Life-history variation and evolved response to food stress in *Oncopeltus fasciatus* (Hemiptera: Lygaeidae)
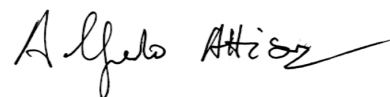
Submitted by Alfredo Attisano, to the University of Exeter as a thesis for the degree

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I certify that all material in this thesis which is not my own 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.

Abstract

Every organism needs to survive and successfully reproduce in the face of changing environmental conditions in which variation in resource availability can seriously limit performance. Organisms can respond to the variation in quality or availability of food resources with behavioural and physiological accommodations going from the baseline physiological response to environmental stressors to complex life-history strategies like migration and diapause. In insects, one avenue to cope with the resources' variation is to plastically tune the reproductive system to the environmental conditions in order to shift resources away from reproduction during unfavourable periods but maximize it when resources are abundant. I studied the role of reproductive physiology in both males and females in mediating a response to challenging conditions determined by a lack of food resources or the presence of qualitatively different diets using the milkweed bug, *Oncopeltus fasciatus*, as model species. I studied the role of oosorption, a plastic physiological response through which resources can be recovered and redirected to body maintenance and survival, in shaping behavioural strategies to cope with challenging environments. I also studied the effects of diet quality on male's sexual behaviour and how these modulate the trade-offs between reproduction and survival. I then investigated how the effects of diet quality, sexual maturation and rearing conditions influence the occurrence of reproductive diapause in both males and females. I found that females exposed to different diets plastically adapt their schedule of reproduction depending on diet quality: this also influences the occurrence of oosorption in the ovary mediating the amount of resources that are directed to reproduction or survival. Diet quality influences males' sexual behaviour so that even after a long-term adaptation on an alternative artificial diet, they invest more in reproduction at the expenses of survival

when fed on an ancestral high quality diet; this is achieved with a shift in the trade-off between reproduction and survival. The occurrence of reproductive diapause in both males and females is a function of several factors: the quality of food resources ultimately modulates sexual maturation in adult individuals determining the occurrence of diapause or reproduction. Finally, oosorption may be involved in the evolution of alternative condition-dependent strategies as an adaptive physiological mechanism to cope with stressful environments; thus females from different populations may be able either to migrate in favourable areas where they can exploit abundant food resources or remain residents and perform high levels of oosorption to cope with the seasonal shortage of food.

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Author's declarations

Introduction

The views presented in the introduction are my own and were developed under the guidance of Prof. Patricia J. Moore.

Chapter 1: Oosorption in response to poor food: complexity in the trade-off between reproduction and survival.

Prof. Patricia J. Moore designed the experiment. Prof. Patricia J. Moore and I collected the data, analysed the results, co-wrote the manuscript and I am second author of the manuscript.

Chapter 2: Reproduction-longevity trade-off reflects diet, not adaptation.

Prof. Patricia J. Moore, Prof. Allen J. Moore and myself contributed extensively to the design, analysis and interpretation of the data and to the writing of the manuscript. I performed the experiment and collected the data and I am first author of the manuscript.

Chapter 3: Observations on the reproductive diapause in wild and laboratory adapted strains of the milkweed bug, *Oncopeltus fasciatus*

Prof. Patricia J. Moore provided comments on the manuscript. I designed and performed the experiment, collected and analysed all data and I am the only author of the manuscript.

Chapter 4: A simple flight mill for the study of tethered flight in insects.

Dr. Andy W. Vickers and I conceived and wrote the MatLab script. I designed and build the flight mill device, collected and analysed the data and I am first author of the manuscript.

Chapter 5: Reproductive physiology and the evolution of a partial migratory strategy.

Prof. Patricia J. Moore, Prof. Allen J. Moore and Prof. Tom Tregenza provided guidance during the data analysis and writing of the manuscript. I designed the experiment, collected and analysed the data and I am first author of the manuscript.

Concluding comments

The views presented in the conclusions represent my own interpretation of the data presented in the previous chapters, under the guidance of Prof. Patricia J. Moore.

Introduction

Every organism needs to face the challenges of constantly changing environments, including temporal variation in the availability of resources such as prey, host-plants or mates which can seriously limit performance in a given environment. Thus the ability to survive and successfully reproduce in the face of environmental heterogeneity and variation is a prerequisite for any living organism. A crucial feature for survival and reproduction is the ability to maintain physiological integrity under environmental variation that tends to lead the balance away from a self-regulatory equilibrium state, i.e. situations that can lead to conditions of physiological stress. This maintenance can be achieved through both intrinsic physiological adjustments and behavioural strategies to deal with a lack of resources. These two levels of response are of course not independent from each other but rather show a high level of interaction.

My introduction is organised into six parts. I will begin with a general introduction to the biology of stress and the definition of terms like stressors, homeostasis, allostasis and the physiology of the stress response. The next section reviews the basic mechanisms underlying the physiology of insect's reproductive systems. The third section focuses on the key concepts of life-history theory and the role physiology plays in shaping phenotypically plastic responses. I then move to migratory behaviour as an adaptation to the environmental variation in resource availability. Finally, I introduce the model species used in my thesis research, focusing on behavioural and reproductive physiological adaptations to the seasonal availability of food resources before describing my thesis plan.

1. An introduction to the biology of stress

Stress is a highly debated term in biology and medicine due to the complexity of the physiological processes that mount, maintain and terminate a stress-induced physiological response. Selye (1950, 1976) defined stress as a nonspecific response to any demand that is caused by a stressor and that tends to disturb the physiological equilibrium of an organism, its homeostasis. A stress-inducing response determines the onset of a "general adaptive syndrome" that can be divided in three phases: 1) the alarm response, characterized by the release of stress hormones and neurotransmitter (epinephrine and glucocortocoids) that generates a response to help restore homeostasis, 2) a resistant stage in which homeostasis is restored and defence and adaptation are optimal and, if the stressor persists, 3) an exhaustion stage in which adaptation ceases and pathology can arise.

In recent years has become increasingly evident that the definition of stress, stressors and homeostasis needed a revision in light of the recent discoveries about the mechanisms determining the stress response and the role of the physiological mediators, i.e. the physiological response to stress, in the modulation of the stress-induced response. The first debate is about the meaning of the word stress and the circularity that surrounds its definition (Romero et al., 2009). The second fundamental point is that the stress response is modulated by the release of physiological mediators that in the short term help an organism to cope with the challenging situation and to respond adaptively to it (McEwen, 2005), but in the long-term the exposure to stressful conditions and the consequent high levels of physiological mediators cause illness and pathological conditions (Sapolsky, 1996; McEwen, 1998). However, if certain conditions are met (fertility differences between genotypes, small group sizes and limited dispersal), alleles that increase mortality under stressful conditions might succeed in a population, giving rise to a selective advantage of the long-term stress

exposure even if this causes the death of the individual exposed to the stress agent (Hadany et al., 2006). Also, individuals can be characterized by a differential ability to cope with stress through a set of behavioural and physiological stress responses that are consistent over time and characteristic of certain individuals in a population, the "coping style" (Koolhaas et al., 1999), raising interesting questions about the possible correlation between individual differences in physiological stress response and personality traits (Carere et al., 2010).

Recognition of the complexity of the stress response and mechanisms involved lead to a reformulation of the terms and a better understanding on how a stress response is modulated. Two recent proposed formalization of the stress-induced response are the allostatic model (McEwen and Wingfield, 2003) and the reactive scope model (Romero et al., 2009). The main point of both models is that animals are able to cope with periodical perturbations above the level of the normal homeostatic equilibrium but within the range in which the mediator, i.e. the physiological response to stress determined by the physiological systems, do not reach potentially harmful levels or the energy to perform such accommodations do not exceed the available energy in the environment. This mechanism is under the influence of natural selection and has adaptive advantages because it allows organisms to respond to predictable variations in the environment and to physiologically and behaviourally accommodate to it. Such a range corresponds to the allostatic state of McEwen and Wingfield (2003) and to the reactive scope of Romero et al. (2009). These are then divided in two sub-ranges: a lower range in which homeostasis is maintained and an upper range in which allostasis, i.e. the ability to reach homeostasis through the effect of physiological mediators, is performed. Above these ranges of adaptive physiological response lies the range in which a short-term stressful event can be counteracted by

adaptive physiological responses but a sustained long-term physiological response to stress will lead to pathologies and eventually death of the organism. The difference between the two models is the way in which the thresholds between these ranges are defined. The allostatic model measures such a response in terms of energy required for the animal to cope with the stress and how much energy can be obtained from the environment to perform the response (McEwen and Wingfield, 2003). Thus an allostatic overload type 1 is reached when the energy required to cope with the stress exceed the energy that can be acquired from the environment and the animal enters an adaptive life-history emergency state (Wingfiel et al., 1998) until the stressful event ends. An allostatic overload type 2 is reached when the production of physiological mediators is sustained over time and lead to deleterious effects (McEwen and Wingfield, 2003). The reactive scope model uses the level of the physiological mediator involved in the stress response, rather than the energy level, and how this affects the range in which the stress response will fall (Romero et al., 2009). Both models have the advantage of giving a temporal scale to the occurrence of stressful events by considering the different effects of a stressor under different life-history stage and seasonal environmental conditions. Thus the type and intensity of the stressor can depend on, for example, season and reproductive state (McEwen and Wingfield, 2003; Romero et al., 2009) and individuals can respond differently to the same stressor (Wingfield et al., 1999; Carere et al., 2010; Koolhaas et al., 2011).

The conceptualization of these two models leads to a different and more rounded definition of the term stress. With the introduction of the concept of allostasis, the term stress describes "events that are threatening to an individual and elicit physiological and behavioural responses as a part of the allostasis in addition to that imposed by the normal life cycle" (McEwen and Wingfield, 2003; McEwen and

Wingfield, 2010). Koolhass et al. (2011) also pointed out that the term stress should be restricted to unpredictable and uncontrollable situations in which the environmental demands exceeds the natural regulatory capacity of an organism.

In vertebrates the stress response is a complex physiological reaction modulated via the hypothalamic-pituitary-adrenal axis (HPA). The first wave of hormones production happens within seconds of the stressor: catecholamines (epinephrine and norepinephrine) are produced by the adrenal medulla and released into the general circulation, the hypothalamus releases corticotrophin-releasing hormone (CRH) and arginine vasopressin and the anterior pituitary gland releases adrenocorticotropic hormone (ACTH). These hormones exert their effects within seconds to a few minutes from exposure to stressor. A second wave happens within a few minutes with the release of glucocorticoids (GC) by the adrenal cortex into the general circulation while the production of gonadal steroids is suppressed. The effects of GC are not evident until about one hour of the stressor whereas the consequences of decreasing reproductive steroid do not occur for several hours. The role of GC in the stress response is complex and can have permissive, stimulant or suppressive effects depending on the physiological or behavioural reaction we consider (Sapolsky et al., 2000). One of the main GC in vertebrates is the corticosterone hormone (CORT) and it can be involved in different processes derived from the exposure to a stressor. For example, CORT can elicit behavioural responses (Kitaysky et al., 2001; Thaker et al., 2010), modulate the metabolism and distribution of fat reserves (Rebuffè-Scrive et al., 1992) or delay the reproductive investment (Salvante and Williams, 2003).

Different neurotransmitters and hormones are involved in the stress response in insects. Biologic amines are involved in the energy metabolisation in insects and

exposure to stressors causes an increase in octopamine (homolog to norepineprhine) and dopamine (Davenport and Evans, 1984; Hirashima and Eto, 1993; Rauschenbach et al., 1993; Hirashima et al., 2000). The synthesis and metabolism of octopamine and dopamine is under the influence of the JH release by the corpora allata (Gruntenko et al., 2000; Gruntenko et al., 2012). Corazonin is a neuropeptide homolog to gonadotropin-realeasing hormone (GnRH) of vertebrates, is highly conserved between insects and seems to be involved in the modulation of the stress response (Veenstra, 2009; Boerjan et al., 2010; Zao et al., 2010). Adipokinetic hormones (AKHs), glucagon and glucagon-like peptides are involved both in the regulation of insect metabolism and in the stress response (Kodrik, 2008; Bednarova et al., 2013).

Heat shock proteins (Hsps) are expressed by a set of genes activated when a cell is exposed to stressful events. This group of proteins represent a highly conserved defence mechanism (Lindquist, 1986; De Maio, 1999) and includes different families of proteins with the Hsp70 being one of the most conserved from bacteria to man (Lindquist and Craig, 1988). Hsps act as chaperones, binding to proteins in a non-native conformation whether due to a stress effect or because peptides have not been fully synthesized, folded, assembled or localized to an appropriate cellular compartment (Feder and Hofmann, 1999). Thus in a sort of "housekeeping" function Hsps help to maintain the cell's internal organization and to restore unfolded proteins. The heat shock response is induced from a wide variety of environmental and genetic stressors (Lindquist, 1986; Santoro, 2000; Sorensen et al., 2003; Richter et al., 2010). In addition to being an important part of the response to sudden stress, the Hsps is also of ecological and evolutionary importance as a mechanism of adaptation to the environment being continuously expressed as a response to less severe but regular forms of stress (Sorensen et al., 2003).

An interesting question is how, if and in what direction a stress response influences the evolution of life-history traits. Given the adaptive nature of accommodating an organism to its environment, a stress response is expected to have a strong effect on life-history traits like survival and reproduction and consequently on fitness. Indeed physiological mediators are known to have quite a wide range of effects on physiology and behaviour (Sapolsky et al., 2000). However, two recent reviews highlighted the fact that the correlation between stress response and fitness is far from simple. Bonier et al. (2009) reviewed works dealing with the baseline levels of corticosteroids hormones, normally used to indicate the physiological conditions of individuals and populations, and found that this could be positively, negatively or non-significantly correlated with estimates of fitness. Breuner et al. (2008) reviewed works in which the acute stress response was correlated with reproduction and survival and found that the level of glucocorticoids can be positively or negatively associated with reproduction and survival. Both the reviews debated with the proxies of fitness used in these works and pointed out that few experimental works have been actually performed on this topic. Nevertheless this field of research seems particularly promising and the complexity and variation of the response open up even more interesting questions about the role of stress in the evolution of life-history traits (Sorensen et al., 2003; Crespi et al., 2012).

2. Insect reproductive physiology

The main control of reproduction in insects is under the influence of the endocrine system triggered by stimulatory or inhibitory brain factors, which are turned on by sensory inputs from the environment. Juvenile hormone (JH), produced and released by the corpora allata (CA), is a primary mediator of reproductive

physiology in insects and exerts a wide range of functions on different tissues and organs targeting the expression of genes that induce, stimulate or repress reproductive functions (Wyatt, 1997). Allototropic (stimulating) and allatostatic (inhibiting) factors from the central nervous system modulate the activity of the CA stimulating the synthesis and release of JH. The factors modulating the CA activity are diverse and can have a varying degree of overlap. Environmental factors like host quality and availability (Papaj, 2000) and nutrients provided by the diet (Wheeler, 1996) can have a profound effects on the endocrine system, hormone production and consequently on sexual activity. Feeding behaviour also leads to activation of the CA (Davey, 1997). The ingestion of a blood meal in mosquitoes is the main factor triggering the endocrine cascades that leads to the maturation of a batch of eggs (Klowden, 1997). In the burying beetle *Nicrophorus tomentosus* the behavioural cues of assessing, preparing and burying a carcass trigger ovary development (Scott and Traniello, 1987). Social factors also exert an important effect on the activity of the CA and on the hormonal cascades initiated by its activation (Schal et al., 1997). The CA is normally inhibited by a control factor from the brain and mating activity leads to the activation of the CA or a prolongation of its active period (Davey, 1997; Chiang, 1998). Sexual interactions also have a major role apart from the activation or inhibition of the CA. Ejaculate components from male accessory sexual glands can affect virtually every aspect of the female's reproductive activity (Gillot, 2003) with the common result that fecundity, ovulation and/or egg laying in females are stimulated or increased by male seminal fluid proteins (Avila, 2011).

The classical model of endocrine control of reproduction involves a series of steps in which JH exerts a major role in oogenesis. The primary effects of JH are to stimulate the transcription of vitellogenin genes and the synthesis of vitellogenin

proteins from the fat body that are exported into the hemolymph and to regulate the vitellogenin uptake in developing oocytes (Strambi et al., 1997; Davey, 1997). JH also influence the metabolism of pupal fat body, the hypertrophy of adult fat body and the ontogeny of sexual behaviour (Yin and Stoffolano, 1997). In the burying beetle *N. orbicollis* the JH titres increase upon finding a carcass and remain high at the time when young hatch, a physiological response that could be associated either with the behavioural demand of parental care or with the willingness to replace the brood in case of failure (Trumbo, 1997). The titres of JH influence the behaviour of migratory species in which reproduction and migration need to be separated in time (Rankin and Riddiford, 1978) due to trade-offs in resource allocation of nutrients, particularly proteins (Rankin and Burchsted, 1992; Harshman and Zera, 2007). JH exerts a major role in male adult insects as well, influencing spermatogenesis (Dumser, 1980), the development of accessory glands and renewal of secretory production after depletion during mating (Happ, 1992).

In general JH leads to the development of fully mature ovaries and its action is in balance with the effects of other hormones. In *Phormia* and *Sarcophaga* flies the oostatic hormone acts as an antigonadotropin shutting off the action of the JH and application of 20-hydroxy-ecdisone causes the primary oocytes to degenerate with concomitant development of secondary oocytes (Fraenkel and Hollowell, 1979). In sexually mature *Drosophila melanogaster* females JH stimulates vitellogenic oocyte progression while 20-hydroxy-ecdisone induce apoptosis in nurse cells, thus regulating whether oocytes will progress through maturation or undergo apoptosis (Soller et al., 1999). This show that ovarian maturation in female insects is a plastic process well suited for adjusting to environmental conditions. The quantity of eggs to be laid can be adjusted at the individual level and this is true particularly for

synovigenic females that produce eggs continuously during their adult life (Papaj, 2000).

The reproductive system in insects is tuned to respond effectively to environmental conditions and thus to obtain the maximum reproductive success when conditions are favourable and to recover resources when conditions for reproduction are poor. The main environmental cues for insects are photoperiod and temperature because they represent reliable cues of a seasonally changing environment. Generally insects are able to reproduce only between a certain range of temperature and photoperiod, tuning their reproductive efforts on the seasonal cycles that mark the appearance and availability of food resources. Outside of these ranges the reproductive activity is halted and resources are allocated to survival. Diapause represents such a process of stopping the reproductive cycle to overcome the unfavourable seasonal conditions.

Diapause is a complex alternative developmental pathway characterized by behavioural, morphological and physiological features that allow insects to cope with unfavourable environmental conditions (Derlinger, 2002). The distinctive feature of diapause is the slow rate of senescence which derives from the absence of a mature reproductive system due the very low JH titres (Saunders et al., 1999; Tatar and Yin, 2001), a slower metabolic rate (Tatar and Yin, 2001), utilization of resources accumulated during the pre-diapause period (Sonoda et al., 2006; Hahn and Denlinger, 2010) and an increased tolerance to environmental stress (MacRae, 2010). Factors like copulation (Hayes and Dingle, 1983; Sillen-Tulberg, 1984), starvation and host quality (Dingle et al., 1977; Hunter and McNeill, 1997) and moisture (Tanaka, 2000) influence the onset of diapause, but these are always more or less dependent from the ongoing changes of environmental temperature and photoperiod

marking a seasonal change (Dingle, 1974; Masaki, 1980), making the diapause an eco-physiological process in its nature (Kostal, 2006). In the milkweed bug *Oncopeltus fasciatus* JH titres determine the occurrence of reproduction, migration and diapause through a higher and lower threshold of sensitivity (Rankin and Riddiford, 1978): JH titers above the higher threshold allow reproduction while JH titers below the lower threshold initiate entry into diapause. Migration takes place when JH titres are between the two thresholds (Rankin and Riddiford, 1978; Nijhout, 1994).

Insect eggs represent a huge investment for a successful reproduction, but they are a source of nutrients and resources as well (Raikhel and Dhadialla, 1992). These can be recycled and resources reallocated to body maintenance and survival when reproduction is not the best option. Indeed the ability to resorb oocytes in development (oosorption) has been indicated as a physiological mechanism to cope with adverse environmental conditions (Bell and Bohm, 1975) that are unfavourable for a successful reproductive event. The resources, in the form of yolk proteins, can be recovered from the eggs in development and shifted to body maintenance (Kotaki, 2003; Guo et al., 2011), thus oosorption is central to the trade-off between reproduction and survival (Boggs and Ross, 1993; Ohgushi, 1996). One of the cellular mechanisms by which oocytes are resorbed and resources reallocated to the soma is apoptosis (Hopwood et al., 2001; Moore and Sharma, 2005; Barrett, 2009; Clifton and Noriega, 2011). Apoptosis is a conserved mechanism of genetically programmed cell death involved in the cell turnover of healthy adult tissues and responsible for the focal elimination of cells during embryonic development (Kerr et al., 1972). There are different types of cell's death and apoptosis is characterized by the presence of cystein-dependent proteases called caspases (Van Cruchten and Van den Broeck,

2002). Cells undergoing apoptosis shows distinct morphological features like shrinking and formation of processes (budding) (Majno and Joris, 1995). Environmental factors like food availability and social context can induce apoptosis in different tissues like for example in the midgut of starved *Periplaneta americana* (Park et al., 2009) or in the ovarioles of females *Nauphoeta cinerea* forced to delay mating (Moore and Sharma, 2005). The apoptotic pathway may be regulated by the expression of Hsps, which could constitute a protective mechanism against damaging events leading to a cell's death by apoptosis (Beere, 2004). Autolysis is another example of cellular mechanism that determines oosorption of unlaid eggs (Hinsch, 1992; Asplen and Byrne, 2006). The level of oosorption is affected by JH titres (Clifton and Noriega, 2011) while 20-hydroxy-ecdysone is involved in oocyte degeneration (Fraenkel and Hollowell, 1979) and apoptosis induction in nurse cells (Soller, 1999). The most common factors triggering oosorption are the lack of available food resources (Bell and Bohm, 1975; Papaj, 2000; Kotaki, 2003; Osawa, 2005; Barrett et al., 2008a; Kajita and Evans, 2009; Clifton and Noriega, 2011; Guo et al., 2011) and food quality (Lopez-Carretero et al., 2005; Moore and Attisano, 2011). Oosorption is also induced by social factors (Moore and Sharma, 2005; Barrett et al., 2008b) and parasitic load (Hopwood et al., 2001). Oosorption can have an adaptive role both in maintaining a constant supply of mature eggs (RiveroLynch and Godfray, 1997) and inhibiting the production of further eggs until environmental conditions improve (Barrett et al., 2008b). In the cockroach *Nauphoeta cinerea* the physiological ability to resorb oocytes is a heritable traits in unfavourable, but not in favourable, environmental conditions (Edvardsson et al., 2009). The ability to resorb oocytes represents an important plastic physiological response to cope with unfavourable

environments and in the same time is an efficient way to reallocate resources to competing organismal activities.

3. Life-history theory, trade-offs and phenotypic plasticity

Organisms differ in how they develop, mature, reproduce and survive. Age, size and stage specific patterns of development, growth, reproduction, survival and lifespan define the life history of an organism (Daan and Tinbergen, 1997). Through mathematical formalization, life-history theory attempts to understand how natural selection shapes organisms to maximize their reproductive success, i.e. fitness, in the face of extrinsic (environmental) and intrinsic (genetic, physiology, trade-offs) factors that constrain the evolution of life-history traits (Stearns, 1992; Fabian and Flatt, 2012).

Fitness can be defined as "a measurable feature of alleles, genotypes or traits of individuals that predicts their numerical representation in future generation" (Hunt and Hodgson, 2010). The two commonly used measures of population growth are represented by r, the intrinsic population growth rate or Malthusian parameter, and R_0, the net number of daughters per female during a lifetime or net reproductive rate. Both these measures are mathematically related to the fecundity and probability of survival of an organism at a given age. There is an ongoing debate about which and in which conditions one or the other measure should be used. The population growth rate r can be used only for non-stationary populations and it refers to the growth rate of a genotype while normally survival and fecundity are related to a population, which is not formed by a single genotype (Stearns, 1977; Kozlowski, 1993). The net reproductive rate R_0 can be used only in case of stationary populations and is affected by the environmental heterogeneity, but it offers a good way to compare the life-

history differences between age classes (Kozlowski, 1993). Another issue of the fitness concept is that it refers to individual organisms but it is obtained through demographic parameters estimated from groups of individuals thus representing components of fitness rather than individual fitness itself (McGraw and Caswell, 1996). Using different measures of fitness to answer the same question can lead to different and contrasting interpretations (Brommer, 2000). The concept of fitness is a relative one and is context-dependent thus the measure to be used should be considered cautiously depending on the question one wants to ask (Kozlowski, 1993; Hunt and Hodgson, 2010). Common models of life-history theory assumes that fecundity and survival are functions of organism's age, while it is also important to consider what is defined as the organism's "state", i.e. the physiological and environmental conditions that determine survival and reproduction (Houston and McNamara, 1992; McNamara and Houston, 1996).

A life-history trait is any character correlated with total fitness when all other traits are held constant (Schutler et al., 1991). The heritability for life-history traits is usually small (Houle, 1992). However, there is ample genetic and residual variability that can be explained by the great number of genetic and environmental events that affect life-history traits or by lack of stabilizing selection to reduce the phenotypic variance (Houle, 1992; Fabian and Flatt, 2012). The expression of genetic variation for a trait may increase in unfavourable environmental conditions, at least there seems to be such a trend for morphological traits (Hoffman and Merilä, 1999). Despite the great variability of life-history traits, their evolution is constrained by trade-offs thus natural selection cannot maximise fitness beyond certain limits.

A trade-off exists when an increase in one trait, thus a benefit in fitness, is linked to the decrease in another trait, thus a cost in fitness (Williams, 1966; Pianka

and Parker, 1975; Daan and Tinbergen, 1997). Trade-offs are usually described as negative correlations between traits and can occur at different levels: at the phenotypic level between traits directly measured on the individual and related to survival and reproduction, at the genetic level between traits related to quantitative, molecular or mendelian genetics, at the intermediate level between physiological and developmental mechanisms under endocrine control that result in allocation of resources among reproduction, growth, maintenance, storage and survival (Stearns, 1989; Fabian and Flatt, 2012). However, the direction of the correlation depends on the relative genetic variation in acquisition and allocation of resources and on the availability of the resources itself: individuals with a high ability to allocate and low ability to acquire resources can show positive correlations between life-history traits when resources are abundant (van Nordwijk and de Jong, 1986; Reznick et al., 2000). Other constraints on the evolution of life-history traits depend on physiological, biochemical, structural, developmental or phylogenetic factors or simply on low levels of genetic variation (Stearns, 1992; Zuk and Stoehr, 2002; Ricklefs and Wikelski, 2002; Shefferson, 2010; Fabian and Flatt, 2012).

Life-history variation is strongly influenced by the characteristics of the environments in which the organisms live. Thus factors like temperature, amount of resources and presence of predators have an effect on fitness-related traits. The term phenotypic plasticity refers to the ability of a genotype to produce alternative phenotypes depending on the environmental conditions during ontogeny (Nylin and Gotthard, 1998). The set of phenotypes produced in a range of environments represents the reaction norm of a genotype (Stearns and Koella, 1986; Gotthard and Nylin, 1995). Nylin and Gotthard (1998) distinguish two levels of plasticity in insects: 1) a high level plasticity in which different phenotypes (morphs) derived from genetic

polymorphism or polyphenism can be present in a population and be characterized by different life-histories and morphology and 2) a low level plasticity in which the developmental pathways are affected by the environmental factors and are under hormonal control. The phenotypic plasticity is the result of the physiological sensitivity of the organisms to the environment and can be modified by secondarily evolutionary responses and phenotype-environment interactions (Ricklefs and Wikelski, 2002). Plasticity in life-history evolution affects the genetic response to selection across environments, can produce optimal reaction norms that maximize fitness across environments and buffer against environmentally induced changes in other traits so that fitness is optimized (Stearns, 1992; Nylin and Gotthard, 1998; Fabian and Flatt, 2012). At the same time phenotypic plasticity can bear also some important costs and be limited by unreliable environmental cues (DeWitt et al., 1998). Behavioural types expressed by individuals represent an important part of phenotypic plasticity: these derive from the underlying genetic and developmental environments that determine the behavioural syndromes expressed by the population (Sih et al., 2004). Life-history trade-offs can explain why personalities differences, like bold and shy individuals, can be observed in a population: individuals with high future expectations, which have much to lose, should be more risk-averse than individuals with low expectations, who have less to lose (Wolf et al., 2007). Behaviour and life-history traits are thus intimately related through intrinsic physiological mechanisms. Some behavioural syndromes or strategies, like migration and diapause, can be considered as complex life-history traits due to their influence on the schedule of reproduction (Dingle, 1978; Dingle, 1996).

4. Evolution of migration and dispersal

The notions of migration and dispersal are often used to indicate the same type of movement and the definitions are usually highly affected by the animal group under study (Stenseth and Lidicker, 1992). However the two terms should be used to define two different processes. Dispersal indicates the increase in distance between individuals in a population and is thus more of an ecological property of a population rather than an individual behaviour (Dingle, 1996). It is promoted by factors such as competition between related individuals (Frank, 1986; Ozaki, 1995; Gandon, 1999), avoidance of inbreeding depression (Motro, 1991; Gandon, 1999; Perrin and Mazalov, 2000; Lehmann and Perrin, 2003; Pasinelli et al., 2004) and temporal variability in population size, habitat quality and availability (Holt and McPeek, 1996; Doebeli and Ruxton, 1997; Paradis, 1998; Travis and Dytham, 1999; Arlt and Part, 2008). Dispersal may be sex-biased depending on the availability of breeding options (Arlt and Part, 2008), sex differences in reproductive potential (Perrin and Malazov, 2000) and on the choosiness of female related to the inbreeding loads (Lehman and Perrin, 2003). Both foraging and migratory movements can result in either dispersal or aggregation depending on the distribution of resources (Solbreck, 1978).

Migration is an adapted behavioural syndrome allowing individuals to cope with temporary habitats that vary in availability and quality in space and time (Southwood, 1962; Denno et al., 1991; Drake et al., 1995; Dingle, 1996; Denno et al., 2001; Dingle, 2001; Alerstam et al., 2003; Dingle and Drake, 2007). The migratory behaviour is not a passive process, but is rather actively initiated, maintained and terminated by environmental cues (Kennedy, 1961; Dingle, 1972; Drake et al., 1995). Migration is distinguished from other types of movement by a characteristic persistent and straight pattern of movement and temporal inhibition of sensitivity to stimuli that would normally produce a station-keeping response (Kennedy, 1961; Drake et al.,

1995; Dingle, 1996; Dingle and Drake, 2007). The migratory syndrome is characterized by the presence of a suite of co-adapted biochemical, physiological, behavioural and life-history traits: the phenotypic variance observed for many of these traits have a substantial additive genetic component that indicates a polygenic inheritance (Berthold, 1991; Dingle, 1991; 1996; Roff and Gelinas, 2003;; Roff and Fairbairn, 2007). Some of the traits are associated by genetic correlations so that they can evolve together (Palmer and Dingle, 1986; 1989; Dingle, 1991; 1996). The migratory syndrome can be divided into two interconnected sets of components: 1) the "narrow sense" syndrome incorporates phenotypically correlated traits associated with migratory movement like fat deposition, migratory restlessness, orientation ability, initiation and termination response and 2) the "broad sense" syndrome which incorporates traits that also function in other contexts like social behaviours, morphology and life-history characters (Dingle, 2006; van Noordwijk et al., 2006). Other important migratory adaptations involve the ability to use wind currents as an aid for a successful displacement to longer distances that would not be possible using only self-powered means of locomotion (Drake and Gatehouse, 1995; Anderson, 2009; Chapman et al., 2011) and the ability to actively orient and navigate to destination using environmental cues (Wiltschko and Wiltschko, 1988; 1996; Mouritsen and Frost, 2002; Cochran et al., 2004; Zhu et al., 2008; Chapman et al., 2010). The definition of migration is then based on a particular suite of traits that determine the onset of a complex behavioural syndrome: it is not dependent on the organism, the distance travelled or the ability to perform a round-trip journey (Dingle, 2006).

There is variation in migratory performance between species, populations and individuals (Dingle, 1996). Some species retain the morphological features necessary

to migrate only during the migratory period, such as insects that lose their wing muscles through hystolisis after migration (Dingle and Arora, 1973; Solbreck, 1986; Marden, 2000). A more extreme case of variation is associated with the presence of distinct morphs in the population: in certain insect species some individuals are macropterous, developing with wings and able to migrate, while others are brachypterous, developing without wings and sedentary (Denno et al., 1991; Roff and Fairnbairn, 1991; Langellotto et al., 2000; Roff and Gelinas, 2003). The most common type of variation is due to variability in behaviour and life-history in the absence of qualitative morphological or physiological differences as is seen in species where different populations show different migratory tendency (Dingle et al., 1980a; McAnelly and Rankin, 1986; Berthold, 1991). This variability can also be at the individual level where the migratory behaviour occurs only in part of the population while the rest remains sedentary, a phenomenon called partial migration. Partial migration is interesting because it can offer some cues about the evolutionary causes of migratory behaviour (Chapman et al., 2011). For example very little is known about the physiological differences between migrants and residents.

Migration in insects can be obligatory but it usually occurs facultatively in response to environmental cues that are indicative of a future change in the quality of the environment (Johnson, 1969; Dingle, 1996). The ability to reach and exploit favourable breeding areas during the spring migration results in increased fitness and is thus an adaptive behaviour (Chapman et al., 2012). Migration in insects happens during the flight capable adult stage and it is usually a pre-reproductive event (Dingle, 1972; 1996) dependent on the trade-off for resource allocation between flight and reproduction (Rankin and Burchsted, 1992). The tendency to show migratory activity before the onset of reproduction has been termed "oogenesis-flight syndrome"

(Johnson, 1960): it is more common in females, thus the name of the syndrome, while males are generally able to show migratory-type response all along their lifespan (Johnson, 1960; Dingle, 1968; Solbreck and Pehrson, 1979; Moriya and Satoshi, 1998). The integration between reproduction and migration is mediated by the neuroendocrine system through the JH titres, which main influence is on the maturation of the reproductive system (Rankin and Riddiford, 1978; Rankin and Rankin, 1980; McAnelly and Rankin, 1986). However, the normal oogenesis-flight syndrome assumption may not fit all the migratory insect species so the model should not be applied uncritically (Sappington and Showers, 1992; Jiang et al., 2010). Migration determines where and when reproduction occurs and thus is a major component of the life histories of migratory species (Dingle, 1982; 1996).

5. Life history of the large milkweed bug, *Oncopeltus fasciatus*

The large milkweed bug *Oncopeltus fasciatus* is distributed from the Caribbean and Mexico to the northern areas of United States up to the border with Canada (Feir, 1974). Specialising on milkweed seeds as a food source, its life history is entirely dependent from its host plants, mainly *Asclepias* and *Calotropis* spp. (Chaplin, 1980; Chaplin and Chaplin, 1981). Hosts are localized through olfactory cues. Once a milkweed patch is detected and settled the probability of remaining and the choice of an oviposition site by females depends on the density of host plants and developing pods (Feir and Becks, 1963; Ralph, 1977a,b). The presence of milkweed seeds seems to provide sensory cues that affect the level of JH titre resulting in promotion of sexual activity, an effect more evident in males than females (Walker, 1978). During growth, nymphs, which are unable to penetrate seed pods, and young adults release a pheromone that promotes the formation of aggregation on milkweed

pods (Aller and Caldwell, 1979). These could help the nymphs to reach the seeds through the wall's pods due the higher amount of salivary secretions that contains digestive enzymes (Ralph, 1976).

Milkweed bugs have a bright aposematic coloration. The aggregation of nymphs and young adults may also have a reinforcement function of the aposematic signal as antipredatory defence (Gamberale and Tullberg, 1998). Milkweed plants contain different amounts and types of cardiac glycosides, cardenolides, and different tissues of the plant have different concentrations of toxins (Duffey and Scudder, 1972; Seiber et al., 1982). Both nymphs and adults feed on seeds and sequester toxic compounds that are used for their own defence against predators (Duffey et al., 1978; Paradise and Stamp, 1991; Hill, 2006). The sequestration of toxins from the host plants occurs through morphological and physiological adaptations that appear to avoid any physiological costs of uptake and storage (Scudder and Meredith, 1982; Scudder et al., 1986). *O. fasciatus* is able to regulate the uptake of cardenolides from milkweed seeds. This happens via an active uptake and concentration of cardenolides, even if the seeds contain low levels of toxins (Vaughan, 1979) and via a regulation of the cardenolide array through metabolism and differential excretion rather than simple sequestration of toxins from the milkweed host (Moore and Scudder, 1985). Nymphal growth and development is not affected by which milkweed species they are fed nor from the cardenolides content: the only few differences are dependent on the amount of seed's endosperm, which differ between milkweed species (Isman, 1977).

Although *O. fasciatus* possess these feeding specializations for milkweed, it can also feed on non-milkweed host plants and it has successfully been raised on a variety of non-natural hosts after several generations of selection for laboratory conditions (Feir, 1974). Outside of laboratory controlled conditions, *Nerium oleander*

is the only non-milkweed host that has been reported in the wild that confers some degree of reproductive success without a long-term acclimation, but it is mainly exploited during the period of the year when milkweed seeds are not available (Klausner et al., 1980; Miller and Dingle, 1982).

The blooming of milkweed plants follows the seasonal northward latitudinal gradient of photoperiod and temperature, beginning from spring in the southern areas and continuing until late summer in northern areas (Woodson, 1954). Seeds are thus produced and available in different areas in different periods of the year. Milkweeds usually form patches that can be very abundant in certain areas (Hartzler and Buhler, 2000; Hartzler, 2010), but very scattered or even absent during certain period of the year in others (Miller and Dingle, 1982; Dingle, 1992). Migration and diapause are behavioural and life-history strategies used by milkweed bugs to cope with the seasonal abundance or lack of food resources and to find available oviposition sites to colonize (Dingle, 1972; 1978; 1996). Thus milkweed bugs migrate northward during the late spring and early summer months, colonizing areas with new blooming milkweed hosts and migrate back in the southern overwintering areas in late summer and early autumn when temperature and photoperiod are decreasing. This migration is a multigenerational event similar to the monarch butterfly colonization of the northern temperate areas of US (Brower, 1995; Dingle, 1996). The main cues triggering the migratory behaviour are the variation in photoperiod and temperature indicative of a future change in environmental conditions: starvation does not seems to increase the proportion of individuals performing a migratory flight rather it increases non-migratory short duration flights (Dingle, 1968).

Individuals from temperate populations show a propensity to migrate under certain conditions of photoperiod and temperature (Dingle, 1966; 1968). Wing length,

a proxy for body size, is positively correlated to the ability to undertake long duration flight (Dingle et al., 1980a). Selection on wing length increases the percentage of individuals undertaking long duration migratory flights and fecundity is positively genetically correlated with wing length and flight propensity while development time is negatively correlated with wing length (Palmer and Dingle, 1986; 1989). These traits have the ability to evolve together in the temperate populations as a migratory behavioural syndrome formed by a suite of genetically correlated traits that allow the successful colonization of available milkweed patches (Dingle, 1996). Tropical populations show lower propensity to migrate, with a lower proportion of individuals performing long duration flights (Dingle et al., 1980a). These populations also lack the positive genetic correlations between wing length, flight propensity and fecundity of temperate populations suggesting that tropical populations of milkweed bugs have not evolved a migratory syndrome (Dingle and Evans, 1987; Dingle et al., 1988). Populations from Florida and Georgia are somewhat between these two extremes (Dingle et al., 1980a).

Milkweed bugs show a facultative reproductive diapause triggered by short photoperiod (Dingle, 1974). The diapause can be broken by high temperature and long photoperiod (Dingle, 1974) and selection under constant conditions leads to a gradual acclimation and disappearance of the diapause response (Dingle et al., 1977). There is great variation in the onset of diapause between different populations of milkweed bugs: temperate bugs enter diapause in short photoperiod and low temperature conditions while tropical bugs do not enter diapause in any conditions (Dingle et al., 1980b). Populations from Florida and Georgia show the highest variation, entering diapause in a wider range of environmental conditions (Dingle et al., 1980b). Male's sexual behaviour can influence the onset of reproductive diapause

in females (Hayes and Dingle, 1983). Maternal effects also influence variation in the diapause response. For example, offspring from diapausing mothers will not enter diapause (Groeters and Dingle, 1987; 1988). Finally, diapause is a strategy to cope with periods of food shortage in areas where milkweed seeds are seasonally unavailable (Klausner et al., 1980).

Diapause and migration are intimately related: the onset of diapause in short photoperiod and low temperature conditions in autumn allow the reproductive system to be shut down, directing resources to migratory behaviour (Dingle, 1974; 1978). The most complete model linking migratory behaviour, diapause and reproduction is related to two threshold of activity of the JH titre (Rankin and Riddiford, 1977; 1978): titres above a higher threshold promote reproduction, while below a lower threshold diapause occurs. Intermediate JH titres promotes migratory behaviour until a sensory cues, like the discovery of a milkweed patch with mature pods, determines a rise in JH titre that promote reproduction.

6. Thesis plan

The aim of the thesis will be to study the role of reproductive physiology as a mediator of a physiological response to cope with unfavourable environmental conditions; in particular I will be using food availability and food quality as environmental stressors to elicit the physiological response. *Oncopeltus fasciatus* represents a good model to ask these questions, being a species with a high fidelity to a particular host plant. The experimental work will be done with a laboratory population adapted to feed on an artificial diet of sunflower seeds, and four wild populations collected in four different geographic locations. These include tropical, subtropical and temperate areas of distribution of the milkweed bug, thus including

populations with different migratory tendencies that exploit different milkweed species. The work will be focused on the role of oosorption in females as an adaptive physiological mechanism to cope with food shortage and shifts to alternative poor quality diets. The work on males will be focused on the effect of alternative diets on the development of sexual behaviour and how this affects male's fitness. I will then investigate the role of diet quality and onset of male's sexual behaviour on the onset of female reproductive diapause in a laboratory and a wild population. Finally, I will investigate the possible role of oosorption in the evolution of alternative strategies to migration in response to decline of the environmental quality, determined by food shortage.

I will test the following hypotheses:

Hypothesis 1: oosorption is an adaptive and plastic physiological mechanism to recover resources in stressful environments. Lack of food or poor diet will shift the distribution of resources from reproduction to survival. Thus under poor food conditions we expect that females of *O. fasciatus* will respond to environmental stress resorbing oocytes through apoptosis.

Hypothesis 2: the trade-off between reproduction and survival depends on the available diet rather than from the long-term adaptation to an alternative diet. After a long-term adaptation on an alternative food source, male's sexual investment will trade-off with survival in direct relation with the energetic balance derived from the adapted diet. A reversion to the ancestral optimal diet will be likely to affect the energetic balance between reproduction and soma in a different fashion than a long-term adaption on alternative food source.

Hypothesis 3: the onset of the female reproductive diapause is dependent on diet quality and onset of sexual behaviour in males. The diapause response will differ between the laboratory and wild population. In particular I predict that the laboratory population will not show diapause due to long-term adaptation to constant conditions of photoperiod and temperature and higher male sexual activity when compared to the wild population.

Hypothesis 4: the ability to resorb oocytes under environmental stressful conditions can have a role in the evolution of alternative behavioural strategies to cope with environmental variation. The physiological mechanism of oosorption is adaptive and can be maintained as a trait in populations living in seasonal stressful environments.

To test these hypothesis each chapter will pursue the following objectives:

Chapter 1. To understand if oosoprtion in *O. fasciatus* females happens through apoptosis in developing oocytes and if this physiological mechanism is a plastic response to poor diet conditions. I tested this question by exposing females to alternative diet conditions and measuring their ovarian physiological response.

Chapter 2. Explore the question of how the trade-off between reproduction and survival is modulated. Does it reflect the long-term adaptation to an alternative diet or is the trade-off dependent on the short-term energy balance provided by the diet to which an individual is exposed? I tested this question through behavioural observations of *O. fasciatus* males' sexual ability and investment when exposed to two alternative diet treatments: long-term adapted diet of sunflower seeds and ancestral diet of milkweed seeds.

Chapter 3. Explore how and if the female reproductive diapause differ between two populations of the milkweed bug, and what are the causes of such difference. I

tested the effect of photoperiod, temperature, diet quality and male sexual behaviour on the female reproductive diapause of a laboratory and a wild population of milkweed bug.

Chapter 4.   This chapter is a technical description of a flight mill for the study of tethered flight in insects that has been used for the behavioural tests performed in chapter 4. Flight in insects is an important behaviour, particularly in migratory species, and its occurrence is related to a multitude of environmental and physiological factors. The combination of flight behavioural observations with physiological data can highly improve our understanding of insect behavioural ecology.

Chapter 5.   Explore the possibility that the physiological plastic response of resorbing oocytes under challenging environmental conditions may underline the evolution of alternative behavioural strategies to cope with food stress. I studied the oosoprtion response of females characterized by alternative migratory strategies when exposed to a food rich or a food poor environment.

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Chapter 1

Oosorption as a response to poor food: complexity in the trade-off between reproduction and survival

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Abstract

Plasticity in reproductive physiology is one avenue by which environmental signals, such as poor quality food, can be coordinated with adaptive responses. Insects have the ability to resorb oocytes that are not oviposited. Oosorption is proposed to be an adaptive mechanism to optimize fitness in hostile environments, recouping resources that might otherwise be lost and reinvesting them into future reproductive potential. We tested the hypothesis that oosorption is an evolved mechanism by which females can reallocate resources from current reproductive effort to survival and future reproduction when conditions for reproduction are poor by examining the reproductive physiology and life history outcome under poor quality food in populations of the milkweed bug (*Oncopeltus fasciatus*) that have adapted to live on sunflower seed. Females fed a diet of pumpkin seeds, known to be a poor host food, had higher levels of ovarian apoptosis (oosorption), lower reproductive output, but no reduction in lifespan under poor nutrition, as predicted under the oosorption hypothesis. However, the schedule of reproduction was surprising given the "wait to reproduce" assumption of oosorption as early fecundity was unaffected.

Keywords: nutrition, oosorption, energy allocation, ovarian apoptosis, reproductive investment

Introduction

One of the fundamental challenges for research in biodiversity is to understand how organisms respond to changing or novel environments. In particular, how do organisms meet the challenge of suboptimal, stressful, or atypical environments? We expect animals to have evolved mechanisms to balance the conflicting energy

requirements for reproduction and survival (Bell and Koufopanou, 1986; Stearns, 1989; Messina and Fry, 2003; Partridge et al., 2005; Cox et al., 2010; Stoltz et al., 2010). Under suboptimal environmental conditions, such as reduced or poor quality food, it is assumed that any energy saved by a reduction in reproduction can be used to increase survival, deferring reproduction in order to survive until conditions improve.

Insects, like many animals, have the ability to respond plastically to environmental stress, resorbing oocytes that are not oviposited (Bell and Bohm, 1975), an adaptive mechanism to optimize fitness in hostile environments by recouping resources that might otherwise be lost (Bell and Bohm, 1975; Papaj, 2000; Barrett et al., 2008; Boggs, 2009). These resources can then be reinvested into somatic functions that increase lifespan and future reproductive potential. Thus, the ability to resorb eggs provides the opportunity to plastically respond to varied environmental conditions throughout a reproductive lifetime.

The predicted positive phenotypic correlation between egg resorption and longevity has been documented, but is somewhat weak. Most studies of oosorption in insects focus on host plant availability and quality (Awmack and Leather, 2002). For example, in butterflies, a reduction in food leads to a reduction in fecundity, accompanied by an increase in oocyte resorption, but no reduction in lifespan (Boggs and Ross, 1993). In ladybird beetles a reduction in host plant availability, resulting in low oviposition due to egg resorption, is correlated to periods of increased survival in females but not in males (Ohgushi, 1996). The conclusions drawn from both of these studies depend on the correlation between egg resorption and subsequent female survival. They suggest, but do not demonstrate, a trade-off between reproduction and

lifespan mediated by the recycling of nutrients invested in oocytes that cannot be used.

The oosorption hypothesis is underpinned by the 'Y model' (van Noordwijk and de Jong, 1986; King et al., 2011), in which negative correlations among traits such as reproduction and longevity, and current versus future reproduction, arise through competition for limiting resources. Under food or host stress, oosorption will lead to nutrients being redirected from eggs to somatic maintenance. While there is support for the 'Y model'(e.g. King et al., 2011), recent work on the molecular mechanisms underlying the trade-off between reproduction and survival demonstrates that it cannot be fully understood through simple competition for resources between reproduction and somatic maintenance (Partridge et al., 2005; Boggs, 2009; Flatt and Schmidt, 2009; Stearns, 2011). Studies are beginning to address the physiological nature of these trade-offs in natural populations (Cox et al., 2010; Stotz et al., 2010). Their results emphasise the potential complexity underlying the trade-off within and across taxa and illustrate the need to examine these in multiple species and environments.

We investigated the potential for oosorption to play a role in the response to novel food in the milkweed bug, *Oncopeltus fasciatus*. The evolutionary ecology of North American populations of *O. fasciatus* has been well documented. Northern populations of *O. fasciatus* are migratory; they overwinter in southern states and migrate north in spring with the flowering and seed set of the host plant *Asclepias syriaca* (common milkweed; Palmer and Dingle, 1986; Dingle et al., 1988; Leslie, 1990; Dingle, 1992) and are adapted to an abundant and reliable food supply (Palmer and Dingle, 1986). Florida populations show a greater variance in migratory and diapause behaviour (Dingle et al., 1980) and show an increased level of host

acceptance, having the ability to feed on alternative hosts when *Asclepias* seeds are temporarily not available (Klausner et al., 1980). Puerto Rican populations, on the other hand, are non-migratory and adapted to an ephemeral and limited food supply as their host plant, *A. curassavica*, provides fewer seeds and is cleared by farmers (Dingle et. al, 1980, Dingle et al., 1992). These adaptations are evident in comparisons of the genetic architecture between populations. Iowa bugs show a "migratory syndrome" with genetic correlations among body size, wing length, flight capacity and early fecundity (Palmer and Dingle, 1986). These genetic correlations are not present in the Puerto Rico populations (Dingle et al., 1988).

Although *O. fasciatus* preferentially uses milkweed when available, a general ability to adapt to new host plants for food has also been shown using experimental evolution. *O. fasciatus* can be reared on a variety of food sources including sunflower, cashew and pumpkin seeds and peanuts (Beck et al., 1958; Gordon and Gordon, 1971; Feir, 1974). Although initial performance on these alternative hosts is reduced in comparison to performance on milkweed, experimental evolution imposed by exclusive use of these hosts for more than 10 generations leads to improved performance (Gordon and Gordon, 1971; Feir, 1974).

To study the role of oosorption in responding to a poor food source use we used a laboratory strain derived from the Iowa population that has undergone experimental evolution and is adapted to live on sunflower seeds. The population we used for this study has been in culture for over 45 years and reared exclusively on sunflower seeds, which corresponds to over 400 generations of artificial selection. This population appears to have expanded its host range, as it performs well on either sunflower or milkweed. Using a novel food, pumpkin seed, to manipulate food quality, we tested the prediction that reduced food quality leads to increased

oosorption, decreased reproductive output and no reduction in lifespan. Because investment in reproduction can include both the production of gametes and mating effort (Stoltz et al., 2010), we measured the impact of diet on the physiological mechanism of oosorption (ovarian apoptosis), reproductive output and lifespan of females, as well as the rate of sexual maturation and mating behaviour. As a control, we also asked whether females that have adapted to a new food source have retained their ability to utilise the ancestral food, milkweed seeds.

Materials and methods

Animal husbandry and experimental set-up

We obtained sunflower adapted *O. fasciatus* cultures (Figure 1) from Carolina Biological Supply (Burlington, NC, USA). We housed colonies at 24° C on a 16/8-hour light/dark cycle. Cultures were supplied with *ad libitum* sunflower seeds and deionised water, and absorbent cotton wool as an oviposition site. We selected newly emerged adults daily from a culture of late instar nymphs. On the day of adult emergence, we placed one female and one male in a petri dish supplied with a cotton dental wick wetted with deionised water, absorbent cotton wool for an oviposition site, and a*d libitum* food. Half of the pairs received organic, unsalted sunflower seeds (adapted diet) and the other half received organic, unsalted pumpkin seeds (novel diet; Goodnessdirect.co.uk, Daventry, Northamptonshire, UK) as a food source. 44 pairs were set up on pumpkin seed and 41 pairs were set up on sunflower seed.

In a separate experiment, we repeated this design using the same stock population but divided the pairs between organic, unsalted sunflower seeds and their ancestral food, milkweed seeds (Educational Science, League City, Texas, USA). We followed the

same rearing protocols and experimental design as described above. 47 pairs were set up on milkweed seed pairs and 44 pairs were set up on sunflower seed pairs.

Effect of diet on development of sexual maturation, mating rate, fecundity and lifespan

We checked petri dishes daily. The first date that copulation was observed in a dish was recorded as a measure of sexual maturation. For the pumpkin versus sunflower experiments, we also recorded daily copulation rates in pairs over 5 consecutive days, from 14 to 19 days post-adult emergence. We chose this time period to ensure that all pairs had reached sexual maturity and were not yet senescing. Every day at the same time, we recorded which pairs were observed *in copula*. For the milkweed versus sunflower experiment, we observed pairs daily until first observed mating, and then at regular intervals over the females' lifespan. We also recorded the dates that eggs were first observed in the dish (typically on the cotton wool oviposition site, but occasionally eggs were found in the food dish), and the date that newly hatched offspring were first observed.

We provided pairs with food and water as needed and recorded the date of death of the female in the pair. Occasionally a male died and then that male was replaced with random male of a similar age that had been fed the treatment diet of his partner. Once per week following the hatching of the first offspring we replaced the dental wick, food dish, and cotton wool oviposition site. We determined reproductive success for each pair by counting the number of eggs present on the cotton wool. Because the first cotton wool wasn't removed until after the first offspring hatched, data for the first reproductive event included both the number of hatchlings and the number of eggs. Under the culture conditions used, eggs took approximately one

week to hatch so the subsequent oviposition sites mainly contained eggs at various stages of development although newly hatched offspring were present on occasion. Thus, reproduction can be examined over the lifespan of the female, and data include the weekly production of eggs from first reproduction until death.

Ovarian apoptosis

A subset of the pairs housed on pumpkin and sunflower was used to examine female ovarian apoptosis at 10 days post-adult emergence. We dissected ovaries from females and stained them using the Vybrant Apoptosis Assay kit #4 (Molecular Probes, Invitrogen, Eugene, OR, USA) as described by Moore and Sharma (2005). This stain contains two dyes: the dye YO-PRO-1 (green fluorescence) can enter apoptotic cells but is excluded from healthy cells, while propidium iodide (red fluorescence) can not enter living or apoptotic cells due to its large molecular size and thus only stains cells which are either necrotic or in the late stages of apoptosis (Willingham, 1999; Moore and Sharma, 2005). Healthy cells are unstained; apoptotic oocytes are green; and oocytes in the late stages of apoptosis or that are necrotic are red (Moore and Sharma, 2005). We examined our slides using an Olympus BX51 epifluorescence microscope (Olympus UK Ltd., London, UK). For each female we examined ten ovarioles and counted the number of ovarioles that displayed either green or red fluorescence. We did not observe any ovarioles that showed exclusively red stain, indicating necrosis or tissue damage due to dissection. Therefore the data collected was the number of ovarioles out of the 10 observed that showed evidence of apoptosis (green and red fluorescence). Staining was done blind relative to food treatment by coding females just prior to dissection. Codes were only revealed after scoring the staining levels of the ovaries.

Data analysis

We used JMP version 5.0.1a (SAS Institute, Marlow, Buckinghamshire, UK) for our statistical analyses. Except where specified, data were analysed using ANOVA. We used repeated measures ANOVA to analyse the change in reproductive output over time between the pumpkin and sunflower seed treatments. The repeated measures output provide information on single degree of freedom contrasts, it does not provide focused pair-wise comparisons. As we had *a priori* specific comparisons we wished to make, contrast analysis of paired comparisons is appropriate. Therefore, we tested specific hypotheses using paired *t*-tests to examine our *a priori* pairwise contrasts between subsequent ages (Rosenthal and Rosnow, 1985). The difference in female longevity between the two treatments was examined using a Wilcoxon rank sum test.

Results

Effect of novel versus adapted diet on development of sexual maturation, mating rate, and egg quality

Pairs of individuals fed the novel diet of pumpkin seed took, on average, about one day longer to develop sexual maturity, as measured as the mean number of days post adult emergence when first copulations were observed (Figure 2a, $F_{1,\ 78} = 7.696$, $p = 0.007$). Once sexually receptive, however, pumpkin-fed pairs are more likely to be observed *in copula* than sunflower-fed pairs (Figure 2b; $F_{1,\ 89} = 4.17$, $p = 0.044$) when observed between 14 and 19 days post-adult emergence.

Once females had mated, they developed and laid the initial group of eggs at the same rate in both pumpkin- and sunflower-fed pairs, measured as the mean number of days between first mating and first eggs observed (Figure 2c; $F_{1,\ 78} = 0.402$, $p = 0.528$). There also was no difference in development rate between the eggs

laid by pumpkin-fed females and sunflower-fed females, measured as the mean number of days between appearance of the first eggs and appearance of the first hatchlings (Figure 2d; $F_{1, 73} = 0.935$, $p = 0.337$). Combined, this resulted in pumpkin-fed pairs producing their first hatchlings with a delay of about one day, with newly hatched offspring observed in pumpkin-fed pairs at a mean of 16.44 days post adult emergence and in sunflower-fed pairs at a mean of 15.55 days post adult emergence ($F_{1, 75} = 10.147$, $p = 0.002$).

Effect of ancestral versus adapted diet on development of sexual maturation, mating rate, and egg quality

Pairs of individuals fed the ancestral diet of milkweed seed took, on average, about one day less to develop sexual maturity, as measured as the mean number of days post adult emergence when first copulations were observed (Figure 3a; $F_{1, 54} = 5.809$, $p = 0.019$). Once sexually receptive, however, milkweed- and sunflower-fed pairs are equally likely to be observed *in copula* (Figure 3b; $F_{1, 54} = 0.001$, $p = 0.973$) when observed at regular intervals over their lifespan.

Once females have mated, they developed and laid the initial group of eggs at the same rate in both milkweed- and sunflower-fed pairs, measured as the mean number of days between first mating and first eggs observed (Figure 3c; $F_{1, 46} = 0.480$, $p = 0.492$). There also was no difference in development rate between the eggs laid by milkweed-fed females and sunflower-fed females, measured as the mean number of days between appearance of the first eggs and appearance of the first hatchlings (Figure 3d; $F_{1, 48} = 0.111$, $p = 0.740$).

Effect of novel versus adapted diet on levels of oosorption

To explore the potential physiological mechanism underlying response to the poor food environment, we compared levels of ovarian apoptosis in females fed the novel diet of pumpkin seed and those fed the adapted diet of sunflower seed. In *O. fasciatus*, each of the paired ovaries contains 7 ovarioles. The anterior end of the ovariole contains the germarium with trophocytes, oogonia and prefollicular tissues (Bonhag and Wick, 1953). Posterior to the germarium is the vitellarium that typically contains 3 follicles that range in maturity from young (anterior) to older (posterior) follicles. Pumpkin-fed females had higher levels of ovarian apoptosis at 10 days (Figure 4a; $F_{1, 22} = 36.664$, $p < 0.001$). The green fluorescent dye that is indicative of apoptotic cells was observed mainly in the anterior germinarium and nutritive cords, rather than in the three developing oocytes (Figure 4b).

Effect of novel versus adapted diet on fecundity and lifespan

When all females were analysed for total numbers of eggs laid over their lifespan, pumpkin-fed females laid fewer eggs than sunflower-fed females (Figure 5a; $F_{1, 75} = 4.501$, $p = 0.037$). The pattern of reproduction over time was examined on the subset of females that laid eggs over at least 6 weeks. In both pumpkin-fed and sunflower-fed females the number of eggs produced changed over time (Figure 5c; within subjects, $F_{5, 17} = 15.370$, $p < 0.001$), although the change did not depend on the food type (within subjects, time*food $F_{5, 17} = 1.198$, $p = 0.352$). There was a significant difference in the pattern of egg-laying between pumpkin-fed and sunflower-fed females (Figure 5c; between subjects $F_{1, 21} = 10.539$, $p = 0.004$). Specific pair-wise comparisons showed no difference in reproductive output occurred in the first three weeks off egg production, but pumpkin-fed females had fewer

offspring in weeks 4, 5 and 6 (Table 1).   The overall result was that in this subset of females, for which the time available for reproduction is controlled, pumpkin-fed females laid fewer eggs over their lifespan than sunflower-fed females (Table 1). When all individuals were considered, there was no significant difference between survival of females fed the novel diet of pumpkin seeds and those fed the adapted diet of sunflower seeds (Figure 6a; Wilcoxon $\chi^2 = 0.022$, d.f. = 1, $p = 0.881$).   While sample sizes are small for an accurate survival analysis, this was also the case for the females used in the repeated measures analyses (Wilcoxon $\chi^2 = 0.608$, d.f. = 1, $p = 0.436$).

Effect of ancestral versus adapted diet on fecundity and lifespan

The total number of eggs laid by milkweed- and sunflower-fed females over their lifetime was the same (Figure 5b; $F_{1,49} = 2.289$, $p = 0.137$).   There was no difference between survival of females fed milkweed seeds and those fed sunflower seeds (Figure 6b; Wilcoxon $\chi^2 = 0.562$, d.f. = 1, $p = 0.454$).

Discussion

As predicted, *O. fasciatus* responded to a novel diet in a manner consistent with the oosorption hypothesis; resorbing oocytes and reallocating resources to somatic maintenance as evidenced by no reduction in lifespan, despite the poor quality of the novel diet.   While overall our results support the hypothesis that oosorption *via* ovarian apoptosis is a mechanism by which females can respond to novel or poor food environments, the details of the impact of a novel food source on life history traits indicated that the ability to respond is likely to be complicated by constraints of development and physiology. Our results provide evidence for a trade-

off between current and future reproduction. This plastic response to food stress is common; poor nutritional environments trigger a physiological state geared toward survival at the expense of reproduction (Rion and Kawecki, 2007). One mechanism proposed to underlie this trade-off is oosorption. Oosorption is considered to be an adaptive mechanism to conserve resources invested in eggs when conditions for reproduction are poor (Bell and Bohm, 1975). It is presumed that these resources can then be allocated to survival until conditions for reproduction improve. While the positive correlation between oocyte resorption and survival has been documented in a few species, it is becoming clear that the trade off between reproduction and survival is more complex than simple competition for limited resources (Partridge et al., 2005; Boggs, 2009; Flatt, 2011; Stearns, 2011).

The population of milkweed bugs we have used in our study has had many generations under experimental evolution to adapt to a novel diet. Our results on life history traits of this population on milkweed seed, the ancestral diet, compared to sunflower seed, the adapted diet, support our observation that this adaptation is an expansion of host range rather than a substitution, as has been seen in other species (e.g. Messina and Jones, 2009). Females fed ancestral and adapted diets do not differ in fecundity or lifespan under laboratory conditions. It may be that the difference in time to sexual maturation, which is shorter on milkweed seeds, may have some advantage under natural conditions and highlights the need to be cautious about interpreting fitness effects in experimental populations in the laboratory.

The difference between adapted and novel diet affected a number of life history traits. Females fed pumpkin seeds were delayed in producing offspring by one day. Females fed pumpkin seed also mate more often than those fed sunflower seed. Given that these females physiologically seem to have shifted away from a

reproductive mode and towards a survival mode, it seems counter intuitive that they would invest in a reproductive behaviour which presumable reduced the amount of time available for foraging. Because of the way we did these experiments, it is impossible to separate out the effect of diet on the behaviour of males and females. Thus, this increase in mating rate may be due to a diet effect on males. It is known in other lygaied bugs that mating influences female life history. In *Lygaeus equestris*, mating is costly and can affect both longevity and fecundity (Shuker et al., 2006). It is possible that in the lygeids these effects could be mediated via accessory gland proteins (Himuro and Fujisaki, 2008), which could vary under different male diets. While we did not observe any difference in egg quality based on development rates of embryos, we did observe a decrease in the number of eggs produced. This reduction in egg production was mirrored by an increase in ovarian apoptosis. However, our observations do not support a straightforward reduction in eggs laid through resorption of eggs that have initiated vitellogenesis. Apoptosis in the ovarioles was observed mainly in the region of the ovary where new oocytes are born rather than in oocytes undergoing vitellogenesis. Thus, the females do not appear to be "raiding the oocyte larder" for resources, but rather constraining future reproductive potential. This is also reflected in the pattern of reproduction observed between the pumpkin and sunflower fed females in which early fecundity is unaffected, but future clutches are smaller. This schedule of reproduction does not fit the "wait to reproduce" assumption of the oosorption hypothesis.

In many ways, the treatment of the novel food approximates dietary restriction, known to result in a shift in investment from reproduction to longevity (Partridge et al., 2005). *O. fasciatus* presented with pumpkin seeds may simply choose not to feed, being highly specific in their food choice (Feir, 1974).

Alternatively they may feed but be unable to digest the constituent components. The transcriptome of the salivary gland from sunflower adapted *O. faciatus* has been sequenced (Francischetti et al, 2007), and it would be interesting to compare the transcriptome of the salivary glands from adapted and ancestral populations to see if this is a target of selection.

It has recently been found that the trade-off between reproduction and longevity under dietary restriction can be broken in *Drosophila melanogaster* by supplementing with a single amino acid (Grandison et al., 2009). Mating status has also been recently shown to influence the longevity response to dietary restriction (Stoltz et al., 2010). Dietary restriction increases longevity in mated females but not unmated females, perhaps due to investment in mate seeking behaviours being prioritised in unmated females. These studies and others showing variation in the response to poor nutritional environments (e.g. Carey et al., 2008) demonstrate that in order to understand how physiological and molecular mechanisms interact with internal and external factors in shaping the trade-off between reproduction and survival we need to examine these trade-offs in a variety of species (Stoltz et al., 2010; Flatt et al., 2011) in order to begin to develop the framework for predicting how shifts in diet availability will impact on insect populations.

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Tables

Table 1. Specific pair-wise contrasts between numbers of eggs laid by females across subsequent weeks of life. The data only include those females that lived for at least 6 weeks post adult eclosion.

Egg production	F statistic	p	Mean number of eggs laid ($\pm$ SE)	
Week 1	$F_{1,21} = 3.578$	0.073	pumpkin	142.9 ± 9.3
			sunflower	168.9 ± 10.1
Week 2	$F_{1,21} = 2.280$	0.146	pumpkin	111.8 ± 8.0
			sunflower	130.1 ± 9.1
Week 3	$F_{1,21} = 1.250$	0.276	pumpkin	127.5 ± 10.9
			sunflower	145.9 ± 12.4
Week 4	$F_{1,21} = 5.094$	0.035	pumpkin	99.6 ± 11.9
			sunflower	140.4 ± 13.6
Week 5	$F_{1,21} = 9.729$	0.005	pumpkin	83.7 ± 9.8
			sunflower	130.1 ± 11.2
Week 6	$F_{1,21} = 5.465$	0.029	pumpkin	62.0 ± 13.6
			sunflower	110.1 ± 15.5
Total eggs laid	$F_{1,21} = 10.539$	0.004	pumpkin	621.5 ± 41.4
			sunflower	825.5 ± 47.3

Figures

Figure 1. A mating pair of milkweed bugs (*Oncopeltus faciatus*).
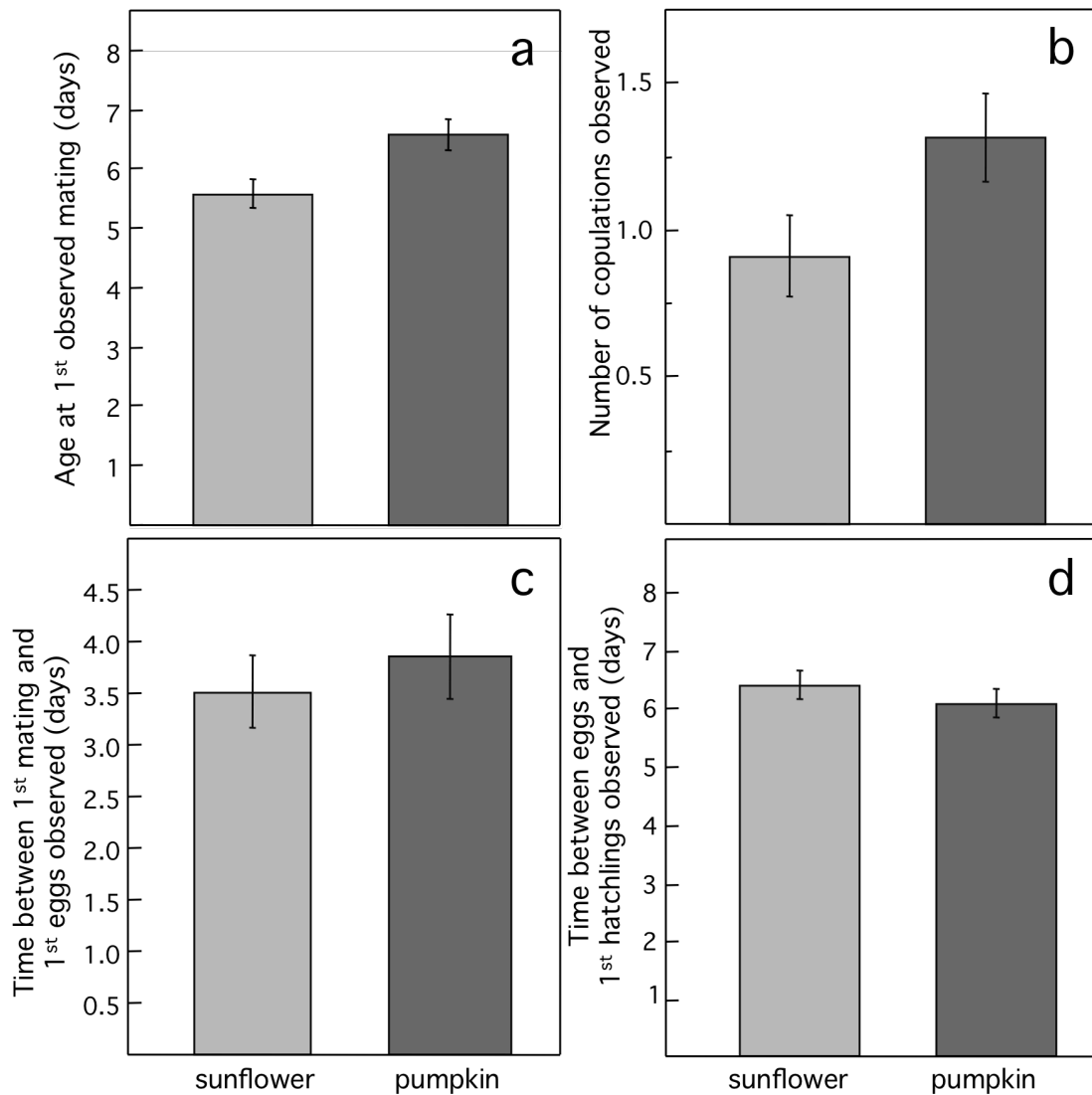
Figure 2. The effect of novel versus adapted diet on development of sexual maturation, mating rate and egg quality. The effect of pumpkin seed (dark grey bars) or sunflower seed (light grey bars) is compared for mean number of days it took for pairs to become sexually mature (a), mean number of copulations observed over 5 days (b), mean number of days it took females to produce a first clutch of eggs following mating (c) and mean development time of offspring (d). Error bars represent ± standard error.
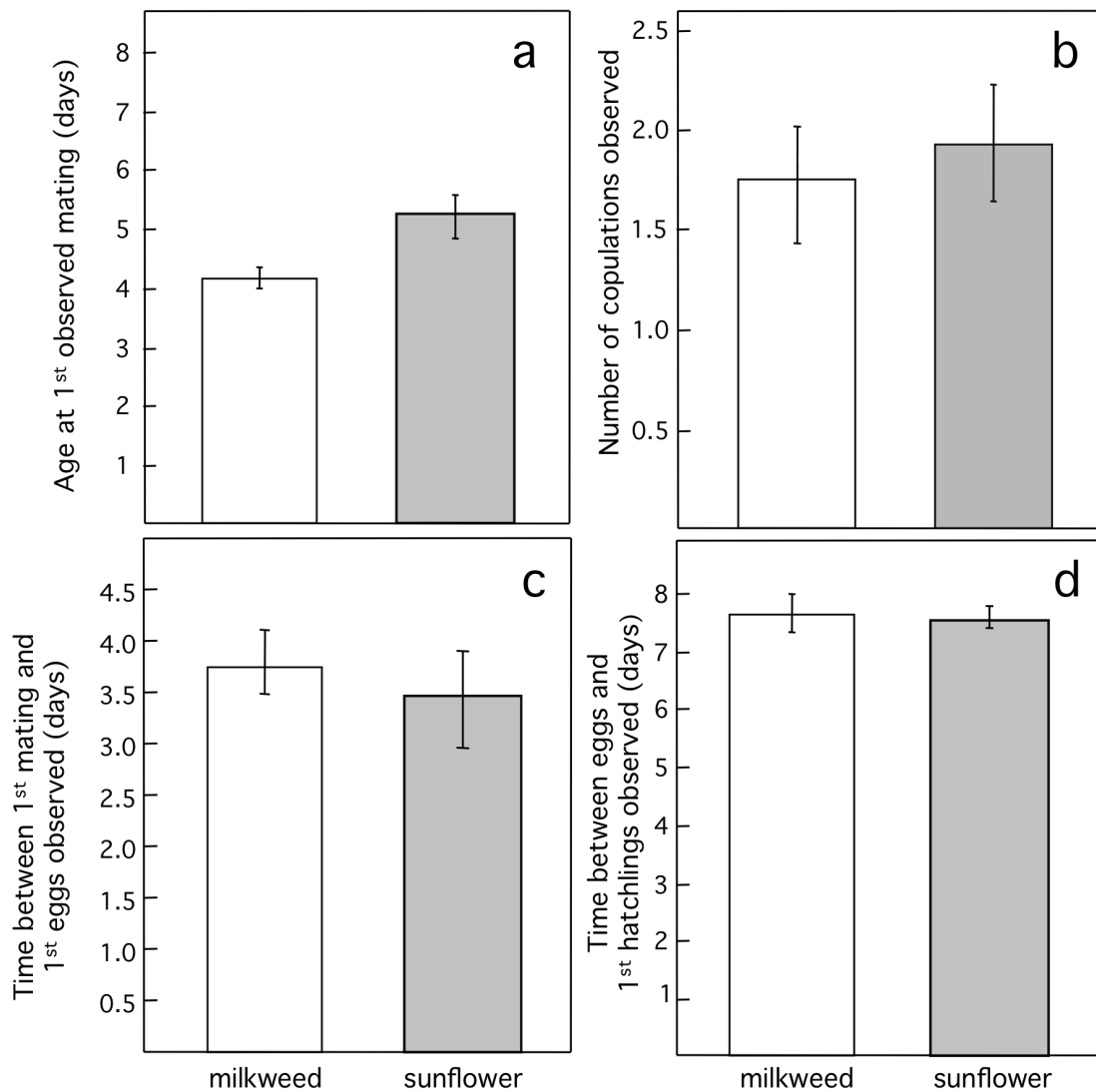
Figure 3. The effect of ancestral versus adapted diet on development of sexual maturation, mating rate and egg quality. The effect of milkweed seed (open bars) or sunflower seed (light grey bars) is compared for mean number of days it took for pairs to become sexually mature (a), mean number of copulations observed (b), mean number of days it took females to produce a first clutch of eggs following mating (c) and mean development time of offspring (d). Error bars represent ± standard error.
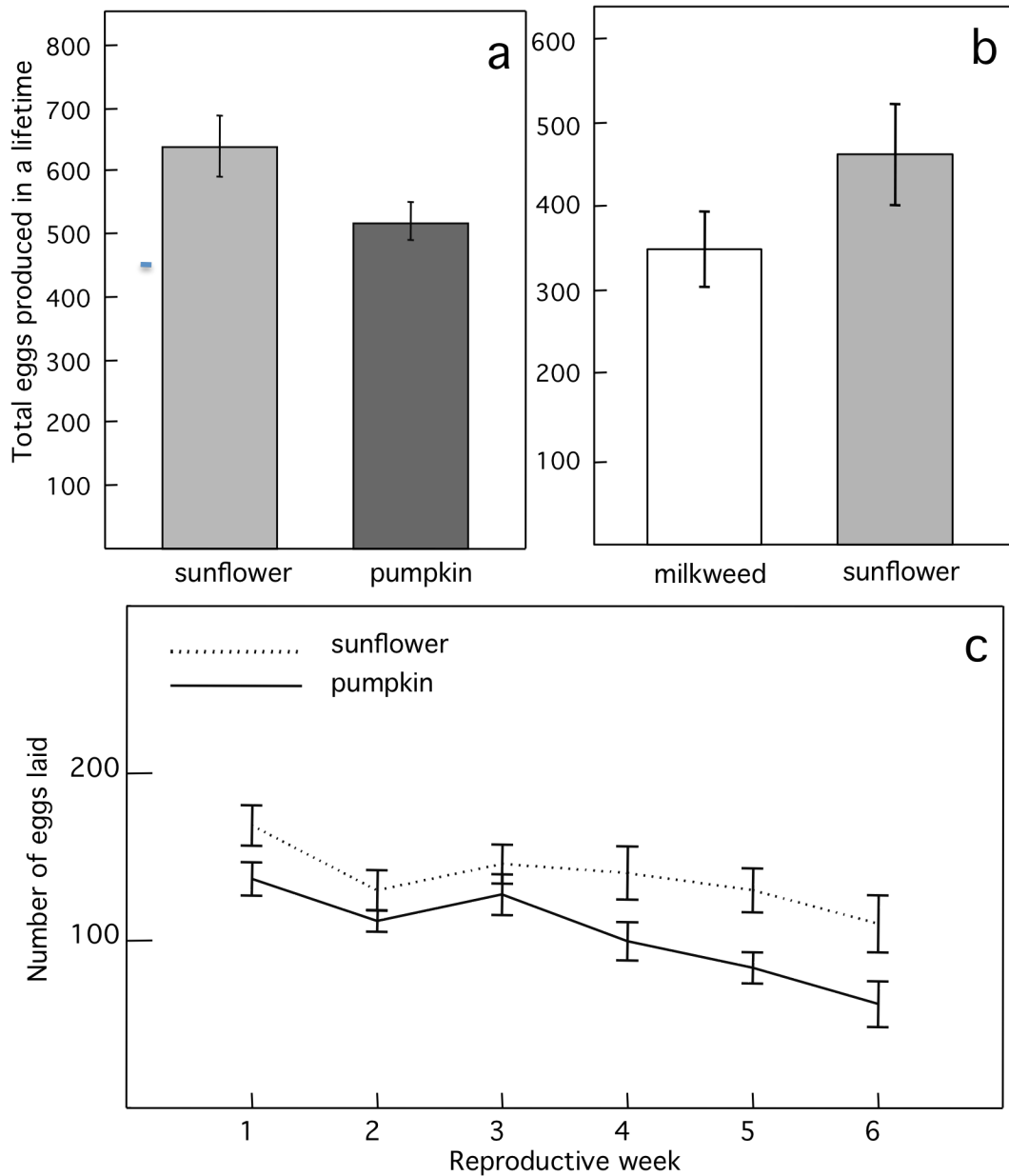
Figure 4. The effect of diets on egg production. The effect of pumpkin seed (dark bars) or sunflower seed (light bars) is compared for mean number of eggs produced over female's lifespan (a). The effect of milkweed seed (open bars) or sunflower seed (light grey bars) is compared for mean number of eggs produced over female's lifespan (b). The pattern of egg production between females fed pumpkin seed (solid line) and sunflower seed (dashed line) is compared among females that lived for at least 6 weeks post adult eclosion (c). Error bars represent ± standard error.

70
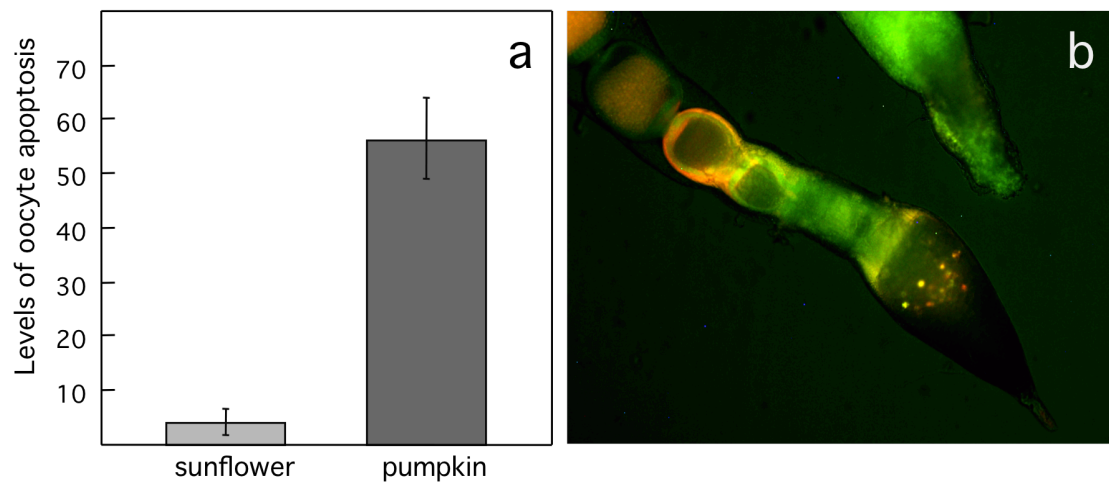
Figure 5. The effect of novel versus adapted diet on ovarian apoptosis. The levels of apoptosis in ovaries of females at 10 days post adult eclosion is compared among females fed pumpkin seed (dark grey bars) and sunflower seed (light grey bar). Error bars represent ± standard error. An example of a stained ovariole from a pumpkin fed female (b) shows that positive apoptotic cells are evident in the germinarium of the ovariole.
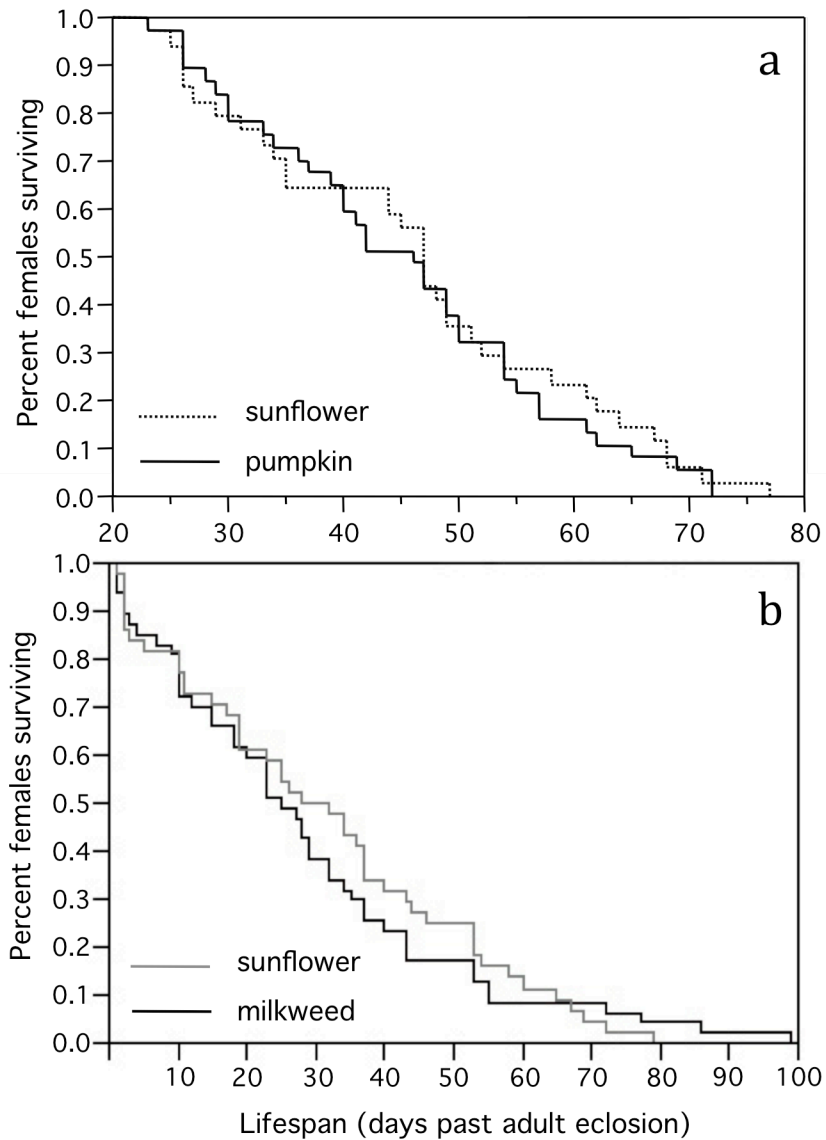
Figure 6. The effect of diet on female's longevity. The survival curves for females fed pumpkin seed (black line) and sunflower seeds (dotted line) are compared (a). The survival curves for females fed milkweed seed (black line) and sunflower seeds (light gray line) are compared (b).

Appendix A1 – oosorption assay on three diet treatments

This appendix report observations on the apoptosis assay performed on virgin females from a laboratory adapted population fed on three diet treatments: sunflower, pumpkin and milkweed seeds. These observations were performed after the completion of the experiment described in chapter 1, thus their exclusion from the published manuscript. Initially they were performed to improve the efficiency of the experimental manipulation and to test the reliability of the kit for apoptosis detection. The data here presented are supplemented with data obtained from milkweed and sunflower fed pairs used in Chapter 1.

Experimental bugs were collected from the same laboratory population described in Chapter 1. Experimental nymphs were collected from mass colonies reared in 16:8 light:dark at 25°C fed on sunflower seeds and placed in the same rearing conditions until eclosion. Newly eclosed virgin adult females were collected and placed in petri dish and a diet treatment of sunflower, pumpkin or milkweed was randomly assigned to each female. All experimental bugs were provided with water *ad libitum* and seeds were replaced every 2-3 days.

Females for the apoptosis assay were dissected at day 10. Body weight of each female was measured on the day of eclosion and again on the day of dissection. After dissection the ovaries were weighed and collected for the apoptosis assay. The ovaries were stained following the protocol described in Moore and Attisano (2011). A total of 106 virgin females were collected: 71 survived to 10 days post eclosion allowing for the collection and staining of the ovaries. Only females with more than 10 ovarioles collected have been included in the analysis for a total of 22 on milkweed diet, 22 on sunflower diet and 20 on pumpkin diet. Stained ovaries were examined for evidence of ovarian apoptosis. The level of apoptosis was scored as the number of

ovarioles showing green or red fluorescence out of the total number of ovarioles in both ovaries. The schedule of fecundity for the first 6 weeks of reproduction and longevity in milkweed and sunflower fed females was obtained using the data from Chapter 1. The repeated measure analysis was performed using only pairs that produced eggs for at least 4 weeks.

Results

Body and ovary mass

There was no difference in body mass at eclosion for all the females collected (ANOVA; $F_{2, 103}$= 0.221; p=0.802) and for the specimens used for the apoptosis analysis (ANOVA; $F_{2, 49}$=0.224; p=0.800). Body mass at death was not different for all females collected (ANOVA; $F_{2, 103}$=0.852; p=0.429) as well as for the females dissected and used for the apoptosis analysis (ANOVA; $F_{2, 49}$ =0.491; p=0.615). Ovaries of sunflower fed females were slightly heavier, but not statistically different, from ovaries of milkweed and pumpkin fed females (χ^2=4.579; d.f.=2; p=0.101; Fig. 2A).

Apoptosis assay

Sunflower fed females had significantly lower levels of apoptosis than milkweed and pumpkin fed females (χ^2=24.730, d.f.=2, p<0.0001; Fig. 3A). There was no difference in levels of apoptosis between milkweed and pumpkin fed females (ANOVA; $F_{1, 40}$=0.189; p=0.666; Fig. 3A).

Schedule of reproduction and longevity on sunflower and milkweed diets

There was no difference in total fecundity between sunflower and milkweed fed pairs (χ^2=0.562; d.f.=1; p=0.454). Milkweed pairs laid more eggs during the first 4 weeks of reproduction (between subjects; $F_{1,\ 25}$=12.747; p=0.001; Fig. 3A). There is no difference from week 5 (between subjects; $F_{1,17}$=2.037; p=0.172; Fig. 3A) and in week 6 only two milkweed pairs were still laying eggs while ten sunflower pairs were still able to lay fertile eggs. In chapter 1 the female longevity was considered using the entire sample size and this show no difference between diets (χ^2 = 0.562; d.f. = 1; p = 0.454; Fig. 6b, Chapter 1) while considering only the females used for the repeated measure analysis reported here, sunflower fed females lived longer than milkweed fed females (χ^2=6.673; d.f.=1; p=0.009; Fig. 4A).

Conclusions

Both pumpkin and milkweed fed females have higher levels of ovarian apoptosis compared to sunflower fed females. As reported in chapter 1, pumpkin fed females cope with a poor quality diet resorbing oocytes through apoptosis and this response affects the schedule of reproductive effort: the decrease in total fecundity is dependent on later rather than earlier fecundity when compared to sunflower fed pairs (Fig. 5c, Chapter 1). Female's longevity is not affected suggesting that resources are shifted from reproduction to survival (Figure 6a, Chapter 1). Total fecundity is not different between sunflower and milkweed fed pairs, but milkweed fed pairs show higher early fecundity than sunflower fed pairs, while later fecundity is similar even if the sample size in this case is quite small. Considering only the females used for the repeated measure analysis, milkweed fed females have shorter lifespan than sunflower fed females. Thus a milkweed diet tend to increase the reproductive effort in early age affecting the female's longevity but not later reproduction, while the pumpkin diet

triggers a physiological state in which higher longevity is achieved to the expense of later fecundity. The milkweed diet seems to offer some important factors that boost the reproductive effort because total fecundity does not differ between treatments even if female's lifespan on milkweed is lower compared to sunflower.

If we want to explain this pattern with the level of ovarian apoptosis then the percentage of ovarioles in apoptosis is probably not the most complete way to offer clear information on this point. Indeed milkweed and pumpkin show similar levels, which are significantly higher than sunflower. This pattern, coupled with the patterns of reproduction and survival, may suggest that the apoptosis affects different areas of the ovarioles. Thus it could be that in ovarioles of pumpkin fed females apoptosis happens mainly in the germinarium and nutritive cord rather than in the developing oocytes, while in milkweed fed females the apoptosis involves mainly the developing oocytes rather than the germinarium. This could explain why early fecundity is higher in milkweed fed females and later fecundity is lower in pumpkin fed females. From the observations of the ovarioles under microscopy there seems to be such evidence for the localization of apoptosis in different areas of the ovarioles, with milkweed fed females more likely to present apoptosis in the oocytes rather than in the germinarium and vice versa in pumpkin fed females. However, no statistics are reported here because such analysis would require a different protocol of data collection than used in this experiment. The counting methods we used previously offers a good way of comparing treatments in which the levels of apoptosis are highly different, such as the case between sunflower and pumpkin fed females. In situation where levels are similar, like in the case of milkweed and pumpkin fed females, a more refined scoring method would be advisable.

From the qualitative point of view, milkweed and sunflower diets confer similar fitness but on a different time scale, while pumpkin represents the lowest quality diet in terms of achieved fitness. Milkweed fed females concentrate their reproductive efforts early in life due to a shorter lifespan, while sunflower fed females can lay eggs for a longer period due to a longer lifespan, but there is no difference in total fecundity between treatments. This is not the case for pumpkin fed females, in which a longer lifespan is not coupled with similar reproductive performances. Thus the oosorption hypothesis holds for pumpkin fed females, but how can we explain similar oosorption levels between pumpkin and milkweed? One way to approach this may be to consider both milkweed and pumpkin as novel diets and sunflower as the adapted diet. Our laboratory adapted population has been on a diet of sunflower seeds for over 400 generations. Thus females from a laboratory population adapted to feed on sunflower seeds may react physiologically to exposure to a novel diet with higher level of oosorption, even if the novel food is represented by the ancestral wild diet of milkweed seeds that can offer some critical factor that is lacking in other diets. This means that the response is similar whatever novel diet is presented to the bugs. The main consideration is that oosorption does not automatically involve a reduction in future reproductive potential and that the level of oosorption could be dependent on the recognition of the diet as host or novel, i.e. a diet to which the bugs are used and adapted to feed on or a diet to which the bugs are never been exposed before. If this is true then it is important to recognize a possible diet effect on the differential localization of the apoptosis in the ovarioles in order to differentiate between a poor or high quality diet.

Another way to explain the pattern of fecundity is related to sexual activity. In Chapter 1 we found that milkweed fed pairs starts to mate earlier than sunflower and

pumpkin fed pairs. The increase in early fecundity and the lack of difference in total fecundity in milkweed females may derive from some mating effects. This may include a higher male's sexual activity or diet-dependent factors transferred by males to females during mating that could help to increase the eggs production. Thus a higher sexual activity by males may help to explain why the survival of the milkweed fed females used in the repeated measure analysis is lower compared to sunflower fed females, while the possibility of some factors transferred during mating may help to explain the increase in early fecundity and the lack of difference in total fecundity. The same reasoning can be applied to explain the patterns for the pumpkin diet: lower male's sexual activity increases female's lifespan and later fecundity is decreased by lack or poor quality of diet-dependent factors transferred during mating.

The female's reproductive physiology can respond in a very plastic way to the exposure to alternative diets and the resulting effects can be possibly dependent on several factors and not only on the categorization of a diet in high or low quality. In future work on the effect of alternative diets, will be advisable to include a more thorough measure of ovarian apoptosis that will include where the apoptosis is localized.

FIGURES
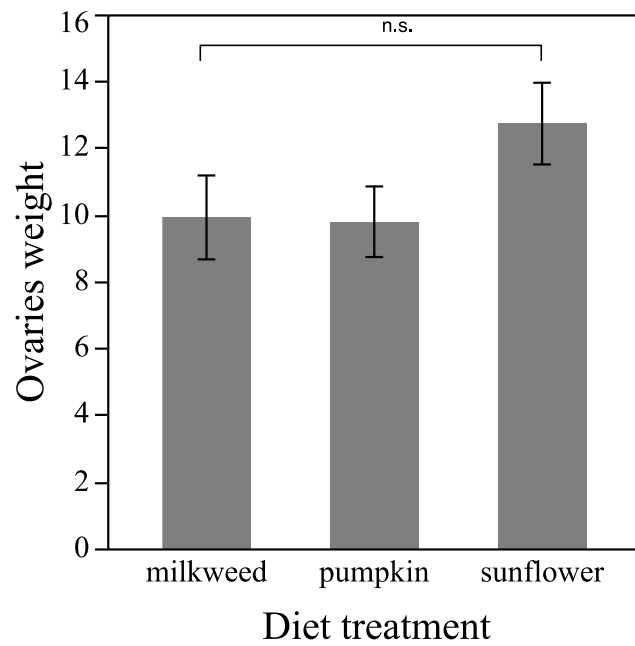
Figure A1. Ovaries' weight under different diet treatments.
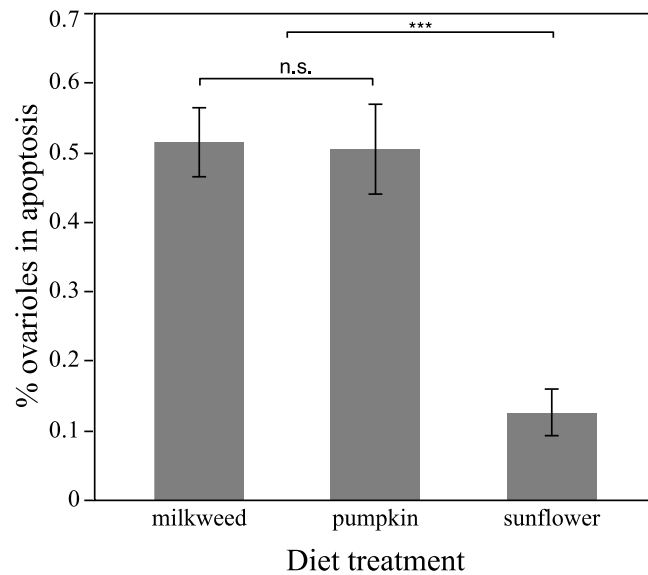
Figure A2. Proportion of ovarioles in apoptosis among females fed on different diet treatments.
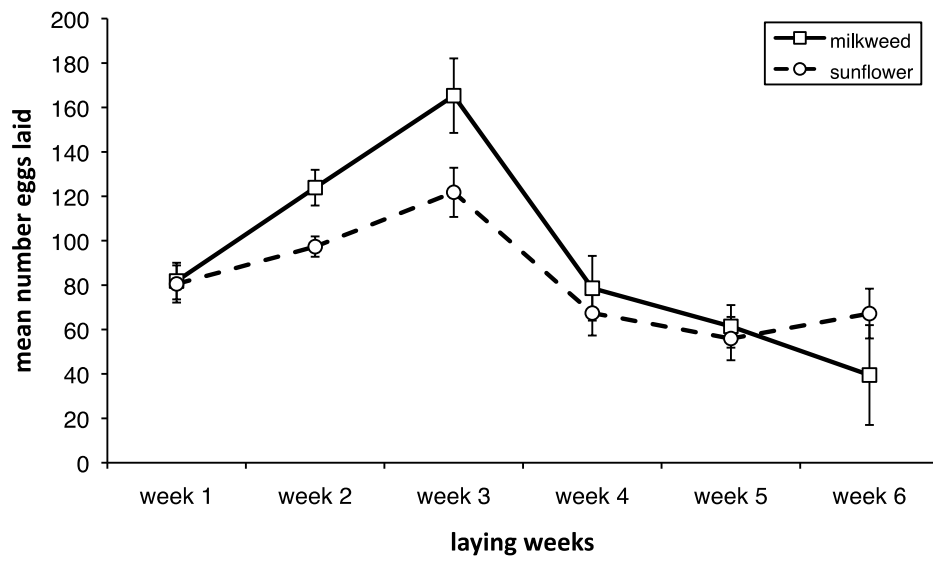
Figure A3. Schedule of fecundity for milkweed and sunflower fed pairs in 16:8 25°C rearing conditions during the first 6 weeks of reproduction.
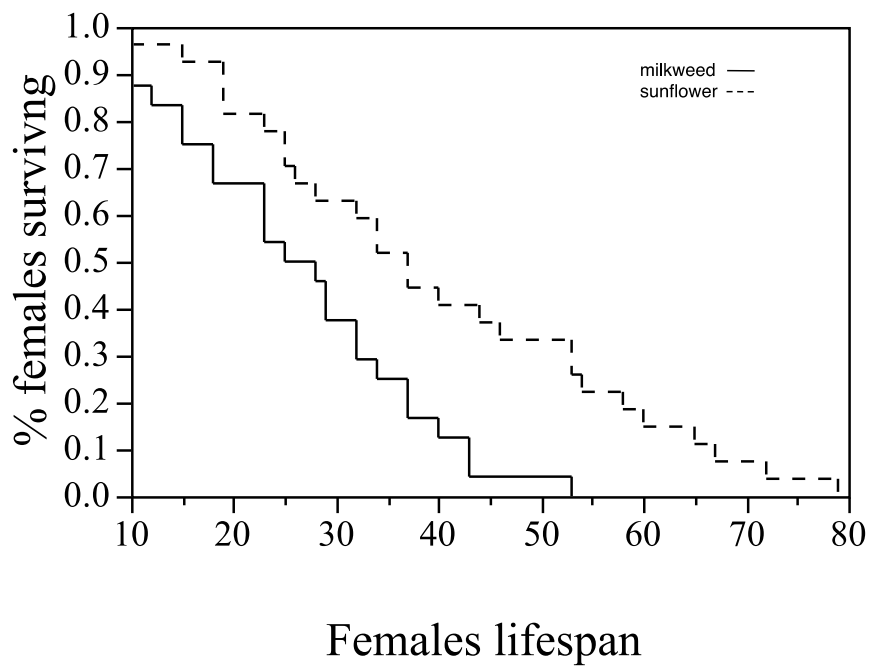
Figure A4. Survival curve for females fed on sunflower and milkweed diet and used for the repeated measure analysis reported in this appendix.

Chapter 2

Reproduction-longevity trade-offs reflect diet, not adaptation

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Abstract

A tenet of life history evolution is that allocation of limited resources results in trade-offs, such as that between reproduction and lifespan. Reproduction and lifespan are also influenced proximately by differences in the availability of specific nutrients. What is unknown is how evolutionary responses to a nutritionally novel diet influence this fundamental trade-off. We tested this by measuring trade-offs in male milkweed bugs, *Oncopeltus fasciatus*, fed either an adapted diet of sunflower or the ancestral diet of milkweed. Sunflower-fed males lived longer but invested less in reproduction, both in mating and fertility. Milkweed-fed males invested in both mating and fertility at the expense of survival. The evolution of an expanded diet was not constrained by the existing trade-off, but instead altered how the trade-off between reproduction and longevity was modulated. We suggest that this occurs because diets differ in promoting germ line development or longevity.

Keywords: reproduction, longevity, trade-offs, life-history traits, diet adaptation, male's investment.

Introduction

The qualitative and quantitative composition of available food has a significant impact on life history traits (Tu and Tatar, 2003; Magwere et al., 2004; Rion and Kawecki, 2007; Lee et al., 2008), including the trade-off between reproduction and longevity. Under a high-quality diet, longevity is likely to be shortened due the allocation of resources to reproductive activity (Hunt et al., 2004; Jacob and Evans, 2000), whereas under a poor diet organisms switch to a

physiological state geared towards survival at the expense of reproduction (Halliday, 1989; Boggs and Ross, 1993; Zera and Harshman, 2001; Kirkwood, 2002). Although the role of dietary restriction has received most attention (Narasimhan et al., 2009), nutrition plays a role in this trade-off (Grandison et al., 2009) and can be influenced by specific nutrients. For example, in *Drosophila melanogaster* experiencing dietary restriction, adding back methionine to the diet maximizes fecundity without reducing lifespan (Grandison et al., 2009). A fundamental but unresolved question is how adaptation to a new diet influences this central trade-off. If nutrition matters, an evolved response to a novel diet that differs in nutrient composition should alter the trade-off between longevity and reproduction.

We tested this hypothesis by examining the effect of ancestral and adapted diet on the reproduction-lifespan trade-off in a laboratory population of milkweed bugs, *Oncopeltus fasciatus*, experimentally evolved to survive on a novel resource of sunflower seed. In other species populations reared for multiple generations under different diet conditions result in genetically based changes in life history traits (Rion and Kawecki, 2007; Warbrick-Smith et al., 2006; Kolss et al., 2009). Wild caught populations of *O. fasciatus* perform poorly on sunflower seeds, but survival and reproduction improve within 10 generations (Feir, 1974). The population we used has been maintained exclusively on sunflower seeds for over 400 generations. Adaptation to sunflower seed in these experimental evolution lines has expanded host range, and has not resulted in the loss of ability to feed on the ancestral milkweed seed diet (Moore and Attisano, 2011). As the two diets differ considerably in nutrition (Walker, 1978), this population provides a unique resource to examine life-history evolution under two diets in a single population. While females from our laboratory population have no difference in fitness on diets of sunflower and milkweed (Moore and

Attisano, 2011), during the course of these experiments, we observed that males paired with females in the milkweed treatment died more rapidly than males paired with females on the sunflower diet. Given the sex specific effects of diet on fitness (Maklalov et al., 2008; Maklalov et al., 2009; Zajitschek et al., 2009; South et al., 2011), we designed a study to explore the potential effect of diet on life history trade-offs in males.

We tested the hypothesis that adaptation to a new diet, sunflower seed, has resulted in a change in the allocation in resources between reproduction and lifespan in male *O. fasciatus*. We examined the mating effort and longevity of males fed on two diets: the ancestral milkweed (*Asclepias syriaca*) and sunflower (*Helianthus annuls*). Our results show that even after a long period of adaptation, the trade-off between reproduction and survival on the ancestral food bears the signature of a high quality diet and that, as predicted, evolutionary expansion of diet is accompanied by a shift in life history trade-offs.

Materials and Methods

Animal husbandry

We obtained colonies of the large milkweed bug, *O. fasciatus*, from Carolina Biological Supply (Burlington, NC, USA). These cultures are adapted to a sunflower seed, having been reared exclusively on this diet for over 45 years, which translates to over 400 generations of experimental evolution in response to a novel host. We have maintained this population in our laboratory for several generations. We housed our colonies at 16L:8D 25°C with a relative humidity ranging from 50 to 65%. We formed experimental groups by placing newly eclosed males in individual petri dishes

with *ad libitum* deionised water. Our experiment was divided into two experimental feeding groups: one group of males that received organic milkweed seeds and the other group that received organic unsalted sunflower seeds. We replaced food weekly, or when the seeds became mouldy. We placed newly eclosed females in a group box with *ad libitum* deionised water and organic unsalted sunflower seeds. Females were kept virgin until the age of 7-10 days post eclosion when they were used for mating trials.

Mating behaviour

We kept males unpaired in petri dishes until they were sexually mature at 3 to 5 days of age. Under our lab conditions, all females are sexually mature by 7 days post eclosion. To ensure that individuals were sexually mature at the time of mating trials, we performed matings when males were 9 to 11 days post eclosion and females 7 and 10 days post eclosion. We paired males once per week for 6 consecutive weeks (from 9 to 46 days of age) with a new sexually mature virgin female provided each week. For each trial, we placed a virgin female in the male's Petri dish. We performed trials in the same petri dish where the males were housed, but removed food to avoid any influence of diet during mating. Short-term food restriction does not have a noticeable influence on the activity of virgin females given that the highest amount of food intake happens on the first week of adult life (Ross, 1978) and mating activity in males was not affected by short-period starvation. We ensured water was available *ad libitum*. We performed mating trials over two consecutive days.

During each mating trial, we made seven observations each day, one each hour starting from 6 hours after the onset of photophase. We chose photoperiod,

temperature and times of observations to maximize the probability of observing mating (Walker, 1979). In weeks 2 through 6 we scored speed of first mating (time from pairing of male and female to start of copulation). We observed pairs on the bench top at room temperature until the start of the first copulation. Pairs were placed in the incubator once they started to mate and we made all subsequent observations on dishes in the incubator. We evaluated mating rate as the proportion of the total number of times mating was observed out of 14 total observations during the two days of the mating trials.   We separated pairs after three days, replaced the experimental diet in the male's dish, and returned the male to the incubator for the next week's trial.   We transferred females to individual dishes for analysis of fecundity and fertility.

Mating behaviour in *O. fasciatus* is mainly under male influence (Hayes & Dingle, 1983). The male's mating capacity was assessed with two behaviours: mating speed, measured as the amount of time from the pair's formation to the mating position, and mating rate, measured as the number of times a male was observed in copula during the 14 observations over the course of the two day mating trial.   In order to compare the mating performance of males, only successful copulations were included (defined as mating speeds less than 3 hours).

Fecundity, fertility and lifespan

We housed females from week 1 of mating (mated with males of 9 – 11 days of age), week 3 of mating (mated with males of 23 – 25 days of age), week 5 of mating (mated with males of 37 – 39 days of age) in individual petri dishes with *ad libitum* organic unsalted sunflower seeds and deionised water once they were

separated from their mates. We provided cotton wool for oviposition and collected all their eggs. We mated each female only once, allowing us to assess the reproductive potential of the same males at different ages. Due to space constraints, we did not score females from week 2 (mated with males of 16 – 18 days of age), week 4 (mated with males of 30 – 32 days of age), and week 6 (mated with males of 44 – 46 days of age), and only used these females to assess the mating behaviour of males. However, we chose the weeks we measured based on the information they would provide. Week 1 represents young males that had just reached sexual maturity and it is a measure of the reproductive potential of very young males. In addition, this is the age at which in the wild bugs are ready to undertake the first migratory flight (Dingle, 1965; Dingle, 1966). Week 3 represents mature males that under natural conditions would have flown to the breeding areas and are thus ready to colonise the newly available habitat (Walker, 1979; Dingle, 1968; Dingle, 1972). Week 5 was included in the analysis because males at this age, especially virgins, show a second peak of migratory flight while females do not (Walker, 1979) and are ageing. Thus, these three weeks represent different stages in the male's life in which there could be a significant influence on his fitness.

Eggs were collected twice a week for four weeks and stored in petri dishes in 16L:8D 25°C. Fertile eggs becomes progressively darker and with an optical microscope (0.63 – 4 X) it is possible to differentiate between fertile and infertile eggs from the colour in the first instance, and then looking through the egg cases to observe the developing embryos. After a minimum period of 4-5 days spent in the incubator it was possible to differentiate fertile from infertile eggs. The number of eggs laid by each female in four weeks and the number of fertile eggs in each clutch were counted. We measured fecundity as the total number of eggs laid by each female

and fertility as the total number of eggs that initiated development (King *et al.,* 2006). We observed all experimental animals daily and recorded date of death. We collected data on lifespan of both males and females to investigate a possible effect of diet and sexual activity on longevity.

Statistical analysis

We performed statistical analyses using JMP 8.0.2 (SAS Institute, 2009). We used a repeated measures ANOVA model to analyze mating speed, mating duration, fecundity and fertility over time between the two experimental food treatments. Given the temporal nature of our experiment, we predicted *a priori* that any overall change would be observed between subsequent ages. Although the repeated measures output provides information on single degree of freedom contrasts, it does not provided focused pair-wise comparisons. As we had *a priori* specific comparisons we wished to make, contrast analysis of paired comparisons is appropriate. Therefore, whenever there was a significant overall significance in the ANOVA, we tested specific hypotheses using paired t-tests to examine our *a priori* pairwise contrasts between treatments at the different ages (Rosenthal & Rosnow, 1985). Shapiro-Wilk W test was used to test for a normal distribution of the data. We used ANOVA when data fitted the assumption of a normal distribution; otherwise we used a Mann-Whitney U test for pairwise comparisons between treatments. We used Wilcoxon χ^2 test to analyse the differences in lifespan between treatments. We used Spearman's rank correlation to analyse the relationship between male lifespan/mating rate and fertilization rate/mating rate.

Results

Dietary effect on mating behaviour

Males fed milkweed seeds initiated copulation more rapidly (Figure 1A; Table 1). Mating speed generally increased with age or experience but this effect did not differ between the diet treatments, and there was no interaction between diet and age (Table 1). Milkweed-fed males also mated more frequently than males fed sunflower (Figure 1B; Table 1). Although there was no significant effect of age, *a priori* pair wise contrasts between males at different ages showed that while the difference in mating rate was not significant between males in the first two mating trials (Table 2), the difference between treatments became evident starting from week 3 when the males were 21-23 days old and continued until week 5. In week 6 (42-44 days old) the difference between treatments approached conventional levels of significance (Table 2).

Dietary effects on male fitness

Male diet did not affect the fecundity of their mates (Figure 2A; Table 1). Male diet did have a significant effect on male fertility. While milkweed-fed males did not stimulate higher egg production by their mates, they had a greater capacity to fertilize eggs than sunflower-fed males (Figure 2B-C; Table 1). However, milkweed-fed males also had a shorter lifespan than males fed sunflower seeds (Figure 3; Wilcoxon χ^2 = 6.959; d.f. = 1; p = 0.008). Lifespan and mating rate are highly correlated in the males fed on milkweed seeds (Spearman's rank correlation; ρ = 0.672; p < 0.001), while there is no correlation in males fed sunflower seeds (Spearman's rank correlation; ρ = -0.152; p = 0.440).

The pattern of fertility across a male's lifespan was revealing. Milkweed fed males seemed in general able to fertilize more eggs than sunflower fed males (Figure 2B; Table 1) but no difference was evident in the pairwise comparisons between each mating week. Also, there was no difference in fertilization rate among males fed milkweed and sunflower at the two younger life stages, but older males fed milkweed fertilized a higher proportion of the eggs laid by their mating partner than older males fed sunflower seed (Figure 2C; Table 3). In addition, in sunflower-fed males at all ages there is a positive correlation between mating rate and fertilization rate that is lacking in the milkweed-fed males (Table 4).

Discussion

Adapting to a new diet can be accompanied by expanded patterns of life history trade-offs. We found that milkweed bugs adapted to a diet of sunflower seeds have maintained the ability to utilize milkweed seeds as a host, and show differences in how the diets influence life-history traits. Our results show that milkweed-fed males invested in reproduction, both through mating effort and fertilizing ability, at the expense of survival.

Our results on the effect of diet on mating rate and speed correspond to data on the role of nutrition in mating behaviour. For example, male crickets fed low quality food court females less intensively and are less attractive to females than males fed high quality diet (Hunt et al., 2004). Males from a wild-caught population of *O. fasciatus* fed on milkweed have a higher mating activity than bugs from the wild population fed on novel diets including sunflower seeds, cashews, peanuts or almonds (Walker, 1978). Milkweed seeds have stimulatory properties that enhance both the

feeding (Feir and Beck, 1963; Walker, 1978) and mating behaviour (Walker, 1978). It is possible that these properties are not found in the alternative host, resulting in a lower mating activity. However, the mating rate we observed in males from the sunflower seed-adapted population fed sunflower seeds was higher than the values reported by Walker (1978), suggesting that the population we studied has shown further adaptation to the alternative diet.

Researchers have made excellent progress in understanding how dietary inputs affect life history trade-offs (Maklalov et al*.,* 2008; Maklalov et al., 2009; Zajitschek et al*.,* 2009; South et al*.,* 2011). However, while these studies utilise precisely defined dietary inputs to investigate how variation in resources can influence life history outcomes, these researchers still must speculate about the underlying mechanisms through which these effects are mediated and they lack a mechanistic explanation for these trade-offs (Tatar, 2011). Male fitness is maximised with increased carbohydrate while female maximal fitness requires increased levels of protein. The explanation for this is that male fitness requires increased energy for courtship while female fitness requires resources for egg production. What is unique about the study reported here is that, while the dietary inputs are not so well defined, the physiological output from those diets has been described (Nation and Bowers, 1982). Thus we know from previous research how the body composition of males on the two diets differs and we are able to specifically link the changes in life history trade offs to the effect of the different diets. And these outputs, changes in levels of specific fatty acids, allow us to link these results to mechanisms regulating the germ-soma trade-off developed in a model system.

How an evolutionary change in diet might modulate plastic trade-offs among various life history components is poorly understood. In *Drosophila*, females from

populations adapted to a poor diet show complex interactions between current food quality, mating behaviour, fecundity and lifespan (Chapman et al., 1994). Thus, in addition to mating behaviour we examined male fitness under the two diet conditions. That male diet did not affect the fecundity of their mates is perhaps not surprising as we expect the female's diet to have a more direct effect on the number of eggs she is able to produce (Adams, 2000). In the Hemiptera, mating is not always required for production of mature ovarian follicles (Adams, 2000). Thus we might not expect variation in female fecundity based on variation in the quality of her mate, although in *Drosophila melanogaster* diet has been shown to affect traits influenced by male accessory gland proteins (Fricke et al., 2008).

The pattern of plasticity in response to diet, in which old milkweed-fed males maintain fertility at the expense of lifespan in comparison to old sunflower-fed males, indicates that even after many generations of experimental evolution on sunflower seed, the milkweed seed diet maintains the signature of a high quality food, inducing a physiological state that prioritizes reproduction over survival (Rion and Kawecki, 2007). Thus, evolving an expanded diet is not limited by current life history trade-offs, as these can be tailored to different diets.

The pattern of fertility could be explained by sperm production. *Oncopeltus fasciatus* females can store sufficient sperm to fertilize eggs across their lifespan (Walker, 1977). The positive correlation between mating rate and fertilization rate in the sunflower-fed males could occur because each ejaculate is sperm limited. Thus, increased numbers of ejaculates would increase the sperm available to females for fertilizing eggs across her lifespan. Milkweed-fed males may produce sperm above some threshold level such that increased numbers of ejaculates do not result in

increased fertilizing ability. The specific effects of diet on sperm production remain to be investigated.

The observation that milkweed-fed males had improved fertilizing capacity over sunflower-fed males is particularly interesting when we look at the pattern of fertility over time and its relationship to longevity. The difference in fertilization rate between sunflower-fed males and milkweed-fed males was age specific and only apparent in the older males, while in general the ability to fertilize eggs was higher in milkweed fed males but not in such a pronounced fashion. Milkweed-fed males also had a shorter lifespan than sunflower-fed males. Thus, diet affected the trade-off between reproduction and survival, with milkweed-fed males investing in maintaining reproductive capacity across their lifespan, both through mating effort and fertilizing ability, at the expense of longevity.   The evolutionary importance of plasticity in life history trade-offs in response to different diets depends on the perspective we adopt. From a behavioural ecology perspective, variation in diet causes variation in the resource allocation trade-off with males on one diet investing more in reproduction either through mating behaviour or investment in gametes. The elongation in male's lifespan on a sunflower diet might reflect the well know trade-off between sexual activity and longevity (Hunt et al., 2004). What is not as well defined is the real effect mating activity exerts on male longevity. The costs in males are derived mainly from resources invested in courtship (Cordts and Partridge, 1996) or sexual signalling (Hunt et al., 2004). However in *O. fasciatus* there is no evidence of pre-copulatory sexual selection and it seems unlikely that the cost of mating arises from the production of costly signals or courtship behaviour, as neither are apparent. Thus, from a behavioural ecology perspective, increased investment in reproduction arises through energetic costs of mating and the increase in gamete production.

We can also consider the trade-off from a functional perspective. The impact of nutritional quantity and quality on the trade-off between reproduction and longevity is complex, and the idea that the trade-off arises solely from the shifting of resources away from reproduction and into survival during food shortages is almost certainly wrong (Warbrick-Smith et al., 2006; Flatt, 2009). Rather, recent evidence from molecular physiologists interested in ageing suggests that the trade-off between reproduction and survival could be based on signalling between the soma and the germ line (Flatt et al., 2008; Kaczmarczyk and Kopp, 2011). The trade-off between reproduction and longevity can be broken by specific supplementation of restricted diets. In *Drosophila melanogaster*, dietary restriction increases longevity at the expense of fecundity. However, adding back the amino acid methionine to the diet increases fecundity without reducing longevity (Grandison et al., 2009). While we did not specifically manipulate dietary composition, one intriguing suggestion about the underlying mechanism by which variation in diet might cause this variation in life history trade-offs in *O. fasciatus* comes from data on lipid composition of milkweed bugs adapted to sunflower and fed the two diets used in this study. In individuals fed sunflower seeds, a higher proportion of their total fatty acid is composed of C18:1, oleic acid, than individuals fed milkweed seeds (Nation & Bowers, 1982). It has recently been shown that germ line ablation, which increases longevity in *C. elegans*, results in activation of the enzyme stearoyl-CoA desaturase, which converts stearic acid to the longevity promoting oleic acid (Goudeau et al., 2011). While it has been proposed that the trade-off between fecundity and lifespan is mediated by genetic variation in the maintenance of germ line stem cells (Kaczmarczyk and Kopp, 2011), our results also suggest a diet-induced change in the communication between the

germ and the soma, with the milkweed seed diet promoting germ line development and the sunflower seed diet promoting longevity.

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Tables

Table 1. Results of repeated measures ANOVA on male mating behavior and fitness of males fed a diet of milkweed or sunflower seeds across their lifespan.

Trait	Effect of diet (between treatments)		Effect of male age (within treatments)		Interaction between diet and age (diet*age)	
	F statistic	p	F statistic	p	F statistic	p
Mating speed	$F_{1, 33} = 8.722$	0.006	$F_{4, 30} = 3.009$	0.034	$F_{4, 30} = 0.554$	0.698
Mating rate	$F_{1, 46} = 9.881$	0.003	$F_{5, 42} = 1.605$	0.180	$F_{5, 42} = 0.612$	0.691
Female fecundity	$F_{1, 46} = 0.404$	0.528	$F_{2, 45} = 0.721$	0.492	$F_{2, 45} = 0.727$	0.489
Fertility	$F_{1, 40} = 4.089$	0.049	$F_{2, 39} = 0.165$	0.848	$F_{2, 39} = 0.252$	0.778
Fertilization rate	$F_{1, 40} = 5.554$	0.023	$F_{2, 39} = 0.634$	0.536	$F_{2, 39} = 2.238$	0.120

Table 2. Results of Mann-Whitney U test of mating rate of males fed a diet of milkweed or sunflower seeds across their lifespan.

	Sample size milkweed	sunflower	d.f.	χ^2	p
Week 1	35	33	1	0.507	0.476
Week 2	31	31	1	1.591	0.207
Week 3	29	31	1	5.735	0.017*
Week 4	27	30	1	6.237	0.012*
Week 5	24	28	1	5.361	0.021*
Week 6	23	25	1	3.441	0.064

Table 3. Results of *a priori* pairwise contrasts on fertility and fertilization rates of males fed milkweed or sunflower seeds across their lifespan.

	Fertility		Fertilization rate	
	F statistic	p	F statistic	p
Week 1	$F_{1, 59} = 0.541$	0.465	$F_{1, 59} = 1.496$	0.226
Week 3	$F_{1, 48} = 0.321$	0.574	$F_{1, 48} = 0.680$	0.414
Week 5	$F_{1, 48} = 2.778$	0.102	$F_{1, 48} = 10.578$	0.002

Table 4. Correlation between fertilization rate and mating rate for males fed milkweed or sunflower seeds across their lifespan.

Male diet	Week 1	Week 3	Week 5
milkweed	Spearman $\rho = 0.274$ $p = 0.129$	Spearman $\rho = 0.124$ $p = 0.574$	Spearman $\rho = 0.242$ $p = 0.278$
sunflower	Spearman $\rho = 0.559$ $p < 0.001$	Spearman $\rho = 0.598$ $p = 0.001$	Spearman $\rho = 0.423$ $p = 0.025$

Figures
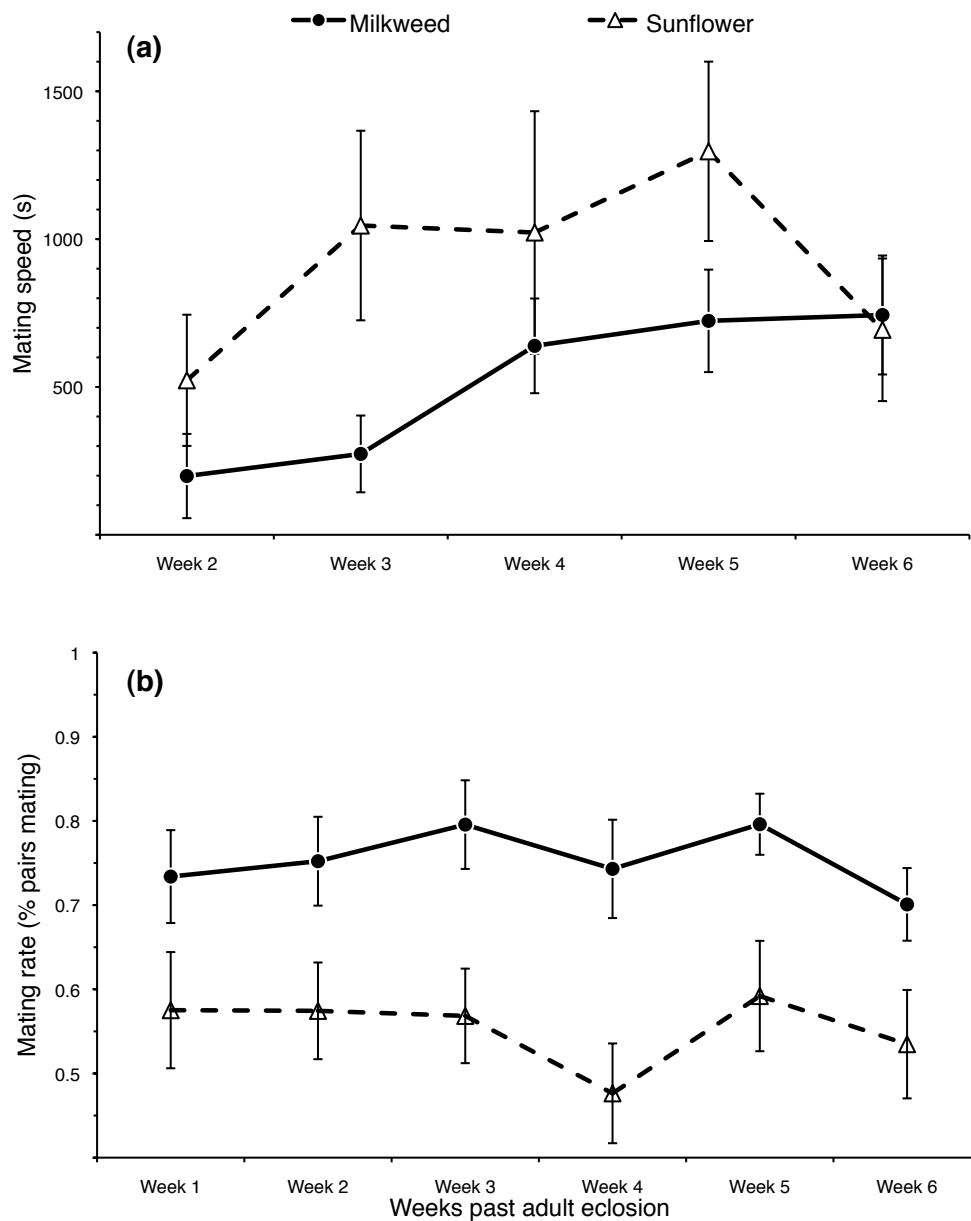
Figure 1. Diet affected two components of reproductive effort, mating speed and mating rate. Milkweed-fed males initiate mating more quickly than sunflower-fed males (A). Mating speed was measured as the time (in seconds; s) from pairing of males and females to the start of copulation. Milkweed-fed males were more likely to be observed copulating with females (B). Mating rate is represented as a measure of the average number of times that mating pairs were observed during the sampling period. Error bars represent SE.
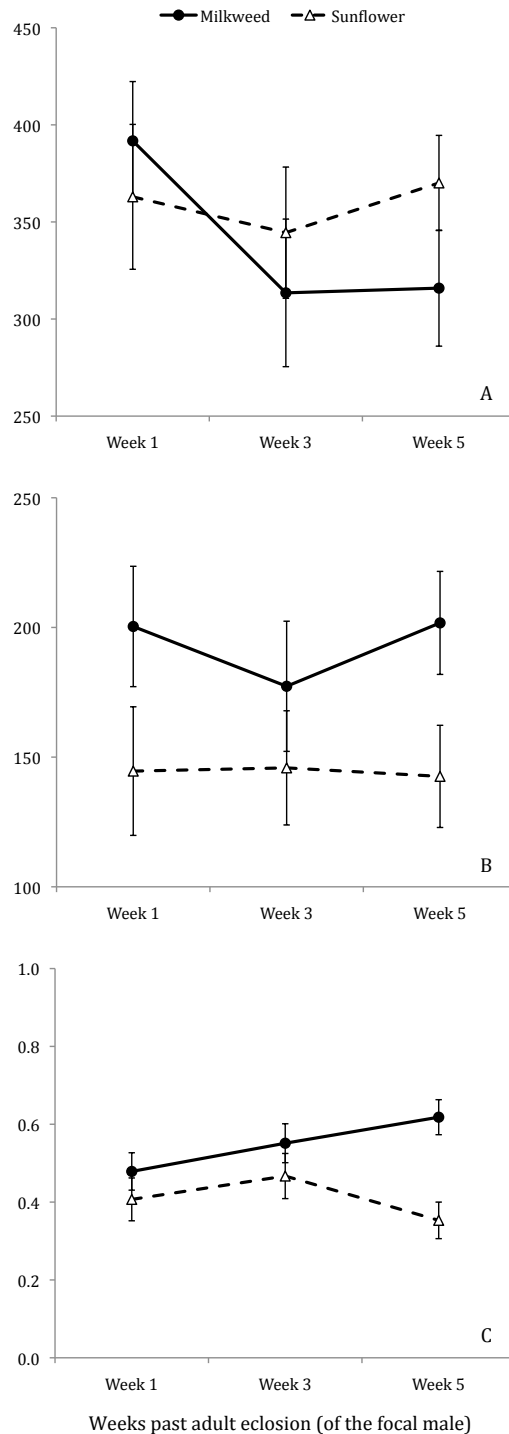
Figure 2. *Diet affected male fertility but not fecundity. Male diet did not affect the* number of eggs laid by the females to which he has mated (A). However, male diet did have an influence on the number of fertilized eggs (B) and did affect the frequency with which eggs laid by the female were fertilized (C); fertility was higher in pairs in which the male was fed on milkweed seeds. Error bars represent SE.
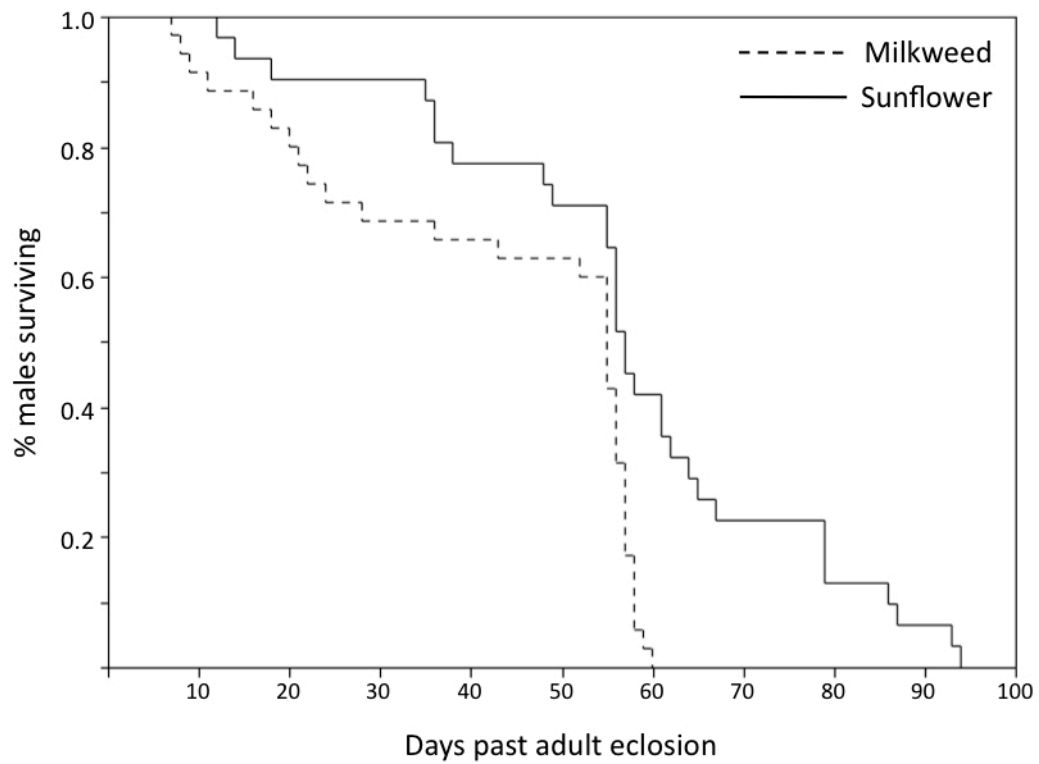
Figure 3. Diet affected male longevity. Milkweed-fed males (dashed line) had a shorter lifespan than sunflower-fed males (solid line).

Chapter 3

Observations on the reproductive diapause in wild and laboratory adapted strains of the milkweed bug, *Oncopeltus fasciatus*.

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Manuscript

Abstract

Diapause is a remarkable and complex trait in insect. It allows individuals to escape unfavourable conditions through a quiescent state and then shifting to reproduction in favourable periods when the chances of offspring survival are maximised. Diapause and migration are tightly linked in insect life history strategies and need to be considered as fundamental life history traits due their influence on where and when to reproduce. In this study two strains of the milkweed bug, *Oncopeltus fasciatus*, were tested for the occurrence of diapause: a wild population from Kentucky and a laboratory-adapted population adapted to feed on an alternative diet of sunflower seeds. Bugs were tested in non-diapause (14L:10D, 25°C) and diapause-inducing (11L:13D, 23°C) environmental rearing conditions, and fed on alternative diets of milkweed or sunflower seeds. The Kentucky population shows a typical delay of reproduction in diapause-inducing environmental conditions while the laboratory-adapted population do not show such a response in any of the rearing conditions. The sunflower diet increased the length of reproductive diapause in the Kentucky strain, while in the laboratory-adapted population diet did not induce diapause but affected some of the other life-history traits measured. The small sample size in some of the treatments in this study do not allow for a definitive comparison between strains. The presence or the absence of the diapause response and its effect on other life-history traits are presented and discussed.

Keywords: diapause, life-history traits, reproductive physiology

Introduction

Diapause is a remarkable feature in insect life histories. It allows to survive in seasonal environments and to synchronize the life cycle with periods suitable for growth, development or reproduction. There is extreme variability in the diapause characteristics and in the activity cycles used by different species (Masaki, 1980; Derlinger, 2002; Kostal, 2006). Diapause represents an alternative developmental pathway with behavioural, morphological and physiological features that allow insects to cope with adverse conditions (Derlinger, 2002). It is not a simple state but rather it involves different active phases in which the animal goes through a series of physiological processes in which expression is significantly modified by environmental factors, making diapause an eco-physiological process in its nature (Kostal, 2006). The steps leading to diapause are characterized by the expression of a set of genes, some of which are common to non-diapausing stages, while others are uniquely expressed during the diapause phases (Derlinger, 2002). A distinctive and important feature of diapause is the very slow senescence rate compared to non-diapausing individuals, in which the delay in maturation of the reproductive system is mediated by very low titres of JH (Saunders et al., 1990; Tatar and Yin, 2001). This is achieved not only by the simple resource allocation trade-off between survival and reproduction (Harshman and Zera, 2007) but also by a slower metabolic rate (Tatar and Yin, 2001) that allow a better utilization of the resources accumulated in the pre-diapause period (Sonoda et al., 2006; Hahn and Denlinger, 2010) and by an increased tolerance to stress (MacRae, 2010). Most of the recent attention on the diapause response has been indeed focused on the increased tolerance to environmental stressors during the diapause stage. Stress resistance could be mediated through the expression of several heat shock proteins (Rinehart et al., 2000; Hayward et al., 2005;

Rinehart et al., 2007; Gkouvitsas et al., 2008; Lopez-Martinez and Derlinger, 2008; Qiu and MacRae, 2008; Aruda et al., 2011) although this may not be a general feature of the insect diapause response (Goto et al., 1998; Goto and Kimura, 2004; Tachibana et al., 2005).

Diapause represents an important trait in the life-history strategies of insects because it determines in which environmental conditions reproduction will take place and it should therefore be considered as a life history trait in itself (Solbreck, 1978). In different latitudinal clines of *Drosophila melanogaster*, the variation in life-history traits like fecundity, lifespan and mortality rates across environmental gradients is mostly explained by the genetic variance for diapause expression (Schmidt and Paaby, 2008). In *Neacoryphus bicrucis* post-diapausing females survive and reproduce at the same rate as non-diapausing females, while their lifespan is greatly increased by the diapause dormancy (Solbreck, 1978). In other examples, the evolution of the diapause response does not seem to be constrained by changes in other life-history traits (Ito, 2009). Migration and diapause are intimately related in insect migratory syndrome allowing them to escape the unfavourable seasonal environmental conditions and to exploit the seasonal resources once they are available (Dingle 1972; Dingle, 1978; Dingle 1996). The onset of diapause allows a delay in reproduction and a time window in which migration to a suitable habitat can happen (Dingle, 1978; Herman, 1981; Dingle, 1996). Thus both migration and diapause can be considered as fundamental life-history traits in insect life-history strategies (Dingle, 1978; Dingle and Drake, 2007).

Photoperiod and temperature offer information about changes in environmental conditions and thus are fundamental cues for the onset of the diapause response (Dingle, 1974; Masaki, 1980). Other factors such as copulation (Hayes and

Dingle, 1983; Sillen-Tulberg, 1984), starvation, quality of available hosts (Dingle et al., 1977; Hunter and McNeill, 1997), and moisture (Tanaka, 2000) can contribute or, more commonly, interact with these main cues in determining the diapause response.

In the milkweed bug *Oncopeltus fasciatus* and other migratory insects, diapause is a prerequisite to undertaking migratory flight to overwintering areas where survival is more likely (Dingle, 1974; Dingle et al., 1980; Herman, 1981; Dingle and Drake, 2007). The main environmental cues determining the onset of both the migratory and diapause response are photoperiod and temperature (Dingle, 1972; Dingle, 1974). Starvation (Dingle et al., 1977) and copulation (Hayes and Dingle, 1983) can also have an influence on the onset of the diapause response. The delay in first oviposition, which represents a measure of the diapause response, shows an irreversible decline in populations maintained in constant diapause-inducing photoperiod and temperature to the point in which no diapause response is evident in those conditions (Dingle et al., 1977). However, diapause can be restored by moving the bugs to lower photoperiod and temperature rearing conditions showing that the response is not eliminated from the population but rather the threshold cues for the onset of diapause are flexible (Dingle et al., 1977). The JH titre determines the occurrence of reproduction, migration and diapause through a higher and lower threshold of sensitivity (Rankin and Riddiford, 1978). When the JH titre is above the higher threshold, reproduction takes place. If the titre is below the lower threshold then bugs enter diapause; migration takes place when the JH titre falls between the two thresholds (see Nijhout, 1994).

Oncopeltus fasciatus shows a latitudinal gradient in diapause. Populations from tropical areas do not enter diapause whatever the environmental conditions to which they are exposed, while temperate populations enter diapause in short day and

low temperature conditions (Dingle et al., 1980). Populations from Florida and Georgia show a higher variation in the diapause response when compared to the populations from the northern areas of USA. This could be the result of the variability in temperature and rainfall conditions that determine if food resources are available or not, while photoperiod conditions are less variable compared to northern areas (Dingle et al., 1980). Indeed the temporal unavailability of food resources can be a particular important stimulus for the diapause response during the summer, when photoperiod and temperature are not triggering a migratory response (Masaki, 1980). Thus *O. fasciatus* may be able to undergo a summer diapause in southern Florida due the lack of available seeds of the *Asclepias spp.* hosts and bugs could be able to break diapause to exploit alternative host plants like *Nerium oleander* (Klausner et al., 1980).

The differences in diapause incidence could derive from genetic flow among different populations adapted to local conditions that can contribute to maintenance of genetic variation in the response to environmental conditions (Dingle et al., 1977; Hayes et al., 1987; Leslie, 1990) and the response seems to be under the influence of one or two major genes (Hayes et al., 1987). Diapause is also under maternal influence thus offspring from a diapausing mother will not enter diapause (Groeters and Dingle, 1987; Groeters and Dingle, 1988).

In the study reported here I used two populations of the milkweed bug: a wild population formed by descendants of bugs collected in Kentucky (KY) and a laboratory adapted population (LAB) which has been subjected to a long-term adaptation to laboratory rearing conditions on an alternative diet of sunflower seeds. The study was conducted to obtain information about the diapause response, including the effect of alternative diets and life-history traits such as mating

behaviour, fecundity and fertility in diapause-inducing conditions in these two populations. Details of sexual maturation, mating activity and fecundity of the KY population were unknown to our group before the beginning of these observations, so this information helped us to fill these gaps in knowledge. The LAB population has never been tested before for the diapause response, so my interest was in understanding if this population was still able to show a diapause after a very strong selection to laboratory rearing conditions and adaptation to alternative diet.

Unfortunately the data collection has been affected by factors that were unpredictable at the beginning of the experiment. The KY population took longer than expected to establish a stable population able to withstand the removal of experimental individuals. KY pairs also had a lower degree of mating activity and higher mortality rate on the sunflower diet compared to the milkweed diet and this affected the eggs collection for the set up of the experimental clutches and pairs for data collection. The LAB population had higher levels of mating activity, reproduction and survival on both diets. Another limiting factor was the supply of milkweed seeds, limiting set up of new experimental pairs on the milkweed diet. This is particularly evident for the long photoperiod and high temperature treatment in the KY sample and the analysis of traits like first oviposition occurrence, mating rate, fecundity and fertility were greatly affected by this small sample size. This influenced some general patterns and limits the comparison between strains and food treatment across rearing conditions. However, statistical analyses were performed including all the data collected from all experimental pairs, even if formed by very small sample sizes, in an attempt to offer the most comprehensive information available. In the following sections it is stated when the sample size for one or more treatments is small and caution needs to be taken in the interpretations of the results.

Materials and Methods

Animal husbandry

The laboratory adapted population (LAB) was obtained from Carolina Biological Supply (Burlington, NC, USA) and has been maintained in laboratory conditions since 45 years on a diet of organic unsalted sunflower seeds before being housed in our laboratory conditions. The wild population (KY) was collected in the University of Kentucky Arboretum, Lexington, KY in September 2009 from *Asclepias syriaca* (Charles Fox, personal communication) and housed in laboratory conditions on a diet of organic common milkweed seeds. The LAB population experienced different photoperiodic rearing conditions during the time it has been housed in our laboratory, ranging from 12L:12D to 16L:8D, while generally the temperature has been constantly maintained to 25°C. These rearing conditions are above the threshold for the onset of diapause in wild bugs (Dingle et. al., 1980), thus the LAB population had never experienced diapause-inducing rearing conditions. Before the beginning of the observations both populations have been reared for at least 6 months in mass colonies formed by random numbers of mixed adults and nymphs and in constant rearing conditions of 14L:10D 25°C. I collected a random number of newly eclosed males and females from the mass colonies to form the parental pairs from which experimental individuals have been collected and reared since the egg stage.

Parental pairs were housed in petri dishes in 14L:10D 25°C and provided with water *ad libitum* and the food they have been exposed while in the mass colonies: thus LAB parental pairs were provided with sunflower seeds and KY pairs with common milkweed seeds. A total of 18 KY pairs and 25 LAB pairs were set up. From each

parental pair the first fertile clutch laid was collected to form the experimental F1 pairs. Eggs were placed in a small square box (110mm x 110mm x 30mm) and housed in 11L:13D 23°C rearing conditions, to allow the nymphs to develop in diapause inducing conditions and to form the experimental pairs at adult eclosion. Nymphs were fed the parental diet: LAB nymphs were fed on sunflower seeds and KY nymphs were fed on milkweed seeds. Water was provided *ad libitum* and seeds changed every three days. Once the box became too crowded due the increasing size of the developing nymphs, these were split into different similar sized boxes, maintaining a maximum density of 20 nymphs per box. Hatching time, survival to 3rd instar stage, time to adult eclosion and survival to adulthood of the experimental nymphs were collected. Hatching time was measured as the number of days from the clutch collection to the observation of the first hatching nymphs, adult eclosion was measured as the number of days from the first observed hatching to the observation of the first eclosed adults. The sensitive stage for diapause induction in *O. fasciatus* is from the 4th instar to the early adult stage (Dingle, 1974) thus the survival to the 3rd instar measured the effects of diapause inducing conditions on clutch survival between strains.

Newly eclosed adults from the F1 were randomly paired to form the experimental mating pairs. These were formed using newly eclosed unrelated individuals collected randomly from different clutches. The newly formed pairs were assigned to one of the experimental environmental conditions: diapause inducing 11L:13D 23°C (short-low) or non diapause inducing 14L:10D 25°C (long-high) rearing conditions and placed on a diet of sunflower (S) or milkweed diet (M). Pairs were checked daily to assess for the occurrence of first mating and first oviposition indicative of the presence or absence of a diapause stage. First mating occurrence was

measured as the time from adult eclosion to the first mating observed. In some pairs the mating was observed after the first oviposition; in these cases if the clutch was fertile then the date of first oviposition was considered as date of first mating as well. First oviposition was measured as the time from adult eclosion to the oviposition of the first fertile clutch. The time from 1st mating occurrence to 1st oviposition was also measured to compare it with the occurrence of 1st oviposition from females' adult eclosion, in order to disentangle the effects of male or pair's sexual activity on the occurrence of reproductive diapause in females. Mating rate of pairs was collected using a single daily observation for a period of 20 days from the occurrence of the first mating, performed between 6 and 10 hours from the start of the light cycle. The total number of eggs laid (fecundity), number of clutches and total number of hatching nymphs (fertility) during the first week of oviposition was also recorded. Fertility was measured both as total number of hatching nymphs and as fertilization rate from the eggs collected from F1 pairs placed in long-high rearing conditions. A control group of females collected from the F1 nymphs was kept unpaired and each female was placed randomly on a diet of sunflower or milkweed seeds in short-low rearing conditions, in order to have a control group to assess for the effect of male presence on the onset of female reproductive diapause. However, only LAB virgin females ended up being included in this control group given the lack of experimental KY females or the complete absence of oviposition response in KY virgin females fed on sunflower. A second control group was formed by KY males and females collected at eclosion from the mass colonies and housed in long-high rearing conditions. This control group was set up in order to obtain information about the timing of first mating and first oviposition in the KY population in our laboratory conditions and to have a standard reference for the F1 experimental pairs.

Statistical analysis

Normal distribution of the data was tested with Shapiro-Wilk W test. For the analysis of the pairwise comparisons, ANOVA was used when data fitted the assumption for a normal distribution and Mann-Whitney U test when data were not normally distributed. Pearson moment correlation was used to measure the correlation between clutch size and survival to adult eclosion of F1 nymphs, while Spearman rank correlation was used to measure the correlation between clutch size and hatching time in F1 nymphs. First mating, first oviposition, number of clutches and clutch size were count data and showed a non-normal distribution thus GLMs with poisson distribution and log link function were fitted to test for the effects of strain (KY, LAB), food (milkweed, sunflower) and rearing conditions (short photoperiod-low temperature, long photoperiod-high temperature) on these traits. First week fecundity, number of hatchings and mating rate were normally distributed thus GLMs with normal distribution and identity function were fitted to test the effect of strain, food and rearing conditions on these traits. All statistical analyses were performed using JMP 8.0.2 (SAS Institute Inc., 1989-2009).

Results

Hatching time, survival to 3rd instar stage and to adult ecdysis of the F1 experimental nymphs in short photoperiod and low temperature rearing conditions

Parental KY pairs laid bigger clutches than parental LAB pairs (χ^2=8.030; d.f.=1; p=0.005). There was no difference between the two strains in hatching time (χ^2 p=0.663; d.f.=1; p=0.415; Figure 1A); hatching time was weakly correlated with clutch size in LAB (Spearman rank correlation; ρ=-0.393; p=0.052) but not in KY

nymphs (Spearman rank correlation; ρ=0.309; p=0.211). KY nymphs began to eclose into adults earlier than LAB nymphs (χ^2=21.450; d.f.=1; p<0.0001; Figure 1A) but fewer KY nymphs survived to the adult stage than LAB nymphs (ANOVA; $F_{1,39}$=14.731; p<0.001; Figure 1B) with no difference in survival to the 3rd instar stage (χ^2=2.111; d.f.=1; p=0.146; Figure 1B). The survival to adult eclosion is correlated with clutch size in KY (Pearson ρ=-0.533; p=0.023) but not LAB nymphs (Pearson ρ=-0.074; p=0.724), but the significance did not hold after removing the two biggest clutches of the KY sample from the analysis (Pearson ρ=-0.406; p=0.1186). However, this did not eliminate the significant difference in survival to adult eclosion between strains (ANOVA; $F_{1,38}$=9.237; p=0.004) and this was true even eliminating from the analysis the smallest LAB clutches in order to eliminate the significant difference in clutch size between strains (ANOVA; $F_{1,32}$=5.344; p=0.027).

Life-history traits of F1 experimental adults under alternative rearing and diet conditions

  a) First mating occurrence and mating activity

Strain (χ^2=38.011; d.f.=1; p<0.0001), diet (χ^2=7.695; d.f.=1; p=0.005) and rearing conditions (χ^2=92.667; d.f.=1; p<0.0001) all affected the time from adult eclosion to occurrence of the first mating. LAB bugs started to mate earlier than KY in both rearing conditions of photoperiod and temperature, milkweed fed bugs started to mate earlier than sunflower fed bugs, and short photoperiod and low temperature delayed the occurrence of first mating (Figure 2). The sunflower diet delayed the occurrence of first mating only in the LAB strain in both short photoperiod and low temperature (χ^2=6.409; d.f.=1; p=0.014) and long photoperiod and high temperature (χ^2=16.947; d.f.=1; p<0.001). The same effect was not evident in the KY strain

exposed to short-low (χ^2=1.158; d.f.=1; p=0.282) and long-high (χ^2=1.636; d.f.=1; p=0.201) rearing conditions. However, the sample size for the KY strain in long-high conditions and milkweed diet is small, thus the comparison may not be accurate (Table 1). There was no diet effect on first mating occurrence in the KY control group pairs reared in long photoperiod and high temperature (χ^2=1.238; d.f.=1; p=0.267).

The number of matings observed was mainly dependent on the strain with LAB pairs mating more than KY pairs in both rearing conditions and diet (χ^2=26.227; d.f.=1; p<0.0001; Figure 3). There was an almost significant effect of diet (χ^2=3.121; d.f.=1; p=0.077) but this is likely to be affected by the small sample size of KY pairs in long-high rearing conditions and milkweed diet (Table 1). Indeed no diet difference was evident between the four LAB experimental conditions (χ^2=0.238; d.f.=1; p=0.626), but diet had a significant effect between the four KY experimental conditions (χ^2=4.992; d.f.=1; p=0.025) although this may be dependent on the difference in sample sizes between treatments in the KY sample (Table 1).

b) First oviposition occurrence

Strain (χ^2=44.967; d.f.=1; p<0.0001), diet (χ^2=11.069; d.f.=1; p<0.001) and rearing conditions (χ^2=81.532; d.f.=1; p<0.0001) all affected the time from adult eclosion to 1st oviposition. Thus LAB females laid their eggs earlier than KY females and sunflower diet and short-low conditions delayed oviposition (Figure 2). There was also a significant interaction between strain and rearing conditions (χ^2=5.766; d.f.=1; p=0.016), but this result was likely affected by the very low sample size of the milkweed fed KY bugs in long-high conditions (Table 1). The time from 1st mating to 1st oviposition was dependent on strain (χ^2=58.241; d.f.=1; p<0.0001), diet (χ^2=5.281; d.f.=1; p=0.022), rearing conditions (χ^2=11.773; d.f.=1; p<0.001) and the interactions

between strain and rearing conditions (χ^2=4.447; d.f.=1; p=0.035) and strain and number of matings (χ^2=7.093; d.f.=1; p=0.007). The comparison between mated and virgin LAB females reared in short-low conditions showed that the sunflower diet significantly delayed the occurrence of the first oviposition compared to milkweed (χ^2=5.452; d.f.=1; p=0.019), but the presence or absence of a male did not affect the time to first oviposition (χ^2=1.305; d.f.=1; p=0.253). The similar comparison between mated and virgin KY females was impossible to analyse given the very low or totally absent sample size of virgin KY females (Table 1).

c) 1st week fecundity, number of clutches, clutch size and fertility

Strain (χ^2=5.743; d.f.=1; p=0.016), food (χ^2=6.038; d.f.=1; p=0.014) and rearing conditions (χ^2=12.619; d.f.=1; p<0.001) affected the total number of eggs produced in first week of oviposition. However the KY sample size was very small in almost all the experimental conditions and diet (Table 1), so the result was likely to be affected by this issue (Figure 4). Considering only the LAB strain, rearing condition was the factor with the greatest effect on fecundity (χ^2=20.443; d.f.=1; p<0.001). A comparison between mated and virgin LAB females showed that the main factor affecting the first week of fecundity was mating status (ANOVA; $F_{1, 78}$=57.201; d.f.=1; p<0.0001) while diet did not have any significant effect (ANOVA; $F_{1, 78}$=0.109; p=0.742).

Strain (χ^2=44.327; d.f.=1; p<0.0001) and rearing conditions (χ^2=23.285; d.f.=1; p<0.0001) affected the number of clutches laid in 1st week of oviposition. However, as in the case of 1st week fecundity, the small KY sample size is likely to affect the result. Considering only the LAB strain, both rearing conditions (χ^2=31.573; d.f.=1; p<0.0001) and food*rearing interaction (χ^2=3.906; d.f.=1;

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p=0.048) affected the number of clutches. The comparison between LAB mated and virgin females showed that the number of clutches is affected by the mating status with mated females laying more clutches than virgin (χ^2=28.013; d.f.=1; p<0.0001) but not by diet (χ^2=1.028; d.f.=1; p=0.311).

Strain (χ^2=21.610; d.f.=1; p<0.0001) and rearing conditions (χ^2=4.761; d.f.=1; p=0.029) affected the clutch size (Figure 5A). KY females laid bigger clutches than LAB females (Figure 5B) and females laid bigger clutches in short photoperiod and low temperature conditions (Figure 5C). The result of rearing conditions on clutch size is likely to be affected by the small sample size of KY experimental pairs, which limited the number of laying females across the rearing conditions' comparison. However, LAB females in short-low conditions fed on milkweed laid bigger clutches (χ^2=5.007; d.f.=1; p=0.025) suggesting that the rearing conditions could interact with food in determining the clutch size and that this could be a pattern likely to be present in KY pairs as well (see Figure 5A). Mated LAB females laid bigger clutches than virgin females (χ^2=7.833; d.f.=1; p=0.005) but the difference was mainly dependent from the comparison between mated and virgin females in short-low conditions (ANOVA; $F_{1,\ 78}$=7.957; p=0.006), while mated females in long-high conditions laid clutches of similar size to virgin females (ANOVA; $F_{1,\ 80}$=3.186; p=0.078).

Strain (χ^2=6.958; d.f.=1; p=0.008) and rearing condition (χ^2=7.433; d.f.=1; p=0.006) explained the variation in number of hatching nymphs. LAB pairs hatched more nymphs compared to KY pairs and pairs reared in long-high rearing conditions hatched more nymphs compared to pairs reared in short-low rearing conditions (Figure 6A). The number of hatching nymphs was correlated to clutch size only in short-low rearing conditions in both KY (Spearman rank correlation; ρ=0.531; p=0.028) and LAB (Spearman rank correlation; ρ=0.331; p=0.03). Again in this case

the KY sample was very small in some of the rearing conditions. There was no difference across rearing conditions in hatching rate (Kruskal-Wallis; χ^2=8.349; d.f.=7; p=0.301; Figure 6B), even after eliminating the KY treatments with low sample size from the analysis (Kruskal-Wallis; χ^2=7.770; d.f.=5; p=0.169).

Discussion

The results obtained in this study are limited by the low sample sizes in some of the treatments, in particular for the data from the KY strain in long-high conditions on milkweed seeds. The factors that determined such a low sample size were unpredictable at the beginning of the data collection and it has been impossible to collect additional data to fill these gaps. Thus the following discussion attempts to address the results in light of the fact that the statistical power of the performed tests is weak in some cases and a more detailed analysis for some of the measured traits is certainly needed.

F1 clutches reared in diapause-inducing conditions

There is no difference in hatching time between strains, and previous observations with LAB clutches housed in 16L:8D 25°C rearing conditions show that no difference is evident with the hatching time measured in 11L:13D 23°C in both Kentucky and LAB nymphs (Appendix, Figure A1). This could be explained by the presence of physiological or developmental constraints on hatching time. In our laboratory the Kentucky bugs have never been reared in very long photoperiod conditions like the LAB population, so a comparison for the KY population cannot be made at the moment. In contrast to embryonic development time, the time to adult eclosion differs between strains, with KY nymphs eclosing earlier into adults

compared to LAB nymphs in contrast with the general correlation between body size and developmental rate (Stearns, 1992). The prolonged development time of the LAB nymphs can be the result of coping with sub-optimal food (Slansky, 1993), suggesting that the sunflower diet still bears the signature of a low quality diet in the long-term adapted LAB population. The interaction between low temperature and low quality diet can be a factor affecting developmental rate as well as feeding rate (Kingsolver and Wood, 1998), and can even act differentially across developmental stages (Petersen et al., 2000). More detailed experimental observations are needed to clarify this point.

The sensitive stage for diapause induction in *O. fasciatus* goes from the 4th instar to the early adult (Dingle, 1974). Thus the 3rd instar marks the passage to the stage in which the developing nymphs rely on environmental cues to undertake or not the physiological response leading to a possible adult reproductive diapause. There is no difference between strains in survival to the 3rd instar stage, whilst the survival to adult eclosion is significantly different between strains and in general the survival rate for the KY clutches is low in diapause-inducing conditions. This suggests that the factors affecting the survival of the nymphs during their development are stronger during the diapause sensitive period rather than all along the developmental period and KY nymphs are more affected than LAB nymphs by the diapause-inducing conditions. Intra-clutch cannibalism could be a factor affecting the nymph's survival but it is more likely to happen during the early hatching stages or when seeds are unavailable (Ralph, 1977). I noticed that cannibalism occurs in the clutches and the first hatched nymphs are likely to feed on both fertile and infertile un-hatched eggs but usually this happens at a very low level or when seeds are not available. Sometimes I even observed early 2nd instar nymphs cannibalizing just hatched 1st

instar nymphs. However, I never observed nymph's cannibalism after the 3rd instar stage and Ralph (1977) stated that this is a very rare event in the field. Thus cannibalism could affect in a certain degree the survival rate to 3rd instar but is very unlikely it affected the survival in this case because the nymphs were not food-deprived, there is no difference between strains and the difference in survival rate to adult eclosion arises only after the period in which the cannibalism is likely to happen. Some developmental and/or physiological factors seem to affect the nymphs' survival during the period in which they respond to the diapause-inducing conditions. It is not clear if this affects the 4th or 5th instar stage or both and if there is a sex-biased effect on survival rate. An interesting observation would be to divide the nymphs on milkweed and sunflower diets, but in this study nymphs were raised on the respective population adapted diet and the diet effect remains an open question. Further observations in this direction are needed in future.

The difference in size of the F1 clutches collected from parental pairs of the two strains could be dependent from the bigger body size of KY females (Appendix, Figure A2) and it could have possibly affected the survival in the most crowded KY clutches. Clutch size is correlated with the survival of the nymphs to adult eclosion only in the KY, but not in the LAB strain and the correlation loses its significance if the biggest KY clutches are removed from the sample, suggesting a clear density-dependent effect on survival in the KY clutches. Even if the maximum density has been maintained to a threshold value, this does not rule out a density effect on survival because I did not use a standard number of nymphs for each box. However, the difference in survival to adult eclosion between strains is significant even eliminating the difference in clutch size by removing the smallest LAB and the biggest KY clutches. Thus there are three possible explanations for the survival

difference: a) a difference in ability to survive in diapause-inducing conditions dependent on the strain 2) an interaction between strain and nymphs' density which does not come out significant because of the small sample size 3) a density effect that probably interact with the strain. Further observations are needed to help answering this question.

Reproductive diapause in KY and LAB strain

The three traits used to quantify the occurrence of reproductive diapause offer different but integrating information. The occurrence of first mating activity could mark the sexual maturation of both individuals in the pair, but in the same time it could mark only the sexual maturation of the male ready to copulate with a female. The occurrence of the first fertile oviposition certainly indicates when the female is physiologically mature enough to lay eggs, but this does not necessarily mark the time of female sexual maturation given that the first oviposition happens past the time in which a female becomes sexually mature and receptive to a male. The time from first mating to first oviposition indicates when a female, assumed as sexually mature given that mating activity has already happened, it is actually ready to lay the first fertile clutch following mating. This trait gives information about the actual delay in reproduction by females so it includes all the factors that could determine a delay in oviposition following mating. Previous works on the diapause of the milkweed bug focused only on the occurrence of first oviposition (Dingle, 1974; Dingle et al., 1980). Hayes and Dingle (1983) reported that males of *O. sandarachatus* are more sexually active than males of *O. fasciatus* and that their higher mating rate decreases the length of reproductive diapause in *O. fasciatus* females in the inter-specific matings.

Similarly in the Lygaeidae *Neacoryphus bicrucis*, males with higher mating activity decrease the length of the reproductive diapause in females (Sillen-Tulberg, 1984).

In the present observations two groups of pairs can be distinguished: a) pairs that in average mate before 20 days and lay the first clutch before 30 days and b) pairs that in average delay mating later than 20 days and lay the first clutch after 30 days. Thus, the first group is formed by pairs showing sexual activity and reproduction in the average time range for the species, while the second group is formed by pairs in reproductive diapause, both for mating activity and oviposition. The two strains show a different response to diapause-inducing conditions. Only KY pairs reared in short-low conditions falls into the diapause category whatever the diet they are fed, while all other pairs do not enter diapause, and in particular the LAB population never shows diapause whatever the conditions they are exposed to and the strains differ in the time from first mating to first oviposition. Also, LAB pairs have higher mating activity compared to KY pairs. Thus the absence of reproductive diapause in the LAB strain seems to be dependent on the higher levels of sexual activity showed by LAB pairs when compared with KY pairs. However, LAB virgin females reared in short-low conditions do not delay oviposition when compared to mated LAB females reared in the same conditions, suggesting that LAB females are naturally able to start mating and ovipositing earlier than KY females and not because they are exposed to more sexually active males. The difference in body size could reflect a difference in development time to sexual maturity (Stearns, 1992) leading the smaller LAB bugs to start mating and ovipositing earlier than bigger KY bugs.

As already found previously, diet affects the occurrence of first mating in the LAB pairs (see Chapter 1) but not in the KY pairs. Diet does not affect the first mating occurrence in the KY control pairs either, suggesting that the result is not

simply a result of the poor statistical power of the KY sample. In previous work (see Chapter 2) has been suggested that the difference in mating activity in males fed on a diet of milkweed or sunflower could depend on the different qualitative composition of fatty acids the two diets provide and this in turn modulates the resources' allocation to reproduction or longevity. Thus LAB males fed on milkweed have higher mating activity than sunflower fed males while sunflower fed males live longer (Attisano et al., 2012). However, why this does not happen in the KY strain is difficult to say and it is actually puzzling given that KY bugs, as opposed to the LAB strain, are not adapted to the sunflower diet and so it would be reasonable to expect a lower activity on this diet compared to the milkweed diet. More detailed observations with a bigger sample size should help to clarify this observation.

The patterns obtained from the analysis of first week fecundity are quite difficult to analyse thoroughly given the very low sample size in some of the KY treatments. What the results suggest is that the rearing conditions affect the number of eggs laid by a female, more than diet does. Indeed in the LAB population, the difference in fecundity is between rearing conditions and not within. Body size cannot be accounted for as the main reason for this difference and in general the relationship between body size and fecundity in insects is not straightforward (Leather, 1988). Also, in *O. fasciatus* the selection for wing length, used as a measure of body size, increases the flight propensity in the migratory Iowa population and, as a genetic correlated response, the fecundity (Palmer and Dingle, 1986; Palmer and dingle, 1989). The non-migratory Puerto Rico population do not show the same correlated response to wing length selection (Dingle et al., 1987; Dingle et al., 1988). Thus the correlation body size-fecundity in this species seems to derive mainly from correlated responses to flight activity rather than dependent solely from a body size effect. The

KY population has a high level of flight activity (see Chapter 5) while observations on the flight behaviour of the LAB population are only preliminary and have not been performed in as much detail, but this strain does not seems to reach the same level of flight activity as the KY strain. However, in diapause-inducing conditions the pattern of fecundity is the opposite of what one could expect with the smaller and less active flyer LAB strain showing higher fecundity than the bigger and active flyer KY strain. As already found in previous works,females need to mate to boost reproductive output (Gordon and Bandal, 1967; Gordon and Loher, 1968), indeed virgin LAB females are less fecund than mated LAB females. As already noticed with the parental strain, KY females lay bigger clutches than LAB females and this could be dependent form the difference in body size. What seems interesting is that females lay bigger clutches in short-low compared to long-high conditions. This result needs to be considered cautiously because the difference between rearing conditions is quite small and can simply derive from the small sample size. However, LAB females in short-low conditions feeding on milkweed lay bigger clutches than LAB females on the other rearing conditions and KY females in short-low seems to lay bigger clutches compared to long-high conditions. Also the clutch size of virgin LAB females do not differ with mated LAB females in long-high conditions, but do differ when compared with mated LAB females on short-low conditions.

The fertility pattern is somewhat puzzling. Using the mean number of hatching nymphs as a measure of fertility shows that LAB seems to produce more nymphs than KY, but this could be dependent on the sample size, and that rearing conditions affects the number of hatching nymphs, while parental diet do not have any influence on the number of hatching nymphs. Using the hatching rate as a measure of fertility does not return any difference at all between the experimental

conditions. Interestingly the clutch size is correlated with the number of hatching nymphs only in short-low conditions on both strains. Thus clutch size and its interaction with rearing conditions seems an interesting trait to look at in more detail in the future because, as seen previously, it seems to have some still unclear influences on the survival rate of the nymphs in the KY population and on the fertility of the pairs in diapause-inducing conditions.

Conclusions

Even if these observations are characterized by a very small sample size and thus based on an incomplete dataset, it is possible to note that the response to diapause-inducing conditions between the strains is very different. These two strains could offer some interesting raw material to set up comparative analysis for the study of the evolution of the diapause response. In the future it could be interesting to perform studies at the molecular level to understand what mechanisms are likely to influence the presence or absence of diapause in these two strains of milkweed bug. Some of the approaches could include the study of the regulation of heat shock proteins (Hsps) or JH titre in the modulation of the diapause response and the role of diet in mediating these responses. Both these approaches seems a good starting point in light of the particular life-history strategy of the milkweed bug which include both migration and diapause in which both Hsps and JH could play a fundamental role in shaping the responses to seasonal unfavourable environments.

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Tables

		KY		LAB		KY control		KY virgin females		LAB virgin females	
		M	S	M	S	M	S	M	S	M	S
1st mating	11:13 23°C	15	12	23	24						
	14:10 25°C	5	18	23	23	27	24				
1st oviposition	11:13 23°C	14	12	22	23			4	0	23	19
	14:10 25°C	2	17	22	23	24	21				
Mating rate	11:13 23°C	14	11	19	19						
	14:10 25°C	2	18	23	23						
Fecundity	11:13 23°C	11	6	22	21			0	0	23	14
	14:10 25°C	2	17	22	23						
Hatching nymphs	11:13 23°C	11	6	16	19						
	14:10 25°C	2	17	22	23						

Table 1. Sample size of the experimental and control pairs used in the analyses. The slow pace of the KY mass colonies during the data collection and the high mortality of the experimental KY pairs greatly affected the sample size of the experimental KY individuals. Also a shortage in the supply of fresh milkweed seeds made difficult to set up new experimental pairs. KY=Kentucky experimental population, LAB=laboratory adapted experimental population, KY control=Kentucky pairs collected from mass colonies and reared in 14:10 25°C, virgin females=experimental individuals reared in 11:13 23°C, M=milkweed, S=sunflower.
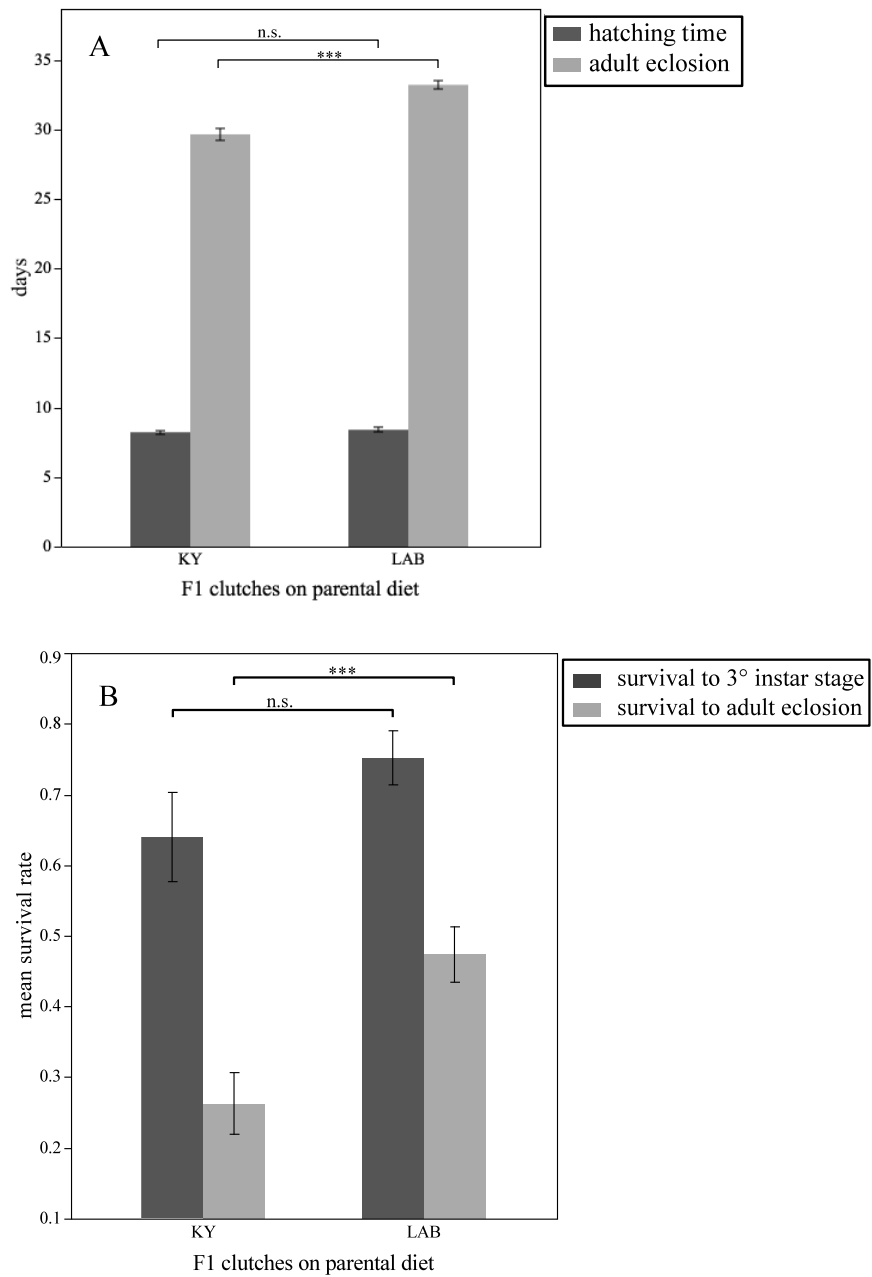
Figures

Figure 1. Mean number of days to hatching and adult eclosion (A) and survival rate to 3rd instar stage and to adult eclosion of F1 nymphs reared on parental diet in short photoperiod and low temperature conditions (B).
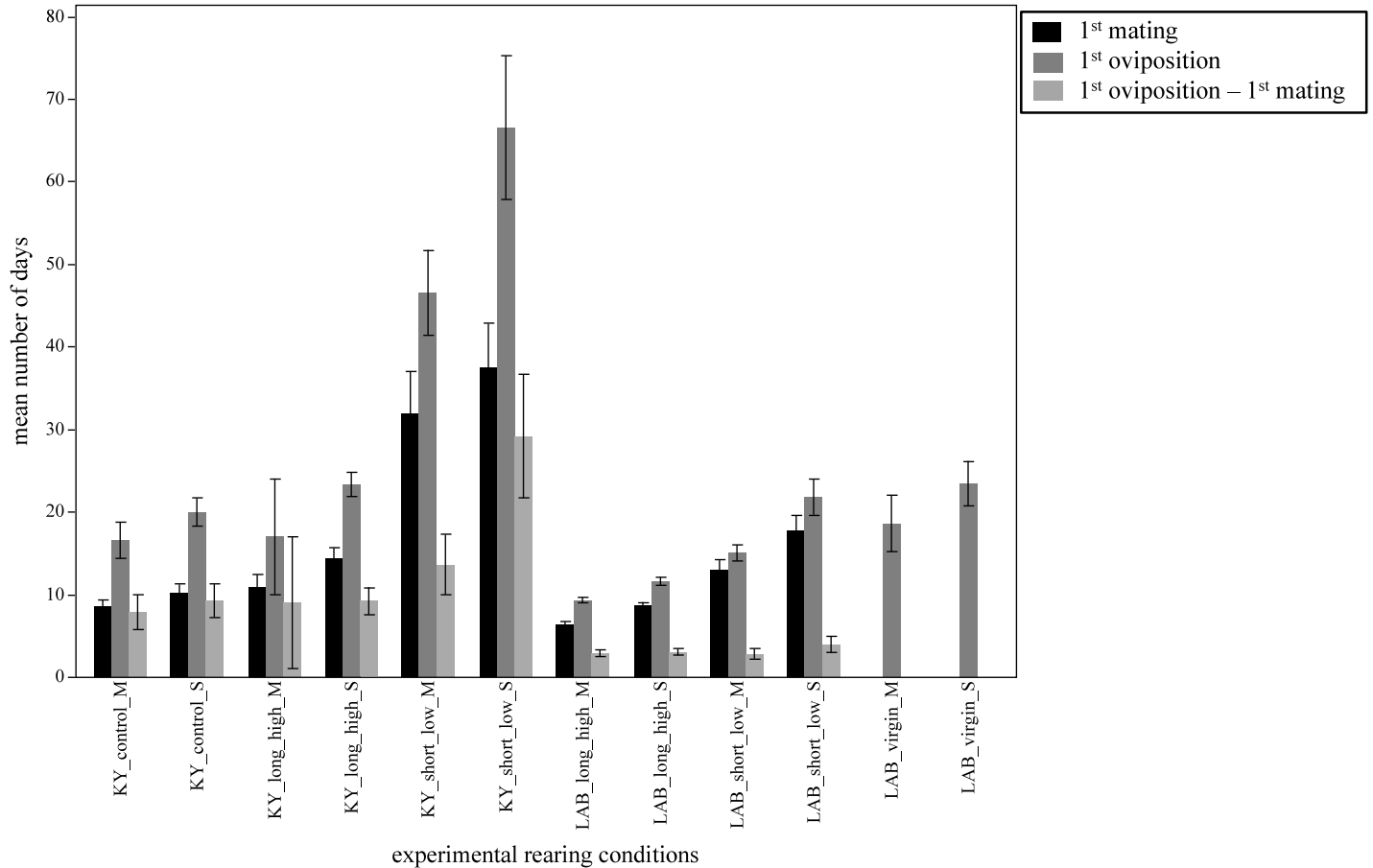
Figure 2. Mean number of days from adult eclosion to 1st mating and 1st oviposition in different rearing and diet conditions. The mean number of days from 1st mating to 1st oviposition is also showed. Strain, rearing conditions and diet affect the time to sexual maturation and 1st oviposition. The labels on the x axis indicate the strain (KY; LAB; KY control; LAB virgin), the photoperiod (long 14L:10D; short 11L:13D), temperature (high 25°C; low 23°C) and the experimental diet (M milkweed; S sunflower).
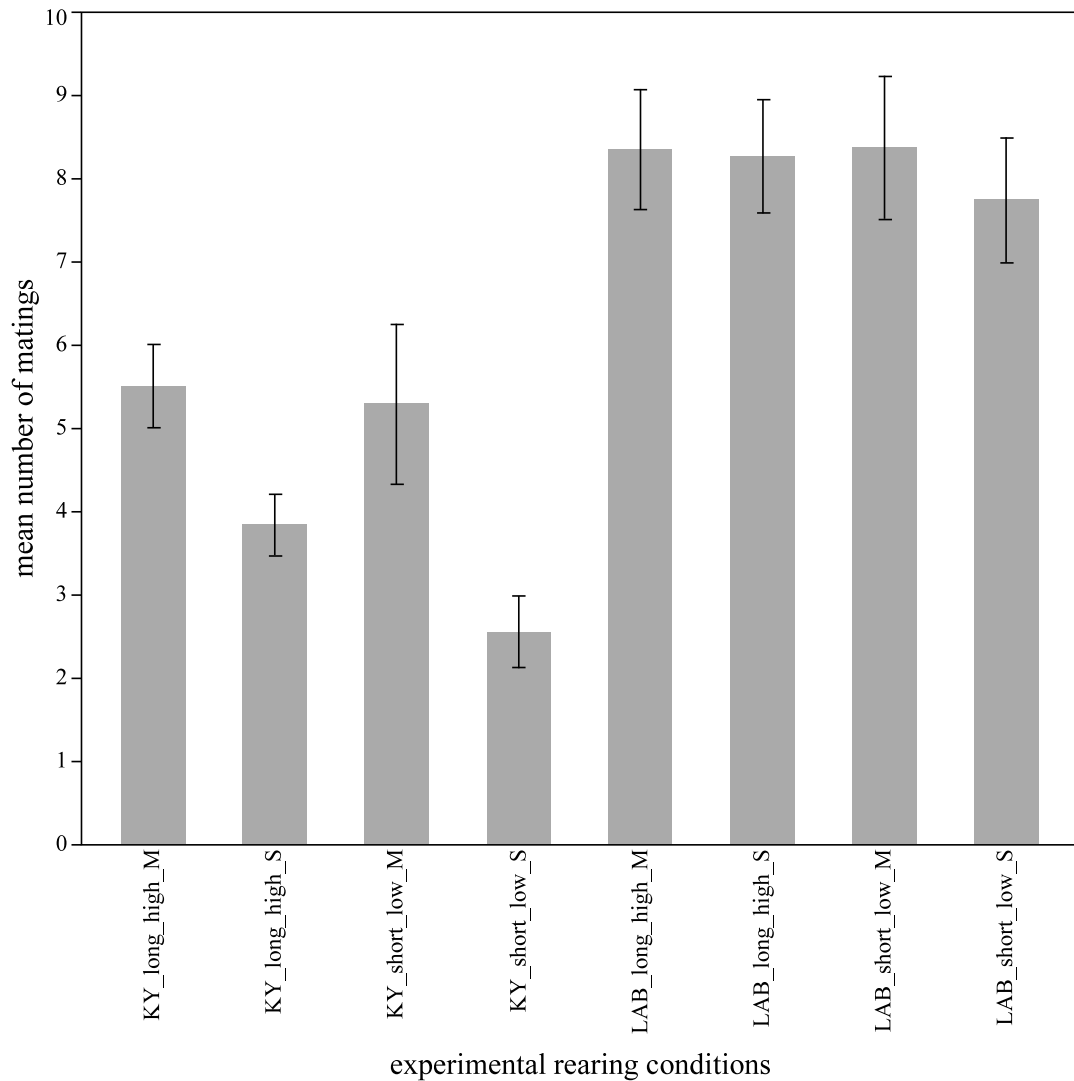
Figure 3. Mating activity measured as the number of matings observed over a 20 days period from the occurrence of 1st mating. Strain and diet affects the amount of mating activity. The labels on the x axis indicates the strain (KY; LAB), photoperiod (long 14L:10D; short 11L:13D), temperature (high 25°C; low 23°C) and the experimental diet (M milkweed; S sunflower).
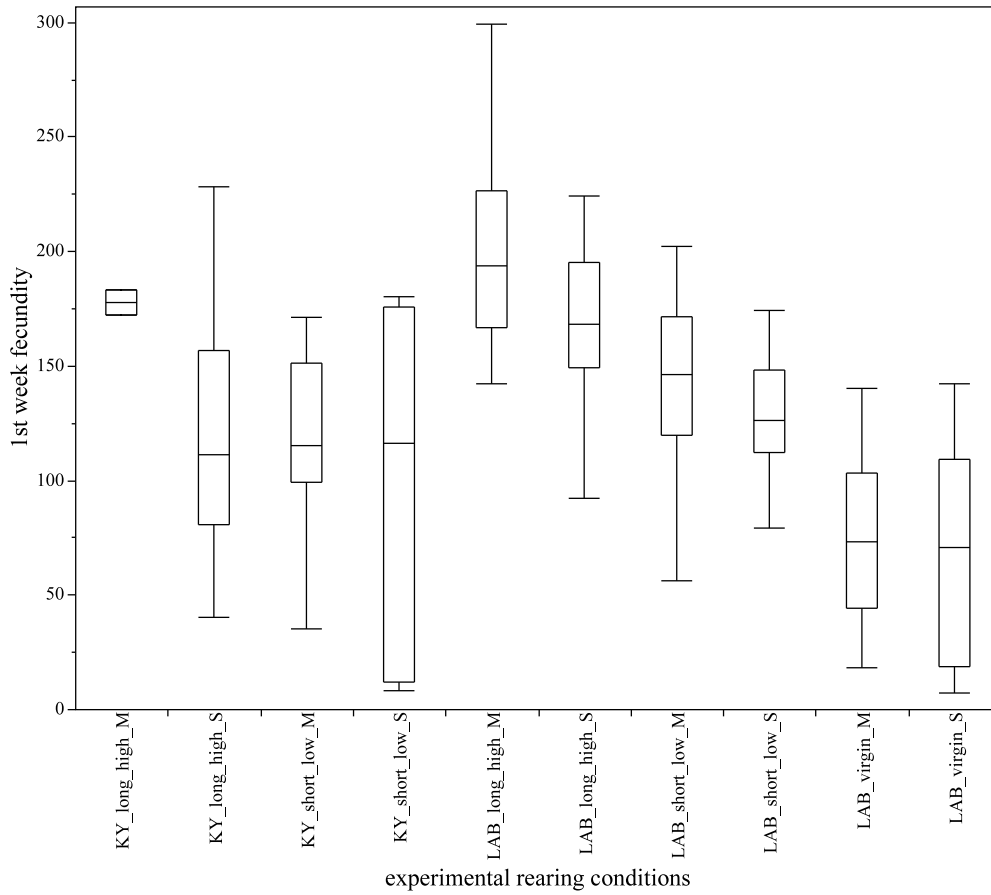
Figure 4. Fecundity of females measured as the number of eggs laid in the first week of oviposition. The labels on the x axis indicates the strain (KY; LAB), the photoperiod (long 14L:10D; short 11L:13D), temperature (high 25°C; low 23°C), the experimental diet (M milkweed; S sunflower) and the LAB virgin group.

Figure 5. Mean clutch size comparison between experimental strains and rearing conditions (A). KY females laid bigger clutches than LAB females (B) and short photoperiod and low temperature seems to increase the clutch size (C). See results for description. The labels on the x axis in figure A indicates the strain (KY; LAB), the photoperiod (long 14L:10D; short 11L:13D), temperature (high 25°C; low 23°C), the experimental diet (M milkweed; S sunflower) and the LAB virgin group.
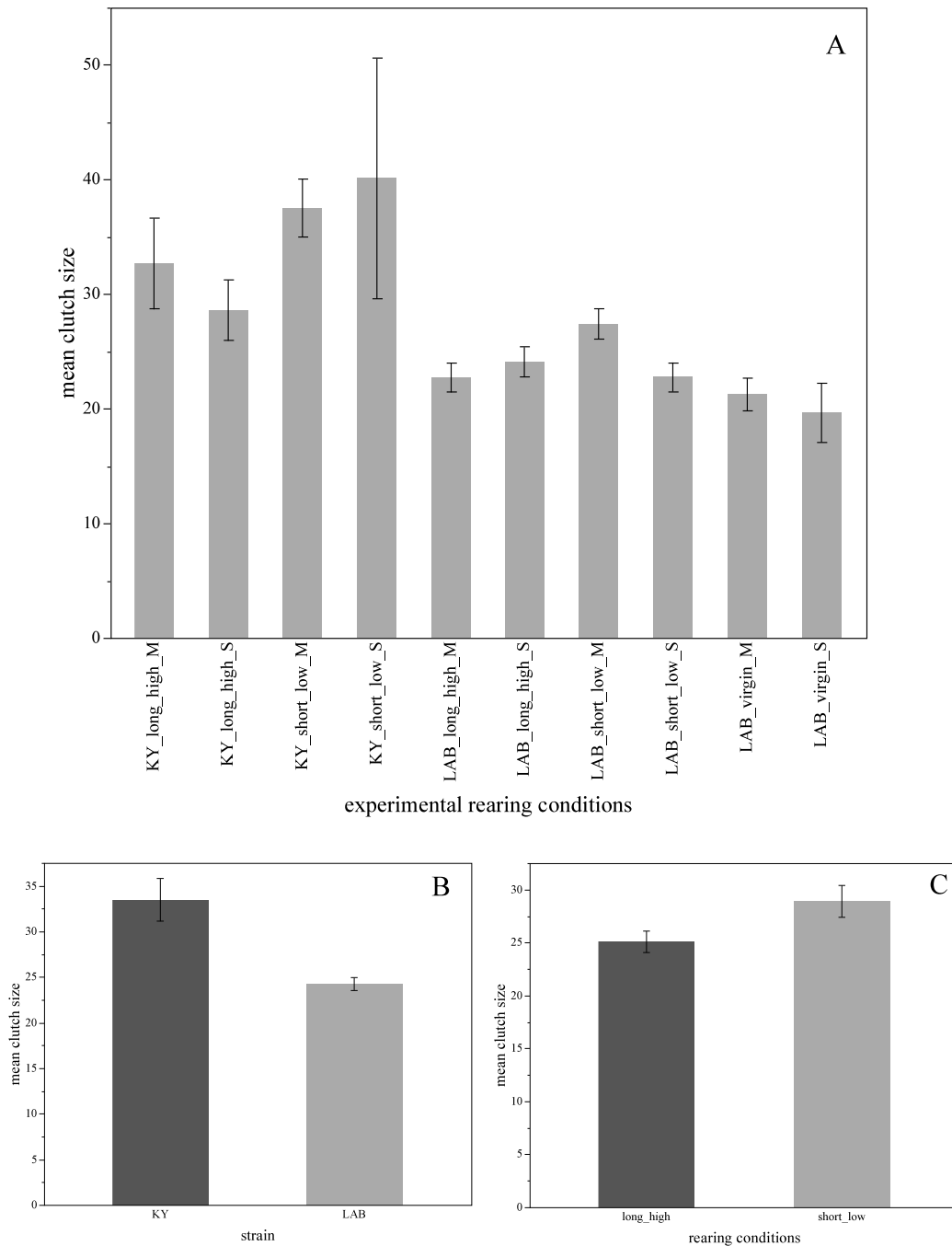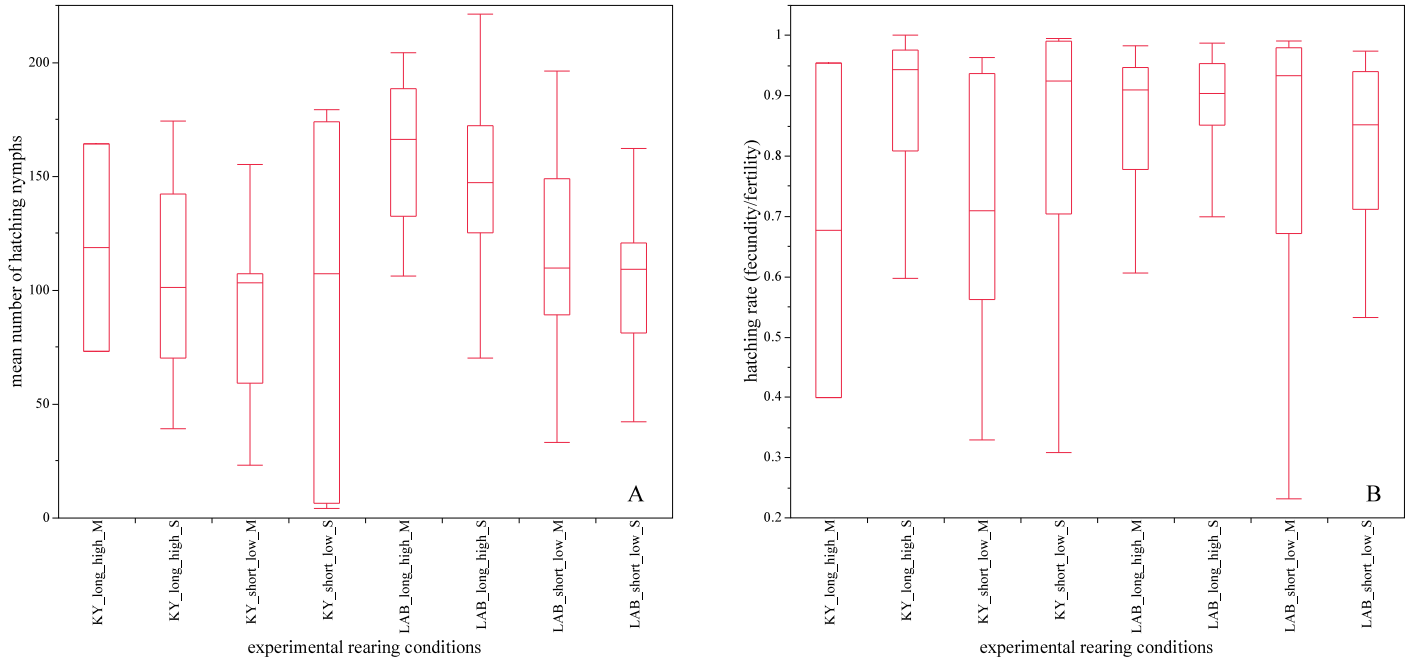
Figure 6. Fertility of KY and LAB pairs measured as the mean number of hatching nymphs (A) and the hatching rate (B) between rearing conditions and diets. The labels on the x axis indicates the strain (KY; LAB), the photoperiod (long 14L:10D; short 11L:13D), temperature (high 25°C; low 23°C) and the experimental diet (M milkweed; S sunflower).

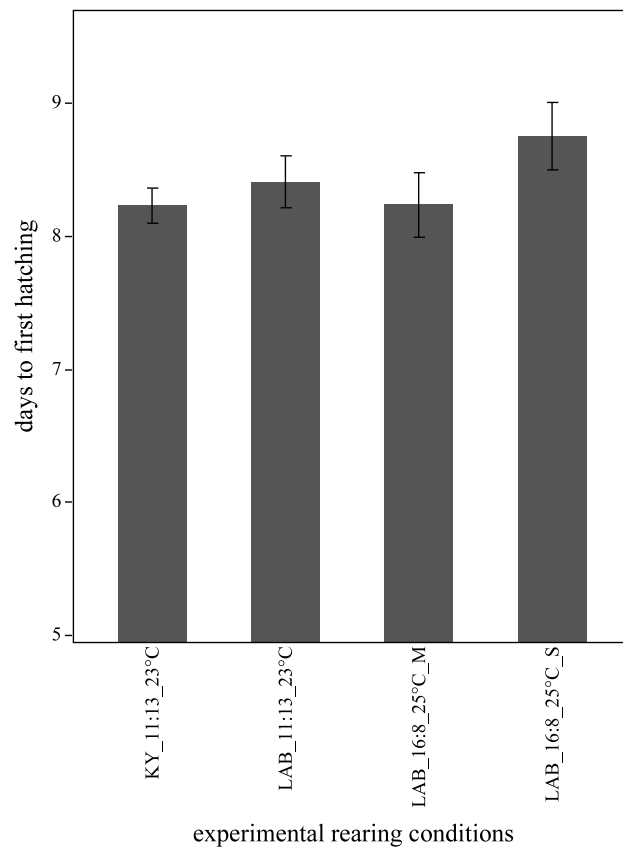## Appendix A2. Chapter 3 supplementary data

Figure A1. Mean number of days to first hatching of milkweed bugs reared in different experimental conditions. The labels on the x axis indicate the strain (KY, LAB), the rearing conditions (photoperiod, temperature) and diet (milkweed, sunflower). There is no difference across treatments (χ^2=3.029; d.f.=3; p=0.387).
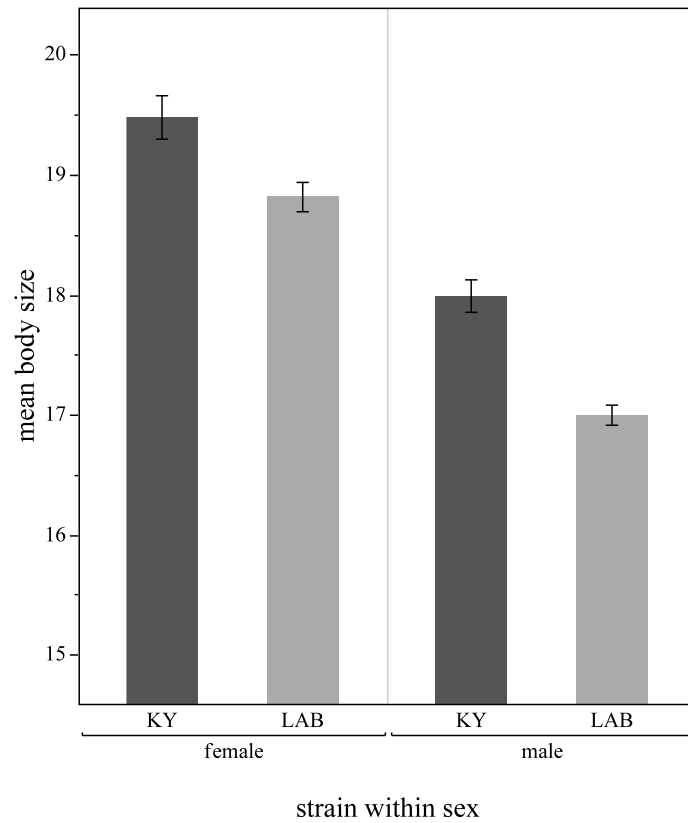
Figure A2. Body size comparison between a random set of males and females from the KY and LAB strains collected from the mass colonies reared in 14L:10D 25°C. Body size was measured as the length from the tip of the head to the posterior margin of the wing. Both LAB females (ANOVA; $F_{1, 38}$=9.043; p=0.005) and males (ANOVA; $F_{1, 38}$=38.597; p<0.0001) are smaller than the respective sex of the KY strain.

Chapter 4

A simple flight mill for the study of tethered flight in insects

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Manuscript

Abstract

Previous studies have shown that flight mills are valuable tools for the experimental study of insect flight behaviour. Lack of detailed information about how to build such a device can make their construction appear to be prohibitively complex. We present a simple and inexpensive flight mill for the study of tethered flight in insects. Experimental insects can be tethered with a non-toxic and reusable glue adhesive and revolve around an axis by means of a very low friction magnetic bearing. The mill is designed for the study of flight in controlled conditions as it can be used inside an incubator or environmental chamber. The strongest points are the very simple electronic circuitry, the ability to adjust the mill to accommodate different species of insects and potential to use the device in a very limited workspace. Sixteen insects can fly simultaneously allowing the collection and analysis of a large number of samples in a short time.

Keywords: flight mill, insects flight behaviour

Introduction

Several different techniques have been developed for the study of insect flight behaviour in laboratory conditions (Hardie, 1993; Reynolds and Riley, 2002). These techniques range from the simple static tethering (Dingle et al., 1980; Davis, 1986) to more sophisticated devices that allow greater freedom of movement for the tethered insect (Gatehouse and Hackett, 1980). To date flight chambers, most notably the "Kennedy flight chamber" and its successive modifications (Kennedy and Booth, 1963; Kennedy and Ludlow, 1974; Laughlin, 1974; Grace and Shipp, 1988) represent the devices allowing the highest level of freedom of flight in controlled conditions.

Although this technique allows free flight of the experimental insects, it has two major drawbacks: it is quite difficult to use for the study of large insects, unless using a very large device, and the manual procedure of data collection is quite time consuming.

Flight mills represent one of the most common and affordable techniques for the study of insect flight in laboratory conditions (Krell et al., 2003; Wang et al., 2009; Liu et al., 2011). Use of a flight mill implies that the experimental insects need to be tethered, intrusively handled and the flying path is constricted on a circular trajectory. This technique is preferable to static tethering because it offers moving stimuli (Dingle, 1996), but it differs from a free flight behavioural response (Riley et al., 1997; Blackmer et al., 2004; Taylor et al., 2010). However, some aspects of the flight behaviour on the mill and in the wild are similar (Gatehouse and Hackett, 1980; Cooter and Armes, 1993) so despite some limitations, flight mills represent a viable option to investigate questions regarding the occurrence of particular flight behaviour responses, as is the case of migratory flight type. Also, from the technical point of view, flight mills are easier to realize compared to wind tunnels or flight chambers and the data collection can easily be automated. Finally flight mills can be more easily managed when space is a limiting factor for the experimental work.

Several authors describe alternative designs for flight mill devices. In general the main part of the flight mill system, i.e. the pivoting mill's arm, is quite simple to realize. Less straightforward is the electronic part of the flight mill system, which allows the recording of the data. Dealing with electronic circuits design could be more challenging, especially for the entomologist or the behavioural ecologist lacking in background knowledge of electronics. We found that in the literature some authors describe a complicated or out of date electronic circuit component in their flight mill

design (Chambers et al., 1976; Clarke et al., 1984; Resurreccion et al., 1988; Taylor et al., 1992), or the description of the electronic part of the flight mill is missing (Schumacher et al., 1997; Bruzzone et al., 2009). Other designs describe mechanically complicated actographs, which are quite complicated to realize but can help investigators to undertake more complex behavioural observations (Gatehouse and Hackett, 1980). All these factors can only have the effect of decreasing the interest of the researcher in the design and manufacture of an experimental apparatus for behavioural study. A recent flight mill design is the one developed by the V. Jones group at the Washington State University for the study of flight behaviour in codling moths and leafrollers (http://entomology.tfrec.wsu.edu/VPJ_Lab/Flight-Mill.html). In this design each flight mill is composed by a single unit formed by a teflon support and a magnetic bearing by which an axis, formed by a hypodermic tubing, is revolving. The drawback of this design is the space required for the mills' experimental setting (Thea Smith, personal communication). Given that we were restricted to a very limited experimental space, the single mill's unit design was not the most functional one in our case. However, this design suggested us the idea of using hypodermic tubing as the revolving axis but the resulting design of the final device is quite different. We opted for a solution with cheaper and easier to find materials (plexiglas vs. teflon) and for a design in which the mills were stacked upon each other rather than being organized in single units. This allowed us to optimize our available laboratory space. A similar design to ours, which we were unaware of before the realization of our flight mill, is the one designed by Jason Lim at Rothamsted Research (Jason Lim, personal communication, http://www.rothamsted.ac.uk/agec/JL_PEG_facilities_tetheredflight.php). However, this device includes a more complex circuital design and the entire mill's structure is

fixed once set up in place. We opted for a design that could be rearranged and fitted in several experimental spaces, easily disassembled once not needed for experimental work and with a very simple circuital design that could be easily realized without the assistance or supervision of an electronic expert.

Thus, we present an easy and relatively cheap option to set up a flight mill for the study of tethered flight in insects. All researchers interested in the study of insect flight can easily realize this device, due the fact that the only technical knowledge required is limited to a few basic electronic components. Together with the extremely simple electronic part, our design has some other main strong points: 1) the structure is made out of transparent acrylic plastic so that a single light source can evenly reach every individual in separate chambers of the mill; 2) given the transparency of the material and the limited size, the flight mill can be used in incubators of adequate size; 3) the entire structure can be assembled and disassembled in a very short time and, once disassembled, it can be stored in a small space; 4) the design of the structure can be modified to allow the study of insects of different size and using different revolution distances 5) the data can be collected using 8 simultaneous channels at once for each of the data-loggers used so that a high number of individuals can be analysed at the same time, and large numbers of samples can be handled in the same day; 6) the entire device can be mounted and used in a very limited space, like a normal small office desk, thanks to the stacked design of the flight mill's chambers; 7) no expensive software is needed to record and visualize the data; 8) the custom written script for the data analysis can be modified following the specific needs of the experimental design.

Materials and methods

Flight mills device

The design involved the realization of two structures formed by eight stacked cells each for a total of 16 flight cells. Each structure was produced from 3mm thick transparent acrylic plastic (poly-methyl-methacrylate) sheets. This material was chosen for its transparency and light-weight. The plastic sheets were shaped in a way that each one fitted into one another along pre-cut linear junctions allowing the build up of the stacked cell configuration in order to form a squared 56cm x 56cm structure with eight mill cells, with each cell measuring 12.5cm x 25 cm x 25cm in size (Fig. 1). The stacked configuration of the flight mill cells was chosen to allow the device to fit into limited space areas whilst at the same time be easily disassembled once all the data have been collected. Each plastic sheet ended with a ~2.5cm "flap" on both sides: when the structure is formed these flaps are situated on the external sides of each chamber along the entire structure, allowing supports for the junctions between the sheets and for the entire structure itself. Eight 12.8cm x 4cm x 4cm polystyrene columns were placed in the external backside corner of each single cell providing increased stability to the structure. Alternatively an external support of polystyrene could be allocated on the lateral sides using the external "flaps" of the acrylic sheets (Fig. 2). An alternative solution would have been to use thicker acrylic sheets, but this approach came at the cost of increasing the total weight of the structure. We found that the best trade-off was to use 3mm thick plastic sheets and include some sort of removable support into the structure, which still allowed an easy handling of the device.

Two pieces of circular plastic piping tubes of 1cm diameter were fixed in the central part of the top and bottom internal faces of each cell forming the two main supports for the magnetic pivot (top: 5cm, bottom: 2cm). A couple of 10mm x 4mm

N42 neodymium magnets (Magnet Expert Ltd, Nottinghamshire, UK) were fixed with hot glue to each of the plastic tubes forming the magnetic bearing for the mill's arm. An entomological pin was inserted into a 20μm pipette tip and held in place with hot glue. The pin was positioned such that both ends extended out of the pipette tip. This assembly formed the armature of the flight mill where the top of the pin attached itself to the top set of magnets. The bottom set of magnets was used to maintain the attitude of the armature. Since the only connection was formed by the armature and the top magnets, the friction in the system was extremely low. Non-magnetic hypodermic steel tubing needle 220mm x 20mm gauge 19 was used as the mill's arms (Tomlinson Tube & Instrument Ltd, Warwickshire, UK, Fig. 3). These were found to be more effective than alternative mill's arms like drinking straws or wooden sticks because even though they were heavier (~1g), the drag produced was much lower due the small diameter of the hypodermic tubing. Thus, the tethered insects needed a slightly stronger effort to start the first revolution of the arm around the axis compared with a more lightweight arm, but all the successive revolutions were effortless compared to arms with higher levels of drag, due to the higher kinetic energy of the steel arm's itself.

The steel arm was curved to an angle of 95° at 2cm distance from one end and thus divided in two sides of 10 and 12cm. The 10 cm short curved side was used as a tethering point. The curved side and the gauge of the steel arm allowed a comfortable point of insertion for an entomological pin to which the insect could then be tethered to the pinhead side. Bostik® Blue-Tack was found to be an optimal non-toxic material to secure the insects to the entomological pin. This was as effective as other more powerful glues or dental waxes but in the same time it made easier to tether the experimental individuals without extensive handling and to remove the insects at the

end of the flight tests. In this configuration the entire revolution distance covered by a tethered insect was 62.8cm (10cm x 2π). A square shaped flag made with foil was attached on the 12 cm long end side of the arm providing both a counterweight and an optimal reflective surface for the passage through the IR sensor's beam during each revolution. By removing the vertical central plastic sheet, the cells could be enlarged to double the size. Using a longer steel arm would allow to test larger insects and to have revolution distances up to 1.20m.

The IR sensors (OPB800W, Optek Technology Inc., Texas, USA) were fixed with Bostik® Blue-Tack on the external sides of each cell (Fig. 3). The passage of the foil flag through the infrared beam allowed the recording of each arm's revolution. This allowed collecting data like speed, total distance and duration of the flight. The output of each sensor was sent as input into a data-logger (DI-149, DATAQ Instruments, Ohio, USA). These particular data-loggers were chosen for two main reasons: they offered a ready-to-use connection via USB with the main computer and also were provided with their own free software (WinDaq®/Lite, DATAQ Instruments, Ohio, USA) for the visualization and recording of the analog output signals from the infrared sensors. This turned out to be particularly useful in maintaining a very limited budget for the entire device with no need to purchase more expensive software to visualize and record the data. Each data-logger can read 8 channels simultaneously, so two data-loggers and two stacked structures of 8 cells each were used to obtain data from a total of 16 individuals at the same time. The recorded data were easily converted into *.csv files, which could be then analysed using commonly available software (Excel, R, MatLab).

Electronic circuit design

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For the sake of keeping the realization to an easily achievable design, the circuit did not include any complex electronic component but only simple resistors and the IR sensors. The circuit was constructed following the information available in the IR sensor's datasheet. The main consideration to take into account was to choose the right resistor's value to apply on the input and output of the sensors to obtain the right current intensity to power all the sensors in the 16 channels circuit: we used a 180Ω in input to limit the input forward current into the sensor and a 2.2kΩ for the output current from the sensor to the data-loggers (Fig. 4). A power unit with variable output voltage was used as power source. This allowed us to vary the power input and to adjust the power to obtain the optimal working voltage for each sensor and the best signal visualization in the software's recording user interface. The sensor's output was visualized by a combination of base and peak voltage. The base voltage was represented by the output signal coming from the sensor at rest, i.e. when the IR beam was not interrupted by the passage of the flag, plus the low electric noise level deriving from passive currents inside the circuit. The peak voltage was visualized as an electric peak of ~300mV from the base voltage and obtained from the interruption of the IR beam by the passage of the flag. A voltage of ~7V was used as standard because allowed for the best discrimination of base and peak voltage. The entire circuit was mounted and maintained on a two-sided breadboard for circuital design.

Software

The recorded data were visualized through the WinDaq/Lite software and then converted into a *.csv file. The file was then analysed with a custom-designed software written in MatLab (MatLab, MathWorks R2011b; Appendix 1). The main core of the software is to count the number of revolutions of the mill. This is done by

automatically identifying the peaks in the voltage trace due to the IR beam being interrupted by the flag. The magnitude of any data in the voltage trace below a threshold value is given a value of zero. The threshold value represents the average electrical noise in the system the magnitude of which is prescribed by the user. This leaves a voltage trace where only peaks are present separated with zero values. The peaks were then identified by checking the data to find two types of occurrences: 1) if a data point is preceded and followed by data points of lower values; 2) if a data point is preceded by a data point of a lower value and followed by a data point with the same value.

Each of these peaks indicated a distance travelled by the end of flymill arm of 62.8cm. At this point there is no time signature to the data. To add a time element to the signal the number of points and the sampling frequency (prescribed by the user) are used as each data point will represent 1/ (sampling frequency) seconds in time. With the time vector alongside the distance travelled the signal can now be differentiated to produce the velocity of the mill. The output of the software are variables in the MatLab workspace which can be further analysed in MatLab as well as *.csv files. The *csv files contain distance against time and velocity against time which can then be imported into Excel, R or other software for further analysis.

Experimental test

The experimental test was conducted using a population of milkweed bugs collected in Kentucky during the summer of 2009 (Charles Fox, personal communication). These bugs have been maintained in laboratory conditions for several generations but did not show a decrease in flight response (see Chapter 5). Newly eclosed adults were collected from house colonies maintained in 14:10 25°C

on a diet of milkweed seeds and water *ad libitum*. Individuals were put in 13:11 24°C

on a diet of milkweed seeds and water *ad libitum*: these rearing conditions were

chosen to maximize the number of bugs likely to show a flight response (Dingle et al.,

1980). A total sample of 65 females and 60 males were collected and tested for the

flight response. Both females and males were flight-tested at 8, 10, 12 and 14 days of

age in order to include young and sexually mature adults. We followed the flight

assessment technique described in Chapter 5. Flight tests were performed at room

temperature conditions (range 19°C-21°C). In addition the flight mills have been

tested with a range of insects of variable size: *Coccinella septempunctata,*

Nicrophorus vespilloides, N. interruptus and *N. investigator* in order to test the

reliability of the design in dealing with insects of different size, weight and flight

ability. In all situations the system showed itself to be suited for the collection of

flight data from a wide range of different experimental species.

Results

     The recorded signals were converted by the software into three human-

readable graphs showing: 1) the number and voltage of each single peak recorded

during the flight duration plotted against the time spent in flight 2) the overall trend of

the flight speed and total distance travelled plotted against time 3) the detailed speed

variation for the entire flight duration (Fig. 5). The label in the middle graph reports

distance travelled, average speed, minimum and maximum speed. It was then possible

to obtain detailed information about the characteristics of the flight behaviour and to

combine these data with variables like sex, food regime and temperature. Furthermore

the visualization offers other interesting points that could be analyzed like the detailed

individual variation in speed during the time spent in flight, flight patterns like bursts

of flight activity or long term sustained flights, variation in the flight behaviour related to individual characteristics like sex, age, body size or body mass (Fig. 5). The script can be converted in different programming languages and in the same time customized to obtain the graphs configuration and visualization that better suit the experimental questions.

Discussion

We designed our flight mills with three main aims in mind: 1) to maintain the complexity of the design to the minimum level possible 2) take into account factors like small workspaces, use in controlled experimental conditions, possibility to work with big sample sizes and possibility to adapt the device to different experimental settings 3) reliability in the collection of the behavioural data. The second point is the strongest in our opinion. In opposite to previous flight mills, our structure can be arranged to work with different species and the software can be changed according to the experimental questions.

The simple design makes the device also quite easy to realize. In particular we tried to maintain the circuital scheme to the possible lowest grade of complexity in order to make it easy to understand and to set up. This is, in our opinion, extremely valuable for the experimenter who does not have electronic skills but require this kind of experimental tools for his/her research needs. In addition the design is customizable enough in the structural and software parts to be adjusted to different experimental needs. Flight mills represents a useful device to ask questions related to flight behaviour in insects. The combination of life-history with flight behavioural data will give more insights into the behavioural strategies of those insect species in which flight is an important behavioural trait.

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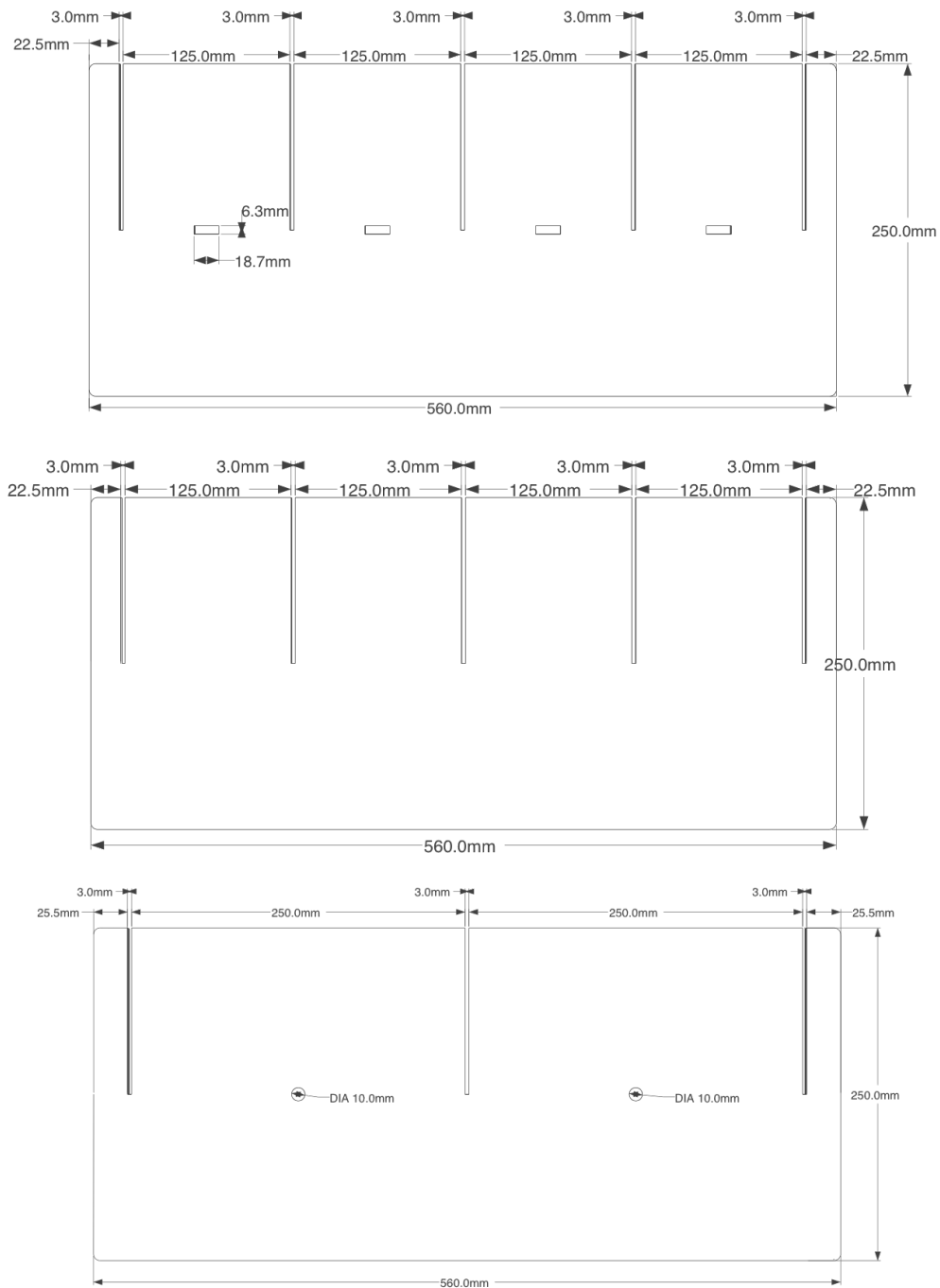
Figures

Figure 1. The design of the plastic sheets used to build up the 8 cells stacked flight mill: 2 vertical sides with holes for the allocation of the IR sensors (upper figure), a central vertical support (middle figure) and 5 horizontal flat surfaces to divide the 8 cells. The axes with the magnetic bearing are allocated in the centre of the horizontal surfaces.

Figure 2. Flight mills structure operating during one of the experimental tests. The second structure on the side mounts two lateral polystyrene supports.
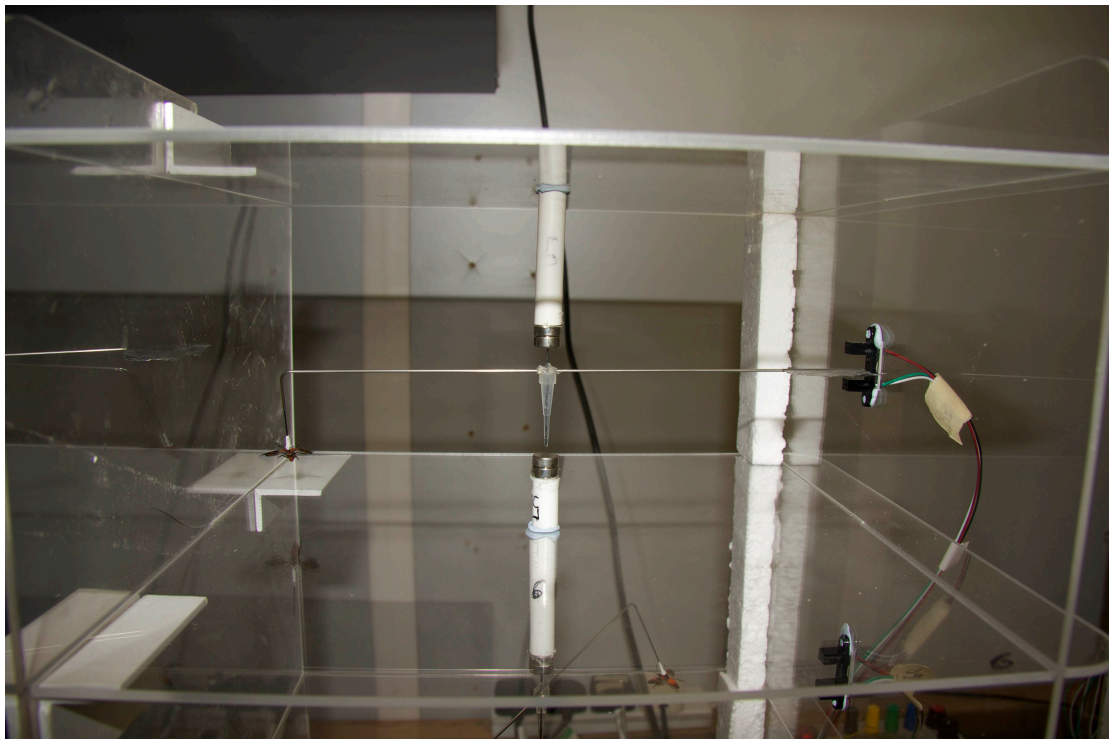

Figure 3. Single flight cell during one of the experimental tests. The arm is made out of a 22cm hypodermic steel needle curved at one side to allocate the tethering. The IR sensor is placed on the left side of the cell.
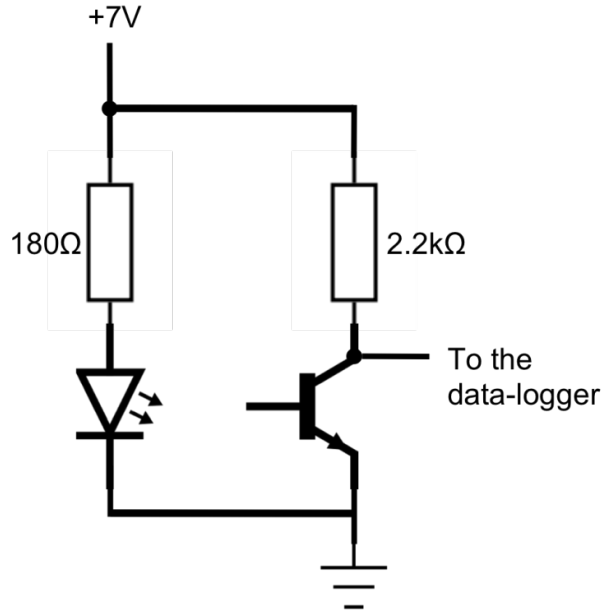
Figure 4. The simple electronic circuit scheme used to power each sensor.
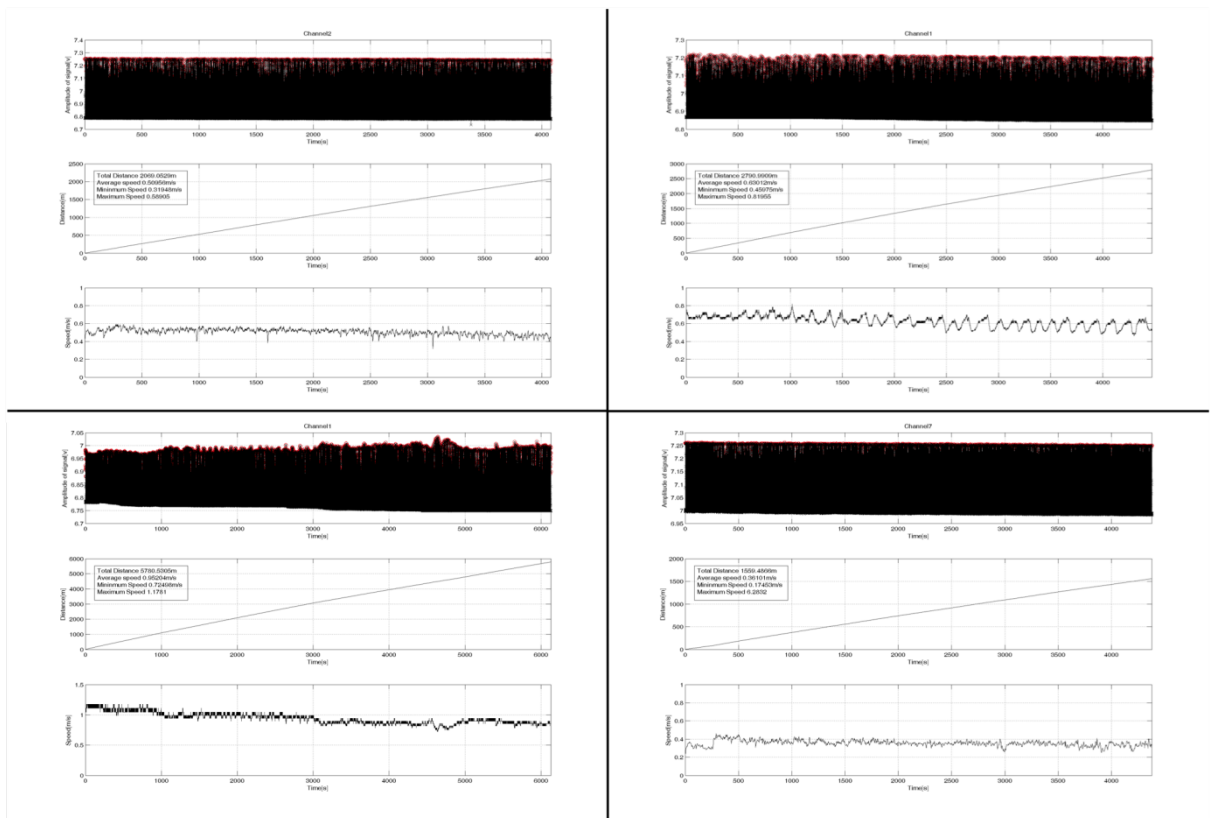

Figure 5. Graphs of flights data from 4 individuals. Each window is formed by three graphs: the recorded peaks (upper), the trend of the speed and the distance travelled (middle) and the detailed speed variation during the flight duration (lower). Notice the overtime speed variation between the two upper graphs and the difference in average speed between the two lower graphs.

Appendix A3 – MatLab script for the analysis of the flight mill's data

Flymill.m

This script will allow the analysis of data files from a flight mill measuring the number of revolution of the mill's arm due to an insect in flight. To use the script simply navigate the "current directory" in MatLab to the folder where the script is stored. Next, from the "command window" run the script typing "run flymill".

The script will run and a dialog box will appear asking the user to select the *.dat file containing the data recorded with the flight mill.

Next a dialog box will appear asking for information about the noise level of the voltage signal. This noise level indicates the level below which the data is considered noise and not required in the analysis.

Also in the same dialog the sample rate of the data is required. This is needed to calculate a time vector for the data to make the differentiation possible for the flight speed as well as the total time in flight.

The script will output a *.png file of the resulting data as well as *.csv for each channel of the velocity against time and the distance against time.

%% Get file name. Opens a dialog to allow the input file to be selected
```
[filename,path] = uigetfile('*.DAT','enter the DAT file');
```

%% read file
```
data=csvread([path,filename]);
[a b]=size(data);
```

% opens dialog for information on the max noise and sample rate
```
prompt={'Input max noise voltage [v]', 'Input sample Rate [Hz]'};
defans={'7', '30'};
fields = {'noise_v','samp_rate'};
info = inputdlg(prompt, 'Input noise level and sampling rate', 1,
defans);
if ~isempty(info)
   info = cell2struct(info,fields);
   voltag_ind = str2num(info.noise_v);
   samp_rate = str2num(info.samp_rate);
else return
end
clear defans
clear info
clear fields
clear prompt
Status= 'Finished reading data'
```

%% build time vector
```
time=(1/samp_rate:1/samp_rate:a*1/samp_rate)';
```

%% Finding the peaks in the voltage time history

```
peakind=cell(1,b);
for i =2:a-1
 for j=1:b
  if or(data(i-1,j)<data(i,j) && data(i,j)>data(i+1,j),data(i-
  1,j)<data(i,j) &&
  data(i,j)==data(i+1,j));
  peakind{:,j}=[peakind{:,j};i];
 end
end
end
Status= 'Voltage peaks found'
```

%% finding the distance and velocity time histories
```
peaks=cell(1,b);
channel=cell(1,b);
velocity=cell(1,b);
for i=1:b
 peaks{:,i}=[time(peakind{:,i}),data(peakind{:,i},i)];
 peaks{:,i}(peaks{:,i}(:,2)<voltag_ind,:)=[];
 channel{:,i}=[peaks{:,i}(:,1),[2*pi/10:2*pi/10:length(peaks{:,i})*
 2*pi/10]'];
 velocity{:,i}(:,2)=diff(channel{:,i}(:,2))./diff(channel{:,i}(:,1));
 velocity{:,i}(:,1)=channel{:,i}(1:length(velocity{:,i}));
end
Status= 'Finished calculating motion stats'
```

%% Writing data
```
for i=1:length(velocity)
 csvwrite([filename,'_Velocity_channel',num2str(i),'.txt'],
 velocity{i});
 csvwrite([filename,'_Distance_channel',num2str(i),'.txt'],
 channel{i});
end
Status= 'data files written'

for i=1:b
  if isempty(channel{i})==0
  figure
  set(gcf,'Position',get(0,'Screensize'));

 subplot(3,1,1)
  plot(peaks{:,i}(:,1)-(peaks{i}(1,1)),peaks{:,i}(:,2),'ro')
  hold on
  plot(time-(peaks{i}(1,1)),data(:,i),'kx-')
  title(['Channel',num2str(i)])
  xlabel 'Time[s]'
  ylabel 'Amplitude of signal[v]'
  xlim([0 max(channel{i}(:,1))-(peaks{i}(1,1))])
  grid on

 subplot(3,1,2)
  plot(channel{:,i}(:,1)-(peaks{i}(1,1)),channel{:,i}(:,2),'k')
  xlabel 'Time[s]'
  ylabel 'Distance[m]'
  xlim([0 max(channel{i}(:,1))-(peaks{i}(1,1))])
  grid on

 subplot(3,1,3)
  plot(velocity{:,i}(:,1)-(peaks{i}(1,1)),velocity{:,i}(:,2),'k')
  xlabel 'Time[s]'
  ylabel 'Speed[m/s]'
  ylim([0 1.5])
```

```matlab
  xlim([0 max(channel{i}(:,1))-(peaks{i}(1,1))])
   grid on

 annotation('textbox',...
  [0.144777573529412 0.515063110264349 0.184128676470588
   0.0933786078098472],...
  'String',{['Total Distance ',num2str(max(channel{i}(:,2))),'m'],...
  ['Average speed ',num2str(mean(velocity{i}(:,2))),'m/s']...,
  ['Minimum Speed ',num2str(min(velocity{i}(:,2))),'m/s'],...
  ['Maximum Speed ',num2str(max(velocity{i}(:,2)))]},...
  'FitBoxToText','on');

 set(gcf,'Position',get(0,'Screensize'));
 set(gcf,'PaperPositionMode','auto')
 print(gcf, '-dpng ', [filename,' channel ',num2str(i),'.png'])
end
end
Status= 'Processing complete'
```

%close all

Chapter 5

Reproductive physiology and the evolution of a partial migratory strategy

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Abstract

Partial migration is a puzzle. If migration allows individuals to overcome locally poor environmental conditions, why do not all individuals migrate? Little is known about the physiological mechanisms underlying individual variation in propensity to migrate. We tested the hypothesis that compared to migrants, residential behaviour is associated with a higher degree of phenotypic plasticity in oosorption, an adaptive physiological mechanism that allows individuals to recoup resources from developing oocytes. Reallocation from reproduction to survival in stressful conditions would allow individuals to avoid migration and allow them to cope with unfavourable environments. If this plasticity is evolved, we further predicted it would vary among as well as within populations. We examined variation associated with migratory behaviour in females from four populations of the milkweed bug, *Oncopeltus fasciatus*. We used a behavioural assay to categorise females as either migrant or resident and examined differences among these groups in oosorption. As expected, food availability, source population and wing length all influenced the propensity for migratory flight, and food availability influenced levels of oosorption. We also found support for our key prediction that resident females are characterized by higher levels of oosorption than migrant females. Our study provides support for the hypothesis that partial migration evolves as alternative strategic responses to physiologically stressful environments.

Keywords: insect reproductive physiology, oosorption, flight mill, behavioural strategy, partial migration, phenotypic plasticity.

Introduction

Migration is a complex behavioural syndrome that evolves in response to temporal fluctuations in resource availability (Dingle, 1996; Alerstam et al., 2003; Dingle and Drake, 2007; Pulido, 2011). Partial migration, when a population includes both migrant and non-migrant individuals, has attracted attention from researchers interested in animal migratory movements due to the realization that partial migration is a more widespread phenomenon than previously assumed (Chapman et al., 2011). The presence of individuals willing to migrate as well as individuals willing to remain *in situ* raises the question of how these seemingly opposite tactics evolve and are maintained. Patterns of partial migration can develop as a consequence of resource limitation due to extreme weather conditions (Boyle, 2011), trade-offs between resource quality and predation (Hebblewhite and Merrill, 2007; Hebblewhite et al., 2008; Hebblewhite and Merrill, 2009; Skov et al., 2011), interacting ecological factors like snow cover and topography (Ball et al., 2001; Cagnacci et al., 2011) and intra-specific competition (Lundberg, 1985; Mysterud et al., 2011). Behavioural variation in migratory tendency can have a genetic basis (Biebach, 1983; Berthold, 1991; Pulido, 2011). This indicates that the degree of behavioural variation is determined by a combination of genetic, social and ecological interactions (Gillis et al., 2008). However, few authors have focused on the physiological mechanisms which may influence individual variation, mainly because previous work has focused on model species for which the collection of these type of data is challenging.

Much of the partial migration literature to date has examined vertebrate species, leaving a large gap regarding invertebrate examples, particularly insects (Chapman et al., 2011), even though insects represent a large proportion of the biomass involved in animal movements (Holland et al., 2006). Insect migration

differs from vertebrate migration in that it is a multi-generational event (Dingle, 1972; Brower, 1995; Holland et al., 2006), thus individuals are likely to adopt the migrant or resident tactic only once in their lifetime. Moreover, insects may provide a model for understanding the proximate causes of variation in migration tactics, and therefore addressing why partial migration evolves. For example, insects are capable of a high degree of life-history plasticity in response to environmental cues (Nylin and Gotthard, 1998), making them excellent candidates for asking how individuals with different migration tactics vary. Indeed, most migrant insects can be categorized as facultative migrants (Dingle, 1996).

The decision to migrate in insects typically is viewed from the perspective of the oogenesis flight syndrome (Johnson, 1969) which involves a trade-off between migratory ability and reproductive maturation (Dingle, 1996; Zera and Denno, 1997; Roff and Gélinas, 2003), and thus adult life in insects can be divided into a migratory pre-reproductive phase followed by a non-migratory reproductive phase (Dingle, 1972, 1996). When faced with a challenging environment, individuals can either allocate resources to flight, foregoing offspring in favour of survival until new resources have been found or they can allocate current limited resources to reproduction at the expenses of survival and future reproductive potential (Roff and Gélinas, 2003).

In some insect species, it has been observed that individuals show behavioural variation in propensity to fly with individuals categorized as flyers or non-fliers (Dingle, 1965,1968; Cooter, 1982; McAnelly, 1986). Despite the known variation in propensity to fly, to our knowledge no study has focused on explaining why in species without wing dimorphism, nor wing muscle histolysis, some individuals fly long distances while others never fly at all. Yet evidence for partially migrant insect

species does exist (Dingle et al., 1980a; Corbet, 1999), although the term partial migration, while is widely used and accepted in the vertebrate migration literature, has not been used to describe this phenomenon (Chapman et al., 2011). Furthermore, insects provide a potential model system for understanding migratory strategies because of the potential to conduct manipulative experiments that are impractical in vertebrate systems. Such experiments allow us to address the lack of knowledge about the underlying physiological mechanisms allowing the adoption of different life-history strategies (Chapman et al., 2011). Such experimental tests are badly needed. To date, only Nilsson et al. (2011) have identified physiological differences between migrants and residents in blue tits.

The link between migration and reproduction suggests a mechanism that may influence partial migration in insects, oocyte resorption (oosorption). Oosorption in insects is a physiological response to unfavourable environments (Bell and Bohm, 1975). Females can resorb nutrients from developing oocytes, to invest resources in survival rather than reproduction (Boggs and Ross, 1993; Ohgushi, 1996). Social factors (Moore and Sharma, 2005; Moore et al., 2007; Barrett et al. 2009), food availability and starvation (Kotaki 2003; Osawa 2005; Barrett et al., 2009; Kajita and Evans, 2009; Park et al., 2009) and parasitic loads (Hopwood et al., 2001) all influence oosorption. Thus, there is a link between poor environmental conditions and oosorption, suggesting that oosorption may be an alternative to migration when conditions are poor.

We have shown that the presence of suboptimal food and starvation increases the amount of oosorption through apoptosis in females compared to a optimal diet allowing them to shift available resources to survival rather than reproduction in the large milkweed bug, *Oncopeltus fasciatus* (Moore and Attisano, 2011; Attisano,

unpublished results). Thus, this bug presents an ideal candidate to address the question of how oosorption might be related to migratory polymorphism. *O. fasciatus*, is distributed from the Caribbean to southern areas of Canada (Feir, 1974). It commonly feeds on milkweed, mainly *Asclepias sp.,* also *Sarcostemma sp.* and naturalized *Calotropis sp.* in southern areas, but can feed on alternative hosts like *Nerium oleander* (Klausner et al., 1980) in areas where milkweed plants are temporarily unavailable (Miller and Dingle, 1982). Nymphs develop better on a diet of milkweed seeds (Chaplin, 1980; Chaplin and Chaplin, 1981) compared to alternative diets (Feir, 1974), but alternative hosts can allow nymphs' development (Klausner et al., 1980), particularly after a few generations of adaptation (Feir, 1974; Moore and Attisano, 2011). *O. fasciatus* perform an annual colonization of northern areas of US migrating from the southern overwintering areas following the seasonal blooming of milkweed hosts (Dingle, 1972, 1996). Populations from different areas of US show a geographical variation in flight behaviour; highly migrant bugs from northern areas and sedentary bugs from tropical areas of Mexico and Caribbean represents the two extremes along this geographical gradient, while bugs in southern US areas are somewhat in the middle (Dingle et al., 1980a). Specimens from Iowa show the greatest tendency to perform long distance flight and a migratory syndrome in which traits like wing length, fecundity, development time and flight duration are genetically correlated (Palmer and Dingle, 1986, 1989). The Puerto Rico population shows instead the lower propensity to fly long distances and traits like wing length, fecundity and flight duration are not genetically correlated suggesting the absence of a similar migratory syndrome (Dingle and Evans, 1987; Dingle et al., 1988). However, only a proportion of bugs from northern populations perform long distance flight (Dingle, 1965, 1966; Dingle et al., 1980a), and these are either individuals that can

perform long duration flights in consecutive days or individuals that fly just once (Dingle, 1966). This percentage can be increased by factors like temperature, photoperiod and lack of mating (Dingle, 1966) and selection on wing length and flight duration (Dingle and Evans, 1987; Dingle et al., 1988). Short periods of starvation do not increase the number of long duration migratory flights, but only the number of short duration flights (Dingle, 1966). Thus, even in these northern migratory populations, there are bugs that never fly (Dingle, 1965, 1966; Dingle et al., 1980a) and this sort of variation has never been taken into account and explained. Although wing muscle histolysis has been reported in at least two lygaeid bugs (Solbreck and Pearson, 1979; Solbreck, 1986), it has never been reported for *O. fasciatus,* which probably have the best-described migratory syndrome of all Lygaeidae (Dingle, 1996). Brachiptery has been observed only in laboratory cultures of tropical *O. fasciatus*, but never observed in the field, and could be derived from a single recessive Mendelian unit (Klausner et al., 1981). Thus neither of these processes seems to be involved in the behavioural polymorphism observed in *O. fasciatus*.

We studied females from 4 populations of the large milkweed bug, *O. fasciatus*, characterized by different migratory tendencies (Dingle et al., 1980a). In our first experiment we examined the effects of population, wing length and food availability on the proportion of virgin females exhibiting migratory flight behaviour. Using the data collected in the first experiment, we divided females into two categories. Females that undertook long distance flights were classed as migratory and those that did not fly were classed as resident. We then tested the hypothesis that resident and migrant females differed in the level of ovarian apoptosis induced when food is removed. Our hypothesis was that females that are exposed to poor nutritional conditions could respond either behaviourally, by migrating to a new food source, or

physiologically, by resorbing eggs and waiting to reproduce. Our second hypothesis was that this is an evolutionary adaptation, and therefore the plasticity in this response will depend on the population from which the female has been derived. Hence we predict that migratory females will show a lower tendency to use apoptosis to re-allocate resources from reproduction to survival than non-migratory females. If this is an adaptation, populations with high levels of migration will be less likely to show oosorption.

Materials and methods

Collection and rearing

Four populations of milkweed bugs were obtained from different locations in USA: Puerto Rico, Florida, Kentucky and Iowa. Apart from Kentucky bugs, all individuals were collected during August 2011. Milkweed bugs migrate northward during late spring–summer months (Dingle, 1972, 1996) and during the summer months the entire USA population is divided into migratory northern populations and residential southern populations. Thus the collection period has been chosen to maximize sampling from populations formed mainly by migrant or by resident individuals. Kentucky bugs were collected in the University of Kentucky Arboretum, Lexington, KY in September 2009 from *Asclepias syriaca* (Charles Fox, personal communication). Puerto Rico bugs were collected from *Calotropis procera* in pastures sites in the southern area of the island near Santa Isabel. Florida bugs were collected in Homestead, south Florida, from *Nerium oleander*, *Calotropis gigantea*, and from *A. curassavica* and *A. tuberosa* growing in a plant nursery. Iowa bugs were collected in eastern, central and western Iowa from *A. syriaca* and *A. verticillata*.

In the laboratory, all populations were reared in 14h:10h Light:Dark, 25°C and relative humidity ranging from 50% to 65% and fed on *A. syriaca* seeds (Educational Science, League City, TX) and *ad libitum* deionized water for 4-5 generations before the beginning of the experiment. Kentucky bugs had been reared in the same conditions since October 2009. The longer rearing period of Kentucky bugs did not affect their ability to perform migratory flights (Attisano, unpublished results), confirming observations by Dingle (Dingle, 1965, 1966).

Only female bugs were used for the experiment, given the nature of the ovarian physiological response we wanted to measure. Females are also more likely to fly than males (Dingle, 1966). Newly eclosed adult females were collected daily from nymph colonies reared at 14:10 25°C. Focal females were placed in small square plastic boxes (110 mm x 110 mm x 30 mm) with *A. syriaca* seeds and a dental wick wetted with deionised water. The dental wick was rewetted daily. Focal females were placed in 13h:11h Light:Dark, 24°C. Density was controlled at five females per box. The rearing conditions were chosen to prevent females from entering diapause (Dingle et al., 1980b).

Females were maintained in these rearing conditions for 6 days. At 7 days of age each female was randomly placed in a petri dish (90 mm x 15 mm) and assigned to one of the experimental food availability treatments: food present or food removed. Food present females had *ad libitum* deionized water and milkweed seeds available for the entire duration of the experiment while food removed females had only deionized water. The amount of food in the food present treatment was not measured, but seeds provided were more than required to sustain individuals for the duration of the experiment.

Effect of food availability, source population and wing length on flight behaviour

We were first interested in the factors of food availability and source population and the covariate of wing length underlying flight behaviour. Based on previous data, we predicted that both factors would increase the proportion of females flying and that propensity to fly would be greater with longer wing length. Females were flight tested at 8 and 14 days of age using a custom built tethered flight mill (Attisano and Vickers, *in review*). The ages tested were chosen based on previous observations about the emergence of the flight response in milkweed bugs (Dingle, 1965). Flight tests were performed following a standardized procedure. A female was attached to an entomological pin using Bostik Blu-Tack ® white, which gave similar performances than more powerful glues or waxes but was not toxic to the bugs, did not need any particular preparation before the tethering and allowed bugs to be safely removed from the apparatus at the end of the flight trial. Once secured to the pin, the bug was lifted and stimulated to fly with a puff of air directed to the head. Usually bugs flew immediately after stimulation, but in some cases needed to be waved gently in the air to stimulate a flight response. Each female was tested in such a way for 3 consecutive times. If in at least one of these tries the flight was longer than 5-10 seconds, the pin was inserted into the flight mill's arm to record the flight. Otherwise the bug was categorized as a non-flyer. Bugs that performed a flight burst of at least 10 seconds during the stimulation trials were likely to fly when attached to the mill's arm, while bugs that gave a flight burst for less than 5 seconds never flew once tethered. Bugs that were willing to fly responded readily to even a minimal stimulation and the experimental manipulations like lifting and waving the bug, or inserting the pin to the mill's arm did not stop their flight. Thus the pre-flight stimulation distinguished between flying and non-flying bugs. Females that did not

fly were tested again one hour after the first stimulation to verify the reliability of the first response. In all cases, bugs that did not respond to the first stimulation did not fly in the same day, even if tested twice. Flight tests were performed at 25 ± 1 °C.

We characterized the individual migratory tendency as either migrant or resident based on propensity to engage in sustained flight. Our assay for migratory flight performance was fixed at 1 hour. Bugs flying for at least 1 hour are likely to fly for longer periods (Attisano, personal observations). In our experience, this flight length was a more reliable indicator than the 30 minutes flight indicated as a threshold by Dingle et al. (1980a). Females that flew for more than 1 hour in at least one of the flight tests were designated as migrants with all others designated as residents. In total 645 bugs were flight-tested and 343 classed as migrants. Only 42 females flew for less than 1 hour in either or both the flight tests after being attached to the flight mill's arm and in the analysis these were not considered as migratory flights (Table 1).

At day 15, following the last of the flight trials, the right forewing of each focal female was collected and wing length was obtained by measuring the longest distance from the proximal to the distal point passing through the centre of the wing marked by the conjunction between radial and median veins.

Effect of migratory behaviour, food availability and population on levels of ovarian apoptosis

Once females were classified as either migratory or resident, we examined whether these two classes of females differed in levels of oosorption under food stress. We predicted that the level of ovarian apoptosis would be higher in resident females. A random subset of migratory and resident females from each population and for each treatment was dissected on day 15 from adult emergence to assess for the

level of ovarian apoptosis (Table 2). It has been shown previously that oocyte resorption in *O. fasciatus* under nutritional stress occurs through apoptosis (Moore and Attisano, 2011).

Ovaries were dissected into PBS (Phosphate Buffered Saline) and stained with the Vybrant® Apoptosis Assay Kit #4 (Molecular Probes, Invitrogen, Eugene, OR) as described by Moore and Sharma (2005). The kit contains two dyes: the YO-PRO1 can enter apoptotic cells but not healthy cells giving a green fluorescence, while the propidium iodide (PI) can enter only cells that are either necrotic or in the late stages of apoptosis giving a red fluorescence (Willingham, 1999; Moore and Sharma, 2005). Thus healthy cells remain unstained, while apoptotic oocytes show green fluorescence and oocytes in late stages of apoptosis or necrotic show red fluorescence. The ovaries were observed using an Olympus BX61 epifluorescence microscope (Olympus UK Ltd., London, UK). Ovaries of *O. fasciatus* females are formed by 7 ovarioles each. We did not collect observation from ovarioles showing tissue damage due to dissection. Therefore the data collected were the total number of ovarioles showing evidence of apoptosis through green or red fluorescence out of the total number of ovarioles collected intact for each female. Prior to the dissection a volunteer unaware of the population, treatment and flight response was chosen to randomly code each female. Thus staining and observation were done blindly in relation to population, treatment and flight behaviour. Codes were only revealed after scoring the staining levels of the ovaries.

Statistical analysis

Our first test examined the factors associated with migratory tendency. We used logistic regression to examine the effect of population, food availability and their

interaction on migratory tendency. Wing length was included as a covariate. Our second test examined the factors associated with oosorption assayed by the extent of ovarian apoptosis. We used ANOVA to analyse the effect of migratory tendency, population, food availability and all interactions on levels of ovarian apoptosis. Wing length was again included as a covariate. All analyses were performed using JMP® 8.0.2 (SAS Institute Inc., 1989-2009).

Results

Effect of food availability, source population and wing length on migratory tendency

Food availability, source population, and wing length all had significant effects on the migratory tendency of females. Fed females were less likely to engage in migratory flight than starved females (χ^2 = 5.19, d.f. = 1, p = 0.022; Figure 1). Populations also differed in their propensity to undertake migratory flights (χ^2 = 48.04, d.f. = 3, p < 0.001; Figure 1), but there was no interaction between food availability and population (χ^2 = 5.70, d.f. = 3, p = 0.127). The populations from Iowa and Florida were less likely to show sustained flight. Wing length also had a significant effect on flight behaviour (χ^2 = 19.17, d.f. = 1, p <0.001). As has been found in the past, females with longer wings are more likely to exhibit migratory flight.

Effect of migratory behaviour, food availability and population on levels of ovarian apoptosis

Migratory tendency, food availability and population all had statistically significant effects on levels of ovarian apoptosis (Table 3). In addition there was a weakly significant interaction between population and treatment but no other

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interactions were significant (Table 3). Wing length was not a significant covariate (Table 3). The pattern of the effects of these factors can be seen in Figure 2. There was a very strong influence of food availability, as expected, with less food resulting in more ovarian apoptosis. There was also a strong influence of migratory tendency with resident females having higher levels of apoptosis than migrants. Population was also a strong statistically significant factor, with generally higher levels of ovarian apoptosis in Florida and Iowa populations than in Kentucky or Puerto Rico populations. The differences among the source population were greater under the food available treatment than the food removed treatment, and this effect was stronger in Iowa and Puerto Rico than in Kentucky and Florida.

Discussion

We found both between and within population variation in two alternative strategies for coping with poor food conditions. Females that find themselves in an environment that does not provide adequate nutrients can either migrate to a new habitat (Roff and Fairbairn, 2007) or they can resorb eggs, allocate resources to survival and wait for conditions to improve (Papaj, 2000). Typically these two strategies have been examined independently. However, our data show that both may be operating in our populations of *O. fasciatus*, with females opting to either fly or resorb their eggs when faced with an absence of food. The population differences suggested that there are adaptive differences in adopting these alternative tactics. These results are particularly interesting in light of the question of partial migration: why do some individuals migrate under poor conditions and why do some remain resident?

Behavioural variation in flight response

As expected, food availability, source population and wing length all affected the propensity for migratory flight. All source populations contained females that demonstrated migratory behaviour. Based on previous work by Dingle and colleagues (Dingle et. al, 1980a) we predicted that we would observe differences in migratory tendency among the four populations if partial migration is an alternative strategy. Bugs from Puerto Rico, in particular, belong to a "resident" population (Dingle et al., 1980a), with a very low proportion of individuals showing long duration flight. Our experimental conditions were set to maximise flight response from all populations: photoperiod, temperature, virginity and prolonged starvation are all factors triggering an increase in flight response even if not expressly migratory flight (Dingle, 1966, 1968). We found that populations varied in migratory tendency, but in an unexpected pattern. Puerto Rico and Kentucky were the most active flyers, while Iowa and Florida the less active. Furthermore, Puerto Rico and Iowa showed a very similar flight propensity response on the food available treatment. This proportion of individuals showing sustained flights was expected for Iowa but not for Puerto Rico. It may be that Puerto Rico bugs are not truly migratory, but still able to perform long duration flights in search for available patches of food around the island. Nevertheless, this result differs from Dingle et al. (1980a). However, our experimental conditions are different from those previously used to determine the flight behaviour in milkweed bugs. For instance, the use of flight mills, which could provide more appropriate cues to maintain a flight response, rather than static tethering is likely to provide a more accurate assay of migratory tendency. Indeed, all of our populations showed a greater proportion of fliers than any population measured by Dingle et al. (1980a). Also, we used virgin females rather than mated females and

mating might affect the trade-off between searching for new habitat and waiting to reproduce. Kentucky females were the most active flyers. Kentucky bugs have never been studied before, making it difficult to compare with the literature. This population was reared in laboratory conditions longer than the other populations, but there is no reason to suspect this has resulted in selection for migratory traits, which are more likely to be selected against.

The behavioural response to stress showed a more expected pattern, and is consistent with migration in response to poor food. While all populations contained a proportion of individuals that showed migratory behaviour, only the southern populations of Florida and Puerto Rico showed an increase in flight response when starved. In *O. fasciatus* collected from Iowa, starvation is likely to increase the number of trivial but not long duration migratory flights, thus is more likely to be a local search for food rather than a migratory movement (Dingle, 1968). Other studies have shown that starvation can decrease flight performance (Cooter, 1982; Wang et al., 2009; Johnson et al., 2011), increase the propensity to fly in search for new resources (Dingle and Arora, 1973; Obha and Takagi, 2005), or have no effect on the propensity to fly (Moriya and Satoshi, 1998). Milkweed seeds represent the main source of food for developing nymphs of *O. fasciatus* (Chaplin and Chaplin, 1981). Milkweed plants are scattered and quite rare in southern Florida, particularly in summer (Miller and Dingle, 1982), and locally common, but patchy, in Puerto Rico (Dingle, 1992). In southern Florida alternative hosts are available in summer when milkweeds are not producing pods (Miller and Dingle, 1982). In Puerto Rico the alternative host *C. procera* is available all year round but is present in big patches only in the southern dry areas of the island (Weaver and Chinea, 2003). Both these situations are in contrast with the abundance of milkweed, *A. syriaca* in particular,

during the summer months in Iowa and Kentucky. Thus, southern populations may have evolved a stronger propensity to disperse in response to cues like food availability compared to northern populations, which could rely mainly on cues like temperature and photoperiod. Host plant phenology may therefore create selection for long duration flights in southern areas where food is seasonally scarce or unsuitable, while the same selective pressure is lacking in northern areas where host plants can be reached within shorter distances. Thus migration in this species is not a distinctive feature only of northern populations, but might happen in southern populations as well as a response to the unpredictable distribution of resources.

What determines which tactic a female adopts when faced with poor nutritional conditions?

Both the selective pressure on individuals to migrate when conditions deteriorate (Southwood, 1962; Denno et., al 2001; Alerstam et., al 2003; Dingle and Drake, 2007) and the physiological mechanisms underlying the transition to migratory behaviour (Rankin and Rankin, 1980; Rankin and Burchsted, 1992; Dingle, 1996; Dingle and Winchell, 1997; Paez et al., 2011) have been discussed in some detail. However, what happens to the individuals that do not migrate? In this and in earlier studies of flight behaviour individual *O. fasciatus* can be divided into two distinct categories. Previous authors have focused their attention on the bugs that did fly, explaining the variation with genetic differences between populations, environmental stimuli or poor detection power of the experimental procedure (Dingle, 1966; Rankin and Riddiford, 1977; Dingle et al., 1980a). While this work has helped us to understand the nature of migratory behaviour, our aim is to understand the nature of the residents. Do these individuals have an alternative mechanism to respond to

difficult conditions? Our results suggest that residents respond via an alternative strategy, physiological plasticity, in which resources are conserved and reallocated until conditions improve.

Given the two tactics that we have identified, either reallocate resources and wait, or move to a new habitat, what determines which an individual female should use? The decision to migrate or remain resident could be due to a conditional strategy; all females may be capable of both tactics and the decision to migrate or remain resident may depend on individual state, such as size at maturity. As has been observed previously (Dingle et al., 1980a), resident populations tend to be smaller than migrant populations, but this result should be considered cautiously (see Roff and Fairbairn, 1991). Larger individuals expend relatively less energy in flight than smaller individuals (Roff and Fairnbairn, 1991), and may be in better physiological condition than smaller individuals. This may enable them to afford the costs involved in migration (Rankin and Burchsted, 1992). Smaller females may not have the resources required to undertake a migratory flight and thus must find an alternative strategy to cope with the absence of food.

It has previously been shown that in *O. fasciatus* females oosorption is a plastic physiological response to poor nutritional environments (Moore and Attisano, 2011). Such a mechanism could allow resident females to cope with sub-optimal sources or even total lack of food. In southern Florida, individuals have to rely on alternative sources of food, if and where they are available. This situation is likely to have led to the evolution of mechanisms to cope with sub-optimal conditions. Diapause has been hypothesised as such a mechanism (Klausner et al., 1980). However, in *O. fasciatus*, reproduction reduces flight, but diapause permits flight (Dingle, 1978; Dingle et al., 1980b). We cannot completely rule out the possibility

that some bugs entered diapause in our rearing conditions, in particular Florida females, which show a wider threshold for diapause initiation (Dingle et al., 1980b). Females would be able to perform a less radical physiological response that would allow finer tuning of the plastic response without a long-term shut down of the reproductive ability.

Variation among females in response to a lack of food through either behaviour (migratory flight) or physiology (oosorption) could also be purely genetic. Northern populations of *O. fasciatus* show a "migratory syndrome" with genetic correlations among body size, wing length, flight capacity and early fecundity (Dingle and Evans, 1987; Dingle et al., 1988), while these correlations are not present in the Puerto Rico populations (Palmer and Dingle, 1986, 1988). This suggests there might be a "non-migratory syndrome" with similar genetic correlations associated with the ability to survive in a limited food environment, including the ability to resorb eggs under nutritional stress. While we have not undertaken this type of study in *O. fasciatus*, we have shown that the level of ovarian apoptosis under stressful conditions is heritable in a cockroach (Edvardsson et al., 2009).

Conclusion

Regardless of whether the partial migration strategy is conditional or genetic, our results demonstrate that individuals differ in their propensity to respond both behaviourally and physiologically to environmental stimuli. Populations differ in this propensity, suggesting it can evolve. This variation plays a role in determining whether an individual will move to an alternative habitat or remain in the same area and face the challenging conditions with physiological accommodation. Our study

illustrates the potential benefits of investigating basic physiological processes in order to understand the mechanisms driving the evolution of partial migratory strategies.

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Tables

Table 1: Sample sizes of the females' flight response.

	Puerto Rico	Florida	Kentucky	Iowa	Total
Food present	90	60	85	88	323
Food removed	90	59	85	88	322
Food present, 1 hour flight	42	17	58	38	155
Food removed, 1 hour flight	60	32	59	37	188
Food present, <1 hour flight	10	3	1	3	17
Food removed, <1 hour flight	6	5	7	7	25
Food present, no flight	38	40	26	47	151
Food removed, no flight	24	22	19	44	109

Note. Based on the result of two flight tests, only females that flew for at least 1 hour in at least one of the flight tests were classed as migrants. Females that flew always less than 1 hour (N=42) and females that never flew (N=260) were classed as residents.

Table 2: Sample size of the female subset used for the oosorption analysis.

	Puerto Rico	Florida	Kentucky	Iowa	Total
Food present, migrant	22	16	23	26	87
Food present, resident	23	24	21	23	91
Food removed, migrant	23	27	24	24	98
Food removed, resident	22	14	22	22	80

Table 3: Factors affecting the variation in levels of oosorption.

Explanatory variable	df	F	p
Migratory tendency	1	12.78	< 0.001
Food availability	1	61.83	< 0.001
Population	3	9.72	< 0.001
Food availability*population	3	3.04	0.029
Food availability*migratory tendency	1	0.07	0.786
Migratory tendency*population	3	0.92	0.431
Migratory tendency*food availability*population	3	0.91	0.439
Wing length	1	0.20	0.651

Note. Resident and food deprived females have higher levels of apoptosis and differences among the source populations were greater under high food availability.
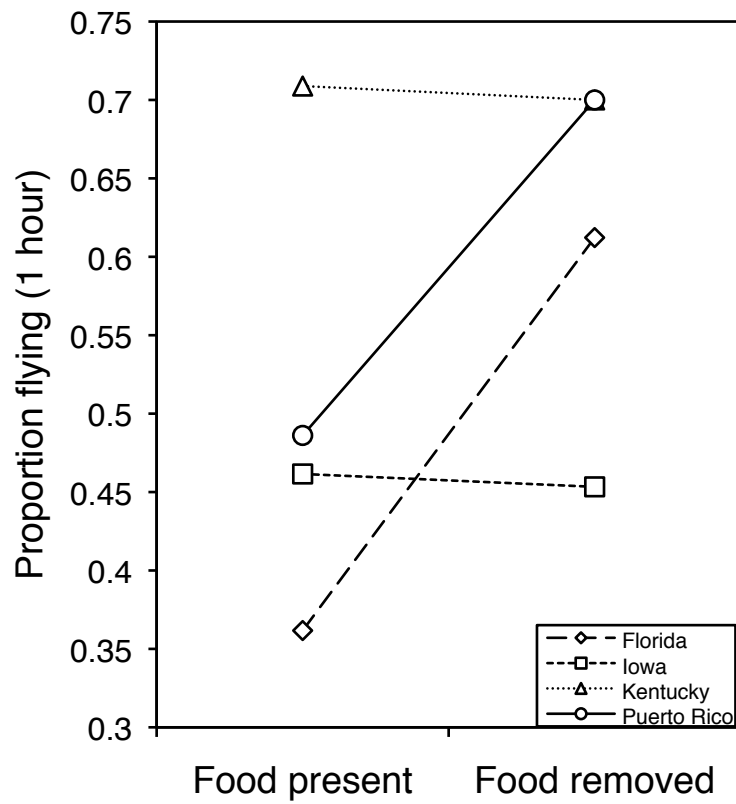
Figures

Figure 1: Migratory tendency, measured as at least 1 hour continuous flight, in response to food availability. Flight response differs between populations on similar food regimes and removal of food increases the percentage of females showing migratory flight behaviour.
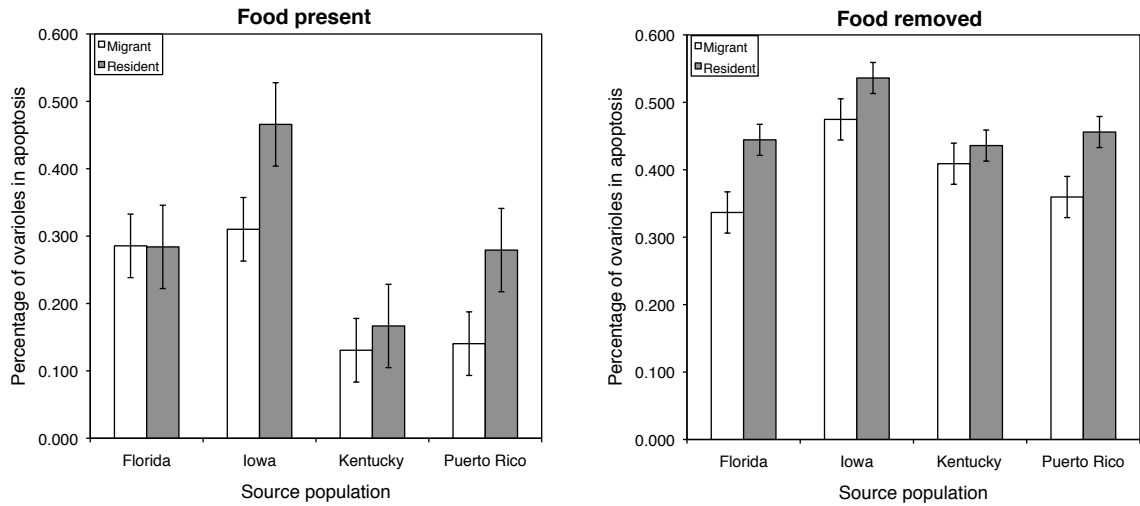
Figure 2: Levels of ovarian apoptosis (± SE) for females from the source populations divided as migrants or residents on alternative food regimes. Source population, migratory tendency and food availability have significant effects on the level of ovarian apoptosis (see Table 3). The overwhelming pattern is that resident individuals have a higher proportion of ovarioles undergoing apoptosis, and that populations vary in this trait.

Concluding comments

My thesis focused on plasticity in insect reproductive physiology in response to unfavourable environments. In particular my aim was to study the role of oosorption as a physiological mechanism for coping with resource-limited environments. The work focused on milkweed bugs with different evolutionary histories, which provided different kinds of information. The laboratory adapted population allowed me to ask questions about the evolution of trade-offs and life-history variation due to diet shifts and a long-term adaptation to an artificial diet. Both ovarian physiology and sexual behaviour in males have been investigated. The work on this population exposed bugs to different diets in order to explore how the presence of resources of variable quality affected the physiological and behavioural response to a change in diet. I exposed these bugs to three diets of different quality: sunflower, pumpkin and milkweed seeds. Sunflower is the adapted diet: populations have had at least 400 generations in laboratory conditions, thus we can expect that this population is well adapted to exploit and optimize their fitness with the nutrients provided by this diet. Pumpkin and milkweed represent two new diets to which the laboratory population has never been exposed before, but bear different quality signatures. Pumpkin is a low quality food that results in a decrease in both reproductive effort and sexual activity and thus a decrease in total fitness. Milkweed represents the ancestral food for the population from which the lab adapted bugs are derived. In wild populations, this is the diet that allows for maximal fitness. Milkweed and sunflower differ both in the array of fatty acids and also the presence of cardenolides in milkweed but not in sunflower. So, if the laboratory population has to exploit this food, one can expect an increase in some component of fitness due to the nutrient signature provided by milkweed and the evolutionary relationship with

this diet, but at the same time one might also predict some costs related to the exposure to a new and unknown, possibly toxic, food source.

The second set of experimental bugs was made up of four wild milkweed bug populations collected in different geographic areas, representative of the life-history variation found in natural conditions for this species. My sample included populations collected in tropical, subtropical and temperate areas, which are characterized by a different availability and quality of milkweed host plants. These populations also differ in their propensity to migrate and to enter diapause. Each population is thus characterized, both at the physiological and behavioural level, by its own life-history strategy to cope with the seasonal abundance or shortage of food resources. I exposed these bugs only to milkweed, the wild diet, but simulated contrasting environmental conditions of extremely abundant or totally absent food resources. I explored the question of how the physiological ability to cope with food shortage can mediate the evolution of complex behavioural strategies, in particular how this affects the variation in migratory propensity between populations.

Based on the main initial hypothesis delineated at the beginning of the thesis, I will add some concluding comments to summarize the main findings and suggest ideas for future work.

Hypothesis 1: oosorption occurs in females *Oncopeltus fasciatus* as an adaptive physiological mechanism to cope with environmental stressful conditions dictated by sub-optimal diets and it happens through apoptosis.

Conclusion 1: I found that *O. fasciatus* females from a laboratory population adapted to feed on sunflower seeds, show variation in life-history traits like sexual maturation, lifespan reproductive effort and longevity when exposed to new alternative diets of

pumpkin and milkweed seeds. Females tend to resorb oocytes through apoptosis when exposed to both new diets and this process does happen through apoptosis. However the signature of pumpkin and milkweed diet is quite different. Pumpkin fed females show a decrease in fitness with a lower late life reproductive output compared to sunflower fed females. Milkweed fed females have similar total fecundity to sunflower fed females but the schedule of reproduction is opposite to pumpkin: milkweed fed females have higher early fecundity than sunflower fed females, while later fecundity is similar between the two diets. The longevity on the two treatments is also different from sunflower fed females: when considering only females that laid eggs for at least 6 weeks on pumpkin and 6 weeks on milkweed, pumpkin fed females live as long as sunflower fed females while milkweed fed females have a reduced lifespan compared to sunflower fed females. However, both treatments show similar levels of oosorption. An explanation for the difference in the schedule of reproduction and longevity between pumpkin and milkweed may be that the apoptosis is localized in different areas of the ovarioles in the two treatments. So, in pumpkin fed females the apoptosis may be more likely to be localized in the germinarium, where new oocytes are produced, while in milkweed fed females the apoptosis may be mainly localized on the oocytes. The end result is that pumpkin fed females have decreased later reproductive potential, while in milkweed fed females such a decrease is not evident. Instead milkweed shortens female's lifespan and reproductive effort is higher at early adult age. At the moment this point remain a circumstantial observation given that the method used to score the ovarian apoptosis did not allow a clear and univocal way to obtain a statistical data on the localization of the apoptosis in the ovarioles. The counting procedure used previously offers a good way of comparing treatments with extreme outcomes like in the case of sunflower and pumpkin treatments in which

oosorption levels are clearly different, while with similar levels of oosorption and different life-history outcomes a more refined method that could reveal fine-scale differences in apoptosis occurrence is needed. This hypothesis will require future work on the refinement of the apoptosis scoring procedure.

Another possible explanation depends on the sexual activity modulated by diet. Pumpkin fed bugs start to mate later than sunflower and milkweed fed bugs, while milkweed bugs starts to mate earlier than the other two diets. If the occurrence of first mating is a good proxy for the amount of sexual activity, the milkweed diet results in the highest reproductive activity. The reduction in female life span could be mediated through increased exposure to ejaculate components rather than directly through exposure to components of the milkweed seeds themselves. The possible role of diet on male's sexual activity has been the subject of the work in Chapter 2 and I found that milkweed diet does increase sexual activity in males. Thus this factor may explain, at least in part, the shorter lifespan of milkweed females. Possible future work should be focused on characterizing the composition of male's ejaculate and to test the effect of alternative diets on the ejaculate components.

Another possible explanation for the variation in apoptosis levels is that laboratory bugs adapted to sunflower simply may not recognize pumpkin and milkweed as hosts and oosorption plays a role in the physiological adaptation to a new host. In our laboratory, we observed that laboratory bugs are more readily adaptable to new conditions, like new diet, photoperiod and temperature. Individuals from a Kentucky wild population put on a diet of sunflower seeds have very low reproductive output when compared to a diet of milkweed seeds, unless this population experience several generations of adaptation to the new host. Laboratory bugs instead do not show such very low performance when put on alternative diets so

their response to a new host is not as drastic as a total halt of the reproductive capacity. Thus oosorption may help individual females to optimize fitness in presence of suboptimal diets, even if this means recovering resources from developing oocytes. Considering milkweed as a suboptimal diet seems counterintuitive because it is the ancestral food. However, even if it provides essential chemical components that boost reproduction and fecundity in wild milkweed bugs, it is still a new and never experienced before diet for the laboratory adapted bugs. And it also contains a toxic compound that may have a still unrecognized effect in the physiology of the sunflower adapted bugs. Further work on this point may focus on the molecular and genomic basis of adaptation to new hosts using the laboratory and a wild population as models, but for now it remains an untested hypothesis.

Hypothesis 2: the trade-off between reproduction and survival is dependent on the energy balance dictated by the available diet rather than evolutionary changes derived from a long-term adaptation to alternative diet.

Conclusion 2: *O. fasciatus* males from the laboratory adapted population show two alternative life histories when exposed to the adapted sunflower diet or the ancestral milkweed diet. The two diets bear different signatures determining alternative condition-dependent sexual investments derived from the allocation of the available resources. Milkweed fed males have shorter lifespan in which resources are allocated to optimize the reproductive efforts at the expenses of survival, while sunflower fed males have a longer lifespan in which the reproductive efforts are decreased and distributed over a longer time.

When paired with sunflower fed females, the higher sexual investment of milkweed fed males is not related to an increase in female's fecundity, suggesting that this trait is limited by constraints on eggs production. Indeed the increase in male's

sexual activity is mirrored by an increase in early fecundity and decrease in female's lifespan only if females are fed on a milkweed diet as well (see Chapter 1 and Appendix 1). However, milkweed increases male fitness due to a higher fertilization rate in older males when compared with a sunflower diet. Thus despite the long-term adaptation on sunflower, milkweed still bears the signature of a better host. In addition, milkweed stimulates an increase in sexual behaviour in males. This is probably due to a hormonal regulation of the behaviour, with the level of JH titres being the first candidate. To further elucidate this point, future experimental work could be directed to characterize the JH titres in sexually active sunflower and milkweed fed males.

Mating rate and fertilization rate were highly correlated in sunflower fed males while this correlation was absent in milkweed fed males. This suggests a likely effect of diet on ejaculate components. Thus ejaculate produced by males on a sunflower diet seems to be limited in the ability to fertilize eggs unless the amount of time spent in copula is increased, suggesting that fertilization ability is dependent on diet quality and that, in limiting conditions, the fertilization ability can be increased by longer copulations. The proximal mechanism for the observation that in this species a longer copulation is correlated to a higher sperm load transfer and higher fertilization ability remains to be elucidated. Future work may be focused on the characterization of the ejaculate components as modulated by the nutrients array provided by the alternative diets.

Hypothesis 3: the onset of the female reproductive diapause is dependent on diet quality and onset of sexual behaviour in males and response differs in laboratory and wild populations.

Conclusion 3: this experiment has been characterized by a series of issues that limited the number of experimental pairs from the Kentucky population and on the milkweed treatment. Thus, I cannot yet draw a complete picture for the Kentucky population because most of the comparisons between diets and rearing conditions are highly affected by the small sample size. There is, however, a certain degree of confidence in the results for the laboratory adapted population. A complete discussion on the life-history traits comparison between the post-diapause stages of the experimental populations is dependent on a more detailed future study that will rely on a bigger sample size.

The laboratory adapted population do not show signs of reproductive diapause in any of the experimental conditions. Differences in body size between the two populations, and thus development time, may underline the variation in occurrence of first mating and oviposition when comparing non-diapausing long-high conditions between populations. Thus the bigger Kentucky bugs may simply take longer to develop sexual maturity than smaller laboratory bugs. However, in diapause-inducing short-low conditions, Kentucky bugs show a clear delay in reproductive activity in net contrast with pairs reared in non-diapausing long-high conditions. Laboratory bugs show a delay of reproduction in short-low compared to long-high conditions, but not as strongly as Kentucky bugs and generally this delay is below the threshold to be considered as reproductive diapause. Thus I can be safely assumed that laboratory bugs do not enter reproductive diapause, and the longer time to first occurrence of sexual activity in this population is dependent on a slower developmental rate in the lower temperature condition.

A factor that could determine the absence of female reproductive diapause in the laboratory population is the higher level of male's sexual activity and laboratory

bugs have higher mating rates than Kentucky bugs in all experimental conditions. The time from first mating to first oviposition indicates the amount of time the female needs to develop and lay the first batch of eggs following mating. In laboratory bugs this period is not different between treatments and across environmental conditions, suggesting that the time to first oviposition is mainly dependent on the occurrence of first mating, and thus on male's sexual maturity. In the Kentucky population this period is longer and generally more variable between treatments and across environmental conditions suggesting that the first female reproductive attempt is less dependent on the first mating occurrence, but is rather a function of the environmental conditions. To test this possibility, future work may be focused on the study of diapause occurrence in mixed pairs of laboratory and Kentucky bugs to further elucidate the influence of male's sexual activity on the onset of female's reproductive effort.

Diet has an effect on the occurrence of reproduction and this is quite evident in the laboratory population while the result of the Kentucky population still needs to be verified with a reliable sample size. However, the comparison with Kentucky pairs reared in our laboratory standard rearing conditions does suggest that diet does not affect the timing of reproduction in this population. Why this happens is quite difficult to say at the moment. It may be that laboratory bugs are more adaptable to a new food source and can more readily accommodate sexual investment compared to Kentucky bugs. Thus the availability of a food source that can increase investment in reproduction is readily recognized and exploited in laboratory bugs, while Kentucky individuals are not as able to do so.

Future work may be focused on the molecular and hormonal characterization of the diapause response in these two populations, based on their opposite response to

diapause-inducing conditions. Some of the physiological mechanisms determining the diapause response in insects are still not completely understood. For example, further investigations could be directed to characterize the array of Hsps proteins expressed during diapause and how these are regulated and mediate the stress response in these model populations.

Hypothesis 4: the physiological ability to resorb oocytes can play a role into the evolution of condition-dependent strategies to cope with unfavourable environmental conditions.

Conclusion 4: migration in *O. fasciatus* is an important life-history strategy to escape unfavourable conditions dictated by lack of food resources. It involves the evolution of a series of co-evolved traits that shape the migratory behavioural syndrome. However the presence of both resident and migrant in the same population raises the questions of how these strategies are maintained in a population and if they represent two alternative solutions for coping with seasonal shortage of food. I found that resident and migrant females *O. fasciatus* may adopt alternative condition-dependent strategies to cope with the environmental stress derived by food shortage, showing the ability to respond to a lack of food resources through either a specialized behaviour or a physiological accommodation. Migrant females shows higher levels of behavioural response determined by the capacity to engage in long duration flights when compared with resident females. On the other end resident females shows higher levels of ovarian apoptosis, indicative of oosorption, when compared with migrant females. Both the behavioural and physiological responses are present and vary at the individual and population levels, indicating that these alternative strategies coexist and can evolve in population of *O. fasciatus*.

The presence of alternative condition-dependent strategies to migration opens the possibility of the presence of a "non-migratory" strategy, in which a suite of traits can originate in a "non-migratory" behavioural syndrome in which oosorption can be one of these traits. To shed light on this point further investigations are needed. In particular a quantitative genetic experiment would help in quantifying the levels and patterns of heritability of both the migratory behavioural character and the physiological ability to resorb oocytes. Also such an experiment will offer evidence that certain environmental conditions could influence the heritability of a trait, denoting an adaption to unfavourable environments.

Another point to consider in future work is the possibility that the resident strategy is actually dependent on wing muscles polymorphism, and thus that residents are unable to fly because they histolyze their wing muscles. Such a possibility seems unlikely because most of the resident bugs were able to open the wings and fly for short periods, thus they seems more inclined to not show a long sustained fly rather than being unable to fly. Also, for this species no evidence of wing muscle histolysis has been reported. However, histolysis of wing muscle is present in some closely related species, thus it is not entirely a hypothesis to discard.

Understanding the mechanisms that determine how organisms react to challenging and stressful environments is a central topic in evolutionary biology. It addresses the key question of how organisms respond to environmental conditions and which factors lead to adaptation. The integration of behavioural and physiological studies represents a comprehensive approach to shed some light into how organisms respond to challenging conditions, how much plasticity is present and expressed in such responses and how the life-history strategies are affected by the physiological

and behavioural accommodations. In my thesis I attempted to combine behavioural and physiological traits in order to reveal the mechanistic basis of the behavioural outcomes and life-history variation determined by quality and availability of food resources as proxy for a common type of environmental stressor. Even if the work here presented has gaps and limitations, it also offers some ideas and suggestions for future research that may be further developed from these foundations. Plasticity in response to poor environments is an important aspect of the adaptation to the environmental conditions. In this time of increasing environmental challenges for all living beings on the planet, it is also increasingly important to understand how, how much and in what direction organisms respond to both predictable and unpredictable changes in environmental quality and how the mechanisms involved in such responses affect life-histories.

Acknowledgments

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