Maternal effects in the large milkweed bug

Oncopeltus fasciatus

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

Maternal effects are the non-genetic contributions of mothers (or fathers) towards the phenotype of their offspring. Maternal effects are now well recognised as a facilitator for evolutionary change in offspring phenotypes and life history strategies which can have effects on population dynamics, population divergence and even speciation. Furthermore, maternal effects have been shown to have a heritable genetic basis and that they are genetically variable, which suggests that they contribute to maintaining phenotypic variation. Maternal effects may impede or accelerate responses to selection which has implications for adaptive evolution and making predictions about their evolutionary potential. The importance of their contribution to phenotypic variation and life history evolution has made maternal effects an important consideration in fields such as conservation and population biology, evolutionary ecology and evolutionary genetics.

The aim of this thesis is to investigate if maternal effects can influence offspring life history traits and fitness parameters through maternal resources via the egg. Main questions that are asked include: can maternal effects help facilitate transition to a novel host-diet (Chapter 2); does maternal diet influence egg composition and, if so, does this have an effect on offspring life-history parameters (Chapter 3); is there a genetic basis to egg composition and is there potential for egg composition to evolve (Chapter 4); and are defensive compounds from the diet transferred into the eggs, if so, are these uni- or biparentally transferred and does this offer protection against predation (Chapter 5)? To address these questions we used a specialist insect herbivore, the large milkweed bug *Oncopeltus fasciatus* (Hemiptera: Lygaeidae). In the wild, *O. fasciatus* feed on plants from the genus *Asclepias* (Apocynacea). However, *O. fasciatus* can be reared successfully in laboratories on sunflower seeds *Helianthus annus*. For our experiments we used two populations of *O. fasciatus*, one population has been maintained on dry seeds of *A. syriaca* while the other population has been reared and maintained on sunflower seeds.

The results of Chapter 2 were suggestive of a maternal host-diet effect on egg mass and hatching success, but we did not find evidence that maternal host-diet was significant in influencing a transition to a novel host. In Chapter 3 we found that there was variation in the free amino acid profiles of the eggs between our treatments suggesting that amino acid profiles may be influenced by maternal diet. The results of our multivariate selection analysis to examine linear and nonlinear

(quadratic) relationships between maternal diet and the free amino acid profiles of the eggs suggest that there may be population-specific responses which can influence specific amino acid profiles in relation to hatchling mass. In Chapter 4 we used only the milkweed-adapted population to determine if there was a genetic basis to amino acid profiles in the eggs. We constructed a genetic variance-covariance (**G**) matrix to determine the strength and direction of the relationships between amino acids and to assess the potential for amino acid profiles to evolve. While we found genetic variation for amino acids, and that there was evidence for positive moderate to strong genetic correlations between many of them, we also found evidence for constraints for the potential for amino acid profiles to evolve as evidenced by the calculation of g_{max} (which represents the linear combination of components that has the highest genetic variance and which is the most accessible to evolution). In Chapter 5 we found maternal, but not paternal, transmission of cardenolides into the eggs. However, this did not confer protection of all eggs against predation from larvae of the green lacewing *Chrysoperla carnea*.

Overall, results suggest that for our populations of *O. fasciatus*, maternal effects are significant in influencing early life history traits such as egg mass and hatchling mass. However, we did not find any significant effects on other offspring life history or fitness parameters that we measured. This may be surprising as positive, and negative, effects of non-genetic contributions of females (and males) to their offspring has been widely reported in many taxa. The patterns and implications of maternal resource allocation and their effects on offspring life history evolution are explored and discussed, as are the limitations of our experimental designs. I hope that this research can be used to stimulate further investigations into maternal effects and the relationships between host-plant, maternal allocation strategies and life history evolution.

Table of Contents

List of tables and figures

Chapter 2

Chapter 3

List of plates

Author's declaration

This thesis was made possible by funding from the European Social Fund (ESF) and the University of Exeter. All the chapters presented in this thesis were written by D. Newcombe with additional editing from Prof. Allen J. Moore, Prof. Patricia J. Moore, Dr. John Hunt, Dr. Jonathan Blount and Christopher Mitchell. Views presented are those of D. Newcombe and developed under guidance from supervisors Allen J. Moore, Patricia J. Moore and John Hunt. Individual contributions for each chapter are listed below.

Chapter 2

Prof. A. Moore and Prof. Patricia Moore designed the experiment. D. Newcombe carried out the experiment and collected data. Data were analysed by D. Newcombe, Prof. Allen Moore and Patricia J. Moore. The manuscript was written by D. Newcombe with editing from Prof. Allen Moore and Patricia J. Moore.

Chapter 3

D. Newcombe developed the idea of investigating amino acids in eggs. Prof. Allen Moore designed the experiment. D. Newcombe carried out the experiment and collected data. Chris Mitchell gave assistance and guidance for amino acid extraction from eggs and operated the GC/MS. Data were analysed by D. Newcombe, Prof. Allen Moore and Dr. J. Hunt. D. Newcombe wrote the manuscript with editing from Prof. Allen Moore, Dr. J. Hunt and Christopher Mitchell.

Chapter 4

This experiment was designed by Prof. Allen Moore and Dr. John Hunt. D. Newcombe carried out the experiment with assistance from undergraduate students Bethany Gratton, Stephanie Hopkins, Amy Rowley and Jo Solino. Christopher Mitchell did the amino acid extractions and operated the GC/FID. Data were analysed by D. Newcombe, Prof. Allen Moore and Dr. J. Hunt. D. Newcombe wrote the manuscript with editing from Prof. Allen Moore, Dr. J. Hunt and Christopher Mitchell.

Chapter 5

D. Newcombe developed the idea of investigating parental transmission of cardenolides into eggs of *O. fasciatius* from papers written by Prof. Thomas Eisner and colleagues. Experimental design was based on these papers with guidance from Prof. Allen Moore. D. Newcombe carried out the experiment and collected data. Chris Mitchell gave assistance and guidance for extracting cardenolides and operated the HPLC. Data were analysed by D. Newcombe with guidance from Prof. Allen Moore. Manuscript was written by D. Newcombe with editorial contributions by Allen J. Moore, Dr. Jonathan Blount and Chris Mitchell.

Chapter 1 Maternal effects: a general introduction

1.1 Defining maternal effects

In general, maternal effects are the non-genetic contribution of parents to their offspring (Bernardo, 1996a; Wolf & Wade, 2009) and have been shown to be evident in a wide variety of taxa across different phyla (Lacey, 1998). The ubiquity of maternal effects has led to their increasing acknowledgement and acceptance by researchers in various fields of biology as a stimulating area of research. Over the years, the concept of maternal effects has become increasingly integrated into empirical and theoretical studies of evolutionary biology including life history evolution, evolutionary genetics, population biology, evolutionary ecology and conservation biology due to recognition of their importance in facilitating evolutionary change (Mousseau & Dingle, 1991; Bernardo, 1996a; Mousseau & Fox, 1998b; Badyaev, 2008; Mousseau *et al.*, 2009).

The diversity of maternal effects across disciplines demonstrates the pervasiveness of maternal effects as a phenomenon that has many ways of influencing offspring phenotypes with potential evolutionary implications (Mousseau & Fox, 1998b). However, the diversity of contexts in which maternal effects can be regarded is also reflected by the diversity of approaches and interpretations utilised by different research disciplines (Mousseau *et al.*, 2009). This has led to some inconsistencies and confusion as to how maternal effects may be characterized (Lacey, 1998; Badyaev & Uller, 2009; Bonduriansky & Day, 2009; Wolf & Wade, 2009).

As maternal effects covers such an extensive array of perspectives it is impossible to cover every aspect here. Rather, I would like to include here a brief synopsis to clarify the context in which maternal effects is considered within this thesis. Considering the vast amount of attention now given to this topic, I feel that to prevent confusion to the reader it would be useful to provide a functional and cohesive definition within which a framework for this thesis can be set. I will then outline the main themes for this thesis and why they are important to studies in evolutionary biology. My hope is that the information here on within can contribute to further insights into the mechanisms behind such an influential facilitator of evolutionary change.

While the term ‘maternal effects’ is typically used to encompass effects of the mother, it is sometimes used to denote effects from either parent (e.g. Wolf & Wade, 2009)(e.g. Wolf & Wade 2009). Some authors prefer to use the term parental, maternal or paternal effects to denote specifically the effects derived from either parent, the mother or the father, respectively. Maternal effects also come under the general umbrella of indirect genetic effects (IGEs) whereby there is an indirect effect of an individual’s genotype on the phenotype of another interacting individual (e.g. offspring) through a shared environment (Moore *et al.*, 1998; Wolf *et al.*, 1998).

Non-genetic inheritance encompasses any influence on offspring phenotype by vertical transmission (i.e. from parents to offspring) of factors other than DNA sequences (Bonduriansky & Day, 2009; Jablonka & Raz, 2009; Richards *et al.*, 2010). While IGEs include horizontal transmission (i.e. transmission of factors from non-related individuals that share a common environment), non-genetic effects are a specific type of IGE that only includes factors resulting from interactions between ancestor-descendants (e.g. parents and offspring) (Bonduriansky & Day, 2009). Nongenetic effects may also include epigenetic inheritance which are heritable phenotypic changes resulting from changes in gene expression other than changes in DNA sequences and includes processes such as DNA methylation and histone modification (Jablonka & Raz, 2009; Richards *et al.*, 2010).

In a broad sense epigenetic inheritance can include phenotypic variations that stem from developmental interactions between mother and offspring. Phenotypic plasticity is where the same genotypes can express different phenotypes in response to environmental cues. Phenotypic plasticity is a result of underlying epigenetic mechanisms and can include transgenerational phenotypic effects (Richards *et al.*, 2010). So, in this sense there is a relationship between epigenetic inheritance, maternal environment and phenotypic plasticity (Richards *et al.*, 2010). Epigenetic inheritance can be linked with maternal effects as they both relate to the vertical transmission of nongenetic factors that result in transgenerational phenotypic change (Bonduriansky & Day, 2009; Richards *et al.*, 2010).

To help clarify the situation further, Wolf and Wade (2009) proposed that definitions of maternal effects should imply a causal influence on offspring phenotype so that the maternal genotype, maternal environment or their interaction influences the phenotypes of offspring over and above

any genetic contributions (i.e. maternal inheritance). Parental influence of offspring phenotype through the environment may be either indirectly through the ecological environment experienced by the parental generation or directly though the environment that the parent provides for their offspring (e.g. provisioning, care). By developing a framework by which maternal effects can be better defined allows the distinction between phenomena where parental genotypes or phenotypes have a causal relationship in influencing offspring phenotype from those that do not and that this will result in evolutionary outcomes that are distinct from that of other functionally distinct phenomena (Wolf & Wade, 2009).

For the purposes of this thesis, maternal effects are regarded specifically as a non-genetic contribution towards the offspring (unless otherwise stated). The main theme covered within this thesis investigates if maternal effects can influence offspring life history traits and fitness parameters through maternal resources that are passed on to offspring via the egg.

1.2 Why study maternal effects?

Maternal effects have been increasingly recognised as an important facilitator for evolutionary change rather than as a nuisance component in evolutionary studies that can confound heritability estimates thereby biasing predictions on evolutionary responses to selection (Futuyma *et al.*, 1993; Mousseau & Fox, 1998b; Räsänen & Kruuk, 2007; Wolf & Wade, 2009). Its acceptance may stem from the realisation that the non-genetic contribution of mothers (or fathers) can significantly influence the phenotypes of offspring which may ultimately have repercussions on offspring life history traits including fitness parameters and survival. In addition, the development of more advanced genetic models and experiments and increasing computational statistical power have also allowed maternal effects to be partitioned and estimated independently from other genetic and non-genetic components.

There have been an increasing number of studies investigating the role of maternal effects in organismal life histories (see reviews: Roach & Wulff, 1987; Mousseau & Dingle, 1991; Bernardo, 1996a; Mousseau & Fox, 1998b; Marshall *et al.*, 2008). A wide range of field studies and laboratory experiments have covered a variety of taxa to gather evidence of how, and under what circumstances (environmental or ecological), maternal effects generate patterns of phenotypic

change. Maternal effects may also be a mechanism for phenotypic plasticity whereby the maternal genotype adjusts offspring phenotypes in response to environmental cues. Maternal effects can therefore be attributed, in part, as a source of phenotypic variation that has impacts not only on just an individual level but, potentially, also up to the population level.

Maternal effects can result in adaptive transgenerational phenotypic plasticity when predictable environmental cues allow maternal adjustment of offspring phenotypes to suit the environment in which the offspring will encounter to maximise both parent and offspring fitness (Bernardo, 1996a; Mousseau & Fox, 1998a,b; Marshall & Uller, 2007; Uller, 2008; Mousseau *et al.*, 2009). However, maternal effects may not always result in offspring phenotypes that are well-suited to the offspring ecological environment (Bernardo, 1996a; Marshall & Uller, 2007). For example, maternal provisioning is a dynamic process and can change depending on environmental factors, nutritional condition, maternal age, the mating system and the interactions between parents, offspring and their environment (Marshall & Uller, 2007; Plaistow *et al.*, 2007; Uller, 2008). Many studies usually only portray a 'snapshot' of maternal provisioning under certain conditions over restricted time periods (Marshall & Uller, 2007; Plaistow *et al.*, 2007). Therefore, when considering the 'adaptive' significance of maternal effects careful considerations must be taken with experimental designs and data interpretation (Bernardo, 1996a,b; Marshall & Uller, 2007). Demonstrating the potential adaptive role for maternal effects will depend on their influence on offspring traits, the consequences on both maternal and offspring fitness and if any of these differ over time or with changing environmental conditions (Marshall & Uller, 2007; Plaistow *et al.*, 2007).

Increasing empirical evidence also suggests that maternal effects not only have a heritable genetic basis (Mousseau & Fox, 1998b; Wilson *et al.*, 2005; Räsänen & Kruuk, 2007) but that they are genetically variable (Donohue & Schmitt, 1998; Shaw & Byers, 1998). This means that not only are maternal traits themselves genetically variable but that they can be passed onto the next generation and contribute to the maintenance of heritable variation (Räsänen & Kruuk, 2007; Bonduriansky & Day, 2009). Heritable genetic effects can be important when making predictions about the response of phenotypic traits to selection (Wilson *et al.*, 2005). Appropriately quantifying genetic variation (Mousseau & Fox, 1998a; Blows & Hoffmann, 2005; Wilson *et al.*, 2005; Walsh & Blows, 2009) underlying maternal effects will enable us to make predictions on phenotypic responses to selection, the strength and direction of selection and their evolutionary potential (McGuigan & Blows, 2010).

However, maternal effects may accelerate or impede phenotypic responses to selection depending on the relationship between them i.e. a positive or negative covariance (Wolf *et al.*, 1998; Wilson *et al.*, 2005).

Evolutionary biology aims to gain an understanding of how variation is maintained between individuals and populations (Stearns, 1992). Maternal effects may be one mechanism by which phenotypic variation is maintained. Research designed to address how maternal effects influence phenotypes will assist us in gaining more knowledge about the processes underlying maternal effects and their role in life history evolution and to make evolutionary predictions regarding population responses to selective pressures.

1.2.1 Maternal provisioning: linking maternal diet, reproductive investment and offspring performance

The ecological environment can play a big part in parental nutrition and this may provide cues as to the amount of resources that are allocated to reproduction with effects on the number, size and quality of offspring that are produced (Mousseau & Dingle, 1991; Stearns, 1992; Bernardo, 1996a,b; Donohue & Schmitt, 1998; Lindström, 1999; Marshall *et al.*, 2008; Zas *et al.*, 2013). Therefore parental investment, such as maternal provisioning, represents an important strategy that can have repercussions on pre- and/or postnatal development with effects on both maternal and offspring fitness (Bernardo, 1996a,b; Kaplan, 1998; Dzialowski & Sotherland, 2004; Marshall *et al.*, 2008). Indeed, nutritional experience of the parental generation has been shown to influence (pre- and post natal) provisioning of offspring which can have direct and indirect consequences on life history traits and fitness parameters in many taxa (Rossiter, 1991a; Mousseau & Fox, 1998b; Lindström, 1999; Marshall *et al.*, 2008; Bonduriansky & Day, 2009), but these effects can be particularly evident in species that do not demonstrate parental care (Rossiter *et al.*, 1993).

Offspring size has been a well-studied trait in a variety of taxa as it represents an important feature between parental reproductive investment and reproductive effort. Traditionally, for oviparous organisms, egg size been extensively used as a proxy for egg quality in investigations into life histories and evolutionary ecology (Bernardo, 1996a,b; Fox & Mousseau, 1998; Fox & Czesak, 2000; Sloggett & Lorenz, 2008; Krist, 2011; Pöykkö & Mänttäri, 2012) as it is considered to be both a maternal and offspring trait and therefore has potentially significant effects on both maternal

and offspring fitness parameters (Bernardo, 1996a,b; Fox & Mousseau, 1998; Fox & Czesak, 2000). However, there has been much debate on the appropriateness of using egg mass as a measure for egg quality (Rossiter, 1991a,b; Bernardo, 1996b; Diss *et al.*, 1996; McIntyre & Gooding, 2000; Giron & Casas, 2003; Lock *et al.*, 2007; Geister *et al.*, 2008; Krist, 2011) and there has been growing interest in using egg composition as a more detailed parameter for measuring maternal resource investment (Rossiter, 1991b; Bolton *et al.*, 1992; Rossiter *et al.*, 1993; Diss *et al.*, 1996; Fox & Mousseau, 1998; Royle *et al.*, 1999; Blount *et al.*, 2000; Dzialowski & Sotherland, 2004; Sloggett & Lorenz, 2008; Pöykkö & Mänttäri, 2012). There are many (non-genetic) constituents that can be maternally transferred into eggs such as amino acids, antibodies, carotenoids, defensive compounds, hormones, lipids, proteins, steroids and vitamins (Boggs, 1995; Heath & Blouw, 1998; McCormick, 1998; Royle *et al.*, 1999; Blount *et al.*, 2000; Izquierdo *et al.*, 2001; Thompson *et al.*, 2001; Blum & Hilker, 2002; Eisner *et al.*, 2002; Verboven *et al.*, 2003; Uller & Olsson, 2006; Uller *et al.*, 2007; Groothuis & Schwabl, 2008; Ho *et al.*, 2011; Müller *et al.*, 2012), many of which have been shown to have an effect on offspring phenotypes. Therefore, the quality and quantity of resources females allocate into their eggs is an important determinant for the development, and possibly the survival, of her offspring.

Until recently, there has been little research into how maternal nutrition directly influences offspring fitness through egg provisioning in herbivorous insects (Rossiter, 1991a; Geister *et al.*, 2008). As maternal diet is likely to be a key determinant in the type and quality of nutrients that a female will be able to allocate into her eggs, therefore, the quality of the parental host plant could influence egg composition via maternal provisioning with subsequent effects on offspring development and/or fitness parameters (Mousseau & Dingle, 1991; Futuyma *et al.*, 1993; Rossiter *et al.*, 1993; Diss *et al.*, 1996; Rossiter, 1996; Awmack & Leather, 2002; Giron & Casas, 2003; Bonduriansky & Day, 2009). The quality of a host-plant can depend on the amount and type of nutrients it contains and this will also determine its acceptance as a suitable host and/or food source (Bernays & Chapman, 1994). Carbon-based (e.g. carbohydrates and lipids), nitrogen-based (e.g. amino acids) and defensive compounds are particularly important in influencing insect life history parameters and reproductive strategies (Awmack & Leather, 2002). For example, carbohydrates may increase female lifespan, fecundity and reproductive investment while lipids are an important energetic reserve for embryonic development (Bauerfeind & Fischer, 2005; Geister *et al.*, 2008) and yolk proteins have also been shown to be a good predictor for offspring fitness (Geister *et al.*,

2008). Furthermore, water may also improve hatching success where there is a risk of desiccation (Bauerfeind & Fischer, 2005; Geister *et al.*, 2008).

Lepidoptera are well represented in studies of female reproductive investment and the link between maternal larval and/or adult nutrition and egg composition. For example, Geister *et al.* (2008) found that in a fruit feeding butterfly *Bicyclus anynana* maternal diet had a significant effect on lipid, protein, glycogen and free carbohydrate content of eggs. However, egg hatching success was largely determined by absolute energy investment (calculated as average caloric values for free carbohydrates, proteins, glycogen and lipids per mg of dry egg mass) and water content. In their study of a moth *Lymantria dispar* (L.), Diss *et al.* (1996) found that prepupal females exposed to nutritional stress as larvae had higher levels of two haemolymph proteins arylphorin & vitellogenin (a precursor to vitellin, the predominant egg storage protein in *L. dispar*), but that nutritional stress resulted in decreased reproductive output (i.e. fewer eggs). They also did not find any differences in the amount of the egg storage proteins vitellin or glycine-rich protein (GRP) between females that were nutritionally stressed or unstressed. However, they did find that vitellin and GRP (but not egg mass) influenced the survival of starved post-hatched larvae which supports other findings that maternal host-diet effects may be only be apparent when offspring are subject to adverse conditions (e.g. nutritional stress) (Fox & Mousseau, 1996; Kyneb & Toft, 2006; Pöykkö & Mänttäri, 2012). Interestingly, they also found that while maternal nutritional experience influenced larval dispersal this was not reflected by vitellin or GRP levels in the eggs which suggests that there may be some other unmeasured component within the egg, a physiological response within the insect and/or an environmental component that may influence dispersal behaviour (Diss *et al.*, 1996).

However, other nutrients such as amino acids have also been shown to be essential to the reproductive biology of some insect species. While there has been some debate on the fitness effects of amino acids, amino acids are recognised to feature highly in the reproductive biology of Lepidoptera (Mevi-Schutz & Erhardt, 2005; Cahenzli & Erhardt, 2012). Several isotopic studies have shown that amino acids from the larval and/or adult stages can be incorporated into eggs (O'Brien *et al.*, 2000; O'Brien *et al.*, 2003, 2005). Furthermore, other insects such as aphids (Liadouze *et al.*, 1995), crickets (Brown, 2011; Gershman *et al.*, 2012) and mosquitos (Uchida, 1993) may also depend on the intake of amino acids as part of their reproductive biology. Amino acids are the building blocks of proteins and many other biomolecules that are important for

embryonic development and are themselves an important energy source for metabolic pathways throughout the developmental process (Finn & Fyhn, 2010). However, few studies that we are aware of have investigated maternal allocation of amino acids specifically as a maternal effect and the relationship with offspring life history and fitness parameters (Geister *et al.*, 2008; Cahenzli & Erhardt, 2012; Pöykkö & Mänttäri, 2012).

In their study, Cahenzli and Erhardt (2012) investigated the effects of high and low larval and adult nutrition effects on female reproductive parameters and offspring life history parameters in the small heath butterfly *Coenonympha pamphilus* L., Satyrinae. Larval in the high diet food treatments were fed fertilised host-plants *ad libitum* while those in the poor food treatment were fed limited amounts of unfertilised host-plants. Likewise, in the high adult food treatment females were provided with a nectar diet either with or without amino acids. The authors found that larval food quality was highly important for the total number of eggs laid whereas adult food quality was not although there was a slight interaction between larval food quality and nectar quality. They also found that adults that had amino acids in their diet produced heavier offspring whereas there was no effect of larval diet. Hatching time and hatching success did not differ significantly between treatment groups and the authors did not find any trade-offs between adult nectar diets and offspring hatching mass and egg number. The authors also emphasised that fitness advantages of heavier hatchling mass may still need to be explored. So, while their study demonstrates that amino acids have a role in influencing female reproduction parameters it also demonstrates the complexity between maternal nutrition and the allocation of resources and the effects on offspring life history parameters.

There are also other types of plant compounds that can be provisioned to the eggs which may protect the eggs against predation (Blum & Hilker, 2002; Opitz & Müller, 2009). Some insects become adapted to utilising specific secondary plant compounds of their host-plants which remain toxic to many other insects and potential predators (Bernays & Chapman, 1994). These compounds can then be sequestered and used as a defence mechanism against predation (Blum & Hilker, 2002; Opitz & Müller, 2009). Chemical protection of eggs has been widely reported for many insect taxa (see Blum & Hilker 2005 and references therein). Defensive chemicals can be biosynthesised by the insects themselves or ingested through the host-plants on which they feed (Blum & Hilker, 2002). While defensive chemicals supplied to eggs are usually maternally transferred, male transference

through seminal ejaculate has been recognised to make a significant contribution in some insect species (Blum & Hilker, 2002).

These studies demonstrate the diversity of ways in which maternal effects can influence female reproductive investment and the effects on offspring life history parameters in a variety of insect taxa. Through these studies it is possible to gain information on the importance of dietary compounds, how they may be allocated, their roles in reproduction and their influences on offspring life histories. However, there is also evidence that many life history traits, including egg composition, have a genetic basis (Partridge *et al.*, 1991; Rossiter *et al.*, 1993; Mousseau & Fox, 1998b; Arcos *et al.*, 2004). Few studies have explored the genetics underlying maternal allocation of resources into eggs (Fox *et al.*, 1999; Arcos *et al.*, 2004) and this is being increasingly recognised as a potentially important area for investigations into how maternal effects contribute to variation in the expression and evolution of phenotypes.

1.3 Thesis outline

The aim of this thesis is to investigate if maternal effects can influence offspring life history traits and fitness parameters through maternal resources that are passed on to offspring, via the egg, using a specialist insect herbivore, the large milkweed bug *Oncopeltus fasciatus*. The following is a brief summary of each chapter which aims to address specific questions relating to this main theme.

Chapter 2: Can maternal effects facilitate a transition to a novel host diet by influencing the responses of offspring life history traits? One of the most crucial aspects of maternal effects is their effects on the developing phenotype (Badyaev & Uller, 2009). In insects, maternal host diets have been well documented to influence offspring phenotype and fitness parameters such as egg mass and offspring size, growth, development and survival (Rossiter, 1991a,b; Fox & Mousseau, 1998; Kyneb & Toft, 2006). We were interested investigating if maternal effects influenced the response of offspring life history traits when introduced to a novel food source, thereby facilitating a transition to a novel host diet. This chapter therefore makes a good starting point to explore the influence of maternal diet on offspring life history parameters in our two populations of *O. fasciatus*. For instance, given that the populations have been reared on two different host diets, does maternal diet influence the growth and development of her offspring when they are raised on a

novel diet? The aim was to test if maternal effects can facilitate a transition to a novel host-diet and if maternal effects are different between our populations, i.e. can maternal effects evolve?

Chapter 3: Does maternal diet influence the composition of eggs, and are there selective forces that operate on this to influence offspring performance parameters? Oviparous animals must invest all their resources required by the developing embryo as a 'box lunch' strategy (Izumi *et al.*, 1994; Wheeler, 1996; Blount *et al.*, 2002b; Trougakos & Margaritis, 2002). There are many constituents that are incorporated into eggs such as proteins, lipids, hormones, and other nutrients (Awmack & Leather, 2002; O'Brien *et al.*, 2005; Geister *et al.*, 2008; Pöykkö & Mänttäri, 2012). However, it has been increasingly recognised that it is not just the composition that is important, but that specific combinations of constituents are also important for offspring pre- and post-natal development (Bolton *et al.*, 1992; Uchida, 1993; Boggs, 2009). Therefore, maternal investment of resources into eggs is particularly important in organisms where there is no parental care and constitutes a vital component to offspring development and fitness (Rossiter *et al.*, 1993; Bernardo, 1996b; McIntyre & Gooding, 2000). This chapter investigates if maternal diet influences egg composition, with a particular focus on amino acids, and if this has an effect on offspring life history traits. Furthermore, we used multivariate selection analysis to investigate the relationships between the amino acid profiles of the eggs and offspring performance traits.

Chapter 4: What is the underlying genetic architecture to maternal allocation and is there potential for the composition of eggs to evolve? This chapter extends on the theme of egg composition by asking if there is a genetic basis to the amino acid composition of eggs. Furthermore, if amino acid allocation demonstrates genetic variability then this could tell us more about its evolutionary potential. Comparisons of the egg protein, vitellogin, has shown a potential for vitellogins to evolve distinct amino acid profiles which may be species-specific and relate to the functioning requirement of the developing embryo (White III, 1995). This is a little explored area of maternal effects (Fox & Mousseau, 1998; Mousseau & Fox, 1998a; Arcos *et al.*, 2004) and may give useful insights to assist in making predictions regarding evolutionary responses to selection (Arnold *et al.*, 2008).

Chapter 5: Are there defensive constituents that are maternally and/or paternally transferred from the diet into eggs, and if so, does chemical defence of the eggs provide protection from predators? Chemical defence of insect eggs has been widely reported and usually this occurs through maternal

transmission of compounds that have been ingested. However, there have been reports of paternal transmission of defensive compounds that afford better protection than if the eggs were only maternally provisioned. Here we use a two part experiment to investigate if there is uni- or bi-parental transmission of defensive compounds into the eggs of *O. fasciatus* and to assess if transmission of cardenolides protect the eggs against predation from an oophagous invertebrate predator, the common green lacewing *Chrysoperla carnea* (Stevens) (Neuroptera: Chrysopediae) **(**Error! Reference source not found.**)**.

By integrating the results of these chapters I hope that this thesis will not only make a valuable contribution towards extending our knowledge on the contribution of maternal effects in the evolution of life histories but also generate further questions to stimulate future studies.

1.4 Study System

The large milkweed bug *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae) are hemipterans or 'true bugs' in that they have a modified proboscis ('rostrum') which they use to feed on plant parts by piecing them and sucking up their contents. While native to North and Central America, *O. fasciatus* is highly specialised to feed on milkweed plants from the genus *Asclepias* (Apocynaceae) (Duffey *et al.*, 1978; Scudder & Meredith, 1982; Detzel & Wink, 1995). These plants contain secondary plant alkaloids (cardiac glycosides or cardenolides) which to many vertebrates are bitter tasting, toxic and can induce vomiting, cardiac arrhythmias and possibly death (Isman *et al.*, 1977; Vaughan, 1979; Opitz & Müller, 2009). Like many insects which are able to feed on food sources that are toxic to their predators, *O. fasciatus* sequester these compounds and have been shown to be chemically protected against potential predators who are not adapted to tolerate cardenolides (Scudder *et al.*, 1986; Opitz & Müller, 2009). While there may be some metabolism of the cardenolides (Duffey & Scudder, 1974; Detzel & Wink, 1995), *O. fasciatus* also have specialised epidermal cells which are used for cardenolide storage (Duffey & Scudder, 1974; Duffey *et al.*, 1978; Scudder & Meredith, 1982). Special weak areas of the thorax and abdomen allow release of the bitter tasting cardenolides onto the body surface when the insect is squeezed (Scudder *et al.*, 1986; Detzel & Wink, 1995).

There are 5 instar stages before nymphs undergo a final moult (adult ecdysis) with each instar stage lasting 4-6 days (Feir, 1974)**Error! Reference source not found.**. Males become sexually mature at 2-3 days post adult ecdysis while females take a little longer at 5-12 days (Feir, 1974). Mating can last from one to several hours, and repeated mating can stimulate egg production and fertility in females (Gordon & Loher, 1968; Feir, 1974)**Error! Reference source not found.**. Oviposition can start several hours post mating and females can lay more than 30 eggs per day (Feir, 1974)**Error! Reference source not found.**. Females can lay multiple clutches with variations in the number of eggs laid per clutch (Feir, 1974). Sperm can be stored by females for 4-5 weeks following a single mating. Lifespan of mated *O. fasciatus* in laboratory settings can be up to 4 weeks (Feir, 1974). While they can be reared and maintained on a variety of alternative seeds and nuts these are found to be inferior to milkweed seeds and may have adverse effects on female fecundity and/or fertility and decrease nymph growth and survival (Beck *et al.*, 1958; Gordon & Loher, 1968). Maternal effects can be highly evident in organisms that do not exhibit parental care (Rossiter *et al.*, 1993), and therefore makes *O. fasciatus* a suitable organism in which to study nutritionally based maternal effects.

Plate 1.4.1 *Oncopeltus fasciatus* nymph feeding on milkweed seeds.

Photo taken by D. Newcombe

Plate 1.4.2 Milkweed bugs mating.

Photo taken by D. Newcombe

Plate 1.4.3 Female milkweed bug ovipositing eggs in cotton wool.

Photo taken by D. Newcombe

Plate 1.4.4 Larva of green lacewing *Chrysoperla carnea* consuming an *O. fasciatus* egg.

Photo taken by D. Newcombe

Chapter 2: Evolving an expanded diet: can maternal effects facilitate transition to a novel host?

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2.1 Abstract

Previous studies have demonstrated that parental rearing host-plant can influence offspring phenotypes that can be either adaptive or non-adaptive in relation to host suitability. We were therefore interested in investigating if maternal effects can facilitate a transition to a novel host-diet using a specialist insect herbivore, the large milkweed bug *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae). In the wild, *O. fasciatus* are highly specialised to utilise plants from the genus *Asclepias* (Apocynaceae). For this experiment, we used two different populations of *O. fasciatus*. One population has been maintained on milkweed seeds *Asclepias syriaca* while the other has been adapted to utilise sunflower seeds *Helianthus annus*. We manipulated maternal adapted-diet and used a nested full-sib, split brood design to test if maternal effects can facilitate a transition to a novel host-diet, and if maternal effects would differ between populations as a consequence of evolving the ability to use a new diet effectively. We measured egg mass, hatching success, offspring development time (time from hatching to adult eclosion) and offspring adult size (pronotum width). We predicted that maternal (host-diet) effects between our populations would differ in respect to how females allocate resources to offspring via the egg and that this would be reflected by the response of offspring life history traits to a novel host-diet. While we found evidence for an effect of maternal diet on egg mass and hatching success, offspring (rather than maternal) diet effects were more important in influencing later offspring life history traits. Differences in egg mass reflected a maternal host-diet effect and may suggest a change in maternal effects with adaptation to different hosts. However, this was not central to evolving the capacity to use a new host in *O. fasciatus*.

Key-words: Insect herbivore, life history evolution, Hempitera, host-specialisation, maternal resource allocation, offspring performance

2.2 Introduction

Host-plant specialization in insects is a common phenomenon whereby insects have a narrow diet breadth in relation to the number of plant species on which they feed in their geographical range (Fox & Morrow, 1981; Bernays, 1991; Schoonhoven *et al.*, 2005). Even while a variety of potential host-plants may be available to herbivorous insects at any given time, selection of which plants to utilise may only include a small subset of those available, thus suggesting a high degree of specialization of these insects for their food sources (Fox & Morrow, 1981; Schoonhoven *et al.*, 2005). Proximate and ultimate questions regarding insect host-plant specialisation have been widely researched in recent years (Cates, 1980, 1981; Jermy, 1984; Futuyma & Peterson, 1985; Bernays & Graham, 1988; Jaenike, 1990; Via, 1990; Mayhew, 1997; Quental *et al.*, 2007; Laukkanen *et al.*, 2013). There may be various causes for host-plant specialisation such as ecological, chemical, morphological and genetic factors (Fox & Morrow, 1981; Schultz, 1988) and selection pressures (Cates, 1980; Quental *et al.*, 2007).

However, equally important may be the role of parental nutritional experience and the transgenerational effects on their offspring that may influence offspring life history and fitness parameters in response to novel diets. Nutrition plays a central role in the reproductive biology of oviparous insects (Wheeler, 1996). Nutritional experience of the parental generation can influence life history parameters and fitness in the offspring through maternal effects (Rossiter, 1991a; Fox *et al.*, 1995; Mousseau & Fox, 1998b; Kyneb & Toft, 2006). Maternal effects occur when maternal genotype or phenotype has a causal influence on offspring phenotype over and above any genetic contributions (Bernardo, 1996a; Mousseau & Fox, 1998b; Wolf & Wade, 2009). A common maternal effect is when the environment experienced by the mother affects the phenotypes of her offspring (Bernardo, 1996a,b; Mousseau & Fox, 1998b; Badyaev, 2008). Quality of the parental host-plant can influence maternal allocation of nutrients into the eggs thereby altering egg composition with subsequent effects on offspring growth and development (Rossiter, 1991a,b; Fox *et al.*, 1995; Fox *et al.*, 1997b; Mousseau & Fox, 1998b; Fox & Czesak, 2000; Fox & Savalli, 2000; Awmack & Leather, 2002; Amarillo-Suárez & Fox, 2006; Cahenzli & Erhardt, 2013)..

However, maternal effects are also a mechanism by which organisms can ameliorate environmental uncertainty or novelty (Mousseau & Fox, 1998a; Crean & Marshall, 2009) or spatial or temporal uncertainty (Spitzer, 2004; Spitzer, 2006; van Asch *et al.*, 2010). Maternal effects could provide an influence on the ability to expand host use. Given the growing recognition of the role of maternal effects in life history evolution and as a mechanism that facilitates evolutionary change (Mousseau & Dingle, 1991; Bernardo, 1996a; Mousseau & Fox, 1998b; Badyaev, 2008; Mousseau *et al.*, 2009), we were interested in investigating if maternal effects can facilitate a transition to a novel host-diet and if maternal effects would differ as a consequence of evolving the ability to use a new diet effectively.

In the wild, *O. fasciatus* are specialised to feed on plants from the genus *Asclepias* (Apocynaceae). We used an experimentally evolved population reared exclusively on sunflower seeds *Helianthus annus* for over 400 generations and compared this experimental population to a population that has been reared on dried milkweed seeds of *Asclepias syriaca*. We hypothesised that maternal effects can facilitate the ability to use a novel host. We used a nested, full-sib, split brood design and manipulated both maternal and offspring diet to examine the effects of maternal and offspring diet on offspring performance. Traits measured included egg mass, egg hatching success, nymph development time (time taken from hatching to adult development) and offspring adult pronotum width (as a proxy for size). We predicted that maternal (host-diet) effects between our two populations would differ and that this would be reflected by the response of offspring to the novel host-diet. That is, a significant population by maternal diet interaction would indicate population-specific adaptation in maternal effects associated with evolved ability to use a new host.

2.3 Materials and methods

2.3.1 Study system

The large milkweed bug *O. fasciatus* is widespread across North America and is also found in parts of central and northern South America (Feir, 1974). In the wild, *O. fasciatus* feed and reproduce mainly on milkweed plants from the genus *Asclepias*, which contain toxic cardiac glycosides (cardenolides) (Feir, 1974; Ralph, 1976). Cardenolides are secondary plant substances that are bitter tasting and toxic to many vertebrates (Ralph, 1976; Isman *et al.*, 1977; Agrawal, 2005).

These toxic compounds are sequestered by *O. fasciatus* and used as a defence strategy against potential predators (Duffey *et al.*, 1978; Scudder *et al.*, 1986). *Oncopeltus fasciatus* can be reared in the laboratory on a variety of food sources including sunflower, cashew and pumpkin seeds and peanuts (Beck *et al.*, 1958; Gordon & Gordon, 1971; Feir, 1974; Scudder *et al.*, 1986). Although initial performance on these alternative hosts is poor, continued exclusive rearing selects for improved performance (Gordon & Gordon, 1971; Feir, 1974).

2.3.2 Experimental populations & rearing

The aim of our study was to investigate if maternal effects could aid transition to a novel host-diet by way of influencing offspring life history parameters via maternal egg effects. To investigate this, we reared and tested two different populations of *O. fasciatus*. One population derived from individuals collected from the wild at the University of Kentucky Arboretum, Lexington, KY, USA, in September, 2009. We maintained this population on a diet of dried milkweed seeds of *A. syriaca* (Educational Science, League City, TX, USA). As we have never exposed this population to another food-source, this population is denoted as the 'milkweed-adapted' population. The other population is a long-standing laboratory reared population obtained from Carolina Biological Supply House (Burlington, NC, USA) and has been maintained on sunflower seeds for over 400 generations. During this time they have only been reared on sunflower seeds and are therefore denoted as the 'sunflower-adapted' population. We reared these populations separately at 25°C with a light:dark regime of 16:8 hours and in mass colonies in multiple transparent boxes (36cm X 28cm X 18cm). Every week fresh seeds (as per adapted diet) and demineralised water were supplied to all colonies. Absorbent cotton wool was provided for oviposition sites in all boxes.

Eggs from the main populations (above) were collected as required and used to start experimental colonies for the current study. Four treatments were created and maintained for this experiment: Treatment 1: milkweed-adapted bugs maintained on milkweed seeds (KYMW); Treatment 2: milkweed-adapted bugs maintained on sunflower seeds (KYSF); Treatment 3: sunflower-adapted bugs maintained on milkweed seeds (LABMW); Treatment 4: sunflower-adapted bugs maintained on sunflower seeds (LABSF). These experimental colonies were kept in an incubator L:D 16:8 at 25° C. All boxes were routinely moved around the incubator to account for incubator effects. Fresh

seeds (as per allocated treatment) and water were provided *ad libitum* and changed weekly or as necessary.

Newly eclosed adults were collected daily from each of the above 4 treatments. Males and females were kept separate according to treatment and day of eclosion. All experimental adults were housed in boxes (11 X 11 X 3 cm) and were provided with their allocated diet (sunflower or milkweed seeds) and a cotton wick moistened with demineralised water. Seeds and water were refreshed at least twice a week, reflecting *ad libitum* conditions.

Adults were mated when females were at least 7 days old but no older than 10 days, and males were mated between the age of 5 days and 10 days (see Gordon and Loher (1968)). Pairs were left together for 3 days to encourage egg production in the female and to ensure sufficient fertilisation of eggs (Gordon & Loher, 1968). Several copulations would have occurred between the pair during this time. Females that originated from the milkweed-adapted population were only mated with males from the same population that had been maintained on milkweed seeds. Likewise, females that had originated from the sunflower-adapted population were only mated with males from the same population that been maintained on sunflower seeds.

Mated pairs were placed into a Petri dish with a food dish containing allocated female diet, cotton wick moistened with demineralised water and cotton wool for ovipositing. The male was removed after the end of the mating period. Cotton wool was removed four days after separation and replaced with a fresh piece of cotton wool. Cotton wool was then checked at least twice daily for clutches until a minimum of 20 eggs could be collected from each female. Any clutches laid overnight were discarded. Where 20 eggs could be collected from a single clutch (or from multiple clutches laid one the same day) these were kept for weighing and hatching.

We used a Mettler Toledo UMX2 microbalance to weigh the selected eggs and a small piece of foil was used to contain the eggs during weighing. After recording weight and to standardise rearing conditions, the 20 weighed eggs from each female were equally divided into two small pieces of cotton wool and placed into two separate boxes (11 x 11 x 3 cm) (i.e. 10 eggs in each box) with appropriate labelling (family, maternal treatment and date laid). Eggs were checked daily from day 5 for hatching. When nymphs had hatched, the date of hatching and the number of hatchlings were

recorded. If eggs did not hatch by day 8 or did not show any signs of development then the eggs were scored as unfertilised.

Once hatched, each box containing nymphs was randomly allocated a diet of either sunflower or milkweed seeds, i.e. so that for each female, 10 of her offspring were reared on milkweed seeds while the other 10 were reared on sunflower seeds. Nymphs were then maintained on the allocated diet along with a cotton wick moistened with demineralised water in Petri dishes. All nymphs from all treatments were housed under standard rearing conditions (as above) in a separate incubator from the adults, eggs and colonies. Fresh food and water was provided twice weekly and as necessary. Sibling offspring were reared together in their respective family treatment (milkweed or sunflower) until adulthood but analyses were always on family means. Any deaths were recorded and used to calculate proportion of a clutch that survived to adult. Dead nymphs were removed to prevent remaining nymphs feeding on the corpses.

Once nymphs had reached their fifth instar stage, they were checked daily for final (adult) eclosion. When new adults had eclosed, the date of eclosion and sex were recorded to calculate development time (i.e. hatch to adult eclosion). Adults were then labelled and frozen for later image analysis of size. A Leica MZ6 stereomicroscope was used to obtain an image of the bugs ensuring the same magnification for each individual (magnification set at 0.63). Photographs were taken using PixeLink software (PixeLink Capture Standard Edition 1394 Camera (colour) 90602. When taking the images, individuals were all positioned in the same way so as to minimise any positioning effects. We used ImageJ software (http://rsbweb.nih.gov/ij/download.html) to measure the pronotum width of each individual as a proxy for size. Width was taken as the widest point at the base of the pronotum for each individual. A test of repeatability on five measurements made on 342 individuals showed that only 0.4% of the variation was due to measurement error.

2.3.3 Statistical analyses

We measured egg mass as a proxy for maternal allocation while offspring performance parameters were measured as hatching time (time taken from date eggs laid to hatching), development time (time taken for newly hatched nymphs to develop into adults) and pronotum width (as a proxy for size). We also measured proportion of a clutch within a family that survived and the proportion of a

clutch that was fertilised. All analyses were performed using JMP version 8. Statistical significances was set at $\alpha = 0.05$.

We used Model III ANOVA and first tested for differences between these two populations, maternal or parental effects and their interaction (we use the term "parental effects" here to denote cross-generational influences that cannot be attributed to just one parent). We used a full-factorial design with both factors as fixed effects. Only fertile clutches were included in the analysis for egg mass to ensure that we were testing for differences of maternal treatments on viable clutches. For the analysis of hatching success, a standard ANOVA is not appropriate as the data are binomial. We therefore used GLM with the same factors fitted into the model and with clutch fertility as the binomial response variable (infertile = 0, fertile = 1). Clutches that resulted in at least one nymph were classed as fertile while clutches that did not produce any offspring were classed as infertile. Females that did not produce any eggs after 4 weeks were excluded.

We next used a Model III ANOVA and tested for differences in maternal and offspring diet effects on offspring performance. We again used a full-factorial design with population (milkweed-adapted or sunflower-adapted), maternal diet (milkweed or sunflower) and offspring diet (milkweed or sunflower) as fixed factors to investigate effects on family mean offspring performance: development and size. For the analysis of development we also included sex as a fixed factor because we expected, but did not know a-priori, that sexes might differ. On the other hand, females are known to be larger than males and so for the analysis of size we analysed sex separately. Family mean values were used for development, size and survivorship, to protect against inflated sample sizes. We did not analyse family effects, given all of the families were full sibs, confounding unmeasured environmental effects, genetics, and common environmental effects. Survivorship was taken as the mean proportion of offspring survival from hatching to adult ecdysis for each population/maternal diet/offspring diet treatment combination. As nymphs are unable to be sexed the number of adult females and males in each treatment did not affect analysis. However, as family means were used, only data that included at least two offspring at hatching from all treatment combinations were included. A fully factorial generalised linear model was conducted, but as survivorship was taken as a proportion of nymph survivorship the model was run with binomial errors.

2.4 Results

We mated a total of 306 pairs, with 223 dams laying eggs. In the milkweed-adapted population 59 females on the milkweed diet from 70 matings laid eggs (84.3% of females) while eggs were collected from only 28 females out of 80 matings from females fed sunflower seeds (35%). In the sunflower-adapted population eggs were collected from 69 females from 75 matings in the milkweed diet group (92%) and from 67 females out of 81 matings in the sunflower diet group (82.8%). A total of 2,539 F1 nymphs (1,306 females and 1,235 males) were reared to adulthood from 199 dams.

2.4.1 Effects on egg mass

For egg mass we found a significant difference between populations and a significant interaction between population and maternal diet (Population X Maternal diet, $F_{1,197} = 9.043$, $P = 0.003$; **Figure 2.1**). Overall, the milkweed-adapted population laid lighter eggs than the sunflower-adapted population **(Figure 2.1)**. However, females from the milkweed-adapted population that were reared on milkweed laid lighter eggs than the females fed sunflower **(Figure 2.1)**. In the sunflower-adapted population, females reared on milkweed seeds laid heavier eggs **(Figure 2.1)** than females reared on sunflower. Unfortunately, we do not have data on female mass so we are unable to use female mass as a covariate (e.g. we cannot ask if larger females lay larger eggs). We have evidence that female size does not influence egg mass in our populations (see Chapter 3), but also see the Discussion section this chapter.

In the milkweed-adapted population 98% of clutches laid by females on the milkweed diet resulted in hatchlings compared to 82% of clutches laid by females on the sunflower diet. In the sunflower-adapted population 89.9% of clutches laid by females on the milkweed diet had hatchlings and 86.6% from females on the diet of sunflower seeds. Therefore, while maternal diet had a significant influence on egg hatching success (GLM maternal diet: $X^2_1 = 24.775$, $P = 0.029$) there was no significant effect of population (GLM maternal diet: $X^2_1 = 0.699$, $P= 0.403$) nor was there an interaction between population and maternal diet (GLM maternal diet: $X^2_1 = 2.305$, $P = 0.129$).

2.4.2 Effects on offspring development

Development time: Development time is reported as mean days from hatch to final adult moult. There was a strong effect of the interaction between population and offspring diet on nymph development time to final (adult) eclosion **(Table 2.1)**. The effects of offspring milkweed diet were similar across populations while offspring from the milkweed-adapted population on the sunflower diet took longer to reach adult moult than the laboratory/sunflower combination. There was a very weak population by maternal diet interaction on offspring development time **(Table 2.1)** suggesting a weak relationship between offspring development time, maternal host-diet and the adapted host-diet. None of the other higher-order interactions were statistically significant **(Table 2.1)**.

Main effects confirmed the overall differences of population, offspring diet and sex on nymph development **(Table 2.1)**. There was a highly significant effect of population on nymph development time indicating differences between the two populations and the time it took for nymphs to attain final adult moult. That is, offspring from the milkweed-adapted population took longer to reach final moult than offspring from the sunflower-adapted population **(Table 2.1, Figure 2.2)**. In both populations, offspring were faster to develop when fed milkweed than when fed sunflower. However, regardless of population or maternal diet, offspring reared on the milkweed diet reached final moult more rapidly than did offspring reared on the sunflower diet **(Figure 2.2)**. Overall, females took slightly longer than males to reach final adult moult when on the sunflower diet **(Figure 2.2**Error! Reference source not found.**)**. There was no statistically significant effect of maternal diet on nymph development time to final moult **(Table 2.1)**.

Size: As only 20 eggs from each female were collected rather than whole clutches (see methods section), not all families produced both males and females so there was a small difference in sample sizes for each sex **(Table 2. 2)**. The direction and the size of the effects were virtually identical on the sexes. None of the interactions reached statistical significance for either sex **(Table 2. 2)**. Both population and offspring diet had statistically significant and large effects on adult pronotum size in both females **(Table 2. 2)** and males **(Table 2. 2)**, milkweed-adapted bugs are much larger than the sunflower-adapted bugs **(Figure 2.3)**. In addition, in all treatments offspring fed milkweed seeds were much larger than offspring fed sunflower seeds **(Figure 2.3)**. Maternal diet was not a significant effect **(Table 2. 2)**.

2.4.3 Effects on offspring survival

Population (GLM: $X^2_1 = 20.939$, $P<0.001$), offspring diet (GLM: $X^2_1 = 18.737$, $P<0.001$), and their interaction (GLM: $X^2_1 = 10.471$, $P<0.001$) were significant influences on offspring survival. Maternal diet had no effect on offspring survival (GLM: $X^2_1 = 0.021$, $P = 0.883$). Furthermore, none of the other interactions approached statistical significance (maternal diet x offspring diet: GLM: $X^2_1 = 0.008$, $P = 0.929$; population x maternal diet x offspring diet: GLM: $X^2_1 = 1.020$, $P = 0.313$). A larger proportion of offspring from a family within the sunflower-adapted population survived compared to the milkweed-adapted population and a greater proportion of offspring reared on milkweed lived compared to those reared on sunflower **(Figure 2.4)**. Although sunflower had a small negative effect on survivorship in the sunflower-adapted population, within the milkweed-adapted population the effect of a diet of sunflower on survivorship was profoundly negative **(Figure 2.4)**.

2.5 Discussion

We were interested in investigating if maternal effects can facilitate a transition to a novel host-diet and if maternal host-diet effects between our two populations would differ as a consequence of evolving the ability to use a new diet effectively. Our study was motivated by the prediction that maternal host-diet effects between our two populations would differ in respect to how females allocate their resources to offspring and that this would be reflected by the response of offspring life history traits to a novel host-diet. That is, we would see a significant population by maternal diet interaction which would indicate population-specific adaptation in maternal effects associated with an evolved ability to use a new host (Magalhães *et al.*, 2007).

We found some evidence of a population x maternal diet effect on offspring development. In the sunflower-adapted population there was no difference in the development time of offspring whose mothers were reared on milkweed or sunflower, while in the milkweed-adapted population offspring from mothers reared on milkweed had marginally shorter development times than offspring whose mothers were fed sunflower. As sunflower is an inferior food source for *O. fasciatus*, this suggests that the introduction of a poor quality host in the maternal generation may still adversely affect offspring development. However, in the sunflower-adapted population it is

likely that there has been intense selection, over a number of generations, for improved performance to an inferior food (Fox *et al.*, 1995).

If parental rearing host is indicative of future nutritional conditions for offspring then it would be advantageous for offspring to be conditioned to the same host as the parental generation (Fox *et al.*, 1995). If adaptive maternal effects are involved then we would expect to observe evidence of offspring having higher fitness parameters when raised on the same host as the mother – a maternal host x offspring host interaction (Fox *et al.*, 1995). Transgenerational acclimation, or conditioning, is an adaptive maternal effect whereby maternal environment gives reliable cues as to the anticipated environment of offspring so that females are able to adjust offspring phenotype accordingly to suit that environment (Fox *et al.*, 1995; Spitzer, 2004; Cahenzli & Erhardt, 2013). We found only weak evidence for conditioning to sunflower in the sunflower-adapted population, as offspring raised on sunflower had a slightly shorter development time when their mothers were also raised on sunflower than for offspring raised on sunflower whose mothers were raised on milkweed **(Figure 2.2)**. In their study, Fox *et al.* (1995) found no evidence that maternal rearing host acclimated offspring to perform better when raised on the same host as their mother. Likewise, Via (1991) found that maternal experience of host diet in the pea aphid *Acyrthosiphon pisum* (Harris) did not influence performance parameters of offspring and she suggests that population differences in use of host crops are likely due to genetic differentiation and local adaptation.

As populations become adapted to novel conditions, mean trait values increase resulting in increased performance in local conditions but decreased performance in alternative conditions (local adaptation hypothesis) (Agrawal, 2000; Amarillo-Suárez & Fox, 2006; Magalhães *et al.*, 2007). In our study there was a strong population effect on all offspring life history parameters measured (except for hatching success). That our two populations have diverged in their life history traits may not be surprising given the historical differences between them (see Methods section) (Rios *et al.*, 2013). However, while offspring in the milkweed-adapted population did poorly on sunflower, the sunflower-adapted population performed well on both hosts. This implies that while initial exposure to a novel host may adversely impact on offspring performance, given sufficient time selection for genotypes that are able to survive and reproduce on this novel host will allow the population to persist on the poor quality host (Fry, 1989; Via, 1991; GarcÍa-Robledo & Horvitz, 2012).

Gordon and Gordon (1971) showed that in the early generations of selection, *O. fasciatus* adapted to diets of cashew and sunflower seeds in the laboratory easily revert back to a diet of milkweed seeds. However, long term evolution on a single host may be expected to lessen the ability to accept a novel host (Magalhães *et al.*, 2007). Our results demonstrate that although our sunflower-adapted population has been reared over 400 generations without exposure to milkweed, the bugs not only readily accept milkweed, examination of life history traits and survivorship indicate a favourable response towards a re-introduction of their toxic ancestral diet. This suggests that *O. fasciatus* have not lost their ability to handle and store cardenolides that are present in *A. syriaca* seeds and are able to perform just as well to the re-introduction of their ancestral host.

Intense selection to an inferior food source can lead to local adaptation and this has been reported in the seed beetle *Stator limbatus* (Fox *et al.*, 1995). Here, the authors found population differences in the ability of *S. limbatus* to use a poor quality host *Cercidium floridum* over a higher quality host *Acacia greggii*. The authors found population differences in acceptance of females from one population (Scottsdale) to oviposit on *C. floridum* and that the offspring had faster development times and higher survivorship on this host. However, they suggest that the differences in survivorship may be related to variation in egg size between populations and host substrate. As larvae need to be able to penetrate the thicker seed coat of *C. floridum* to burrow into the seed, larger larvae on this host could be expected to have higher survivorship than smaller larvae (Fox & Mousseau, 1996). Females from the Scottsdale population lay larger eggs, therefore, larvae from this population are expected to be larger and more successful in penetrating the seed coat than smaller larvae produced by smaller eggs in the other population (Fox *et al.*, 1995).

Maternal host experience provides the female with nutritional information regarding the quality of nutrients available for reproduction. Furthermore, females may adjust nutritional allocation to eggs in response to the type of quality of their host. While the introduction of a novel diet resulted in heavier eggs in both our populations of *O. fasciatus* **(Figure 2.1)**, the effects of milkweed appears to have driven this result. While females from the milkweed-adapted population that were maintained on milkweed seeds laid the lightest eggs, sunflower-adapted females fed milkweed seeds laid the heaviest eggs. However, there did not seem to be any significant difference in egg mass when females were fed sunflower seeds, regardless of the population from which they were derived.

Allocation to eggs could, therefore, reflect a maternal effect related to host-diet and for a change in maternal effects with adaptation to different hosts (maternal diet x population interaction). Milkweed-adapted females reared on sunflower and sunflower-adapted bugs reared on milkweed laid relatively heavier eggs. The specific adaptive nature of this maternal effect is not necessarily interpretable from egg size, although egg size has traditionally been considered a good predictor of offspring fitness with larger eggs being positively correlated with offspring fitness (Fox, 1994a; Fox & Czesak, 2000; Krist, 2011). For example, in their investigation on *S. limbatus*, Fox *et al.* (1995) found that maternal rearing host-plant produced a maternal effect on egg size and/or quality affecting offspring body size and development time.

However, egg size is often only a crude measure of maternal allocation of nutrient resources to offspring (McIntyre & Gooding, 2000; Giron & Casas, 2003; Lock *et al.*, 2007). Egg weight does not always correlate with nutritional composition, and nutritional components such as lipids, proteins and carbohydrates may change over the course of egg development (Giron & Casas, 2003). However, sunflower and milkweed differ in composition, and milkweed bugs differ in fatty acid composition depending on their food source (Nation & Bowers, 1982). In some insect species certain fatty acids are required for the production of prostaglandins, without which may result in down regulation of ovarian uptake of protein yolk bodies required for oogenesis (Stanley-Samuelson *et al.*, 1988; Stanley, 2006). We found that egg mass changes with maternal diet and that there are slight differences in egg colour of newly laid eggs between populations (personal observation). Differences in egg colour have also been observed in *S. limbatus* (Fox *et al.*, 1995) which could indicate variations in maternal allocation of resources into eggs via maternal host-diet effects (Fox, 1994b; Fox *et al.*, 1995).

Maternal diet also affected the proportion of the clutch that hatched. A greater proportion of the eggs hatched if the parents were fed milkweed, regardless of their population of origin and despite that we found milkweed-adapted females laid the lightest eggs. Some maternally derived egg constituents, such as vitamin E and amino acids, have been related to enhanced fertility and hatching success in other taxa (Ramsay & Houston, 1998; Guisande *et al.*, 2000; Møller *et al.*, 2008), so it may seem reasonable to surmise that milkweed remains a superior food source over sunflower in terms of egg viability. Furthermore, we were not able to distinguish between eggs that were not fertilised and eggs that were fertilised but failed to develop successfully. Males can also

transfer nutritive compounds to the female via ejaculate which can be used to provision the eggs (Zeh & Smith, 1985; Futuyma *et al.*, 1993; Mousseau & Fox, 1998b; Wedell & Karlsson, 2003; Roth *et al.*, 2010). As we did not measure male contribution, we do not know if our results reflect a paternal effect.

Population and offspring diet had, by far, the greatest effects. As discussed above, our two populations demonstrated differentiation in their use as sunflower as a host and this was evidenced by population being a significant factor for all the traits that we measured. However, offspring diet was found to be a profound factor in nymph post-hatching growth and development in both populations. Offspring fed a diet of milkweed seeds had shorter development times and they developed into larger adults than offspring fed sunflower seeds regardless of the population from which they were derived. Faster development may reflect better performance for offspring as eclosion in *Oncopeltus* has been shown to be a size-dependent trait (size triggered metamorphosis) (Blakley & Goodner, 1978; Blakley, 1981). Fifth instar nymphs must reach a critical size before they are able to eclose into adults (Blakley & Goodner, 1978; Blakley, 1981). While sunflower had a detrimental effect on performance traits in the milkweed-adapted population, the sunflower-adapted population performed just as well, if not better for some traits, on milkweed.

Overall, our study suggests that adaptation of *O. fasciatus* to a novel host-diet has been accomplished by the ability to utilise an alternative host (host expansion). Contrary to our predictions, this expansion and adaptation in offspring performance was not reflected in changes in maternal effects beyond differences in the eggs produced by mothers and a small effect on the total time of offspring development. Our findings are in line with other studies that maternal effects tend to be more pronounced early in life (Cheverud & Moore, 1994; Bernardo, 1996a,b; Mousseau & Fox, 1998b; Badyaev & Uller, 2009) through the allocation of resources via maternal egg effects.

However, the role of maternal effects in the process of adaptation requires further investigation, especially as maternal effects are known to confer plasticity which can facilitate change in an ecological timescale (Agrawal, 2001). Detecting the adaptive role of maternal effects in diet expansion will depend on measuring both the environmental maternal effects and genetic basis of maternal effects during the exploration of and subsequent colonisation onto novel diets. It would

also be helpful to begin to identify the proximate factors that cause maternal effects, such as specific diet-related differences in allocation of specific nutrients into eggs.

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Table 2.1 ANOVA table for effects of treatments on development time. All factors are fixed effects. The full model explains 62% of the variance in development time. Development time was measured as number of days from hatching to adult eclosion.

Factor	MS	df	F	P
Population	876.9642	1	188.440	**<.0001**
Maternal diet	0.8941	1	0.192	0.661
Offspring diet	2854.5840	1	613.385	**<.0001**
Sex	18.5714	1	3.991	**0.046**
Population x Maternal diet	19.8954	1	4.275	**0.039**
Population x Offspring diet	351.2733	1	75.481	**<.0001**
Maternal diet x Offspring diet	6.4299	1	1.382	0.240
Population x Sex	0.0188	1	0.004	0.949
Maternal diet x Sex	0.5736	1	0.123	0.726

Table 2. 2 ANOVA tables detailing effects of population, maternal diet and offspring diet (all fixed effects) on pronotum width separately for females and males. The full model explains 43% of the variance for female size and 52% of the variance for male size. We measured a total of 310 families (means female size) and 312 families (means male size) due to some families lacking one or the other sex.

	Female Size					**Male Size**			
Factor	**MS**	**df**	**F**	**P**		**MS**	**df**	**F**	**P**
Population	1.4668	1	73.606	**<0.0001**		1.9931	1	136.322	**<0.0001**
Maternal diet	0.0320	1	1.606	0.206		0.0052	1	0.359	0.550
Offspring diet	2.3067	1	115.754	**<0.0001**		1.9026	1	130.128	**<0.0001**
Population x Maternal diet	0.0154	1	0.771	0.381		0.0039	1	0.265	0.607
Population x Offspring diet	0.0617	1	3.097	0.080		0.0017	1	0.119	0.730
Maternal diet x Offspring diet	0.0001	1	0.010	0.922		0.0002	1	0.011	0.915
Error	0.0199	302				0.0146	304		

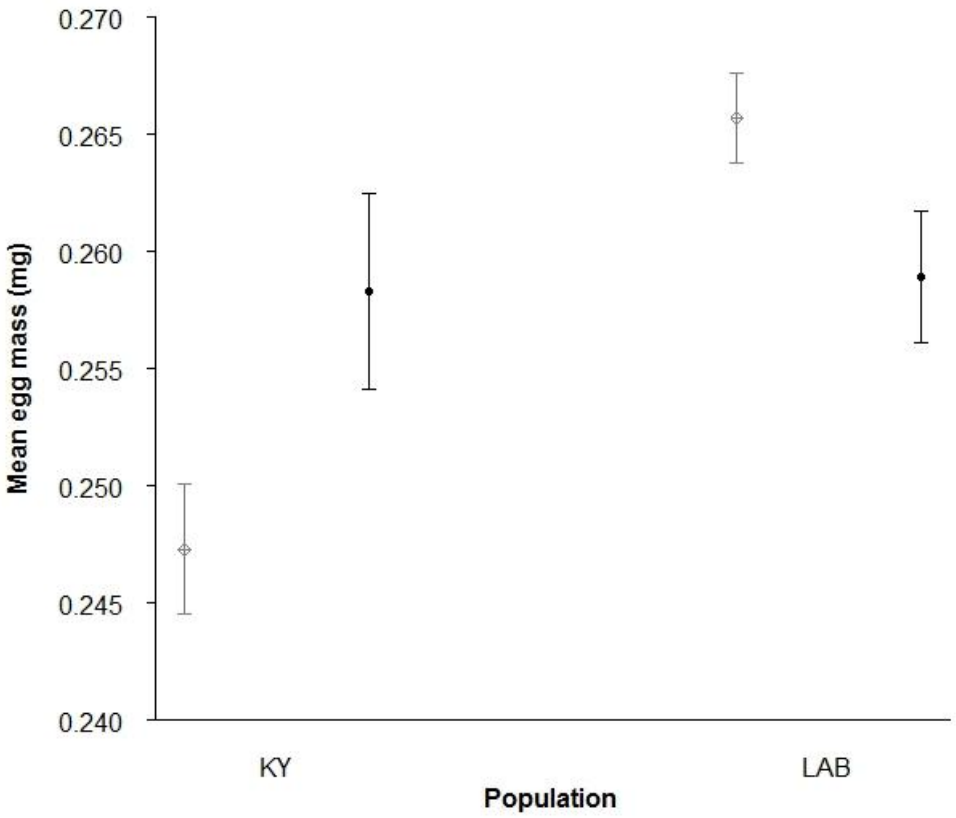
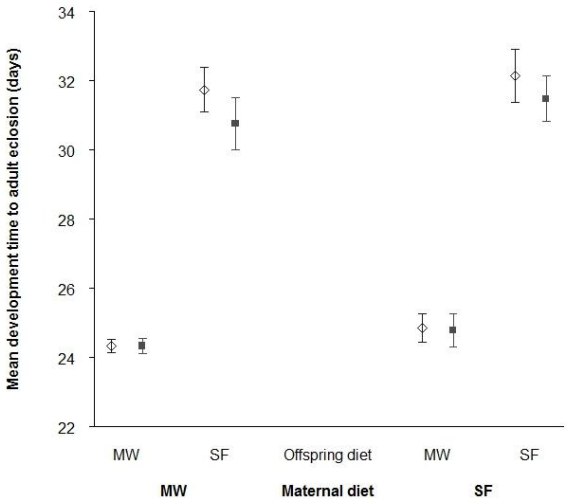


Figure 2.1 Mean (± SE) mass of eggs from fertile clutches laid by females from the milkweed-adapted population (KY – wild population collected from Kentucky, USA) and the sunflower-adapted population (LAB – laboratory population obtained from Carolina Biological Supply) fed on a diet of milkweed seeds (open circles) or sunflower seeds (filled circles).

A. Milkweed-adapted population



B. Sunflower-adapted population

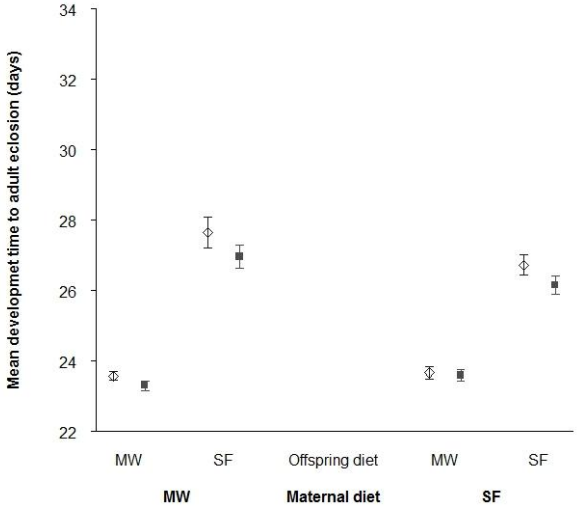


Figure 2.2 Development time in our populations of *O. fasciatus*. Mean number of days (± SE) from hatch to final adult moult. Females (open diamonds) and males (filled squares) are presented in both figures. Figure **2.2A** shows development time for the milkweed-adapted population, with offspring diet (milkweed; MW or sunflower; SF) under corresponding maternal diet (MW or SF). Figure **2.2B** provides the same data for the sunflower-adapted population.

A. Females **B**. Males

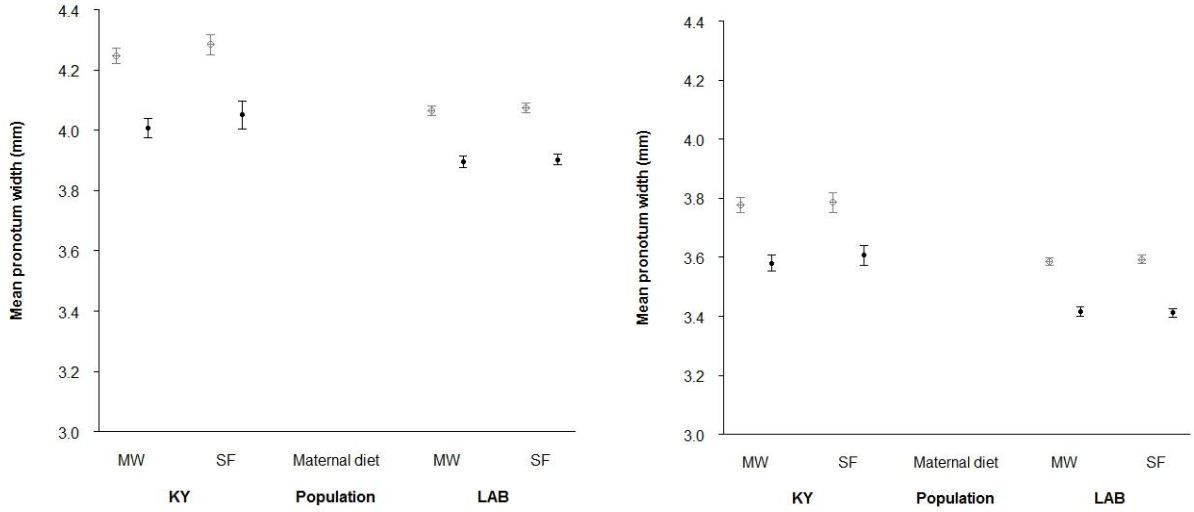


Figure 2.3 Size of (**A**) female and (**B**) male offspring in all treatment groups. Size measured as mean pronotum width (mm) ± SE. Bars are grouped in pairs for maternal diet of milkweed (MW) or sunflower (SF) under corresponding population: milkweed-adapted (KY) or sunflower-adapted (LAB). The different coloured bars illustrate offspring diet, with milkweed (open circles) or sunflower (filled circles) diets. The y-axes are the same range for males and females to show differences between the sexes.

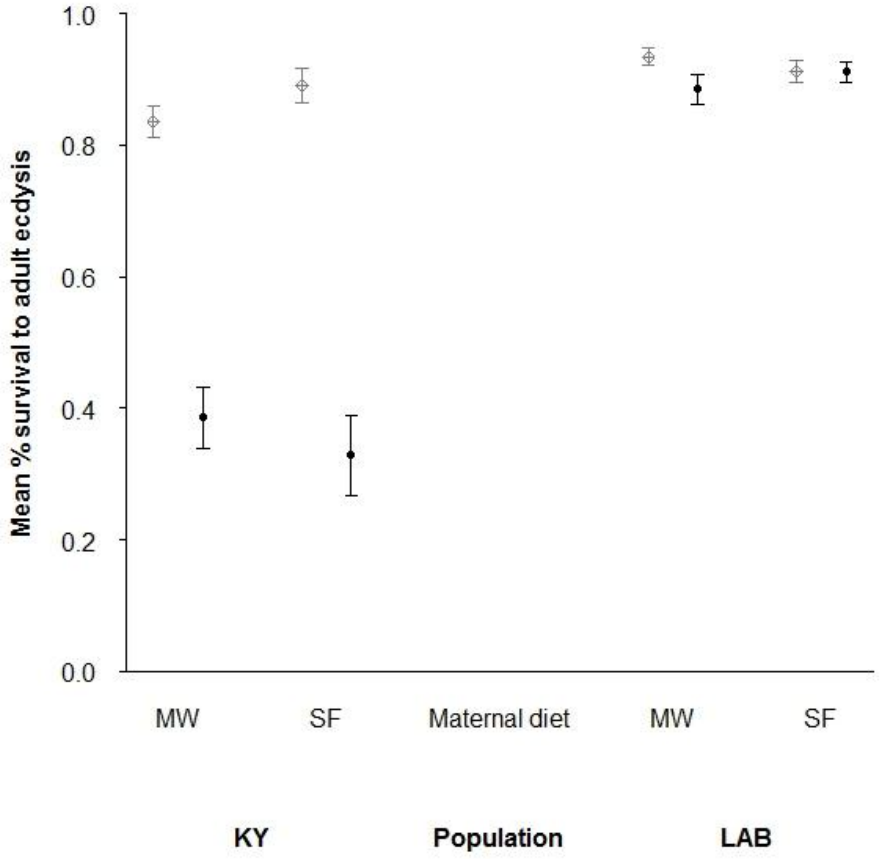


Figure 2.4 Mean (± SE) offspring survivorship as a proportion of nymph survival to adult ecdysis within each family. Bars are grouped in pairs for maternal diet of milkweed (MW) or sunflower (SF) under corresponding population (milkweed-adapted: KY; sunflower-adapted: LAB). Offspring diets are shown as different coloured bars for milkweed (open circles) or sunflower (filled circles).

Chapter 3: Effect of diet on free amino acids in eggs of *Oncopeltus fasciatus*

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3.1 Abstract

Maternal diet has been shown to have significant consequences for offspring performance traits through the allocation of resources incorporated into the eggs. While there are many dietary compounds that can be incorporated into eggs, amino acids offer a novel insight into investigating the effects on offspring performance traits. Here, we investigate amino acid profiles of eggs in two different populations of the large milkweed bug *Oncopeltus fasciatus*. In its natural habitat, *O. fasciatus* are specialist herbivores that feed on plants from the genus *Asclepias* (Apocynaceae). In our study we have one population that has been reared exclusively on dried milkweed seeds *A. syriaca* while the other population has been reared exclusively on sunflower seeds *Helianthus annus*. We wanted to test if changes to maternal diet influenced the free amino acid profiles of whole eggs of *O. fasciatus* and if the amino acid profiles of the eggs affected offspring performance (for purposes of this paper, offspring performance traits were measured as hatching success and egg/hatchling mass). We also asked if the patterns we observed were due to population differences and if there was any evidence that eggs from our two populations exhibited population-specific differences in amino acid profiles related to maternal diet (i.e. that there would be an interaction between population and maternal diet on amino acid profiles of eggs). We manipulated maternal diet so that females from both populations were fed either milkweed or sunflower seeds. We used Gas Chromatography Mass Spectrometry (GC/MS) to examine quantitative differences in the amino acid profiles of eggs. We also measured egg mass, hatching success and hatchling mass to test for a relationship between the amino acid profiles of the eggs and offspring performance traits. We conducted a multivariate selection analysis to determine linear and nonlinear (quadratic) selection on amino acid profiles and any relationships between offspring performance traits (hatching success and hatchling mass). We used thin-plate splines to visualize the performance surface and assess the relationships between hatchling mass and amino acid profiles. Our results suggest that amino acid profiles of eggs in *O. fasciatus* differ between maternal diet treatments and that population-specific responses can alter amino acid profiles. We discuss the implications of our results in relation to complex interactions between evolutionary history, host-diet effects and egg composition.

***Keywords*:** maternal effects, offspring performance, *Oncopeltus fasciatus* (Hemiptera), Principal Component Analysis

3.2 Introduction

Parental investment in offspring via the resources and nutrients that are provisioned during the early stages of development can dramatically affect offspring phenotypes with potential repercussions on fitness parameters (Lindström, 1999). Therefore, parental nutritional environment is an important aspect of life history evolution as it can be mediated via maternal effects to influence the expression of offspring phenotypes and fitness components (Mousseau & Fox, 1998b and references therein). Maternal effects can be defined as a non-genetic environmental influence of the mother (or father) on offspring phenotype (Wolf & Wade, 2009) and are particularly evident in affecting early rather than late life history traits (Bernardo, 1996a,b; Mousseau & Fox, 1998b; Heath *et al.*, 1999).

As egg content provides the earliest environmental experience for offspring (Kyneb & Toft, 2006), egg provisioning can be an environmental influence on offspring phenotype (Bernardo, 1996a) and a potential source of nutritionally-based maternal effects (Rossiter, 1991b). Constituents of the eggs determine the initial resources available to the offspring for embryonic growth and post-hatching size so that maternal allocation of the quantity and quality of resources to the eggs can have profound effects on early offspring development (Bernardo, 1996b; Fox *et al.*, 1997a; Mousseau & Fox, 1998b). Egg size has traditionally been used as a proxy for egg quality and a source of the common environment for the embryo provided by the mother (Bernardo, 1996b; McIntyre & Gooding, 2000), with many studies investigating the relationship between maternal environment, egg size and offspring performance traits (Mousseau & Dingle, 1991; Mousseau & Fox, 1998b). However, egg size is often only a crude measure of maternal allocation of nutrient resources to offspring (McIntyre & Gooding, 2000; Giron & Casas, 2003; Lock *et al.*, 2007) and therefore, may not be a reliable predictor for offspring fitness (McIntyre & Gooding, 2000) or egg composition (Bernardo, 1996b; Giron & Casas, 2003; Geister *et al.*, 2008). Rather, the relationship between maternal diet and the nutritional quality of eggs may correlate more closely to some offspring life history parameters (Bernardo, 1996b).

Therefore, investigating effects of specific egg constituents is also important for understanding the full impact of maternal effects on evolution, which is facilitated by measuring specific maternal traits (Kirkpatrick & Lande, 1989). It can be hard to identify potential maternal traits, but one clear source of maternal effects is the composition of the egg that provides the raw materials for a

developing embryo. Maternal provisioning in eggs and the effects on offspring performance parameters has been well studied in birds (Groothuis & Schwabl, 2008; Müller *et al.*, 2012), amphibians (Kaplan, 1998), reptiles (Thompson *et al.*, 2001; Uller & Olsson, 2006; Uller *et al.*, 2007) fish (Heath & Blouw, 1998) and marine animals (Marshall *et al.*, 2008). However, few studies have explored how variation in egg composition is determined in arthropods and how this effects female and offspring life histories (Amarillo-Suárez & Fox, 2006).

Studies into the biochemical constituents by which mothers can indirectly influence offspring performance may give insights into how these processes contribute towards insect development and the evolution of insect life histories (Mousseau & Dingle, 1991). There are many constituents that females can incorporate into their eggs such as hormones, steroids, antibodies, carotenoids and vitamins (Thompson *et al.*, 2001; Verboven *et al.*, 2003; Uller & Olsson, 2006; Uller *et al.*, 2007; Groothuis & Schwabl, 2008; Ho *et al.*, 2011; Müller *et al.*, 2012) that have been shown to effect offspring phenotypes. However, relatively well-less studied constituents are amino acids and their effects on offspring. Amino acids are the building blocks of proteins and are therefore vital to the growth and development of many insects (Zhang *et al.*, 1997; Berg *et al.*, 2002). There has been a growing body of evidence that amino acids play an important role in the reproductive biology of herbivorous insects and that having the right balance of specific amino acids may be required to promote embryogenesis (Bolton *et al.*, 1992; Uchida, 1993; Boggs, 2009). Some studies have shown that female butterflies (Alm *et al.*, 1990; Mevi-Schütz & Erhardt, 2003; Mevi-Schutz & Erhardt, 2005; Cahenzli & Erhardt, 2012) and honey bees (Alm *et al.*, 1990) can demonstrate a preference for nectars with amino acids added artificially when raised on a low quality larval diet which may be a compensatory mechanism for overcoming nutritive deficiencies in the juvenile stage. In Lepidoptera, it has been shown that amino acids from both larval and maternal diets are incorporated into eggs (O'Brien *et al.*, 2002; O'Brien *et al.*, 2003, 2005) and that amino acids can be used to enhance female fecundity (Mevi-Schutz & Erhardt, 2005; Cahenzli & Erhardt, 2012). So, while egg cytoplasm is maternally derived (Mousseau & Dingle, 1991) the quality and amount of resources that are maternally allocated can have profound effects on offspring fitness traits (Mousseau & Fox, 1998b).

Finally, understanding amino acid composition may help in the understanding of the evolution of life histories and dietary specialization (O'Brien *et al.*, 2005). Around 10 amino acids are considered

to be essential for the growth and development of insects (Sandström & Moran, 1999) but the specific amino acids, and the concentrations required, varies within and between species (House, 1962). While amino acids are well known to have essential roles during embryogenesis (Bellés *et al.*, 2005) such as DNA synthesis (Quesney-Huneeus *et al.*, 1979), the specific roles of individual amino acids during embryogenesis are difficult to establish. They are all important precursors to biomolecules (Berg *et al.*, 2002) which are involved in a variety of biochemical processes such as the regulation of metabolic pathways, signal transductions and for the synthesis of proteins such as hormones and enzymes (Meijer, 2003). However, while many studies have investigated the relationship between dietary amino acids and adult female fecundity in herbivorous insects, few studies have explored the relationship between maternal diet, amino acid profiles of eggs and offspring life history evolution (Geister *et al.*, 2008).

The large milkweed bug *Oncopeltus fasciatus* is an herbivorous insect that is highly specialized to feed on milkweed seeds from the genus *Asclepias*. Milkweed and diet play an important role in the life history and reproductive biology of *O. fasciatus* (Beck *et al.*, 1958; Gordon & Gordon, 1971; Ralph, 1976; Isman, 1977; Chaplin, 1980; Slansky, 1980b,a; Blakley, 1981). As maternal diet can have significant consequences for the provisioning of embryos, then it may be reasonable to predict that when challenged with a novel food source, a specialist insect such as *O. fasciatus* may alter allocation of resources into the oocytes. In a previous experiment (Chapter 2) we showed that maternal diet had an effect on egg mass in eggs of *O. fasciatus*. We interpret this as a differential allocation of resources into the eggs resulting in early environmental maternal effects with effects on the developing embryos and potentially manifested by differences in early offspring life history parameters.

The aim of the current study is to investigate if changes to maternal diet influence amino acid profiles of eggs of *O. fasciatus* and if the amino acid profiles of the eggs affect offspring performance (for purposes of this paper, offspring performance traits were measured as hatching success and egg/hatchling mass). We hypothesized that 1) maternal diet influences amino acid profiles of eggs and 2) amino acid profiles of eggs lead to early environmental maternal effects on offspring life history traits. We tested for this in two populations that have different evolutionary histories. One population derives from individuals collected from the wild and has been reared for fewer than 10 generations in the laboratory on milkweed seeds *Asclepias syriaca*. The other

population has been exclusively reared on sunflower seeds *Helianthus annus* for over 400 generations in the laboratory. We expected that amino acid profiles would differ between our two populations of *O. fasciatus* and, given their recent evolutionary histories, that there would be population specific differences in response to diet. We also expected to see a relationship between amino acid profiles of eggs and offspring performance parameters. To investigate the effects of amino acid profiles of eggs of *O. fasciatus* on offspring performance, we used a conventional multivariate selection analysis based on multiple regression analysis (Lande & Arnold, 1983; Gershman *et al.*, 2012). As we were interested specifically on the effects on early life history traits, for purposes of this paper, offspring performance is taken as hatching success (proportion of a clutch that hatch) and hatchling mass. Fitness surfaces are where phenotypic traits are plotted against a surrogate measure of fitness/performance (Pigliucci, 2012). Our results suggest that amino acid profiles of eggs in *O. fasciatus* differ between maternal diet treatments and that population-specific responses can alter amino acid profiles. We discuss the implications of our results in relation to complex interactions between evolutionary history, host-diet effects and egg composition.

3.3 Materials and methods

3.3.1 Study system

The large milkweed bug *O. fasciatus* is found across North America and in parts of central and northern South America (Feir, 1974). *Oncopeltus fasciatus* feed and reproduce mainly on milkweed plants from the family Asclepiadaceae, which contain toxic cardiac glycosides (cardenolides) (Feir, 1974; Ralph, 1976). Although milkweed is the preferred and natural host, in the laboratory *O. fasciatus* can be reared on a variety of food sources including sunflower, cashew and pumpkin seeds and peanuts (Beck *et al.*, 1958; Gordon & Gordon, 1971; Feir, 1974; Scudder *et al.*, 1986). While initial performance may be poor, there is selection for relatively improved performance when they are continually reared on these alternative food sources (Gordon & Gordon, 1971; Feir, 1974).

To test for differences of free amino acid profiles of eggs, we used two different populations of *O. fasciatus* that have been reared and maintained on different host-diets. One population derived from individuals collected from the wild at the University of Kentucky Arboretum, Lexington, KY, USA.

We maintained this population on a diet of dried milkweed seeds *Asclepias syriaca* purchased from Educational Science, League City, TX, USA. The other population was supplied from Carolina Biological Supply House (Burlington, NC, USA). This is a long-standing laboratory population, which has been reared on de-husked sunflower seeds *Helianthus annus* (purchased from Goodness Direct) for over 400 generations. Both the milkweed-adapted ('Kentucky', 'KY') and the sunflower-adapted ('Laboratory', 'LAB') populations, while kept separate, are reared in mass colonies in multiple boxes. Colonies are kept in incubators at 25°C with a light:dark regime of 16:8. We used upturned glass jars (with a base made from paper towels and the base of a Petri dish) filled with demineralized water as water receptacles. Fresh seeds (as appropriate), water and cotton wool were supplied every week and as necessary.

3.3.2 Experimental populations & rearing

For the purposes of this study, eggs were collected from the two main populations to create 4 experimental colonies that were reared in boxes (28 X 16 X 9 cm). Each colony was reared and maintained on either milkweed seeds or sunflower seeds thereby creating 4 treatments: 1) Kentucky population on milkweed seeds (KYMW), 2) Kentucky population on sunflower seeds (KYSF), 3) sunflower-adapted population on milkweed seeds (LABMW) and 4) sunflower-adapted population on sunflower seeds (LABSF). These colonies were housed in an incubator at 25°C with a light:dark regime 18:6 hours. Boxes were routinely moved around the incubator to minimize incubator effects.

3.3.3 Experimental design

Newly eclosed adults were harvested daily from each of the 4 experimental colonies (as above). Females were housed as per their treatment and collection date in either standard Petri dishes or boxes (11 X 11 X 3 cm). Males were only retained if they had been raised on their original host-diet, i.e. milkweed-adapted males raised on milkweed and sunflower-adapted males raised on sunflower. Adults were provided with their allocated diet (sunflower or milkweed seeds) *ad libitum* and a cotton wick moistened with demineralized water. Seeds and water were replenished as necessary.

To ensure that adults were fully sexually mature, females were mated when they were between 7 and 10 days old while males were mated when they were between 5 and 10 days old (see Gordon &

Loher, 1968). Females, regardless of diet treatment, were only mated with a male from her respective population that had been reared on their original diet, i.e. milkweed-adapted females (KYMW or KYSF) were only mated with milkweed-adapted males that had been reared on milkweed and sunflower-adapted females (LABMW or LABSF) were only mated with sunflower-adapted males that had been reared on sunflower. Each female was only mated with one male, and to encourage egg production and ensure fertilization pairs were left together for 72 hours (Gordon & Loher, 1968). Mated pairs were housed in standard Petri dishes and maintained on the allocated diet of the female. Moist cotton wicks were also provided and changed as required. Small pieces of cotton wool were provided as substrate for oviposition. Males were discarded after mating.

Upon separation of mated pairs, the number of clutches laid was noted and these eggs were then discarded and the female given fresh cotton wool. Cotton wool was checked every few hours until 20 eggs could be collected from either a single clutch or multiple clutches if laid at the same time. Clutches laid overnight were discarded. The numbers of eggs from each female were counted and up to 20 eggs were collected from each female. Ten eggs were selected for weighing and amino acid analysis while the other 10 eggs were kept for hatching. We used a Mettler Toledo UMX2 microbalance to weigh the eggs and nymphs. A small piece of foil was used as a weigh boat. Weighed eggs were frozen at -80°C until analysis could be conducted. Clutches that were found to be infertile (see below) were removed from any analysis. Eggs for amino acid analysis were collected from a total of 201 females (33 from KYMW, 48 from KYSF, 50 from LABMW and 69 from LABSF).

Eggs that were kept for hatching were placed in cotton wool and placed in an incubator L:D 18:6 at 25°C (± 1°). We checked daily from day 5 for hatching, and recorded the date of hatching and the number of hatchlings. Nymphs were chilled in a refrigerator for up to one hour before weighing. If eggs did not hatch by day 8 then the eggs were scored as unfertilized and the corresponding eggs from that clutch that had been set aside for amino acid analysis were not included in any further analysis.

3.3.4 Free amino acid extraction

Eggs were prepared for analysis following modified methods of Gelman *et al.* (2000). Ten eggs were collected from each female and placed in an Eppendorf tube. 100µl of 75% ethanol was

pipetted into each sample. Samples were sonicated for 3 minutes then mashed with a manual pestle and further washed with another 100 µl of 75% ethanol. Samples were then pulse vortexed for 5 seconds and then placed on ice for 30 minutes. Following this, samples were centrifuged for 10 minutes at 4°C at maximum speed. Samples were then placed in liquid nitrogen and the supernatant removed and placed into fresh Eppendorf tubes. Samples were stored at -80°C ready for amino acid analysis.

Amino acids were extracted using a Phenomenex EZ:faast^{TM} kit for free amino acid analysis. 100µl of each sample was pipetted into a sample preparation vial along with 100µl of Reagent 1 (internal standard solution; Norvaline 0.2nM, N-propanol 10%). The solution was then passed slowly through a sorbent tip attached to a 1.5 ml syringe. Any liquid that was passed into the barrel and not kept within the sorbent tip was discarded. 100 µl of Reagent 2 (wash solution; N-propanol) was pipetted into the sample preparation vial, which was then drawn slowly through the sorbent tip and into the syringe barrel. Liquid that accumulated in the barrel was again discarded, leaving only the solution contained within the tip. 200µl of freshly prepared eluting medium (a 3:2 mix of sodium hydroxide and N-propanol) was pipetted into the preparation vial. After drawing air into the barrel of a 0.6ml syringe, the syringe was attached to the sorbent tip and the eluting medium was slowly passed through until the liquid reached the filter plug within the sorbent tip, thereby wetting the sorbent with the eluting medium. The liquid and sorbent particles were then ejected out of the tip and into the sample preparation vial until only the filter disk remained within the empty tip. Using the adjustable Drummond Dialamatic microdispenser 50 µl of Reagent 4 (chloroform) was transferred into preparation vial. The vial was then vortexed for 5-8 seconds in pulse mode at 80% of maximum speed. The reaction was then left to proceed for at least one minute. The vials were then vortexed again for 5 seconds and left for the reaction to proceed for another minute. The microdispenser was then used to transfer 100µl of Reagent 5 (iso-octane) and the vial was vortexed for 5 seconds and the reaction left to proceed for one minute.

These reactions allowed separate layers to develop and 50-100µl of the organic layer was pipetted into the insert of an autosampler vial. The solvent was then slowly evaporated under a nitrogen stream. Amino acid derivatives were then re-dissolved in 100µl of Reagent 6 (hydrochloric acid) and vortexed for 10 seconds. Vials were capped and stored at -80°C until analysis using the GC/MS.

3.3.5 Quantification of free amino acid composition

Using the EZ:faast^{TM} kit we measured the following 17 amino acids: alanine, glycine, leucine, isoleucine, threonine, serine, proline, asparagine, aspartic acid, methionine, glutamic acid, phenylalanine, glutamine, lysine, tyrosine and tryptophan. The amino acids α-aminobutyric acid, valine, cystathionine, orthinine and glycyl-proline were removed from the analysis, as they were not detected in all of our samples. Sarcosine, β-aminoisobutyric acid, alloleucine, thiaproline, 4-hydroxyproline, hydoxylysine, and proline-hydroxyproline were not detected in any of our samples.

3.3.6 Statistical analysis

All statistical analyses were conducted using IBM SPSS Statistics version 19 (license supplied by the University of Exeter). To standardize amino acid values with clutch size from each female, the amino acid values were divided by the total mass of eggs from each sample. For each female, we used mean egg mass and mean offspring (hatchling) mass in our statistical analyses.

Due to the large number of individual amino acids found in our samples, we used Principal Component Analysis (PCA) to reduce the number of individual variables into fewer dimensions (Tabachnick & Fidell, 2001; Gershman *et al.*, 2012). We extracted the Principal Components (PCs) using the correlation (rather than covariance) matrix, to minimize the effects of differences in scale on the PCs extracted (Tabachnick & Fidell, 2001). PCs with an eigenvalue of greater than 1 were retained for further analysis (Gershman *et al.*, 2012). We interpret factor loadings of 0.3 or above as biologically relevant (Tabachnick & Fidell, 2001). Four multivariate outliers were removed prior to analysis based on Mahalanobis distances (Tabachnick & Fidell, 2001). Once the PCs were extracted, we used a multivariate analysis of covariance (MANCOVA) to test for any effects of population and maternal diet, as well as their interaction on the PCs describing variation in amino acids, including female body size as a covariate. Univariate analysis of covariance (ANCOVA) was used to determine which PCs contributed to any overall significant multivariate effect detected.

3.3.7 Egg amino acid profiles and effects on offspring performance traits

Selection can affect the phenotypic distribution of a population through the effect that the phenotype has on an individual's fitness, that is, while selection can act on individuals, it has its

effects on distributions (Brodie III *et al.*, 1995). While selection can affect the mean of a population (linear selection) it can also affect variances (quadratic/nonlinear i.e. disruptive or stabilizing selection) or covariances (correlational selection) (Brodie III *et al.*, 1995). Estimates of selection can be applied to PCA (Schluter & Nychka, 1994) and quadratic regression can be made on the PCs to gain estimates of nonlinear and correlational selection gradients to gain information on the strength and direction of nonlinear selection acting on them (Blows *et al.*, 2003). When eigenvalues are positive the curve will be upward (concave – disruptive selection) but down (convex – stabilizing selection) if the eigenvalue is negative (Blows *et al.*, 2003). Major axes of the response surface (consisting of different combinations of PCs) can be determined by performing a canonical analysis (Brooks *et al.*, 2005). Local features of the response surface can then be used to assess the complex relationships between the amino acid profiles (Blows *et al.*, 2003). The smoothing parameter, lambda, determines how rough the fitness surface will be (Blows *et al.*, 2003).

As recommended by Lande and Arnold (1983), our performance measures (hatching success and hatchling mass) were transformed to a relative measure by dividing by the mean of the population. All PC scores were standardized to a mean of zero and standard deviation of 1 (Lande & Arnold, 1983). We then fitted a linear regression including the PCs describing the amino acid composition of eggs and offspring performance to estimate the vector of standardized linear selection gradients ($\boldsymbol{\beta}$) for each performance measure. A quadratic regression model including all the linear, quadratic and cross-product terms was then used to estimate the matrix of nonlinear selection gradients ($\boldsymbol{\gamma}$). Quadratic regression coefficients were doubled, as recommended by (Stinchcombe *et al.*, 2008).

The strength of nonlinear selection is greatly under-estimated if the size and significance of $\boldsymbol{\gamma}$ terms are interpreted individually (Blows *et al.*, 2003). We therefore used canonical analysis (Phillips & Arnold, 1989) to locate the major axes of nonlinear selection acting along the performance surface for each offspring measurement. The strength of linear selection along each of the eigenvectors ($\boldsymbol{m_i}$) is given by theta ($\boldsymbol{\theta_i}$) and the strength of nonlinear selection is given by their eigenvalues ($\boldsymbol{\lambda_i}$). We estimated $\boldsymbol{\theta_i}$ and $\boldsymbol{\lambda_i}$ using the double regression method of (Bisgaard & Ankenman, 1996).

As our offspring performance measures were not normally distributed, we tested the significance of our standardized selection gradients using a resampling procedure where relative offspring performance measures were shuffled randomly across individuals in the dataset to obtain a null

distribution for each selection gradient where there is no relationship between the PCs describing amino acids in the eggs and offspring performance. Probabilities are the number of times (out of 9,999 permutations) in which the gradient pseudo-estimate was equal to or less than the original estimated gradient. We conducted separate randomization tests for the multiple regression models for linear selection and for the full quadratic model. We used the same re-sampling procedure to assess the significance of $\boldsymbol{\theta_i}$ and $\boldsymbol{\lambda_i}$ for each eigenvector after the canonical analysis of $\boldsymbol{\gamma}$.

We used thin-plate splines (Green & Silverman, 1994) to visualize the major axes of the performance surface extracted from the canonical analysis of $\boldsymbol{\gamma}$. We used the *Tps* function in the FIELDS function of R (v. 2.12.2, www.r-project.org) to fit the thin-plate splines and to visualize them in contour view. We used the value of the smoothing parameter ($\boldsymbol{\lambda} = 0.021$) that minimized the generalized cross-validation scores when fitting the thin-plate splines.

The above selection analysis was conducted across our four treatments (KYMW, KYSF, LABMW and LABSF). We used a sequential model building approach (Draper & John, 1988; Chenoweth & Blows, 2005) to determine if maternal selection on the amino acid composition of eggs differed across our four treatments. There was no significant differences in linear ($F_{4,176} = 1.988$, $P = 0.098$), quadratic ($F_{4,160} = 1.059$, $P = 0.379$) or correlational ($F_{6,136} = 1.272$, $P = 0.284$) selection across our treatments indicating that our pooling of data was justified (Chenoweth *et al.*, 2012).

3.4 Results

3.4.1 Offspring performance

We found that population ($F_{1,193} = 10.450$, $P = 0.0014$) and maternal diet ($F_{1,193} = 33.577$, $P < 0.0001$) had a significant effect on egg mass, with a marginally significant interaction between population and maternal diet ($F_{1,193} = 3.727$, $P = 0.055$). The sunflower-adapted population laid heavier eggs than the milkweed-adapted population **(Figure 3.1)**. In both populations, females fed milkweed seeds laid heavier eggs than females fed sunflower seeds **(Figure 3.1)**. Female size significantly covaried with egg mass ($F_{1,192} = 9.989$, $P = 0.0018$), as bigger females had heavier eggs.

There was a statistically significant effect of population on hatchling mass ($F_{1,191}$ = 4.607, P = 0.033). Maternal diet was again a highly significant effect ($F_{1,191}$ = 40.4601, P = 0.0001). There was no significant interaction between these main effects ($F_{1,191}$ = 0.0137, P = 0.907). Consistent with the effects on egg mass, hatchlings from females fed milkweed were significantly larger than hatchlings born to females fed sunflower **(Figure 3.2)**. We found no significant covariance between female size and hatchling mass ($F_{1,191}$ = 1.607, P =0.207).

3.4.2 Free amino acid profiles of eggs

Our analysis of free amino acid content of the eggs identified variation in the composition of 16 amino acids. We found four PCs with eigenvalues greater than one that collectively explained 66% of the total variation in free amino acid content of our egg samples **(Table 3.1)**. PC1 explains 29.85% of the variance of amino acid content and is positively loaded with leucine, isoleucine, threonine, serine, asparagine, methionine, glutamic acid, phenylalanine, glutamine, lysine and tyrosine. This suggests that these amino acids constitute the free amino acid pool of the eggs at this stage of development, and PC1 reflects overall quantitative differences in the total amount of free amino acids. While alanine, glycine, aspartic acid and tryptophan were present, they were not deemed as biologically relevant in our samples as they had loadings of less than 0.3 (see Methods). Only proline had a negative loading but, again, it is not deemed biologically relevant to our samples.

PC2 explains 16.42% of the variance in amino acid composition in the eggs and demonstrates a trade-off between glycine, proline, tyrosine and tryptophan (positive loadings) and glutamic acid and lysine (negative loadings). PC3 accounts for a further 11.73% of the variation with a trade-off between alanine, aspartic acid, phenylalanine, lysine and tryptophan (positive loadings) and threonine and asparagine (negative loadings). PC4 accounts for 7.9% of variation in amino acid composition with a trade-off between alanine, asparagine, aspartic acid and glutamine (positive loadings) and serine and methionine (negative loadings). Thus, PC2, PC3 and PC4 indicate differences in the specific composition of free amino acids and reflect variation in allocation patterns, independent of quantitative differences.

3.4.3 Effects on egg free amino acid profiles

Overall, there was a significant effect of population, maternal diet, as well as a significant interaction between these main effects, for all four PCs describing the amino acid composition of the eggs **(Table 3.2)**. In contrast, the amino acid composition of eggs did not significantly vary with female size **(Table 3.2)**. Given the overall significance of our MANCOVA, we investigated the univariate effects on each PC using ANCOVA **(Table 3.2)**. The overall multivariate effect of population was driven by significant differences in PC1, PC2 and PC4. On average, PC1 and PC4 values were higher in the sunflower-adapted population than the milkweed-adapted population, but this pattern was reversed for PC2 **(Figure 3.3)**. The overall multivariate effect of maternal diet was driven by significant differences in PC1 and PC3 **(Table 3.2; Figure 3.3)**. On average, PC1 values were higher on the sunflower diet than the milkweed diet, while the reverse pattern was observed for PC3 **(Figure 3.3)**. The overall multivariate effect of the interaction between population and maternal diet was driven by significant differences in PC2, PC3 and PC4 **(Table 3.2; Figure 3.3)**. The significant interaction for PC2 occurs because there was a difference in PC2 values across maternal diets in the sunflower-adapted population but not in the milkweed-adapted population **(Figure 3.3)**. The significant interaction for PC3 occurs because although PC3 values are higher for milkweed than the sunflower diet in both populations, this difference is more pronounced in the sunflower-adapted population **(Figure 3.3)**. The significant interaction for PC4 occurs because of the opposing effects maternal diet has on PC4 values across populations **(Figure 3.3)**.

3.4.4 Amino acid profiles of eggs and offspring performance

None of the standardized selection gradients for the relationship between the PCs describing amino acid composition of the eggs and hatching success were significant **(Table 3.3)**, nor were the eigenvectors extracted from the canonical analysis of γ significant **(Table 3.4)**. In contrast, we found that there was significant positive linear selection for PC3 and PC4, as well as significant quadratic (stabilizing) selection on PC3 associated with offspring hatching mass **(Table 3.4)**. There was also significant correlational selection for negative covariance between PC1 and PC3, and between PC2 and PC3 **(Table 3.4)**. Canonical analysis of the λ matrix found three eigenvectors with significant linear selection on $\mathbf{m_1}$ (heavily weighted by PC3 with decreased values of PC1 and PC2), $\mathbf{m_2}$ (heavily weighted by PC2 with decreased values of PC1) and $\mathbf{m_4}$ (heavily weighted by

PC4) **(Table 3.4)**. Two eigenvectors, $\mathbf{m_1}$ and $\mathbf{m_4}$, were found to have significant quadratic selection acting along them **(Table 3.4)**. The positive eigenvalue of $\mathbf{m_1}$ indicates disruptive selection operating along this eigenvector and the negative eigenvalue for $\mathbf{m_4}$ indicates stabilizing selection. This performance surface can be visualized as a thin-plate spline in **Figure 3.4**. The combination of positive and negative eigenvalues for these eigenvectors indicates the presence of a multivariate saddle on the response surface with a pronounced peak of performance at high positive values of $\mathbf{m_1}$ and intermediate values of $\mathbf{m_4}$ **(Figure 3.4)**. We plotted the mean **m** scores for each treatment on the contour view of the performance surface **(Figure 3.4)** to visualize local features of the response surface and assess the relationships between the treatments.

3.5 Discussion

In this study we examined how free amino acid profiles of eggs may vary in relation to maternal diet in two different populations of *O. fasciatus*. As our populations have persisted on different diets (i.e. either milkweed seeds or sunflower seeds) for many generations we would expect that they would differ in their egg amino acid profiles when challenged with a novel food source. We found a significant relationship between egg mass and the two diets (milkweed and sunflower) suggesting that resource allocation differs between the diets **(Figure 3.1)**. Furthermore, we found that the free amino acid profiles of eggs differed between our two populations suggesting evolved differences in patterns of egg amino acids **(Table 3.2)**.

3.5.1 Maternal diet and egg mass – quantity or quality?

As with other studies, we found that maternal diet can have significant consequences on egg size with potential implications for egg quality (Rossiter *et al.*, 1993; Awmack & Leather, 2002; Kyneb & Toft, 2006). Larger eggs can result in larger offspring with faster development times and higher survival rates (Rossiter, 1991b; Fox, 1994a; Fox & Mousseau, 1996). It could be that offspring from large eggs are able to assimilate resources more quickly than offspring from smaller eggs, which may then give them a competitive advantage when competition is high (Fox, 1994a). Alternatively, larger eggs may represent a higher maternal investment to provide the developing embryo with nutritive compounds thereby resulting in larger offspring. Offspring size is considered an important life history trait as it is the product of both maternal and offspring phenotype (Bernardo, 1996b; Fox

& Mousseau, 1996; Mousseau & Fox, 1998b). However, Cahenzli and Erhardt (2012) emphasise the point that while heavier hatchling mass may have fitness benefits in some species this is not always the case, and that further studies need to assess any fitness benefits. For *O. fasciatus*, body size and mass do seem to be positively related to fitness. Body size has been shown to be positively correlated to reproductive success (Blakley & Goodner, 1978). Furthermore, eclosion is a size-dependent trait (size triggered metamorphosis) and 5th instar nymphs must reach a critical size before they are able to eclose into adults (Blakley & Goodner, 1978; Blakley, 1981). Smaller offspring may take longer to reach a critical size threshold to undergo adult ecdysis. They may also eclose into smaller adults with a decreased reproductive output (Blakley & Goodner, 1978). Delayed moulting times can also increase the risk of mortality before the adult stage is reached (Blakley & Goodner, 1978). Indeed, it has been observed that occasionally nymphs from the milkweed-adapted population reared on sunflower are so slow to develop that they were not likely to survive if after a three weeks full maturation has not taken place (personal observation).

Many studies have investigated relationships between maternal environment, egg size, offspring size and offspring performance (fitness) traits in various insect species (Rossiter, 1991b; Fox, 1993, 1994a,b; Fox *et al.*, 1995; Bernardo, 1996b; Jann & Ward, 1999; Kyneb & Toft, 2006; Bonduriansky & Head, 2007; Geister *et al.*, 2008). While findings have varied as to any correlations between egg size and offspring performance, some studies have shown that egg size would only influence offspring performance when offspring were raised under adverse conditions (e.g. poor quality host diet) (Fox & Mousseau, 1996; Fox *et al.*, 1997b; Jann & Ward, 1999; Bonduriansky & Head, 2007). Females may also demonstrate variations in allocation strategies when challenged with a poor quality diet. For example, a study on zebra finches *Taeniopygia guttata* by Rutstein *et al.* (2004) found that females laid progressively heavier eggs when maintained on a high quality diet and progressively lighter eggs when maintained on a low quality diet. Therefore, egg mass could be seen to be related to hatching success as heavier eggs were found to have lower hatching mortality than lighter eggs. Furthermore, the authors found that differences in diet quality also influenced the sex ratio with more males than females being produced when mothers were reared on a low quality diet.

Egg size has traditionally been considered a predictor of offspring fitness with larger eggs being positively correlated with offspring performance traits (Fox, 1994a; Fox *et al.*, 1997b; McIntyre &

Gooding, 2000; Giron & Casas, 2003), yet there is evidence that egg size is not always a good indicator of maternal allocation of nutrient resources to offspring (Rossiter, 1991b; McIntyre & Gooding, 2000; Giron & Casas, 2003). Egg size was generally related to hatchling mass in this experiment, but it was not a perfect predictor. It has been argued that egg mass may not always correlate to nutritional composition as that egg mass and nutritional components such as amino acids, lipids, proteins and carbohydrates change over the course of egg development (Favalora & Kalicki, 1973; McGregor & Loughton, 1976; Chaubey & Bhatt, 1988; Gelman *et al.*, 2000; Giron & Casas, 2003).

Growing evidence over the years in a variety of taxa has shown that maternal diet quality can affect offspring phenotypes through resource allocation (Bernardo, 1996b). Resources obtained from the diet and which are incorporated into the eggs are important components for successful embryonic development. The quality of resources that are incorporated into the eggs are an important determinant of offspring performance (Verboven *et al.*, 2003) and host quality has been shown to be contribute to offspring phenotypic variation (Amarillo-Suárez & Fox, 2006). For example, carotenoids are pigments produced by photosynthesizing organisms that can serve as antioxidants and immunostimulants (Blount *et al.*, 2000). As very few animals can manufacture carotenoids *de novo* they must ingest them (Müller *et al.*, 2012). Maternally ingested carotenoids are known to be found in eggs of a variety of taxa including birds, amphibians, reptiles and invertebrates (Goodwin, 1986; Koutsos *et al.*, 2003) and have been implicated to enhance antioxidant and immune function and may even increase survivorship probability of offspring (Blount *et al.*, 2000; Karadas *et al.*, 2005).

3.5.2 Maternal diet and amino acid profiles of eggs

We found that, as expected, free amino acid content of eggs was related to maternal diet. Consistent with the egg size results, the overall quantity of amino acids (PC1) reflected the maternal diet. In addition, PC3 and PC4, both of which suggest differences in the specific composition of free amino acids, were influenced by diet. Other studies have also shown, or implied, that variation in maternal host-diet may influence maternal allocation of resources into eggs, thus leading to variation in egg composition. For example, (Rossiter *et al.*, 1993) demonstrated significant maternal effects in response to diet in the gypsy moth *Lymantria dispar*. They found that temporal and spatial variation

in parental nutritional experience can lead to maternal variation in allocation of the egg protein, vitellogenin, into eggs resulting in variation in gene expression and phenotypic plasticity in natural populations. In the eggs of the seed beetle *Stator limbatus* differences in egg color between females fed different host diets suggests differential allocation of resources through maternal host diet effects (Fox *et al.*, 1995). Maternal host effects were also evident in offspring performance traits suggesting that maternal resource allocation may be an important factor influencing some offspring life history traits in *S. limbatus*. Furthermore, Amarillo-Suárez and Fox (2006) found that maternal host experience influenced offspring development time in *S. limbatus*. However, as there was no influence of maternal host on egg size the authors propose that maternal host plant effects on offspring development time were mediated through maternal adjustment of egg composition, especially when exposed to a poor quality host. There is some evidence to suggest that the carotenoid profiles of eggs could be important in maximizing antioxidant protection of developing embryos of lesser black-backed gulls *Larus fuscus* (Blount *et al.*, 2002a).

In our study, the population differences we see in free amino acid profiles suggest that the evolutionary history of populations may influence these profiles. Comparative studies on a vertebrate egg protein, vitellogin, suggest that vitellogins may evolve specific amino acid profiles that are related to the requirements of the developing embryo (White III, 1995). If nutritional input from milkweed and sunflower are significantly different to influence embryonic development then it may be that the two populations have evolved in relation to the amino acid profiles required to utilize the different diets during embryonic development. However, this would require further investigations as we do not have any information regarding the amino acid content of the seeds or of the females. It may also be possible that some amino acids may be derived from males. Amino acid transfer from males to females via nuptial gifts has been reported in crickets (Gershman *et al.*, 2012) and some of these amino acids may then be incorporated into the female's reproductive tissues (Brown, 2011). Thus, patterns of allocation may not simply be a function of maternal diet.

However, our results suggest that as populations evolve in response to different host use, maternal effects may evolve alongside a dietary shift perhaps because of selection. This is supported by the significant population x maternal diet interaction term. There was a significant interaction for all of the composition characters (PC2, PC3 and PC4) **(Table 3.2)** indicating that, even given the same resources, populations with different evolutionary histories can, potentially, evolve different egg

amino acid profiles. A study by Springer and Boggs (1986) also found that maternal resource allocation could evolve between two ecologically separate populations of the butterfly *Colias philodice eriphyle*. Comparative vertebrate studies have found that egg proteins may have specific amino acid profiles that are specific to the requirements of the developing embryo (White III, 1995). Our results support the suggestion of O'Brien et al. (2002, 2005) that by investigating a range of nutrients, including amino acids, could help illuminate understanding of nutrient allocation, the evolution of life histories and dietary specialization (O'Brien *et al.*, 2005).

3.5.3 Egg amino acid profiles and relationship with offspring performance

Given the differences of amino acid profiles we see alongside the different evolutionary histories of the populations we studied **(Figure 3.3)**, we wished to know if these differences were a response to selection. We therefore used multivariate selection analysis to investigate the relationships between the amino acid profiles of the eggs and offspring performance traits (hatching success and hatchling mass). Performance surfaces can be used to plot phenotypic traits to a surrogate measure of fitness and to visualize a multivariate non-linear function (Phillips & Arnold, 1989; Pigliucci, 2012; Rice, 2012) – also see Methods section.

Hatching success

Amino acids have been reported to have a number of effects on reproductive systems in a variety of taxa. For example, amino acids have been shown to enhance egg viability and hatching success in a marine copepod (Guisande *et al.*, 2000) and egg protein synthesis in fish (Izquierdo *et al.*, 2001), increase ovulation rates in ewes (Dunn & Moss, 1992) and egg production in blue tits (Ramsay & Houston, 1998). Furthermore, human embryos with specific amino acid turnover profiles may be used to determine viable embryos for IVF (Houghton *et al.*, 2002). While we did not find evidence of selection for amino acid profiles on hatching success, it may be that other egg constituents that we did not measure, such as vitamin A, vitamin E, carotenoids, hormones or fatty acids, may influence egg viability and hatching success (Wilson, 1997; Price, 1998; Izquierdo *et al.*, 2001; Karadas *et al.*, 2005; Møller *et al.*, 2008) in our populations of *O. fasciatus*. However, a study on pied flycatchers *Ficedula hypoleuca* did not find any relationship with egg components and hatching success (Ruuskanen *et al.*, 2011). Furthermore, we did not measure male contributions. Paternally derived nutrients have also been shown to influence female fecundity (Vahed, 1998;

Gillott, 2003; Wedell & Karlsson, 2003) so therefore we cannot exclude the possibility of an unmeasured paternal contribution towards hatching success.

Hatchling mass

The effects of specific amino acid profiles may be a reflection of population differences in the way that they utilize the two different diets, as indicated by the significant population x maternal diet interaction **(Table 3.2)**. This is supported by the selection analysis on the effects of amino acid profiles on hatchling mass which suggests that specific profiles may be selected for in relation to developing into heavier hatchlings **(Table 3.2)**. Specific amino acid profiles may also be interactive, (in this case PC3& PC1 and PC3 & PC2), in the way that they influence hatchling mass as indicated by significant correlational selection **(Table 3.3)** (Sinervo & Svensson, 2002). We also found evidence of disruptive and stabilizing selection which may favour the maintenance of variation for specific amino acid profiles and indicate a complex interplay between amino acid profiles and hatchling mass. A study on male guppies *Poecilia reticulate* by Blows *et al.* (2003) found that stabilizing and disruptive selection contributed to variations in male attractiveness, while interpretation of the fitness surface revealed complex interactions between morphological and colour traits that may contribute to the maintenance of males having multiple sexual ornaments. Our results suggest that while selection has influenced free amino acid profiles of eggs, this has not evolved equally in both populations. It is possible that the evolution of amino acid profiles in one population may be constrained from reaching the same performance optima as the other population depending on maternal diet. This can be visualized in the performance landscape for hatchling mass as a function of the amino acid profiles **(Figure 3.4)**. However, further work on the genetics of the amino acid composition of eggs in this species is needed to assess this possibility.

While we have used multivariate selection analysis in exploring relationships between free amino acid profiles of eggs and offspring performance parameters, free amino acids are only one aspect of egg composition. As discussed above, other components that females allocate to their eggs could supplement offspring development to influence offspring performance parameters. Due to the complex nature of the environmental, genetic (Rossiter *et al.*, 1993; Boggs, 2009) and biochemical processes (Izumi *et al.*, 1994) underpinning maternal allocation of egg provisioning and embryonic development, no single compound may well be associated with egg/offspring viability (Bauerfeind

& Fischer, 2005; Geister *et al.*, 2008). Rather, a complex suite of compounds in an appropriate balance (Bolton *et al.*, 1992; Uchida, 1993; Wilson, 1997; Devreker *et al.*, 2001; Blount *et al.*, 2002a; Geister *et al.*, 2008; Boggs, 2009) may be required to optimize offspring fitness via maternal egg effects. As amino acids may have essential roles in embryonic development (Devreker *et al.*, 2001), our work provides a novel insight to examining their role as a maternal (diet) effect. How nutritional resources influence egg production can give further insights into the relationships between nutritional ecology, resource allocation and life history traits (O'Brien *et al.*, 2002; Boggs, 2009) that influence population dynamics and life history evolution (Mousseau & Dingle, 1991).

Acknowledgments

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Table 3.1 Principal Component Analysis (PCA) of the free amino acid composition of eggs in *Oncopeltus fasciatus*. We retained PCs with eigenvalues exceeding 1 for further analysis and interpret factors loadings of 0.30 or above as biologically important (in bold). Amino acids in italics are essential amino acids. Those amino acids marked with * are potentially important intermediaries for the mevalonate cycle and those marked with + are biosynthesized from phenylalanine.

	Principal Component			
	PC1	PC2	PC3	PC4
Eigenvalues	4.775	2.663	1.877	1.268
% Variance Explained	29.85	16.46	11.73	7.93
Amino Acids				
Alanine*	0.110	0.258	**0.522**	**0.311**
Glycine	0.098	**0.844**	-0.181	0.024
Leucine	**0.755**	0.194	0.206	-0.102
*Isoleucine**	**0.843**	0.293	-0.178	-0.093
*Threonine**	**0.652**	0.105	**-0.575**	0.139
Serine*	**0.476**	-0.108	0.158	**-0.616**
Proline	-0.122	**0.826**	0.057	-0.037
Aspargine	**0.509**	0.113	**-0.478**	**0.480**
Aspartic acid	0.178	-0.158	**0.677**	**0.307**
Methionine+§	**0.654**	-0.070	-0.213	**-0.346**
Glutamic acid	**0.764**	**-0.412**	0.140	0.052
Phenylalanine	**0.694**	0.110	**0.311**	0.208
Glutamine	**0.619**	-0.187	-0.083	**0.423**
*Lysine**	**0.702**	**-0.339**	**0.308**	-0.154
Tyrosine+	**0.377**	**0.393**	0.032	-0.242
Tryptophan	0.057	**0.705**	**0.434**	-0.018

Table 3.2 Multivariate Analysis of Covariance (MANCOVA) examining the effects of population and maternal diet (milkweed or sunflower) and their interaction on four PCs describing free amino acid composition of the eggs of *Oncopeltus fasciatus*, with females size as a covariate. We follow the overall MANCOVA with univariate analysis of covariance (ANCOVA) to determine how each of the PCs contributes to the overall multivariate effects.

	MANCOVA		
Model term	Pillai's Trace	$F_{4,188}$	*P* value
Population	0.426	34.870	0.000
Maternal diet	0.553	58.063	0.000
Population x Maternal diet	0.120	6.379	0.000
Female size	0.029	1.415	0.230
	Univariate ANCOVAs		
	F	*df*	*P*
Population			
PC1	31.666	1,191	0.0001
PC2	25.933	1,191	0.0001
PC3	3.630	1,191	0.058
PC4	42.826	1,191	0.0001
Maternal diet			
PC1	21.639	1,191	0.0001
PC2	0.114	1,191	0.736
PC3	171.989	1,191	0.0001
PC4	2.005	1,191	0.158
Population x Maternal diet			
PC1	0.430	1,191	0.513
PC2	4.664	1,191	0.032
PC3	7.440	1,191	0.007
PC4	16.161	1,191	0.0001
Female size			
PC1	1.963	1,191	0.163
PC2	2.366	1,191	0.126
PC3	1.143	1,191	0.286
PC4	0.845	1,191	0.359

Table 3.3 The vector of standardized linear selection gradients (β) and the matrix of standardized quadratic and correlational selection gradients (γ) for the four PCs describing the amino acid composition of the eggs in *Oncopeltus fasciatus* and their effects on **(A)** offspring hatching success and **(B)** hatchling mass. Randomization test: * $P < 0.05$, ** $P < 0.01$, ** $P < 0.001$.

A.		**γ**			
	β	PC1	PC2	PC3	PC4
PC1	-0.004	0.002			
PC2	-0.007	-0.045	0.026		
PC3	-0.004	-0.011	0.030	0.018	
PC4	-0.015	-0.026	0.009	-0.038	-0.028
B.		**γ**			
	β	PC1	PC2	PC3	PC4
PC1	-0.015	-0.010			
PC2	-0.006	0.003	0.008		
PC3	0.051***	-0.028*	-0.023*	0.016	
PC4	0.029**	-0.015	0.003	-0.003	-0.030****

Table 3.4 The M matrix of eigenvectors from the canonical analysis of γ for the four PCs describing the amino acid composition of the eggs in *Oncopeltus fasciatus* and their effects on **(A)** offspring hatching success and **(B)** hatchling mass. The linear (θ_i) and quadratic (λ_i) gradients of selection along each eigenvector are given in the last two columns. The quadratic selection gradient (λ_i) of each eigenvector (m_i) is equivalent to the eigenvalue. Randomization test: * $P < 0.05$, ** $P < 0.01$, ** $P < 0.001$.

	M					**Selection**	
A.	PC1	PC2	PC3	PC4		$\boldsymbol{\theta_i}$	$\boldsymbol{\lambda_i}$
$\mathbf{m_1}$	-0.515	0.728	0.452	0.027		-0.005	0.038
$\mathbf{m_2}$	0.387	-0.140	0.702	-0.581		0.005	0.018
$\mathbf{m_3}$	0.667	0.669	-0.314	-0.096		-0.005	-0.017
$\mathbf{m_4}$	0.375	-0.045	0.453	0.808		-0.015	-0.031
	M					**Selection**	
B.	PC1	PC2	PC3	PC4		$\boldsymbol{\theta_i}$	$\boldsymbol{\lambda_i}$
$\mathbf{m_1}$	-0.417	-0.492	0.763	0.033		0.049***	0.023**
$\mathbf{m_2}$	-0.567	0.743	0.155	0.319		0.021*	0.001
$\mathbf{m_3}$	0.379	0.447	0.522	-0.620		0.001	-0.010
$\mathbf{m_4}$	0.600	0.078	0.348	0.716		0.029**	-0.024**

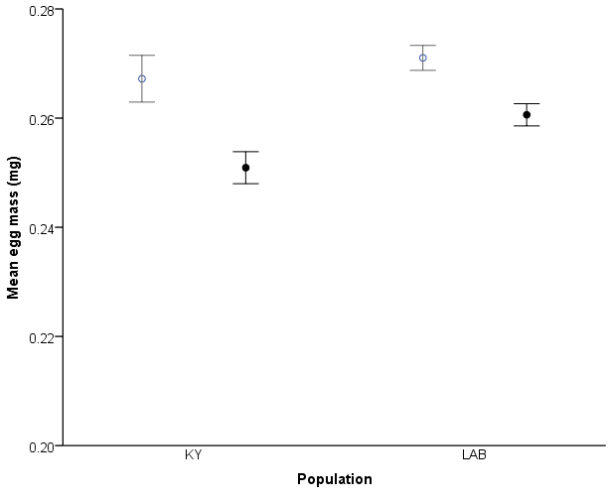

Figure 3.1 Mean egg mass (±SE) across the milkweed-adapted (‘KY’) and sunflower-adapted (‘LAB’) populations when females reproduce on milkweed (open circles) and sunflower (closed circles) diets.

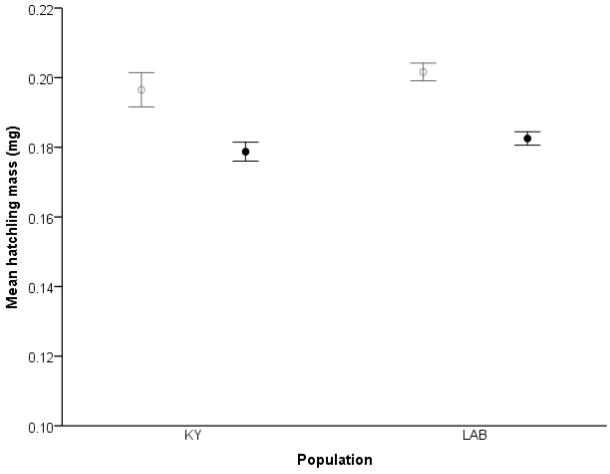

Figure 3.2 Mean hatchling mass (±SE) across the milkweed-adapted (‘KY’) and sunflower-adapted (‘LAB’) populations when females reproduce on milkweed (open circles) and sunflower (closed circles) diets.

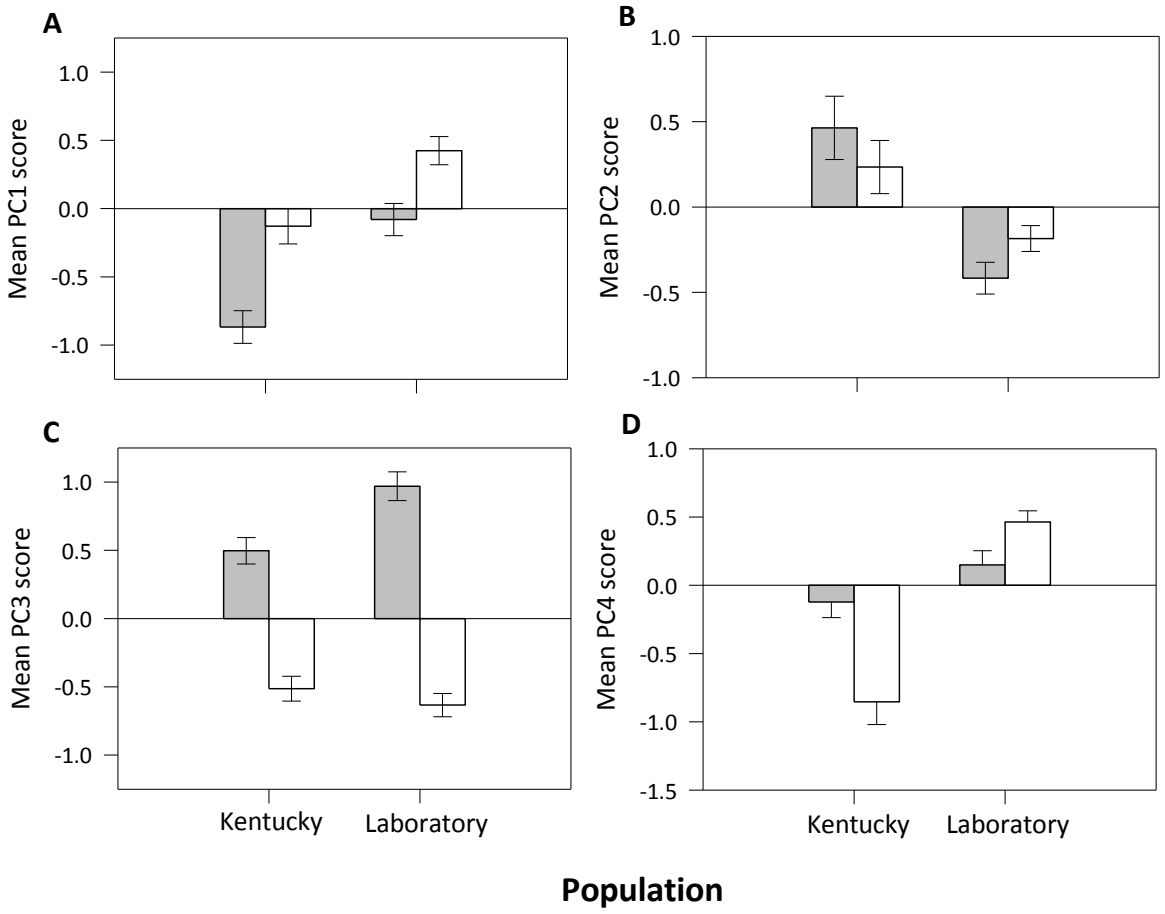

Figure 3.3 Mean PC scores (±SE) describing the amino acid composition of eggs across the milkweed-adapted ('Kentucky') and sunflower-adapted ('Laboratory') populations when females reproduce on milkweed (grey bars) and sunflower (white bars) diets. (A) PC1, (B) PC2, (C) PC3 and (D) PC4.

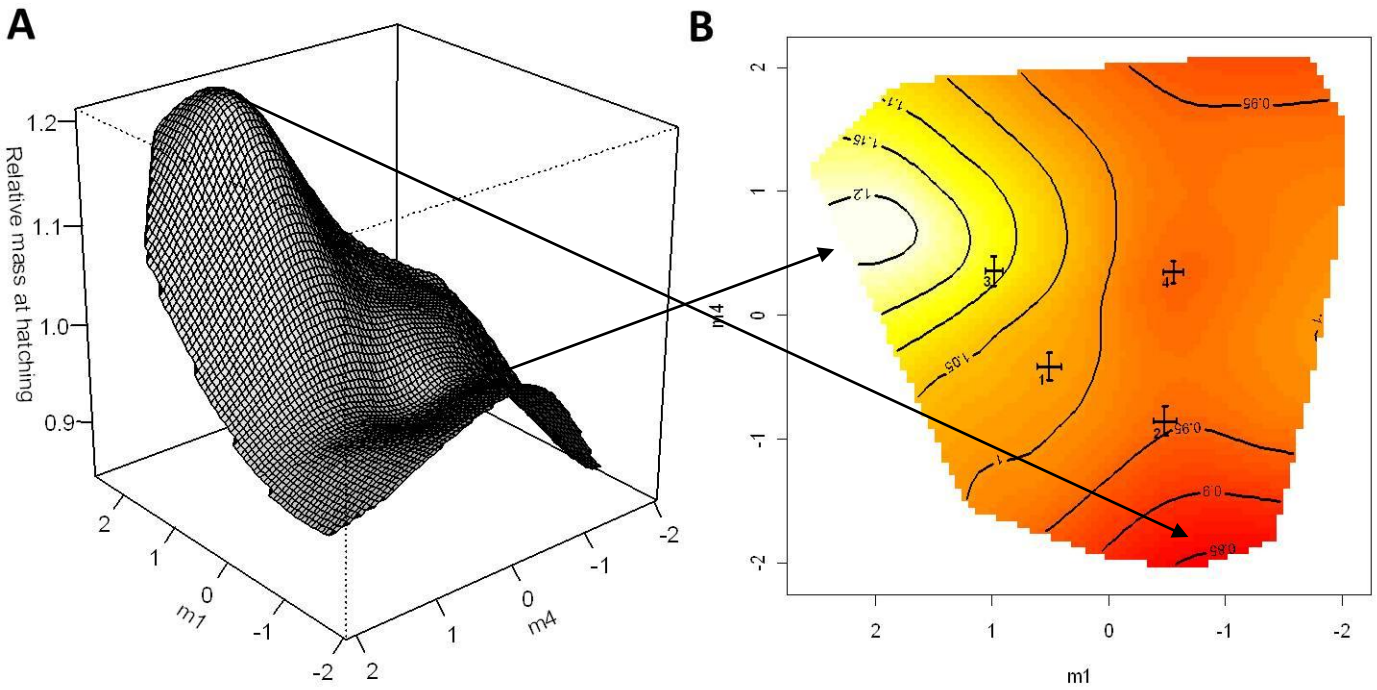

Figure 3.4 Thin-plate spline **(A)** perspective and **(B)** contour view visualization of the fitness surface along the two major axes of nonlinear selection ($\mathbf{m_1}$ and $\mathbf{m_4}$). In (B) the mean $\mathbf{m_1}$ and $\mathbf{m_4}$ scores for each treatment combination are provided where 1 = KYMW; 2 = KYSF; 3 = LABMW; 4 = LABSF. Note that (A) and (B) rotated.

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Chapter 4: Quantitative genetics of maternal allocation of amino acids into eggs of an insect herbivore

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4.1 Abstract

The importance of maternal effects on evolutionary trajectories depends on heritable genetic variation underlying the maternal trait that influences the offspring phenotype. Very often we lack information on the specific maternal trait that contributes to a maternal effect, and only get indirect estimates of heritable effects from offspring performance. However, evolutionary quantitative genetic models of maternal effects show that a full understanding of the evolutionary consequences requires examining the specific maternal traits that contributes to differences in offspring performance. Here we examine the quantitative genetic architecture underlying free amino acids in eggs of the large milkweed bug *Oncopeltus fasciatus*. Although we found evidence for genetic variation in 16 of the 19 free amino acids measured, our results suggest that there is low evolutionary potential in amino acids as evidenced by low evolvability, I_A, (a standardised measure of evolutionary potential). Further genetic constraints were found by examining the genetic variance-covariance (**G**) matrix. The most evolutionary accessible linear combination, as reflected by $\mathbf{g}_{max}$, involved only a small subset of the 16 heritable amino acids. Although $\mathbf{g}_{max}$ explained only 50% of the variance in **G**, in combination with our information on evolvability and heritability we suggest that genetic constraints may limit the rate of evolutionary change for free amino acid profiles in eggs for our milkweed-adapted population of *O. fasciatus*. Our study presents a novel insight into the genetics of amino acids in eggs and highlights the importance of considering maternal traits in a multivariate context when investigating their evolutionary potential as adaptive maternal effects.

Keywords: Evolvability, genetic variance-covariance (**G**) matrix, maternal effects, *Oncopeltus fasciatus* (Hemiptera: Lygaeidae)

4.2 Introduction

There has been an explosion of interest in the evolutionary importance of maternal effects and their potential role in adaptation following the volume published by Mousseau and Fox (1998b). Maternal effects, defined as any environment provided by a mother that affects her offspring; i.e., the causal influence of the maternal genotype or phenotype on the offspring phenotype (Wolf & Wade, 2009), can have profound influences on development and phenotypic plasticity (Galloway & Etterson, 2007), behaviour (Storm & Lima, 2010; Giesing *et al.*, 2011), and life history (Mousseau & Fox, 1998b; Allen *et al.*, 2008; Badyaev & Uller, 2009; Galloway *et al.*, 2009; Head *et al.*, 2012). The traditional view in quantitative genetics was that maternal effects were a source of unwanted complication (Falconer *et al.*, 1996), but as Fox and Mousseau (1998) point out, maternal effects can themselves evolve and therefore can facilitate (rather than hinder) adaptation (Kirkpatrick & Lande, 1989; Cheverud & Moore, 1994). Experimental work confirms that maternal effects may evolve to facilitate or constrain adaptation to new environments (Mousseau & Fox, 1998b; Marshall & Uller, 2007; Crean & Marshall, 2009). For maternal effects to evolve, however, there has to be a genetic basis to the variation among mothers (or fathers) creating the maternal effect. There is considerable empirical evidence that maternal effects have a heritable genetic basis and that maternal effects have the potential to evolve (Mousseau & Fox, 1998b; Fox *et al.*, 1999; Rauter & Moore, 2002a; Rauter & Moore, 2002b; Wilson *et al.*, 2005; Räsänen & Kruuk, 2007; Head *et al.*, 2012).

In theory, maternal effects can constrain as well as facilitate adaptation, so understanding how the maternal effect itself may evolve is essential (Cheverud & Moore, 1994). An understanding of the pattern of genetic variation underlying multiple specific egg constituents may be especially revealing, as the presence of multiple components gives rise to potential genetic correlations that may constrain evolution (Lande & Arnold, 1983; Roff, 2000; Blows, 2007; Arnold *et al.*, 2008). The genetic variance-covariance matrix, **G**, should reveal constraints between characters by way of low additive genetic variances or correlations between characters indicating a limited potential for response to selection (Arnold, 1992; Stearns, 1992). Through an examination of **G** it is therefore possible to gain information on the rate and direction of selection on multiple traits (Blows *et al.*, 2004) and make predictions about their evolutionary responses (Arnold *et al.*, 2008) through the determination of evolutionary lines of least resistance (Schluter, 1996).

The consequences of maternal effects are most evident in early life history stages of offspring (Wilson & Réale, 2006), so one aspect of maternal effects that should be of particular interest is maternal allocation of resources to the developing embryo. Eggs provide the earliest environmental experience of developing offspring (Rossiter, 1991a,b; Kyneb & Toft, 2006), and therefore the quality of nutrients or resources that females allocate to her eggs determine quality of her offspring when they hatch (Fox *et al.*, 1997b; Fox & Mousseau, 1998). This can reflect variation in the way that females acquire and allocate resources (Rossiter *et al.*, 1993). Maternal host-diet effects can influence early offspring life history traits through maternal provisioning of nutrients taken in from the maternal diet (Fuiman & Ojanguren, 2011; Itonaga *et al.*, 2011) or through nuptial gifts provided by males (Bonduriansky & Day, 2009). For example, amino acids are important in reproductive biology especially in relation to oogenesis (Wheeler, 1996; Devreker *et al.*, 2001; O'Brien *et al.*, 2002) as they are vital to embryonic development and are the basis for biomolecules that are involved in biochemical processes. In a previous experiment (Chapter 3) we showed a relationship between maternal diet and free amino acid profiles of eggs in the large milkweed bug *Oncopeltus fasciatus*, and that amino acid profiles can, potentially, impact on early offspring life history traits. However, we wanted to ask if there is genetic basis to the amino acid composition of eggs and, if so, are there genetic constraints which may reflect limitations in the way that amino acid profiles may respond to selection? Here we follow this work with an examination of the genetic basis of variation in the specific components of eggs.

The aim of our current study is to extend on our previous experiment to determine if there is genetic variation of free amino acids in eggs of the large milkweed bug *O. fasciatus* and, if so, does the genetic architecture of amino acids constrain the potential response of amino acid profiles to selection? If there are genetic constraints, then this may have implications for the evolvability of amino acid allocation with potential impacts on offspring phenotypes and life history traits. Given there are two major sources of genetic influences on patterns of evolution – evolvability reflecting overall genetic variation, and constraint reflected in patterns of genetic covariation – we measured genetic variation and covariation through a quantitative genetic study of free amino acids in eggs of *O. fasciatus*. We hypothesised that there is a genetic basis to the amino acids found in eggs and that this would suggest evolutionary potential for a response to selection. As amino acids are vital to the developing embryo we make the assumption that they are closely related to fitness and therefore we predict that they would exhibit higher additive genetic variance (V_A) (Houle, 1992). Following on

from this, if there is evidence of high V_A then we would also expect there to be a potential for evolutionary change. We discuss our findings in relation to amino acids in eggs as a maternal trait and the implications for evolutionary responses to selection.

4.3 Materials and methods

4.3.1 Study species

Our base population of large milkweed bugs *O. fasciatus* (Hemiptera: Lygaeidae) was founded by individuals collected from the University of Kentucky Arboretum, Lexington, KY, USA, in September 2009. In the wild, *O. fasciatus* feed on plants from the genus *Asclepias*. We reared and maintained our population on dried milkweed seeds *Asclepias syriaca* purchased from Everwilde Farms, Inc. They were housed in several plastic boxes (36cm X 28cm X 18cm) and bugs were shuffled between boxes weekly. Demineralised water and seeds were also changed weekly. Absorbent cotton wool was provided as an oviposition substrate.

Eggs were collected from the base population and kept in boxes (28 X 16 X 9 cm) with *A. syriaca* seeds, *ad libitum*, and demineralised water changed weekly and as required. Nymphs were reared until adults. Newly eclosed (virgin) adults were harvested daily. Males and females were kept separate and housed in boxes (11 X 11 X 3 cm) with *A. syriaca* seeds and demineralised water.

4.3.2 Breeding design

We used a standard paternal half-sib design to estimate additive genetic variance (V_A) and phenotypic variance (V_P) of free amino acids in eggs of *O. fasciatus*. Families were created by randomly mating 54 sires with up to five females each (see **Figure 4.1**). Males were kept with each female for 2 days. Males were allowed to rest in individual Petri dishes for 24 hours between mating each female (*ad libitum A. syriaca* seeds and demineralised water supplied). Matings were set up in Petri dishes with *A. syriaca* seeds and demineralised water (changed as required). Cotton wool was supplied as an oviposition substrate. After separation, fresh cotton wool was supplied for the female. Once a clutch was laid, 10 eggs from each female were collected and placed in boxes (11 X 11 X 3 cm) with *A. syriaca* seeds and water. The F1 nymphs that hatched from these eggs were reared until they became adults. Newly eclosed virgin (F1) adults were then collected daily

and housed in boxes (11 X 11 X 3 cm) as per family and eclosion date (males and females were housed separately). Five F1 virgin females from each family were randomly mated with a single non-related male as above. Pairs were allowed to mate for 3 days after which the male was removed. Females were checked every 2 hours during the daytime for freshly laid eggs. Eggs that had been laid overnight were discarded. Freshly laid eggs were collected when there were more than 20 eggs in a clutch. 10 eggs from each clutch were weighed. Weighed eggs were then placed in a 1.5ml Eppendorf tube and stored at -80° until eggs had been collected from each female, after which the eggs were then prepared for amino acid analysis (see methods below).

4.3.3 Amino acid extraction

Ten eggs from each female were collected and placed in 1.5ml Eppendorf tubes which were stored at -80°C until eggs had been collected from all females. Once all the eggs had been collected they were prepared for amino acid extraction. To prepare them for amino acid analysis we modified methods following Gelman *et al.* (2000). Firstly, 100µl of 75% ethanol was pipetted into each sample tube. Samples were then sonicated for 3 minutes so they could then be mashed with a manual pestle and further washed with another 100 µl of 75% ethanol. They were then pulse vortexed for 5 seconds after which they were placed on ice for 30 minutes. Following this, samples were centrifuged for 10 minutes at 4°C at maximum speed. Samples were then placed in liquid nitrogen. The supernatant from each sample was pipetted into a fresh Eppendorf tube and all samples were stored at -80°C ready for amino acid analysis.

Amino acids were extracted and analysed using a EZ:faast GC-FID physiological amino acid analysis kit (Phenomenex Inc., Elville House, Macclesfield, SK10 2BN, UK) using their protocol. 100µl of each prepared sample was pipetted into a sample preparation vial along with 100µl of the internal standard solution (Norvaline 0.2nM, N-propanol 10%). A 1.5ml syringe with a solid phase extraction (SPE) sorbent tip was used to separate the solution by slowly passing the solution through the tip into the barrel. Any fluid that was passed into the barrel was discarded and only solution within the tip was retained. 100 µl of wash solution (N-propanol) was then pipetted into the sample preparation vial. The solution was passed slowly through the tip and into the barrel and air was drawn through to dry the sorbent particles. Again, only solution within the tip was retained while any solution in the barrel was discarded.

200µl of freshly prepared eluting medium (a 3:2 mix of sodium hydroxide and N-propanol) was then pipetted into the preparation vial. A 0.6ml syringe was attached to the sorbent tip and the eluting medium was passed slowly through to the tip thereby wetting the sorbent particles. The liquid and sorbent particles were then ejected back into the sample preparation vial. 50 µl of chloroform was then added into the sample preparation vial. The vial was then vortexed for 5-8 seconds in pulse mode at 80% of maximum speed. The reaction was then left to proceed for at least one minute. The vials were then vortexed again for 5 seconds and left for the reaction to proceed for another minute. After adding 100µl of iso-octane, the vial was vortexed for 5 seconds and the reaction left to proceed for one minute. This series of reactions allowed the solution to separate into an aqueous and an organic layer from which 50-100µl of the organic layer was pipetted into the insert of an autosampler vial. The solvent was then slowly evaporated under a nitrogen stream for no more than 10 minutes. Amino acid derivatives were then re-dissolved in 100µl of hydrochloric acid and vortexed for 10 seconds. Vials were then capped and stored at -80°C until analysed using the GC-FID.

For the GC-FID analysis, 2µl of each sample was injected onto an Agilent 7890A GC fitted with a Zebron ZB-AAA column (10m x 0.25mm). The inlet temperature was set at 250°C, and hydrogen was used as the carrier gas at a flow rate of 1.5ml/min. The oven temperature was increased at 32°C/min from 110°C to 320°C, and amino acid peaks were detected using a flame ionization detector.

4.3.4 Quantification of free amino acid composition

Using the EZ:faast kit we quantified the amount of the following 19 amino acids in each sample: alanine (ALA), glycine (GLY), valine (VAL), leucine (LEU), isoleucine (ILE), threonine (THR), serine (SER), proline (PRO), aspargine (ASN), methionine (MET), 4-Hyrdoxyproline (4-HYP), α-Aminoadipic acid (AAA), phenylalanine (PHE), glutamine (GLN), lysine (LYS), Histidine (HIS), tyrosine (TYR), tryptophan (TRP) and cystathionine (CTH). The amino acids α-aminobutyric acid (ABA), aspartic acid (ASP), orthinine (ORN), glycyl-proline (GPR), hydoxylysine (HLY), proline-hydroxyproline (PHP) and Cystine (C-C) were not included in our analyses as they were not detected in most of our samples, while sarcosine (SAR), β-aminoisobutyric acid (β –Aib), allo-isoleucine (aILE), thiaproline (TPR), glutamic acid (GLU) were not detected in any samples.

4.3.5 Statistical analyses

Given the cost of amino acid analysis, we reduced our data to a more balanced design. Out of the 54 sires that were originally mated, we collected data on eggs where sires had mated with a minimum of three dams (maximum of 4) and each dam produced a minimum of three offspring (maximum of 5). This left us with data from 30 sets of half-sib families with a total of 117 dams and 416 offspring that could be analysed for amino acid concentration in the eggs. We estimated variance components from a nested ANOVA of our half sib data set using the model: $Y_{ijk} = \mu + \alpha_i + \beta_{ij} = e_{ijk}$, where Y_{ijk} is the phenotype of the k-th progeny of the j-th dam mated to the i-th sire; μ is the common mean; α_i the effect of the i=th sire, β_{ij} is the effect of the j-th dam mated to the i-th sire and e_{ijk} - uncontrolled environmental and genetic deviations attributable to the individuals (i.e., "error") (Becker, 1992; Lynch & Walsh, 1998). From this model we used restricted maximum likelihood (REML) to obtain estimates for sire and dam genetic variances and co-variances and genetic and phenotypic correlations to construct the additive genetic variance-covariance matrix (**G**). We used the genetic variances to calculate narrow-sense heritabilities and evolvabilities. Evolvability I_A, was calculated as $4(V_A)/m^2$, where m is the mean of the trait of interest (Hansen *et al.*, 2011). Evolvability, measured this way, scales additive variance by the mean, and may be more appropriate for estimating the potential for evolutionary change than is heritability (which scales additive genetic variance by phenotypic variance) (Hansen *et al.*, 2003b; Kirkpatrick, 2009; Hansen *et al.*, 2011). I_A can be interpreted as expected proportional evolutionary response of a trait to a unit strength of selection (Hansen *et al.*, 2003b). Standard errors for I_A were determined by $4(V_A)/V_P$ (Hansen *et al.*, 2003b).

The genetic relationships between the amino acids we tested are summarised by the genetic variance-covariance (**G**) matrix (Hine & Blows, 2006) where the diagonals of **G** represent additive genetic variances and the off diagonals represent additive genetic covariances (Arnold, 1992; Steppan *et al.*, 2002; Revell, 2007). However, the information contained within **G** is limited in what it can tell us about traits in multivariate trait space. To reduce dimensionality of our amino acids to a smaller number of factors and to be able to summarise patterns between our variables, we use Principal Components Analysis (PCA) (Tabachnick & Fidell, 2001). A PC can be defined as a linear combination of variables that still retains most of the variance within the data set (Joliffe, 2002). While the actual number of PCs extracted should be equal to the number of observed

variables being analysed, not all PCs extracted will be deemed important in relation to the amount of variance that they contribute (Tabachnick & Fidell, 2001; Joliffe, 2002). In PCA, eigenvalues represent variance and each standardised variable contributes 1 unit of variance to the PC extraction and, therefore, any values less than 1 are deemed unimportant (Tabachnick & Fidell, 2001). We therefore present higher order estimates with eigenvalues greater than 1 **(Table 4.5)**. Furthermore, variables with factor loadings greater than 0.3 can be deemed biologically significant (Gershman *et al.*, 2012). We conducted a PCA on **G** to produce PCs, the first of which gives $\mathbf{g_{max}}$. This first eigenvector represents the linear component of highest genetic variance and the combination of traits most accessible to evolution (Schluter, 1996).

4.4 Results

Table 4.1 presents the descriptive data for the 19 free amino acids from *O. fasciatus* eggs. There was variation in the amount of all amino acids allocated. The largest quantities were for proline (PRO) and glutamine (GLN). There was significant heritable variation for all amino acids except threonine (THR), methionine (MET) and phenylalanine (PHE). Heritability refers to the proportion of total phenotypic variation (V_P) between individuals within a population that is due to genetic influences (V_G) (Stearns, 1992; Visscher *et al.*, 2008). Narrow-sense heritability, h^2, partitions V_G into additive and non-additive genetic (dominance, gene interactions, environment and maternal effects) effects (Stearns, 1992; Visscher *et al.*, 2008). We give estimates of h_2 in **(Table 4.1)**. Where significant, heritabilities ranged from low (0.15) to moderate (0.46) - with 4 amino acids (alanine ALA, cystathionine CTH, serine SER and proline PRO) displaying moderate heritabilities - implying that only a small proportion of amino acid variation is down to genotypic variation (Visscher *et al.*, 2008). Similarly, evolvabilities (I_A) were low (0.01 - 0.19) for all amino acids **(Table 4.1)** suggesting that amino acids in eggs of *O. fasciatus* have low evolutionary potential in response to (weak) selection and may reflect genetic constraints (Hansen *et al.*, 2003b).

Phenotypic correlations among the different amino acid concentrations were mostly positive and moderate to high in magnitude (0.3-0.84). There were only three amino acid combinations (α-Aminoadipic acid-histidine AAA-HIS, α-Aminoadipic acid-phenylalanine AAA-PHE and cystathionine-methionine CTH-MET, **Table 4.2**) whose phenotypic correlations, although significant, were weak and negatively correlated. Nearly all the phenotypic correlations between the

amino acids are significant, with the exception of α-Aminoadipic acid (AAA), which did not have significant phenotypic correlations for 10 out of the 18 possible combinations.

Threonine (THR), methionine (MET) and phenylalanine (PHE) were excluded from estimations of genetic correlations due to non-significant heritabilities **(Table 4.1)**. There were fewer significant genetic correlations than there were significant phenotypic correlations. Of the genetic correlations between amino acids that were significant, all were moderate to high (0.3-1.35, **Table 4.3**). Valine-isoleucine, valine-lysine, valine-tyrosine, tyrosine-isoleucine, tyrosine-glycine, tyrosine-histidine and tyrosine-lysine all had genetic correlations close or equal to +1. Only 4 combinations were negatively genetically correlated (α-aminoadipic acid-serine, alanine-cystathionine, alanine-leucine, cystathionine-glycine). 4-hydorxyproline was the only amino acid that had only one positive, high genetic correlation (with α-aminoadipic acid) and was not correlated to any other amino acid.

To better visualise the pattern of genetic constraint, we calculated $\mathbf{g}_{max}$ and other eigenvectors from the G matrix **(Table 4.4 & Table 4.5)**. We found four with eigenvalues greater than 1, collectively explaining 86.5% of total variation of free amino acid content. $\mathbf{g}_{max}$ explained 50% of variation and was positively loaded with 5 amino acids: valine (VAL), leucine (LEU), isoleucine (ILE), histidine (HIS) and tyrosine (TYR). $\mathbf{g}_2$ explained 17.4% of variance and is positively loaded with asparagine (ASN), α-aminoadipic acid (AAA), glutamine (GLN) and cystathionine (CTH). $\mathbf{g}_3$ explained 12% of variance and is positively loaded with alanine (ALA), 4-Hydroxyproline (HYP) and tryptophan (TRP). $\mathbf{g}_4$ explained 7.1% of variance and is positively loaded with ALA, glycine (GLY) and AAA. There were no suggestions of trade-offs by way of significant negative values between any of the amino acids within any of the 4 PCs.

4.5 Discussion

Maternal allocation of resources to eggs is a fundamental trait that can have an effect on offspring performance traits and fitness. In most cases, we have little information on how specific components of eggs create a maternal effect. Previously (Chapter 3), we have shown that specific amino acid profiles may be important in eggs of *O. fasciatus* in that they can potentially influence the developing embryo with cumulative effects on early offspring life history traits such as hatchling mass. In this study we examined the pattern of genetic variation of free amino acids in the

eggs of *O. fasciatus*, and how this might influence the potential for evolutionary change as a maternal effect, in other words, is there genetic variation in the way that free amino acids are found in the eggs and are there constraints to their evolutionary potential? Given that there is selection for specific combinations of amino acids in eggs (see Chapter 3), for this to be an adaptive maternal effect then these combinations must be accessible to evolution. The results of our current study show that both heritabilities and evolvabilities are low for amino acids in eggs of *O. fasciatus*. This suggests that only a small proportion of the variation in amino acids in the eggs in our experimental population of *O. fasciatus* can be contributed to genetic variation (Visscher *et al.*, 2008) and furthermore, that the potential to evolve may be constrained (Hansen *et al.*, 2003b).

In the first instance, the short term evolutionary potential of maternal effects depends on additive genetic variance (Hansen *et al.*, 2011) underlying the trait that causes the maternal effect. We used a standard half-sib design to estimate V_A and used it to determine I_A, a standardised measure of evolvability (Hansen *et al.*, 2011). We found that all the amino acids that we detected have low evolvability **(Table 4.1)** suggesting that even though there is additive-genetic variance, there may be limitations on the rate of evolutionary change (Hansen *et al.*, 2003b) for amino acid profiles .

In their study of reproductive traits and egg composition in white shrimp *Penaeus (Litopenaeus) vannamei*, Arcos *et al.* (2004) found moderate to high heritabilities for the egg components triacylglycerides and vitellin, but a low heritability for protein, suggesting variation for genetic variance for egg components. They also found moderate to high positive genetic correlations between egg total proteins with both vitellin and triacylglycerides but negative genetic correlations between day to first spawn with both vitellin and triacylglycerides. As developmental, biochemical and regulatory pathways are interdependent we may expect to find strong genetic correlations between traits that are involved in these processes (Arcos *et al.*, 2004). For example, vitellin and triacylglycerides may contribute to the amount of total egg proteins found in eggs while negative correlations between growth to maturation (taken as days to first spawn) and egg composition may imply trade-offs between allocation to soma and allocation to reproduction. While we found that in our study, a number of amino of amino acids demonstrated positive genetic correlations and, in general, demonstrated high phenotypic correlations **(Table 4.3)** this may also suggest that they are highly interdependent in relation to the biochemical processes during embryonic development. Furthermore, as our heritability estimates are low to moderate and our phenotypic and genetic

correlations demonstrate similar patterns, this suggests that both genetic and environmental factors may produce similar patterns of amino acid variation (Cheverud, 1988).

Positive correlations between certain traits may form under selective pressures to increase functional integration (Conner & Via, 1993). Alternatively, if functional independence between individual traits or groups of traits increases fitness then selection pressures may reduce the number of correlations (Conner & Via, 1993). For example, Conner and Via (1993) found that plant traits related to reproduction had higher phenotypic and genetic correlations than floral traits not related to reproduction in a wild radish *Raphanus raphanistrum*. They also found reduced correlations between floral and vegetative traits. The authors suggest that different parts of the plant may be more likely to evolve under selection pressures where there were low to moderate genetic correlations, but that evolution of spatial relationships between the reproductive parts of the flower may be impeded by their high genetic correlations, depending on if the correlations are caused by pleiotropy or linkage disequilibrium.

Hansen *et al.* (2003b) found generally low evolvabilities in floral traits in a neotropical vine *Dalechampia scandens* (Euphorbiaceae) despite being evidence of additive genetic variance for most of the traits measured. However, as their study examined these traits in a number of different populations they also found that there was less variation between populations in the traits with lower evolvabilities than in the traits with higher evolvabilities, i.e. traits with very low evolvabilities did not demonstrate variance between populations. The authors suggest that each population may experience different local environments and therefore the floral traits may be subject to different selection pressures.

We found a number of genetic correlations between the amino acids that were moderate to strong **(Table 4.3)** suggesting limited potential for amino acid constituents in eggs to evolve independently (Hansen *et al.*, 2003a). This is further supported by an examination of the PCA that we conducted on the **G** matrix. g_{max} explained only 50% of the variance, and while this does not represent an absolute constraint to evolving a different pattern of allocation, our results suggest that there are combinations of amino acids that are more accessible to evolutionary change than others. While we have evidence of some additive genetic variance **(Table 4.1)**, which is a prerequisite for trait evolution, individual traits that display genetic variance may not necessarily demonstrate genetic

variance in multivariate trait space (Blows, 2007; Walsh & Blows, 2009). So, while individual traits may still harbour additive genetic variation, multivariate genetic constraints may still arise due to genetic associations between individual traits (McGuigan & Blows, 2010) as evidenced by the $\mathbf{g}_{max}$ for the traits we have measured. Correlations between traits can impede evolution as fewer traits will be able to respond to selection due to pairwise genetic correlations that limit evolution to specific trait combinations (Kirkpatrick, 2009). In a previous experiment (Chapter 3) we showed maternal diet influenced amino acid profiles of eggs in *O. fasciatus* and that there was evidence for multivariate selection acting on specific amino acid combinations. Hatchling mass from the various treatments attained different optima, suggesting population-specific responses in attaining a maximum mass at hatching. We could speculate that the results of our current experiment suggest that constraints in amino acids of eggs of *O. fasciatus* may act to impede the attainment of a performance optima. However, this would require further empirical studies as **G** can vary within and between populations at different times and can also be affected by mutation, selection, drift and migration (Steppan *et al.*, 2002; Jones *et al.*, 2003).

Furthermore, we did not take into account paternal effects. For example, males may transfer more than just genes to the female when mating (Vahed, 1998; Gillott, 2003). Male-derived nutrients transferred to the female during mating may influence female fecundity and reproductive effort (Wedell & Karlsson, 2003). Mating may also have costs for males as multiple matings in bush crickets has been shown to decrease spermatophore quality which can have an effect on female fecundity and offspring fitness (Wedell & Ritchie, 2004). As males were sequentially mated with females (see Methods), age effects may need to be taken into account as male age can also affect sperm quality by decreasing the volume of ejaculate and sperm motility and morphology with effects on fertility rates (Kidd *et al.*, 2001; Wedell & Ritchie, 2004).

Eggs are constituted by the maternal phenotype and genotype and provide crucial environmental conditions for prenatal development of her offspring (White III, 1995; Bernardo, 1996b; Dzialowski & Sotherland, 2004). Our results suggest that amino acids are one such constituent in eggs that may demonstrate some evolutionary constraints. This may, potentially, have repercussions on adaptive evolution. By investigating the genetics behind the maternal allocation of dietary compounds and their evolutionary potential we can gain deeper insights into the evolution of maternal effects and

the mechanisms that influence offspring development and the evolution of life histories (Mousseau & Dingle, 1991).

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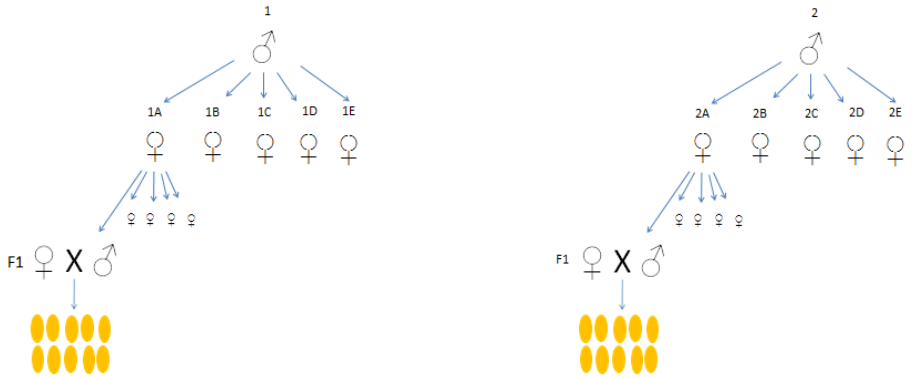

Figure 4.1 Diagram of quantitative genetic design. One sire mated up to 5 females each. Each male was paired with a single female for 48 hours and was allowed to rest for 24 hours between mating each dam. Ten nymphs from each female were raised until adult ecdysis upon which up to 5 F_1 female offspring from each female were selected to randomly mate with a non-related male. Ten eggs were collected from each F_1 female for amino acid analysis.

Table 4.1 Descriptive statistics, heritabilities and evolvabilities. Genetic estimated based on 30 sires each mated to 3-4 dams (mean = 3.9), and 417 total offspring. Genetic estimates obtained by REML**.**

Amino Acid	Mean (ug)	$\mathbf{V_P}$	$\mathbf{V_A}$	**SE (V_A)**	$\mathbf{h^2}$	**SE(h^2)**	I_A
ALA	34.782163	120.79741	13.299151	6.4068221	**0.44**	0.21	0.04
GLY	28.179471	43.419995	1.8327517	1.4780314	**0.17**	0.14	0.01
VAL	9.6009856	9.9153475	0.398397	0.3699399	**0.16**	0.15	0.02
LEU	8.3792788	6.3315786	0.4770477	0.2696172	**0.30**	0.17	0.03
ILE	7.062524	3.7983959	0.1774764	0.1263677	**0.19**	0.13	0.01
THR	9.2310377	11.39567	0.1914409	0.32671	0.07	0.11	0.01
SER	22.896995	81.28965	9.3803895	4.3443395	**0.46**	0.21	0.07
PRO	200.10305	2454.2593	207.14198	97.910804	**0.34**	0.16	0.02
ASN	9.6111538	8.5968058	0.2977855	0.2952532	**0.14**	0.14	0.01
MET	4.6655971	3.0441571	0.0637711	0.0883931	0.08	0.12	0.01
4-HYP	9.1579327	4.4095096	0.162581	0.1369265	**0.15**	0.12	0.01
PHE	2.9027404	2.1243048	0.0138712	0.0476539	0.03	0.09	0.01
AAA	4.9344952	6.8023703	0.3211786	0.1894832	**0.19**	0.11	0.05
GLN	257.78529	4642.1158	271.2233	187.80627	**0.23**	0.16	0.02
LYS	18.839183	16.493643	0.6142731	0.479185	**0.15**	0.12	0.01
HIS	8.2932452	4.0315468	0.1730437	0.1298797	**0.17**	0.13	0.01
TYR	5.4496394	1.8755817	0.0831267	0.0641074	**0.18**	0.14	0.01
TRP	1.6096394	0.3406388	0.0163616	0.0122098	**0.19**	0.14	0.03
CTH	2.8486298	3.8648068	0.3865472	0.1789022	**0.40**	0.19	0.19

Table 4.2 Phenotypic correlations (below the diagonal) and significance (above the diagonal) for all traits.

	ALA	GLY	VAL	LEU	ILE	THR	SER	PRO	ASN	MET	4-HYP	PHE	AAA	GLN	LYS	HIS	TYR	TRP	CTH
ALA		<.0001	<.0001	<.0001	<.0001	<.0001	0.0527	<.0001	<.0001	<.0001	<.0001	0.2678	0.19	<.0001	<.0001	<.0001	<.0001	<.0001	0.024
GLY	**0.41**		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0003	<.0001	<.0001	0.2251	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
VAL	**0.27**	**0.59**		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.402	<.0001	0.0056	0.6639	<.0001	<.0001	<.0001	<.0001	<.0001	0.0037
LEU	**0.29**	**0.57**	**0.74**		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0002	0.2834	<.0001	<.0001	<.0001	<.0001	<.0001	0.0159
ILE	**0.28**	**0.61**	**0.78**	**0.84**		<.0001	<.0001	<.0001	<.0001	0.0606	<.0001	0.0009	0.164	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
THR	**0.32**	**0.45**	**0.49**	**0.50**	**0.60**		<.0001	<.0001	<.0001	0.2322	<.0001	0.0074	0.5676	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
SER	0.10	**0.33**	**0.37**	**0.48**	**0.54**	**0.32**		0.0001	<.0001	0.4386	<.0001	0.0256	0.006	0.0035	<.0001	<.0001	<.0001	<.0001	0.0061
PRO	**0.34**	**0.44**	**0.32**	**0.36**	**0.38**	**0.32**	**0.19**		<.0001	<.0001	<.0001	0.0054	0.1118	<.0001	<.0001	<.0001	<.0001	<.0001	0.0016
ASN	**0.29**	**0.61**	**0.61**	**0.66**	**0.74**	**0.58**	**0.48**	**0.24**		0.1774	<.0001	<.0001	0.6758	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
MET	**0.50**	**0.18**	0.04	**0.19**	0.09	0.06	0.04	**0.40**	0.07		<.0001	0.029	0.0131	<.0001	0.0006	<.0001	<.0001	<.0001	0.0112
4-HYP	**0.34**	**0.62**	**0.35**	**0.48**	**0.51**	**0.45**	**0.45**	**0.53**	**0.44**	**0.28**		<.0001	0.2817	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
PHE	0.05	**0.25**	0.14	**0.18**	**0.16**	**0.13**	0.11	**0.14**	**0.21**	**0.11**	**0.21**		0.0123	<.0001	<.0001	<.0001	0.0004	0.3741	0.0928
AAA	0.06	-0.06	-0.02	0.05	0.07	0.03	**0.13**	0.08	**0.02**	**0.12**	**0.05**	**-0.12**		0.073	0.0113	0.0002	0.1925	<.0001	0.2582
GLN	**0.46**	**0.79**	**0.44**	**0.44**	**0.45**	**0.35**	0.14	**0.42**	**0.55**	**0.19**	**0.49**	**0.21**	-0.09		<.0001	<.0001	<.0001	<.0001	<.0001
LYS	**0.31**	**0.58**	**0.55**	**0.67**	**0.75**	**0.53**	0.57	**0.46**	**0.64**	**0.17**	**0.64**	**0.20**	**0.12**	**0.48**		<.0001	<.0001	<.0001	<.0001
HIS	**0.26**	**0.66**	**0.45**	**0.48**	**0.50**	**0.38**	0.40	**0.41**	**0.47**	**0.20**	**0.61**	**0.29**	**-0.18**	**0.56**	**0.54**		<.0001	<.0001	<.0001
TYR	**0.29**	**0.65**	**0.55**	**0.56**	**0.59**	**0.30**	0.46	**0.43**	**0.49**	**0.23**	**0.55**	**0.17**	0.06	**0.52**	**0.58**	**0.59**		<.0001	0.0004
TRP	**0.29**	**0.52**	**0.47**	**0.54**	**0.66**	**0.41**	**0.38**	**0.35**	**0.55**	**0.20**	**0.57**	0.04	**0.19**	**0.41**	**0.58**	**0.38**	**0.57**		0.0037
CTH	**0.11**	**0.26**	**0.14**	**0.12**	**0.24**	**0.26**	**0.13**	**0.15**	**0.26**	**-0.12**	**0.23**	0.08	0.06	**0.23**	**0.25**	**0.25**	**0.17**	**0.14**	

Table 4.3 Genetic correlations below the diagonal; SE of the genetic correlations above the diagonal. All correlations in bold are > 1 SE. Genetic correlations were not calculated for THR, MET or PHE due to lack of significant heritability in these traits.

	ALA	GLY	VAL	LEU	ILE	SER	PRO	ASN	4-HYP	AAA	GLN	LYS	HIS	TYR	TRP	CTH
ALA		0.54	0.54	0.36	0.48	0.39	0.41	0.51	0.53	0.50	0.48	0.54	0.62	0.48	0.48	0.28
GLY	-0.05		0.46	0.55	0.56	0.44	0.54	0.22	0.57	0.68	0.54	0.69	0.74	0.02	0.64	0.42
VAL	0.09	**0.60**		0.23	0.05	0.44	0.45	0.32	0.71	0.68	0.64	0.05	0.14	0.56	0.46	0.51
LEU	**-0.41**	0.19	**0.78**		0.33	0.28	0.38	0.38	0.53	0.43	0.47	0.35	0.61	0.32	0.52	0.37
ILE	-0.11	0.37	**1.04**	**0.60**		0.27	0.48	0.24	0.64	0.62	0.57	0.28	0.20	0.25	0.35	0.47
SER	-0.07	0.36	0.39	**0.56**	**0.64**		0.39	0.33	0.50	0.38	0.46	0.41	0.18	0.32	0.44	0.38
PRO	-0.05	0.13	-0.42	-0.36	-0.16	-0.12		0.51	0.51	0.43	0.49	0.54	0.63	0.41	0.46	0.34
ASN	-0.24	**0.83**	**0.75**	**0.57**	**0.79**	**0.60**	-0.24		0.62	0.28	0.37	0.33	0.40	0.19	0.63	0.51
4-HYP	-0.13	-0.45	-0.11	-0.25	-0.04	-0.11	0.23	-0.34		0.33	0.54	0.68	0.82	0.59	0.57	0.51
AAA	0.22	0.07	0.12	**0.47**	0.08	**-0.48**	0.42	**0.77**	**0.72**		0.09	0.12	0.78	0.62	0.58	0.31
GLN	-0.09	0.38	-0.03	-0.27	0.05	0.02	-0.05	**0.65**	-0.37	**0.92**		0.63	0.73	0.36	0.58	0.36
LYS	0.04	0.16	**1.04**	**0.62**	**0.75**	**0.45**	-0.02	**0.73**	-0.20	**0.91**	-0.07		0.38	0.04	0.49	0.50
HIS	0.08	0.31	**0.91**	0.26	**0.85**	**0.84**	-0.07	**0.71**	0.06	-0.16	-0.04	**0.74**		0.41	0.64	0.50
TYR	0.24	**0.99**	**1.35**	**0.63**	**1.19**	**0.58**	**0.46**	**0.85**	0.32	-0.20	**0.63**	**0.97**	**1.24**		0.50	0.49
TRP	-0.14	-0.09	**0.55**	0.03	**0.64**	0.25	0.28	0.15	0.33	0.29	-0.04	**0.49**	0.39	0.43		0.47
CTH	**-0.55**	**-0.43**	-0.16	-0.35	-0.06	-0.01	**0.38**	0.12	-0.14	**0.62**	**0.47**	-0.19	-0.42	0.02	-0.13	

Table 4.4 Additive genetic variance-covariance matrix (**G**) of amount of free amino acids allocated to eggs.

	ALA	GLY	VAL	LEU	ILE	SER	PRO	ASN	4-HYP	AAA	GLN	LYS	HIS	TYR	TRP	CTH
ALA	1122.95797	-19.609693	179.792243	-963.29001	-154.519	-76.350836	-22.64824	-439.31706	-166.49026	372.104389	-43.760174	10.6420432	76.5768899	228.551521	-60.88171	-1187.0614
GLY	-19.609693	148.1509881	413.635952	162.421681	190.657215	135.116421	23.8678962	553.387483	-212.77853	40.9150414	70.494835	15.1334043	111.087631	338.420967	-13.28561	-338.71732
VAL	179.792243	413.635952	3246.66359	3103.05748	2537.85882	687.71682	-357.71345	2333.21122	-240.89593	362.472072	-29.222726	446.667287	1518.20492	2168.40597	393.309665	-581.84944
LEU	-963.29001	162.421681	3103.05748	4923.32008	1792.35516	1200.15911	-377.36435	2178.02556	-676.29059	1679.24456	-289.30879	329.785453	530.270258	1242.59166	23.9988387	-1567.129
ILE	-154.519	190.657215	2537.85882	1792.35516	1833.40434	836.229915	-100.48006	1857.85639	-66.221221	183.027505	29.652539	241.493401	1066.41658	1432.94021	344.819592	-165.09708
SER	-76.350836	135.116421	687.71682	1200.15911	836.229915	935.134404	-54.609017	1002.70226	-137.45474	-752.62605	11.3469181	104.401176	748.103068	497.094072	95.3080631	-10.156215
PRO	-22.64824	23.8678962	-357.71345	-377.36435	-100.48006	-54.609017	224.325733	-194.84115	137.76972	320.693178	-12.004737	-2.6439525	-29.197214	192.274908	53.1305999	368.614058
ASN	-439.31706	553.387483	2333.21122	2178.02556	1857.85639	1002.70226	-194.84115	3005.02498	-722.4153	2151.65112	544.32476	301.973618	1144.64225	1304.52811	102.83064	430.487417
4-HYP	-166.49026	-212.77853	-240.89593	-676.29059	-66.221221	-137.45474	137.76972	-722.4153	1538.12173	1438.8533	-222.51044	-58.461933	71.7292324	355.980243	163.900096	-341.8839
AAA	372.104389	40.9150414	362.472072	1679.24456	183.027505	-752.62605	320.693178	2151.65112	1438.8533	2602.38132	721.556987	349.24249	-238.30179	-181.50043	182.852962	2032.96238
GLN	-43.760174	70.494835	-29.222726	-289.30879	29.652539	11.3469181	-12.004737	544.32476	-222.51044	721.556987	234.988268	-8.1470836	-19.684009	272.419667	-7.6077609	460.502551
LYS	10.6420432	15.1334043	446.667287	329.785453	241.493401	104.401176	-2.6439525	301.973618	-58.461933	349.24249	-8.1470836	57.1067173	162.624406	206.786077	46.2911493	-94.71444
HIS	76.5768899	111.087631	1518.20492	530.270258	1066.41658	748.103068	-29.197214	1144.64225	71.7292324	-238.30179	-19.684009	162.624406	854.347779	1018.82143	143.823139	-786.33816
TYR	228.551521	338.420967	2168.40597	1242.59166	1432.94021	497.094072	192.274908	1304.52811	355.980243	-181.50043	272.419667	206.786077	1018.82143	792.716189	150.536182	44.9634607
TRP	-60.88171	-13.28561	393.309665	23.9988387	344.819592	95.3080631	53.1305999	102.83064	163.900096	182.852962	-7.6077609	46.2911493	143.823139	150.536182	157.325237	-107.87482
CTH	-1187.0614	-338.71732	-581.84944	-1567.129	-165.09708	-10.156215	368.614058	430.487417	-341.8839	2032.96238	460.502551	-94.71444	-786.33816	44.9634607	-107.87482	4147.85569

Table 4.5 Eigenvectors of the **G** matrix for amino acid allocation in the eggs of *O. fasciatus*. Loadings greater than 0.3 were interpreted to be biologically significant (in bold). g_{max}, indicates the genetic line of least resistance to evolutionary change and explains 50% of the variance in amino acid composition.

	g_{max}	g2	g3	g4
ALA	-0.03251	-0.2576	**0.50482**	**0.32015**
GLY	0.04237	-0.11719	-0.12719	**0.78607**
VAL	**0.34427**	0.05761	0.01846	-0.00105
LEU	**0.30791**	0.14182	-0.00135	-0.03934
ILE	**0.34482**	0.0844	0.02018	-0.02363
SER	0.29433	-0.08945	-0.29606	-0.10375
PRO	-0.29885	0.16539	0.15671	0.04192
ASN	0.27373	**0.35044**	0.00207	0.17686
4-HYP	-0.15191	0.07083	**0.55473**	-0.17866
AAA	-0.05311	**0.48191**	-0.06497	-0.16017
GLN	-0.07847	**0.48757**	0.07927	**0.37563**
LYS	0.29331	0.23199	0.21996	0.0776
HIS	**0.32176**	-0.09703	0.14866	0.06096
TYR	**0.33118**	-0.01407	-0.00831	-0.07264
TRP	0.21791	0.03854	**0.46696**	-0.1413
CTH	-0.1878	**0.43737**	-0.09583	0.00701
Eigenvalue	7.996	2.7819	1.9206	1.1378
% Variance Explained	50	17.4	12	7.1
Cumulative	50	67.4	79.4	86.5

Chapter 5: Maternal, not paternal, transmission of cardenolides into eggs of the large milkweed bug *Oncopeltus fasciatus* (Dallas)

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5.1 Abstract

Many herbivorous insects sequester defensive compounds from their host-plants and incorporate them into their eggs to protect them against predation. Here we investigate if transmission of cardenolides from the host-diet to the eggs is maternal, paternal or biparental in the large milkweed bug *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae). We reared individual bugs on either milkweed seeds (MW) *Asclepias syriaca* (contain cardenolides) or sunflower seeds (SF) *Helianthus annus* (do not contain cardenolides). We mated females and males so that all four maternal/paternal diet combinations were represented: MW/MW, MW/SF, SF/MW, SF/SF. Using larvae of the common green lacewing *Chrysoperla (=Chrysopa) carnea* (Stevens) (Neuroptera: Chrysopidae), we conducted two-choice predation trials to assess if maternal, paternal or biparental transmission of cardenolides into the eggs of *O. fasciatus* increased protection against predation by *C. carnea*. Furthermore, we used High Performance Liquid Chromatography (HPLC) to assess putative cardenolide content of eggs from our different parental diet treatment groups.

We did not find any significant effect of parental diet treatment in protecting the eggs against *C. carnea*. However, not all eggs were consumed from parental diet treatments that contained cardenolides and our test clutch sizes were small. We therefore discuss the possibility that while chemical defence of eggs does not guarantee protection to eggs on an individual basis, for large clutches they may increase the probability that some eggs in a clutch are left intact thereby potentially conferring a fitness advantage to more offspring than if eggs are left unprotected. Our predation trials also suggest that many eggs containing a maternal contribution of cardenolides are distasteful, as while many were pierced and sampled by *C. carnea* they were not fully consumed. This is supported by our HPLC analysis that suggests that maternal contribution of cardenolides into eggs is significantly greater than paternal contribution.

***Key words*:** Cardenolides, defensive compounds, Hemiptera, High Performance Liquid Chromatography (HPLC), maternal effects

5.2 Introduction

Insect eggs are potentially highly vulnerable as they are immobile and often laid on resources that attract potential predators and parasites (Blum & Hilker, 2002). Predation pressures may have selective influences on egg characteristics to enhance protection (Orians & Janzen, 1974; Hilker *et al.*, 2002). Many insects are known to bestow chemical protection into their eggs as a defence against predators (Dussourd *et al.*, 1988; Eisner *et al.*, 1996; González *et al.*, 1999a; Blum & Hilker, 2002; Camarano *et al.*, 2009). While defensive compounds can be ingested and sequestered via the parental host-diet, they may also be synthesised de novo or by endosymbionts (Blum & Hilker, 2002; Opitz & Müller, 2009). They can be applied as barriers onto the egg surface such as in the form of hairs, secretions and faeces, or incorporated into the egg contents or egg shell (Blum & Hilker, 2002).

As there are many different types of toxic plant metabolites (allelochemicals), insect herbivores are known to have developed a diverse array of physiological mechanisms to be able to overcome specific compounds in order to be able to ingest and sequester them for defensive purposes (Després *et al.*, 2007; Opitz & Müller, 2009; Agrawal *et al.*, 2012). Furthermore, distinct groups of these allelochemicals may be important determinants in insect host-plant interactions (Dobler *et al.*, 2011) as many members of the same insect orders can be found feeding on host-plants with similar classes of compounds (Opitz & Müller, 2009). Allelochemicals can include aromatic compounds (e.g. flavonoids, phenolic acids and tannins), sulphur-containing metabolites, nitrogen-containing metabolites (e.g. pyrrolizidine alkaloids, cyanogenic glycosides and glucosinolates) and isoprenoids (e.g. cardiac glycosides and iridoid glycosides) (Després *et al.*, 2007; Opitz & Müller, 2009).

Cardenolides are a class of the cardiac glycosides that are derived from the metabolism of terpenoid plant steroids (Dobler *et al.*, 2011; Agrawal *et al.*, 2012). More than 500 cardenolides have been characterised which are distributed across 12 botanical families (Agrawal *et al.*, 2012). The type and concentrations of cardenolides can vary in geographical space and time, and may also vary between different plant parts (Duffey & Scudder, 1972; Isman *et al.*, 1977; Nelson *et al.*, 1981; Brower *et al.*, 1982; Schoonhoven *et al.*, 2005; Agrawal *et al.*, 2012). Cardenolides are highly represented in the Apocynaceae. This family of flowering plants contains over 400 genera, of which cardenolides are found in more than 30 of these (Agrawal *et al.*, 2012).

Several insect species from different orders including Lepidoptera, Coleoptera, Diptera, Hemiptera, and Orthoptera, are known to successfully feed on plants containing cardenolides (Betz *et al.*, 1997; Agrawal, 2005; Opitz & Müller, 2009; Agrawal *et al.*, 2012). However, there is much variation in the methods, and to the extent, that they are able to sequester, store, metabolise and secrete different cardenolides. Various morphological and physiological mechanisms allow them to become insensitive to cardenolides and/or to sequester and use these toxins as a defence against potential vertebrate and invertebrate predators that are not able to tolerate the ingestion of cardenolides (Opitz & Müller, 2009; Dobler *et al.*, 2011; Petschenka *et al.*, 2011). Species often have aposematic colouration to advertise their unpalatability and toxicity (Pasteels & Grégoire, 1983; Opitz & Müller, 2009; Rafter *et al.*, 2013) as cardenolides are bitter tasting and are known to have repellent, emetic and cardio-toxic effects on non-adapted vertebrate and invertebrate predators (Rothschild & Kellett, 1972; Isman *et al.*, 1977; Berenbaum & Miliczky, 1984; Hilker *et al.*, 1992). For example, it has been shown that Chinese mantids *Tenodera sinensis* (Mantodea: Mantidae) regurgitate after being fed aposematic milkweed bugs *Oncopeltus fasciatus* (Hemiptera: Lygaeidae) which have been raised on milkweed seeds *Asclepias syriaca* (Apocynaceae), but that they may learn to adapt their behaviour to reject them as unpalatable prey items (Berenbaum & Miliczky, 1984; Paradise & Stamp, 1991). Malcolm (1989) found altered web construction behaviour in an orb-weaving spider *Zygiella x-notata* (Clerk) (Araneae: Araneidae) when fed the aposemtic oleander aphid *Aphis nerii* Boyer de Fonscolombe (Hemiptera: Aphididae) which sequesters cardenolides after feeding on *Asclepias curassavica* L. (Apocynaceae). Malcolm (1989) also found that toxic aphids altered predatory behaviour of *Z. x-notata* towards non-toxic aphids.

The response of predators that have ingested prey that contain cardenolides is often dependent on the type of cardenolides that are present and the body part(s) that have been sampled. For example, emesis can be produced in blue jays *Cyanocitta cristata bromia* (Oberholser) (Passeriformes: Corvidae) when fed caterpillars of the monarch butterfly *Danaus plexippus* L. (Lepidoptera: Nymphalidae) that have been fed *A. curassavica*, but emesis is not seen when fed caterpillars that have been fed *A. syriaca* (Rothschild *et al.*, 1970). Eggs from fireflies of the genus *Photinus* (Coleoptera: Lampyridae) have been shown to contain sequestered steroidal pyrones (lucibufagins) and autogenously produced defensive betaine (González *et al.*, 1999a). While lucibufagin was demonstrated to deter an ant *Leptothorax longispinosus* (Hymenoptera: Formicidae), a ladybird larva *Harmonia axyridis* (Coleoptera: Coccinellidae) and an earwig *Forficula auricularia*

(Dermaptera: Forficulidae), betaine only produced a deterrent response in the ant and the ladybird larva (González *et al.*, 1999a). Various gutting or dismembering behaviour has also been reported for vertebrate and invertebrate predators when fed toxic larvae or adults of *D. plexippus* (Rafter *et al.*, 2013). This suggests that different predators have varying degrees of preference for, or avoidance of, body parts that contain different types, and/or concentrations of, cardenolides (Rafter *et al.*, 2013).

Previous studies (Berenbaum & Miliczky, 1984; Sillén-Tullberg, 1985; Paradise & Stamp, 1991) have shown that nymphs and adults of the large milkweed bug *Oncopeltus fasciatus* (Hemipetra:Lygaeidae) are distasteful to predators when fed *A. syriaca* seeds. Although cardenolides have been reported to be present in the eggs of *O. fasciatus* (Duffey & Scudder, 1974; Blum & Hilker, 2002), it is not known if these compounds are uni- or bi-parentally transferred, or if cardenolides provide the eggs with protection against potential oophagous predators. While defensive chemicals are usually maternally incorporated into eggs (Pasteels & Grégoire, 1983; Blum & Hilker, 2002), there is some evidence that males also can have an important role in transferring chemicals (González *et al.*, 1999a; González *et al.*, 1999b; Eisner *et al.*, 2002; Camarano *et al.*, 2009; Opitz & Müller, 2009). Paternal transference of defensive compounds usually occurs during courtship or mating where they can be passed to the female via nuptial gifts or within male ejaculate and then incorporated into the eggs (Dussourd *et al.*, 1989; Eisner *et al.*, 2002; Camarano *et al.*, 2009; Opitz & Müller, 2009). It has further been shown that these toxins confer protection on the eggs against predation and parasitism (Dussourd *et al.*, 1988; Eisner *et al.*, 2002; Bezzerides *et al.*, 2004). We were therefore interested in investigating parental allocation of toxic compounds into the eggs of *O. fasciatus* and the effectiveness of the transmission in protecting eggs.

Oncopeltus fasciatus is widespread across North America and also found in parts of central and northern South America (Feir, 1974). In the wild, *O. fasciatus* feed and reproduce mainly on milkweed plants from the genus *Asclepias* (Apocynaceae), which contain cardenolides (Feir, 1974; Ralph, 1976; Dobler *et al.*, 2011; Agrawal *et al.*, 2012). Sequestration of cardenolides by *O. fasciatus* has been well documented (Feir & Suen, 1971; Scudder & Duffey, 1972; Duffey *et al.*, 1978; Vaughan, 1979; Scudder & Meredith, 1982; Detzel & Wink, 1995), and *O. fasciatus* have several morphological and physiological adaptations to sequester, store and release cardenolides

(Scudder *et al.*, 1986) that are subsequently used as a defence strategy against predators (Duffey *et al.*, 1978; Vaughan, 1979; Scudder *et al.*, 1986). Although milkweed is the preferred and natural host, in the laboratory *O. fasciatus* can be reared on a variety of food sources including sunflower, cashew and pumpkin seeds and peanuts (Beck *et al.*, 1958; Gordon & Gordon, 1971; Feir, 1974; Scudder *et al.*, 1986). Although initial performance on these alternative hosts is poor, continued exclusive rearing selects for improved performance (Gordon & Gordon, 1971; Feir, 1974).

In order to test for maternal and/or paternal transmission of cardenolides into the eggs of *O. fasciatus*, we manipulated parental diet so that females and males were raised on either milkweed seeds *Asclepias syriaca* (contain cardenolides) or sunflower seeds *Helianthus annus* (lacking cardenolides). We used sunflower seeds as these are commonly used as a food source to rear *O. fasciatus* in the laboratory (Gordon & Gordon, 1971; Feir, 1974) and they do not contain cardenolides (Berenbaum & Miliczky, 1984; Petschenka *et al.*, 2011). We conducted mating experiments so that eggs could be collected from 4 different parental diet treatments: 1: both females and males raised on milkweed seeds (MW/MW); 2: females raised on milkweed seeds and males raised on sunflower seeds (MW/SF); 3: females raised on sunflower seeds and males raised on milkweed seeds (SF/MW); and 4: both females and males raised on sunflower seeds (SF/SF). We performed two-choice bioassay tests with clusters of eggs from the first 3 treatments each against a cluster of eggs from the fourth treatment using common green lacewing larvae *Chrysoperla (= Chrysopa) carnea* (Stevens) (Neuroptera: Chrysopidae) as a test predator on the eggs . We also quantified the concentration of cardiac glycosides of the eggs in the different treatments using High-Performance Liquid Chromatography (HPLC).

5.3 Materials and methods

5.3.1 Experimental populations and rearing

We reared and tested eggs from two populations of *O. fasciatus* that differed in their host food source. One population (KY) was collected from the wild at the University of Kentucky Arboretum, Lexington, KY, USA, in September 2009. We maintained this population on a diet of dried milkweed seeds of *A. syriaca* (Educational Science, League City, TX, USA). The other population (LAB) is a long-standing laboratory-reared population obtained from Carolina Biological Supply House (Burlington, NC, USA). This population has been reared for over 400 generations

exclusively on sunflower seeds. We obtained organic sunflower seeds from Goodness Direct (goodnessdirect.co.uk). We reared these two populations separately, as our main stock populations, in mass colonies in multiple boxes (38 cm X 28 cm X 16 cm) all at 25°C with a light:dark regime of 16:8 hours. Every week fresh food from their allocated diet (i.e. KY bugs on milkweed seeds and LAB bugs on sunflower seeds) and demineralised water were supplied to all colonies. Absorbent cotton wool was provided for oviposition sites in all boxes. From these colonies, experimental colonies were set up for the current study (see below).

Eggs were collected from the stock populations (see above) and used to start 4 experimental colonies for the current study. Four colonies were maintained for this experiment: Colony 1: KY bugs maintained on milkweed seeds (KYMW), Colony 2: KY bugs maintained on sunflower seeds (KYSF), Colony 3: LAB bugs maintained on milkweed seeds (LABMW), Colony 4: LAB bugs maintained on sunflower seeds (LABSF). These experimental colonies were reared in separate transparent boxes (28 cm X 16 cm X 9 cm). Fresh food from the allocated diet (milkweed or sunflower) and water were provided, *ad libitum*, and changed as necessary. Experimental colonies were kept in an incubator L:D 16:8 at 25° C. All boxes were routinely moved around the incubator to account for incubator effects.

5.3.2 Parental diet treatment groups

Newly eclosed adults were collected daily from each of the above four experimental colonies. Adults from both populations were kept separately. Males and females were kept separate according to treatment and day of eclosion. All adults were housed in either transparent 90 mm diameter triple vent Petri dishes (for a maximum of two adults) or small transparent boxes (11 cm X 11 cm X 3 cm) for a maximum of 5 adults. Adults were provided with their allocated diet (sunflower or milkweed seeds) and a cotton wick moistened with demineralised water *ad libitum*. Matings were conducted so that eggs could be collected from four different parental diet combinations: females fed milkweed mated to males fed milkweed (MW/MW), females fed milkweed mated to males fed sunflower (MW/SF), females fed sunflower mated to males fed milkweed (SF/MW) and females fed sunflower mated to males fed sunflower (SF/SF).

To ensure sexual maturation, adults were mated when females were at least 7 days old but no older than 10 days and males were not mated until they were at least 5 days old (Gordon & Loher, 1968).

Only single-pair matings were conducted (i.e. females were only ever mated with one male, and males were disregarded after mating with one female). Matings were conducted in transparent 90 mm diameter Petri dishes. Pairs were fed the allocated female diet and were given moist cotton wicks as a water source and cotton wool for the female to oviposit her eggs. To encourage egg production in the female and to ensure sufficient fertilisation of eggs (Gordon & Loher, 1968), pairs were kept together for 72 hours, within which time pairs can engage in several copulations. Pairs were recorded as mated when observed to be in copulation. In some instances, pairs may be actively engaged in copulation at the 72 hour check point. In this case they were separated when they had ceased copulating provided they had not been together for more than 96 hours. However, when males reared on sunflower were paired with females reared on milkweed, pairs were discarded if they mated for more than 72 hours to minimise the probability of sequestering toxins from the maternal diet into their ejaculate.

Eggs were collected on the day the choice test was conducted. Only eggs that were within 4 days of development (yellow to orange in colour) were used. As well as 5 eggs being collected from each female for the bioassay trials, an extra 10 eggs were collected from each female and stored at -80°C for later cardenolide analysis (see below). However, eggs stored for cardenolide analysis were only used if corresponding eggs from the same female were successful at the bioassay trial (see below).

5.3.3 Bioassay: predation of eggs by green lacewing larvae Chrysoperla carnea

The larvae of green lacewings *C. carnea* are known to be voracious predators of aphids and other insect pests and their eggs; for example, they are widely used as biocontrol agents and commercially available (Chang *et al.*, 2000; Abd El-Gawad *et al.*, 2010). A previous study by Eisner *et al.* (2000) used lacewing larvae to test for parental transmission and the protective capability of pyrrolizidine alkaloids in moth eggs.

We used green lacewing larvae supplied from Agralan Ltd., Wiltshire, UK, for our experiment. Larvae were housed individually in small Petri dishes (5cm diameter) to prevent cannibalism. Larvae were fed irradiated mealworm eggs (supplied by Agralan Ltd., Wiltshire, UK) until used for predation tests. Larvae used for the predation experiments were at least at their third instar so they were large enough to be able to pierce the egg shells.

Lacewing larvae were starved for 48 hours prior to experiment (Eisner *et al.*, 2000). Each trial was a two-choice test which consisted of a cluster of 5 eggs from one of the different parental diet treatments: 1: MW/MW, 2: MW/SF or 3: SF/MW tested against a cluster of 5 eggs from parental diet treatment 4: SF/SF treatment. A small (5 cm diameter) transparent Petri dish was used as an arena for each trial. Egg clusters were placed at opposite sides of the Petri dish. A single lacewing larva was placed in the centre of each Petri dish. Several tests were conducted consecutively, depending on the number of eggs and lacewing larvae available. The behaviour of each lacewing larva was observed throughout the duration of the tests. The maximum time allocated for each test was 4 hours. After 4 hours, the test was stopped and eggs were scored according to how they were treated by the lacewing larvae (see below). At the end of the predation tests, to ensure larvae had not stopped consuming eggs due to satiation and were still sufficiently motivated to eat (Eisner *et al.*, 2000) each larva was given a further 5 eggs from the SF/SF treatment. Data from the tests were only included if the larva consumed at least 3 of these eggs (Eisner *et al.*, 2000). A dissecting microscope was used for monitoring events and to check the fate of eggs. Each larva was used only once.

The fate of each egg was scored using three categories (following Dussourd *et al.* (1988)): 'Intact': egg shell and contents remain intact without any signs of being pierced; 'Partially eaten': eggs pierced by larval jaws and the larva has fed on the egg contents but egg contents still discernible; 'Completely eaten': eggs pierced by larval jaws and egg contents sucked out completely leaving only the empty transparent shell **(Plate 1.4.4)**.

5.3.4 Quantification of cardenolide content of eggs using High Performance Liquid Chromatography (HPLC)

For each female, eggs for the cardenolide analysis were collected on the day that the bioassay was performed. Ten eggs from each female were stored in 1.5ml Eppendorf tubes at -80°C until all eggs had been collected from all the conducted tests. Once all bioassay trials had been conducted, eggs were prepared for cardenolide extraction using the HPLC.

Firstly, 100 µl of hexane was added to each sample and the samples were ground manually with a hand-held plastic pestle for 30 seconds. Capped samples were placed in a water bath for 30 minutes at 35°C. Following this, samples were centrifuged in an Accuspin Micro^{TM} for 5 minutes at 16200

g. The hexane containing the fats was removed from the samples using a pipette and discarded. 1ml of ethanol containing 20µg digitoxin (Sigma-Aldrich, D5878) was added to the defatted pellet. Samples were sonicated for 60 minutes at 60°C and centrifuged for 5 minutes at 16200 g. The supernatant was removed and the samples transferred over to fresh 1.5 ml Eppendorf tubes. Samples were dried down in a Savant ISS 110 Speedivac^{TM} concentrator. The dried extract was dissolved in 100µl ethanol and transferred to a 0.3ml autosampler vial and stored at -80°C until used in the HPLC analysis.

Samples (50µl) were injected onto a Dionex HPLC system fitted with a Waters Spherisorb 5µm ODS2 column (4.6 x 150mm). A multistep gradient of acetonitrile and water was used as the mobile phase as follows: initial concentration 20% ACN held for 5 minutes, ramp to 70% ACN at 20 mins until 25 mins, ramp to 95% ACN at 30 mins until 35 mins, returning to 20% ACN at 40 mins, with an end time of 50 mins. The flow rate was 0.7ml/min. Cardenolide peaks were quantified relative to the area of the digitoxin peak at a known concentration of 20µg using a PDA detector at an absorbance value of 218nm (Malcolm & Zalucki, 1996). Spectral data between 200-400nm were collected, and cardenolides were identified as peaks with a symmetrical absorbance band with a maximum absorbance between 217nm – 222nm (Malcolm & Zalucki, 1996). Representative output of spectral data are presented in **Plate 5.1**.

5.3.5 Statistical analyses

Eggs from each predation test were scored according to fate, i.e. 'Intact', 'Partially' consumed or 'Completely' consumed, and tallied according to parental diet treatment. As embryos from eggs that have been pierced will not survive (Eisner *et al.*, 2000), we combined eggs that had been 'partially eaten' and 'completely eaten' and classed these as 'assaulted'. We then tested for the null hypothesis that there was no effect of parental diet treatment and the probability of an egg being left 'intact' or 'assaulted'. We used the lme4 package in R (http://cran.r-project.org/) to perform a generalized linear mixed model (GLMM) (population as the random factor) fit by the Laplace approximation of likelihoods.

Cardenolide content of eggs was calculated by dividing the total cardenolide content of samples from each female's pooled eggs by the number of eggs sampled to give "micrograms per egg" (µg/egg) values. This standardisation was necessary because there were 4 samples that had fewer

than 10 eggs. While eggs from 148 females were collected for cardenolide extraction, eggs from only 131 females could be used for cardenolide analysis due to incomplete data. Eggs from both the KY and LAB populations were pooled into each respective parental diet treatment group for simplicity of presentation, as there was no population effect. There was variation in the number of cardenolides found in each sample with one to six peaks per sample indicating the presence of different cardenolides. We could not identify the individual cardenolides, so samples with more than one cardenolide were pooled to obtain a total cardenolide content for each sample. As the data were not normally distributed, a Kruskall-Wallis test was used to test for differences between the cardenolide concentrations of eggs from the different parental diet treatments. Statistical analysis was conducted using IBM SPSS version 20 (licence supplied by the University of Exeter).

5.4 Results

5.4.1 Predation bioassays

A total of 199 predation tests were conducted of which 101 tests were successfully completed; i.e., lacewing larvae consumed eggs during the trial and control eggs were consumed post-test. As results for both populations were identical, we combined results for predation tests for eggs from the same parental diet treatments. Therefore, the number of predation tests using eggs where both parents were raised on milkweed (MW/MW) n = 34; females on milkweed and males on sunflower (MW/SF) n = 37; and females on sunflower and males on milkweed (SF/MW) n = 30. As eggs for the SF/SF treatment were used in each predation test there were n = 101 for this group. We found no significant effects of any of the parental diet treatments in protecting eggs from predation by *C. carnea* (Treatment 1 (MW/MW): z = -0.169, P = 0.866, Treatment 2 (MW/SF): z = -0.344, P = 0.731; Treatment 3 (SF/MW): z = -0.546 P = 0.585; Treatment 4 (SF/SF): z = -0.809; P = 0.419).

5.4.2 Cardenolide content of eggs

Cardenolide content of eggs differed significantly between parental diet treatment groups (Kruskall-Wallis, $p < 0.0001$, **Figure 5.1**). Figure 5.3 illustrates a box plot of egg cardenolide content for each parental diet treatment group. The treatments where both parents were fed milkweed (MW/MW) (n = 23) and where females were fed milkweed and males were fed sunflower (MW/SF) (n = 35) had higher egg cardenolide content than the parental diet treatments where females were fed sunflower

and males were fed milkweed (SF/MW) (n = 38) or where both parents were fed sunflower (SF/SF) (n = 35) **(Figure 5.1)**.

5.5 Discussion

Although cardenolides have previously been reported to be present in the eggs of *O. fasciatus* (Duffey & Scudder, 1974; Blum & Hilker, 2002), we investigated if there was maternal, paternal or biparental transmission of cardenolides into the eggs and if uni- or biparental transmission of cardenolides conferred protection against an oophagous predator, *C. carnea*. Our HPLC analysis found that maternal transmission of cardenolides into the eggs was far greater than paternal contribution, which was minimal **(Figure 5.1)**. However, we did not find any statistical significance for any of the parental diet treatments in protecting the eggs against predation from *C. carnea*. While maternal endowment was not a guarantee against predation, eggs from this treatment group contained more cardenolides than eggs from the paternal diet treatment (SF/MW) and the control group (SF/SF) **(Figure 5.1)**.

While our results did not demonstrate that cardenolides were a guarantee against predation for each egg on an individual basis, not all eggs that contained cardenolides were assaulted within a clutch. As we only used clutches with 5 eggs, for larger clutches it may still be possible that maternal contribution of cardenolides increases the probability of some eggs within the clutch being left untouched, thereby potentially conferring an overall fitness advantage (Blum & Hilker, 2002). Not all eggs from the cardenolide treatment groups were completely consumed suggesting some degree of unpalatability compared to control eggs. Bitter tasting eggs may deter oophagous predators from attempting to consume other eggs within a clutch (Stamp, 1980; Hare & Eisner, 1993; Eisner *et al.*, 2000; Blum & Hilker, 2002). Results of previous studies (Hare & Eisner, 1993; Eisner *et al.*, 2000) have shown that invertebrate predators may destroy a number of eggs while sampling a clutch but that endowment of chemical protection, such as alkaloids, into the eggs confers greater protection overall in comparison to clutches of eggs without chemical protection.

We did not measure the cardenolide content of individual bugs or their diets as we made some a priori assumptions. Firstly, as sunflower seeds are cardenolide free (Isman *et al.*, 1977; Vaughan, 1979; Moore & Scudder, 1985; Petschenka *et al.*, 2011), we did not expect to detect cardenolides in any of our control (SF/SF) egg samples. The trace readings of cardenolides in some of the eggs

from treatments where females were fed sunflower (SF/MW or SF/SF), were likely to be biologically irrelevant. It is possible that there was some carry-over of trace amounts of cardenolides from previous samples during analysis in the HPLC, but more likely the reading was something other than cardenolides. Absorption between 217-222 nm is not specific for cardenolides, but rather it is the lactone ring of the molecule that absorbs the UV light. Any lactone and other related compounds could have produced a weak signal. In the case of the SF/MW treatment (i.e. only males reared on milkweed), it could have been that males transferred small quantities of cardenolide via their ejaculate to the female, which was then incorporated into the eggs. This has been reported in other insect species, but mainly within Coleoptera and Lepidoptera (Pasteels & Daloze, 1977; González *et al.*, 1999a; Eisner *et al.*, 2002; Camarano *et al.*, 2009). However, if males did transfer cardenolides via their ejaculate, it was in such small quantities that it did not significantly influence feeding by *C. carnea* larvae.

De novo synthesis of cardenolides by males is also possible but unlikely. De novo synthesis of defensive compounds has been reported in a number of species within Coleoptera and Lepidoptera (Pasteels & Grégoire, 1983; Pasteels *et al.*, 1995; Blum & Hilker, 2002; Opitz & Müller, 2009). However, de novo synthesis of defensive compounds usually involves modification of alkaloids that are sequestered through the diet (Hilker *et al.*, 1992; Termonia *et al.*, 2002), which suggests this is an unlikely explanation for our results. Furthermore, we know of no reports of *O. fasciatus* being able to biosynthesize cardenolides de novo. If there is de-novo biosynthesis, it appears to contribute very little towards the cardenolide content within the eggs.

It has been reported that when reared on various seeds of *Asclepias*, the cardenolide content of adults reflects that of the seeds (Isman *et al.*, 1977; Scudder *et al.*, 1986). However, it has also been found that *O. fasciatus* can selectively sequester and concentrate cardenolides in response to the cardenolide content of their food source (Vaughan, 1979; Scudder *et al.*, 1986). Furthermore, female *O. fasciatus* are also reported to contain higher concentrations of cardenolides than males (Duffey & Scudder, 1974; Isman, 1977; but see Moore & Scudder, 1985). That females have higher amounts of toxins than males has been reported in species of Lepidoptera (Pasteels & Grégoire, 1983; Opitz & Müller, 2009). However, while Moore and Scudder (1985) found only minor differences between the cardenolide profiles of adult female and male *O. fasciatus*, they found that adult females had higher concentrations of cardenolides stored in the fat body relative to the fat

body of adult males. As fat bodies have a role in synthesising yolk proteins (Izumi *et al.*, 1994; Trougakos & Margaritis, 2002), this may then partly explain the role of the female fat body as a contributing factor towards the incorporation of sequestered defensive compounds into the ovaries and eggs (Opitz & Müller, 2009). All our bugs that were allocated to the milkweed diet were fed dried whole seeds of *A. syriaca*, *ad libitum*, with fresh seeds provided as necessary. As we were testing for uni- or biparental transfer of cardenolides into the eggs, we therefore made the assumption that the cardenolide content of adults would be reflected in the cardenolide content of eggs if both parents contributed equally to the transmission of cardenolides into the eggs.

Additionally, although we do not have data to test for intra-clutch variation of females, Eisner *et al.* (2000) suggest that low within-clutch variation of alkaloid content in eggs could be an adaptive strategy whereby potential predators may inspect and assess alkaloid-laden egg clusters without having to kill all the eggs. In any case, maternal contribution of alkaloids into her eggs may ultimately increase the probability of her offspring surviving to hatch over egg clutches that do not contain any defensive compounds (Eisner *et al.*, 2000).

While some insects cover their eggs with defensive secretions (Blum & Hilker, 2002), there was no evidence of this from our test results using eggs of *O. fasciatus*. We observed *C. carnea* larvae holding and attempting to pierce egg shells without any evidence of rejecting the eggs due to the egg shell being unpalatable. Potential rejection of the egg only occurred once the contents had been sampled. In a study by Eisner *et al.* (2000), the authors showed that egg shells of eggs from the moth *Utethesia ornatrix* (family Arctiidae) that were raised on an alkaloid diet were endowed with pyrrolizidine alkaloids (PAs). While these egg shells would be consumed by *U. ornatirx* larvae that were devoid of alkaloids (presumably to endow themselves for protection), this endowment did not necessarily prevent an oophagous predator green lacewing *Ceraeochrysa cubana* (family Chrysopidae) from piercing and sampling the eggs. The authors suggested that the PAs contained within the eggshell may be too low in concentration to deter *C. cubana* larvae or that the PAs may be in a non-active form. As *C. carnea* larvae use their mouth parts for piercing and sucking **(Plate 1.4.4)**, they may be less exposed to cardenolides contained within the egg shell as they pierce it, but are able to make an assessment once they have sampled the egg contents contained within the egg.

As plants can synthesise a diverse variety of hormones (phytohormones) and steroids (phytosteroids) (Janson *et al.*, 2009) that can be sequestered by phytophagous insects and used to synthesise compounds related to growth, development and defence purposes there may be other compounds (Hilker *et al.*, 2002; Tooker & De Moraes, 2007; Opitz & Müller, 2009 and references therein), some of which may not necessarily be toxic (Harborne, 2001), that can be incorporated into insect eggs and that may deter potential predators. For example, compounds that provide bright colouration to eggs, such as beta-carotene, may also serve as a warning signal to deter oophagous predators (Howard *et al.*, 1982; Blum & Hilker, 2002). Lipids, such as oleic acid, have been detected in the eggs of a chrysomelid beetle *Gastrophysa cyanea*, and, while not toxic, have been shown to deter predatory ants (Howard *et al.*, 1982; Blum & Hilker, 2002). Jasmonic acid (JA), benzoic acid (BA) and salicylic acid (SA) are phytohormones that are commonly found in the eggs in a number of insect species (Tooker & De Moraes, 2007). In their study, Tooker and De Moraes (2007) found that eggs of *O.fasciatus* reared on sunflower seeds contained substantial amounts of BA, small amounts of JA but were absent of SA. While BA and SA are known to have antimicrobial and antifungal properties the role of JA in insect eggs remains unclear (Tooker & De Moraes, 2005, 2007). However, the authors did not detect JA within the sunflower seeds and suggest the possibility that JA may be synthesised de novo. Despite BA being detected in their results, if BA was present in the eggs of our SF/MW or SF/SF treatments then it could be that either it does not act as a deterrent against *C. carnea* larvae in our predation trials or was not present at levels to act as a deterrent against *C. carnea* larvae.

Our results suggest that maternal endowment of cardenolides into eggs may make the eggs more distasteful to an oophagous predator than eggs that are not endowed with cardenolides. Similarly, Eisner *et al.* (2000) and Dussourd *et al.* (1988) demonstrated that eggs of *U. ornatrix* that were devoid of pyrrolizidine alkaloids were more vulnerable to predation than eggs that were endowed with these alkaloids. In contrast with our findings however, Dussourd *et al.* (1988) demonstrated that while maternal provision of alkaloids to the eggs is greater than paternal contribution, male contribution of alkaloids is significant in providing additional protection of the eggs against predation. Paternal contribution of defensive compounds into eggs has been increasingly recognised as an important factor towards not only chemical defence against predation in insect eggs but in some instances may also benefit the female (Dussourd *et al.*, 1988; Dussourd *et al.*, 1989; González *et al.*, 1999a; González *et al.*, 1999b; Camarano *et al.*, 2009).

In summary, the results of our study suggest that eggs from females fed a diet of milkweed seeds are endowed with cardenolides and that female *O. fasciatus* make a significant contribution of cardenolides into their eggs compared to the males which make little or no contribution **(Figure 5.1)**. Furthermore, eggs derived from adults on a cardenolide free diet were potentially more vulnerable to predation by an oophagous predator. While individual eggs may be at risk of being destroyed through the sampling process of the predator, the alkaloids within them may deter predators from persisting in feeding and destroying the whole clutch, thereby conferring a fitness advantage to the developing embryos in other eggs. However, we recognise that insects may sequester or biosynthesise an array of defensive compounds that can be applied onto or incorporated into eggs which can affect predator responses in different ways (Rothschild & Kellett, 1972; Tooker & De Moraes, 2007; Opitz & Müller, 2009; Petschenka *et al.*, 2011).

While chemical endowment of insect eggs with defensive compounds has been widely reported, and is recognised to confer protection from potential predators, the response of the predator to the eggs will depend on the types and overall profiles of the defensive compounds present on the eggshell and/or within the egg contents (Rothschild & Kellett, 1972; González *et al.*, 1999a; Blum & Hilker, 2002; Eisner *et al.*, 2002). Using a variety of biochemical detection methods and different taxa for predation trials may enhance our knowledge of the relationships between insects, their host-plants and their predators.

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Figure 5.1 Box plots of putative cardenolide content of eggs from each parental diet treatment. MW/MW: both female and male parents were fed milkweed seeds; MW/SF: females fed milkweed seeds and males fed sunflower seeds; SF/MW: females fed sunflower seeds and males fed milkweed seeds; SF/SF: both females and males fed sunflower seeds. There are no significant differences between MW/MW and MW/SF treatments, or between SF/MW and SF/SF treatments, while both treatments where females were fed milkweed are significantly different from both treatments where females were fed sunflower. Medians, 1st and 3rd quartiles (boxes) reflecting 75th and 25th quartiles, and 1.5 x interquartile range (whiskers) are presented. The median value for the SF/MW and SF/SF treatments is zero. Number of samples in each treatment: MW/MW n = 23; MW/SF n = 35, SF/MW n = 38, SF/SF n = 35. (Each sample consisted of 10 eggs from each female.)

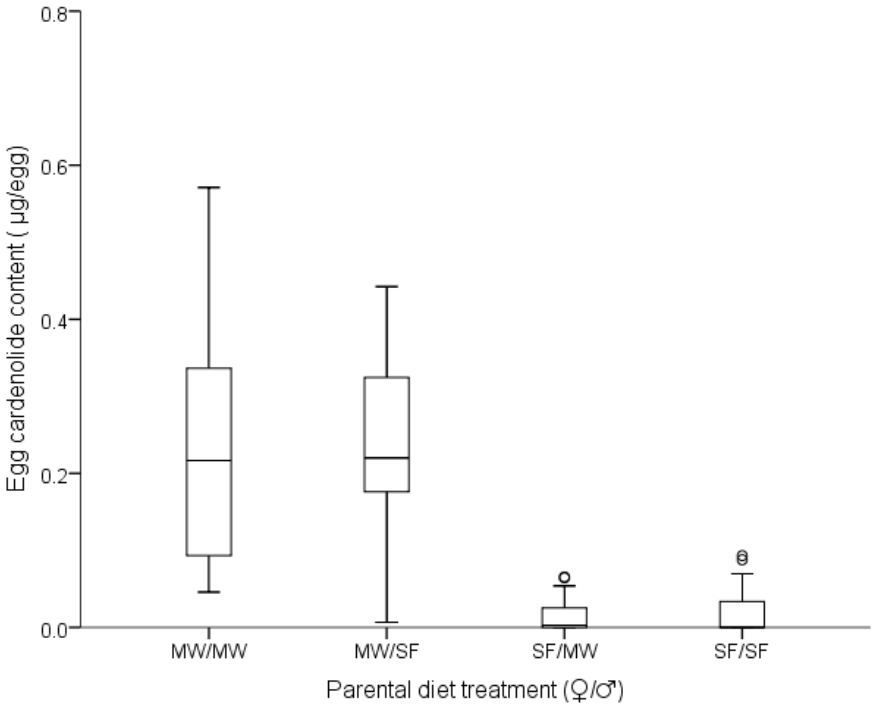
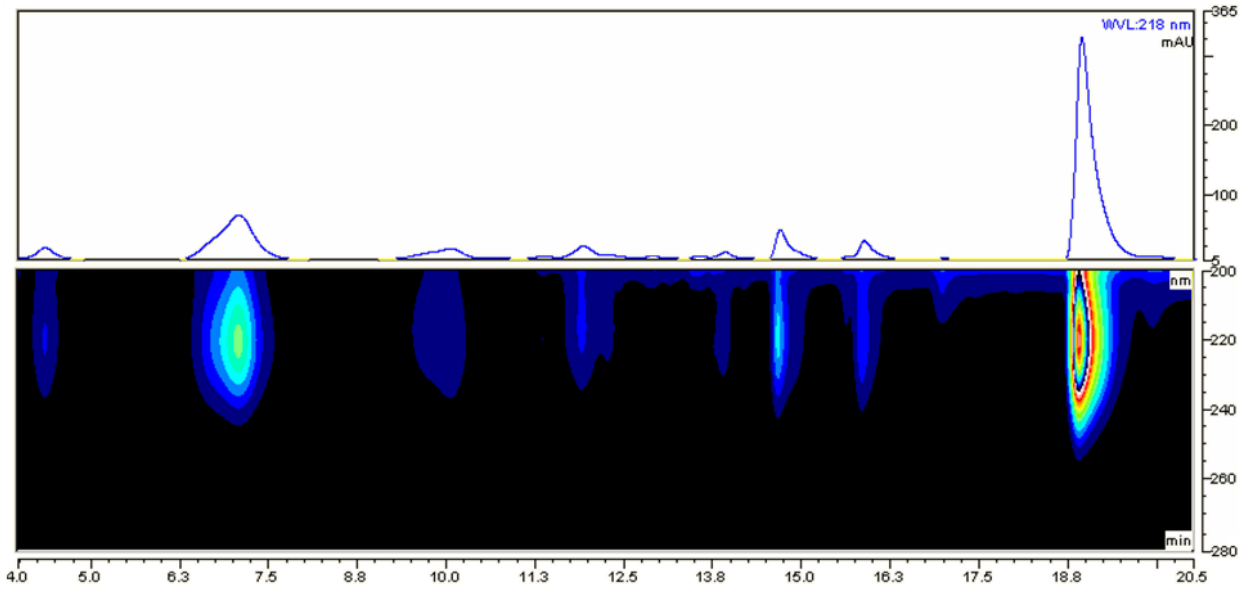


Plate 5.1 Chromatogram representative of cardenolide peaks found in our egg samples. Spectral data between 200-400nm were collected, and cardenolides were identified as peaks with a symmetrical absorbance band with a maximum absorbance between 217nm – 222nm as per Malcolm & Zalucki (1996). We used 20 µg digitoxin as our standard, which is the peak at 19.13 min.



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Chapter 6: General discussion

The overall aim of this thesis is to contribute to our understanding of how maternal (diet) effects can influence offspring life history traits and fitness parameters via the egg using a specialist insect herbivore *Oncopeltus fasciatus*. Main questions that were asked were 1) can maternal effects help facilitate transition to a novel host-diet; 2) can maternal diet influence egg composition and, if so, does this have an effect on offspring life-history/fitness parameters; 3) is there a genetic basis to egg composition and, if so, do egg amino acid profiles have the potential to evolve; and 4) can males contribute compounds into eggs that may influence offspring fitness parameters?

6.1 Maternal effects and novel host-diets

While we did not find evidence that maternal effects can facilitate the transition to a novel host diet (Chapter 1) we did find that maternal diet can influence egg composition with potential impacts on early life history traits (Chapters 3 & 5). While in some instances maternal effects have been found to have long lasting effects in offspring (Reinhold, 2002; Maestripieri & Mateo, 2009), even carrying over to subsequent generations as grand parental effects (Mousseau & Dingle, 1991; Bernardo, 1996a; Shaw & Byers, 1998; Hunter, 2002), they are often more evident in affecting early rather than late life history traits in many organisms (Mousseau & Dingle, 1991; Cheverud & Moore, 1994; Bernardo, 1996a; Rossiter, 1996; Shaw & Byers, 1998). This is particularly the case for organisms that do not display parental care (Rossiter *et al.*, 1993).

The results of our studies suggest that while maternal diet is highly significant in influencing egg and hatchling mass (Chapters 2 & 3), offspring diet is more important than maternal diet in relation to offspring post-hatching growth, adult body size and survivorship in our populations of *O. fasciatus* (Chapter 2). That maternal effects are more evident in early life history traits may be expected as developing offspring are reliant on maternal resources during the embryonic stage, and therefore the initial resources allocated to offspring must meet the requirements of the developing embryo to ensure successful development and hatching (Mousseau & Dingle, 1991; Wilson, 1997; Izquierdo *et al.*, 2001; Finn & Fyhn, 2010).

Females may be able to adjust the size (or quality) of their eggs in response to host quality (Mousseau & Fox, 1998a; Royle *et al.*, 2003; Spitzer, 2004). While we found that females adjusted

egg size in response to a novel host (Chapters 2 & 3), we did not find that this translated into an overall improvement in offspring performance parameters, as evidenced in the milkweed-adapted population (Chapter 2). In contrast, Fox and Savalli (2000) found evidence that exposing females of the generalist seed beetle *Stator limbatus* to a native plant aided in transition to a non-native host. By rearing females on either *Cercidium floridum* (native plant) or *Chloroleucon ebano* (non-native plant) then forcing them to lay eggs on *C. ebano*, females laid larger eggs on *C. ebano* when reared on *C. floridum* than females who had only been exposed to *C. ebano*. Furthermore, offspring produced had higher survivorship on *C. ebano* when their mothers were reared on *C. floridum* than offspring whose mothers were not reared on *C. floridum*. The authors suggest that being able to utilise a novel host is a result of egg size plasticity, and possibly nutritional composition, mediated via the females' ability to respond to variation in host-plants available to her offspring (Fox & Savalli, 2000). Springer and Boggs (1986) tested the hypothesis that there should be a positive correlation between the number of (provisioned) oocytes and available oviposition time in a butterfly, *Colias philodice eriphyle* Edwards, from two different ecological populations (high altitude versus low altitude). While they found evidence of genetic variation in the number of oocytes per female, the authors also found population differences in the number of oocytes suggesting that populations may evolve adaptive reproductive strategies in response to their ecological environment. However, we did not find evidence for adaptive plasticity in our populations of *O. fasciatus*.

A reason for not finding an adaptive response in *O. fasciatus* may be due to its high affiliation with its ancestral (natural) host. Specialization on host-plants is expected to increase performance on one host with a concurrent decrease in the ability to use other hosts (Thompson, 1996). While this was highly evident in the milkweed-adapted population, we also found some evidence for this in the sunflower-adapted population. For example, offspring who were fed sunflower had slightly slower development times **(Figure 2.2)** and lower survivorship **(Figure 2.4)** when their mothers had been raised on milkweed than offspring fed sunflower when their mothers had also been raised on sunflower suggesting a reduced ability to utilise sunflower even after only one generation.

In contrast, Rios *et al.* (2013) found maternal host plant influenced feeding preference of offspring in the specialised tortoise beetle *Chelymorpha varians* Blanchard. However, the strength of offspring preference for the same host-plant as their mother was more evident with the higher

quality host-plant. The authors suggest that individuals may opt to switch host if the quality of the host-plant improves performance and fitness parameters. They did not find evidence that maternal host directly influenced offspring fitness. If mothers oviposit on a poorer quality host when higher quality hosts are available (or accessible) to offspring, host fidelity would be maladaptive if the offspring chooses the poorer quality host (Rios *et al.*, 2013).

According to Spitzer (2004), certain criteria must be fulfilled to demonstrate that maternal effects result in adaptive transgenerational phenotypic plasticity: Firstly, there must be environmental variation between offspring environments; secondly, the maternal environment must be a reliable indicator for offspring environment; thirdly, females must be able to adjust offspring phenotype to suit the predicted environment. Furthermore, demonstrating the potential adaptive role for maternal effects will depend on their influence on offspring traits, the consequences on both maternal and offspring fitness and if any of these differ over time or with changing environmental conditions (Marshall & Uller, 2007; Plaistow *et al.*, 2007). While we have made some cautious inferences as to the adaptive significance of maternal effects in our populations of *O. fasciatus*, our experimental methods may restrict any general inferences regarding the adaptive significance of maternal effects in the adaptation to novel host-diets (Bernardo, 1996a; Marshall & Uller, 2007).

6.2 Maternal diet and egg composition

Nutritional maternal effects are an important consideration in life history studies (Bernardo, 1996a). The effect of maternal nutrition on offspring via maternal egg effects has been widely investigated in birds (Price, 1998), reptiles (White III, 1995; Warner *et al.*, 2007) and commercial livestock (Lopez & Leeson, 1994; Wilson, 1997; Green, 2008). Our results in Chapter 2 supports findings of other studies on arthropods that eggs may differ in their composition as a response to maternal host-diet (Cahenzli & Erhardt, 2013) and that there is evidence for genetic variation for egg composition (Chapter 3) . While it is not possible to speculate on how our results may affect wild populations of *O. fasciatus*, evidence for a link between maternal nutritional experience, genetic variation in egg composition, offspring phenotypes and reproductive strategy comes from a series of experiments on the gypsy moth *Lymantria dispar*. While Rossiter (1991a) found that maternal diet influenced larval development time and pupa mass in *L. dispar*, Rossiter (1991b) found genetic variation for egg

mass. As egg and pupal mass influence hatching and development time *L. dispar* this could have potential impacts on population dynamics for this species (Rossiter, 1991a,b). Furthermore, Rossiter *et al.* (1993) found that variation in host-plant quality contributed to variation in levels of the egg yolk protein vitellogin but that there was also genetic variation in female allocation of vitellogin. The authors suggest that variation in non-genetic material supplied to the egg may result in the expression of different offspring phenotypes which may be beneficial as a bet-hedging strategy in the face of environmental heterogeneity.

The effects of amino acids on reproductive traits and amino acid allocation strategies have recently been reported in a number of different taxa. In line with Cahenzli and Erhardt (2012) and their study on the small heath butterfly *Coenonympha pamphilus*, we found that amino acid profiles were significant for hatchling mass but not hatching success. Food quality has also been shown to affect egg production and egg viability in the marine copepod *Euterpina acutifrons*, with specific effects of amino acids. Furthermore, while amino acids of adult *E. acutifrons* remained relatively stable over time, egg amino acids were shown to vary with maternal diet and influence hatching success more than egg size (Guisande *et al.*, 2000). A study by (Ramsay & Houston, 1998) suggests that amino acid imbalance may be a constraint to egg production in blue tits *Parus caeruleus*.

Using a multivariate analysis approach we also found that maternal diet not only influenced the composition of amino acids in the eggs, but that evolutionary history of the population also had an effect on egg amino acid profiles in our populations of *O. fasciatus*. A comparison of amino acid profiles of egg vitellogins in vertebrates demonstrated variation between species, suggesting that there is a potential for vitellogins to evolve distinctive amino acid profiles which may be species specific and which correspond to the requirements of the developing embryo (White III, 1995). It is being increasingly recognised that specific combinations of constituents may be important to development and life histories (Bolton *et al.*, 1992; Uchida, 1993; Boggs, 2009). Royle *et al.* (1999) found that even though maternally provisioned vitamin E and carotenoids decreased with laying order in lesser black-backed gulls *Larus farus*, the ratios of specific carotenoids within the eggs did not differ substantially, possibly, as the authors suggest, as a reproductive strategy to enhance offspring fitness and reproductive success.

While we investigated the effects of amino acid profiles of eggs, there are many other compounds that also influence the composition of eggs with effects on offspring pre- and post-hatching development. For example carotenoids are powerful antioxidants and immunostimulants that are naturally occurring in plants and synthesised by some algae and bacteria (Blount *et al.*, 2000; Costantini *et al.*, 2005). Carotenoids, which are then ingested through the diet, have been found in eggs of insects, birds, fish (Goodwin, 1986), reptiles (Dierenfeld *et al.*, 2002) and amphibians (Ogilvy *et al.*, 2012) and may enhance the antioxidant properties of the egg during embryonic development (Blount *et al.*, 2000; Karadas *et al.*, 2005) with potential benefits to offspring post-hatching (Karadas *et al.*, 2005). Vitamin E and some fatty acids have also been shown to increase fertilization rates in fish (Izquierdo *et al.*, 2001) and enhance embryonic development and hatchability in avian eggs (Wilson, 1997). While a deficiency of vitamin A was found to decrease avian egg production, there have been contrasting reports on its effects to improve hatchability (Wilson, 1997). A study on pied flycatchers *Ficedula hypoleuca* did not find any relationship with egg components and hatching success (Ruuskanen *et al.*, 2011).

There have been a variety of studies that have investigated quantitative changes of egg composition and their relationship with maternal investment. For example, Giron and Casas (2003) investigated the reproductive investment of a parasitoid wasp *Eupelmus vuilletti* (CRW) (Hymenoptera, Eupelmidae), which use larvae of a seed beetle *Callosobruchus maculatus* as a host. They quantified both egg composition (lipids, carbohydrates and protein) and egg mass over the life time of female wasps. They found that protein and carbohydrate concentrations decreased over oviposition rank (laying sequence) but that the concentration of lipids, after decreasing slightly, remained relatively stable. Proteins and carbohydrates were also found to be highly correlated with egg size, with lipids constituting the highest energetic contribution. Sloggett and Lorenz (2008) investigated changes in egg lipid, glycogen, free carbohydrate and protein content of ladybirds from different feeding clades. The authors found differences in how the compounds changed during the course of egg development and that egg mass and total energetic content of eggs differed between species.

In their study of a geometrid moth *Cleorodes lichenaria* (Hufnagel), Pöykkö and Mänttäri (2012) investigated how egg composition may change over the oviposition period as a female ages and the effects on egg and offspring life history parameters. That is, if a decrease in egg size during the

oviposition period of aging females was related to absolute provisioning (measured as micrograms of nutrients in an egg i.e. egg mass) or relative provisioning (measured as percentage of nutrients of total egg fresh weight). Despite being capital breeders (i.e. feeding occurs during the larval but not the adult stage), Pöykkö and Mänttäri (2012) found that egg composition remained relatively stable as adult female *C. lichenaria* age. While fresh and dry egg mass, as well as absolute provisioning of proteins and lipids, changed over the oviposition period (i.e. eggs became smaller), relative provisioning (i.e. concentrations) of proteins and lipids did not. However, larvae that hatched from later laid (lighter) eggs had lower survivorship than larvae that hatched from earlier laid (heavier) eggs when under nutritional stress. This suggests that later laid eggs may not provide adequate nutrients for larvae to survive under nutritional stress (Diss *et al.*, 1996; Fox & Mousseau, 1996; Kyneb & Toft, 2006). The authors suggest that the decrease in egg size relates to an overall decrease in the total (absolute) amount of nutrients that are supplied to the eggs.

The above studies are a good demonstration of how changes in egg composition can vary between species, resource acquisition strategies (e.g. income versus capital breeders), female age and feeding clades (e.g. specialists versus generalists), and highlight the importance of studying a wide variety of taxa to further our knowledge on the different strategies used in resource allocation (Boggs, 2009). As discussed above, and in the previous sections, maternal allocation of egg provisioning is a complex interplay of environmental, genetic and biochemical processes that can influence embryonic development and the expression of offspring phenotypes (Springer & Boggs, 1986; Rossiter *et al.*, 1993; Izumi *et al.*, 1994). Compounds that can be maternally provisioned into eggs may be highly diverse in their type, quality and quantity. Restricting investigations into any single one compound in a single environment would not provide a complete picture of how maternal allocation may influence egg development and offspring life history/fitness parameters (Bauerfeind & Fischer, 2005; Geister *et al.*, 2008).

6.3 Genetics of maternal allocation

While traditionally, maternal effects were regarded as a nuisance component in quantitative genetic models, it is now widely recognised that they are an important component in generating and maintaining phenotypic variance. Evidence that maternal effects have a genetic basis, are heritable and can themselves evolve suggests the potential for influencing responses to selection (Mousseau

& Fox, 1998b; Räsänen & Kruuk, 2007). Furthermore, it has been found that maternal effects can evolve and can either constrain or facilitate adaptation, so determining the underlying genetic architecture of maternal (and paternal) traits that cause maternal effects may enable predictions of phenotypic evolutionary responses to selection.

The genetics of maternal allocation of resources into eggs represents an interesting and important insight into maternal effects as selection on egg composition could lead to microevolution of egg components, which may then have effects on trait evolution with potential effects on population differentiation (Ruuskanen *et al.*, 2011). While selection studies to determine heritabilities and genetic correlations between maternal traits, egg components and offspring phenotypes have been widely used for domestic and commercial livestock (Hartmann *et al.*, 2003; Wilson & Réale, 2006), evidence of egg components under genetic control in wild bird populations has recently been reported (Tschirren *et al.*, 2009; Ruuskanen *et al.*, 2011). A Europe-wide study by Tschirren *et al.* (2009) on collared flycatchers *Ficedula albicollis* found high within-population variation for egg components which the authors suggest could be due to population-specific environmental and/or social factors or genetic factors. The authors demonstrated high heritabilities for the maternal yolk hormone testosterone, egg mass and yolk mass signifying a genetic basis for prenatal maternal effects in *F. albicollis*. Furthermore, the authors found evidence for genetic discrimination for the regulation of maternal hormones and that females also demonstrated variability for hormone deposition depending on environmental conditions.

As maternally derived egg components can be important in both pre- and postnatal development with lasting consequences into adult life stages (Tschirren *et al.*, 2009 and see previous section), studies such as these can make important contributions towards understanding how genetic variation in maternal allocation can influence phenotypic variation and how they may respond to selection. However, a caveat to quantitative genetic statistics is that generalisations cannot be made regarding the heritablities and evovabilites of traits as these are population and context-dependent and, as such, are specific to the population(s) being tested and cannot be transferred to other populations or environments (Hansen *et al.*, 2011). Therefore, more studies into the genetics behind maternal allocation, and their effects on offspring phenotypes and life history/fitness parameters, are needed to be able to illuminate any patterns in this understudied, and potentially important, maternal effect.

6.4 Male contributions

While this thesis has been focused primarily on the non-genetic contribution of females, males have also been recognised to make non-genetic contributions to offspring phenotypes and fitness parameters (Mousseau & Fox, 1998b; Roth *et al.*, 2010). In Chapter 5 we investigated if there was uni- or biparental contribution of cardenolides into the eggs of *O. fasciatus*. Our results supported that of many other studies (see Blum & Hilker, 2002) in that we found maternal contribution to be greater than paternal contribution **(Figure 5.1)**. However, paternal contribution of defensive compounds has been reported for other species of arthropods. For example, while female fireflies from the genus *Photuris* can endogenously produce betaine, male fireflies from the genus *Photinus* are laden with a chemical steroid lucibufagin (González *et al.*, 1999a; Blum & Hilker, 2002; Eisner *et al.*, 2002). Female *Photuris* fireflies attract and devour male *Photinus* fireflies thereby consuming lucibufagin from the male (González *et al.*, 1999a; Eisner *et al.*, 2002). Furthermore, female *Photuris* can endow their eggs with both compounds which contributes to their protection against some insect predators (González *et al.*, 1999a). In other species, males may contribute defensive compounds via their ejaculate. Biparental endowment of endogenous alkaloids has been reported in ladybird beetles *Epilachna paenulata* (Camarano *et al.*, 2009) and several species of meloid beetles and chrysomelid beetles, while reports for sequestered defensive compounds has been reported in a number of Lepidoptera (Blum & Hilker, 2002; Eisner *et al.*, 2002). Interestingly, female meloid beetles of *Lytta vesicatoria* do not produce their own chemical defence but rely on a supply of cantharidin from the males which they then incorporate into eggs (Eisner *et al.*, 2002).

There is also evidence that maternal provisioning of eggs may be mediated by a male's influence on females, either through accessory gland products, nuptial gifts or even through the number of mating partners she may have (Zeh & Smith, 1985; Gillott, 2003; Kotiaho *et al.*, 2003; Andersson & Simmons, 2006; Simmons & Garcìa-González, 2007; Sprenger *et al.*, 2010). Males may transfer nutrients to females as nuptial gifts before/during/after courtship/mating and, while this has been well-documented in insects (Vahed, 1998), can occur in other animal taxa (Tryjanowski & Hromada, 2005). Gift giving may include prey items, parts of the male's own body and glandular products (secretions, spermatophores and substances within the ejaculate) (Vahed, 1998).

Males can also provide direct parental care to eggs and/or offspring. Demonstration of uni- or biparental care in nature is diverse across taxa. For example, biparental care is common in birds, maternal care is common in mammals and invertebrates, where fish demonstrate care it is usually paternal, and some animals such as mammals, crocodiles and some fishes may have one or both parents help look after eggs/offspring (Klug *et al.*, 2013). Anurans can demonstrate a variety of parental care strategies depending on the species (Delia *et al.*, 2013). For oviparous animals, parents can adjust frequency of their care in response to embryonic condition and environmental cues and this has been reported for invertebrates, fish, reptiles and birds (Delia *et al.*, 2013). So, while for animals that do not demonstrate parental care of offspring, females and males can influence offspring phenotypes at the egg stage which can have dramatic effects on offspring phenotypes (Rossiter *et al.*, 1993).

6.5 Concluding remarks

Maternal effects, that is the non-genetic influences of females (and males) on offspring phenotypes, are now widely recognised to have dramatic effects on life-history traits that can enhance or impede adaptive responses to selection. The overall aim of this thesis was to investigate if maternal (diet) effects can influence offspring life history/fitness parameters via the egg in a specialist insect herbivore *Oncopeltus fasciatus*. As maternal effects can be dramatic for species that do not demonstrate parental care (Rossiter *et al.*, 1993), we would expect to find strong maternal effects in *O. fasciatus*. However, while we found maternal diet influenced egg and hatchling mass, this did not translate into strong effects on other life history traits that we measured. It may be that our experimental designs were too constrictive in terms of the number of populations that we used for our experiments. In addition, due to differences in their recent evolutionary histories (i.e. the milkweed-adapted population derived from wild individuals while the sunflower-adapted population have long being adapted to laboratory conditions) differences that we observed between our populations may have been due to drift rather than evolved differences. As some wild populations of *O. fasciatus* are migratory, other factors such as temperature and photoperiod have been shown to produce a range of responses in diapause, migration and breeding strategies (Dingle *et al.*, 1980). Furthermore, there is evidence of positive genetic correlations (pleiotropy) between body size, flight behaviour and reproductive output in migratory, but not non-migratory, populations of *O. fasciatus* (Palmer & Dingle, 1986). Therefore, it may be that maternal effects in

O. fasciatus could be more evident in producing a variation of offspring phenotypes when responding to environmental cues.

Although we found a maternal contribution of a defensive compound into the eggs, we did not find a significant effect on protection against an invertebrate predator. As nymph and adult *O. fasciatus* (when raised on milkweed seeds but not sunflower seeds) have been found to be repellent to invertebrate and vertebrate predators (Berenbaum & Miliczky, 1984; Paradise & Stamp, 1991), and as chemical egg defence in insects is common (Blum & Hilker, 2002), it would seem likely that cardenolides in the eggs of *O. fasciatus* could serve as a defence mechanism. It may be that *C. carnea* was more tolerant to the cardenolides in the eggs or that our test clutches were too small to observe a true effect. As different defensive compounds can produce different responses in (invertebrate and vertebrate) predators (Rothschild & Kellett, 1972; González *et al.*, 1999a; Blum & Hilker, 2002; Eisner *et al.*, 2002) it may be useful to test a natural predator that could potentially feed on eggs of *O. fasciatus* in order to determine if cardenolides within the eggs are an adaptive defensive strategy.

Evidence from the literature demonstrates that females can allocate various nutrients into eggs and that these can have both positive and negative effects on offspring phenotypes and life history/fitness parameters. The non-genetic contribution of males can also affect maternal allocation of resources into eggs as well as influencing female reproductive effort and reproductive strategies (Vahed, 1998; Gillott, 2003; Wedell & Karlsson, 2003). Females may also adjust resource allocation and reproductive strategies in response to environmental cues (Mousseau & Fox, 1998b). Therefore, to understand how maternal effects influence the responses of life history parameters through the allocation of resources via maternal egg effects, it is necessary to investigate female responses in a number of different conditions (ecological, nutritional and/or environmental) (Bernardo, 1996a; Mousseau & Fox, 1998b; Marshall & Uller, 2007). It is also important to distinguish how maternal effects may influence maternal fitness, as well as offspring fitness, as this will give more appropriate insights into the adaptive nature of maternal allocation of dietary resources and phenotypic plasticity (Mousseau & Fox, 1998b; Marshall & Uller, 2007; Crean & Marshall, 2009). As more evidence is being gathered on the genetics of maternal allocation, this will provide insights into the contribution of maternal effects to evolutionary patterns for the

generation and maintenance of phenotypic variation and to life history evolution (Cheverud & Moore, 1994; Mousseau & Fox, 1998b; Wilson *et al.*, 2005; Räsänen & Kruuk, 2007).

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