Transgenerational effects: nutrition, immunity and infection dynamics in *Plodia interpunctella*

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Statement of originality

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Multiple infections result in a complex response to transgenerational immune priming. Littlefair, J; Laughton, A; Knell, R. J *Functional Ecology* (submitted)

The development of a synthetic diet for investigating the effects of macronutrients on the development of *Plodia interpunctella.* Littlefair, J; Nunn, K. A; Knell, R. J *Entomologia Experimentalis et Applicata* (in review)

Abstract

Phenotype can be shaped by transgenerational effects from parental and even grandparental environments. Parental resource allocation strategies can alter offspring condition to increase fitness. Alternatively, environmental influences such as stress, pollution, or pesticides, can be transmitted as non-adaptive "developmental noise". This thesis explores the roles that nutrition and infection play as transmitted effects to offspring, using the Indian meal moth *Plodia interpunctella* as a model host.

Parents acquire more resources to provision offspring with through their diet. However, interactions with offspring environment often go unexamined due to the complex nature of these factorial studies. Using good and poor diet qualities, I demonstrate the relative importance of parental and offspring environments and how this changes for offspring disease resistance, weight and longevity. I find that parental effects are present but are modest in comparison to the effects of the offspring's own environment.

I then develop a synthetic artificial diet to examine which macronutrients (such as protein and carbohydrate) within parental diet influence offspring phenotype. These diets allowed both the total nutritional content to be diluted, and the ratio of protein and carbohydrate to change. I find that offspring are relatively robust to extreme changes in parental dietary macronutrient content, which possibly explains the success of *Plodia interpunctella* as a widespread pest species.

Vertical transmission of upregulated immune function has recently been demonstrated in insects, which is reliant on parental exposure but functions without an obvious acquired transmitted cue. To date, experiments have been conducted using single infections under laboratory conditions. However, this study shows that in a more ecologically realistic scenario involving multiple infections and resource limitation,

maternal pathogen exposure results in environmentally-contingent decreases in offspring survival in response to infection. Transgenerational immune priming may not as common as previously thought, and any benefits accrued to offspring should be viewed within an inclusive framework that includes potential costs to the parents.

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Chapter 1: General introduction

1.1 Defining transgenerational effects

Transgenerational effects are a form of non-genetic inheritance that arise when the phenotype or environmental variation experienced by previous generations influence the phenotype of the progeny (Mousseau & Fox 1998). They are now believed to act as important sources of phenotypic variation, interacting with both genetics and the present environment to create the overall phenotype (Laland *et al.* 2014). Transgenerational effects have consequences for both the fitness of individuals and the dynamics of entire populations. Parental effects can be mediated through epigenetics, culture, behaviour, cytoplasmic factors or somatic factors such as nutrients or hormones (Bonduriansky & Day 2009). There are numerous types of environmental information that can be communicated through these mechanisms, including parental exposure to changes in temperature (Willott & Hassall 1998; Garbutt *et al.* 2014), heat shock (Sikkink *et al.* 2014), diet (Frost *et al.* 2010; Valtonen *et al.* 2012; Franzke & Reinhold 2012; Triggs & Knell 2012; Shikano *et al.* 2015), exposure to pollutants (Marshall 2008), light regimes in plants (Galloway & Etterson 2007), immune challenges (Zanchi *et al.* 2011; Trauer & Hilker 2013) and infection with parasites (Tidbury, Pedersen & Boots 2011) . The increasing use of insect models by ecologists has allowed for the easy experimental manipulation of major sources of environmental variation and large experimental sample sizes to investigate the impact of transgenerational effects on multiple generations of animals.

1.1.1 The evolutionary significance of transgenerational effects

Parental effects can be interpreted non-exclusively as adaptive strategies or fitness constraints at the level of the individual. I will focus here on defining the resource

allocation hypothesis, parental stress hypothesis, and anticipatory parental effects, as these are the predominant interpretations discussed in the literature. Under a resource allocation hypothesis, high-quality or resource-rich parents are able to transfer their condition to their progeny through the partitioning of resources to reproduction (Qvarnström & Price 2001; Franzke & Reinhold 2012). This may be particularly beneficial when offspring themselves face low-quality environments, where condition is most important (Bonduriansky & Head 2007). However, in poor conditions, the limited resource pool means that parents may not be able to allocate sufficient resources in order for offspring to reach their phenotypic optima.

Resource acquisition from the environment will determine the total level of resources for allocation, for example capital breeding animals have an initial fixed amount of resources with which to produce eggs (Calvo & Molina 2005; reviewed in Jervis, Boggs & Ferns 2005). Resources can be acquired through mechanisms other than direct consumption: radio tracing experiments have shown that males can transfer nutrients to the female during mating, and that these can be reflected in the egg composition, or indeed males can contribute directly to offspring, for example through nutrient provisioning to fledgling chicks (Boggs & Gilbert 1979; Zera & Harshman 2001; Boggs 2009). Parental resource levels will also change with age: typically mothers have fewer resources to allocate to eggs as they age (Giron & Casas 2003; Jervis *et al.* 2005), although some insect species have shown terminal investment of their resources in the face of death (Sadd *et al.* 2006).

Resource allocation-based maternal effects have interesting implications for parent-offspring conflict, which will occur when parents and offspring have different optimal strategies for allocation. When we consider the adaptive nature of maternal effects, we

must consider which party they are adaptive for. For example, if a mother invests resources to impose her own optimal allocation between her present and future offspring, conflict could exist between them because individuals are selected to maximise their own fitness and not that of the parent (Uller 2008). If this is detrimental to the fitness of offspring, selection will favour reduced sensitivity to manipulative parental signals. The evolutionary dynamics of conflict will change depending on whether manipulation is expensive or cheap (for example, the transfer of information through hormones or epigenetic marks is not considered expensive, whereas the transfer of resources is) (Uller 2008). To date very few studies have investigated the possibility that modification of offspring phenotype could be a compromise between parent and offspring strategies (Poulin & Thomas 2008).

Not all instances of transmitted parental phenotype are adaptive for offspring or parents. There can be a huge burden of stress from the transgenerational effects of malnutrition, infection, pesticides and pollutants (Lewis 1991), which Jablonka and Lamb term non-adaptive "developmental noise" (Jablonka & Lamb 1995). Negative consequences extend throughout the offspring phenotype and can be far-reaching into maturity. For example, offspring of the bryozoan *Bugula neritina* had poor performance (measured by survival × colony size) even six weeks after spawning after their mothers had been exposed to heavy metal pollution (Marshall 2008). Similar transmitted inheritance of stress factors has severe consequences for human health and well-being globally, as they cause metabolic disorder, a combination of diabetes, high blood pressure and obesity (Stanner *et al.* 1997). Typically experiments have demonstrated a negative effect on offspring fitness, but we should also consider interactions between the transgenerational effects of stress and the offspring's own environment. Although difficult to demonstrate experimentally, offspring may be able to compensate for a poor

start in life in a similar manner to compensation for stress in early development (Metcalfe & Monaghan 2001).

In addition to resource allocation and parental stress, anticipatory parental effects (APEs) are another non-exclusive hypothesis to explain influences on offspring phenotype. They occur when parents adjust offspring phenotype to the local environment, based on the idea that parental environment is a good predictor of future environmental experience (Mousseau & Fox 1998; Fox *et al.* 1999; Marshall & Uller 2007; Burgess & Marshall 2014). For example, experiments using the herb *Campanulastrum americanum* show that offspring fitness is enhanced when seedlings grow under the same lighting regime as their mothers, allowing adaptation to variable local lighting conditions through transgenerational plasticity (Galloway & Etterson 2007). Marshall and Uller define a set of specific conditions under which this type of parental effect is predicted to occur: 1) conditions change between generations, 2) offspring environment can be predicted by the mother, 3) the costs of maternal cue detection and transmission of the information are low, and 4) the level of parent-offspring conflict is low (Marshall & Uller 2007). Although there are a few demonstrable examples of APEs, a recent meta-analysis highlights the overall lack of experimental evidence (Uller, Nakagawa & English 2013), implying that researchers have yet to investigate many experimental systems where these conditions are met. However, the authors also highlight a lack of fully-factorial experimental designs in the appropriate candidate systems.

Parental effects have consequences beyond the life history of individuals. Nongenetic inheritance can decouple phenotypic change from genetic change over multiple generations, allowing populations to respond to selection even in the absence of genetic

variation for traits affecting fitness (Badyaev 2008; Bonduriansky, Crean & Day 2012), although sometimes interacting in combination with allelic inheritance. For example, parental effects could allow organisms to respond quickly to fluctuating conditions in the environment, at a faster rate than genetic evolution (Rossiter 1996). Recently, it has been suggested that transgenerational plasticity could even help to buffer species against the rapid effects of anthropogenic warming or habitat change, for example as host plants and prey ranges shift (Bonduriansky *et al.* 2012; Burgess & Marshall 2014). However, it should be recognised that transgenerational plastic responses can be disadvantageous when environments are highly unpredictable, because parental environment is a poor predictor of offspring environment under these conditions (Reed *et al.* 2010).

Another population-level consequence of parental effects will be variation in the competitive environment of offspring, driven by differential parental investment (Beckerman *et al.* 2006). Parents invest different amounts according to changing resource allocation as they trade-off maternal per-offspring investment with their own somatic condition and future reproductive success. For example, the size-number trade-off, which is defined by a negative relationship between the size and number of progeny, is influenced by resources available to the mother (Fox & Czesak 2000). In abundant conditions, mothers produce a greater number of small offspring, but in times of hardship mothers generally produce fewer but larger progeny. This plasticity in maternal strategies will affect offspring weight, but further effects (sometimes termed "indirect maternal effects") will result from the altered population density and changing competition for resources (Beckerman *et al.* 2006). Parental effects also act as a proximal source of time-lagged effects on population dynamics, especially if they affect fecundity in adult offspring (Rossiter 1996). Developmental lags, birth-order effects and

changing population density could also activate cannibalistic behaviour in older instars or adults, further altering the age-structure of the population.

1.1.2 Mechanisms of transgenerational effects

The mechanisms by which parental effects act are largely unknown and their discovery is important as they will affect the evolutionary dynamics of parental effects. Here, I will predominantly emphasise examples such as epigenetics and transferred molecules in the eggs of invertebrates, as they are the focus of this thesis. Cultural or behavioural parental effects are of course shaped by behaviours post-hatch or post-birth which are less common in invertebrates (although burying beetles are an important exception (Sloan Wilson & Fudge 1984)), and are well reviewed elsewhere (Day & Bonduriansky 2011).

Mammals have a protracted period of intimate contact between mother and offspring in which substances can be transferred either through the placenta or lactation. Most invertebrates lack this period of contact (although an exception is the "lactation" of the cockroach *Diploptera punctata* (Roth & Willis 1955; Ingram, Stay & Cain 1977)) but both female- and male-derived compounds can be directly transferred into eggs which can be classified as maternal or paternal effects if they alter the offspring phenotype. Transferable molecules include nutrients (Boggs & Gilbert 1979), anti-microbial peptides which are thought to be a mechanism for transgenerational immune priming (Sadd & Schmid-Hempel 2007) and protective anti-predator alkaloids (Dussourd *et al.* 1988). The transfer of such molecules can form part of a differential resource allocation strategy, subsequently affecting egg size or composition. For example, yolk proteins such as vitellin are important for newly developing larvae and are a good predictor of neonate fitness (Diss *et al.* 1996). Radiolabelling experiments in three butterfly species

showed that paternally derived nutrients can also be incorporated into the eggs, which benefit offspring fitness with no extra cost to the mother (Boggs & Gilbert 1979). However, males can also produce compounds that increase resource allocation from females at a cost to her net fitness, for example male accessory proteins passed across in ejaculate have been shown to influence virtually all aspects of female reproductive activity (Chen 1984; Gillott 2003; Crean & Bonduriansky 2014).

Epigenetic regulation of the genome is another likely mechanism to explain transgenerational effects in invertebrates. In DNA methylation, specific CpG dinucleotides are targeted for *de novo* methylation by the enzyme DNA methyltransferase 3, or by DNA methyltransferase 1, which propagates existing patterns of methylation. More widely studied in vertebrates, the primary focus in invertebrate studies has been the functional CpG methylation systems of social insects. Evidence is accumulating for the control of reproductive caste fate and behaviour in eusocial insects by DNA methylation (Yan *et al.* 2014, 2015), and possibly the control of solitary and gregarious phase polyphenisms in locusts (Ernst *et al.* 2015). Other insect orders have not been widely studied, although we know that functional DNA methylation has been lost in the Coleoptera and Diptera, but does exist in Lepidoptera despite the loss of DNA methyltransferase 3 in the lineage (Glastad *et al.* 2011). In contrast to vertebrates, DNA methylation in invertebrates appears to be a more dynamic process, changing with age and developmental stage (Jones & Takai 2001; Wang *et al.* 2006; Yan *et al.* 2014). Methylation patterns have also been shown to change with variation in environmental factors, providing a putative mechanism for inherited environmental variation from diet, infection and drug exposure (McKay & Mathers 2011). Parental diet, for example, alters the methylation profile of mouse offspring and subsequently their lipid and cholesterol metabolism (Carone *et al.* 2010; Li *et al.* 2011; Ferguson-Smith & Patti 2011). *Daphnia*

exposed to the pharmaceutical 5-azacytidine reduced overall levels of DNA methylation as well as body length. This pattern of reduced DNA methylation was transferred to two subsequent non-exposed generations, demonstrating possible transgenerational epigenetic inheritance of environmental exposure (Vandegehuchte *et al.* 2010).

Other forms of epigenetic modification of the genome contribute to environmentally acquired transgenerational effects. Nucleic DNA is packaged around globular proteins known as histones, which undergo many types of post-translational modification, such as acetylation (the addition of an acetyl group to lysine residues, which is associated with the activation of transcription) (Kouzarides 2007), which could possibly transmit parental effects. In conjunction with other epigenetic marks, these modifications switch chromatin between transcriptionally silent and active states, thus providing sophisticated control over gene expression (Jenuwein & Allis 2001), which like DNA methylation can also be influenced by specific dietary components (McKay & Mathers 2011). Non-coding RNAs are receiving increased attention as an increasingly likely mechanism for transgenerational effects. Little attention has been focused on them in insects, but RNAs are known to populate both mammalian sperm and eggs, possibly silencing genetic elements containing retrotransposons or by directing post-transcriptional silencing (Daxinger & Whitelaw 2012)

1.2 Insect immunity

One aspect of parental effects that has received considerable recent interest is the phenomenon of transgenerational immune priming, in which the offspring of a parent exposed to a pathogen exhibit raised resistance themselves. To fully understand this we need first to consider the various mechanisms by which immune systems operate, and

therefore I will provide a brief introduction to cellular and humoral components of the invertebrate immune system here.

Parasites are organisms that live within or on the host (including macroparasites such as helminths and parasitoids, and micro-parasites such as bacteria, fungi and viruses), and by definition inflict an overall fitness cost on the host (Wilson & Cotter 2013). Hosts have evolved defences, including the immune system, to protect the soma from the detrimental aspects of parasites long enough for the organism to reproduce. Before the immune system is invoked, mechanical barriers limit the degree of parasite exposure in many species (Chapuisat *et al.* 2007; Cotter & Kilner 2010; Otti, Tragust & Feldhaar 2014). The cuticle is one such example of a defensive barrier against penetrating pathogens such as fungi, and can be strengthened by deposits of cuticular melanin (Wilson *et al.* 2001). Insects can also use antimicrobial compounds as external defences, which are either environmentally-derived or secreted (Otti *et al.* 2014), such as the secretion of antimicrobial peptides in the exudates of the burying beetle or from the meta-pleural gland of fungus-growing ants (Fernández-Marín *et al.* 2006; Arce *et al.* 2012).

If pathogens evade external immune defences, pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide or peptidoglycan are recognised by receptors. Signal transduction pathways including Toll (mainly activated by gram positive bacteria and fungi) and Imd (mainly activated by gram negative bacteria) respond to the invading parasite's pattern recognition receptors. For example, Toll-like receptors in *Drosophila* respond to bacterial, fungal and viral pathogens, resulting in cascades of transcription factors that produce rapid increases in the expression of antimicrobial peptides (Zambon *et al.* 2005). JAK/STAT is a third pathway, activated in response to

viruses and fungi (Souza-Neto, Sim & Dimopoulos 2009; Dong *et al.* 2012). After pathogen recognition, haemocytes are recruited to the site of infection and proteolytic cascades are initiated, resulting in the production of cytotoxic molecules (Tzou, De Gregorio & Lemaitre 2002).

The immune system has traditionally been divided into humoral and cellular branches but in reality these are intimately linked, with feedback from each linking in to the other. Cellular responses are enacted by haemocytes, the highly specialised effector cells of the immune system. Their density is tightly correlated with the ability of an insect to resist bacterial and viral attack (Eslin 1996). Types of differentiated haemocytes differ between insect lineages (see figure in Strand 2008); lepidopteran larvae contain granulocytes (which recognise and phagocytose pathogens), plasmatocytes (capsule-forming haemocytes), spherule cells (function mostly uncertain but they may contain cuticular components) and oenocytoids (contain and release phenoloxidase) (Gillespie, Kanost & Trenczek 1997; Strand 2008). Phagocytosis is an evolutionary conserved response whereby pathogens, or abiotic particles such as glass beads, are engulfed. Multiple plasmatocytes can surround a pathogenic target if it is too big to be engulfed by a single cell, known as encapsulation, and the capsule is often subsequently melanised by phenoloxidase. The pathogen subsequently dies of a lack of oxygen or food, or from the release of toxic reactive oxygen species by the host (Moreno-García *et al.* 2013).

Humoral responses in invertebrates complement cell-based immunity and mainly consist of the production of antimicrobial peptides (AMPs), complement-like proteins and cascades of enzymes involved in melanisation (Strand 2008). AMPs and lysosomes are produced in the fat body, and to a lesser extent in circulating haemocytes (Lavine, Chen & Strand 2005). Effective against a broad variety of invading organisms, AMPs

are an inducible response (Lavine, Chen & Strand 2005), which kill or inhibit bacterial growth. For example, attacin disrupts the synthesis of the outer bacterial membrane, lysozyme hydrolyses glycosidic bonds in the peptidoglycan of bacterial cell walls (Gillespie *et al.* 1997), and cecropin interacts with lipid bilayers to disrupt the inner bacterial membrane (Bulet *et al.* 1999; Ganz 2003).

Part of the humoral immune system, phenoloxidase (PO) is a key enzyme in wound repair, parasite encapsulation and cuticle defence (Soderhall & Cerenius 1998), which are essential processes to limit the spread of pathogens. Pro-PO is released by haemocytes in response to the detection of non-self particles (Ling & Yu 2006). Serine protease cascades cleave proPO into active PO, the enzyme that converts dopamine into quinones, which are subsequently polymerised into melanin (Nappi & Vass 1993). Cytotoxic reactive oxygen species are also produced by this reaction and can play a part in combating infection (Tzou *et al.* 2002), but may also be toxic for the host. PO activity can easily be assayed in invertebrate haemolymph by combining the sample with dopamine substrate and measuring the resulting colour change over time (Moreno-García *et al.* 2013).

Disease ecologists have called for measures of disease resistance to be performed alongside measuring the titre of constitutive immunity (Graham *et al.* 2011). In some cases the intuitive scenario that a stronger immune response leads to greater resistance against pathogens prevails, but strong immune responses can also be detrimental, causing immunopathology from autoimmunity (Graham, Allen & Read 2005; Schulenburg *et al.* 2009; Pursall & Rolff 2011). High titres of immune molecules or cells may actually be associated with the host being overwhelmed by high numbers of infective parasites. For example, wild *Daphnia* infected with the sterilising parasite

Pasteuria ramosa have upregulated haemocyte counts but these do not appear to be helping to combat the infection (Auld *et al.* 2012). Assaying direct resistance to pathogens will therefore give us a greater understanding of whether hosts will survive attack by pathogens (and then go on to reproduce), in comparison to solely taking measures of the constitutive immune system (Graham *et al.* 2011). Finally, the majority of studies that examine invertebrate immunity and resistance take place in captive animals reared in laboratory settings, and more studies of wild systems are welcomed to better understand the response to the huge variety of wild pathogens and how infection dynamics will interact with the environment (Maizels & Nussey 2013).

1.3 Transgenerational immune priming

Transgenerational immune priming (TGIP) is a form of parental effect that transmits transgenerational resistance to pathogens (Sadd & Schmid-Hempel 2007). A series of experimental studies in invertebrates have reported that mothers and, in some cases, fathers who are exposed to a pathogen produce offspring with raised resistance to that pathogen or upregulated constitutive immunity (Little *et al.* 2003; Sadd *et al.* 2005; Moret 2006). For example, strain-specific transgenerational immunity has been found in *Daphnia magna* to the pathogenic bacterium *Pasteuria ramosa,* and a challenge of heat-killed bacteria to *Bombus terrestris* queens upregulated the antibacterial activity of their offspring (Little *et al.* 2003; Sadd *et al.* 2005). This process is usually interpreted as an anticipatory, protective parental effect functionally homologous to the maternal transfer of antibodies in vertebrates, although antibodies themselves are not present in invertebrates (Kurtz & Armitage 2006; Hasselquist & Nilsson 2009). The discovery of TGIP has profound implications for our understanding of invertebrate host-pathogen

dynamics, for example by providing a new source of heterogeneity in host susceptibility and therefore influencing infection prevalence (Tate & Rudolf 2012).

Research on TGIP has been conducted for about 10 years, and it is therefore a relatively new concept in invertebrate host-pathogen dynamics and much is still unknown. For example, many TGIP studies use heat-killed bacteria or bacterial PAMPs to challenge the mother, and measure the strength of the constitutive immune system in offspring as a response with phenoloxidase or antibacterial assays (Sadd *et al.* 2005; Sadd & Schmid-Hempel 2007; Freitak, Heckel & Vogel 2009; Zanchi *et al.* 2011), whereas few studies have used live pathogens, which is essential for understanding the true nature of host-pathogen dynamics as well as answering questions about the pathogen specificity of TGIP (although see Little *et al.* 2003; Tidbury *et al.* 2011; Eggert, Kurtz & Diddens-de Buhr 2014). Furthermore, there has been little research into environmentally-dependent TGIP, or developmental stage-specific TGIP. One study found that the transmission of TGIP is dependent on the combination of parental and offspring diets (Shikano *et al.* 2015), which could be a possible explanation for the lack of TGIP found in certain species, such as *Aedes aegypti*, or *Drosophila melanogaster* (Voordouw, Lambrechts & Koella 2008; Linder & Promislow 2009). Finally, an overall understanding of the mechanism by which TGIP acts is unknown, although two recent papers suggest that the transfer of antimicrobial activity in eggs is likely to upregulate the immune system of offspring (Sadd & Schmid-Hempel 2007; Zanchi *et al.* 2012). Whether this is the case for other host-pathogen combinations must still be investigated, and this hypothesis also cannot explain demonstrated specificity in transgenerational immunity (Little *et al.* 2003).

1.4 The biology of Plodia interpunctella

Plodia interpunctella, commonly known as the Indian meal moth, is a small pyralid moth measuring approximately 1cm in length. This is the study organism that will be used in this thesis. *P. interpunctella* is widely distributed globally, and found on every continent on earth except Antarctica (Rees 2004). The first scientific description of its life stages was given by Hamlin (Hamlin, Reed & Phillips 1931), detailing the change from egg through five larval instars (distinguished by the size of the head capsule) to pupae and eclosion as adults. The entire life cycle takes approximately 40-45 days in laboratory conditions at QMUL. However, stadium duration is flexible, and is strongly influenced by food quality, temperature, and geographic strain (Mohandass *et al.* 2007). *P. interpunctella* is a tractable lab organism, as it can easily be kept on grain-based diets. Major areas of research using *P. interpunctella* as an insect model have included sexual selection (Gage 1998; Lewis, Wedell & Hunt 2011), host-parasite dynamics (Sait, Begon & Thompson 1994a; b; Knell, Begon & Thompson 1996), and their status as agricultural pests.

P. interpunctella is an economically important pest of stored products and is commonly found in post-harvest settings such as freight containers, warehouses and grain mills. The fact that they are found in warehouse environments means that lab environments can easily simulate their ecology. Lewis *et al* suggest that this means that measures of fitness taken in the laboratory will reflect those in the wild (Lewis *et al.* 2011). *P. interpunctella* larvae are dietary generalists, infesting food as diverse as grain, dried products such as fruit, nuts and seeds, and animal feed. Larvae create a support structure for feeding by spinning a silken web on top of and within the food. Further degradation occurs as frass and pupal cases are mixed within the web. Commercial

costs occur due to the requirement for discarding contaminated food, as well as the need for monitoring and extermination of the pest (reviewed in Mohandass *et al.* 2007).

A number of control options are available for the management of stored product pests, including fumigation (Campbell & Arbogast 2004), . However, the agricultural industry is beginning to move away from the use of inundative chemical pest control due to its environmentally damaging effects. Chemical pesticides are being increasingly regulated, for example the use of the fumigant methyl bromide is now controlled by the Montréal protocol in order to protect the ozone layer (*The Montreal protocol on substances that deplete the ozone layer*. 2000), and the U.S. Environmental Protection Agency had to re-evaluate all registered pesticides as a result of the passing of the Food Quality Protection Act in 1996, resulting in some products being removed from the market. A growing consumer demand for pesticide-free products coupled with the evolution of resistance to chemical insecticides means that finding alternatives is an ever-pressing concern for the future of grain production (Arthur 1996). Integrated pest management (IPM) is an environmentally-sensitive alternative which takes into account ecological information about the pest and combines multiple approaches to minimise health hazards, cost and environmental damage (Kogan 1998). IPM options for the management of *P. interpunctella* include the use of pheromone lures combined with traps (Campos & Phillips 2014), ultrasound to disrupt mating and oviposition (Svensson, Skals & Löfstedt 2003), inert dusts, and the release of parasitoids to control the egg and larval stages (Grieshop, Flinn & Nechols 2006). Biopesticides are also being explored as a control option, using bacterial and fungal entomopathogens in commercial formulations (Schnepf *et al.* 1998; Shah & Pell 2003; Sanahuja *et al.* 2011).

1.5 Life history traits in capital breeding Lepidoptera

In capital breeders, the production of progeny is financed by stored capital (i.e. resources that have already been obtained prior to breeding), as opposed to income breeders who reproduce using resources obtained as feeding adults, although in reality many species appear on a continuum between these two definitions (Tammaru & Haukioja 1996; Stephens *et al.* 2009). In capital breeding Lepidoptera such as *P. interpunctella*, the resources available for egg production should be present at the time of eclosion, so that female body size will be tightly linked to fecundity. Indeed, Tammaru *et al* found that larger capital breeding Lepidoptera produced 3.5 times more eggs than smaller females, and found no evidence for any costs of becoming larger, such as longer larval development time and therefore higher mortality (Tammaru, Esperk & Castellanos 2002). Honek 1993 found that this relationship held across all insect taxa, quantifying a 0.81% increase in ovariole number for each 1% increase in dry body weight (Honek 1993). As there is no need for adult foraging, capital breeders tend to display a shorter adult lifespan. The dichotomy between capital and income breeding has consequences for population dynamics; capital breeders tend to display eruptive patterns of population dynamics similar to those seen in *P. interpunctella* (Tammaru & Haukioja 1996).

While studies specifically in *P. interpunctella* have confirmed that female body size is correlated with fecundity, male pre- or post-copulatory mating success does not follow the same relationship (Lewis, Lizé & Wedell 2013), but is instead positively related to increased adult longevity. Male fitness is more likely to be determined by gaining paternity through sperm competition, and fitness is therefore associated with rapid

development time and increased longevity as adults, possibly leaving more time to court and mate with females (Lewis *et al.* 2011).

1.6 Aims of this thesis

The aim of this thesis was to gain a better understanding of how parental nutrition and exposure to pathogens influences the phenotype of offspring in *Plodia interpunctella.* Specifically, my aims were to:

1. Investigate the effects of maternal and paternal diet quality on offspring phenotype, and how this interacts with the offspring's own environment.
2. Develop a synthetic artificial diet for the rearing of *P. interpunctella* larvae to facilitate experiments in manipulating macronutrient content of the diet.
3. Determine the effects of manipulating macronutrient content and ratios of macronutrients within the diet on the phenotype of *P. interpunctella*.
4. Use the artificial diet to examine the transgenerational consequences of manipulating macronutrient resources available to the mother.
5. Examine how resource limitation and infection interacts with maternal immune priming with multiple pathogens.

Chapter 2: Methods – The development of a synthetic diet for investigating the effects of macronutrients on the development of *Plodia interpunctella*

Katherine Nunn performed the majority of lab work in section 2.5 with some assistance from me as part of her undergraduate honours project in 2014-2015.

2.1 Maintenance of the stock population of Plodia interpunctella at QMUL

A stock population of *Plodia interpunctella* has been maintained at Queen Mary since December 2011, which originated from an outbred population at the University of Leeds. The stock population is maintained in autoclave-proof plastic pots (Thermo Scientific™ Nalgene™, 13.5cm height × 11cm width) covered with net and secured with elastic bands, in a temperature controlled room at 27°C on a 12:12 light/dark cycle. To create the next generation, over 200 adults are placed in a funnel with both ends secured with net, and allowed to mate. The resulting eggs are collected and placed on laboratory food consisting of organic wheat bran (Mount Pleasant Mill, Lincolnshire), brewers' yeast and glycerol thoroughly mixed together in a 10:1:1 ratio, and the larvae are allowed to grow on unlimited food until adulthood. In between generation cycles, the pots are autoclaved to prevent the spread of disease. The population was maintained for 16 generations before the beginning of the first experiment (see chapter 4).

Figure 2.1: The stock population of *Plodia interpunctella* is fed on a bran-based diet.

2.2 Artificial diets

"Much of future entomology will likely continue to depend on diet-based programs." (Carson-Cohen 2004). Mass rearing programmes for insects already have important applications, which will only increase in importance as global food insecurity grows, and we are forced to become more reliant on insects within the human food chain. Artificial diets are a crucial aspect of mass rearing programmes for insects, and maximise cost- and time-effectiveness by eliminating the need for the maintenance of multiple trophic levels within a laboratory environment (e.g. supporting herbivorous insects and their plant hosts, or three trophic levels for the production of predatory insects). Ideally, both the yield and quality of insects would be maximised by the diet, while costly ingredients (i.e. protein) and waste from the metabolism of the diet would be minimised. Examples of the commercial importance of mass rearing programs using artificial diets are briefly discussed here; along with an outline of their use in evolutionary ecology studies.

Billions of individual insects are reared using artificial diets as part of integrated pest management strategies using sterile insect technique (SIT). Here, sterile males are released to mate with native females, reducing their reproductive success and hopefully leading to the suppression or elimination of a pest species (Knipling 1955; Krafsur 1998), and reducing the need for environmentally damaging chemical pesticides. For example, six artificial diets were used to maintain the larvae and adults of the New World screwworm in a mass rearing program for SIT. 100-500 million sterile insects were produced weekly, eventually resulting in the eradication of the screwworm from north and Central America, and North Africa (Chen *et al.* 2014). The maximisation of cost-effective rearing procedures, including the nature of the insect's laboratory diet, is

of crucial importance and has been described as a major obstacle to the implementation of further area-wide control programmes using insects which are otherwise amenable to SIT (Robinson & Hendricks 2005). Other industrial applications for using artificial diets to mass-rear insects include the production of insect predators to control greenhouse pests (Riddick 2008), and mass-rearing insects for the human food chain (entomophagy), which would limit the environmentally damaging effects of vertebrate meat production and over-harvesting of wild insects for consumption (van Huis 2011; van Huis *et al.* 2013).

On a smaller scale, artificial diets can used within a scientific research setting to rear insects as model organisms to answer a number of evolutionary and ecological questions (Vanderzant 1974). Diets can be completely chemically defined, so that the ratio of macronutrients (i.e. protein, carbohydrate, or lipid) is known and is also adjustable, which has facilitated recent research in nutritional ecology on the importance of various components of nutrition on different aspects of fitness such as lifespan, total egg production, or immune responses (Simpson & Abisgold 1985; Lee *et al.* 2006; Cotter *et al.* 2011; Povey *et al.* 2013; Harrison *et al.* 2014). Simpson and Raubenheimer have introduced the widespread use of the geometric framework, a visualisation and space-state modelling tool which allows life history traits to be modelled over a response surface of different intake quantities of macronutrients. This is only made possible by the use of multiple artificial diets containing different ratios of macronutrients while also varying the total available nutrition (Raubenheimer & Simpson 1999; Ponton *et al.* 2011b). Using the geometric framework, it has been possible to visualise the relationship between different macronutrients and the performance consequences of consuming them, even locating the individual nutritional optima for different traits such as reproduction or longevity, facilitating our knowledge

of resource-based life history trade-offs (Lee, Raubenheimer & Simpson 2004; Simpson & Raubenheimer 2009; Povey *et al.* 2009).

The limitations of artificial diets predominantly lie in the synthetic nature of their construction and structure. For example, chemically defined diets lack the complex structure of natural food items, such as plants, prey items or seeds. In these items nutrients are often highly compartmentalised, allowing insects to select their own nutritional profile according to their changing requirements such as during pathogen infection or at different stages of development (Carson-Cohen 2004). This is often not possible with artificial diets with many of them relying on a homogenous blend of nutrients, as the structural complexity of natural products is very difficult to mimic. Furthermore, plant chemicals often stimulate feeding or metabolic responses, which are not present in artificial diets if they are uncharacterised in the original plant or unavailable in a synthetic form. The texture or smell of the diet may differ from natural products, with the potential to affect the desirability or rate of consumption of the diet (Carson-Cohen 2004). Finally, it is not always possible to rear some species of insects on artificial food, especially predatory bugs, which thrive much better on natural or factitious prey items possibly because it is not yet possible to mimic their mode of consumption (Riddick 2008).

2.2.1 Development of an artificial diet suitable for rearing Plodia interpunctella

To investigate the effects of changing macronutrients ratios, a novel diet suitable for rearing *Plodia interpunctella* larvae was created. Artificial diets for *P.interpunctella* have been created in the past as it is a well-studied insect model organism, but these have not been chemically defined, and predominantly were created for ease of rearing *P.interpunctella* in laboratory conditions (Fraenkel & Blewett 1946; Silhacek & Miller

1972; Silhacek & Murphy 2006). For example, the diet described in Silhacek and Miller 1972 consists of a blend of whole wheat flour, yeast, glycerol, honey, wheat germ, white cornmeal, rolled oats, and dog food.

2.2.2 Essential components of insect diets

A significant proportion of material that insects consume is indigestible, and therefore the inclusion of non-nutritional components when creating an artificial diet is important in order to mimic natural food items. Inert material such as cellulose is indigestible to the majority of insects due to the presence of the β1-4 bonding sequence, although a few species such as termites possess cellulolytic symbionts which enable them to digest it (Martin, Jones & Bernays 1991). It possibly aids peristalsis in insects with larval stages (Carson-Cohen 2004), but predominantly functions in plant tissue as a mechanical barrier to herbivorous insects, which will have to chew through large amounts of plant matter in order to access the nutrients in the cytoplasm. This is even thought to be defensive by the plant, as large amounts of cellulose and a very dilute nutritional content increase stadium duration and decrease pupal mass (Lee *et al.* 2004).

Protein is the only macronutrient to contain nitrogen; an essential element for growth and reproduction. Most female insects require protein to matures their ovaries and eggs (Nation 2008). Disease resistance also requires protein, most likely due to the high protein-cost of immune defence. For example, *Spodoptera littoralis* have higher levels of constitutive immune defence, and are also more resistant to nucleopolyhedrovirus when they feed on a protein-rich diet, even selecting diets with a higher protein content once they have been infected (Lee *et al.* 2006). Although some artificial diets have been entirely constructed from purified free amino acids, Carson-Cohen recommends the use of bulk protein, as individual amino acids powders can desiccate the insect. Casein

contains all the essential amino acids in well balanced proportions (Carson-Cohen 2004).

Carbohydrates are a major energy source for insects, although most of them do not have a requirement for a specific quantity in order to grow. Carbohydrates can also be synthesised from amino acids and lipids (Nation 2008). Experiments in cockroaches have shown that carbohydrate is an important limiting factor for male pheromone production, and by increasing carbohydrate in the diet, male cockroaches became more attractive to females (South *et al.* 2011). In a similar vein, it is possible that carbohydrate may also be a limiting factor for males which have energy intensive courtship rituals, such as dancing in moths (although see Cordes *et al.* 2015).

Typical lipids in insects include free and bound fatty acids, steroids, acylglycerols, and phospholipids (Nation 2008). Specific fatty acids are critical for pupation and ecdysis, and without them metamorphosis will fail. The requirement for polyunsaturated fatty acids is usually fulfilled by linolenic or linoleic acid (Canavoso *et al.* 2001). In the diet formulations described in this chapter, fatty acids have been supplied in the form of linseed oil, which contains both of these in adequate proportions as well as other fatty acids such as palmitic acid and oleic acid. Fatty acids are also a rich source of energy (greater than protein sources) and their accumulation is particularly important in organisms which have non-feeding adult stages, such as some Lepidoptera species (Stockhoff 1993). Granivorous Lepidoptera, such as *Plodia interpunctella*, have quite a different lipase profile to folivorous Lepidoptera due to the prominent presence of triacylglycerol (TAG) lipids in their diet (Christeller, Amara & Carrière 2011). The fat body functions as a lipid storage facility where 90% of fat is stored as TAG – in which case no conversion is necessary from dietary fat to storage fat. All insects require

dietary sterols (a major difference from vertebrate nutrition) as a necessary precursor to the synthesis of ecdysone, the hormone necessary for moulting, and for a structural component in cell membranes (Canavoso *et al.* 2001). Finally, vitamins are needed as co-factors in glycolysis for the production of ATP. Vanderzant vitamin mixture for insects is typically used as a supplement in many artificial diet formulations (Carson-Cohen 2004).

There are several methods available for preserving artificial diets, including heating, freezing and the use of preservatives. Heating is recommended for killing bacteria and fungi; however it may also denature proteins and the structure of vitamins, or result in the Maillard reaction which reduces the nutritional value of the protein (Carson-Cohen 2004). Very rapid flash heating, similar to the pasteurisation process, can remove this problem because the ingredients are only heated for a very short amount of time. However, the expense of the equipment renders it unavailable for an academic project such as this one. Freezing could also change the structure and texture of the diets. Chemical preservatives were therefore selected as an appropriate method of diet preservation.

The physical state of the diet is important in achieving a high rearing success (e.g. solid, liquid etc.). For example, insects with sucking mouthparts are likely to be more successful on a liquid or micro-encapsulated diet, although the former can lead to high mortality if insects become trapped in a sticky liquid and drown (Tan, Wang & Zhang 2013). *Plodia interpunctella* larvae consume solid food, and often burrow and prefer to pupate within it (pers obs), possibly as a defensive mechanism. They also often drown in small quantities of liquid and appear unable to free themselves from it (pers obs). It was therefore important to create a solid diet to prevent drowning and to mimic natural

food conditions, but which would allow the soft-bodied larvae to burrow through the food.

2.3 Diet development using pilot experiments

2.3.1 Pilot 1: using desiccation as a method of preservation

This pilot experiment aimed to create a fine granular diet using desiccation as a method of preventing the growth of bacteria and fungi on the diets (Simpson & Abisgold 1985; Ponton *et al.* 2011a). Three diets were created using the formulations in Table 2.1. All the ingredients except the protein and vitamin mix were weighed out and added to a glass 150ml Duran. The ingredients were suspended in a 1% solution of autoclaved agar in a 1:2 ratio. Once the agar cooled to 37° the casein powder and vitamin mixture were added, and the entire solution was blended thoroughly. Diets were spread out on autoclaved foil and dried in a drying oven overnight, and in the morning crushed to create a fine powder. A third instar larva from the stock population was added to a 55 mm Petri dish with 1.2 grams of diet (n = 5 per treatment, including a control treatment consisting of the normal laboratory diet). The larvae were observed every three days; during which larval weight, mortality and metamorphosis was recorded.

Table 2.1: Percentage of each dietary component in Pilot 1.	30% cellulose	50% cellulose	70% cellulose
Cellulose	30	50	70
Casein	28.5	18.5	8.5
Sucrose	28.5	18.5	8.5
Wesson's salts	1	1	1
Vanderzant vitamin mixture	1	1	1
Cholesterol	1	1	1
Linseed oil	5	5	5
Glycerol	5	5	5

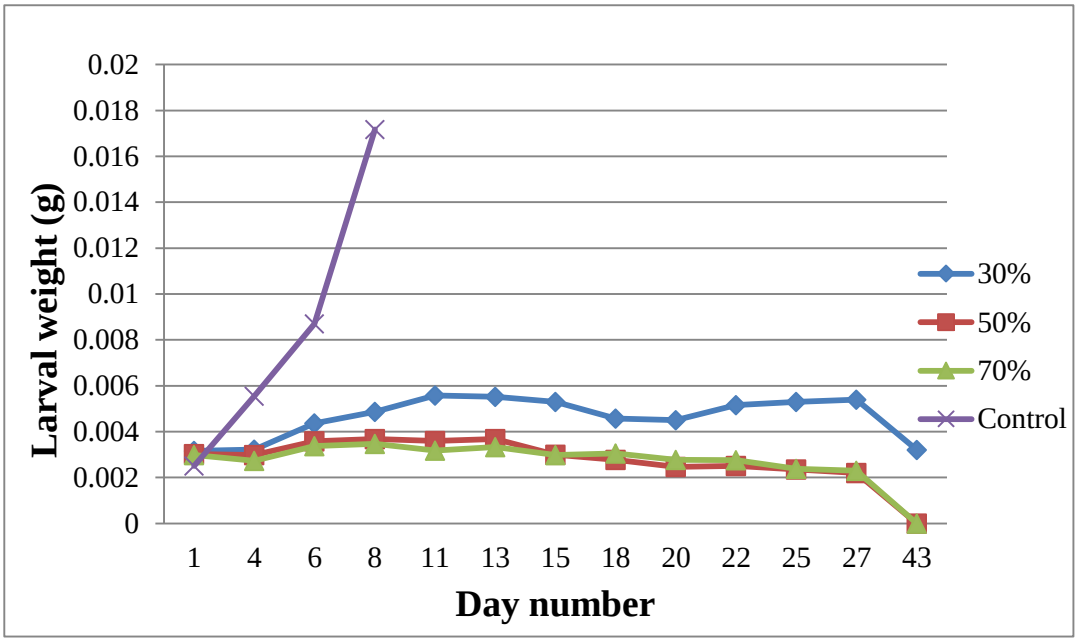

Figure 2.2: Larval weight of *P. interpunctella* larvae on the three dried artificial diets. Individuals that died were removed from the experiment. After eight days, all the control larvae (on the standard bran-based laboratory diet) had pupated. N = 5 per treatment.

As the mortality on the 50% and 70% cellulose diet was 100% before pupation (table 2.2), this method of dietary preservation was discarded because adult life history traits could not be observed, and when compared to the control larvae it was obvious that normal growth rate was not occurring. The poor growth rate could be due to the inaccessibility of the nutrients as the diet was too hard and dry for the larvae to consume. Several other formats were therefore experimented with.

Table 2.2: Percentage of larvae that pupate and eclose in Pilot 1

% of cellulose in diet	% larvae that pupate	% larvae that eclose
30	80	80
50	0	0
70	0	0
Control	100	100

2.3.2 Pilot 2: using gel format diets (dry ingredients suspended in agar)

Table 2.3: Percentage of each dietary component in Pilot 2	40% cellulose	50% cellulose	70% cellulose
Cellulose	40	50	70
Casein/peptone/albumen	7.83 each	6.17 each	2.83 each
Sucrose	23.5	18.5	8.5
Wesson's salts	1	1	1
Vanderzant vitamin mixture	1	1	1
Cholesterol	1	1	1
Linseed oil	5	5	5
Glycerol	5	5	5

The components (except the protein powders and vitamin mix) were weighed as before and placed in an autoclavable pot. The ingredients were suspended in a 1% solution of autoclaved agar in a 1:4 ratio. Once the agar cooled to 37° the casein powder and vitamin mixture were added, and the entire solution was blended thoroughly using a magnetic stirrer. The diets were placed in the fridge to set overnight. Problems were initially experienced with the gel format diets due to heavy mould growth, but this was remedied by adding methyl paraben (1g/1L food). Third instar larvae were added to a 55 mm Petri dish secured with net and elastic bands with 1g diet. Third instar larvae are used throughout the experiments using gel-based diet because smaller larvae have a tendency to drown in the moist diet, although complete growth from the egg stage to adulthood is possible (pers obs). Larvae were examined every 2-3 days until their death as adults for weight gain, metamorphosis and mortality (n = 12 per treatment, except the control bran diet where n = 8). When they were examined, the diet was changed simultaneously to prevent desiccation from the airflow in the temperature controlled room.

Table 2.4: Percentage of larvae that pupate and eclose in Pilot 2		
% of cellulose in diet	% larvae that pupate	% larvae that eclose
40	91.7	83.3
50	91.7	91.7
70	100	91.7
Control	100	75

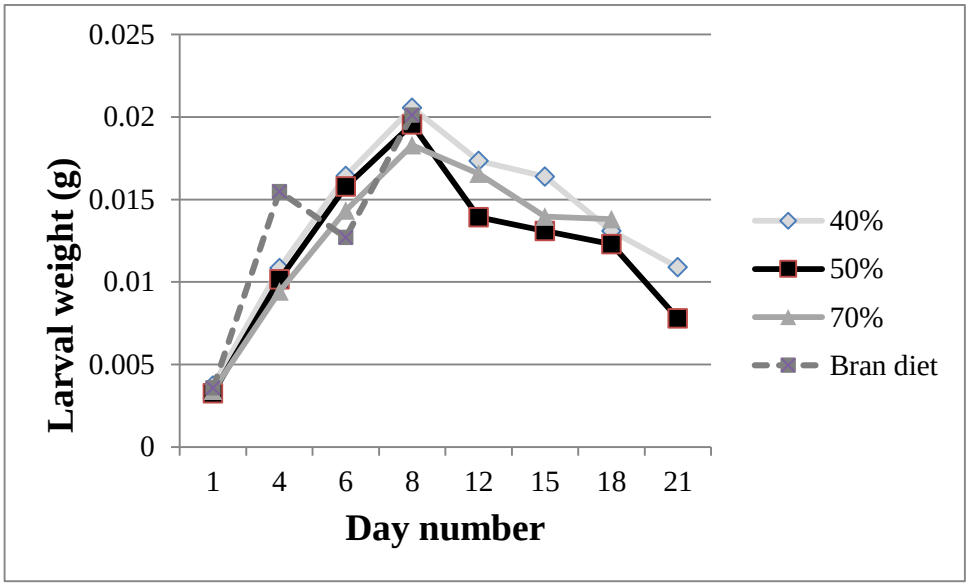

Figure 2.3: Larval weight in Pilot 2, consisting of the three diets in gel format (suspended in agar). All the larvae in the control bran diet had pupated by day 8. All the larvae in the 70% cellulose diet had pupated by day 18. In the 40% and 50% diet treatments one larva died before pupation (n = 12 per treatment, except the control bran diet where n = 8).

These artificial diets were successful in supporting the growth of larvae into adulthood, and were much more successful than the dried diets (table 2.4, figure 2.3). As the growth rate between the 40%, 50% and 70% cellulose diets did not seem to differ substantially, a third pilot experiment was carried out to examine whether *P. interpunctella* larvae can survive on agar alone, or agar supplemented with essential vitamins and minerals. It was important for my subsequent experiments to select diets

with a sufficient quantity of nutrients that the larvae would survive to adulthood, but at the same time produce measurable differences in life history traits that could be attributable to diet.

2.3.3 Pilot 3: can P. interpunctella *larvae survive on very low nutrient diets?*

A third pilot experiment was conducted to investigate whether *P. interpunctella* larvae can be supported by diets consisting solely of agar, as the macronutrient composition of previous gel-based formulations appeared to have little effect on larval growth rate. The diets were produced as above using the formulations in table 2.5; two diet treatments contained supplementary minerals, vitamins and lipids but no protein or carbohydrate. Third instar larvae were placed in Petri dishes with 1g of diet as before, and the diet changed every 2-3 days to prevent desiccation (n = 11 per treatment). They were observed every 2-3 days until they died.

Table 2.5: Percentage of each dietary component in Pilot 3	100% cellulose	93.5% cellulose	87% cellulose
Cellulose	100	93.5	87
Casein/peptone/albumen	0	0	0
Sucrose	0	0	0
Wesson's salts	0	0.5	1
Vanderzant vitamin mixture	0	0.5	1
Cholesterol	0	0.5	1
Linseed oil	0	2.5	5
Glycerol	0	2.5	5

All the larvae in this experiment died before they pupated, although larvae in the 87% cellulose group lived for substantially longer and gained more weight than the other treatments (figure 2.4). Larvae therefore cannot be supported by a solely agar-based diet.

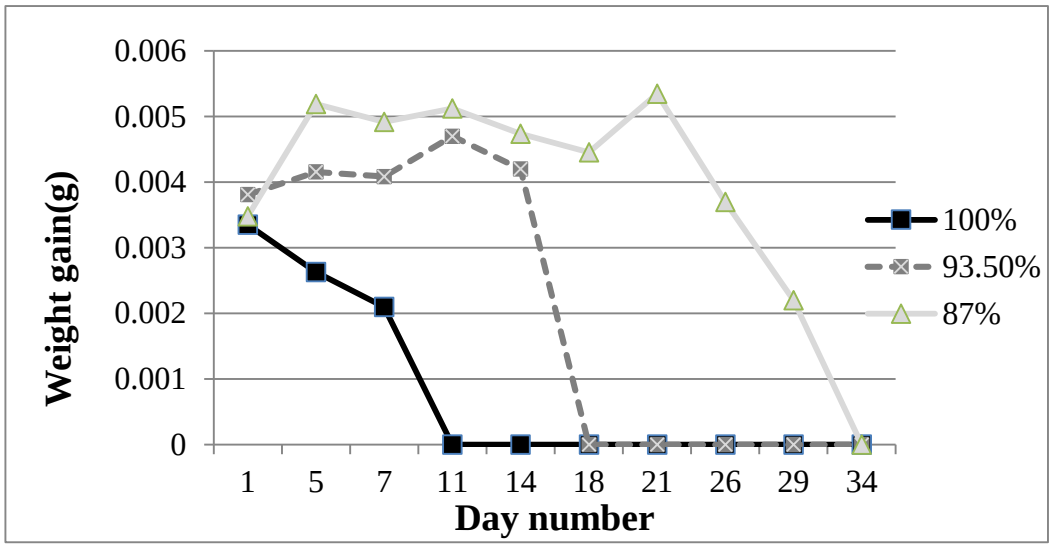

Figure 2.4: Weight gain in pilot 3; diet treatments refer to the percentage of cellulose (inert material) in the diet, i.e. 87% = 87% cellulose; n = 11 per treatment.

2.4 Final method for creating artificial diet suitable for rearing *P. interpunctella*

The dry ingredients excluding the protein and vitamin powders were weighed and added to an autoclavable pot, totalling 75 g per diet (see table 2.6). A 1% solution of agar was added in a 4:1 ratio to dry ingredients (300 mL of agar per diet). The mixture was thoroughly blended with a magnetic spinner and the solution left to cool to 37°C. The protein powders, methyl paraben preservative (1 g/1 L diet dissolved in ethanol) and vitamin mix were then added; the solution was blended again and set in the refrigerator at 4°C overnight. Using this artificial diet formulation, both the total quantity of nutrients and the ratio of individual nutritional components can be varied. As the percentage of cellulose (inert non-nutritive component) increases, the total amount of available nutrition decreases. Within each level of non-nutritional inert material (e.g. 30%, 50%, 70%), the amount of protein and carbohydrate can vary with respect to each other. Here high (2/3 protein, 1/3 carbohydrate) and low protein (1/3 protein, 2/3 carbohydrate) levels were nested within each total nutrition amount.

Table 2.6: Formulation for final artificial diet for the rearing of *P. interpunctella*

Diets (e.g. C30HP is 30% cellulose, High Protein) Quantities given in percentages (1st column) and grams (2nd).												
Ingredients	Diet 1: C30HP		Diet 2: C30LP		Diet 3: C50HP		Diet 4: C50LP		Diet 5: C70HP		Diet 6: C70LP	
Cellulose	30	22.5	30	22.5	50	37.5	50	37.5	70	52.5	70	52.5
Casein	13.11	9.83	6.56	4.92	8.67	6.50	4.33	3.25	4.22	3.17	2.11	1.58
Peptone	13.11	9.83	6.56	4.92	8.67	6.50	4.33	3.25	4.22	3.17	2.11	1.58
Albumen	13.11	9.83	6.56	4.92	8.67	6.50	4.33	3.25	4.22	3.17	2.11	1.58
Sucrose	19.67	14.75	39.33	29.50	13	9.75	26	19.5	6.33	4.75	12.67	9.50
Cholesterol	1	0.75	1	0.75	1	0.75	1	0.75	1	0.75	1	0.75
Wesson's salts	1	0.75	1	0.75	1	0.75	1	0.75	1	0.75	1	0.75
Glycerol (ml)	4	3	4	3	4	3	4	3	4	3	4	3
Linseed oil (ml)	4	3	4	3	4	3	4	3	4	3	4	3
Vitamin mix	1	0.75	1	0.75	1	0.75	1	0.75	1	0.75	1	0.75

2.5 Experiment to investigate the effect of artificial diet with different levels of total nutritional content on the longevity of *Plodia interpunctella*

2.5.1 Introduction

An experiment was conducted to examine the effect of the newly created artificial diets on the length of the developmental stages of *P. interpunctella*, their longevity as adults, and whether this differs from the length of life stages in moths reared on wheat bran-based laboratory diet. Three artificial diets were tested with different levels of total available nutrition (30%, 50%, and 70% non-nutritional content), which were selected using the preliminary data from pilot experiments in *2.3.1-2.4* to give a broad range of total nutritional content while still being able to support development. High protein (HP) diets were chosen, rather than a balance of protein and carbohydrate, to make the data comparable to that collected in chapter 4.

2.5.2 Methods

Three artificial diets were created using the methods outlined in section *2.4*, comprising of the 30HP, 50HP and 70HP formulations. Fresh lab diet was also created by mixing organic wheat bran, brewer's yeast and glycerol in a 10:1:1 ratio using a food mixer. Third instar larvae were collected from the stock population, and each larva was added to an individual 55 mm Petri dish with 0.85g (±0.05g) of one of the four diets (n = 50 per treatment). The diet was changed every 2-3 days which was sufficient to prevent desiccation. The larvae were monitored daily and time to pupation, eclosion and death as adults was recorded.

2.5.2.1 Statistical analysis

Data were analysed in R (version 3.0.1) using an ANOVA and post-hoc Tukey test to examine the differences between factor levels. Development time was analysed as the amount of time until eclosion (i.e. as larvae and pupae). Any larvae that did not eclose were removed from the analysis. Model validation plots were examined for violations of the assumptions of normality and homoscedasticity; and data in the analysis of development time were square-root transformed to meet these assumptions.

2.5.3 Results

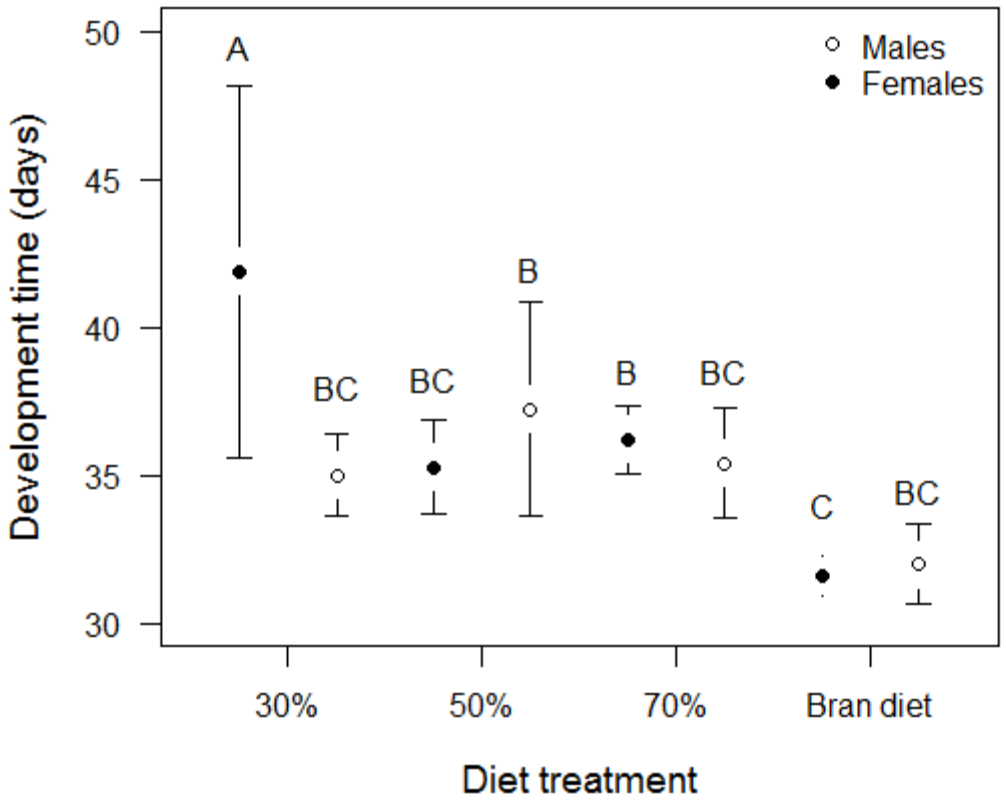

Figure 2.5: Effect of the interaction between sex and diet treatments on development time (days spent in the larval and pupal stages) (ANOVA, $F_{3,137} = 4.12$, $p = 0.00778$). Diet treatments are the percentage of non-nutritive inert material, i.e. 30% = 30% cellulose; filled circles are females, open circles are males; data points with a different

letter are significantly different from each other according to the results of a post-hoc Tukey test; bars are 95% confidence intervals.

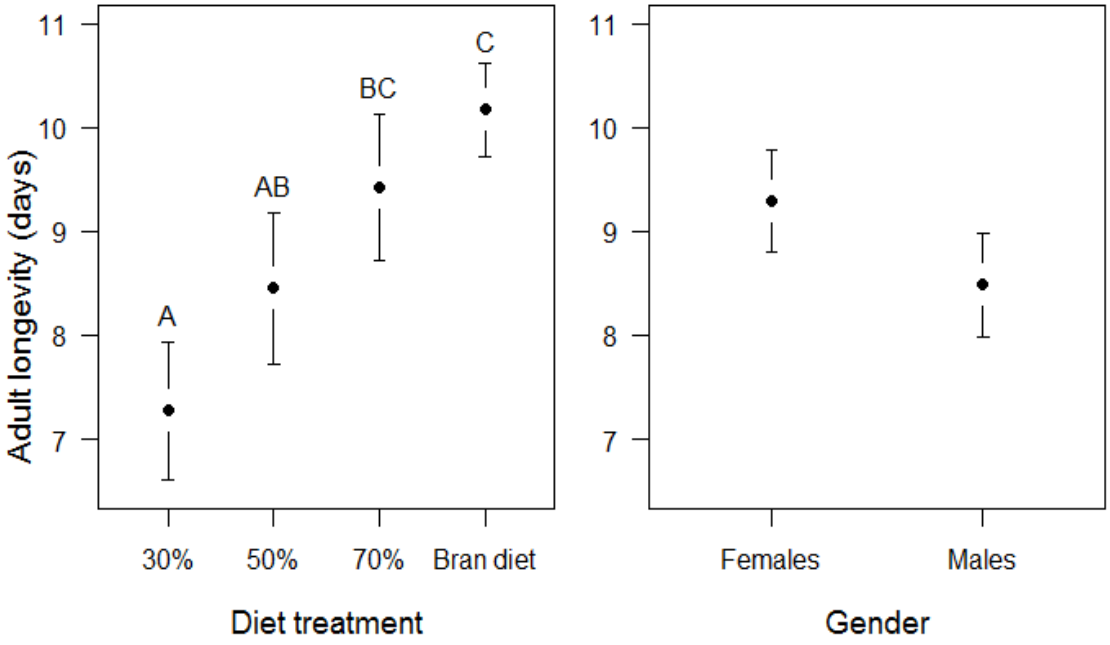

Figure 2.6: Left: There is a significant effect of larval diet treatment on adult longevity (ANOVA, $F_{3,137} = 16.99$, $p = <0.0001$); significant differences as a result of a post-hoc Tukey test are marked with a different letter; diet treatments are the percentage of non-nutritive inert material, i.e. 30% = 30% cellulose. Right: There is a significant effect of sex on adult longevity, with females living longer than males (ANOVA, $F_{1, 137} = 7.01$, $p = 0.00904$). The interaction between sex and diet treatment was not significant (ANOVA, $F_{3,137} = 2.095$, $p = 0.104$). Bars are 95% confidence intervals.

2.5.4 *Discussion*

Using gel-based formulations, I successfully created artificial diets that produced similar effects on longevity and development time when compared with the grainivorous diet that *P. interpunctella* usually consume. The larval and pupal development time on the 50% cellulose diet was not significantly different from the

bran diet in females. In males, none of the diets were significantly different from each other in terms of effects on development time. For both sexes, the 70% cellulose diet allowed *P. interpunctella* to achieve a similar adult lifespan comparable to the bran-based diet. Therefore, the artificial diets created are not so dissimilar to a grainivorous diet that we cannot infer realistic facts about the biology of *P. interpunctella* when we artificially manipulate their nutrition.

However, there were several interesting effects produced by the range of artificial diets examined here, which bodes well for experiments using *P. interpunctella* as a model organism to examine the effects of artificial macronutrient manipulation. For example, increasing the total amount of available nutrition decreased the adult lifespan. Individuals reared on the bran-based diet had the longest adult lifespan, and the artificial diet with the least available nutrition (70% cellulose) was comparable to this. This is surprising given that a greater ratio of nutritionally available material to inert components would increase the amount of resources available for acquisition during the larval stage, and therefore could intensify investment in traits such as longevity during the adult stage, potentially leaving more time to find a mate.

Several possible explanations for this are gaining traction within the nutritional ecology literature. Dietary restriction experiments have shown that limiting a component of nutrition can increase the organism's lifespan, sometimes with dramatic effects. This is an evolutionarily conserved mechanism with taxa as diverse as yeast, rotifers, insects, rats and primates showing similar effects of lifespan extension (Yu *et al.* 1982; Partridge, Piper & Mair 2005; Weithoff 2007; Colman *et al.* 2009). As yet there is still debate as to the precise nature of the limitation needed, for example caloric restriction or the limitation of one particular macronutrient, or even amino acid (Mair, Piper &

Partridge 2005; Masoro 2005; Grandison, Piper & Partridge 2009; Piper *et al.* 2011). This is extremely interesting on medical grounds as the Western world struggles to balance a lifestyle where surplus food is available with the health costs of eating too much.

Certain macronutrients may in fact be toxic when consumed in excessive quantities, and their presence could limit development in the same way as nutrients in dietary restriction. In this experiment, the artificial diet with the most protein and carbohydrate produced a detrimental effect on adult lifespan. Although we cannot say for certain in this study which macro- or indeed micro-nutrient is responsible for a toxic effect, the literature has pointed to an excessive amount of protein as detrimental to lifespan, possibly mediated by higher levels of reactive oxygen species, or DNA damage (Simpson & Raubenheimer 2009; Solon-Biet *et al.* 2014). Another interesting effect of the high macronutrient diet is the dramatic extension of the development time required by females to reach eclosion, which is longer than on any other diet treatment. This effect is particularly increased by four individuals that took longer than 50 days in the development period, possibly entering a diapause state induced by toxic elements in this diet. *P. interpunctella* larvae are able to enter a facultative pre-pupal diapause which can be induced by photoperiod, temperature, strain of origin or food quality (Williams 1964; Bell 1994; Wijayaratne & Fields 2012); and diapause seems to be an important life stage in most stored-product Lepidoptera (Bell 1994), where they are able to diapause in crevices of warehouses undisturbed. Termination of diapause then occurs when more favourable conditions resume.

To conclude, a synthetic artificial diet was created to support the development of *P. interpunctella* larvae. The ratio of available macronutrients within it can be

manipulated, increasing the tools available to us to use *P. interpunctella* as a model organism for the study of resource-based life history trade-offs and the effects of macronutrient imbalance.

2.6 Summary

This chapter describes the development of a synthetic diet that supports the development of *P. interpunctella* to adulthood. It is possible to vary both the total nutritional content of the diet, as well as the ratio of protein: carbohydrate macronutrients to each other. This will facilitate subsequent experiments in nutritional ecology examining the effects of macronutrients on life history traits and transgenerational effects of varying macronutrients. The artificial diet was created in a gel rather than dried format, after preliminary experiments showed that dried diets could not support development to pupation. I also tested whether larvae could survive on diets containing only micronutrients, but found that this would also not support their development to pupation. The final version of the diet was used to examine the effects of variation in total macronutrient content on development time and adult lifespan. Larvae that consumed the lowest total nutrient content had the longest adult lifespan, known in the literature as "dietary restriction". The diet with the highest protein content caused some larvae to have extended development times before eclosion, possibly as a result of entering diapause, which is common in unfavourable conditions amongst stored-product Lepidoptera.

Chapter 3: Transgenerational effects of diet in a stored product pest

I received technical assistance with sorting larvae for sampling from James Westmancoat in this chapter.

3.1 Introduction

Parental, or transgenerational, effects are defined as transfer of non-genetic information from parental environments which alters offspring phenotype (Mousseau & Fox 1998). Once regarded as an annoying source of variation to be eliminated in experiments, a slew of recent work has brought parental effects into prominence due to their probable role in providing a mechanism for the transgenerational inheritance of immunity in invertebrates (Bernardo 1996; Little *et al.* 2003; Sadd *et al.* 2005; Roth *et al.* 2010). However, it is important to investigate parental effects in the context of variation in the offspring's own environment, as it is intuitive that the majority of an organism's fitness will continue to be defined by its current environment (Beckerman *et al.* 2006). The present environment could also determine the influence of parental environments, for example resource-abundance could mask any detrimental constraints induced by parental stress (Jann & Ward 1999; Plaistow, Lapsley & Benton 2006). Research examining the interactions between parental and offspring resource levels on the phenotype of offspring have been severely neglected in the literature, which this study will address.

One such interaction is the adaptationist view that parents can predict the environment their offspring will experience, and alter offspring phenotype to maximise their fitness. Known as anticipatory parental effects, parents can benefit offspring when resources are spatially or temporally variable by the transfer of environmental cues (Marshall & Uller 2007). For example, seed beetle mothers allocate greater resources to eggs when they

are laid on seeds with thicker coats, helping offspring to bore through the extra distance (Fox *et al.* 1999). We therefore expect offspring to do better in a matched, rather than mismatched, environment to their parents. There is convincing evidence for this prediction from medical studies where mismatched levels of nutrition result in a higher prevalence of damaging non-communicable diseases; an immense public health concern given the rapid changes in global diet quality between generations (Roseboom, de Rooij & Painter 2006; Cleal *et al.* 2007). However, the quality of information transfer is uncertain in taxa which do not have intimate and prolonged contact between the generations through the shared phenotype of the mammalian placenta. Although the eggs of invertebrates are a shared phenotype of mother and offspring, it remains to be seen whether the shorter development window allows for adequate time for information transfer about the mother's external environment before the eggs become chorionated, although a snapshot of conditions at the time of egg creation could be transferred (Bonduriansky & Head 2007; Crean, Kopps & Bonduriansky 2014).

Non-adaptive parental effects are commonly reported in the literature, as by-products of changes in the parental environment. Defined as the parental stress hypothesis, parents constrained by the resources available to allocate to reproduction may produce offspring with lower fitness (Vijendravarma, Narasimha & Kawecki 2009). Examples have been found demonstrating the impact of poor parental nutrition acting to decrease offspring size, reduce the immune system, or decrease the capacity for sexual signalling in offspring (Frost *et al.* 2010; Franzke & Reinhold 2012; Triggs & Knell 2012). If widespread, the parental stress hypothesis has interesting implications for parent-offspring conflict as a parental resource investment will maximise the fitness of an individual, at the expense of other trade-offs within parental life history such as the production of future siblings (Russell & Lummaa 2009). However, it is important to

note that reported detrimental parental effects on the offspring phenotype have often been relatively modest, for example a 3-6% change in egg weight when parents experience a poor diet (Vijendravarma *et al.* 2009). Individuals may also be able to compensate for any detrimental parental effects if their own environment is resource abundant, in a similar manner to compensation for early-life nutritional stress (Jespersen & Toft 2003), or by trade-offs with other traits.

The transgenerational effects of maternal diet have been reasonably well-characterised in invertebrates, particularly as a factor affecting the progeny size-number trade-off (Mousseau & Dingle 1991; Fox & Czesak 2000). In good conditions induced by plentiful resources, classical life history theory predicts that mothers will invest in more numerous but smaller progeny, while the reverse situation in adverse conditions ensures that larger progeny have the best chance of surviving under greater competition (Smith & Fretwell 1974; Roff 1992). The influence of maternal diet is far reaching and can extend to shaping other fitness traits later in life, such as reproductive output, disease resistance and developmental time (Rossiter 1991; Bonduriansky & Head 2007; Frost *et al.* 2010; Stjernman & Little 2011; Hafer *et al.* 2011). By contrast, the ecological and evolutionary effects of paternal diet remain relatively understudied in invertebrates, perhaps due to the traditional lack of obvious mechanisms through which they can influence the egg (but see Hafer *et al.* 2011; Valtonen *et al.* 2012). Again, there are relatively few multifactorial designs that examine the interactions between maternal, paternal and offspring resource availability.

Although invertebrates do not typically demonstrate parental care in comparison to other taxa (although there are exceptions – see Wilson & Fudge 1984), there is plenty of scope for parental effects to be mediated by other means. Many experiments have investigated egg provisioning, as a maternal mechanism to allocate nutritional resources

to offspring, as the egg itself is a shared maternal and offspring trait (Bernardo 1996). mRNAs, antimicrobial compounds or even polyunsaturated fatty acids are also candidate molecules for mothers to transmit information about the future offspring environment within the eggs (Zanchi *et al.* 2012; Schlotz, Ebert & Martin-Creuzburg 2013). DNA methylation or histone modification comprise other likely mechanisms for reprogramming offspring phenotype, although research on these epigenetic changes were hindered until the relatively recent discoveries of functional CpG methylation systems in insects, and the apparent loss of DNA methyltransferases in some insect orders (Wang *et al.* 2006; Glastad *et al.* 2011). Fathers could contribute to offspring fitness through epigenetic information in the DNA, factors in the seminal fluid, or both – as a recent study shows that different aspects of resistance to *Bacillus thuringiensis* are transferred to genetic and step-offspring through these two pathways (Krawetz 2005; Crean & Bonduriansky 2014; Eggert, Kurtz & Diddens-de Buhr 2014). The paternal transfer of information also has a relationship with the mother's investment. By supplementing her reproductive resources with courtship gifts or nutrients, he will be able to increase her provisioning either to their immediate progeny through inclusion in the egg, or by increasing her somatic condition for future reproductive bouts, and thus her own resources can be expended at a later opportunity (Vahed 1998).

Owing to their different roles in the production and provisioning of offspring, the two sexes have different macronutrient requirements during their lives. Invertebrate females need to create and provision eggs, requiring protein, the only macronutrient to contain nitrogen (Wheeler 1996). Invertebrates are capable of self-selecting diets that contain the required macronutrients according to their needs, for example mated female *Drosophila* will choose a diet with a much higher protein:carbohydrate ratio than that chosen by males (Lee, Kim & Min 2013). Males are possibly less constrained by

specific macronutrient requirements, but at least one study indicates that they select diets for energy content in order to produce sexually attractive traits such as courtship rituals or pheromone production (South *et al.* 2011). If specific resources are limited, traits with similar nutritional optima may be involved in trade-offs. For example, protein is needed for growth and weight gain, but has also been implicated in disease resistance, probably due to the high protein costs of manufacturing immune cells and enzymes (Lee *et al.* 2006; Povey *et al.* 2009). By measuring multiple life history traits when we manipulate different parental and offspring environments, we begin to gain an understanding of the sensitivity of each trait to the nutritional environment of each generation.

3.1.1 Bacillus thuringiensis

Bacillus thuringiensis (Bt) was selected to be the model pathogen used to measure disease resistance in host offspring in this study. Bt is a gram positive bacterium commonly found in the soil, in insect cadavers, and less frequently, on plant surfaces (diagram in Raymond *et al.* 2010). Developed as a successful commercial biopesticide (Sanahuja *et al.* 2011), by 2011 100 million accumulated hectares of Bt transgenic crops had been planted in a bid to control mostly lepidopteran pest species (James 2011). It is therefore important to study this pathogen in conjunction with pest species such as *Plodia interpunctella*, which is particularly tractable as a model organism as the natural route of infection can be mimicked in laboratory conditions by oral inoculation (Raymond *et al.* 2010). After consumption, inactive protoxins are cleaved by host midgut proteases, resulting in protease resistant Cry and Cyt δ-endotoxins. These cause cell lysis in the midgut epithelium by binding to specific midgut receptors, resulting in pore formation in the cell membranes. Disruption of the midgut epithelium and lysis releases the cell contents, allowing Bt spores to germinate, leading to widespread

septicaemia and host death (Maagd, Bravo & Crickmore 2001; Bravo, Gill & Soberón 2007). In host larva, the midgut becomes paralysed and feeding stops (Heimpel & Angus 1959; Abdel-Razek *et al.* 1999). Death is easily assayed in larval *P. interpunctella* by the black appearance of the larvae, which preliminary tests show occurs within 48 hours.

While much research on Bt has focused on the applied aspects of pesticide development, its host-pathogen dynamics have also been examined in an evolutionary ecology context. Raymond et al do not find evidence for vertical transmission of Bt in the closely related diamondback moth *Plutella xylostella* (Raymond, Elliot & Ellis 2008), but experiments have shown that parental exposure to Bt contributes to offspring resistance to Bt in both *Ephestia kuehniella* and *Tribolium castanuem* (Rahman *et al.* 2004; Roth *et al.* 2010). Increased offspring survival against Bt as a result of paternal immune priming is attributable to epigenetic changes in the paternal contribution to DNA, while factors in the seminal fluid upregulate different components of the immune system (Eggert *et al.* 2014). To my knowledge, other experiments have not investigated the effects of other parental environmental variables on offspring resistance to Bt, such as diet.

In this experiment I investigate the interactions between maternal, paternal and offspring nutritional environments (‘good’ and ‘poor’ treatments which alter protein, carbohydrate and micronutrient dietary profiles) and examine the effect on multiple offspring life history traits including adult weight, development time, longevity and resistance to the microbial pathogen *Bacillus thuringiensis* (Bt).

3.2 Materials and methods

3.2.1 Multifactorial treatments

The parental generation was created by allowing c200 adult *Plodia interpunctella* from the stock population to mate in a funnel secured with net at both ends. After 24 hours the resulting eggs were placed on the 'poor' quality laboratory food, consisting of 20:1:1 organic wheat bran (Mount Pleasant Mill, Lincolnshire) to brewers' yeast and glycerol. 72 hours later, the process was repeated to collect another batch of eggs which were placed on 'good' quality food, comprising 10:1:1 wheat bran to brewers' yeast and glycerol. Larvae on poor food take longer to develop (unpublished data Laughton & Littlefair), so this method ensured that all the adult moths were ready to mate at the same time. Larvae were left to develop in the temperature controlled room at 27^0C on a 12:12 light/dark cycle. The experiment was performed in two blocks, staggered by approximately six weeks.

On days 25, 28 and 31 after egg laying pots were searched to sex the fifth instar larvae and separated according to sex. *P. interpunctella* larvae can be sexed by the appearance of red gonads on the dorsal plane in males. Upon eclosion, moths were mated in individual small chambers (5.4cm width × 4.5cm depth) in either Good×Good, Poor×Good, Good×Poor or Poor×Poor rearing condition combinations (Figure 3.1; n = 203 at the family level). In order not to select for early development in the parental generation, pairs were mated on subsequent days as more moths eclosed. The male was identified and discarded after 24 hours, and each day the female was placed into a new pot and her eggs counted daily, although only eggs from the first 48 hours after mating were used to create the offspring generation. To create fully factorial combinations of parental and offspring diet treatments, each female's eggs were divided between good

and poor food. To remove possible confounding effects mediated through offspring density, pots had a maximum of 25 eggs allocated to them, which allowed the larvae access to plentiful food.

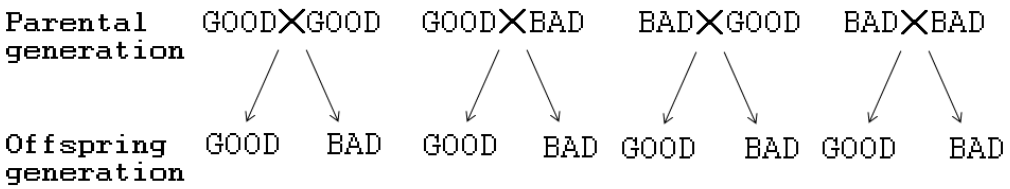

Figure 3.1: Multifactorial experimental design. Parents and offspring were arranged in complete combinations of good (10:1:1 wheat bran: brewers' yeast: glycerol) and poor food (20:1:1).

3.2.2 Disease resistance assay

Offspring larvae were allowed to develop until fourth instar (approximately day 15 in the QMUL population) when the pots were searched to select larvae for the disease resistance assay (n = 1189). Wherever possible five larvae per family were selected, but if this was not available, as many as possible of the appropriate size were used. 0.45mg/ml solution of *Bacillus thuringiensis* (DiPel formulation, ProGreen Weed Control Solutions Ltd, South Fen Business Park, Lincs) was created in a saturated sugar solution with 10% blue food dye (to enable visualisation of successful droplet dosing). Larvae were allowed to consume a 1µl drop of Bt solution (figure 3.2), and following dosing were transferred into the individual cells of 12cm × 12cm square petri dishes with ad lib food in each cell, and kept at 26^0C in an incubator with a 12:12 light/dark cycle. Mortality was assayed 48 hours after the initial infection, as trial experiments had determined that the majority of mortality occurred within this time. Two larvae per

family were dosed with a control sugar solution to assess the background rate of mortality and test for any Bt contamination. No control larvae died within the course of this experiment, or displayed any symptoms of disease, and they were therefore excluded from the analysis.

Figure 3.2: Larvae were infected with Bt suspended in sugar solution and 10% blue food colouring, and inoculated orally.

3.2.3 Offspring weight and longevity

When the remaining larvae had reached fifth instar the pots were searched again, and the larvae were placed into individual 55mm petri dishes with ad lib good or poor food (figure 3.3).

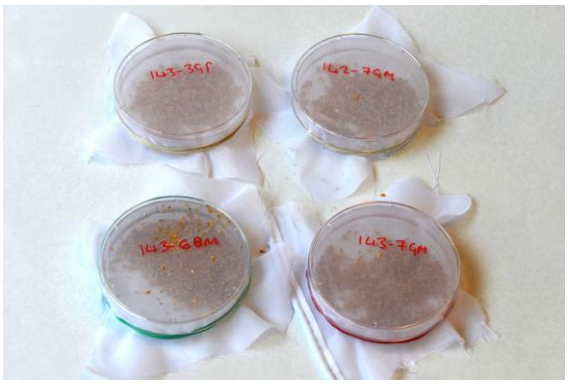

Figure 3.3: Larvae were kept in individual petri dishes whilst longevity was measured, and monitored daily for pupation, eclosion and death.

They were either assigned to be weighed at eclosion (n = 1156) or to a longevity experiment (n = 993). All the moths were checked daily for eclosion, and those to be

weighed were sacrificed by freezing and the weight measured on a Mettler Toledo UMX2 balance (d=0.1ug). Those in the longevity group were kept in the temperature controlled room and checked daily for death at 11am each day.

3.2.4 Statistical analysis

The data from the experiment were analysed using mixed effects models which were fitted using the nlme and lme4 packages (Bates *et al.* 2014; Pinheiro *et al.* 2014) in R (version 3.0.1 R Development Core Team 2014). In all cases family was nested within parental mating date, and included as a random intercept. Block was included as a fixed effect, rather than as a random effect, because there were only two levels and therefore not enough variation to justify including it as a random effect. In all models, higher-order interactions such as three- or four-way interactions were not initially fitted to avoid over-parameterising the models; as such interactions are also difficult to interpret in a biologically meaningful way. However, if preliminary analysis indicated that they contributed towards explaining variation in the model, they were included in the final fit (Zuur *et al.* 2009).

Terms were sequentially dropped from the initial models, with interaction terms first, until the minimal adequate model was reached. During model reduction, models were fitted with the Maximum Likelihood method in order to compare nested models using the likelihood ratio test. Model reduction was not performed on the random effects, because these reflect the design of the experiment. Missing values were excluded from the analyses in order to facilitate model comparison. Graphical model validation was performed on the minimal adequate models, to test for heteroscedasticity, normality and patterns in the model residuals.

3.2.4.1 Models for total fecundity

Although this response variable is count data, Poisson/quasiPoisson distributions were not used in this instance because the Poisson distribution tends towards the normal distribution as the mean becomes larger. The response variable was initially transformed with the square root transformation to meet the assumptions of normality in the model validation plots, but this was eventually removed in the final model because it made no qualitative difference to the terms selected for inclusion. Maternal treatment, paternal treatment and maternal longevity were included as initial fixed terms, along with two-way interactions between maternal and paternal diets, paternal diet and maternal adult lifespan, and maternal diet and maternal adult lifespan.

3.2.4.2 Models for offspring eclosion weight

The response variable offspring eclosion weight was log-transformed to reduce heteroscedasticity that appeared in the residuals when models were fitted to non-transformed data. The initial model was fitted with two-way interactions between maternal diet, paternal diet, offspring diet and offspring sex as the initial fixed terms, but preliminary analysis suggested that a three way interaction between maternal diet, offspring diet and offspring sex would best explained the model variation. Models were fitted with the lme function from the nlme package in R (Pinheiro *et al.* 2014).

3.2.4.3 Models for Bt resistance assay

The binomial response variable of alive/dead was coded as dead=0, alive=1. Survival data were analysed with a binomial mixed effects model using the function glmer from the lme4 package (Bates *et al.* 2014). The model was fitted using the Laplace approximation and a logit link. Maternal diet, paternal diet, offspring diet, offspring sex

and weight at the time of infection were included in the initial model along with their two-way interaction terms.

3.2.4.4 Longevity models

Adult lifespan (time from eclosion until time to death) and developmental lifespan (time from egg laying until time to eclosion) were analysed separately with mixed effects survival models in the R package "coxme" (Therneau 2012).

3.3 Results

3.3.1 Maternal fecundity (table 3.1)

There was no effect of maternal diet quality on female fecundity (likelihood ratio = 0.177, df = 1, p=0.674). Male treatment did not influence female lifetime fecundity (likelihood ratio = 0.571, df = 1, p=0.450), and did not interact with maternal treatment (likelihood ratio = 0.328, df = 1, p=0.567) or female lifespan (likelihood ratio = 0.170, df = 1, p=0.193) to influence female fecundity either. The block term was removed as an explanatory variable, as it had no significant effect on fecundity (likelihood ratio = 2.63, df = 1, p=0.105). Female lifespan had a strong negative correlation with female fecundity (likelihood ratio =13.3, df = 1, p=0.0003, figure 3.4) – for every extra day alive, the female produced approximately 10 fewer eggs overall – indicative of a possible trade-off between reproduction and longevity. However, female lifespan was also strongly influenced by their fecundity (model not shown here), and causality could run in either direction, or also be influenced by a third underlying variable, such as a 'live fast, die young strategy'.

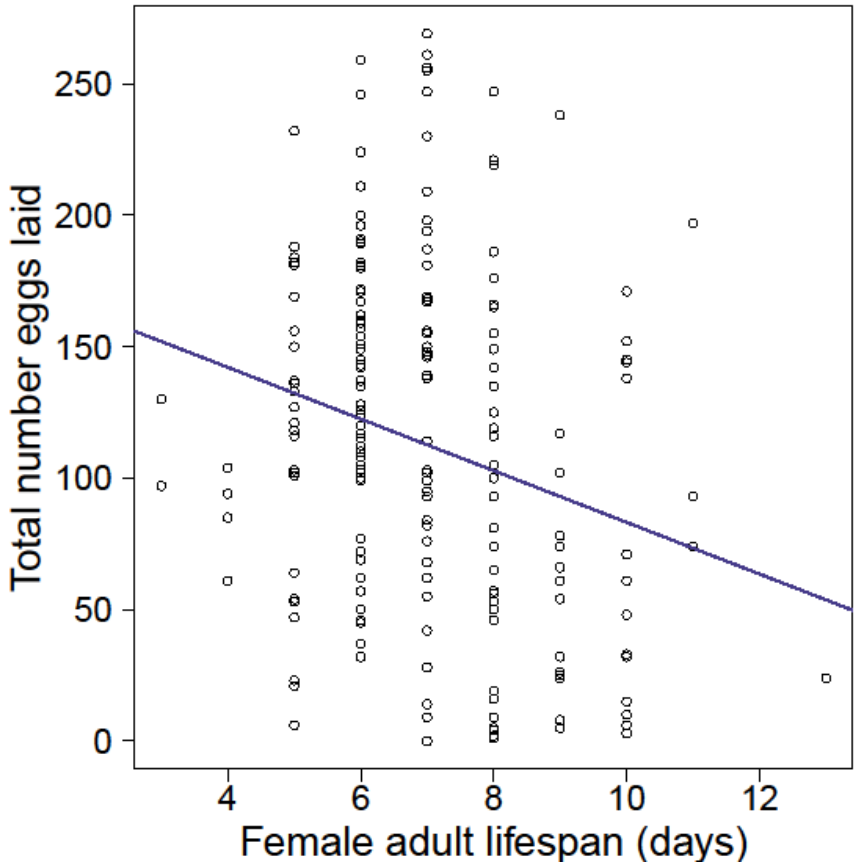

Figure 3.4: There is a negative relationship between lifetime fecundity and maternal lifespan, which is not influenced by maternal diet treatment.

Table 3.1: Total number of eggs laid by mother

Initial model: total eggs ~ maternal diet + paternal diet + female lifespan (and all two way interactions) + mother container + father container + block, random=~1|date

n=203

Standard deviations of random effects

Date

Intercept: 21.6

Residual: 60.4

Fixed effects

Variable	**Estimate**	**Standard error**	**Degrees of freedom**	**Likelihood ratio**	**P value**
Intercept	182	20.0			
Maternal diet:Female lifespan			1	0.0102	0.920
Maternal diet:Paternal diet			1	0.328	0.567
Paternal diet:Female lifespan			1	1.70	0.193
Father container			5	2.93	0.711
Mother container			5	4.88	0.430
Maternal diet			1	0.177	0.674
Paternal diet			1	0.571	0.450
Block			1	2.63	0.105
Lifespan	-9.88	2.64	1	13.3	0.0003
Minimum adequate model: total eggs ~ lifespan, random = ~1|date					

3.3.2 Offspring eclosion weight (table 3.2)

There were very large differences in weight between the sexes (females weighed 64.7% more than males). Eclosion weight of offspring was affected by a three-way interaction term between their own diet, maternal diet and offspring sex (figure 3.5, likelihood ratio = 4.38, df = 1, $p = 0.0364$). Sons appeared to be less affected by nutritional stress either in their own environment or from the maternal environment than female offspring, but the greatest differences in weight were as a result of changing maternal diet irrespective of their own, weighing an average of 5.86mg when laid by mothers on good diets and

5.69mg when laid by mothers on poor diets. In contrast, daughters appeared to be much more sensitive to the interaction between the maternal and current nutritional environment. They did best when both mothers and daughters received a poor diet (weighing 9.83mg), but weighed 6.37% less when eggs from mothers that had received a good diet were allocated to the mismatched poor diet treatment. When offspring and mothers received a good diet, they did better than the mismatched treatments but not as well as when they both received a poor diet, weighing 9.60mg.

Weak interactions between paternal diet and offspring diet quality also influenced weight at eclosion (likelihood ratio = 4.00, df = 1, $p = 0.0454$). When paternal diet was matched to offspring diet, adult offspring of both sexes weighed more than if the diet were mismatched between generations, irrespective of whether this diet quality was good or poor. This could indicate the presence of a possible anticipatory paternal effect, although it should be noted that this result was close to non-significance. Block was retained in the model as it was statistically significant (likelihood ratio = 10.4, df = 1, $p = 0.0013$).

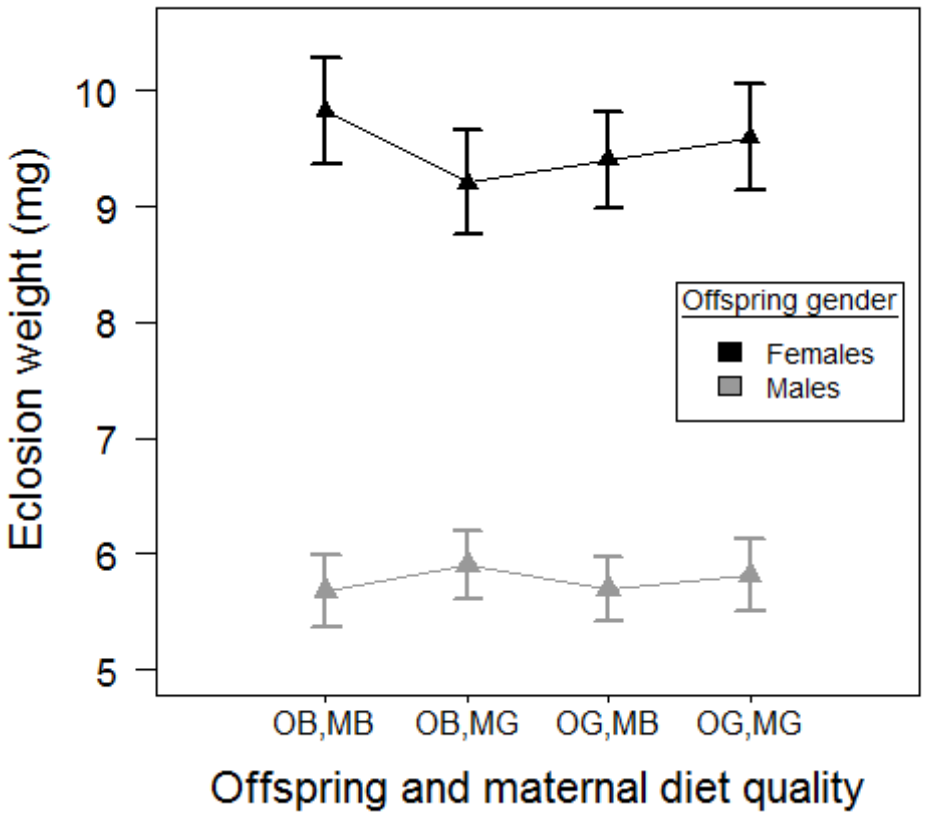

Figure 3.5: There was a significant three-way interaction between maternal diet quality, offspring diet quality and offspring sex influencing larval weight at eclosion in mg. Bars are 95% confidence intervals; X axis displays the interaction between offspring and maternal diets (O = offspring, M = maternal, B = bad diet (20:1:1 wheat bran, Brewer's yeast, glycerol), G = good diet (10:1:1).

Table 3.2: Offspring eclosion weight

Initial model: log(Offspring weight) ~ maternal diet + paternal diet + offspring diet + sex (and all two way interactions) + offspring diet: maternal diet: offspring sex + block, random=~1|date/family

n=1156

Standard deviations of random effects

Date

Intercept: 0.00001

Family code in date

Intercept: 0.069

Residual: 0.217

Fixed effects

Variable	**Estimate**	**Standard error**	**Degrees of freedom**	**Likelihood ratio statistic**	**P value**
Intercept	2.33	0.0274			
Paternal diet: sex			1	0.345	0.557
Maternal diet: Paternal diet			1	3.21	0.073
Offspring diet: sex	0.0515	0.0362			
Maternal diet: Offspring diet	0.0802	0.0361			
Maternal diet (good)	-0.0774	0.0299			
Paternal diet (good)	-0.0332	0.0237			
Offspring diet(good)	-0.0689	0.0279			
Sex (male)	-0.558	0.0261			
Maternal diet (good): sex (male)	0.116	0.0367			
Paternal diet (good):offspring diet (good)	0.0522	0.0262	1	4.00	0.0454
Offspring diet (good): maternal diet (good): sex (male)	-0.108	0.0516	1	4.38	0.0364
Block	-0.0751	0.0200	1	10.4	0.0013

Minimum adequate model: off.weight ~ (maternal diet + sex + offspring diet) – and all their interactions + paternal diet:offspring diet + paternal diet + block, random=~1|date/family

3.3.3 Disease resistance assay (table 3.3)

Offspring were 11.9% more resistant to infection with Bt when they had access to good nutrition compared with poor nutrition (figure 3.6, $\chi^2 = 21.4$, 1df, $p < 0.0001$). Males were less resistant to Bt than females ($\chi^2 = 5.83$, $p = 0.0157$); of males infected 47.1% lived compared to 52.4% of females. Heavier larvae were significantly less resistant to Bt ($\chi^2 = 6.17$, $p = 0.0130$). I fitted additional models to check whether larval weight was influenced by offspring diet, which could be contributing to increased resistance on good diets (model not shown here). In this model, nutritionally stressed larvae were heavier, but only when their mothers also experienced a poor diet, and sex had no effect on weight. It therefore seems unlikely that increased resistance on good diets is attributable to smaller larvae, that for example might drink less of the Bt solution. Furthermore, every effort was made to ensure that larvae drank all of the solution, and they were observed until they had finished the drop. Finally, parental environment did not influence offspring disease resistance, nor were any of the two-way interactions significant (table 3.3).

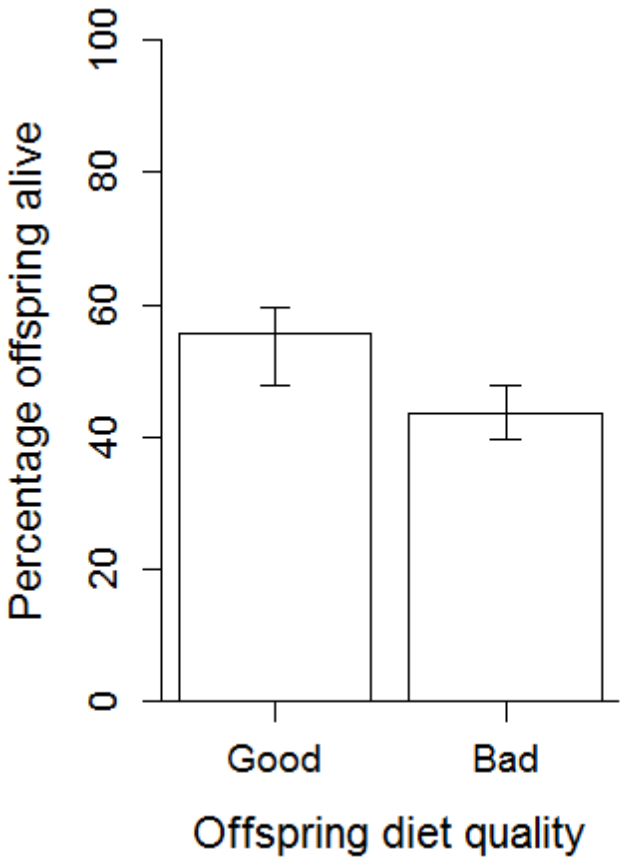

Figure 3.6: Offspring do better when challenged with a Bt infection when they fed on a good diet; error bars are Agresti-Coull intervals calculated in the "binom" R package (Dorai-Raj 2014).

Table 3.3: Survival status after infection with Bt

Initial model: death ~ maternal diet + paternal diet + offspring diet + sex + weight (and all two way interactions) + block + (1|date/family), family=binomial

n=1189

Standard deviations of random effects

Date

Intercept: 0.521

Family code in date

Intercept: 0.305

Fixed effects

Variable	**Estimate**	**Standard error**	**Chisq value (df difference between models)**	**P value**
Intercept	0.399	0.359		
Maternal diet: Offspring diet			0.0011 (1)	0.974
Maternal diet: Sex			0.0071 (1)	0.933
Sex: Weight			0.0211 (1)	0.884
Maternal diet: Weight			0.190 (1)	0.663
Maternal diet: Paternal diet			0.317 (1)	0.573
Offspring diet: Sex			0.471 (1)	0.492
Paternal diet: Weight			0.856 (1)	0.355
Paternal diet: Offspring diet			1.36 (1)	0.243
Paternal diet: Sex			2.90 (1)	0.887
Offspring diet: Weight			2.64 (1)	0.104
Block			0.00220 (1)	0.963
Maternal diet			0.163 (1)	0.686
Paternal diet			0.530 (1)	0.467
Offspring diet (good)	0.572	0.125	21.4 (1)	<0.0001
Sex (male)	-0.296	0.123	5.83 (1)	0.0157
Weight	-125	54.1	6.17 (1)	0.0130
Minimum adequate model: death ~ offspring diet + sex + weight + (1|parent.date/family), family=binomial				

3.3.4 Development time (tables 3.4, 3.5, 3.6)

The initial model showed that males were faster to develop than females (measured as time to eclosion, including the larval and pupal stages) by 0.39 days (figure 3.7, χ^2 = 32.6, $p < 0.0001$). The interaction between diet and sex was marginally non-significant (table 3.4, $\chi^2 = 3.45$, $p = 0.0633$), and when experimental blocks were analysed separately, this interaction was significant in block 1 (table 3.5, figure 3.8, $\chi^2 = 4.19$, $p = 0.0407$). Males had a shorter development time on bad food when compared with the good food, whereas female development time was not affected to the same extent by diet quality. Figure 3.8 shows that block 2 followed the same pattern, although was not significant. There were large quantitative differences between blocks, with larvae in block 1 developing an average of 1.71 days faster than those in block 2 (table 3.4, χ^2 = 13.6, $p < 0.0001$). Finally, parental environment did not affect development time.

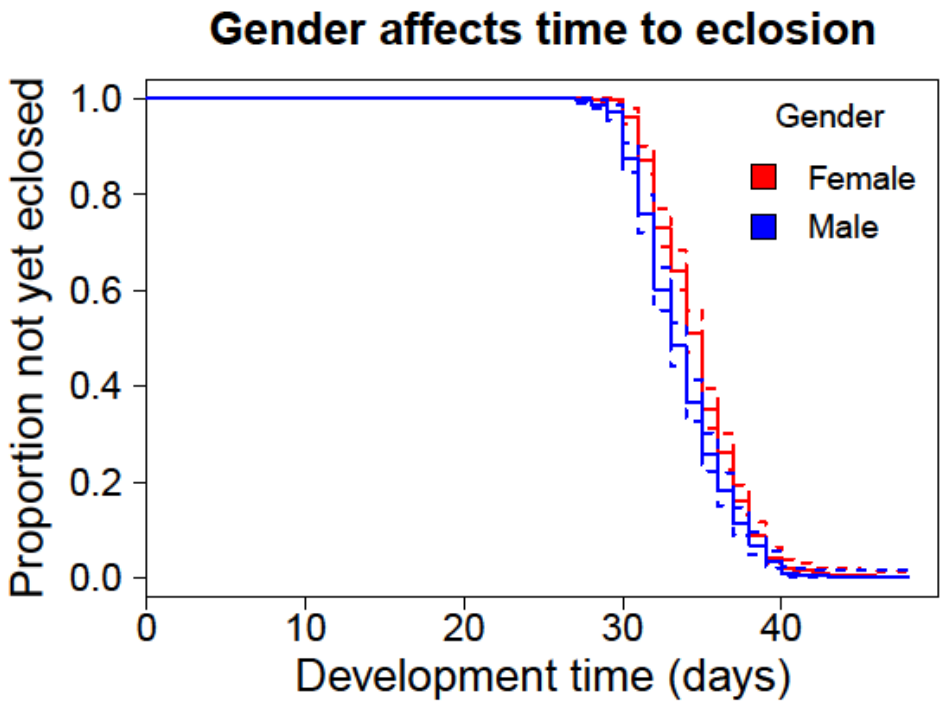

Figure 3.7: Male larvae and pupae have a faster development time compared to females

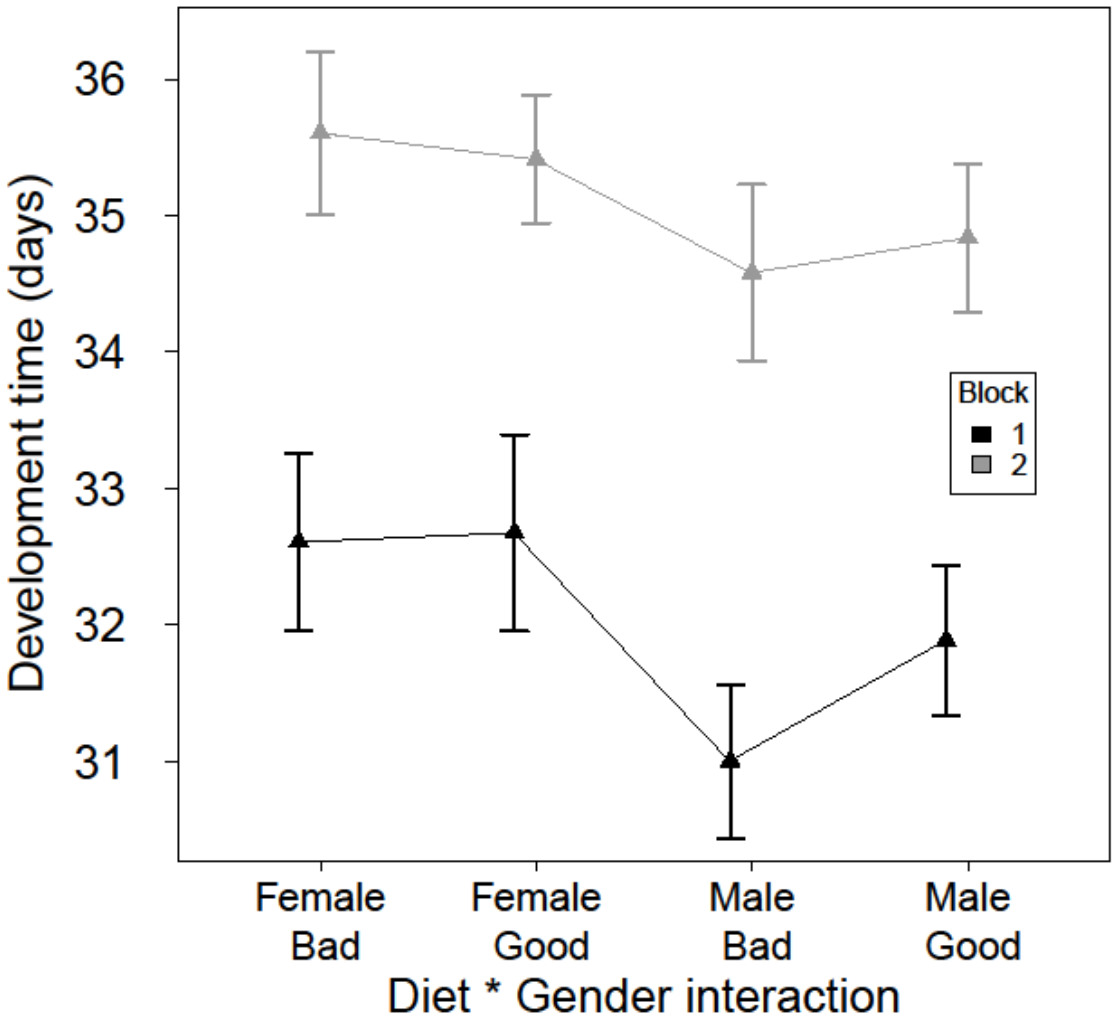

Figure 3.8: Larvae and pupae in block 2 developed slower than those in block 1. Development time was defined as the number of days taken in larval and pupal stages, before eclosion. Males in block 1 developed faster on poor food, but this trend was not significant in block 2 (offspring diet × sex interaction), although the pattern followed a similar trend. Bars are 95% confidence intervals.

Table 3.4: Offspring development time

Initial model: development time ~ maternal diet + paternal diet + offspring diet + sex (and all two way interactions) + block, random=~1|date/family
n=990

Standard deviations of random effects

Date
Intercept: 0.0202

Family code in date
Intercept: 0.635

Fixed effects

Variable	**Estimate**	**Standard error**	**Chisq value (df difference between models)**	**P value**
Paternal diet: Sex			0.0506 (1)	0.822
Maternal diet: Offspring diet			0.107 (1)	0.744
Maternal diet: Paternal diet			0.197 (1)	0.657
Maternal diet: Sex			1.07 (1)	0.300
Paternal diet: Offspring diet			1.95 (1)	0.163
Offspring diet: Sex			3.45 (1)	0.0633
Paternal diet			0.0198 (1)	0.888
Maternal diet			0.305 (1)	0.581
Offspring diet			0.124 (1)	0.724
Sex (male)	0.390	0.0681	32.6 (1)	<0.0001
Block (block 2)	-1.71	0.141	13.6 (1)	0.000227

Minimum adequate model: development time ~ sex + block, random=~1|date/family

NB: Offspring diet: sex interaction is almost significant – p = 0.0633

Table 3.5: Offspring development time – block 1

Initial model: development time ~ maternal diet + paternal diet + offspring diet + sex (and all two way interactions), random=~1|date/family

n=295

Standard deviations of random effects

Date

Intercept: 0.170

Family code in date

Intercept: 0.421

Fixed effects

Variable	**Estimate**	**Standard error**	**Chisq value (df difference between models)**	**P value**
Maternal diet: Offspring diet			0.0022 (1)	0.962
Maternal diet: Paternal diet			0.217 (1)	0.641
Maternal diet: Sex			0.500 (1)	0.479
Paternal diet: Sex			0.616 (1)	0.433
Paternal diet: Offspring diet			1.14 (1)	0.286
Maternal diet			0.743 (1)	0.389
Paternal diet			2.81 (1)	0.0935
Sex (male)	0.909	0.186		
Offspring diet (good)	0.110	0.182		
Offspring diet (good): sex (male)	-0.523	0.248	4.19 (1)	0.0407

Minimum adequate model: development time ~ sex + offspring diet + sex: offspring diet, random=~1|date/family

Table 3.6: Offspring development time – block 2

Initial model: development time ~ maternal diet + paternal diet + offspring diet + sex (and all two way interactions), random=~1|date/family

n=695

Standard deviations of random effects

Date

Intercept: 0.0202

Family code in date

Intercept: 0.622

Fixed effects

Variable	**Estimate**	**Standard error**	**Chisq value (df difference between models)**	**P value**
Maternal diet: Offspring diet			0.00270 (1)	0.958
Maternal diet: Paternal diet			0.150 (1)	0.698
Paternal diet: Sex			0.352 (1)	0.553
Paternal diet: Offspring diet			0.748 (1)	0.387
Offspring diet: Sex			1.59 (1)	0.207
Maternal diet: Sex			3.21 (1)	0.0731
Paternal diet			0.0204 (1)	0.886
Offspring diet			0.173 (1)	0.677
Maternal diet			1.29 (1)	0.256
Sex (male)	0.276	0.0791	12.12 (1)	0.000498
Minimum adequate model: development time ~ sex + diet, random=~1|date/family				

3.3.5 Adult lifespan (table 3.7)

There is a highly significant main effect of offspring diet; larvae reared on poor diets had a longer lifespan after eclosion by approximately 1 day when compared with those receiving good nutrition (figure 3.9, $\chi^2 = 157$, $p < 0.0001$). Adult lifespan is also influenced by a three-way interaction between maternal diet, paternal diet and their sex (figure 3.10, $\chi^2 = 5.62$, $p < 0.0177$). Male offspring had a shorter adulthood by

approximately half a day when both of their parents have a good diet, and the longest adulthood when their mothers had a good diet and fathers were nutritionally stressed. Female offspring have the shortest adult lifespan when both their parents had good diets and the longest when both had a bad diet.

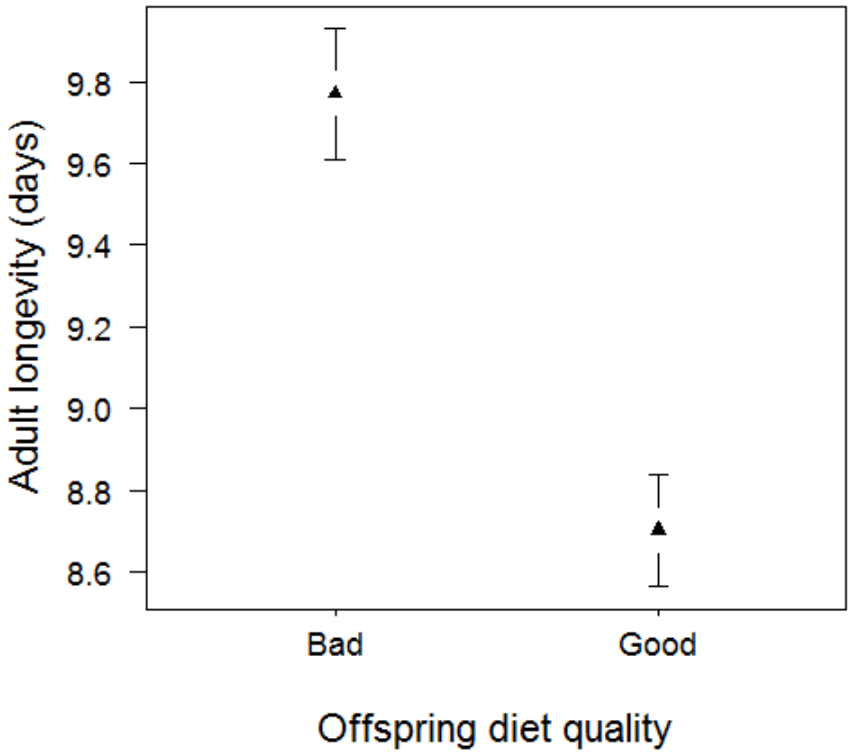

Figure 3.9: There is a highly significant effect of larval diet quality on adult longevity across both sexes and for all combinations of maternal and paternal diet treatments; bars are 95% confidence intervals.

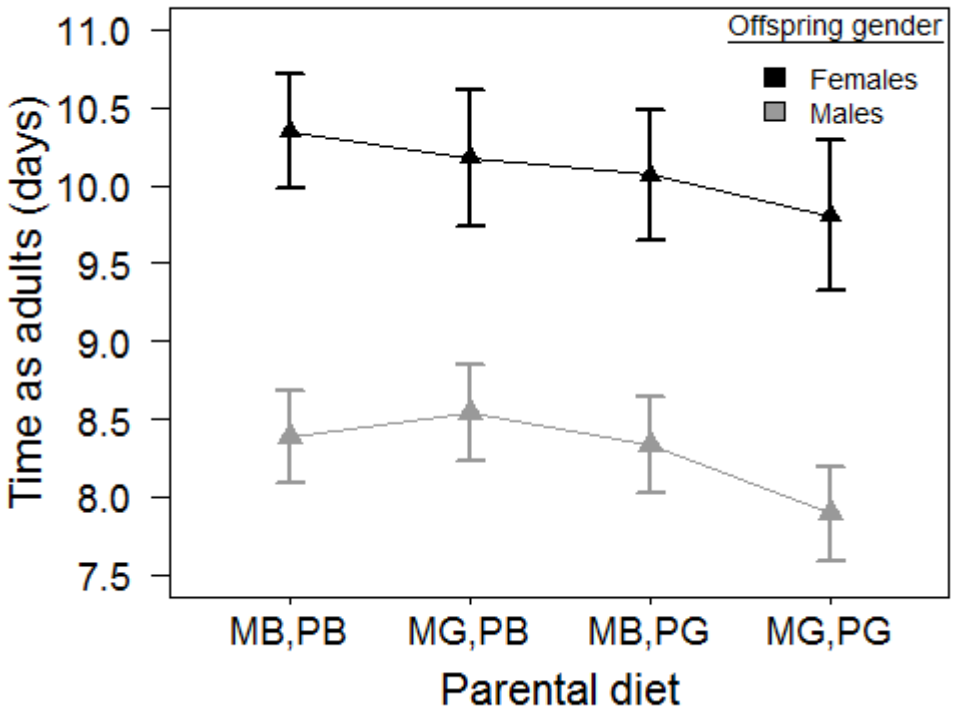

Figure 3.10: Males have a shorter adult lifespan than females. A three way interaction between maternal and paternal diet treatment and offspring sex significantly affected offspring lifespan as adults. Bars are 95% confidence intervals; M = maternal diet quality, P = paternal diet quality, B = bad diet (20:1:1 wheat bran, Brewer's yeast, glycerol), G = good diet (10:1:1).

Table 3.7: Adult lifespan of offspring

Initial model: adult lifespan ~ maternal diet + paternal diet + offspring diet + sex (and all two way interactions) + maternal diet : paternal diet : sex + block, random=~1|date/family
n=993

Standard deviations of random effects

Date
Intercept: 0.0206

Family code in date
Intercept: 0.319

Fixed effects

Variable	**Estimate**	**Standard error**	**Chisq value (df difference between models)**	**P value**
Maternal diet: Paternal diet: Sex	0.625	0.264	5.62 (1)	0.0177
Maternal diet: Offspring diet			1.85 (1)	0.174
Offspring diet: Sex			0.195 (1)	0.659
Paternal diet: Offspring diet			0.247 (1)	0.619
Paternal diet: Sex	-0.145	0.182		
Maternal diet: Sex	-0.284	0.184		
Maternal diet: Paternal diet	-0.0125	0.232		
Block			1.37 (1)	0.242
Maternal diet (good)	0.132	0.163		
Paternal diet (good)	0.155	0.159		
Sex (male)	1.69	0.129		
Offspring diet (good)	0.891	0.0705	157 (1)	<0.0001

Minimum adequate model: adult lifespan ~ maternal diet * paternal diet * sex + offspring diet, random=~1|date/family

3.4 Discussion

This experiment manipulated maternal, paternal and offspring nutrition in a fully factorial combination in order to study the importance of parental effects and progeny

environment on fitness traits in an economically important pest of stored products. Few studies manipulate the environments of both parents and offspring, owing to the large sample sizes required for many treatment combinations, and the complexity involved in interpreting higher-order interactions. This study emphasises the importance of environmental variation in defining traits such as resistance to the bacterial pathogen *Bacillus thuringiensis,* developmental time and adult lifespan. Parental effects tended to contribute more modest amounts of variation in the traits that they did affect, such as developmental time and lifespan after eclosion. In some cases offspring environment mediated the influence of parental effects, for example mismatching the two environments appeared to be detrimental to female offspring weight.

Current nutritional environment was very important in determining the Bt resistance phenotype in *P. interpunctella,* with 11.9% more larvae surviving an LD50 dose of Bt when they were reared on good food. There are a number of hypotheses to explain why good nutrition is beneficial for disease resistance. Increased protein content of the diet can increase the amount of immune effector cells and molecules needed to contribute to pathogen resistance (Lee, Simpson & Wilson 2008; Povey *et al.* 2009). Previous work in this laboratory using the same diets has shown that the good diet composition results in increased levels of constitutive levels of phenoloxidase and haemocytes in *P. interpunctella* (Triggs & Knell 2012), probably due to its increased protein levels derived from the more plentiful ratio of yeast. Bt-resistant cabbage loopers require a high dietary protein:carbohydrate ratio (Shikano & Cory 2014), indicating that increased levels of constitutive immune molecules made available by higher levels of dietary protein is a useful mechanism of resistance to Bt along with other entomopathogenic bacteria or viruses (Lee *et al.* 2006; Povey *et al.* 2009). As well as raised levels of constitutive immunity, there are other mechanisms of pathogen

resistance induced by diet such as an improvement in tolerating the presence of the pathogen (Sternberg *et al.* 2012). Finally, parental nutrition had no demonstrable effect on the resistance phenotype of offspring in this experiment, contrasting with previous work on the *P. interpunctella*-granulovirus system (Boots & Roberts 2012).

Maternal diet influenced the weight of their offspring at eclosion, although these parental effects were mediated by the sex of the individual and their own environment. Female offspring were 64.7% heavier than males, and variation in this trait was determined by different factors for the two sexes, indicating sex-specific life history optima. Male offspring appeared to have similar adult weights on both diet qualities, and the influence of maternal diet was moderate, producing a 2.9% increase in weight when mothers had good diets. Offspring diet quality produced only very small differences in weight for females (0.16% heavier when nutritionally stressed), but it had an important role in mediating maternal effects of diet. Female offspring were heavier when their own diet quality matched their mothers, and did badly when they were mismatched, especially when mothers received a good diet but they themselves were nutritionally stressed. Early life factors in the larval stage in *P. interpunctella* are shaping the adult phenotype, either through the epigenetic control of growth mediated by maternal effects, or egg provisioning by mothers which could provide small amount of nutrition before the larvae begin eating for themselves. It is possible that mothers could be anticipating the environment of their daughters and optimising their phenotype for the predicted environment (Mousseau & Fox 1998; Rotem, Agrawal & Kott 2003; Marshall & Uller 2007). This opens up interesting further avenues of research into the evolution of anticipatory maternal effects in *P. interpunctella*, because although it appears from this experiment that mothers are able to use their own environment as a

predictive cue of future nutritional quality, adult moths are able to disperse away from their own larval substrate, which may therefore be an unreliable cue.

In this experiment, we find a strong effect of larval diet on their longevity as adults. Experiencing nutritional stress as a larva increases life span by, on average, 1 day in comparison to offspring on the good diet. This phenomenon has been described in the literature for about 70 years across a wide range of taxa, and is known as dietary restriction. The proximate mechanisms mediating dietary restriction are still poorly understood (Piper & Partridge 2007). Suggestions have included the restriction of energy intake through fewer calories (Masoro 2005), but are increasingly focussed on the toxicity of certain nutrients, or the ratio of protein to non-protein energy (Mair, Piper & Partridge 2005; Simpson & Raubenheimer 2009; Piper *et al.* 2011; Dussutour & Simpson 2012). In our experiments, we created poor food treatments by diluting the ratio of yeast and glycerol to wheat bran, which decreases the amount of calories in the diet (from bomb calorimeter data – Littlefair, Westmancoat and Laughton, unpubl). However, the amount of protein and carbohydrate available in our diets also decreases which cannot be untangled from the effect of calories. The fact that we demonstrated effects of diet on longevity suggests that *P. interpunctella* is not able to compensate for poor conditions (at least, not entirely) by altering their feeding rate as larvae, or through post-digestive nutrient regulation. It is therefore possible that other aspects of fitness appear in trade-offs with longevity, for example, Triggs and Knell previously found that the same good quality diet resulted in upregulated constitutive immunity, which is also a costly trait (Triggs & Knell 2012).

In addition to these strong effects of larval diet, adult longevity was more moderately influenced by parental nutrition. When maternal and paternal diets were matched, their progeny had the shortest adult lifespan of all treatment groups; whereas when one or

both parents were nutritionally stressed, adult longevity increased. The effects of parental diet quality influenced adult longevity in the same direction as their offspring's own nutrition, i.e. nutritional stress in either parents or offspring always lengthened lifespan. Similar to the effects of parental diet on adult eclosion weight, egg provisioning by parents could influence the nutrition available to first instar larvae, passing on the same macronutrient ratios that result in dietary restriction as in their own diet. However, these parental effects are not apparent in the developmental period in this study, and appear to carry over through the pupa to solely affect the adult stage. Combined with the fact that fathers as well as mothers are producing effects on adult lifespan, epigenetic regulation seems to be a more parsimonious explanation for the effects on lifespan, although differential egg provisioning cannot be ruled out as male-derived nutrients have been shown to provision eggs (Boggs & Gilbert 1979; Ferguson-Smith & Patti 2011).

When we attribute parental effects to either mothers or fathers, as in this experiment, we must be aware that the other parent may have the ability to influence their investment. For example, mothers may appear to invest more in their offspring when they are well-resourced, but these may be male-derived nutrients from courtship gifts (Greenfield 1982; Vahed 1998), and could therefore be classified as paternal effects. In a similarly confounding situation, mothers could be adjusting their own investment according to their perception of their partner's quality (Crean & Bonduriansky 2014). While this possibility needs to be addressed, the evidence suggests that this is unlikely in *P. interpunctella*. Males do adjust ejaculate size in response to female body size (Gage 1998), but females do not show increased fecundity or longevity when they receive more spermatophore material (Cook 1999; Ryne *et al.* 2001). It is possible that males transfer nutrients of variable quality, which could be influenced by diet, but experiments

in the comma butterfly show that female reproductive success does not vary with larval diet (Wedell 1996).

Parental and offspring environments determine the fitness phenotype of *P. interpunctella,* although this study finds that the current environment generally produces greater effects on the phenotype, as in the case of resistance to Bt infection and length of developmental period (Ergon, Lambin & Stenseth 2001; Beckerman *et al.* 2006). This finding is supported by a recent meta-analysis of anticipatory parental effects studies which demonstrated that variation in traits was primarily dictated by manipulation of the offspring, rather than parental, environment (Uller, Nakagawa & English 2013). In our study, the current environment can also mediate the strength of parental effects, interacting to produce effects analogous to dietary restriction on adult lifespan in the offspring's own diet. Interactions between parental and offspring environments are difficult to measure experimentally, but this highlights the importance of examining parental effects under a number of environmental conditions in the offspring generation, including food-deprived environments. Relatively few experiments include this, as it is time-consuming to process large sample sizes in a fully multifactorial design. Unanswered questions remain about how anticipatory parental effects fit in with other aspects of the ecology of Lepidoptera, such as dispersal of adults and larvae. Finally, many questions remain concerning the mechanisms of parental effects and the role of epigenetic regulation in invertebrates.

3.5 Summary

This study uses a fully factorial experimental design to investigate the role of maternal, paternal and offspring diets in shaping offspring phenotype. Despite the emphasis on parental effects in the literature, I found that the environment of offspring was most

important in determining phenotype, and often mediated the influence of parental effects. Offspring, but not parental, diet was very important in determining resistance to infection with *Bacillus thuringiensis*: 11.9% more larvae survived infection when consuming a good diet when compared with a poor one. Offspring diet also had strong effects on adult lifespan, with individuals consuming the poor diet living on average one day longer than those on good diet. The influence of parental environment was generally more subtle throughout the experiments. Both poor maternal and paternal diets lengthened offspring lifespan, but not to the same extent as offspring diet. A three-way interaction between offspring sex, maternal diet and offspring diet defined weight at eclosion, with small influences on weight produced by both offspring and maternal environments.

Chapter 4: A novel artificial diet allows us to study the transgenerational consequences of varying dietary macronutrients

I received technical assistance from James Westmancoat with feeding the larvae with artificial diets in this chapter.

4.1 Introduction

Transgenerational effects are hailed in the current literature as important sources of phenotypic variation, interacting with both genetics and the offspring's own environment to create the overall phenotype (Laland *et al.* 2014). They arise when the phenotype or environmental variation experienced by previous generations influences the phenotype of the progeny (Mousseau & Fox 1998). The quality or quantity of maternal and paternal nutrition is one such source of variation impacting the fitness , as a consequence of changes in resource allocation or epigenetic marks (Smith *et al.* 2007; Li *et al.* 2011; Altmann *et al.* 2013). But what is meant by variation in the quality or quantity of nutrition? Experiments investigating the transgenerational consequences of parental nutrition often rely on starvation or diluted diets as treatments to create variation in nutritional quality. However, if oligidic diets (containing complex organic ingredients of a poorly-defined nature) are used this means that the quantity of different macronutrients in the diet (e.g. carbohydrate, protein, lipids) is unknown, and the effects of the different types of macronutrients are confounded by the total amount of calories within the diet (Karlsson & Wickman 1990; Braby & Jones 1995). Finally, this type of experimental design is limited by allowing for just two diets as different treatment factor levels, which means that we cannot see a full range of responses in investment.

Nevertheless, the use of diluted diets or starvation treatments has highlighted some interesting directions for investigations of the precise effects of parental nutrition on

offspring life history traits. When parents are starved, offspring fitness often suffers, for example, slower growth rate of immature stages, lower weight of the imago, increased mortality and ultimately lower reproductive output (Bonduriansky & Head 2007; Frost *et al.* 2010; Franzke & Reinhold 2012; Frago & Bauce 2014). These transgenerational effects could be mediated by mothers reallocating resources in different nutritional conditions, either affecting egg size or egg composition (Boggs & Gilbert 1979; Dussourd *et al.* 1988; Zanchi *et al.* 2012), so under conditions of resource limitation, mothers will have less to invest in offspring if they are not to compromise their own somatic condition. This is not universal – a few studies have found evidence for adaptive plasticity when mothers experienced poor nutrition, whereby nutritionally stressed mothers produce fewer but larger offspring, perhaps by altering per-offspring investment (Fox & Czesak 2000; Rotem, Agrawal & Kott 2003). These changes have the potential to canalize developing larvae, perhaps through affecting their hatching time or early larval development and go on to have significant, although often small, effects much later in their offspring's life span.

Variation in parental diet could potentially alter other aspects of somatic condition such as resistance to parasites and pathogens. Although much attention has been paid to transgenerational effects of parental immune priming (Little *et al.* 2003; Sadd *et al.* 2005), the relationship between other environmental variables affecting parents and offspring immunity has been neglected. One of the few studies demonstrated that investment in immune function decreased by 26% when both mothers and fathers were nutritionally stressed (Triggs & Knell 2012); such lack of investment in this costly life history trait is likely to make the offspring more susceptible to disease. This is not always a straightforward relationship (Sheldon & Verhulst 1996; Graham *et al.* 2011; Auld *et al.* 2012), as demonstrated by one contrasting study where nutritionally stressed

Daphnia magna produced offspring that were more resistant to the parasite *Pasteuria ramosa* (Stjernman & Little 2011). Subsequent research demonstrated that this was a result of decreased rates of filtering algae from the water, which reduced their exposure to the parasite (Garbutt & Little 2014). Further research into the precise way that parental nutrition produces transgenerational effects can only aid our understanding of whether parental nutrition can alter immunity, or whether other mechanisms such as pre-or post-digestive regulation alter the immune system.

To date there has been no research on the transgenerational consequences of particular macronutrients in invertebrates, although nutritional ecologists have mapped their within-generational effects on traits. For example, it is well established that dietary protein is essential for reproduction and growth (Simpson & Raubenheimer 2012), leading to a number of possible avenues for offspring phenotype to vary with parental protein intake. Invertebrate mothers provision their eggs with yolk proteins, the most abundant of which are vitellin and lipophorin (Arrese & Soulages 2010). Increased dietary protein also raises the level of constitutive immune defence in the haemolymph, and could be providing additional nitrogen resources for the production of immune cells and enzymes (Lee, Simpson & Wilson 2008b; Graham *et al.* 2014). Interestingly, recent papers also hint that excessive protein may in fact be detrimental to individual lifespan in certain circumstances, as well as hastening the collapse of entire colonies (Pirk *et al.* 2010; Goeriz Pearson *et al.* 2011; Dussutour & Simpson 2012). Mechanisms mediating the detrimental effects of excessive protein might include damage to DNA by reactive oxygen species, the toxic effects of nitrogenous breakdown products, and changes in the constitutive immune function (Simpson & Raubenheimer 2012). In mammals, recent research has indicated that exposure to protein-rich diets increases DNA methylation in

the genome, which is another candidate mechanism for the inheritance of dietary-based transgenerational effects (Li *et al.* 2011; Altmann *et al.* 2013).

Carbohydrate is a major macronutrient necessary for energy-requiring processes, such as movement, somatic maintenance and growth (Maklakov *et al.* 2008). Excess carbohydrate can be stored as lipids, resulting in increased pupal mass (Roeder & Behmer 2014). In some cases carbohydrate is also implicated in defence against pathogens, even though generally the immune system is thought to be protein-limited. Studies using the Australian plague locust *Chortoicetes terminifera* and the mealworm beetle *Tenebrio molitor* show that individuals constrained to a protein-rich diet had lower survival against fungal and helminth infections respectively and chose to switch to a carbohydrate-rich diet in choice experiments (Ponton *et al.* 2011; Graham *et al.* 2014). Simultaneously the total lipid storage of the mealworm beetles decreased, suggesting that carbohydrate-based energy was diverted away from storage into the increased energy demands associated with defence and damage repair.

Artificial diets have been used to great effect in studies manipulating the macronutrient content of foods (Carson-Cohen 2004). Laboratory diets can either be supplemented with the macronutrient of interest, or a completely synthetic artificial diet can be created from the molecular components necessary (Warbrick-Smith *et al.* 2006; Lee *et al.* 2008a; Harrison *et al.* 2014). Although the successful development of a synthetic artificial diet can be complex and time-consuming, they allow us to accurately manipulate macronutrient content in comparison to oligidic diets. They also remove the use of yeast as a protein source in diets for Lepidoptera, which could be providing stimulation to the immune system by stimulating the immune response via pathogen associated molecular patterns (PAMPs) on the cell surface or even by acting as an

opportunistic pathogen itself. Finally, isocaloric diets can be created with varying proportions of protein and carbohydrate, allowing us to separate the confounding effects of altering calories and macronutrient intake (Merrill & Watt 1973).

Here I perform an experiment to characterise the effects of varying both the total nutrition (by diluting isocaloric macronutrients with non-digestible cellulose) and the ratio of protein to carbohydrate within each of the three levels of total nutrition, using the synthetic diets developed in Chapter 2. It is the first experiment using artificial diets to examine the effects of changing levels of macronutrients available to the mother on the phenotype of invertebrate offspring. I additionally characterise the direct effect of the six diets on the condition of *P. interpunctella*, measuring their eclosion weight, fecundity and constitutive immune activity. By rearing *P. interpunctella* larvae individually, we could determine the requirement for macronutrients while removing the density-dependent competition for food resources that can shape the population cycles of these moths. Only daughters were examined in the F_1 generation of this experiment due to logistical constraints.

4.2 Materials and methods

4.2.1 Creating the artificial diets

Six artificial diets were created containing three different levels of non-nutritive bulking agent (30%, 50% and 70% cellulose), with either high protein, low carbohydrate or low protein, high carbohydrate levels nested within these (see methods chapter for details). In addition, 1% cholesterol, 1% Vanderzant vitamin mixture, 1% Wesson salts, 4% glycerol and 4% linseed oil were added to each diet. Each diet contained 75g dry ingredients in total and was suspended in 300ml 1% agar with 0.37g methyl paraben added as a preservative, and stored in a fridge.

4.2.2 Experiment 1: How do dietary macronutrients influence life history characteristics?

Approximately 200 adults were taken from the stock population of *Plodia interpunctella* and allowed to mate. Their eggs were collected after 24 hours and placed on standard lab. food consisting of 10:1:1 wheat bran: brewers' yeast: glycerol. After 15-16 days the resulting larvae had reached third instar and were weighed and placed in individual 55mm width petri dishes with a 0.8g block of one of the six artificial diets. Larvae were fed on the artificial diet from third instar onwards because smaller instars tended to drown in the slightly wet diet consistency. The petri dishes were secured with a square of net and elastic band. The artificial diet was removed and replaced with fresh diet every two days to prevent desiccation. Larvae were maintained in a temperature controlled room at 27°C on a 12:12 light/dark cycle for the duration of the experiment.

In four blocks of the experiment, the larvae were allowed to pupate and then eclose (n = 335). The date of pupation, pupal weight, and date of eclosion were all recorded. On the day the adults eclosed, they were killed by freezing and weighed to obtain adult weight (Sartorius BP2215 balance, d=0.1mg).

In four different blocks of the experiment, constitutive levels of larval immunity were measured. Larvae that had been reared on artificial diet were allowed to grow until they reached the wandering phase of fifth instar. A haemolymph sample was taken by pricking the larvae with a sterile needle (BD Microlance, 0.5mm tip, 25mm length) and allowing the haemolymph to pool out on to Parafilm. 1µl haemolymph was transferred into 10ul chilled sterile PBS in a 0.2ml microtube, and stored at -80°C for the phenoloxidase assay (n = 393). 1µl was transferred into a 0.2ml microtube containing 3ul chilled EDTA buffer in PBS and 4µl glycerol, which allows the sample to be frozen

without disrupting the haemocytes (Cotter *et al.* 2004). All samples were collected using sterile technique, and were collected at the same time of day to avoid possible confounding circadian variations in haemocyte numbers (n = 386).

4.2.3 Experiment 2: How do different macronutrient ratios in maternal diets influence offspring life history characteristics?

Approximately 200 adults from the stock population were allowed to mate and the eggs reared until third instar on lab diet. At third instar, larvae were transferred onto the six artificial diets described above. The diet was changed every two days until the larvae began to pupate. Males were discarded from the experiment at fifth instar, when the testes become visible on the dorsal side of the larva. Female moths were weighed as pupae and were mated with a freshly eclosed virgin male from the stock population once they had eclosed. After 24 hours the male was removed, and the number of eggs laid by the female was recorded daily until she died (n = 224 at the family level). Eggs from the first 72 hours of laying were reared on the normal lab food described above and formed the offspring generation of the experiment. Offspring were reared until fifth instar, when they were separated into three treatment groups within each family. Group 1 was reared until eclosion, when they were sacrificed by freezing and weighed to obtain weight at eclosion (n = 266). Group 2 was allowed to eclose and were then monitored daily for survival until they died to obtain longevity data (n = 159). Group 3 was sacrificed as wandering fifth instars to assay immune traits as described above (n = 344 (haemocyte counts), n = 326 (phenoloxidase assay)).

4.2.4 Immunity assays

Circulating haemocyte levels were assayed by pipetting 4µl of sample on each side of a haemocytometer (Neubauer improved brightline haemocytometer, 0.1mm depth,

0.0025mm^2 , Assistent, Germany) and counting the haemocytes in four corner squares and one central square of the grid on each side of the haemocytometer, so that 10 measurements for each individual were made in total.

Phenoloxidase assays were performed by vortexing and spinning down each sample for 15 seconds using a Jencons-PLS mini bench centrifuge and transferring them to flat-bottomed 96-well plates. 100µl of 4mg/ml L-DOPA was added to each sample in the plate. They were incubated in an Ascent v2.6 plate reader (Thermo labsystems) at 30°C for 1 hour and measured every 15 seconds at 492nm. Each plate included two blank wells containing 9µl PBS on each plate, and blank values were subtracted from the experimental wells on each plate at each time point. Vmax of the reaction was calculated using an R script written by Dr Robert Knell (see Appendix 1). Vmax is the maximum linear rate of reaction when an enzyme (in this case phenoloxidase) is saturated with substrate (L-DOPA). The script created an individual enzyme kinetics graph for each sample and allowed the user to manually select at least 20 minutes of the linear portion of the reaction. The script returns the vmax and R-squared value for each sample.

4.2.5 Statistical analysis

All analysis was conducted using R version 3.0.1 (R Development Core Team 2013).

4.2.5.1 Artificial diet and life history traits

The effects of artificial diet on adult weight, pupal weight, haemocyte counts and phenoloxidase activity were analysed with multiple linear regression. The percentage of nutritional dilution and amount of protein (high or low) were coded as factors. Analysis began with three-way interactions between protein level, the percentage of total

nutrition, initial weight when added to the experiment and sex. Block was added as a fixed effect rather than a random factor to the analyses of diet on life history traits (as opposed to the maternal effects experiments) because of the low number of factor levels (n = 4). Data points with missing entries were removed from the analysis, in order to facilitate model comparison. Model reduction was performed with likelihood ratio tests (Zuur *et al.* 2009) by using the R `drop1` function and discarding non-significant terms. Phenoloxidase activity was transformed using a reciprocal transformation with the formula 1/(vmax+1) as the raw data had a strong positive skew, and then analysed with a linear regression.

4.5.2.2 Analysis of maternal effects experiments

The maternal effects experiments were analysed with linear mixed effects models using either the nlme or lme4 packages in R (Bates *et al.* 2014; Pinheiro *et al.* 2014). Family identity was nested within block (n = 7 blocks) and included as a random effect. The random effects were retained throughout model reduction as they reflected the design of the experiment. Model reduction was performed using likelihood ratio tests to compare models fitted using maximum likelihood, and fixed effects terms were discarded if there was no significant difference between models with and without them (Zuur *et al.* 2009). Offspring PO data was square root transformed after examination of model validation plots.

Longevity data (adult lifespan and larval development time) were analysed with mixed effects models in the package "coxme" (Therneau 2012), and model reduction was also performed with the likelihood ratio test, as above.

4.3 Results

4.3.1 Direct effects on animals reared on different diets

4.3.1.1 Effect of artificial diet on weight of pupae (table 4.1)

Males weighed 22.9% percent less than females in the pupal stage ($F_{1,328} = 225$, $p < 0.0001$). The degree of nutritional dilution in the artificial diet also predicted pupal weight, with animals grown on the 70% diet weighing 9.26% less than those consuming the 50% diets, and 6.08% less than those consuming 30% diets (Figure 4.1, $F_{2,328} = 7.59$, $p = 0.000599$). Experimental block was also retained as a term in the model ($F_{3,328} = 3.67$, $p = 0.0126$).

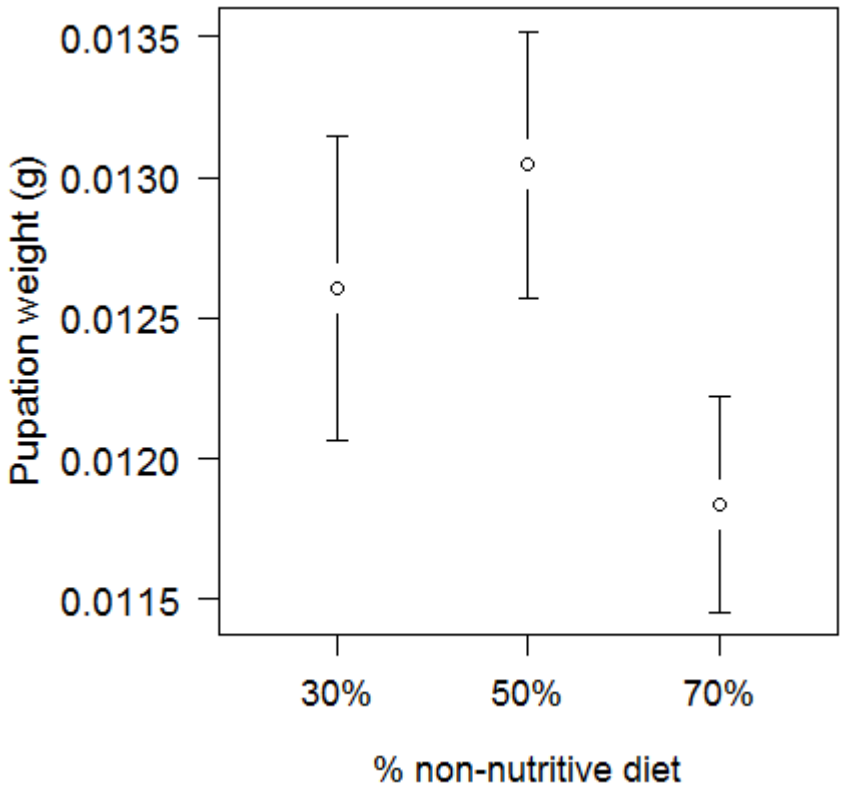

Figure 4.1: The total nutritional content in the artificial diet influenced the weight of pupae. X axis: 30% = 30% cellulose (non-nutritive content) etc; error bars are 95% confidence intervals.

Table 4.1: Pupation weight

Initial model: Pupation weight ~ block + (sex + cellulose treatment + protein treatment + initial weight – and all their third order interactions)

n = 335

Term	Estimate	Standard error	Degrees of freedom	F value	P value
Intercept	0.0146	0.000282			
Cellulose: protein: sex			2, 310	2.03	0.133
Cellulose: protein: weight			2, 310	1.00	0.368
Cellulose: sex: weight			2, 310	0.472	0.624
Protein: gender: weight			1, 310	0.256	0.613
Protein: weight			1, 317	1.51	0.221
Sex: weight			1, 317	2.32	0.129
Cellulose: protein			2, 317	0.0707	0.932
Protein: sex			1, 317	0.509	0.476
Cellulose: sex			2, 317	0.526	0.592
Cellulose: weight			2, 317	0.594	0.553
Weight			1, 326	1.15	0.284
Protein			1, 326	1.70	0.193
Sex (males)	-0.00323	0.000215	1, 328	225	< 0.0001
% cellulose (50) % cellulose (70)	0.000170 -0.000754	0.000257 0.000254	2, 328	7.59	0.000599
Block (B) (C) (D)	-0.0000241 0.00104 0.0000170	0.000280 0.000356 0.000271	3, 328	3.67	0.0126
Minimum adequate model: pupation weight ~ sex + cellulose treatment + block					

4.3.1.2 Effect of artificial diet on weight at eclosion (table 4.2)

A three-way interaction term between the total nutrition, amount of protein and sex explained the eclosion weight of adult moths ($F_{2,320} = 3.15$, $p = 0.0442$), although this term was only weakly significant. From figure 4.2 it can be seen that females weighed less on the diets with the greatest amount of nutritional dilution (70% cellulose) and the 30L diet (30% cellulose, low-protein, high carbohydrate). Block was a highly significant explanatory variable in the model and therefore retained as a fixed effects term ($F_{3,320} = 5.62$, $p = 0.000912$), with individuals from block C weighing 17.2% heavier than individuals from the other blocks.

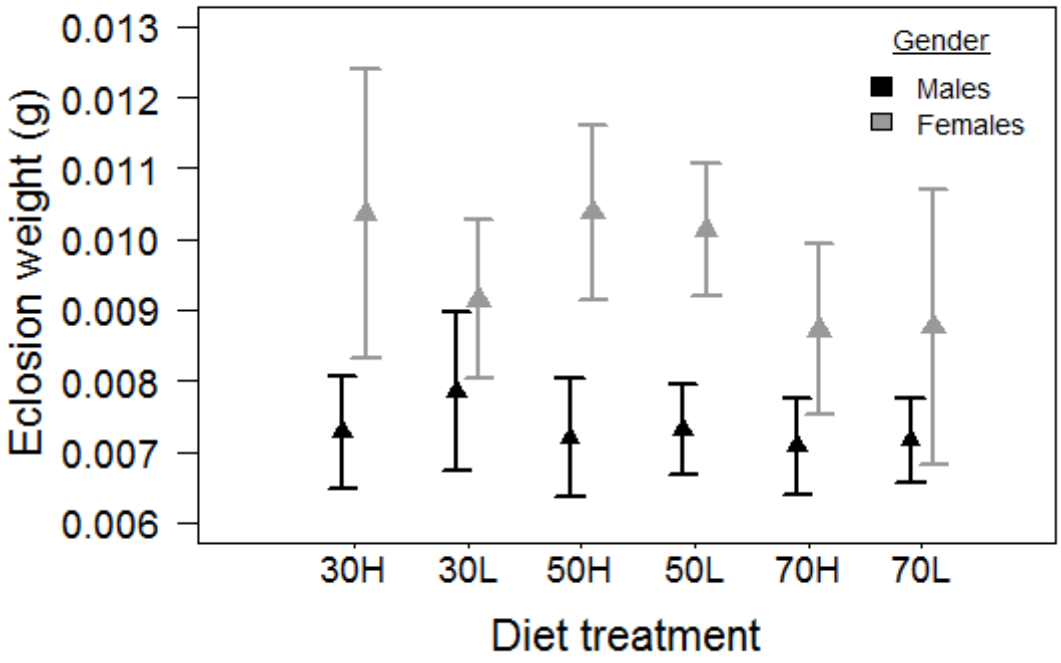

Figure 4.2: A three-way interaction between total nutritional content, level of dietary protein, and sex defined weight at eclosion. Bars are 95% confidence intervals, x axis number refers to percentage of nutritional dilution (i.e. 30 = 30% cellulose, etc), x axis letter refers to level of dietary protein (H = high protein, low carbohydrate; L = low protein, high carbohydrate).

Table 4.2: Adult eclosion weight

Initial model: Eclosion weight ~ block + (sex + cellulose treatment + protein treatment + initial weight – and all their third order interactions)

n = 335

Term	Estimate	Standard error	Degrees of freedom	F value	P value
Intercept	0.0110	0.000321			
Cellulose: protein: weight			2,310	0.542	0.581
Cellulose: sex: weight			2,310	0.938	0.393
Protein: sex: weight			2,310	0.101	0.750
Cellulose: weight			2,315	0.827	0.438
Protein: weight			1,315	0.560	0.455
Sex: weight			1,315	0.114	0.736
Cellulose (50%): protein (low): sex Cellulose (70%): protein (low): sex	0.000196 -0.00138	0.000688 0.000692	2,320	3.15	0.0442
Protein (low): sex (males)	0.000591	0.000497			
Cellulose (50%): protein (low) Cellulose (70%): protein (low)	-0.000145 0.00114	0.000536 0.000556			
Cellulose (50%): sex (males) Cellulose (70%): sex (males)	-0.000102 0.00110	0.000503 0.000492			
Sex (males)	-0.00416	0.000369			
Protein (low)	-0.000468	0.000399			
% cellulose (50) % cellulose (70)	-0.0000124 -0.00143	0.000399 0.000394			
Block (B) (C) (D)	-0.000127 0.000778 -0.0000255	0.000183 0.000232 0.000177	3,320	5.62	0.000912

Minimum adequate model: eclosion weight ~ block + (sex * cellulose treatment * protein treatment)

4.3.1.3 Effect of artificial diet on haemocyte counts (table 4.3)

No interaction terms or main effects of nutritional dilution or amount of protein explained variation in total haemocyte count (% total nutrition: $F_{2,378} = 1.71$, $p = 0.182$, protein: ($F_{1,377} = 0.330$, $p = 0.566$). Block was a significant explanatory variable and retained as a fixed effect term ($F_{3,382} = 12.4$, $p < 0.0001$).

Table 4.3: Haemocyte count
Initial model: Total count ~ block + (sex + cellulose treatment + protein treatment + final weight – and all their second order interactions)
n = 386

Term	Estimate	Standard error	Degrees of freedom	F value	P value
Intercept	110	4.48			
Cellulose: protein: sex			2, 361	0.344	0.709
Cellulose: protein: final weight			2, 361	0.793	0.453
Cellulose: sex: final weight			2, 361	1.19	0.304
Protein: sex: final weight			1, 361	0.204	0.652
Sex: final weight			1,368	0.0127	0.910
Protein: final weight			1,369	0.644	0.423
Cellulose: sex			2,370	0.744	0.476
Cellulose: final weight			2,372	0.827	0.438
Protein: sex			1,374	1.78	0.183
Protein: cellulose			2,375	1.44	0.238
Protein			1,377	0.330	0.566
Cellulose			2,378	1.71	0.182
Final weight			1,380	2.07	0.151
Sex			1,381	2.00	0.158
Block (E) (F) (G)	-13.8 -21.6 -39.1	6.37 7.72 6.53	3,382	12.4	p<0.0001
Minimum adequate model: Total count ~ block					

4.3.1.4 Effect of artificial diet on phenoloxidase activity (table 4.4)

As the weight of the fifth instar larvae increased, phenoloxidase activity also increased ($F_{1,392} = 42.9$, $p < 0.0001$). The interaction between sex and the degree of nutrient dilution was close to significance and therefore retained as an interaction term in the model ($F_{2,393} = 2.80$, $p = 0.0621$); if this term is removed then the degree of nutrient dilution is not retained as a significant term but sex is. Experimental block also explained the level of phenoloxidase activity ($F_{3,393} = 22.1$, $p < 0.0001$).

Table 4.4: Phenoloxidase activity
Initial model: Vmax ~ block + (sex + cellulose treatment + protein treatment + final weight – and all their third order interactions). Data was transformed with reciprocal + 1
n = 393

Term	Estimate	Standard error	Degrees of freedom	F value	P value
Intercept	0.0628	0.0876			
Cellulose: Protein: Sex			2, 378	0.913	0.402
Cellulose: Protein: Weight			2, 378	0.300	0.741
Cellulose: Sex: Weight			2, 378	0.182	0.834
Protein: Sex: Weight			1, 378	0.718	0.397
Cellulose: Protein			2, 385	1.10	0.333
Cellulose: Weight			2, 385	1.40	0.249
Protein: Sex			1, 385	0.0427	0.836
Protein: Weight			1, 385	0.0833	0.773
Sex: Weight			1, 391	2.80	0.0953
Cellulose (50%): Sex (males) Cellulose (70%): Sex (males)	0.0276 -0.115	0.0637 0.0630	2, 393	2.80	0.0621
Protein			1, 392	1.06	0.304
Weight	27.2	4.15	1, 393	42.9	<0.0001
Block	0.0242 -0.00165 -0.233	0.0353 0.0412 0.0346	3, 393	22.1	<0.0001
Cellulose (50%) (70%)	-0.0761 -0.0146	0.0468 0.0436			
Sex (males)	0.146	0.0479			
Minimum adequate model: Vmax ~ block + sex + cellulose treatment + final weight + cellulose treatment: sex					

4.3.1.5 Total fecundity (table 4.5)

When maternal pupal weight increased, total fecundity also increased ($F_{3,382} = 12.4$, $p < 0.0001$). There was also a significant three-way interaction between macronutrient dilution, the amount of protein and maternal longevity ($F_{2,199} = 4.76$, $p = 0.00953$). As can be seen from figure 4.3, this interaction is probably due to the effect of the 70%

dilution, low-protein diet, which has a much flatter negative relationship between maternal longevity and fecundity. It appears that during the first few days of female life they produce fewer eggs, possibly due to the low amount of protein present in the diet.

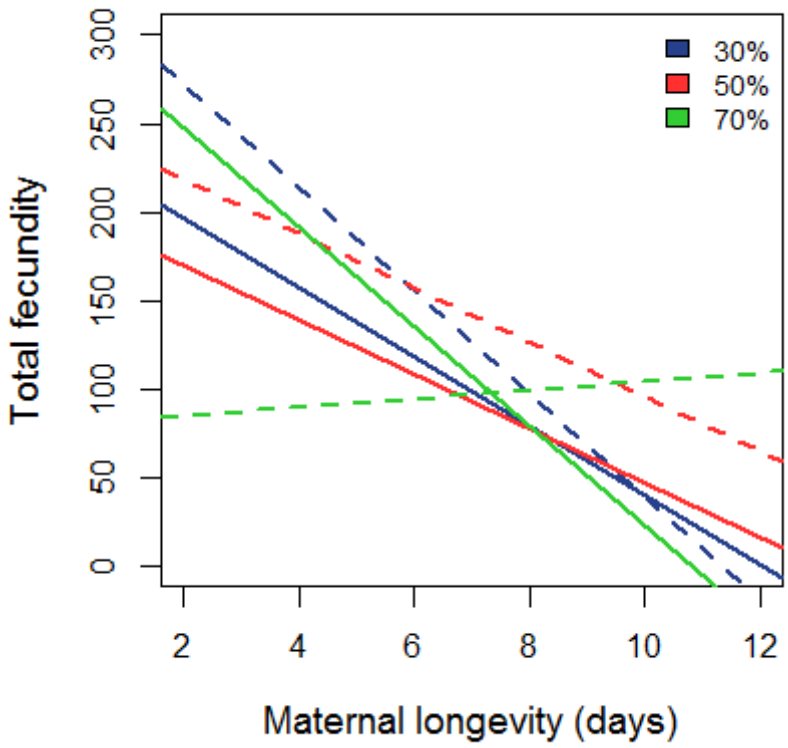

Figure 4.3: There was a significant three-way interaction between macronutrient dilution, amount of protein and maternal longevity on maternal fecundity; legend refers to the percentage of non-nutritive content in the diet (i.e. cellulose material); solid lines are the high protein treatment; dashed lines are the low-protein treatment.

Table 4.5: Total fecundity

Initial model: Fecundity ~ (cellulose treatment + protein treatment + longevity + maternal pupal weight) – and all their second order interactions, + block, plus third order interactions between cellulose treatment + protein treatment + longevity, and cellulose treatment + protein treatment + maternal pupal weight.

n = 224

Term	Estimate	Standard error	Degrees of freedom	F value	P value
Intercept	121	51.8			
Cellulose treatment (50%) (70%)	-38.1 70.5	66.5 72.5			
Protein treatment (Low)	85.1	66.6			
Longevity	-19.5	6.17			
Maternal pupal weight	7949	2360	1,199	11.3	0.000911
Cellulose treatment (50%): Protein treatment (Low) Cellulose treatment (70%): Protein treatment (Low)	-39.7 -306	92.0 99.4			
Cellulose treatment (50%): Longevity Cellulose treatment (70%): Longevity	4.19 -8.51	9.27 10.3			
Protein treatment (Low): Longevity	-9.59	8.90			
Cellulose treatment (50%): Protein treatment (Low): Longevity Cellulose treatment (70%): Protein treatment (Low): Longevity	6.28 40.0	12.4 13.7	2,199	4.76	0.00953

Minimum adequate model: Fecundity ~ maternal pupal weight + cellulose treatment * protein treatment * longevity

4.3.2 Effects on the offspring of animals reared on different diets

4.3.2.1 Maternal effects on offspring pupal weight (table 4.6)

No significant main effects of maternal weight or maternal diet treatments were retained in the model as explanatory variables (maternal weight: likelihood ratio = 0.0912, df = 1, $p = 0.763$, % macronutrient dilution: likelihood ratio = 4.22, df = 2, $p = 0.121$, amount of protein: likelihood ratio = 0.00483, df = 1, $p = 0.945$). No other terms were significant.

Table 4.6: Offspring pupation weight
Initial model: Offspring pupation weight ~ (Cellulose treatment + protein treatment + maternal weight – and all their second order interactions) + (1|block/family ID)
n = 266

Standard deviations of random effects

Block: 0.000000163
Family nested within block: 0.00149

Fixed effects

Term	Estimate	Standard error	Degrees of freedom	Likelihood ratio	P value
Intercept	0.0145	0.000211			
Maternal weight: Cellulose			2	0.719	0.698
Protein: Cellulose			2	0.311	0.856
Protein: Maternal weight			1	0.630	0.427
Protein			1	0.00483	0.945
Maternal weight			1	0.0912	0.763
Cellulose			2	4.22	0.121
Minimum adequate model: Offspring pupation weight ~ (1|block/family ID)					

4.3.2.2 Maternal effects on offspring eclosion weight (table 4.7)

There were also no significant main effects of maternal weight or maternal diet treatments in the model of offspring eclosion weight (maternal weight: likelihood ratio = 0.0252, df = 1, $p = 0.874$, % macronutrient dilution: likelihood ratio = 3.81, df = 2, $p = 0.149$, amount of protein: likelihood ratio = 0.0384, df = 1, $p = 0.845$). No other terms were significant.

Table 4.7: Offspring weight as adults
Initial model: Offspring eclosion weight ~ (Cellulose treatment + protein treatment + maternal weight – and all their second order interactions) + (1|block/family ID)
n = 266

Standard deviations of random effects

Block: 0.0000000805
Family nested within block: 0.000985

Fixed effects

Term	Estimate	Standard error	Degrees of freedom	Likelihood ratio	P value
Intercept	0.0109	0.000145			
Maternal weight: Cellulose			2	0.0110	0.946
Protein: Cellulose			2	0.341	0.843
Protein: Maternal weight			1	1.70	0.192
Protein			1	0.0384	0.845
Maternal weight			1	0.0252	0.874
Cellulose			2	3.81	0.149

Minimum adequate model: Offspring weight ~ (1|block/family ID)

4.3.2.3 Maternal effects on offspring longevity (tables 4.8 & 4.9)

Maternal diet did not explain the adult longevity of their progeny (defined as the time from eclosion to death) (table 4.8; figure 4.4, % macronutrient dilution: likelihood ratio = 1.24, df = 2, $p = 0.539$; amount of protein: likelihood ratio = 0.0554, df = 1, $p =$

0.814; % macronutrient dilution × protein: likelihood ratio = 2.12, df = 2, p = 0.346). Nor were there any effects of maternal diet on the development time of the larvae (defined as the time from egg laying to pupation) (table 4.9; % macronutrient dilution: likelihood ratio = 2.73, df = 2, p = 0.256; protein: likelihood ratio = 1.95, df = 1, p = 0.163; % macronutrient dilution × protein: likelihood ratio = 1.48, df = 2, p = 0.478).

Table 4.8: Offspring adult longevity
Initial model: Offspring adult longevity ~ (Cellulose treatment + protein treatment – and second order interaction) + (1|block/family ID)
n = 159

Standard deviations of random effects

Block: 0.0117
Family nested within block: 0.406

Fixed effects

Term	Estimate	Standard error	Degrees of freedom	Likelihood ratio	P value
Cellulose: Protein			2	2.12	0.346
Protein			1	0.0554	0.814
Cellulose			2	1.24	0.539
Minimum adequate model: Offspring adult longevity ~ (1|block/family ID)					

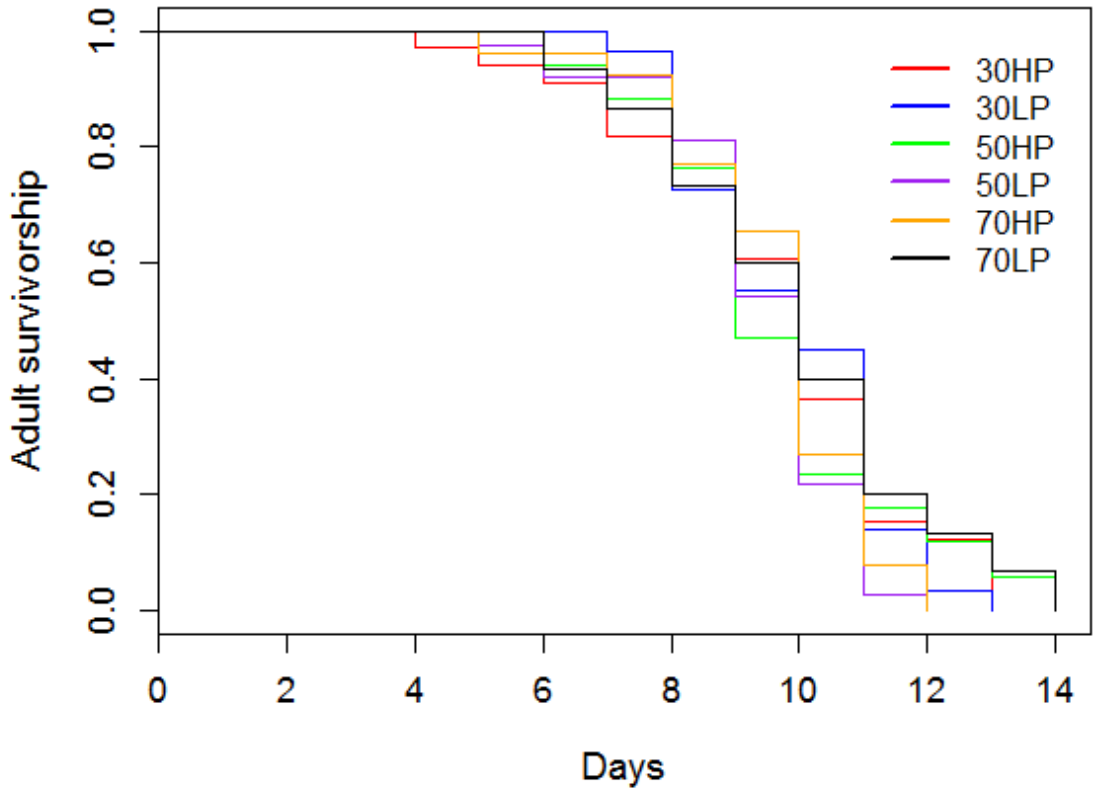

Figure 4.4: No effect of maternal diet on adult longevity of offspring (defined as days from eclosion to death)

Table 4.9: Offspring larval development time
Initial model: Larval development ~ (Cellulose treatment + protein treatment – and second order interaction) + (1|block/family ID)
n = 220

Standard deviations of random effects

Block: 0.225
Family nested within block: 0.805

Fixed effects

Term	Estimate	Standard error	Degrees of freedom	Likelihood ratio	P value
Cellulose: Protein			2	1.48	0.478
Protein			1	1.95	0.163
Cellulose			2	2.73	0.256
Minimum adequate model: Larval development ~ (1|block/family ID)					

4.3.2.4 Maternal effects on offspring haemocyte count (table 4.10)

An interaction between offspring weight and the % of macronutrient dilution in the maternal diets affected offspring haemocyte number (figure 4.5, likelihood ratio = 7.62, df = 2, $p = 0.0222$). Mothers with diets containing high or low macronutrient dilution (30% and 70%) produced larvae with similar levels of total haemocyte counts, but mothers feeding on the diet with 50% macronutrient dilution produced heavier larvae with fewer haemocytes and lighter larvae with more haemocytes. Neither maternal weight nor the protein in the maternal diet additionally affected haemocyte counts (maternal weight: likelihood ratio = 1.10, df = 1, $p = 0.293$, maternal dietary protein: likelihood ratio = 1.99, df = 1, $p = 0.159$).

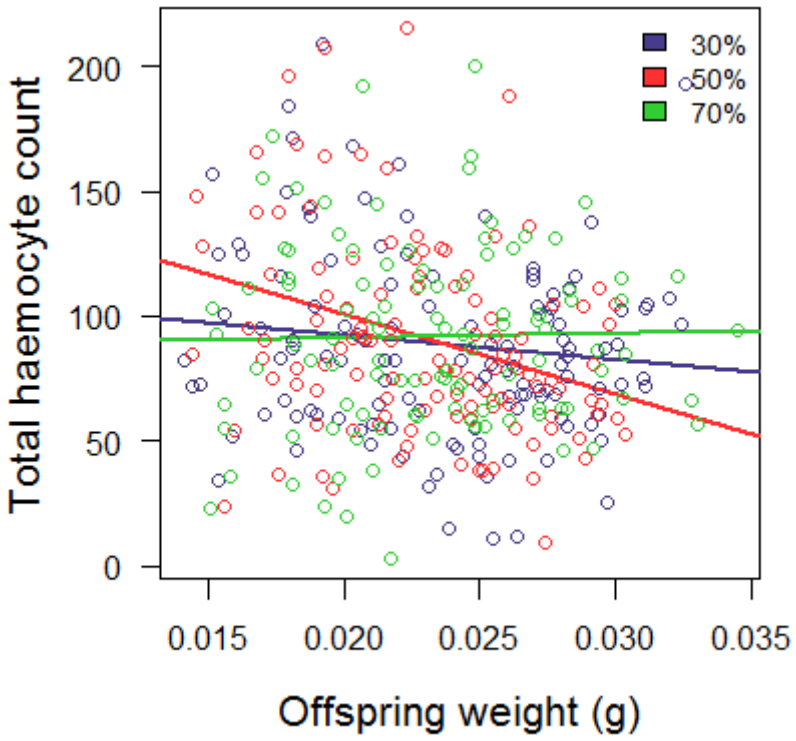

Figure 4.5: A two-way interaction between the percentage of macronutrient dilution in the maternal diet and offspring larval weight at the time they were sampled for immune measures (fifth instar) affected total haemocyte count. Legend refers to the percentage of non-nutritive content in the diet (i.e. cellulose material).

Table 4.10: Offspring haemocyte counts
Initial model: Offspring haemocyte count ~ (Cellulose treatment + protein treatment + offspring weight + maternal weight – and third order interactions) + (1|block/family ID)
n = 344

Standard deviation of random effects

Block: 9.20
Family nested within block: 0.00767

Fixed effects

Term	Estimate	Standard error	Degrees of freedom	Likelihood ratio	P value
Intercept	112	17.5			
Maternal weight: Offspring weight: Protein			1	0.00285	0.957
Maternal weight: Cellulose: Protein			2	0.827	0.661
Offspring weight: Cellulose: Protein			2	0.355	0.838
Maternal weight: Offspring weight: Cellulose			2	4.13	0.127
Offspring weight: Protein			1	0.00141	0.97
Maternal weight: Protein			1	0.790	0.374
Cellulose: Protein			2	2.34	0.310
Maternal weight: Offspring weight			1	1.60	0.205
Maternal weight: Cellulose			2	3.18	0.204
Offspring weight: Cellulose (50%) Offspring weight: Cellulose (70%)	-2027 1229	1115 1075	2	7.62	0.0222
Offspring weight	-1048	711			
Cellulose (50%) Cellulose (70%)	48.3 -25.6	26.3 25.7			
Maternal weight			1	1.10	0.293
Protein			1	1.99	0.159

Minimum adequate model: Offspring haemocyte counts ~ cellulose * offspring weight + (1|block/family ID)

4.3.2.5 Maternal effects on offspring phenoloxidase activity (table 4.11)

A weak interaction between maternal dietary protein and offspring weight explained offspring phenoloxidase activity (figure 4.6, likelihood ratio = 3.92, df = 1, $p = 0.0477$). When maternal diets had high levels of protein (and simultaneously, low levels of carbohydrates), heavier larvae had less haemolymph PO activity while lighter larvae had increased levels of PO when compared with larvae from mothers on low protein, high carbohydrate diets. No other fixed effects terms were retained in the model.

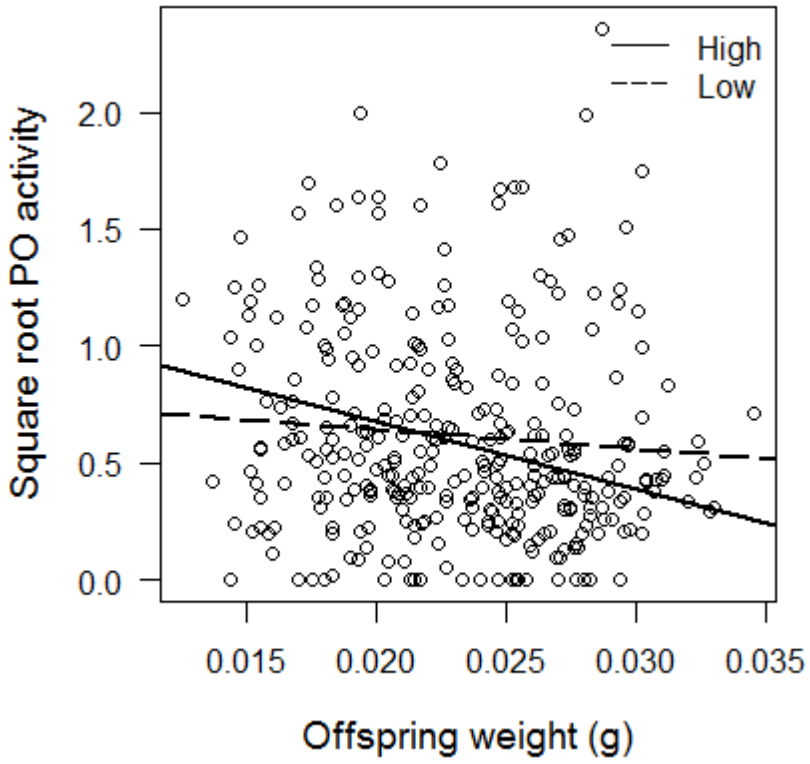

Figure 4.6: A two-way interaction between the level of dietary protein in the maternal diet and offspring larval weight at the time they were sampled for immune measures (fifth instar) affected offspring phenoloxidase activity. Key refers to the level of maternal dietary protein.

Table 4.11: Offspring PO activity
Initial model: Vmax (square root transformed) ~ (Cellulose treatment + protein treatment + weight – and third order interactions) + (1|block/family ID)
n = 326

Standard deviation of random effects

Block: 0.124
Family nested within block: 0.228

Fixed effects

Term	Estimate	Standard error	Degrees of freedom	Likelihood ratio	P value
Cellulose: Weight: Protein			2	1.38	0.502
Cellulose: Weight			2	0.510	0.775
Cellulose: Protein			2	4.21	0.122
Protein: Weight	20.7	10.2	1	3.92	0.0477
Cellulose			2	0.747	0.688
Weight	-29.1	7.40			
Protein	-0.444	0.243			

Minimum adequate model: Vmax (square root transformed) ~ (protein treatment * weight) + (1|block/family ID)

4.4 Discussion

Using artificial diets as tools to manipulate macronutrient content, I generally find that *Plodia interpunctella* is extremely robust to changes in the protein and carbohydrate content of its diet. Whilst other studies have found carbohydrate: protein ratios and the total amount of nutrition to have important effects on life history traits in insects, my data suggest that this is not universal. Parental immune measures and many aspects of offspring phenotype including weight and longevity were unaffected by dramatic variation in both the total content and type of macronutrients in the parental diet. As a dietary generalist, *P. interpunctella* is a successful pest species of many stored and processed grain products that vary in nutrient content. Here I discuss this interesting

aspect of the biology of *P. interpunctella* and relate dietary robustness to possible pre-- and post-digestive regulatory processes that might allow it to be successful in a wide variety of nutritional conditions.

Pupal and eclosion weight varied according to the total nutritional content of diets, for example pupae that consumed the 70% nutritional dilution diet weighed 9.26% less than those on the 50% dilution diet. By eclosion, male adults had generally compensated for their larval diet which caused changes in pupal weight, and as can be seen from figure 4.2, there was little variation in adult weight across all the diet treatments. However, there was a sex-specific difference in eclosion weight in response to diet dilution: females were lightest on the 70% diet dilution. The change in female weight has consequences for other aspects of lepidopteran life history, for example, pupal and adult weight is strongly linked to fecundity in other capital breeding Lepidoptera and insects generally (Honek 1993; Calvo & Ma Molina 2005). The allocation of nitrogen and carbon from the diet into the abdomen as opposed to other areas of the soma increases in heavier capital breeding Lepidoptera, which probably facilitates this increase in fecundity (Karlsson & Wickman 1990). Female weight also influences sexual selection dynamics in *P. interpunctella*: males invest more sperm in larger females, probably as a result of increased sperm competition (Gage 1998).

Neither of the two measures of immune reactivity varied according to diet, which was surprising when contrasted with results from previous studies in our laboratory and elsewhere (e.g. Triggs & Knell 2011). It is possible that this is partly a consequence of the specific assays chosen: while the relationship between dietary protein and haemolymph antibacterial activity seems to be well supported elsewhere, the effect of dietary protein on constitutive phenoloxidase seems to be less strong (Lochmiller &

Deerenberg 2000; Lee *et al.* 2008b; Povey *et al.* 2009; Cotter *et al.* 2011; Srygley & Lorch 2013). There are also few cases of experiments which examine the effect of dietary protein on haemocyte counts - in fact the addition of dietary protein has been shown to decrease haemocyte counts in two studies, possibly due to differential investment in different types of haemocytes, or changes in their metabolic activity (Szymas & Jedruszuk 2003; Alaux *et al.* 2010). Finally, these experiments only measure constitutive immunity only, and a protein cost might be elucidated if the animals had been challenged with a pathogen.

Life history traits of *P. interpunctella* female progeny were generally robust even to extreme dietary changes affecting the mother. Maternal dietary protein varied from 39.33% to just 4.75% but neither offspring pupal nor eclosion weight or longevity or development time were affected. There was also no effect of varying the total nutritional content in the diet from 30% to 70% on any of these life history traits, despite previous experiments finding that weight and longevity are influenced by maternal nutrition (Mousseau & Dingle 1991). However, not all life history traits were robust to the dramatic variation in maternal diet. An interaction between larval weight and maternal macronutrient dilution influenced circulating haemocyte cell counts. Very high and low levels of nutrient dilution in the maternal diet resulted in a flat relationship between offspring weight and haemocyte count, whereas the 50% dilution meant that there was a negative relationship between weight and haemocyte count. Here, mothers may be trading off their investment in the immune activity of larger offspring with investment in their own somatic condition, as they weigh more at eclosion in this treatment group. Phenoloxidase titre was influenced by a weak interaction between larval weight and maternal dietary protein. When dietary protein level increased, the negative relationship between phenoloxidase activity and larval weight steepened. We should also consider

the possibility that, since these effects are weak and the results are close to non-significance they could be Type I errors, which would mean that there are no significant transgenerational effects produced by variation in the maternal diet.

It is possible that mothers or larval offspring in *P. interpunctella* compensate for poor nutritional conditions, such as a lack of dietary protein, by pre-or post-digestive regulation of food intake which would explain their robustness to variation in food quality. It is already known that *P. interpunctella* can enter larval diapause in conditions of dietary stress (Bell 1994; Wijayaratne & Fields 2012). As a prolific generalist pest species which infests a huge variety of unprocessed and processed grain products, other protective or compensatory mechanisms would be useful for *P. interpunctella* when faced with a poor diet (Mohandass *et al.* 2007). Regulation mechanisms in other successful generalist insects include the preferential consumption of depleted macronutrients, the increased passage rate of food through the gut, or the excretion of more nitrogen in the case of excessive protein consumption (Lee, Raubenheimer & Simpson 2004; Raubenheimer & Jones 2006). Other aspects of the soma can alter to increase food intake, for example *Daphnia* feeding screens enlarge up to 83% under conditions of nutritional stress in order to increase the filter feeding rate (Lampert 1994). These mechanisms have important implications for the plastic biology of successful generalist pests, and in turn influence human food security as stored and processed food products are damaged or lost to pest species.

Although the artificial diet varied from the bran-based diets in chapter 3 in terms of precise calorie and macronutrient content, it was expected that the diets with 70% nutrient dilution would produce effects similar to nutritional stress. However, our results here contrast with my findings in chapter 3 in which nutritionally stressed parents

produced offspring that lived longer as adults, and where maternal and offspring diet quality was matched, offspring mass was greater than when they were mismatched. Here the offspring diet did not vary as they all consumed the standard laboratory diet which may have been nutritionally rich enough to mask any detrimental effects of maternal macronutrient deprivation in all aspects of fitness except the immune measures. Further investigations using nutrient depletion in offspring diets may have uncovered maternal effects of varying macronutrients, similar to my results in chapter 3, resulting in maternal × offspring environment interaction terms.

4.5 Summary

Using artificial diets I have investigated both the within- and trans-generational effects of variation in total nutritional content and the relative ratios of dietary macronutrients. Generally I find that phenotype in *P. interpunctella* is extremely robust to changes in dietary macronutrients, although there were some modest but convincing within-generational effects. For example, at the lowest dilution (70% non-nutritional content) of diet, weight at eclosion decreased by 9.26% when compared with the 50% dilution. Mothers feeding on the diet with the lowest amount of protein also experienced an altered relationship between their fecundity and longevity, which was flat as opposed to a negative relationship. However, there were no effects of diet on the constitutive immune system, which included measures of phenoloxidase activity and haemocyte counts. Parental diet did not produce any transgenerational effects on offspring weight or longevity, and only weak influences on offspring phenoloxidase and haemocyte activity. I suggest that as *P. interpunctella* may be able to compensate for even large dietary variation through pre- or post-digestive nutrient regulation.

Chapter 5: multiple infections and the specificity of transgenerational immune priming

Alice Laughton provided assistance with lab work during the offspring infection phase of the experiment. Thanks to Dr Jason Baverstock for supplying the strain of Beauveria bassiana *from Rothamsted Research, and also for much advice regarding culturing and infection with fungal pathogens. Also thanks to Dr Ben Parker for advice on infection with* B. bassiana.

5.1 Introduction

Parental effects have recently attracted much interest in ecological immunology for their role in the transmission of transgenerational resistance to pathogens. Known as transgenerational immune priming (TGIP), typically experiments have shown that invertebrate parents are able to use their experience of the pathogen environment to upregulate their offspring's response to disease (Little *et al.* 2003; Sadd *et al.* 2005; Moret 2006; Freitak, Heckel & Vogel 2009; Roth *et al.* 2010; Tidbury, Pedersen & Boots 2011). This has been interpreted as an anticipatory, protective parental effect functionally homologous to the maternal transfer of antibodies in vertebrates, which do not exist in invertebrates. The discovery of TGIP has important implications for our understanding of host-pathogen dynamics, for example by providing a new source of heterogeneity in host susceptibility and therefore influencing infection prevalence (Tate & Rudolf 2012), but since only a few invertebrate host-pathogen systems have been examined, we know little of how widespread the phenomenon is. There is also little understanding of how TGIP will interact with other environmental stressors to impact fitness. Here, I consider an as yet unstudied aspect of TGIP – the dynamics of coinfection by bacterial and fungal pathogens, combined with changing resource

availability, to examine whether parental effects on the immune system are always beneficial to fitness when combined with other stressors.

One common interpretation of TGIP is as an adaptive anticipatory parental effect which increases parental fitness by transferring cues which cause increased investment in immunity and defend offspring against pathogens. In order to be classified as an anticipatory parental effect, parents must be able to predict the pathogen environment that will be experienced by their offspring from their own environment (Marshall & Uller 2007), which is experimentally introduced in TGIP studies by initiating a low-level infection or immune stimulus in the parental generation. If parents do not predict correctly (i.e. there is no threat from pathogens), the resulting induced up-regulation of immunity may result in costs for the next generation by restricting resources that would otherwise be used for other aspects of their fitness. This interpretation is supported by evidence that up-regulation of the immune system is generally costly (Lochmiller & Deerenberg 2000; Hasselquist & Nilsson 2012), and also that TGIP leads to costs for offspring when there is no exposure to infection: Zanchi *et al* found that *Tenebrio molitor* offspring exhibited increased development time and reduced pupal mass when parents were challenged (Zanchi *et al.* 2011). Under this adaptive interpretation of TGIP, the benefits gained by the offspring by having an immune system in a state of readiness would outweigh the costs paid when mothers fail to correctly predict the likelihood of infection or parasitism. Classical life history theory predicts that such costs might be revealed under resource limitation, which has been traditionally used to study trade-offs made within a single organism's life history strategy (Boggs & Freeman 2005; De Block & Stoks 2008; Diamond & Kingsolver 2011).

An alternative interpretation of TGIP is as a by-product of host-parasite dynamics which is not adaptive for the host (Curno *et al.* 2011). For example, TGIP could involve

the vertical transmission of pathogens through sublethal persistent infection. Burden *et al* showed that RNA transcripts of *Plodia interpunctella* granulovirus persist in the ovaries, testes and offspring of individuals that survived a viral infection (Burden *et al.* 2002). In order to control for this, many studies have removed the possibility for vertical transmission by using heat-killed pathogens, pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPS), or even glass beads to challenge the parents (Sadd *et al.* 2005; Sadd & Schmid-Hempel 2007; Voordouw, Lambrechts & Koella 2008; Roth *et al.* 2010; Zanchi *et al.* 2011; Moreau *et al.* 2012; López *et al.* 2014). This is important to discount the possibility of vertical transmission, but this widespread use of more-or-less artificial immune activators means that few studies have mimicked an ecologically relevant situation by using live pathogens and measuring outcomes in terms of offspring survival (although see Tidbury *et al.* 2011). Typically, pathogen resistance in offspring has been inferred using measurements of general immune markers such as antimicrobial peptide or phenoloxidase activity (Sadd *et al.* 2005; Voordouw *et al.* 2008; Freitak *et al.* 2009; Zanchi *et al.* 2011), but an upregulated immune phenotype does not always correspond to pathogen resistance (Sadd & Schmid-Hempel 2009).

As yet, relatively few experimental designs have investigated a functional mechanism for TGIP, but the transfer of antimicrobial products within the eggs in a similar manner to egg provisioning with nutrients has been demonstrated in the bumblebee *Bombus terrestris* and the mealworm beetle *Tenebrio molitor* (Sadd & Schmid-Hempel 2006; Zanchi *et al.* 2012). This recent discovery could explain why TGIP has not been found in some invertebrate species, because the timing of the immune stimulus in the parental generation relative to the beginning of egg development is likely to be important and should be factored into future studies. Although research to date has concentrated on

maternal immune priming, paternal immune priming has also been confirmed and defined as a form of paternal investment in offspring (Roth *et al.* 2010; Jokela 2010). It seems likely that paternal effects would be mediated through epigenetic changes in the genome, such as CpG methylation, or the transfer of RNAi (Jones & Takai 2001; Glastad *et al.* 2011; Eggert, Kurtz & Diddens-de Buhr 2014), although a recent study found that the immune system could also be primed by substances contained in the seminal fluid (Eggert *et al.* 2014).

New research in invertebrate eco-immunology points to the increasing awareness of homology between the vertebrate and invertebrate immune systems. In the jawed vertebrates, antibody specificity is mediated by the somatic rearrangement of immunological receptors, giving rise to the huge diversity that allows the recognition of nearly any molecule by the immune system. This hallmark of immunological memory allows the rapid recognition of any pathogen which has previously been encountered. The evidence for invertebrate pathogen-specificity and memory is growing for within-generation priming (Sadd & Schmid-Hempel 2006; Roth & Kurtz 2009); however, we are as yet unsure as to the degree to which TGIP is pathogen-specific. An insufficient number of studies have been carried out in order to provide definitive facts, although highly strain-specific transgenerational immunity has been demonstrated once, and may well be more widespread than we realise (Little *et al.* 2003). DSCAM, a hypervariable immunoglobulin domain-encoding gene which can generate as many as 31,000 alternative splice forms (Dong, Taylor & Dimopoulos 2006) has been suggested as being potentially important for the generation of specificity in the invertebrate immune system (Kurtz & Armitage 2006; Armitage, Peuß & Kurtz 2015), and it has also been suggested that DSCAM can mediate specificity in transgenerational immunity (Rolff & Reynolds 2009), but this remains to be tested. Given that one mechanism of transfer

probably involves the inclusion of antimicrobial compounds within eggs, it seems unlikely that all transgenerational immunity is specific, as these compounds are generally effective against a wide range of pathogens including bacteria and fungi (Sadd & Schmid-Hempel 2006; Zanchi *et al.* 2012).

Additionally, no studies to date have considered the role of multiple infections and transgenerational immune priming. Coinfection is very common in nature (Cox 2001), but interactions between pathogens are poorly understood, particularly in invertebrates. There is potential for transgenerational priming against one or both pathogens, which may be dependent on the mechanism of pathogen attack, or the frequency with which parents are likely to encounter the pathogen. In order to test whether TGIP is affected by parental exposure to multiple pathogens, and to investigate the role of resource limitation in the expression of TGIP, I carried out a study of TGIP using a stored product pest, the pyralid moth *Plodia interpunctella,* and two pathogens, the bacterium *Bacillus thuringiensis* (Bt) and the fungus *Beauveria bassiana.*

Bt is a gram-positive soil dwelling bacteria and the primary natural route of infection is oral. Its mode of action involves the creation of pores in the gut using Cry and Cyt toxins, resulting in systemic septicaemia and the rapid onset of death (for more information about Bt, see chapter 3). *B. bassiana* is a general fungal entomopathogen which affects many insect orders (de Faria & Wraight 2007). Entomopathogenic fungi attack insects by penetrating the cuticle through mechanical or enzymatic means. As the haemocoel is colonised, fungal growth switches from hyphal growth to proliferation by yeast-like budding, but will switch back to filamentous growth to invade the internal organs (process reviewed in Castrillo, Roberts & Vandenberg 2005). *B. bassiana* also produces a variety of entomopathogenic secondary metabolites such as Beauvericin, which disrupt the host immune response and progression to metamorphosis (Grove &

Pople 1980; Boucias *et al.* 1995; Gupta, Montillor & Hwang 1995). Finally, there are very few instances of vertical transmission of fungi, and no evidence that Bt can be vertically transmitted, which makes them appropriate models for investigating TGIP as an anticipatory parental effect (Raymond, Elliot & Ellis 2008; Hesketh *et al.* 2010).

Both *B. bassiana* and Bt have been suggested as candidate biopesticides to play a role in integrated pest management (IPM), as part of the shift away from inundative chemical pest control. Although the coinfection dynamics of the two pathogens have not been tested in *P. interpunctella*, the results from within-generation studies from a small number of other pest insects are mixed. Field studies in the Colorado potato beetle suggested a small but significant synergistic effect between the two pathogens, possibly mediated by Bt delaying the host stadium intervals and providing *B. bassiana* with more time to penetrate the cuticle (Wraight & Ramos 2005). A separate laboratory study in the same species found no synergism (Costa, Barbercheck & Kennedy 2001), but the experimental design differed in that the pathogens were applied to different life stages (exposure to Bt δ-endotoxins occurred in the larval stage, whereas the subsequent exposure to *B. bassiana* took place when the beetles were pre-pupae). Generally there is very little information on how fungi interact with other parasites and pathogens (Cox 2001), and its study has traditionally been neglected in eco-immunology.

This study is the first of its kind to investigate the transgenerational transmission of resistance to both single infections and coinfections using the fungus *B. bassiana* and the entomopathogenic bacterium Bt. I exposed mothers to extremely low doses of three pathogen treatments (so as not to select for resistance): Bt, *B. bassiana*, or coinfection with both, and compared the performance of their offspring against one of the three pathogen treatments with larvae from control mothers. In order to examine the interaction between nutritional stress and transgenerational immune priming, offspring

were also divided into two treatment groups receiving either the normal lab diet or a nutritional stress treatment.

5.2 Materials and methods

5.2.1 Creating the parental generation

To create the parental generation, approximately 200 adult *Plodia interpunctella* were taken from the stock population and were allowed to mate. After 24 hours their eggs were placed on the standard laboratory diet comprising of a 10:1:1 ratio of wheat bran, brewers' yeast, and glycerol. 16 days later the female larvae had reached fourth instar and were allocated into the pathogen and control treatments groups (figure 5.1). The food was also searched for four subsequent days for more fourth instar larvae because I did not want to select only the early developing larvae, and these subsequent larvae were also allocated into the treatment groups.

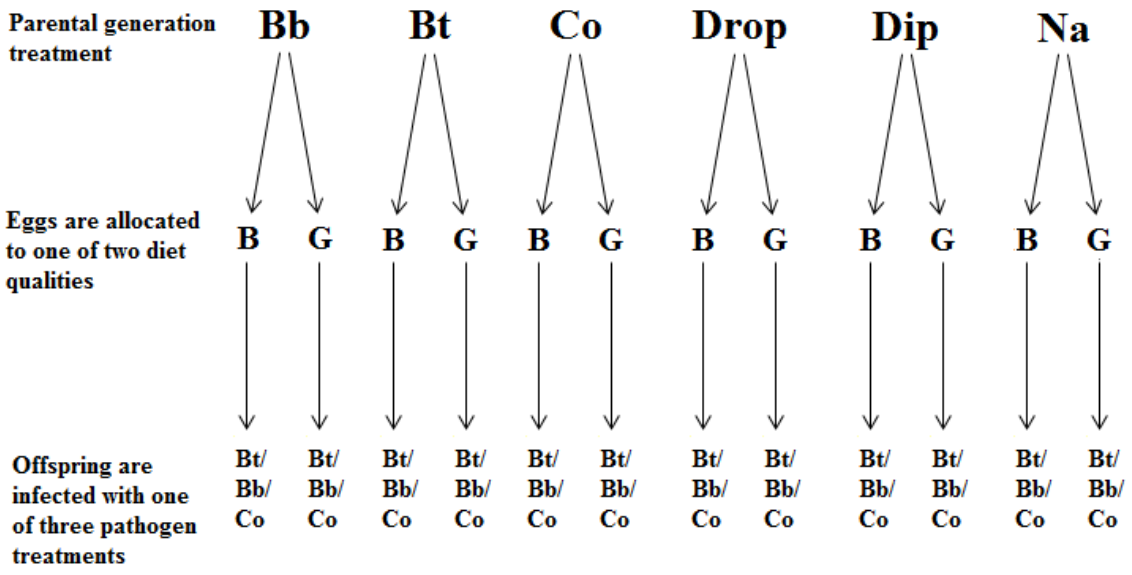

Figure 5.1: Experimental design. The parental generation are dosed with infection or control treatments in the fourth instar (Bb = dip in *B. bassiana* solution, Bt = droplet dose with *B. thuringiensis*, Co = coinfection with both pathogens, Drop = dosed with

control droplet of sugar water and food dye, Dip = control dip in distilled water, Na = naïve larvae are handled only). Mothers were mated with males from the stock population, and the eggs from each family were divided between two diet qualities (B = bad diet (20:1:1 wheat bran, brewers' yeast, glycerol), G = good diet (10:1:1)). When the offspring reached fourth instar, they were assigned to one of three pathogen treatments and assayed for survival (Bb = dip in *B. bassiana* solution, Bt = droplet dose with *B. thuringiensis*, Co = coinfection with both pathogens).

5.2.2 Pathogen cultures and infection in the parental generation

B. bassiana cultures were obtained from Rothamsted Research. Stocks were maintained in 50% glycerol solution at -80°C. The fungus was cultured on Sabouraud Dextrose Agar in Petri dishes sealed with parafilm. After three weeks of growth, the fungus was harvested in sterile conditions with an autoclaved scraper and vortexed for 10 minutes in autoclaved distilled water. This eliminated the need for a surfactant, which preliminary experiments showed affected the growth and survival of the larvae. The solution was then filtered using a Buchner funnel and vacuum pump into a sterile Duran bottle. Larvae were infected by dipping the whole animal into the spore suspension of 7900 spores/µl for 15 seconds (corresponding to an LD05 dose in preliminary experiments). This method was selected rather than injection or feeding, as it mimics the natural route of fungal infection through conidia attachment and penetration of the cuticle. Larvae were dried on a paper towel before being returned to their own individual Petri dish. The stock *B. bassiana* suspension was stored in a 4° C fridge and a fresh dilution for larval dosing was made up each day. The solution was vortexed thoroughly each day and sonicated for two minutes before use to prevent the spores clumping together. Preliminary experiments showed that the resulting mortality level in the larvae remained consistent over a week.

A 0.01 mg/ml suspension of Bt (DiPel formulation, ProGreen Weed Control Solutions Ltd, South Fen Business Park, Lincs) corresponding to an LD05 dose was created using sugar saturated water and 10% blue food dye. Larvae were infected by feeding 1ul droplets, were observed until all the solution had been finished, and were discarded from the experiment if they did not finish it (figure 3.2 in Chapter 3).

The coinfected treatment group was exposed to both *B. bassiana* and Bt with fungal exposure first to prevent contamination of the suspension with Bt. Three control groups were also used including 1) naive larvae that experienced handling with forceps, 2) larvae dipped in distilled water for 15 seconds, and 3) larvae fed a dose of 10% food colouring in sugar solution (figure 5.2). Larvae were stored at 26°C in an incubator on a 12:12 light: dark cycle in individual 55mm Petri dishes with *ad libitum* lab diet. The dishes were checked a week after infection and any dead larvae were removed from the experiment.

5.2.3 Mating and egg collection

Dishes containing pupae were checked daily for eclosion. Freshly eclosed females were mated with a freshly eclosed virgin male from the stock population. Eggs were counted after 48 hours and each family's eggs were split into two groups which were grown on either good (10:1:1 wheat bran, brewers' yeast, glycerol) or poor (20:1:1) diet in plastic pots measuring 5.4cm × 4.5cm. This offspring generation was allowed to develop in the incubator at 26°C, and pots were systematically searched on days 18, 19, 20 and 21 after egg laying for fourth instar larvae. When they reached fourth instar, offspring were assigned to 3 groups, 1) infection with a 0.35mg/ml dose of Bt (corresponding to LD33, n = 908), 2) 20000spores/µl dose of *B. bassiana* (corresponding to LD33, n = 899), or 3) coinfection with both pathogens (n = 903). After infection these larvae were placed in

10 cm ×10 cm 25 cell Petri dishes supplied with either good or poor diet as appropriate, and assayed for mortality 8 days later. Preliminary experiments showed that this is the optimum time to assay complete mortality from the fungus. Mortality from Bt primarily occurs within the first 48 hours after exposure.

5.2.4 Statistical analysis

Analysis was performed in R version 3.0.1 (R Development Core Team 2013) using the package lme4 (Bates *et al.* 2014). Three separate generalised mixed effects models with binomial errors were fitted for each of the types of offspring infection, i.e. infection with *B. bassiana*, Bt, or coinfection with both pathogens. The three different control treatments were collapsed into one control treatment. Models were initially fitted with two-way interactions between offspring sex, maternal Bt treatment, maternal fungus treatment and offspring diet quality. Higher order interactions were generally not included to avoid over parameterisation of the models (Zuur *et al.* 2009), although the interactions between maternal Bt treatment, maternal fungus treatment and offspring sex, and maternal Bt treatment, maternal fungus treatment and offspring diet quality were included to allow the coinfection treatment to interact with offspring diet and sex. Family ID and the date of offspring infection were included as crossed random effects. Terms were sequentially removed from the models, interaction terms first, and the nested models were compared with likelihood ratio tests until a minimal adequate model was achieved (Zuur *et al.* 2009).

5.3 Results

5.3.1 Effect of pathogen exposure on mothers (table 5.1)

There was no significant effect of the maternal treatment on the developmental period of mothers, measured as the amount of time taken in the larval and pupal stages before eclosion (figure 5.2, Bt treatments: $\chi^2 = 2.94$, df = 1, $p = 0.0862$, fungus treatments: $\chi^2 = 1.01$, df = 1, $p = 0.313$). However, there was a trend towards increased development period in treatments that include Bt, including in single exposure treatment and coinfection with the two pathogens. As can be seen from figure 5.2, Bt-infected mothers take on average 0.68 days longer to reach eclosion than their respective control treatment (droplet feeding).

Table 5.1: Maternal development time

Initial model: development time ~ Bt treatment * fungus treatment + (1|date.infected) n=558

Standard deviations of random effects

Date infected: 1.44

Fixed effects

Variable	**Estimate**	**Standard error**	**Degrees of freedom**	**Chi statistic**	**P value**
Bt treatment: fungus treatment			1	1.45	0.229
Fungus treatment			1	1.01	0.313
Bt treatment			1	2.94	0.0862
Minimum adequate model: development time ~ (1|date.infected)					

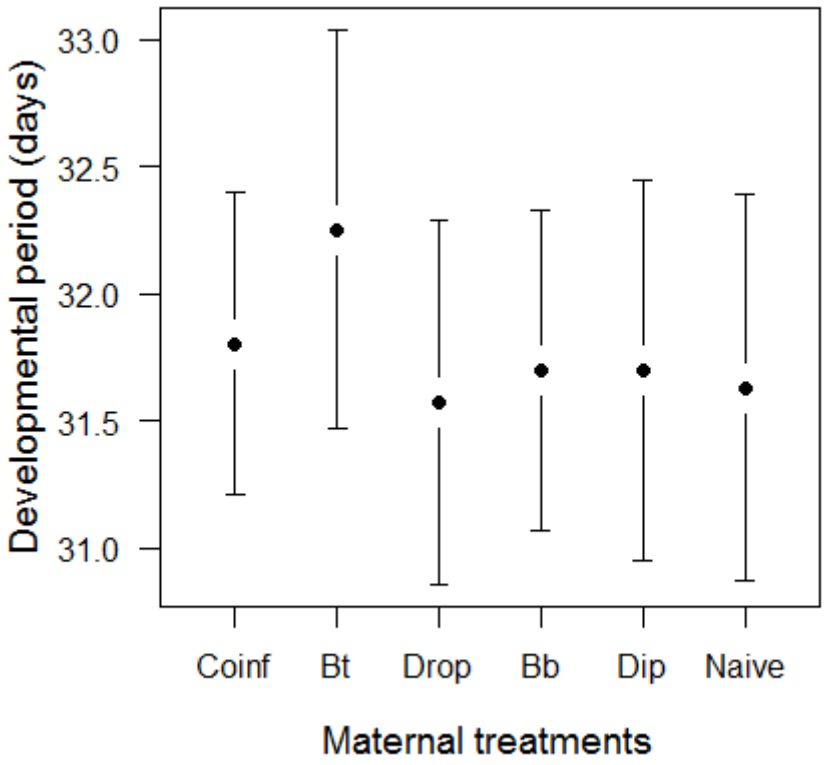

Figure 5.2: Maternal treatment in fourth instar does not significantly affect time to eclosion, measured as the number of days spent in the larval and pupal stages. Bars are 95% confidence intervals; X axis labels are Coinf = coinfected, Bt = infection with Bt, Drop = droplet fed a dose of sugar solution (control), Bb = infection with *Beauveria bassiana*, Dip = dipped in distilled water (control), Naive = just handling of the larva (control).

5.3.2 Offspring infected with Bt (table 5.2)

Offspring mortality from Bt was unaffected by maternal exposure to either Bt or *B. bassiana* or coinfection with the two pathogens (maternal Bt treatment: $\chi^2 = 2.10$, $p = 0.147$, maternal fungus treatment: $\chi^2 = 0.0433$, $p = 0.835$, maternal Bt × fungus interaction (coinfection treatment): $\chi^2 = 0.556$, $p = 0.458$). In contrast to the findings presented in Chapter 3, female offspring were not significantly better at resisting the Bt infection than their brothers ($\chi^2 = 0.0856$, $p = 0.770$).

Larvae had greater survival against Bt infection when they were fed a high-quality diet (figure 5.3, back-transformed values: good diet = 72.3% survival, poor diet = 63.2% survival, $\chi^2 = 6.65$, $p = 0.00993$), which confirms the results obtained in Chapter 3.

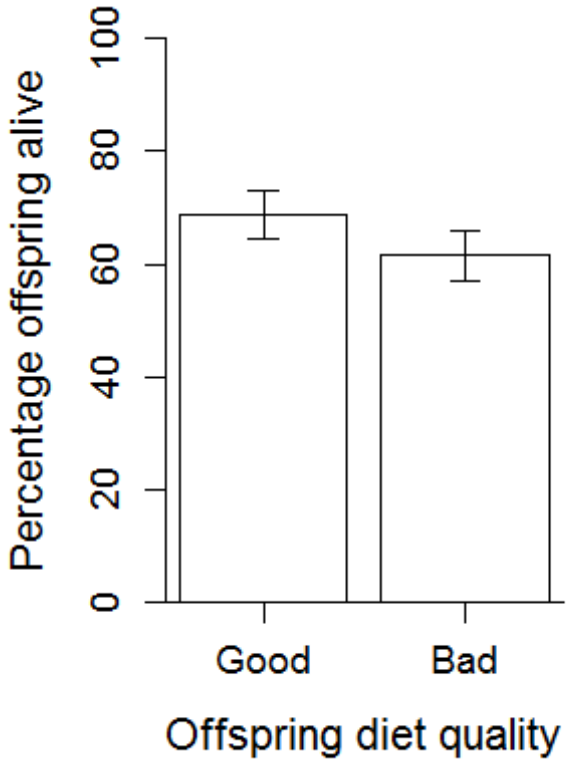

Figure 5.3: More offspring survive Bt infection when on the good diet than when nutritionally stressed; error bars are Agresti-Coull intervals calculated in the "binom" R package (Dorai-Raj 2014).

Table 5.2: Offspring infected by Bt

Initial model: Bt mortality ~ sex + Maternal Bt treatment + Maternal fungus treatment + Offspring diet quality (and all two way interactions) + Maternal Bt treatment:Maternal fungus treatment:Offspring diet quality + Maternal Bt treatment:Maternal fungus treatment:Sex + (1|family) + (1|date.infected), family=binomial.
n=908

Standard deviations of random effects

Family: 0.873
Date infected: 0.000207

Fixed effects

Variable	**Estimate**	**Standard error**	**Degrees of freedom**	**Chi statistic**	**P value**
Intercept	0.545	0.132			
Bt: Fungus: Food			1	0.281	0.596
Bt: Fungus: Sex			1	1.21	0.272
Sex: Bt			1	0.232	0.630
Sex: Food			1	0.250	0.617
Bt: Food			1	0.600	0.439
Sex: Fungus			1	0.429	0.512
Bt: Fungus			1	0.556	0.458
Fungus: Food			1	2.15	0.142
Fungus			1	0.0433	0.835
Sex			1	0.0856	0.770
Bt			1	2.10	0.147
Food (good)	0.417	0.162	1	6.65	0.00993

Minimum adequate model: Bt mortality ~ offspring food + (1|family) + (1|date.infected), family=binomial.

5.3.3 Offspring coinfected with both Bt *and* Beauveria bassiana *(table 5.3)*

When offspring larvae were coinfected with both the bacterial and fungal pathogens, I found a significant effect of maternal Bt treatment on offspring survival (figure 5.4, χ^2 = 6.50, p = 0.0108). This was the same in both the single maternal exposure treatment and coinfection with the two pathogens as indicated by the non-significant interaction between the two maternal pathogen exposures (χ^2 = 0.205, p = 0.651). However, unlike

previous experiments investigating TGIP, I found that maternal exposure to Bt actually decreased offspring survival by 10.8%. This did not occur with the maternal fungal treatment, indicating a pathogen-specific burden on mothers ($\chi^2 = 0.772$, $p = 0.380$). There was no effect of sex on larval survival ($\chi^2 = 0.0077$, $p = 0.930$). Although food quality was a determinant of offspring survival when infected with Bt alone, this was not apparent when offspring were coinfected ($\chi^2 = 1.62$, $p = 0.203$).

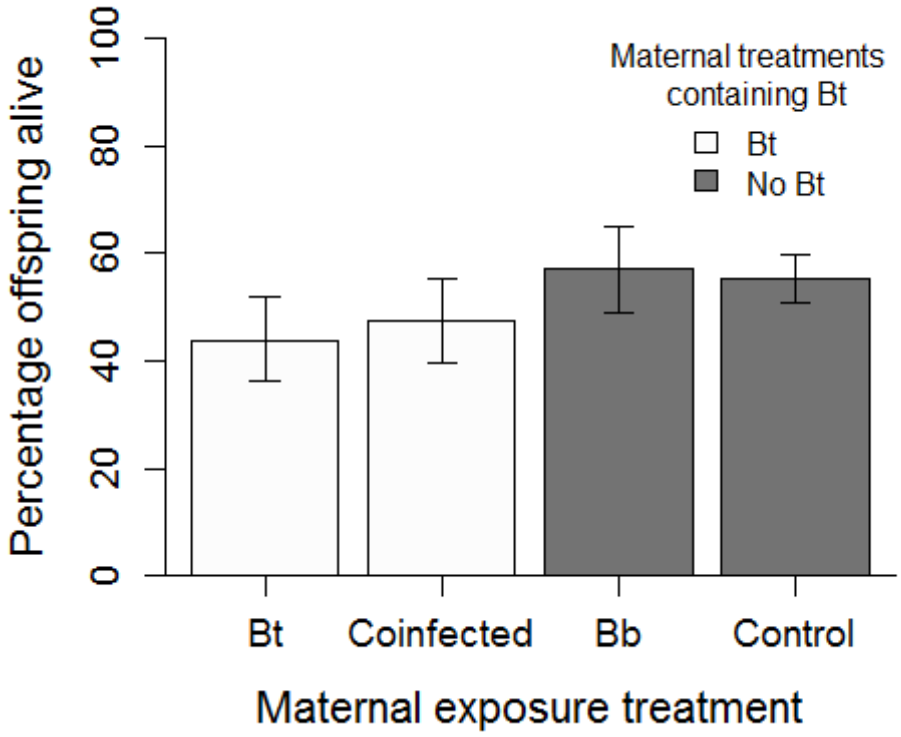

Figure 5.4: Maternal infection with Bt, including both the single exposure treatment and coinfection with two pathogens, resulted in increased numbers of offspring dying when coinfected with both parasites when compared with offspring from mothers given a fungal or control treatment; error bars are Agresti-Coull intervals.

Table 5.3: Offspring coinfected by Fungus and Bt

Initial model: Coinfected mortality ~ sex + Maternal Bt treatment + Maternal fungus treatment + Offspring diet quality (and all two way interactions) + Maternal Bt treatment:Maternal fungus treatment: Offspring diet quality + Maternal Bt treatment:Maternal fungus treatment:Sex + (1|family) + (1|date.infected), family=binomial.
n=903

Standard deviations of random effects

Family: 0.447
Date infected: 0.298

Fixed effects

Variable	**Estimate**	**Standard error**	**Degrees of freedom**	**Chi statistic**	**P value**
Intercept	-0.169	0.153			
Bt: Fungus: Sex			1	0.376	0.540
Bt: Fungus: Food			1	1.15	0.285
Fungus: Food			1	0.0398	0.842
Bt: Fungus			1	0.205	0.651
Bt: Food			1	0.0742	0.785
Food: Sex			1	0.115	0.734
Fungus: Sex			1	0.638	0.425
Bt: Sex			1	2.04	0.154
Sex			1	0.0077	0.930
Fungus			1	0.772	0.380
Food			1	1.62	0.203
Bt (no Bt treatment)	0.433	0.169	1	6.50	0.0108

Minimum adequate model: Coinfected mortality ~ Maternal Bt treatment + (1|family) + (1|date.infected), family=binomial.

5.3.4 Offspring infected with B. bassiana *(table 5.4)*

There was no effect of the heterologous maternal pathogen exposure (Bt) on offspring mortality when infected with *B. bassiana* ($\chi^2 = 0.675$, $p = 0.411$). When mothers had not received a fungus treatment, 75.2% offspring survived when fed the good diet and similarly 76.5% survived when fed the bad diet. Offspring mortality was affected by the interaction between maternal infection with *B. bassiana* and larval diet quality ($\chi^2 =$

6.40, $p = 0.0114$, figure 5.5). Maternal exposure to *B. bassiana* had a slight beneficial effect on the survival on the offspring when they were reared on the good diet, with 81% surviving the dose of *B. bassiana*, but when the larvae experienced nutritional stress maternal fungal priming had a negative effect on progeny survival with only 66.3% surviving. This negative effect was the same for offspring of mothers given a single pathogen exposure and also for those from mothers exposed to Bt as well as *B. bassiana*, as indicated by the non-significant three-way interaction between the two maternal pathogen exposures and diet quality ($\chi^2 = 1.06$, $p = 0.304$). Examination of effect sizes and confidence intervals suggests that this significant interaction is largely a consequence of the increased mortality in the maternal exposure-poor food treatment group Sex had no effect on survival, and was removed as a term in the model ($\chi^2 = 2.89$, $p = 0.0893$).

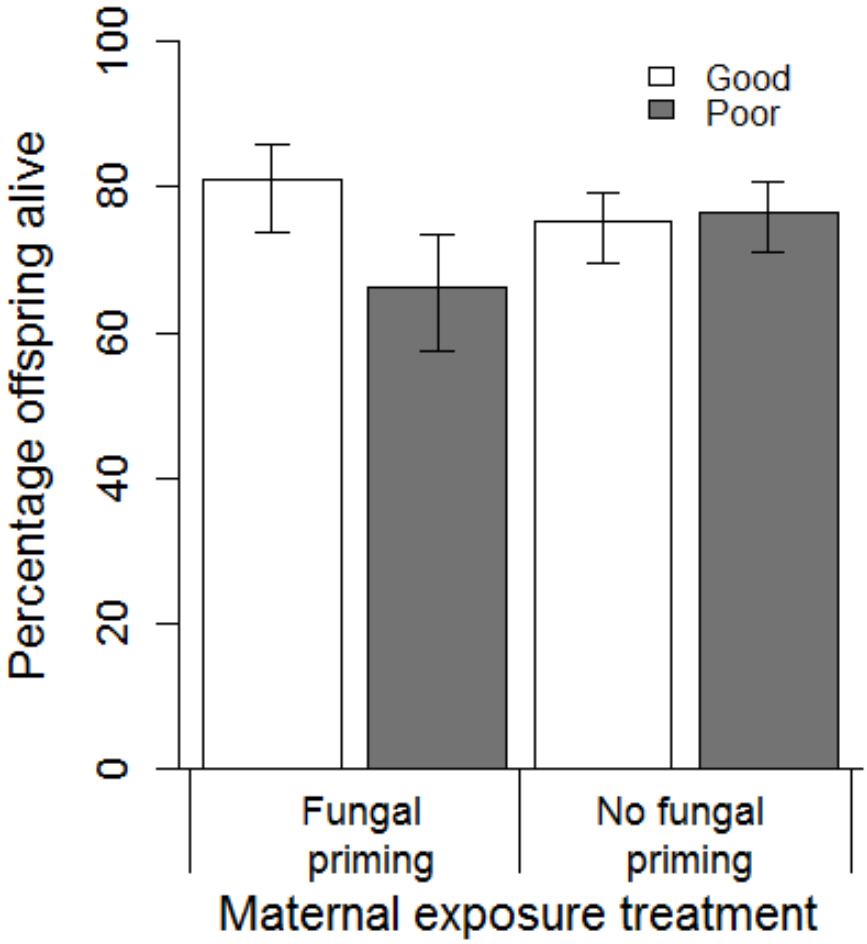

Figure 5.5: There was a significant interaction between maternal exposure to *B. bassiana* and the diet quality available to larvae; error bars are Agresti-Coull intervals.

Table 5.4: Offspring infected by Fungus

Initial model: Fungus mortality ~ sex + Maternal Bt treatment + Maternal fungus treatment + Offspring diet quality (and all two way interactions) + Maternal Bt treatment:Maternal fungus treatment:Offspring diet quality + Maternal Bt treatment:Maternal fungus treatment:Sex + (1|family) + (1|date.infected), family=binomial.
n=899

Standard deviations of random effects

Family: 0.132

Date infected: 0.230

Fixed effects

Variable	**Estimate**	**Standard error**	**Degrees of freedom**	**Chi statistic**	**P value**
Intercept	0.675	0.200			
Bt: Fungus: Sex			1	0.697	0.404
Bt: Fungus: Food			1	1.06	0.304
Bt: Fungus			1	0.0193	0.890
Bt: Sex			1	0.0190	0.890
Food: Sex			1	0.310	0.578
Bt: Food			1	0.882	0.348
Fungus: Sex			1	1.59	0.208
Bt			1	0.675	0.411
Sex			1	2.89	0.0893
Fungus (fungus treatment): Food (good)	-0.848	0.335	1	6.40	0.0114
Fungus (fungus treatment)	0.508	0.236			
Food (good)	0.773	0.275			

Minimum adequate model: Fungus mortality ~ Maternal fungus treatment * Offspring diet quality + (1|family) + (1|date.infected), family=binomial.

5.4 Discussion

This experiment has been the first to investigate coinfection with two pathogens within the framework of transgenerational immune priming. Offspring from each family received either a Bt infection, *Beauveria bassiana* infection, or a coinfection with the

two. Their mothers were exposed to one of the three pathogen treatments, or a control dose. I was therefore able to investigate 1) the possibility of TGIP against a bacterial and fungal pathogen in *Plodia interpunctella*, 2) whether this priming is specific to the pathogen, or whether resistance to a heterologous pathogen is also increased, 3) whether coinfected mothers were able to pass on resistance to one, both, or neither of the pathogens, and 4) how offspring response to infection changes with the nutritional resources they are able to obtain. Rather than a straightforward situation arising from the presence of offspring protection in accordance with maternal exposure, I found that maternal exposure to pathogens either led to no change in offspring resistance to infection (e.g. when mothers were exposed to Bt and the offspring were challenged with either *B. bassiana* or Bt), or led to reduced rather than increased offspring survival (e.g. when mothers and offspring were exposed to *B. bassiana* and the offspring were fed a poor diet, or when mothers were exposed to Bt and the offspring were challenged with both pathogens). Whilst TGIP has been found to occur in a limited number of insect species, I suggest that it is not as common as previously thought, and any benefits accrued to offspring should be viewed within an inclusive framework that includes potential costs to the parents.

5.4.1 Outcomes for Bt-infected progeny

Maternal exposure to Bt did not upregulate their offspring's resistance to this pathogen, so there was no evidence for TGIP in this host-pathogen combination. TGIP is not ubiquitous amongst invertebrate species and has not been found in the yellow fever mosquito *Aedes aegypti* or *Drosophila* (Voordouw *et al.* 2008; Linder & Promislow 2009). However, TGIP has been shown with Bt in a number of other invertebrate species, including the red flour beetle *Tribolium castaneum* and the cabbage looper *Trichoplusia ni* (Roth *et al.* 2010; Eggert *et al.* 2014; Shikano *et al.* 2015). It is

uncertain as to why *P. interpunctella* does not exhibit TGIP to Bt when the other species do. Two other studies have suggested the transfer of a maternal immune phenotype occurs because of protection provided by transferred antimicrobial peptides in the eggs (Sadd & Schmid-Hempel 2006; Zanchi *et al.* 2012). Although the immune response is important in lepidopteran resistance to Bt, modes of defence could include other physiological changes, including the reduction in the receptor affinity in the brush border membrane of the midgut epithelium (Van Rie *et al.* 1990; Ferré *et al.* 1991), or downregulation of protease production that interferes with Cry/Cyt protoxin activation (Ibrahim *et al.* 2010). It may be that these mechanisms of defence cannot be adaptively upregulated in a single generation, although we do know that laboratory resistance to Bt in *P. interpunctella* can evolve in just a few generations (McGaughey 1985). Alternatively, another study of TGIP and Bt has shown that transgenerationally acquired resistance in offspring is environmentally dependent, i.e. when both parents and offspring are nutritionally stressed, or when parental diet is good and offspring diet is poor (Shikano *et al.* 2015), which may be a possible avenue for future exploration.

The nutritional environment significantly affected the percentage of larvae surviving the Bt infection, such that survival amongst larvae experiencing the more nutritious diet was 9.1% higher. The poor diet in this experiment was created by diluting the macronutrient-rich components of the diet (protein-rich yeast, glycerol) with wheat bran, which is not as nutrient-rich and acts as a bulking agent in these diets. One explanation for this pattern is that nutritionally stressed individuals could be more susceptible to infection if the composition of the poor diet resulted in upregulated nutrient processing to maximise the macronutrients from their poor diet. Invertebrates have a wide array of plastic pre- and post-digestive processes to order to obtain the required nutrients from the diet in response to food deprivation or nutrient imbalance.

For example, when macronutrients in the diet are diluted, or when they are present in an uneven ratio, the size of the gastrointestinal tract has been shown to increase (Sørensen *et al.* 2010; Clissold, Brown & Simpson 2012). In *Drosophila*, increased levels of stem cell divisions have been shown to mediate gut remodelling when nutrient levels change (O'Brien *et al.* 2011), which could facilitate this plasticity. However, a side-effect of gastro-intestinal enlargement might be that there are more receptors for Bt to attack the midgut, resulting in an increased level of mortality. Eco-immunology studies using oral inoculation as a route to infection should take note of factors affecting gut size as possible variables to control when administering the desired dose.

Other studies indicate that there is generally a positive relationship between poor diet and decreased levels of immune markers (Siva-Jothy & Thompson 2002). Parasite resistance is energetically expensive and often associated with a protein cost due to the production of immune cells and peptides (Wilson & Cotter 2013). When a fixed amount of resources is allocated between costly traits (Sheldon & Verhulst 1996), if the total pool of acquired resources is decreased under nutritional stress then immunity may suffer. Selection experiments using Bt have shown that resistance is traded-off with development time or pupal weight, and is also lost when selection pressure is relaxed, indicating the costly nature of defence against this pathogen (Oppert *et al.* 2000; Janmaat & Myers 2003). It is therefore also possible that the reduced survival associated with poor diet is a consequence of reduced immune reactivity in those individuals.

5.4.2 Outcomes for coinfected progeny

Maternal infection with Bt was costly when offspring were infected with both pathogens, as 10.8% more offspring died from mothers exposed to single exposure to Bt

and coinfection with both pathogens than larvae from mothers that had experienced the fungus or control treatments. It is possible that there is an increased immune cost from upregulating defences against both the bacterial and fungal pathogens, as they may require different immune molecules for defence such as specific antifungal peptides (Mak, Zdybicka-Barabas & Cytryńska 2010; Arvanitis, Glavis-Bloom & Mylonakis 2013). However, this is confounded by the fact that progeny are also exposed to more infective units of pathogen (the coinfective dose was stronger than the single doses, producing 47.5% mortality in this experiment, in comparison to 34.7% for Bt and 24.9% for *B. bassiana*), which would also require increased resources for immune defence. Further experiments which were beyond the scope of this study could separate these two possible explanations, for example by comparing doses which controlled for the numbers of infective units with additives doses, combined with changing the type of pathogen as a separate factor.

Despite a recent focus on the beneficial and possibly adaptive effects of TGIP in the eco-immunology literature (Sadd *et al.* 2005; López *et al.* 2014), my study highlights the burden of infection on a mother's fitness. It is interesting to consider that my experiments showed that costs for mothers were present even though the maternal dose of pathogen was very low (2.14% mortality of a total of 234 Bt and coinfected mothers). Although TGIP has only been recently confirmed as an aspect of the immune system of some invertebrate species, it is likely that the costs that mothers suffer to their fitness may be more widespread and important in defining the evolutionary dynamics of response to infection. Infection has been shown to result in serious costs to reproductive success, including reduced output and viability of offspring, as well as the mother's future breeding bouts and her own somatic condition (Boots & Begon 1993; Cotter, Kruuk & Wilson 2004; Hurd *et al.* 2005; Reavey *et al.* 2014, reviewed in Zuk & Stoehr

2002; Rolff & Siva-Jothy 2003). The energetic and nutritional costs of infection have been extensively investigated by eco-immunologists, from the deployment and maintenance of the immune system, to the risk of self-reactivity (Stearns 1989; Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Schmid-Hempel 2003; Wilson & Cotter 2013). Here I have shown another cost of infection which is the production of less resistant offspring, although this is specifically dependent on the offspring's own environment and pathogen exposure.

5.4.3 Outcomes for B. bassiana-infected progeny

When larvae were infected with *B. bassiana*, maternal exposure to the fungus was detrimental, causing 33.7% mortality in comparison to 23.5% mortality in larvae descended from mothers who experienced the Bt and control treatments. Unlike the costs paid by coinfected offspring, however, maternal exposure to infection in fungus-infected progeny only decreased survival when the larvae were nutritionally stressed. There is a suggestion that larvae received a moderate beneficial effect of maternal immune priming from mothers infected with *B. bassiana* (i.e. the single maternal fungus treatment and the coinfected maternal treatment) when they experienced plentiful resources, although the effect is weak and not significantly different from control maternal treatments. Overall, our results are indicative of pathogen-specific costs depending on the nature of the challenge received by mothers and offspring. Maternal exposure to *B. bassiana* is costly when offspring are infected with the homologous pathogen and experiencing nutritional stress, and maternal exposure to Bt is costly when offspring are coinfected with both pathogens.

Few studies have emphasised the costly nature of TGIP in terms of higher levels of parasitic infection in offspring or dependence on nutritional resource levels (although

see Sadd & Schmid-Hempel 2009; Vantaux *et al.* 2014; Shikano *et al.* 2015). If TGIP systems are only tested under ideal conditions of maximum food then we may be building a false picture of how common and beneficial TGIP is. I suggest that the production of less resistant offspring and related costs may be more widespread than is currently realised and play a more prominent role in defining host-pathogen dynamics than the beneficial effects of TGIP. Interesting avenues for further experimentation also include investigations into maternal prediction of resource availability for their offspring so that they are able to maintain an increased level of immunity without suffering costs to survival. If the mother also experiences resource limitation, we can speculate that mothers could remove an effect of priming, which would become apparent with an experimental design which also includes maternal diet as an extra factor. This assumes that the maternal experience is a good predictor of offspring environment, which has recently been criticised in the literature (Burgess & Marshall 2014), and may not have evolved in *P. interpunctella* as parents are able to disperse from their larval environment through post-eclosion flight.

Finally, this study has implications for new pest management programs that are taking advantage of the increased popularity of biopesticides. *P. interpunctella* cannot transgenerationally upregulate the resistance of their progeny to Bt, despite having evolved resistance to it in the lab (McGaughey 1985), although due to the prevalence of Bt-induced TGIP amongst other insects, there are good reasons to conduct further experiments examining a greater range of Bt strains and environmental conditions in conjunction with multiple generations of *P. interpunctella*. In some cases maternal infection with Bt decreased offspring fitness by making them more susceptible to disease but only when they were experiencing a significant pathogen burden from coinfection with bacterial and fungal pathogens. *B. bassiana* is effective at controlling

P. interpunctella and this effect is increased when used over two generations but only when the diet quality is poor.

5.5 Summary

This study investigated whether maternal exposure can affect offspring survival when they are infected with the homologous or heterologous pathogen. When mothers were exposed to *Bacillus thuringiensis*, there was no transgenerational immune priming when the offspring were challenged with Bt and fewer offspring survived when coinfected with both pathogens. Maternal exposure to the fungus *Beauveria bassiana* led to increased larval mortality, but only when larvae were nutritionally stressed. When mothers were exposed to both pathogens, no interaction between the two affected offspring survival. When offspring were challenged with Bt, those larvae that consumed good diet had a higher rate of survival than those that consumed poor diet. I did not find evidence for TGIP in this system, but instead discovered that maternal exposure to pathogens results in higher rates of mortality amongst offspring, depending on the combination of maternal and offspring pathogen-specific infections and resource limitation in the offspring generation. The production of less resistant offspring and related costs may be more widespread than is currently realised in TGIP systems.

Chapter 6: General Discussion

6.1 Thesis overview

In this thesis, I have examined the transgenerational effects of diet and infection from an ecological and evolutionary perspective. My aims were to determine the transgenerational effects of parental diet and pathogen exposure status on the phenotype of offspring, whilst defining the particular dietary macronutrients which produced transgenerational effects, as well as the relative importance of the offspring's own environment and infection status. This work represents an important empirical contribution to defining the environmental influences that can be transgenerationally inherited. Although as yet transgenerational inheritance is poorly integrated into current evolutionary synthesis, it is receiving considerable interest as a mechanism which seems to allow populations to make plastic changes in response to the environment at a faster rate than genetic changes (Bonduriansky, Crean & Day 2012; Laland *et al.* 2014).

Much of this thesis examines novel areas within the framework of transgenerational effects of diet and infection. Multiple environmental variables are still understudied in parental effects experiments due to the relatively time-consuming nature of the experiments and large sample sizes needed, but these are important if we are to learn about interactions between different sources of environmental variation. For similar reasons, fully factorial experiments that examine the relative importance of maternal, paternal and offspring environments in contributing to phenotype have also been neglected. The paternal contribution to phenotype is particularly understudied, despite its probable importance in many species where fathers are able to transfer signalling molecules and nutrients packaged within the spermatophore during mating. Whereas the within-generation effects of both the amount and type of macronutrients on life history

traits are relatively well characterised by experiments that use the geometric framework of nutrition (Raubenheimer & Simpson 1999; Ponton *et al.* 2011), here the transgenerational effects of particular macronutrients are investigated which is novel in invertebrates. Previously, only non-chemically defined diets had been used (created, for example, by dilution or substituting in different organically complex food types), which cannot explain the relative importance of each macronutrient.

In the last decade we have built up a body of evidence to confirm the existence of transgenerational immune priming (TGIP) in invertebrates (Little *et al.* 2003; Sadd *et al.* 2005; Sadd & Schmid-Hempel 2007; Roth *et al.* 2010; Zanchi *et al.* 2011). To date, however, studies of TGIP have mostly been restricted to single infections in ideal laboratory environments. Here I addressed these unexamined areas of research by investigating the effects of resource limitation and coinfection with multiple pathogens. In addition, I included the use of live pathogens in my experiment, which to date has not been prominent but is very important if we are to determine real host-pathogen dynamics and their implications for fitness.

6.2 Chapter summaries

Chapter 1: The evolutionary dynamics of transgenerational effects are introduced, including consequences for both individuals and populations. Current speculation around the proximate mechanisms of transgenerational effects is explored. A discussion of insect pathogens and the invertebrate immune system is provided to give context around the disease resistance assays and TGIP experiment presented within the thesis. Finally, the Indian meal moth *Plodia interpunctella* is introduced as the model organism.

Chapter 2: Artificial diets suitable for rearing *P. interpunctella* were developed where both the total nutritional content and the ratios of different macronutrients to each other can change, which have comparable effects to the bran-based lab diet. These diets facilitated a study in Chapter 2 on defining the influence of non-nutritive: nutritive components of diet on development and lifespan, as well as the transgenerational effects of dietary macronutrients in Chapter 4. In the study on development and lifespan, I find that reducing dietary macronutrient content available to larvae extends adult lifespan, which is consistent with a dietary restriction hypothesis. The development of these diets could facilitate further studies of nutritional ecology using *P. interpunctella* as a model organism, for example by use in geometric framework studies *sensu* Simpson and Raubenheimer (Simpson & Raubenheimer 2012).

Chapter 3: This chapter used a fully factorial manipulation of maternal, paternal and offspring diets to determine the relative contribution of each to offspring phenotype. The current environment was the major determinant in defining disease resistance dynamics and adult lifespan, although there were also subtle influences of maternal and paternal nutrition on adult lifespan. Most notably, when mothers and fathers consumed poor diets, their offspring had longer adult lifespans. Eclosion weight was not as strongly defined by the current environment but was influenced by an interaction between the diet of both offspring and mother. For female offspring, when their diet quality matched that of the mother, they were heaviest, than when diet qualities were mismatched, indicating a possible anticipatory parental effect.

Chapter 4: After interesting transgenerational effects of parental diet had been discovered here and in previous work by this lab (Triggs & Knell 2012), I wanted to expand on this work by using artificial diets to investigate the precise effects of dietary macronutrients. There were no effects on phenoloxidase reactivity or haemocyte counts

in the within-generation data, and only very modest transgenerational effects on immune reactivity in offspring. Eclosion weight varied with the macronutrient dilution, with the diets with the lowest concentration of macronutrients producing the lightest moths, but there were no transgenerational effects on eclosion weight in offspring.

Chapter 5: Experiments in this chapter investigated the effects of resource deprivation and maternal pathogen exposure on the ability of offspring to resist infection with either a single infection or coinfection with two pathogens. I found no priming effect when challenging mothers with *Bacillus thuringiensis* (Bt) or *Beauveria bassiana*, in contrast to several other studies of invertebrate immune priming (Roth *et al.* 2010; Eggert, Kurtz & Diddens-de Buhr 2014; Shikano *et al.* 2015). Food quality was the only determinant of larval survival against Bt. When larvae were reared on good quality food, a greater number survived the Bt challenge, which reinforced results obtained in Chapter 3. Maternal pathogen exposure sometimes decreased offspring fitness by rendering them more susceptible to infection, for example, when mothers were infected with *B. bassiana* and offspring were nutritionally stressed, or when offspring were coinfected with both pathogens and mothers were exposed to Bt.

6.3 Discussion and synthesis arising from this thesis

Transgenerational effects of diet

In this thesis I examined effects on offspring phenotype resulting from variation in parental diet in combination with changing the offspring's own environment. Largely, the transgenerational effects of parental diet were relatively modest or non-existent when contrasted with environmental influences from the current environment. This contrasts with previous research which has highlighted the importance of transgenerational influences on phenotype (Mousseau & Dingle 1991; Fox & Czesak

2000; Ferguson-Smith & Patti 2011), and could be a result of the plastic biology of *P. interpunctella* as a generalist pest species.

Results stemming from the artificial diets in Chapter 4 concerning the transgenerational effects of dietary macronutrients point to the apparent robustness of *P. interpunctella* to significant variation in parental diet quality. The experiments involving the bran-based diets in Chapter 3 did produce small transgenerational effects on phenotype, although these were dwarfed by effects produced by variation in the offspring's own diet. This is surprising given results that have previously emphasised the importance of parental diet quality on offspring life history (Bonduriansky & Head 2007; Frost *et al.* 2010; Franzke & Reinhold 2012; Triggs & Knell 2012). *P. interpunctella* is a generalist pest species, living in conditions of varying diet quality, and may have evolved pre-or post-digestive regulatory mechanisms to homeostatically respond to deficits in resource quality. These include consuming larger amounts of food (although this was not obvious (pers obs.)), changes in the length or shape of the gastro-intestinal tract, and the differential release of digestive enzymes (Raubenheimer & Bassil 2007; Clissold *et al.* 2010). If mothers are able to compensate for resource deficits in this way, this could explain a lack of transgenerational effects on phenotype, even in the face of extreme variation in diet quality. Catch-up growth in the early larval instars could also help offspring counteract a lack of parental investment. The plastic biology of pest species could be important for human food security as food products are damaged or lost to pest organisms such as *P. interpunctella*.

Interactions between diet and infection

Good quality diet resulted in greater numbers of larvae surviving infection with Bt (Chapters 3 and 5). Immune measurements were not taken during these experiments,

although it is known from previous work in *P. interpunctella* that bran-based diets with a higher ratio of glycerol and yeast result in raised constitutive phenoloxidase and haemocyte cell titres (Triggs & Knell 2012), indicating that when *P. interpunctella* is provisioned with greater amounts of protein-based resources it is able to upregulate immune defence. We should also consider the fact that when infections occur through the oral and gastro-intestinal route, aspects of digestion and nutrient processing, such as the length, shape or receptors in the gastro-intestinal tract have the potential to interact with pathogen dynamics.

Diet quality determined the effect of maternal pathogen exposure on offspring survival when larvae were infected with *B. bassiana* (Chapter 5). When mothers were exposed to a fungal pathogen, fewer of their resource-deprived offspring survived when infected than those that originated from mothers that were not exposed to *B. bassiana*. Offspring appeared to be unable to pay the costs of raised resistance to an infection under resource deprivation. This highlights the importance of examining TGIP systems under conditions of environmental stress such as reduced diet quality and coinfection with multiple pathogens – if we do not do so we risk over-stating the beneficial effects of TGIP.

Diet is thought to provide resources for inducible immune defences (Zuk & Stoehr 2002; Siva-Jothy & Thompson 2002) which is likely to be occurring here, however, some complexities are apparent within this relationship which could provide interesting avenues for future investigation. The resources obtained through diet could supply the parasite in addition to the host, increasing parasite virulence over the course of infection. Infection-induced anorexia is thought to be an adaptive response to this, as the host increases tolerance to the parasite (Ayres & Schneider 2009; Povey *et al.* 2013). It is also important to consider that diet is not a simple transfer of energy that can be

immediately converted into resources available for expenditure. Essential micronutrients, for example, have shown to be important for health, without which an organism may not be able to develop key traits or pass through particular developmental stages, such as metamorphosis (Wilson & Cotter 2013).

Strategies of parental effects

Anticipatory parental effects occur when parents are able to predict offspring environment from their own environmental experience and use developmental plasticity to adjust offspring phenotype accordingly. Offspring may suffer costs from a mismatched environment in comparison to a matched one, for example if metabolism and development is canalised by parental experience. While overall evidence for anticipatory parental effects is weak (Uller, Nakagawa & English 2013), I demonstrated in Chapter 3 that eclosion weight for female offspring is heavier when they are matched to the maternal environment, even if both the diet qualities are nutritionally poor. Although this is suggestive, other evidence must be obtained before this is deemed an anticipatory parental effect, as we are unable to determine from these experiments whether parental *P. interpunctella* could predict future resource availability.

There was moderate evidence to support a resource allocation hypothesis. Effects on offspring weight were present but subtle: mothers produced heavier sons when they received a good diet, which produced a stronger effect than the male offspring's own diet (Chapter 3). In Chapter 4, mothers consuming a wide variety of diets appeared to be able to compensate for this on most aspects of their offspring's phenotype. Only those on the most extreme diet with the smallest amount of protein laid fewer eggs in the first days after mating. When mothers were exposed to bacterial and fungal pathogens (Chapter 5), their offspring had lower survival when infected themselves, relative to

those from control mothers. This could indicate resource allocation away from offspring quality to maternal ability to resist infection.

6.4 Future directions for research

Linking parental effects with ecology

Burgess and Marshall call for further integration of studies of parental effects with the ecology of organisms (Burgess & Marshall 2014). Some insects have obvious control over the environment of offspring, such as the telescopic generations of aphids, where in effect offspring environment is controlled by the mother (Mousseau & Dingle 1991). However, in other organisms we must consider whether parents would be able to predict the future environment of their offspring, if we are to classify a particular phenomenon as an anticipatory parental effect. Prediction might occur through similarity with the parental environment, or detection of the offspring's future environment, for example through choosing oviposition sites. For example, in the herb *Campanulastrum americanum*, an anticipatory parental effect has been identified in association with environmental light levels, as seeds do not disperse far from the parental tree, and therefore parental environment is a good indicator of progeny light levels (Galloway & Etterson 2007). However, insect parents often disperse away from their natal site where they have been feeding. In *P. interpunctella*, the adult stage is able to disperse by flight away from the larval feeding environment, in addition to dispersal as wandering fifth instar larvae. Therefore the future environment of offspring may be considerably spatially separated from that of the parents, although there is some evidence to suggest that *P. interpunctella* can detect and choose oviposition sites. Some insects can select the environment of their offspring, for example the seed beetle *Stator limbatus* lays larger offspring when the coat of seeds (which they use for oviposition sites) is thicker

(Fox, Thakar & Mousseau 1997). Advances in technology such as data loggers and remote environmental sensing will enable us to more accurately capture data in the field to monitor environmental predictability (Burgess & Marshall 2014).

The impact of parental effects on population dynamics

Beckerman *et al* describe how indirect parental effects could occur when direct parental effects (such as changes in the number or size of offspring as a result of parental resource allocation) drive changes in the density or recruitment rate within the offspring population (Beckerman *et al.* 2006). Increased density could result in greater competition for food and higher levels of pathogen transmission, which then alter the offspring phenotype themselves. Therefore, direct parental effects on the phenotype of an individual have the potential to define the environment of siblings (for example, in a nest) and non-siblings when they are scaled up to population level. Direct parental effects could also influence sibling interactions, such as cannibalism, which is present amongst larval *P. interpunctella*. There is substantial scope for direct and "indirect" parental effects to be investigated together in model systems such as *P. interpunctella* where population dynamics have been extensively investigated, transmission dynamics with co-evolved pathogens are well defined, and the simultaneous replication of multiple populations within a lab can be supported (Boots & Begon 1993; Sait, Begon & Thompson 1994a; b).

Elucidating the mechanisms of transgenerational effects

Multiple reviews of parental effects have called for more information on the proximate mechanisms by which they act. Work is beginning to investigate the mechanisms through which TGIP acts, such as the transfer of antimicrobial compounds within eggs which means that offspring are protected from the outset (Sadd & Schmid-Hempel

2007). However, it seems unlikely that the transfer of antimicrobial compounds would provide strain-specific protection, as seen in *Daphnia magna* (Little *et al.* 2003), because antimicrobial compounds are active against a broad range of parasites.

TGIP can be considered within the broader context of resource allocation by parents, especially if the production of immune molecules is considered to be costly (Lochmiller & Deerenberg 2000). Resource allocation could also include egg provisioning by parents: traditionally egg size has been used as a proxy for nutrient provisioning in invertebrates (Fox & Czesak 2000), although this is a crude measure and cannot take into account the differences in egg composition, for example the inclusion of higher quality proteins. Studies that take into account changes in both egg size and composition (such as Pöykkö & Mänttäri 2012) as mothers respond to the environment, and subsequently link these traits to offspring fitness, will be invaluable in considering how mothers are able to plastically alter their investment.

The epigenome can change throughout the life of an organism in response to environmental stimuli, and changes can be passed on to offspring via the transfer of epigenetic marks (McKay & Mathers 2011). As yet, hypotheses postulating the inheritance of *de novo* methylation in insects are difficult to prove because few insect methylomes are sequenced outside of the Hymenoptera. Technologies such as bisulphite sequencing, in which specific changes are introduced into the genome sequence based on methylation status of cytosine residues, will help us to elucidate this. Some insect lineages including Lepidoptera are missing the *de novo* methylation enzyme, DNA methyltransferase 3 (Glastad *et al.* 2011), which calls into question their ability to methylate their genome in response to environmental change. Further investigation is needed here to ascertain whether this is indeed the case.

6.5 Final conclusions

Using empirical techniques, I have investigated the transgenerational effects of diet and pathogen exposure on the phenotype of offspring. Throughout, the importance of examining transgenerational effects within the context of the current environment to define variation in phenotype is emphasised. I have demonstrated that a generalist pest species is generally robust to nutritional stress across generations, which could lead to interesting future investigations of its feeding ecology. More broadly, I have highlighted the use of insects as tractable model organisms to examine the far-reaching consequences of environmental change for future generations

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Appendix 1: R code to determine vmax of phenoloxidase reaction from output from Ascent v2.6 plate reader (Thermo labsystems)

Authored by Dr Rob Knell

```
#rearranges excel file produced by platereader
#load JLPLPO2b

p2<-read.table(file.choose(),header=F)
attach(p2)

# Then perform the plate rearrange function.

plate.rearrange<-function(p2){
output<-matrix(data=NA, ncol=97, nrow=241)   ##Set up matrix with data in columns
colnames(output)<-
c("Count",paste("A",1:12,sep=""),paste("B",1:12,sep=""),paste("C",1:12,sep=""),paste(
"D",1:12,sep=""),paste("E",1:12,sep=""),paste("F",1:12,sep=""),paste("G",1:12,sep=""),
paste("H",1:12,sep=""))

output[,1]<-1:241  ##count numbers in column 1

  for(row.code in 1:8){

    for (col.code in 1:12){

      for (i in 1:241){

        output[i,1+col.code+(row.code*12)-12]<-p2[4+10*(i-1)+row.code-1,(1+col.code)]
##puts values for rows in columns 3-15

      }
    }
  }
  output
}

# Now save a new object which is the rearranged data

p2a<-plate.rearrange(p2)

##########################################################################
############

#Function to interactively calculate slopes of absorbance reactions from platereader.
Needs data #frame which has been rearranged using plate.rearrange() function. You
need a list of the wells that #were used.

rm(vmaxplottest1a)
```

```
vmaxplotp2a<-function(p2a){

################  Gets user to input the wells that need to be analysed

print("Which cells from row A? Press enter after each number, if no number or when
finished press enter without entering a number")
As<-scan("")
print("Which cells from row B? Press enter after each number, if no number or when
finished press enter without entering a number")
Bs<-scan("")
print("Which cells from row C? Press enter after each number, if no number or when
finished press enter without entering a number")
Cs<-scan("")
print("Which cells from row D? Press enter after each number, if no number or when
finished press enter without entering a number")
Ds<-scan("")
print("Which cells from row E? Press enter after each number, if no number or when
finished press enter without entering a number")
Es<-scan("")
print("Which cells from row F? Press enter after each number, if no number or when
finished press enter without entering a number")
Fs<-scan("")
print("Which cells from row G? Press enter after each number, if no number or when
finished press enter without entering a number")
Gs<-scan("")
print("Which cells from row H? Press enter after each number, if no number or when
finished press enter without entering a number")
Hs<-scan("")

graphs<-c(As,12+Bs,24+Cs,36+Ds,48+Es,60+Fs,72+Gs,84+Hs)   #Converts input from
last step into a list of numbers which are the columns to be analysed

###### Names of the wells
wells<-
c(paste("A",1:12,sep=""),paste("B",1:12,sep=""),paste("C",1:12,sep=""),paste("D",1:12,
sep=""),paste("E",1:12,sep=""),paste("F",1:12,sep=""),paste("G",1:12,sep=""),paste("H
",1:12,sep=""))

###### Set up matrix for output
reg.out<-matrix(data=NA,nrow=length(graphs),ncol=5)

###### Loops through each of the wells to be analysed
for (i in 1:length(graphs)){
  happy<-"N"
   while(happy !="Y"){        ###For each well, will repeat this loop until the user
indicates they're happy

#####Plots graph of absorbance versus time
dev.new()    ###new active graph window
```

```
plot(p2a[,1],p2a[,graphs[i]+1],xlab="",ylab="",main=wells[graphs[i]],type="l",lwd=0.8,
cex.axis=0.8,tcl=0.1,mgp=c(3,0.02,0))

#####Prompts the user to enter the range for the regression. If what's entered is not two
numbers separated by a comma it just loops

    # temp1<-0
    # while(length(temp1)!=2) {
    # temp<-readline(prompt="Enter the x-coordinates to calculate the regression from
and to, seperated by a comma. Press enter when you're done: ")
    # temp1<-as.numeric(unlist(strsplit(temp,split=",")))
    # }

#####Prompts the user to use the mouse to indicate the range for the regression
print("Click twice on the plot to indicate the lower and upper limits for the regression")
coords<-locator(n=2,type="p",pch=16,col="blue")
temp1<-coords$x

range<-temp1[1]:temp1[2]

#####Fits model to absorbance data
mod.temp<-lm(p2a[range,graphs[i]+1]~p2a[range,1])

#####Calculates predicted values over the range indicated
Ystemp<-mod.temp$coefficients[1]+mod.temp$coefficients[2]*range

#####Draws fitted line onto graph in blue
points(range,Ystemp,type="l",col="blue")

#####If the user is happy then go on to the next well
happy<-readline(prompt="Happy? Press Y and enter if OK, any other key if you'd like
to reconsider ")
    dev.off()   ##Close the current graph window
   }

######Assembles all the output
reg.out[i,1:2]<-temp1[1:2]
reg.out[i,3]<-mod.temp$coefficients[1]
reg.out[i,4]<-mod.temp$coefficients[2]
reg.out[i,5]<-summary(mod.temp)$r.squared

  }

reg.out<-data.frame(graphs,wells[graphs],reg.out)
names(reg.out)<-c("Column","Well","From","To","Intercept","Slope","r-squared")
reg.out
}

vmaxplot<-vmaxplotp2a(p2a)
```