring after mating with a perceived high-quality male (Thornhill, 1983, Delope & Moller, 1993, Cunningham & Russell, 2000, Crean & Bonduriansky, 2014).

[2] Nuptial feeding comprises any form of nutrient transfer from the male to the female during or after mating or courtship (Parker & Simmons 1989, Simmons & Parker 1989, Vahed 1998, Gwynne 2008).

1.2.1 The adaptive significance of trans-generational effects

The adaptive significance of transgenerational effects has been a subject of active discussion in the scientific community (Mousseau & Fox, 1998, Marshall & Uller, 2007, Bonduriansky & Day, 2009, Burgess & Marshall, 2014). It is possible that parental experience affects offspring phenotype in a way that improves the performance of the offspring under similar conditions. Such 'anticipatory parental effects' could be beneficial when the parental and offspring environments are likely to correlate, but the effects may be harmful if they do not. For example, undernutrition during the prenatal period can lead to a 'thrifty phenotype', characterised by high energy intake and/or low energy expenditure, which can promote survival under low nutrition conditions but can also increase the risk of obesity and diabetes when food is abundant (Gluckman & Hanson, 2008). Based on such findings, parental effects can be thought of as a form of adaptive transgenerational plasticity. Nevertheless, the evidence for adaptive transgenerational plasticity has been considered to be relatively weak, and it has been suggested that some parental effects are more likely to be physiological side effects without any adaptive value (Uller, et al., 2013, Frago & Bauce, 2014).

1.2.2 The trans-generational effects of nutrition in insects

In insects and other invertebrates, parental diet is found to have variable effects on a number of offspring characteristics, such as fecundity (Futuyma, et al., 1993, Frago & Bauce, 2014), size and development time (Bonduriansky & Head, 2007, Vijendravarma, et al., 2010, Valtonen, et al., 2012), and immune defence (Myers, et al., 2011, Triggs & Knell 2012b, Boots & Roberts, 2012, Saastamoinen, et al., 2013). The diet-induced parental effects can depend on the sex of the parent and that of the offspring (Bonduriansky & Head, 2007, Valtonen, et al., 2012), and the effects may be evident at a particular stage of development only (Saastamoinen, et al., 2013) or persist throughout an individual's lifetime. Little is still known about how parental and offspring nutrition interact in their effect on offspring phenotype in invertebrates. Consistent with the notion of anticipatory parental effects, several studies have found that parents that are subjected to nutritional stress – such as starvation or extreme diets – produce offspring that are more resistant to similar nutritional stress (Gliwicz & Guisande, 1992, Mousseau & Fox, 1998, Rotem, et al., 2003). Other studies suggest that parental effects act as a conduit by which negative effects of poor nutrition are transferred from parents to offspring (Uller, et al., 2013, Frago & Bauce, 2014).

1.3 Ecological immunology in insects

Ecological immunology is a discipline that investigates the causes and consequences of variation in immune function in the context of ecology and evolution. Immunity is increasingly considered to be a life-history trait, which is subject to trade-offs with other important physiological functions, such as reproductive investment, starvation resistance and longevity (Sheldon & Verhulst, 1996, Moret & Schmid-Hempel, 2000, Hoang, 2001, Armitage, et al., 2003, Rolff & Siva-Jothy, 2003, Ye, et al., 2009, Schwenke, et al., 2016). In insects, immune function is found to be costly in terms of energy and specific substrates and to pose a risk to autoimmunity; these are potential factors that create and maintain variation in immune capacity (Rolff & Siva-Jothy, 2003). Research suggests that insects may adaptively increase their investment in immune defence as a result of environmental cues that suggest an increased risk of disease, such as high population density (Wilson, et al., 2002) and previous immune challenges (Contreras-Garduno, et al., 2016). It is widely established that nutritional stress can impair immunity in insects and other animals (Boots & Begon, 1994, Suwanchaichinda & Paskewitz, 1998, Boots, 2000, Muturi, et al., 2011), and research shows that negative correlations between immunity and other life-history traits may become more apparent under limited nutrition (Moret & Schmid-Hempel, 2000, McKean, et al., 2008). However, some studies suggest that nutritional stress can also lead to enhanced immune function in insects (Klemola, et al., 2007, Kelly & Tawes, 2013, Krams, et al., 2015). In some cases, enhanced immunity in response to poor nutrition may be an adaptive response – for example, low nutrition levels may be causally linked to high population density and hence increased risk of disease.

1.3.1 The immune system of insects

The immune system of insects shares many similarities with the innate immune system of mammals, pointing to a common evolutionary origin of these systems (Vilmos & Kurucz, 1998, Hoffmann, et al., 1999). The similarities can be seen in pathogen recognition, signal cascades and the immune effector mechanisms (Vilmos & Kurucz, 1998, Hoffmann, et al., 1999). It was previously assumed that insects possess the innate immune system only, as they lack the antigen-specific lymphocytes that account for the adaptive immunity in mammals. However, research has since shown that the immune system of insects may in fact possess some qualities of an adaptive immune system, because insects are capable of long-lasting and specific immune priming against a wide variety of pathogens (Contreras-Garduno, et al., 2016, Cooper & Eleftherianos, 2017).

The innate immune system of insects can be divided into humoral and cellular branches, though the two are in many respects interrelated (Rolff & Reynolds, 2009).

Humoral immunity consists of a variety of effector molecules such as antimicrobial proteins and peptides, clotting of the haemolymph, and an enzymatic cascade that leads to the formation of melanin (Gillespie, et al., 1997, Rolff & Reynolds, 2009). Cellular immunity consists of mechanisms conveyed by haemocytes: phagocytosis, cellular encapsulation response and the formation of haemocytic aggregates referred to as 'nodules' (Gillespie, et al., 1997, Rolff & Reynolds, 2009). Even prior to an immune challenge, the constitutive immunity – consisting mainly of the prophenoloxidase (proPO) enzyme cascade and circulating haemocytes – is present, which enables a rapid immune response upon a challenge (Gillespie, et al., 1997, Rolff & Reynolds, 2009, Gonzalez-Santoyo & Cordoba-Aguilar, 2012). An important first line of defence is the cuticle, which provides an effective physicochemical barrier to invaders (Vincent & Wegst, 2004, Siva-Jothy, et al., 2005). The process of encapsulation responses and defence mechanisms of insects against fungi are discussed in more detail in sections 2.4.1. and 2.4.2.

1.4 *Galleria mellonella*

The greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae; Linnaeus, 1758, Figure 1) is a widespread pest of the honeybee. It is found in most parts of the world, with the putative native range in Europe and the adjacent Eurasia. Female moths oviposit inside beehives and their larvae consume the honeycomb, pollen, propolis and honey, in addition to the discarded skins of honeybee larvae and pupae (Paddock, 1918, Kwadha, et al., 2017). The wax moth larvae burrow tunnels lined with silk inside the honeycomb, which causes honey to leak and entangles and starves the emerging bees (Paddock, 1918, Kwadha, et al., 2017). Consequently, the number of bees in the colony decreases and the colony may even be abandoned or destroyed. In addition, the moths may act as potential vectors of pathogens (Charriere & Imdorf, 1999, Kwadha, et al., 2017). Indeed, *G. mellonella* is considered to be a possible contributing factor to the significant reduction in honeybee populations (Biesmeijer, et al., 2006, Potts, et al., 2010, Kwadha, et al., 2017). *G. mellonella* adults lack functional mouth parts and do not feed (Kwadha, et al., 2017). The adult lifespan of males is approximately twice as long as that of females (Paddock, 1918, Warren & Huddleston, 1962). Females are larger than males and have a longer development time (Paddock, 1918, Warren & Huddleston, 1962, Kwadha, et al., 2017). Although generation length depends on many factors, including geographical location, it has been estimated that *G. mellonella* undergoes between three and six generations a year (Paddock, 1918, Kwadha, et al., 2017). *G. mellonella* is increasingly considered to be an excellent alternative model host for human pathogens (Purves, et al., 2010, Junqueira, 2012, Ramarao, et al., 2012, Cook & McArthur, 2013), and positive correlations between virulence to mammals and *G. mellonella* have consistently been

found for different bacterial and fungal strains[3] (Jander, et al., 2000, Salamitou, et al., 2000, Brennan, et al., 2002). In addition, *G. mellonella* is widely used as a model host for other significant pathogens, such as an entomopathogenic fungus *Beauveria bassiana* which is used in the biological control of pests and vector insects (Lacey, et al., 2015, McKinnon, et al., 2017, Vilcinskas, 2019).

Figure 1. The greater wax moth, *Galleria mellonella*. The upper images show honeycomb that is infested by the moth larvae (left) and a wax moth larva (right). The lower images show an adult female (left) and an adult male (right). Photographs purchased from Shutterstock.com.

1.5 Aims of the thesis

In this thesis, I investigated the intra- and transgenerational phenotypic responses of *G. mellonella* to larval nutritional conditions. The thesis is based on four experimental laboratory studies, in which I manipulated the nutritional conditions of *G. mellonella* larvae and examined 1) the intra-generational effects of larval nutrition

[3] The increasing use of insects as an alternative to vertebrates as model hosts for human pathogens may be significant from the viewpoint of animal welfare. The existence and degree of pain and suffering in invertebrates remains a question of ongoing investigation (Sherwin, 2001, Elwood, 2011), and continuing development of techniques for anesthesia, analgesia, and euthanasia of invertebrates used in laboratory settings is of great importance (Cooper, 2011). Although further research is crucial before definitive conclusions can be drawn, it is possible that the use of insects in laboratory settings represents a more ethical alternative to the use of mammals or other vertebrates.

on the life-history traits of larvae, pupae and adults, and 2) the trans-generational effects of parental nutrition on the life-history traits of the offspring. I was also interested in the associations between different life-history traits and how nutrition affects these associations. The fitness correlates I assessed were immune function, body size, development time and adult lifespan. The nutritional conditions of the larvae were manipulated by subjecting the larvae to varying lengths of fasting or by reducing the quality of their diet by adding nutritionally inert cellulose. Immune function was measured as susceptibility to an entomopathogenic fungus, *B. bassiana*, or as strength of encapsulation response to a standardised, synthetic immune challenge. *G. mellonella* is an excellent model for studying the effects of developmental nutrition: because the adults do not feed (Paddock, 1918, Kwadha, et al., 2017), all diet-induced phenotypic responses result from the nutritional conditions during the larval stage.

In particular, my research addresses the following questions.

1. How are immune function and other life-history traits affected by larval nutritional conditions in *G. mellonella* at different stages of development? What associations can be observed between different life-history traits, and how does larval nutrition affect those associations?
2. How does parental nutrition affect offspring phenotype? Do parental and offspring nutrition interact in their effect on the characteristics of the offspring?
3. To what extent does variation in susceptibility to an entomopathogenic fungus caused by parental diet result from the maternal diet, the paternal diet or the interaction between the two?
4. How do two environmental stressors – low-nutrition larval diet and immune activation at the pupal stage – interact in their effect on adult immune function?
5. Do larvae exhibit an accelerated growth rate after a period of fasting, and are there costs to this compensatory response?
6. Is there a sex-specific difference in strength of encapsulation response in *G. mellonella*?

In a broad context, the aim of my thesis is to provide further insight into the intra- and trans-generational phenotypic responses of insects to nutritional conditions. Phenotypic plasticity is an important means by which populations can respond to rapid environmental change (Fox, et al., 2019), and research on the subject is especially important at a time when rapid, anthropogenic environmental change is affecting many regions of the world. In addition, due to the abundance, diversity and ecological importance of insects, it is fundamental to understand the effects of

nutrition on insect populations. Finally, although non-genetic inheritance has been an area of active research during the last few decades, there is still much to be learned about the subject. In a narrower context, my purpose is to add to the knowledge on the biology of *G. mellonella*, specifically on the factors that affect immune function in this species. Research on the subject provides a better basis for the use of *G. mellonella* as a model host to study the pathogenesis and the virulence of significant bacterial and fungal pathogens (Junqueira, 2012, Ramarao, et al., 2012, Cook & McArthur, 2013, Vilcinskas, 2019).

2 Materials and Methods

2.1 Stock populations

Two laboratory populations of *G. mellonella* were maintained for the studies of this thesis: one at the University of Turku, Finland (I, II, IV), and one at the University of Daugavpils, Latvia (III). The stock population at the University of Turku originated from individuals collected from natural populations of *G. mellonella* in Novosibirsk, Russia; the laboratory population at the University of Daugavpils originated from moths collected from beehives in south-eastern Estonia. Before the commencement of the studies, each population was maintained at the laboratory for several generations. The rearing temperature for the stock insects was 28 ± 1 °C. The stocks of 5,000 to 10,000 insects were reared in continuous darkness, because in the wild *G. mellonella* larvae live in dark bee nests, protected from sunlight. Previous research shows that both continuous darkness and a 12:12 light cycle are suitable for rearing this species (Bogus, et al., 1987). The stock larvae were fed *ad libitum* with the standard diets described in section 2.3. The moths were kept in several large plastic boxes in densities of several hundred individuals, with wire mesh at the cover for ventilation.

2.2 Study designs

Below are brief descriptions of the experimental setups of studies I–IV (Table 1); more detailed descriptions are given in the original articles. During the experiments, the moths were maintained at a temperature of 28 ± 0.5 °C (III) or 28 ± 1 °C (I, II, IV) in continuous darkness.

In study I, I investigated the intra- and transgenerational effects of larval diet (low-nutrition vs standard; see section 2.3) on the susceptibility of *G. mellonella* larvae to an entomopathogenic fungus *B. bassiana* (see section 2.4.2). The study consisted of two parts. In the first part, the within-generation effect of the larval diet on susceptibility to *B. bassiana* was measured; in the second part, the trans-generational effect of the parental diet on the susceptibility of the offspring to the fungus was assessed. The separate effects of the maternal and paternal diets and their interaction were investigated, and the split-brood study design allowed the

comparison of full siblings. The larvae were infected with the fungus at the fourth larval instar, which was determined on the basis of the head capsule width (Beck, 1970, Srivastava, 1970, Wani, et al., 1994). The sex of the moths was not determined in study I, because distinguishing males and females is difficult at the larval stage due to the absence of sex-specific external morphology (Kwadha, et al., 2017).

In study II, I investigated the independent and interactive effects of two stressors – low-nutrition larval diet and immune activation at the pupal stage – on the strength of adult encapsulation response. *G. mellonella* larvae were reared on either a low-nutrition or a standard diet. As pupae, their encapsulation response was induced using a standardised synthetic immune challenge (see section 2.4.1). Pupae in the sham control group were subjected to a minor cuticular puncture, while pupae in the control group were merely briefly handled. Encapsulation response was measured from all individuals at the adult stage. The egg-to-adult development time and adult dry body mass were also assessed for all individuals. Dry body mass was analysed by keeping the defrosted moths in an oven at 60 °C for 24 hours, after which they were weighed to the nearest 0.01 mg with an electronic microbalance.

In study III, I assessed the effect of a period of fasting on subsequent growth in addition to strength of encapsulation response and adult longevity. Larvae of *G. mellonella* were exposed to fasting periods of varying lengths – 12, 24 or 72 hours (see section 2.3) – starting from day 18 post-hatching, after which they were provided with standard food *ad libitum*. Each larva was weighed daily to obtain individual growth curves. Strength of encapsulation response was measured from the larvae on day 25 post-hatching. The adult moths were observed for mortality once daily to determine their longevity.

In study IV, I examined the intra- and trans-generational effects of larval diet on egg-to-adult development time, adult dry body mass (measured as in study II) and strength of adult encapsulation response. Two generations – parental and offspring – of *G. mellonella* were reared on a low-nutrition diet or a standard diet and the independent and interactive effects of parental and offspring diet on the characteristics of the offspring were assessed. The experimental design of study IV did not allow the separate assessment of the effect of maternal and paternal diet on offspring phenotype.

Table 1. Total N-values, experimental manipulations and outcome measures for studies I–IV.

	Total N	**Experimental manipulations**	**Outcome measures**
Study I	3,380	- Low-nutrition vs standard larval diet - Low-nutrition vs standard maternal diet - Low-nutrition vs standard paternal diet - Topical application of fungal suspension vs control solution	- Survival time in days after the application of fungal suspension or control solution - Final mortality rate of larvae after the application of fungal suspension or control solution
Study II	827	- Low-nutrition vs standard larval diet - Induction of encapsulation response at the pupal stage vs control or sham control treatment	- Strength of pupal encapsulation response - Strength of adult encapsulation response - Egg-to-adult development time - Adult dry body mass
Study III	202	- Fasting period of 12, 24 or 72 hours vs *ad libitum* feeding	- Strength of larval encapsulation response - Larval development time - Body mass at the end of larval period - Body mass increase from end of fasting to end of larval period - Adult longevity
Study IV	1,223	- Low-nutrition vs standard larval diet - Low-nutrition vs standard parental diet	- Strength of adult encapsulation response - Egg-to-adult development time - Adult dry body mass

2.3 Diet manipulations

In my studies, the effects of fasting and a low-nutrition diet were compared with *ad libitum* feeding on a standard diet. In original articles I and II, a standard diet is referred to as a 'high-nutrition diet' and in original article III it is referred to as 'food of high nutritional value'. For clarity, in this thesis the term 'standard diet' is used uniformly to indicate these high-quality diets.

The standard diets used in the experiments were similar to the diets developed by Beck (1960) and Balanzs, et al. (1958), with some minor modifications in the ingredients or their proportions. These diets have been found to be excellent in supporting the growth, development and survival of *G. mellonella* larvae (Balanzs, et al. 1958, Beck, 1960, Marston & Campbell, 1973). The standard diets used in studies I–IV consisted of honey, beeswax, corn meal, wheat flour, dry yeast, glycerol and distilled water, in addition to either dried milk (III) or infant formula powder (I, II, IV), in the proportions shown in Table 2. The same standard diets were used for the feeding of the respective stock populations.

Two methods were used to manipulate the nutritional conditions of the larvae. In study III, sets of larvae were subjected to a period of fasting – lasting for 12, 24 or 72 hours – starting from day 18 post-hatching. The control larvae were provided with the standard diet *ad libitum* throughout their development. The fasting periods accounted for approximately 2%, 4% or 11% of the total duration of the larval stage. No larval mortality was observed, which suggests that the larvae are highly resistant to starvation.

In studies I, II and IV, the quality of the larval diet was reduced by substituting part of the honey, beeswax, wheat flour, dry yeast, corn meal and infant formula powder in the standard diet with α-cellulose powder (Sigma-Aldrich® Chemie GmbH, Munich, Germany, Table 2). Cellulose acts as a nutritionally inert material, as most insects cannot digest it (Martin, 1983, Martin, 1991). Due to the hydrophilic character of cellulose, more glycerol and water had to be added to the diluted diet than to the standard diet in order to obtain a similar consistency between the diets. Indeed, Dadd (1966) suggested that the differences in physical texture and water retentivity in a diet to which cellulose is added may have detrimental effects on the performance of *G. mellonella* larvae, and that mortality may be alleviated by adding extra water. In a preliminary experiment, I determined that the concentration of cellulose that was used did not increase the mortality of the *G. mellonella* larvae. Insects have been found to compensate for dietary dilution with cellulose by such mechanisms as an increased consumption rate, but this is often insufficient to offset the reduced nutrient intake caused by dilution, and it may come at a metabolic cost (Slansky & Wheeler, 1991, Wheeler & Slansky, 1991, Lee, et al., 2004, Lee, 2017).

Table 2. Composition of the diets (expressed in grams per 100 grams of food). The numerical symbols of the studies in which each diet was used are indicated in parentheses.

Ingredient	Standard diet (III)	Standard diet (I, II, IV)	Low-nutrition diet (I, II, IV)
Beeswax	11.1	11.9	5.8
Cellulose	-	-	12.1
Corn meal	22.2	21.4	10.5
Distilled water	11.1	11.9	23.3
Dried milk	11.1	-	-
Dry yeast	11.1	4.8	2.3
Glycerol	11.1	14.5	28.4
Honey	11.1	11.9	5.8
Infant formula	-	11.9	5.8
Wheat flour	11.1	11.9	5.8

2.4 Immune assays

2.4.1 Strength of encapsulation response

Insects use encapsulation response as the main defence mechanism against invaders that are too large to be phagocytosed, such as nematodes, protozoan parasites or parasitoid eggs, although bacteria and fungi may also be encapsulated (Hoffmann, 1995, Carton, et al., 2008, Strand, 2008). During haemocytic encapsulation response, an intruder is isolated from the haemocoel because it is encapsulated with haemocytes (Carton, et al., 2008, Strand, 2008). The structure of this capsule is fairly similar in different insect species, but there are differences in the haemocytes involved; for example, in Lepidoptera, the most common capsule forming haemocytes are plasmocytes (Strand, 2008). Haemocytic encapsulation response is often accompanied by melanotic encapsulation response, which can also occur without the participation of haemocytes (Gillespie, et al., 1997). The synthesis of the melanin pigment is an intricate process, in which tyrosine or phenylalanine is converted into melanin in a sequence of enzymatic steps (Sugumaran, 2002, Gilbert, 2012). An essential component of this process is the phenoloxidase (PO) enzyme (Gonzalez-Santoyo & Cordoba-Aguilar, 2012). An encapsulated intruder typically dies of suffocation and of toxic molecules that are generated during melanogenesis. The cytotoxic molecules created during encapsulation response can react to an

insect's own tissues; this leads to autoreactive damage, which can be an important cost associated with this type of immune response (Sadd & Siva-Jothy, 2006).

In insects, encapsulation response can be artificially induced by inserting a small, standardised object into the haemocoel, to which an insect's immune system reacts by attempting to encapsulate the foreign object. Often, small, identically sized pieces of monofilament are used for this purpose. The method has been used successfully in a variety of insect species (König & Schmid-Hempel, 1995, Rantala, et al., 2000, Rantala, et al., 2002, Rantala & Kortet, 2003, Pölkki, et al., 2012). The object darkens as it is encapsulated by melanin and haemocytes; hence, the intensity of the immune response can be quantified by measuring the darkness of the implant after its removal. The reliability of this method is supported by the findings that the strength of encapsulation response to nylon monofilament implants correlates with other immune measurements, such as levels of PO enzyme (Rantala, et al., 2002) and resistance to an entomopathogenic fungus (Rantala & Roff, 2007).

Strength of encapsulation response was measured from *G. mellonella* larvae, pupae or adults by using monofilament implants in studies II, III and IV. In study II, the insertion of a monofilament implant also acted as the means to activate the immune response of the pupae in order to study the effects of the immune activation on adult immune function. To prepare the implants, the smooth outermost layer of a nylon monofilament (ø 0.18 mm) was removed with sandpaper to facilitate the attachment of haemocytes and melanin to the filament. After that, knotted pieces of 2 mm were cut from the filament and stored in ethanol. Adult moths were anaesthetised with carbon dioxide before the implants were inserted or removed. The implant was inserted between the third and fourth sternite for larvae, on the right side of the sixth abdominal sternum for pupae, and on the right side of the thorax (near the base of the wings) for adults. A light puncture was made in the cuticle with a disinfected insect needle, after which the implant was inserted through this puncture into the haemocoel with pincers. In study III, the implants were removed after ten hours, and in studies II and IV they were removed after one hour. The darkness of the encapsulated implants was later assessed by photographing the implants from two or three angles using a digital camera attached to a microscope, after which the average grey value was measured from the photographs of each implant using the Image J -program (National Institutes of Health, Bethesda, MD, USA).

2.4.2 Susceptibility to an entomopathogenic fungus

Typically, insects are infected by entomopathogenic fungi when the fungal spores (i.e. conidia) attach to the cuticle or are ingested (Vilcinskas, 2019). After attaching themselves to the insect exoskeleton, the spores germinate and form germ tubes and appressoria, which secrete enzymes to digest the structures of the cuticle (Hajek &

Stleger, 1994, Fernandes, et al., 2012). As the proteins of the cuticle are degraded, the PO cascade is induced in the host insect, and in that process, melanin is formed (Ortiz-Urquiza & Keyhani, 2013). In the haemocoel cells of entomopathogenic fungi induce the expression of several different effector molecules, such as proteins and peptides with direct antifungal activity, in addition to proteins that decrease the virulence of the fungus by inhibiting the proteinases produced by the fungus (Vilcinskas, 2019). Fungal cells are also phagocytosed by haemocytes, and larger fungal structures are encapsulated by multiple haemocytes and melanised (Vilcinskas & Götz, 1999). In turn, entomopathogenic fungi have developed a variety of mechanisms to suppress or avoid the immune responses of their host (Vilcinskas, 2019).

B. bassiana (Ascomycota: Hypocreales) is an entomopathogenic fungus found in most regions of the world, and it is found to be infectious to multiple insect and arachnid taxa (Vega, 2008, Rehner, et al., 2011, McKinnon, et al., 2017). *B. bassiana* is increasingly used in the biological control of pests and vector insects and in studies on fungus-insect interactions (Lacey, et al., 2015, McKinnon, et al., 2017). In study I, I measured the susceptibility of fourth instar *G. mellonella* larvae to the fungus *B. bassiana*. The conidia used in the study were obtained from a culture maintained by Dr. Ivan Dubovskiy at the Siberian Branch of the Russian Academy of Science in Novosibirsk, Russia. In a preliminary experiment, I determined a mass concentration of fungal conidia that caused a mortality of 25–50% in *G. mellonella* larvae reared on a standard diet, and this concentration was used in study I. To prepare the fungal suspension, 4.0 mg of conidia were suspended in 1.0 ml of liquid containing 95% phosphate-buffered saline solution (Sigma-Aldrich®) and 5% Tween-20 (VWR®). To infect the larvae, a micropipette was used to place 5.0 µl of the resultant suspension on the dorsal side of each larva. The control larvae were treated with the same solution without the conidia. After the application of the fungal suspension or the control solution, mortality was recorded every day for 21 days, after which the remaining individuals were euthanised. The larvae were considered to have survived the fungal treatment if they were still alive after 21 days or if they had managed to pupate. The observation period of 21 days was chosen on the basis of a preliminary experiment, in which I found that mortality of *G. mellonella* larvae from *B. bassiana* occurred within 14 days of infection. Two outcome measures were assessed. The 'final mortality rate' indicated the proportion of larvae that died during the observation period of 21 days. The 'mean survival time' was analysed from the larvae that died during the observation period, and it indicated the number of days from the application of the fungal suspension or control solution to death. While the final mortality rate indicates the overall risk of mortality from the fungus in each treatment group, the mean survival time may provide rudimentary information on the progression of the fungal disease in the infected individuals.

3 Main Results and Discussion

3.1 The effect of larval nutritional conditions on development time and body mass

The results of my studies show that varying lengths of fasting and dietary dilution with cellulose increased development time and reduced body size in *G. mellonella*. In moths reared on a diet diluted with cellulose (the low-nutrition diet) egg-to-adult development time was about one fifth longer and adult dry body mass about one-fourth to one-third smaller compared with moths reared on a standard diet (II, IV). Fasting prolonged larval development time in relation to the length of the fast, this being longest in larvae that fasted for 72 hours (III). Larvae subjected to fasting also exhibited lower body mass at the end of the larval period (Figure 2). Furthermore, compared to the controls, body mass increase from the end of the fast to the end of the larval period was lower in larvae subjected to 24 or 72 hours of fasting. Interestingly, in contrast to the general pattern, a subgroup of females subjected to 12 hours of fasting exhibited distinctly high growth rates after the fast; this is discussed in more detail in section 3.2. For the most part, the findings of my studies are consistent with a trend that has been observed in nearly all taxonomic and ecological categories of insects: under poor nutritional conditions, maturity is reached later and at a smaller size (Teder, et al., 2014). Accordingly, earlier studies in *G. mellonella* demonstrate that the species shows considerable variation in body size and development time in response to differences in larval nutrition (Mohamed, et al. 2014, Krams, et al., 2015).

Body size and the length of the juvenile period are typically important fitness correlates in insects, which highlights that poor larval nutrition may confer a fitness cost in *G. mellonella*. A long juvenile period can reduce the probability that an individual will survive to the reproductive age (Roff, 1992, Stearns, 1992). In addition, retarded maturation can result in a phenological mismatch with potential mates as well as delayed access to oviposition sites or other resources, leading to reduced reproductive success (Alford & Wilbur, 1985, Nylin & Gotthard, 1998, Doyon & Boivin, 2005). Female body size is a key determinant of fecundity especially in capital breeding insects – such as *G. mellonella* – which rely mostly or entirely on resources accumulated during the larval period for reproduction (Honek,

1993, Tammaru & Haukioja, 1996, Jonsson, 1997, Tammaru, et al., 2002, Calvo & Molina, 2005). In insect males, reproductive success is typically less closely linked to body size than in females, although large males often have a reproductive advantage especially in species which exhibit male-male competition or in which the male body size is the primary criterion on which females base their choice of mate (Andersson, 1994, Nylin & Gotthard, 1998). Interestingly, the mating behaviour of *G. mellonella* is based on acoustic sounds and sex pheromones produced by the male (Leyrer & Monroe, 1973, Kwadha, et al. 2017).

3.2 Sex-specific compensatory growth

Organisms typically grow slower than their physiological potential would allow, even the species in which rapid development would be particularly beneficial (Tammaru, et al., 2004). This may allow the individuals to accelerate their growth rate to mitigate the effects of, for example, poor early growth due to a nutritional deficit. The general pattern is for individuals that have undergone a period of retarded growth to shift to accelerated growth once conditions have improved (Metcalfe & Monaghan, 2001). Such compensatory growth is observed across a wide range of taxa, although the capacity for compensatory growth may depend on such factors as sex and life stage (Arendt, 1997, Metcalfe & Monaghan, 2001). Sometimes the increased growth rate can lead to overcompensation, whereby the affected individual grows even larger than unaffected individuals (Hayward, et al., 1997). However, a high growth rate can have adverse effects on an individual, which sometimes is not expressed until later in life (Metcalfe & Monaghan, 2001). For example, an increased metabolism due to rapid growth can cause oxidative stress (De Block & Stoks, 2008). Fast-growing individuals may also be more susceptible to predators due to their higher foraging activity (Gotthard, 2000) or they may have reduced capacity to tolerate starvation (Arendt, 1997, Blanckenhorn, 2000).

As was mentioned in a previous section, a subgroup of *G. mellonella* females that were subjected to 12 hours of fasting showed distinctly high growth rates after the fast (III, Figure 2). Starting from the end of the fast, the growth trajectories of females subjected to 12 hours of fasting showed bimodal distribution: the fast-growing subgroup of larvae accelerated their growth rate relative to the controls, while the rest of the larvae had body mass increase comparable to controls. Because of their bimodal growth curves, the females that had fasted for 12 hours were divided into two subgroups for the further statistical analysis: ‘the rapid growth 12-hour fast females’ and ‘the typical growth 12-hour fast females’. The rapid growth 12-hour fast females showed a higher increase in body mass from the end of the fast to the end of the larval period and had a larger body mass at the end of the larval period compared with the controls, yet their larval development time did not significantly

differ from that of the controls. The outcome of fasting in this subgroup of females was in contrast with the general trend observed in study III, whereby larvae exhibited reduced growth rate or reduced body mass at the end of the larval period in response to a period of fasting (see section 3.1). Furthermore, all the other groups – the controls, the 12-hour fast males and the 24-hour and 72-hour fast males and females – showed unimodal growth distributions after the fast.

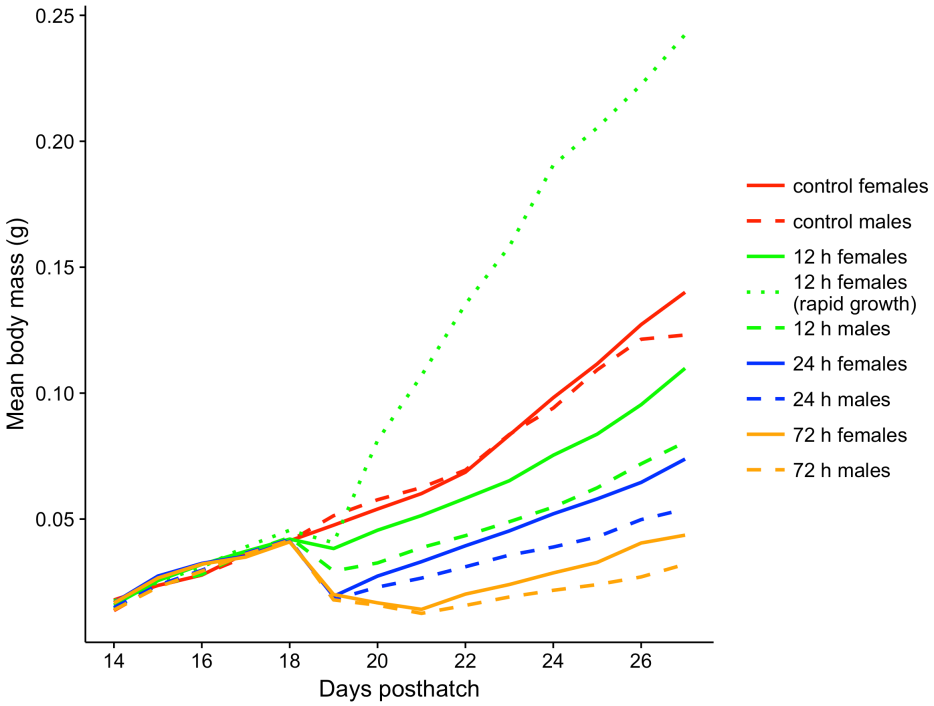

Figure 2. The mean growth curves for males and females of *G. mellonella* that were subjected to fasting periods of varying lengths (12, 24 or 72 hours, starting from day 18 post-hatching) or fed *ad libitum* (the controls). The figure shows, that starting from the end of the fast, the growth trajectories of females subjected to 12 hours of fasting showed bimodal distribution: the fast-growing subgroup of larvae accelerated their growth rate relative to the controls, while the rest of the larvae in this group had body mass increase comparable to controls. The figure is from the original article III.

Perhaps as a result of their high growth rate, the rapid growth 12-hour fast females exhibited weaker encapsulation responses than any other group, including the controls. Similarly, the shortest adult lifespans were observed in this group, although the difference to 72-hour fast females was not statistically significant. Hence, the results of study III suggest that *G. mellonella* females may accelerate their growth considerably as a result of a relatively short period of food deprivation, and that this high growth rate can come at a cost of reduced adult longevity and immune function.

The finding that the compensatory growth occurred in only females may be related to the fact that body size is likely to be more closely linked to fitness in the female moths (see section 3.1). This result is in accordance with some earlier studies, in which compensatory growth was observed in only one sex (Toigo, et al., 1999, Metcalfe & Monaghan, 2001). The underlying factors that caused the individual females within the 12-hour fast group to adopt two distinctly different growth patterns is a matter of speculation. However, in my view it is likely that this observation can be explained by genetic differences between individual females, whereby different genotypes made different growth decisions in response to the fast. The growth curves of the subgroups of females diverged only after the fast, which may reflect the fact that genotypes typically exhibit more divergent growth reaction norms under stressful conditions (Kawecki, 1995, Nylin & Gotthard, 1998).

3.3 The effect of larval nutritional conditions on immune function

The results of my studies indicate that, in general (however, see the previous section), a low-nutrition larval diet or fasting increased immune function in *G. mellonella* larvae and adults (I, II, III, IV). Adult encapsulation responses were about 10-15% stronger in moths reared on low-nutrition diet compared to moths reared on standard diet (IV and II, respectively). Similarly, larvae that fasted for 24 or 72 hours had stronger encapsulation responses at a later stage of their larval development when compared with the controls and the larvae that fasted for 12 hours (III). Strongest encapsulation responses were observed in females that fasted for 72 hours, which exhibited almost three times as strong encapsulation responses as the controls. Furthermore, immune activation at the pupal stage reduced strength of adult encapsulation response in males reared on a standard diet but not in males reared on a low-nutrition diet, which indicates that a low-nutrition larval diet increased the capacity of males to respond to repeated immune challenges (II, see section 3.4). Interestingly, strength of encapsulation response at the pupal stage was not affected by the quality of the larval diet (II). Mortality from the entomopathogenic fungus *B. bassiana* was significantly lower in larvae that were reared on a low-nutrition diet than in larvae reared on a standard diet (16% vs 46%; I; Figure 3). However, the time between exposure to the fungal suspension and death (the mean survival time) was not influenced by the quality of the larval diet (I).

The findings of my studies are, for the most part, parallel to those previously reported in this species. Banville, et al. (2012) found that, compared with unstarved larvae, *G. mellonella* larvae deprived of food for seven days showed higher susceptibility to the yeast *Candida albicans*, in addition to lower haemocyte density and lower expression of antimicrobial peptides and immune proteins in the

haemolymph. However, several other studies on *G. mellonella* larvae have shown that environmental stress factors such as physical stress (Mowlds, et al., 2008), thermal variation (Mowlds & Kavanagh, 2008), microbial priming (Bergin, et al., 2006) or a low-quality diet (Krams, et al., 2015, Krams, et al., 2017) increased several immune parameters such as encapsulation rate, expression of antimicrobial peptides, haemocyte density and antifungal resistance. The results of my thesis add to the previous literature on *G. mellonella* by showing that the upregulation of immune function caused by a poor larval diet can persist across metamorphosis into adulthood (II, IV).

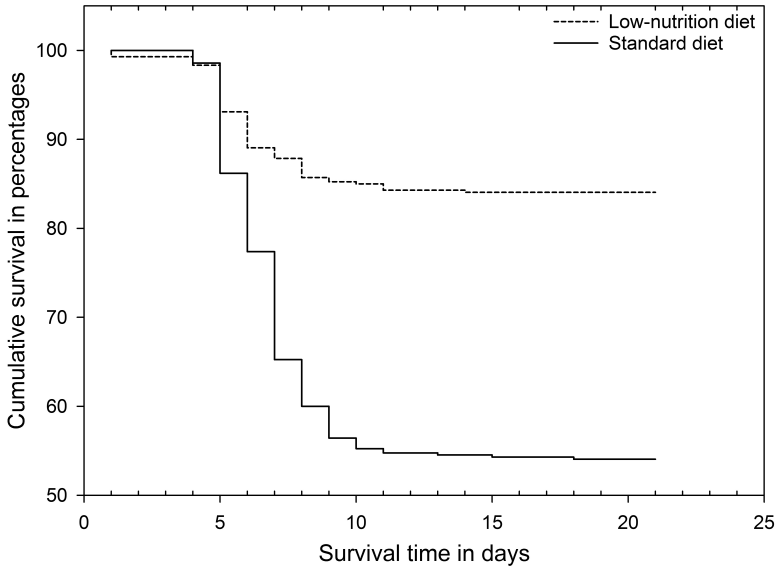

Figure 3. Cumulative survival curves of *G. mellonella* larvae infected with an entomopathogenic fungus *B. bassiana* under low-nutrition and standard conditions. The figure shows that mortality of the larvae reared on a low-nutrition diet was lower compared with the larvae reared on a standard diet. The figure is adapted from the original article I.

A central principle of ecological immunology is that due to the costs associated with immunity and the variation in infection risk in different environments, the optimal investment in immunity differs among environments, which leads to variability in immunity among populations (Sadd & Schmid-Hempel, 2009). In accordance with this, research suggests that insects often show an elevated immune function in response to environmental cues that suggest an increased risk of disease, such as high population density or exposure to immune challenges (Wilson, et al., 2002, Cotter, et al., 2004, Schmid-Hempel, 2005, Sadd & Schmid-Hempel, 2009, Martin, et al., 2011, Contreras-Garduno, et al., 2016). Considering the biological characteristics of *G. mellonella*, increased investment in immunity in response to poor nutrition may

be favoured in the natural habitat of this species. A shortage of food may be indicative of a weak honeybee colony with a reduced number of bees. Bees are known to fend off intruders that try to enter the nest as well as to exhibit hygienic behaviour (Spivak & Downey, 1998); hence, *G. mellonella* larvae may be more protected from pathogens in a healthy nest with a large bee population. A healthy bee nest with a large food supply may provide additional protection from pathogens because components of the bee nest – especially honey – have antimicrobial properties (Viuda-Martos, et al., 2008, Israili, 2014). Furthermore, food shortages may be causally linked to high population density, which in turn is typically associated with increased disease risk, because most pathogens are transmitted in a positive density-dependent manner (Grenfell & Dobson, 1995). Finally, the longer juvenile period associated with poor nutrition may increase the probability of encountering pathogens before maturity is reached. At the physiological level, nutritional stress and other types of stress (such as extreme temperatures and oxidative stress) are known to upregulate heat shock proteins, which are found to activate the immune system (Aly, et al., 1994, Ehrenfried, et al., 1996, Srivastava, 2002, Baruah, et al., 2010).

Although causal links cannot be reliably established using associative observations alone, the results of my studies may suggest that the greater wax moths altered relative investment between growth and immunity in response to different nutritional conditions. This notion is also supported by a finding from study IV, in which a negative relationship between adult body mass and strength of encapsulation response was found in male moths reared on standard diet. On the other hand, in males reared on low-nutrition diet the association between body mass and strength of encapsulation response was the opposite (IV), which suggests that the relationship between the traits is not straightforward. In accordance, a study by Krams, et al (2015) found a negative relationship between growth and immunity only in individuals reared on high-quality – but not on low- or average-quality – diet. Research in other species of animals have found genetic and physiological trade-offs between growth-related traits and immunity (Rantala & Roff, 2005, Cotter, et al., 2008, Vijendravarma et al. 2009), and studies in which one of the traits was directly manipulated confirm a causal relationship (van der Most, et al., 2011).

3.4 Larval nutrition and the outcome of immune activation

The induction of encapsulation response at the pupal stage had a negative effect on strength of adult encapsulation response in *G. mellonella* males reared on a standard diet (about 15–18% reduction compared with the control and sham control groups, respectively; II, Figure 4). However, no effect of the immune activation was

observed in males reared on a low-nutrition diet, indicating that a low-nutrition larval diet increased the capacity of males to respond to repeated immune challenges. In females, the immune activation did not affect adult encapsulation response (see section 3.8). Previous studies in various insect species, including *G. mellonella*, have demonstrated specific or non-specific immune enhancement after an initial immune challenge (Contreras-Garduno, et al., 2016), raising a question as to why the moths did not exhibit stronger encapsulation responses as a result of a previous immune challenge in study II. In my view it is likely that the result is related to the costly nature of encapsulation response as well as the developmental stage at which the immune activation was conducted. Encapsulation response is costly in terms of energy (Freitak, et al., 2003) and the amino acid tyrosine, which is required for melanin synthesis (Nappi & Vass, 1993, Gillespie, et al., 1997). Because insects cannot synthesise tyrosine, it (or its precursor phenylalanine) must be obtained directly from food (Brunet, 1963). Immunity of the moths was activated at the pupal stage, so the moths were unable to compensate for the resources they lost in their initial immune response by feeding. Therefore, the initial immune challenge may have reduced the resources that were available for the second immune response, which led to a trade-off between the consecutive immune responses. In accordance with this, a study on the mealworm beetle *Tenebrio molitor* found indications of a trade-off between two melanin-based processes: encapsulation response and cuticular melanisation (Kangassalo, et al., 2016). In a previous study, exposing *G. mellonella* larvae to non-lethal doses of *Aspergillus fumigatus* fungus has been found to increase the resistance of the larvae to a lethal dose given 24 hours later (Fallon, et al., 2011). Similarly, exposing *G. mellonella* larvae to a non-lethal dose of yeast or polysaccharide 24 hours beforehand protected the larvae from the yeast *Candida albicans* (Bergin, et al., 2006). On the other hand, in a study by Meylaers, et al. (2007), an immune challenge at the onset of, or during, metamorphosis did not affect antibacterial activity measured from the succeeding developmental stages. My research adds to the previous findings in *G. mellonella* by demonstrating that the outcome of immune activation may depend on larval nutritional conditions, highlighting the importance of considering environmental context when the effects of immune activation are examined in insects.

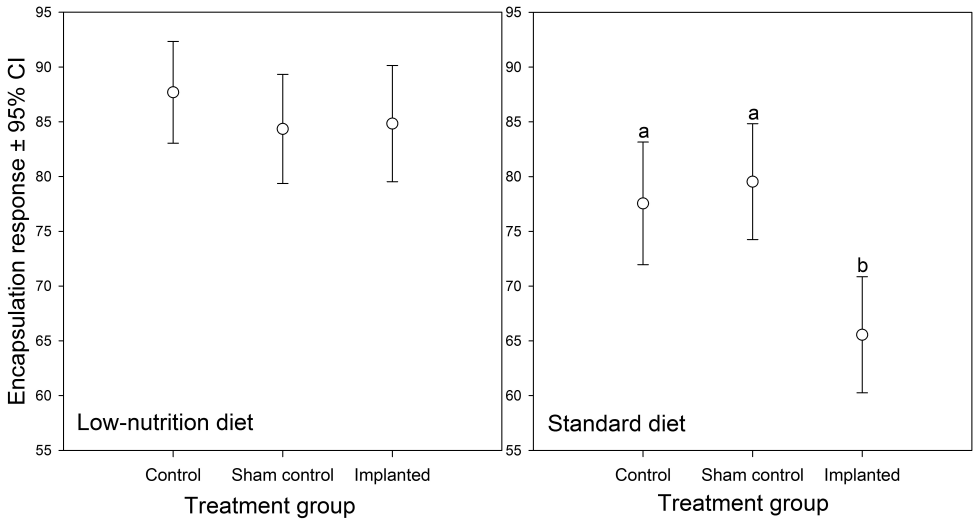

Figure 4. Strength of adult encapsulation response (estimated marginal means ± 95% CI; artificial units) in *Galleria mellonella* males of the three treatment groups: the controls, the males subjected to a minor cuticular puncture (the 'sham control group') and the males whose encapsulation response was induced, using a synthetic immune challenge, at the pupal stage (the 'implanted group'). The moths were reared on a low-nutrition (left) or a standard (right) larval diet. The figure illustrates that the effect of immune activation on adult encapsulation response differed depending on the quality of larval diet – immune activation had a negative effect on adult encapsulation response only in the males reared on a standard diet (different letters indicate a statistically significant difference (Tukey's test: $P < 0.05$)). The figure is adapted from the original article II.

3.5 The effect of fasting on adult longevity

In study III, larval fasting reduced the adult lifespan of the moths in relation to the length of the fasting period (III; however, see section 3.2). In previous studies, the effects of fasting or other types of dietary restriction on the longevity of animals have not been consistent, and the underlying mechanism by which dietary restriction influences the ageing process is not fully understood (Kirkwood, 2005, Inness & Metcalfe, 2008, Longo & Mattson, 2014, Catterson, et al., 2018, Li & Zhang, 2019). A reduction in adult lifespan can have significant negative consequences for fitness, because it typically shortens the time for which an individual is reproductively active.

It is possible that the reduced lifespan of the moths that were subjected to fasting was related to their stronger immune responses (see section 3.3). Previous studies on insects have found that induced immune response or high constitutive immunity reduces the adult lifespan under starvation and continuous feeding (Moret & Schmid-Hempel, 2000, Armitage, et al., 2003, Libert, et al., 2006, Ye, et al., 2009). Maintaining high immune function is costly and may reduce the resources available

for other physiological functions, such as somatic maintenance (Rolff & Siva-Jothy, 2003). Furthermore, immune activity has the potential to cause self-harm in insects. For example, PO enzyme activity produces toxic intermediates, which can lead to oxidative stress and immunopathological effects, and thus eventually to a reduction in lifespan (Söderhall & Cerenius, 1998, Sugumaran, et al., 2000, Sugumaran, 2002, Cerenius & Söderhall, 2004). Reduction in adult lifespan in the moths subjected to fasting can also be associated to their longer juvenile period. Indeed, Tigreros (2013) observed that the cabbage white butterflies (*Pieris rapae*) with a longer development time had shorter adult lifespans, and in the African butterfly *Bicyclus anynana*, a negative genetic correlation was found between development time and adult lifespan (Pijpe, et al., 2006).

3.6 The trans-generational effects of parental diet on development time and body mass

Parental diet had sex-specific effects on development time and body mass of *G. mellonella* offspring (IV; Figure 5). A low-nutrition parental diet extended the development time by about 4% for male offspring under low-nutrition, but not standard, conditions. Under standard conditions, the development time of females with low-nutrition parental diet was about 1% longer than the development time of females with standard parental diet; however, as the effect was very small, it is unlikely to have biological significance. Under low-nutrition conditions, the females with a low-nutrition parental diet were about 6% larger than the females with a standard parental diet. Parental diet did not affect the body mass of the female offspring that were reared on a standard diet.

he finding that under low-nutrition conditions, females with low-nutrition parental diet were able to attain a larger body mass without suffering a corresponding increase in development time is consistent with the hypothesis of anticipatory parental effects, which predicts that parental experience can alter the phenotype of the offspring in a way that increases its fitness in an environment similar to the one experienced by the parent (Mousseau & Fox 1998). However, the result for males does not support this hypothesis. The sex-specificity of the effects may reflect the different selection pressures for age and size at maturity in males and females (see section 3.1). In accordance with my study, some previous research on invertebrates has found that there are sex-specific effects of parental diet on offspring size and development time (Valtonen, et al., 2012, Zizzari, et al., 2016).

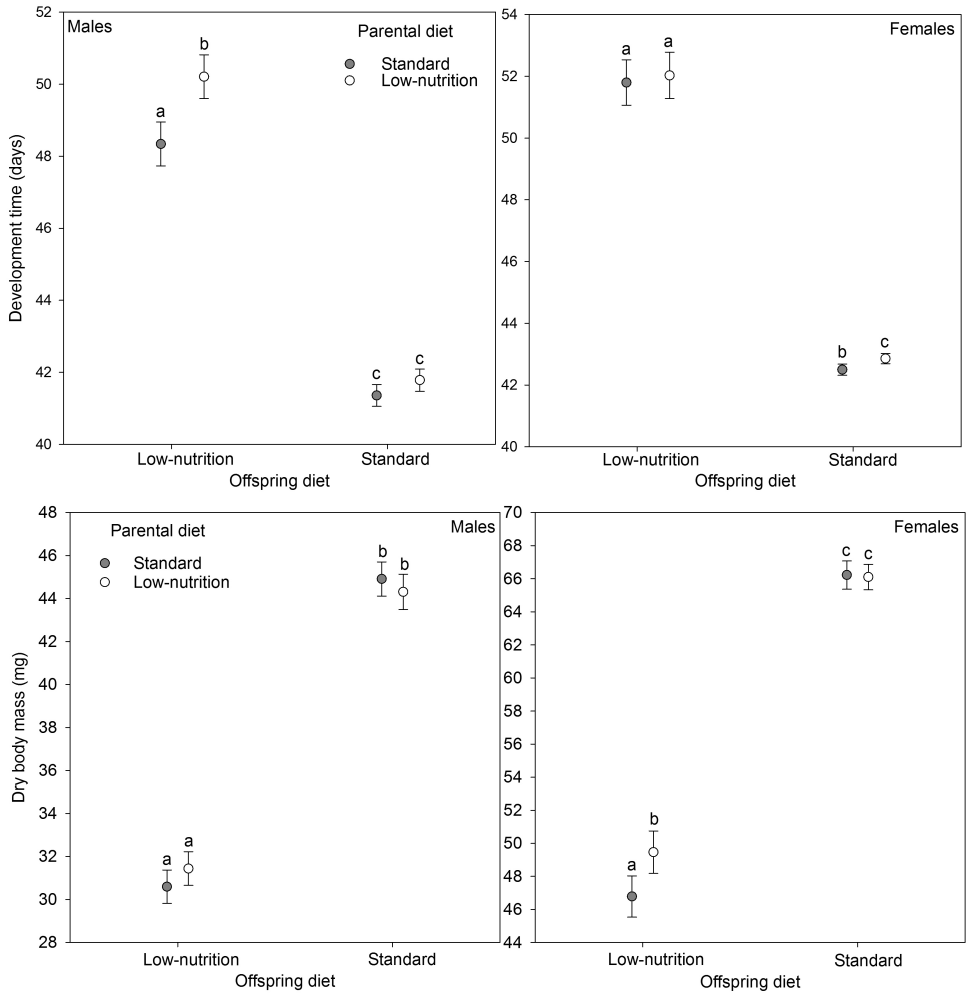

Figure 5. Estimated marginal means and 95% confidence intervals of egg-to-adult development time (the upper figure) and adult dry body mass (the lower figure) in males and females of *G. mellonella* with different parental and larval diets. Different letter above symbols indicate a significant pairwise difference (Tukey's test $P < 0.05$). The figure illustrates, that parental diet had sex-specific effects on the development time and body mass of the offspring, and these effects interacted with the nutritional conditions of the offspring. The figure is adapted from the manuscript IV.

The mechanisms behind the observed parental effects cannot be determined within the framework of the study IV, and it is possible that different mechanisms accounted for the parental effect on the development time of males and the body mass of females. One possible mechanism for the observed effects is diet-induced variation in egg size, as in insects, offspring hatching from larger eggs typically have shorter development time or larger adult size (Rossiter, 1991, Fox, 1994, Azevedo, et al., 1997, Fox & Czesak, 2000, Vijendravarma, et al., 2010). In *G. mellonella* egg size

correlates positively with female body size (Marston & Campbell, 1973), which could explain the shorter development times in offspring of females reared on standard diet. Studies have shown that individuals hatching from larger eggs may be at an advantage particularly under stressful conditions (Fox & Czesak, 2000), which could account for why in males the parental effect on development time was only observed under low-nutrition conditions. However, further studies are needed to clarify the mechanisms underpinning the diet-induced transgenerational effects on size and development time in this species.

3.7 The trans-generational effects of parental diet on immune function

The *G. mellonella* larvae with a low-nutrition maternal diet survived about 5% longer after the infection by the fungus *B. bassiana* than the larvae with a standard maternal diet (I; Figure 6). Interestingly, although maternal nutrition influenced offspring survival time, it did not affect the overall mortality caused by the fungus. In contrast, within a generation a low-nutrition larval diet decreased the final mortality rate without affecting the survival time after the fungal infection (I; see section 3.3). Insects use several defence mechanisms in the cuticle and in the haemolymph to protect themselves from entomopathogenic fungi (Gillespie, et al., 2000), and it is possible that in part, different resistance mechanisms were affected by direct nutritional stress and by a low-nutrition maternal diet. It is also possible that a low-nutrition maternal diet affected tolerance of infection (Schneider & Ayres, 2008) rather than immune resistance mechanisms. However, further investigations are needed to draw any reliable conclusions in this regard. Paternal diet did not influence the final mortality rate or the survival time after the fungal infection in *G. mellonella* larvae, which may suggest that maternal nutrition plays a more important role than paternal nutrition in the susceptibility of *G. mellonella* larvae to pathogens. It is also noteworthy that the maternal and paternal diets did not have an interactive effect on the susceptibility of the offspring to the fungus.

The mechanisms underpinning the observed maternal effect on the survival time of offspring cannot be elucidated within the framework of my study; however, one possibility is that in females, a low-nutrition diet induced epigenetic changes affecting the immune system, which were transferred to the offspring. Environmental factors can induce epigenetic changes in a sex-specific manner (Gabory, et al., 2009), which could explain why only maternal nutrition affected the survival time of the offspring. Previous studies have shown transgenerational immune priming in *G. mellonella*, and epigenetic modifications are involved in transgenerational immune priming against fungal pathogens in this species (Vilcinskas, 2019). However, previous studies have not, to my knowledge,

investigated the trans-generational effects of parental nutrition in *G. mellonella*. In a closely related species, the Indian meal moth *Plodia interpunctella*, both maternal and paternal diet are found to affect the immune function of the offspring (Boots & Roberts, 2012, Triggs & Knell, 2012b).

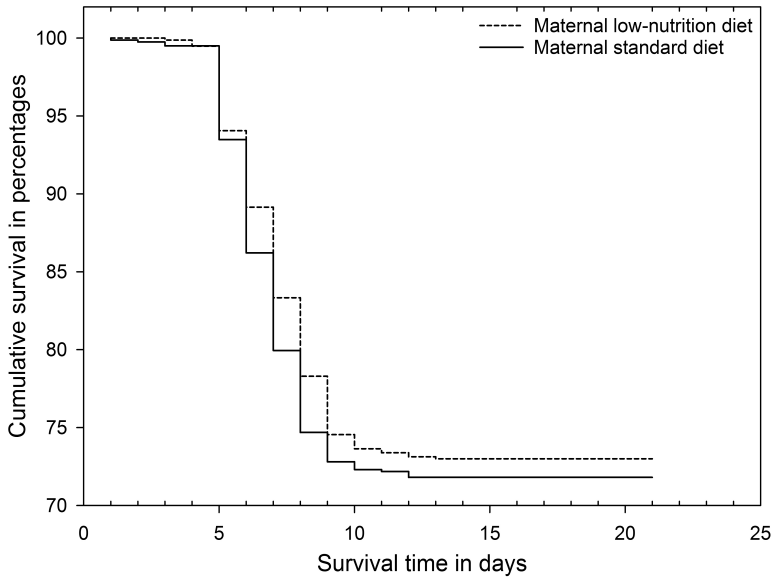

Figure 6. Cumulative survival curves of *G. mellonella* larvae infected with an entomopathogenic fungus *B. bassiana*. The mothers of the larvae were reared on a low-nutrition or a standard diet. The figure shows that the larvae whose mothers were reared on a low-nutrition diet survived longer compared with the larvae whose mothers were reared on a standard diet. The figure is adapted from the original article I.

The quality of the parental diet did not influence strength of encapsulation response of *G. mellonella* offspring at the adult stage (IV). The lack of effect may be related, for example, to the immune parameter that was assayed or to the stage of development at which the measurement was made. For example, it is possible that the effect of poor parental nutrition on offspring immune function in *G. mellonella* is only detectable at early instars in the larval stage, as was observed in the Glanville fritillary butterfly *Melitaea cinxia* (Saastamoinen, et al., 2013). Insects are typically more susceptible to pathogens at the juvenile stage than at the adult stage (Schmid-Hempel, 2011)[4], so selection may be stronger for parental effects that target the immunity of the juvenile stages.

[4] In *G. mellonella*, the risk of infection in adults may be further reduced because the adult moths do not eat, making infection via the alimentary canal unlikely: see Nation & Patton (1961) for description of the alimentary canal of *G. mellonella* adults.

3.8 On the observed sex-specific differences in strength of encapsulation response

In many animals, females are found to be more 'immunocompetent' than males, and this is considered to result from sex differences in optimal life- history strategies (Slatkin, 1984, Kurtz, et al., 2000, Rolff, 2002, Tucker & Stevens, 2003, Schwarzenbach, et al., 2005). Bateman's principle predicts that in general, males can best increase their fitness by improving mating success, whereas for females, investment in longevity and survival is more beneficial (Bateman, 1948, Rolff, 2002). However, the sex difference in immune capacity is expected to shift in favour of males when infection would be relatively more detrimental to male fitness than to female fitness (McKean & Nunney, 2005, Stoehr & Kokko, 2006).

In my studies, I assessed the sex-specific difference in strength of encapsulation response in larval (III), pupal (II) and adult (II, IV) stages of *G. mellonella*. In study III, the female larvae had stronger encapsulation responses than the male larvae in the group that was subjected to a 72-hour fast, whereas in the larvae subjected to shorter fasts or fed *ad libitum* no sex-specific difference in strength of encapsulation response was found (excluding the subgroup of female larvae that exhibited compensatory growth and distinctly weak encapsulation responses; see section 3.2). In pupae, no sex-specific difference in strength of encapsulation response was found (II). In study IV, adult males were found to have stronger encapsulation responses than adult females. On the other hand, in the analysis of study II, which also included individuals whose immunity was activated at the pupal stage (see section 3.4), encapsulation responses of adult females were stronger than those of adult males. A previous study found no indication of a sex-specific difference in immune response to a microbial injection in larvae, pupae or adults of *G. mellonella* (Meylaers, et al., 2007).

Taken together, my studies suggest that *G. mellonella* does not exhibit a fixed sex-specific difference in strength of encapsulation response, but that the difference between males and females in this immune process may be influenced by such factors as developmental stage, larval nutritional conditions and occurrence of previous immune challenges. The sex-specific differences in adult encapsulation response may be related, in part, to differences in tyrosine metabolism between the sexes: female Lepidoptera use tyrosine and PO enzyme – also required for the encapsulation response – for egg production (Ishaaya & Navon, 1974, O'Brien, et al., 2002, O'Brien, et al., 2003, O'Brien, et al., 2005). Finally, it should be noted that the results could have been different had the subject been studied in reproductively active individuals, as reproductive behaviour is known to affect immune function in insects (Adamo, et al., 2001).

4 Concluding remarks

A central principle of ecological immunology predicts that due to variation in infection risk between individuals and environments, and the costs associated with immune function, the optimal investment in immunity varies among individuals and populations (Rolff & Siva-Jothy, 2003). The findings of my studies suggest that fasting and a low-nutrition larval diet induces an upregulation of the immune system in *G. mellonella*, as evidenced by a higher resistance to an entomopathogenic fungus and stronger encapsulation response to a synthetic immune challenge (I, II, III, IV). Increased investment in immune function in response to poor nutritional conditions may be an adaptive response in the natural habitat of *G. mellonella*, as poor nutrition can indicate a weak bee colony, which may provide reduced protection from pathogens. In general, moths subjected to fasting or a low-nutrition larval diet were smaller (II, III, IV), had longer development time (II, III, IV) and shorter adult lifespan (III), which shows that poor larval nutrition can – at least in absence of pathogens – confer a fitness cost in *G. mellonella*. In contrast to the general trend, a small proportion of larvae had unusually high growth rate after a relatively short period of fasting, but perhaps as a result of this compensatory response, exhibited particularly weak immune responses and short adult lifespans (III). Indeed, a basic principle of life-history theory predicts that an individual cannot simultaneously have maximal investment in all costly life-history traits – commonly processes related to growth, survival and reproduction – hence, negative correlations between the traits are observed (Roff, 1992). In accordance, my studies suggest that in response to nutritional stress, the greater wax moths altered their relative investment between the different fitness-related life-history traits.

Nutritional conditions experienced by parents are increasingly recognised to affect the performance of their offspring (Mousseau & Fox, 1998, Valtonen, et al. 2012, Vijendravarma, et al., 2010). The findings of my studies suggest that parental diet has relatively small yet significant effects on immune function (I), body size (IV) and development time (IV) in *G. mellonella*. A low-nutrition maternal – but not paternal – diet increased the survival time of larvae after an infection by an entomopathogenic fungus (I), suggesting that poor nutritional conditions can increase the immune function of *G. mellonella* not only within a generation (I, II, III,

IV) but also across generations. On the other hand, parental diet did not influence strength of encapsulation response of *G. mellonella* offspring at the adult stage (IV). It is suggested that parental experience can alter the phenotype of the offspring in a way that increases its fitness in an environment similar to the one experienced by the parent (Mousseau & Fox, 1998). In my studies, I found support for this hypothesis in female moths, which attained higher body mass under low-nutrition – but not standard – conditions if their parents had also been reared on a low-nutrition diet. However, under low-nutrition conditions, development time was longer in male offspring whose parents were reared on a low-nutrition diet compared with male offspring whose parents were reared on standard diet (IV), suggesting that poor parental diet caused both positive and negative effects on offspring performance. The results of my studies add to the growing body of evidence indicating that environmental conditions experienced by the parents can induce variation in offspring phenotype and alter the way offspring respond to current environmental conditions (Mousseau & Fox, 1998, Bonduriansky & Day, 2009).

Finally, my thesis demonstrates that larval nutrition and individual characteristics such as growth-related traits and sex can influence immune function of *G. mellonella*, highlighting the importance of taking such factors into account when the species is used as a model host to study the pathogenesis and the virulence of significant bacterial and fungal pathogens (Junqueira., 2012, Cook & McArthur, 2013, Vilcinskas, 2019).

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Katariina Kangassalo

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