Effect of rearing conditions on the allocation of larval and adult acquired essential and nonessential fatty acids to flight in two adult Lepidoptera: Danaus plexippus and Mythimna unipuncta

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Abstract

Insects which are nectivorous as adults acquire essential fatty acids, which are important for many biological processes, almost exclusively from the larval diet. Thus, adult allocation of this limited resource may result in trade-offs in migrant insects that delay reproduction. I used the true armyworm, *Mythimna unipuncta*, and monarch butterfly, *Danaus plexippus*, to test the hypothesis that environmental cues (fall migratory or summer reproductive) would influence the use of fatty acids during flight (0-6h). I used larval and adult diets manipulated isotopically (δ¹³C) and chromatographic analyses to determine fatty acid composition and source in the fat body. C4 carbohydrate feeding increased the δ¹³C value of lipid in moths (-29.1 vs -16.5‰) and monarchs (-31.2 vs -22.1‰) and increased total fatty acid concentrations reflecting the important role of adult feeding. Fuel use during flight differed with essential fatty acids being more conserved under fall than summer conditions for moths (0% vs 33% loss) and monarchs (21% vs 33% loss) indicating that the environmental cues responsible for the onset of migration result in physiological changes that modify lipid use. The isotopic composition of fall-reared monarch fat body remained constant during flight but declined in fall-reared moths (-18.7‰) indicating an increased use of adult-derived resources in moths. Extended multi-day flight experiments focused on fall-reared moths with differing diet availability. When diet was available, moths conserved essential fatty acids reflecting the important role nectar availability during migration has on fatty acid allocation. I evaluated the effect of flight under differing diet provisions on egg count and fatty acid composition in virgin female moths. Fasting during flight period reduced egg count compared to fed females, but eggs had similar fatty acid compositions. I explored the role of male-derived essential fatty acids in reproduction. There was incorporation of spermatophore essential fatty acids into eggs produced by mated females and thus male donated fatty acids may play a role in reproductive success. The importance of migratory strategy, nectar availability and life history on the conservation of essential fatty acids during migration and potential mechanisms behind the differential allocation of fatty acids in migratory insects are discussed.
Summary for Lay Audience

Many insects migrate or overwinter in response to potential habitat deterioration. Migration is typically performed by sexually immature adults who reproduce when they arrive at suitable habitats. However, long-distance flights are costly, and if resources cannot be replaced (i.e., limited resource) this could result in reduced reproductive success. This trade-off could be managed by differentially allocating limited resources, thereby reducing overlapping requirements between migration and reproduction. A limited resource for moths and butterflies is essential fatty acids. These insects feed on nectar during their adult stages so essential fatty acids can only come from the larval diet. My goal was to evaluate how reproductive (summer) and migratory (fall) rearing conditions affect the source and use of fatty acids during flight. I tested this by comparing fatty acid use in true armyworm moths (Mythimna unipuncta) and monarch butterflies (Danaus plexippus) using isotopic analysis and gas chromatography. Both insects were used as both migrate similar distances but differ in terms of fuel loading, flight behaviour and migratory strategy. I found that although environmental conditions did not affect how insects accumulated fatty acids, they did affect how insects used them when exercised. Fall-reared insects conserved non-replenishable essential fatty acids vs. other fatty acids for fuel during exercise, especially in moths. Due to this, multi-day flight experiments were conducted on fall-reared moths. The pattern of differential fatty acid use persisted in moths over multiple days of flight but only when provided with food. The impact of diet restriction during flight on subsequent reproduction was assessed in virgin female moths through egg count and fatty acid analysis. I found that the fatty acid composition did not change when insects were flown with no food, but the number of eggs produced declined. The role of male donations of these limited fatty acids was also evaluated. There was a clear incorporation of male-derived essential fatty acids into eggs which could compensate for resources lost during flight by females. This study indicates that environmental cues responsible for migration result
in modified lipid use and the important role nectar availability during migration can have on reproductive success.
Co-Authorship Statement

A version of Chapter 3 from this thesis has been submitted, accepted and published in Frontiers in Ecology and Evolution – Ecophysiology. Dr. Hobson and Dr. McNeil aided in the design of the experiment and edited the manuscript. I conducted the experiments, analysed the data, and wrote the manuscript.

A version of both Chapter 2 and Chapter 4 from this thesis will be published (not yet submitted). Dr. Hobson and Dr. McNeil aided in the design of the experiment and edited the manuscript. I conducted the experiments, analysed the data, and wrote the manuscript.
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Abbreviations

TAW = true army worm
FA= fatty acid
NFA = nonessential fatty acid
EFA = essential fatty acid
PA = palmitic acid
OL = oleic acid
ST = stearic acid
ALA = alpha linolenic acid
LA = linoleic acid
EPA = eicosapentaenoic acid
DHA = docosahexaenoic acid
ARA = arachidonic acid
AKH = adipokinetic hormone
JH = juvenile hormone
Manse-AKH = adipokinetic hormone identified in *Manduca sexta*
5-HT = serotonin
CNS = central nervous system
GI = gastrointestinal
CSIA = compound-specific isotope analysis
ESW = Environmental Sciences Western
TAG = triacylglycerides
DAG = diacylglyceride
ATP = adenosine triphosphate
HDLp= high density lipophorin
LDLp= low density lipophorin
ApoLp III = apolipoprotein III
GC = gas chromatography
BHT = butylated hydroxytoluene
Min = minute
PUFA = polyunsaturated fatty acid
δ^{13}C = carbon isotopic ratio value
^{13}C = carbon-13
SE = standard error
VPDB = Vienna Pee Dee Belemnite
h = hour
D = daylength
RH = relative humidity
COIL = Cornell Stable Isotope Laboratory
ANOVA = analysis of variance
RuBP = Rubisco = Ribulose-1,5-bisphosphate carboxylase/oxygenase
CAM = Crassulacean acid metabolism
PEP carboxylase = Phosphoenolpyruvate carboxylase
spp. = species
δ = delta
Chapter 1

1 General introduction on insect migration

All organisms will face changing and/or deteriorating habitats whether due to stochastic processes (e.g., a fire) or periods of recurring habitat quality change due to seasonality (Dingle, 2014). Insects represent an interesting group of animals that manifest a range of traits allowing them to cope with changing environmental conditions. The factors causing insects to respond to environmental change include potential changes in availability of food as well as their ability to survive local conditions (Southwood, 1962; Menz et al., 2019). Potential changes in habitat quality are perceived using environmental cues that operate prior to environmental decline such as changes in photoperiod (Menz et al., 2019). In temperate regions if conditions are favorable, for example long photoperiods and warm temperatures, insects will typically stay and reproduce. Under unfavorable conditions such as conditions where there are shorter photoperiods and cooler temperatures, insects have evolved to cope by overwintering locally and delaying reproduction to when conditions become more favorable or migrate to locations with more favorable conditions for reproduction (Southwood, 1962; Dingle, 1972; Ramenofsky and Wingfield, 2007; Chapman et al., 2012, 2015).

1.1 Characteristics of migrant insects

Migration is a major life history event that requires many physiological, behavioral, and morphological changes (Johnson, 1963, 1969; Dingle, 2014). For example, many migratory insects undergo phases of hyperphagia, which can lead to fat body hypertrophy, and thus will have larger lipid reserves compared to non-migrant counterparts (Dixon et al., 1993; Ramenofsky and Wingfield, 2007). Lipids are a common fuel source in migratory insects (Beenakkers et al., 1985; Van der Horst et al., 2002) and so this period of hyperphagia allows for fuel accumulation prior to take off. When evaluating lipid gain in gregarious (migratory) or solitary (non-migratory) desert locusts (Schistocerca gregaria), Schneider et al. (1994) found the gregarious individuals stored larger amounts of lipid in the same period compared to their non-migratory counterparts. Migratory and non-
migratory insects can also have differences in the development of flight apparatus. When examining wing polymorphic crickets (*Gryllus rubens*), Mole and Zera (1993) found that long-winged morphs had a significantly larger wing muscle mass compared to the nonmigratory short-winged morph. In a more extreme case, different environmental cues can cause the development of fully winged or wingless morphs as seen in aphids (Hemiptera: Aphididae) (An et al., 2012). Typically, alate (winged) aphids can migrate long distances while apterous (non-winged) aphids are adapted to maximize fecundity (Taylor, 1977; Loxdale et al., 1993).

A major characteristic of migratory insects is reproductive diapause during the migratory phase or the delay of reproduction until after the migratory period (Angelo and Slansky, 1984; Zera and Bottsford, 2001; Ramenofsky and Wingfield, 2007). Several previous studies have found that physical and behavioural migratory/flight characteristics were also associated with sexual immaturity, reduced reproductive structures (i.e., reduced ovarian mass) and reduction in secondary reproductive characteristics such as calling behavior and conspecific aggression (Angelo and Slansky, 1984; Mole and Zera, 1993; Roff et al., 2003; Ramenofsky and Wingfield, 2007; Guerra, 2011; Jiang et al., 2011; An et al., 2012). In the cricket, *G. rubens*, for example, short-winged morphs had greater ovarian development in the initial 14-day adult period compared to long-wing dispersal-selected crickets which was correlated to resources being used for flight muscle development instead of reproduction (Mole and Zera, 1993). The inverse relationship between flight and reproduction was originally coined the “Oogenesis Flight Syndrome” by Johnson (1963, 1969). This relationship between flight and reproduction has formed the basis by which many aspects of insect migration, such as responsiveness to environmental cues and morphology, have been studied.

### 1.2 Migratory cues and endocrine regulation

Typically, during fall migrations in northern temperate regions, the declining photoperiod is a cue for insects that resource quality will change (Dingle, 1972; Johnson, 1995; Roff and Fairbairn, 2007; Chapman et al., 2012; Dingle, 2014). However, temperature can also play a role in delaying sexual maturity in order to allow for migration (Turgeon and McNeil, 1983; Delisle and McNeil, 1987; Hill and Gatehouse, 1992). In migratory oriental
armyworm moths (*Mythimna separata*), cooler temperatures promoted reproductive diapause (longer pre-calling period) compared to warmer rearing temperatures even if photoperiod was fixed (Han and Gatehouse, 1991). In particular, cooler rearing temperatures almost doubled the pre-calling period of female moths compared to warmer temperatures with the same photoperiod.

Changes in temperature and photoperiod are considered important proximate cues for migration but other cues can also influence migratory decisions and the delay of reproductive development. One such cue is the availability or quality of food resources (Goehring and Oberhauser, 2002; Müller et al., 2001; Menz et al., 2019). If abundant food resources are available, insects may remain locally longer than if food resources are scarce. Limiting food availability can trigger different nutritional and hormonal cues that can correlate with an increase in migratory potential. Rankin and Riddiford (1977) found that milkweed bugs (*Oncopeltus fasciatus*) from two different populations (flight selected versus not) that were starved shortly after emergence increased flight duration compared to control insects that were not starved. This trend was present for the first 10-15 days after emergence after which flight duration declined.

Though there are many hormonal signaling pathways and molecules involved in transitioning between migration and reproduction, a major hormone in this migration/reproduction trade-off is juvenile hormone (JH) (Nijhout and Wheeler, 1982; Fairbairn and Yadlowski, 1997; Goodman and Cusson, 2012). There are six different forms of JH produced by the corpora allata which are associated with the insect brain. All JH forms contain an acyclic sesquiterpenoid structure and are considered important to invertebrate development (Röller et al., 1967). Most insects contain only one juvenile growth hormone (JH III), but lepidopterans have more forms (JH 0, JH I, JH II) (Judy et al., 1973). Commonly high concentrations of JH aid in the maintenance of larval/nymphal stages as high levels have been shown to inhibit metamorphosis (Riddiford, 1994; Subramanian and Shankarganesh, 2016). However, in adult stages JH can play a concentration-specific role in transitioning insects between the reproductive and migratory phenotypes (Rankin and Riddiford, 1977; Nijhout and Wheeler 1982; Wyatt and Davey, 1996). It has been hypothesized that many cues, such as photoperiod and diet availability,
mediate the release of insulin like peptides from specialized cells in the insect brain which influence JH expression (Nijhout and McKenna, 2018). JH titers in post-eclosion milkweed bugs indicate that lower levels of JH stimulate migration, whereas high JH levels induce ovarian development and oogenesis (Rankin and Riddiford, 1977). A major response to changing temperature and photoperiod arises in the form of changes in juvenile hormone levels (Rankin and Riddiford, 1977; Cusson et al., 1990; Rankin, 1991). In a study on true armyworm moths (Mythimna unipuncta), insects reared under longer photoperiods and high temperatures produced high levels of JH within a few days post-eclosion, however, there was a significant delay in JH biosynthesis in insects reared in conditions with low temperatures and shorter photoperiods (Cusson et al., 1990). Thus, while JH is required for both migration and reproduction, it is a titer-specific control mechanism to balance between these two life history events (Rankin and Riddiford, 1977). This relationship has also been documented in many other insects (Rankin, 1991). Though hormones play a role in triggering the many morphological, physiological, and behavioral changes required for successful migration, these hormonal pathways must receive signals from the environment (as discussed above) in order to ensure the correct timing of specific life history events (Ramenofsky and Wingfield, 2007).

1.3 Costs and benefits of migration

One of the primary benefits of migrating is avoiding environmental conditions in which the insect or its immediate progeny would not survive (Southwood, 1962, 1977; Dingle, 1972). Many migrant insects are multivoltine (Flockhart et al., 2013; Chapman et al., 2015; Menz et al., 2019) and thus migrant populations could increase reproductive output over subsequent generations (seen in Autographa gamma, Chapman et al., 2012) by exploiting new underused resources and avoiding competition (assuming the mortality during migration is low) (Taylor and Taylor, 1983; Chapman et al., 2015). Migrant populations also tend to have a smaller proportion of individuals infected with pathogens or diseases compared to resident non-migrant populations (Altizer et al., 2011; Menz et al., 2019). A well-studied case of this is seen in monarch butterflies (Danaus plexippus) where long-distance migrant populations have fewer individuals infected with or having high levels of infection with Ophryocystis elektroscirrha compared to residents (Altizer et al., 2000;
Bartel et al., 2011; Altizer et al., 2015). This reduction in pathogen dispersal and loading has been hypothesized to arise through two possible mechanisms: the escape of migrants from areas with pathogens/diseases (migratory escape) or through the loss of infected individuals who were unable to survive the migratory period (migratory culling) (Altizer et al., 2011).

Migration is also associated with considerable costs such as the need for fuel (Rankin and Burchsted, 1992; Kammer and Heinrich, 1978). Flight in particular is considered one of the most energetically expensive modes of transport and insect flight muscles are among the most metabolically active insect tissues known (Dudley, 2002). It has been reported that metabolic rates during flight are 20–100 times higher than at rest (Ellington, 1985). There is also the evident cost associated with developing flight-specific machinery such as wings and flight muscle. Many migratory morphs of insects have been shown to have longer developmental periods due to this requirement (Roff and Fairbairn, 2007). In general, the larger or more structurally complex the migrant morph, the more developmental time is required. In a study conducted on Pyrrhocoris apterus, the development time of the winged morphs was statistically higher than the wingless counterparts by a number of days (Honek, 1985). Migrants have also been hypothesized to potentially have an increased predation risk from novel predators along their migratory route (Chapman et al., 2015). Migrants can hypothetically also face the additional cost of migrating and not finding a suitable habitat (Rankin and Burchsted, 1992; Roff and Fairbairn, 2007; Chapman et al., 2015).

One of the most important costs of migration in insects is a reduction in reproductive success (Rankin and Burchsted, 1992; Bonte et al., 2012). Indeed, many studies on wing polymorphic insects show a correlation with migration and lower fecundity caused by migrant insects laying fewer eggs, having smaller eggs, reduced mating opportunities or having a reduced reproductive window (Penner, 1985; Roff, 1986; Guerra, 2011). Much of the literature has focused on wing polymorphic species. However, another large group of migrant insect species are wing monomorphic, where both migratory and non-migratory morphs are capable of flight. Although many of the costs of migration are present in wing monomorphic insects, in a recent review by Tigreros and Davidowitz (2019) it was found that although a negative association between flight and fecundity is seen in some wing-
monomorphic insects, there was less evidence for a direct trade-off between these two life history events in some of the species studied. This leaves researchers with the question of how some wing monomorphic insects reduce the flight reproduction trade-off.

1.3.1 Adaptations

Migratory insects have developed ways to increase the efficiency of flight and increase reproduction to account for the delays caused by migration. For example, the winged migratory morphs of some insects produce more eggs, though smaller in size, resulting in an increase in overall reproductive output when compared to residents (Solbreck, 1986; Rankin and Burchsted, 1992). Wing shapes can also increase fuel-use efficiency during flight by altering wing loading thereby reducing resources allotted to migration. In migrating and non-migrating monarch butterflies, migrants have larger, more pointed wings and lower wing-loads than residents (Dockx, 2007; Altizer and Davis, 2010), an adaptation that has been hypothesized to result in more fuel-efficient flight (Roff and Fairbairn, 1991; Rankin and Burchsted, 1992; Menz et al., 2019). Migrants can also benefit from larger body sizes reducing water loss (Roff, 1977). After migration has ceased, some insects also break down their flight muscles to allocate protein resources for reproduction (Sahota and Farris, 1980). For example, the pinyon pine beetle (*Ips confusus*) shows an activation of reproduction when appropriate host trees are encountered, a stimulus that results in the rapid catabolism of flight muscle which would result in a reduced capacity to migrate but can provide resources for reproduction (Borden and Slater, 1968).

Post-migration feeding is the most fundamental way by which insects can manage and reduce loss of fitness as many resources can easily be obtained again after the energetically expensive flight. While some resources can be continuously gained through post-migration feeding, others are more limited. Therefore, the allocation of these limited, and sometimes non-replenishable, resources becomes key for the success of migratory insects. An adaptation that has been less studied is the potential for allocating specific physiological resources to different life history events to reduce overlapping nutritional requirements and conserve non-replenishable resources. For example, nonessential amino acids have been shown in many lepidopterans to be used more for fuel and somatic maintenance than essential amino acids which were allocated to eggs (O’Brien et al., 2002, 2005; Levin et
However, even if food is abundant, how different ecological conditions experienced by individuals affects the differential allocation of nutrients has not been well explored.

1.4 Lipid and the fat body

Lipids are a class of organic hydrophobic molecules that act as one of the fundamental components of organismal tissues (Toprak et al., 2020). Chemically, lipids can be categorized as simple lipids (hydrolysis leaves only 2 components) or compound lipids (hydrolysis leaves more than 2 components) (Karasov and Martinez, 2007) but classification has shifted toward being related to the base building blocks that make up the lipid in question (Fahy et al., 2011). The two major classes can be defined as either being derived from keto-acyl groups or isoprene groups. From this base division, eight classes of lipids emerge; fatty acyls (which are a base component to many other classes and store energy), glycerolipids (primary energy storage class), glycerophospholipids (important for cellular membrane integrity), sphingolipids (insulating lipids commonly found on epidermal layers and nerve sheaths), saccharolipids (cell signalling molecules), sterol lipids (hormone synthesis and transport of certain vitamins), polyketides (found in secondary metabolites such as defense secretions) and prenol lipids (cell signalling and hormone synthesis) (Fahy et al., 2005; 2011) (Figure 1.1). Different lipids can play different roles including (but not limited to) energy storage, maintenance of cell membrane integrity, signalling molecules, nutrition of the embryo, synthesis of sex pheromones, cuticular wax, and various defensive secretions (Stanley-Samuelson et al., 1988).
Figure 1.1 Summary of the lipid classes separated by base building blocks. “R” indicates a carbon hydrogen chain of undefined length. “x” indicates the potential space for additions of functional groups on the building block units. Modified after Fahy et al. 2005, 2011.
My study focuses on fatty acids (FAs) as they are the most common class of lipids used for energy and thus act as fuel during migration (Stanley-Samuelson et al., 1988; Van der Horst, 1990). Lipids are commonly used for fuel due to the fact that they can be stored anhydrously and yields the highest amount of energy per unit of mass when catabolized compared to other macronutrient sources such as carbohydrates or proteins (Wu, 2017a). FAs used for fuel are commonly stored as triacylglycerides (TAGs) that are derived from base keto acyl units. As the name implies three fatty acyl chains are attached to a polar 3-carbon compound known as glycerol. In TAGs the glycerol is attached to 3 fatty acid chains via esterification (Wu, 2017b). These FAs can vary in length and saturation and the catabolism of these FAs through β-oxidation provides energy in the form of ATP (Wu, 2017a). The mobility and reductive potential of different FAs can determine their efficacy as fuel. A short chain FA will yield less energy than longer chain ones (Karasov and Martinez, 2007, Wu et al., 2017a,b), while unsaturated FA chains yield less energy than saturated ones of the same acyl length due to the need for additional enzymes (i.e. isomerases) to break them down (Karasov and Martinez, 2007; Wu et al., 2017a,b). However, saturated FAs are more difficult to mobilize initially as they are stored in a more stacked uniform semi-crystalline structure and have higher melting points than unsaturated ones (Karasov and Martinez, 2007, Wu et al., 2017a,b). Thus, it has been predicted in organisms such as birds that intermediate chain length (16-18C) mono- or poly-unsaturated FAs would make ideal fuels during periods of exercise such as flight (Price et al., 2008; Guglielmo, 2018).

Certain FAs can be synthesized from other macronutrients at any stage of development and are deemed nonessential FAs (NFAs) and in insects, including lepidopterans, oleic, palmitic and stearic acids are commonly found NFAs, (Canavoso et al., 2001). The synthesis of NFAs from other macronutrients, such as carbohydrates, is a highly conserved process across various taxa and begins with the glycolysis of molecules such as glucose (Crabtree & Newsholme, 1975; Karasov and Martinez, 2007). The primary product from this catabolism, pyruvate, is then converted to acetyl-CoA and moved as citrate to the cytosol (Karasov and Martinez, 2007) where it is broken down into acetyl-CoA and oxaloacetate. The acetyl-CoA eventually forms fatty acids through various additional reactions (Karasov and Martinez, 2007; Laliotis et al., 2010) performed by the fatty acid
synthetase complex (Crabtree & Newsholme, 1975). Others which cannot be de novo synthesized and must be obtained directly from diet are termed essential FAs (EFAs) and may only be acquired in certain life stages in some insects. EFAs are considered essential due to the irreplaceable role they play in prostaglandin production, brain and eye development, and membrane fluidity (Downer and Matthews, 1976; Stanley-Samuelson et al., 1986; Forte et al., 2002; Wu et al., 2017b; Arrese and Soulages, 2010; Malcicka et al., 2018). The two main EFAs analysed in this thesis were linoleic acid and alpha linolenic acid, which are commonly found in Lepidoptera (Canavoso et al., 2001) and required for the production of other EFAs such as EPA and DHA (from ALA) and ARA (from LA) (Karasov and Martinez, 2007). Migratory flight in insects is primarily fueled by FAs (Beenakkers, 1969; Cenedella, 1971; Brown and Chippendale, 1974; Van der Horst, 1990; Van der Horst et al., 2002; McWilliams et al., 2004). However, FAs, particularly EFAs, are also required for the successful development of eggs, as well as the development of embryos by providing energy, precursors for hormones, prostaglandins and resources for embryonic tissue development (e.g. brain and eyes) (Downer and Matthews, 1976; Stanley-Samuelson et al., 1986; Forte et al., 2002; Arrese and Soulages, 2010; Malcicka et al., 2018).

Migratory insects must balance non-replenishable resources to allocate to migration and maintain enough to still contribute to reproduction (Angelo and Slansky, 1984; Boggs, 2009). In other words, using FAs rather than NFAs to fuel flight, especially when EFAs cannot be acquired by adults, would allow for use of EFAs during reproduction. However, both NFAs (oleic: 18:1ω9) and EFAs (linoleic18:2ω6 and alpha-linolenic: 18:3ω3) and as previously mentioned, 16-18C mono or poly unsaturated FAs make good fuel sources (Price et al., 2008, Guglielmo, 2018) and thus could all potentially be used as migratory fuel. To add to the potential trade-offs FAs used for fuel and reproduction are typically stored in the same place in insects; the fat body (Arrese and Soulages, 2010).

The fat body is a specialized tissue found throughout the insect body, with the majority found in the abdomen. The abdominal fat body tissue is loose in structure and surrounds reproductive and digestive tissues just below the exoskeleton (Arrese and Soulages, 2010). The loose structure allows the fat body to be readily bathed in haemolymph for efficient
response to hormones and transport of different metabolic products including FAs. On average about 50%–70% or more of the fat body by dry weight is lipid and, of this lipid, about 90% is stored as TAG while the rest is found as cholesterol and phospholipids (Gilby, 1965; Arrese and Soulages, 2010; Roma et al., 2010). The fat body is responsible for many different roles beyond just lipid storage and mobilization including, but not limited to, glycogen storage, anti-microbial peptide production, lipophorin production and vitellogenin production for egg laying (Roma et al., 2010; Azeez et al., 2014). I focus on the ability of the fat body to store and mobilize fat for various processes such as migration.

The primary cell in the fat body is a specialized lipid storage cell known as the adipocyte (Dean et al., 1985). Within adipocytes, lipids are stored as TAGs in a structure known as a lipid droplet (Olofsson et al., 2009). Hydrophobic neutral storage TAGs make up the core of the lipid droplet with phospholipids, cholesterol and various proteins making up the outer layer (Bickel et al., 2009). Of particular importance are two lipid droplet storage proteins Lsd1 and Lsd2. Studies on Drosophila melanogaster show that the activation of Lsd2 seems to promote fat accumulation and prevent lipase access to TAGs (Teixeira et al., 2003). In contrast, Lsd1 activation has been shown to promote lipolysis of TAG for transport by allowing lipases access to the TAGs within the core of the lipid droplet (Patel et al., 2005). These lipases convert TAGs into diacylglyceride (DAG) molecules for transport in insects (Van der Horst, 1990). DAG is moved through the hemolymph to target tissues such as muscle or ovaries via a lipid transport lipoprotein known as lipophorin (Beenakkers et al., 1985, Van der Horst, 1990; Van der Horst et al., 2002). Circulating lipophorin is considered high density lipophorin as it does not contain a large lipid component (Shapiro et al., 1988). However, when DAGs are being mobilized from the fat body these high-density lipoproteins associate with apolipoprotein III (Wells et al., 1987) which allows for the accumulation of a much larger proportion of DAG (Wells et al., 1987). This apolipoprotein can interact with and stabilize the DAG core and thus the lipophorin unit is now considered low or very low density lipophorin (Shapiro et al., 1988). At the target tissue DAGs can be released to target tissue without the lipophorin being endocytosed and thus high density lipophorin can readily be recycled into the hemolymph to continue transporting more DAG (Shapiro et al., 1988; Van der Horst, 1990) (Summarized in Figure 1.2). The delicate balance of hormones, proteins and different cell
signaling mechanisms are what allows insects to store and manage fuel use for various energetically demanding processes such as metamorphosis, egg production, and migration. My thesis focuses on lipid within the fat body as it is the primary fuel source for both flight muscles and the reproductive system (Kilby, 1963; Bailey, 1975; Beenakkers et al., 1985; Lorenz and Gade, 2009).
Figure 1.2 Simplified summary of recyclable lipid transport in insects during flight. Abbreviations are as follows: TAG= triacylglyceride, DAG= sn-1,2-diacylglyceride, FA= fatty acid group, HDLp= high density lipophorin, LDLp= low density lipophorin, ApoLp III = apolipoprotein III. Original image but is based off Van der Horst, 1990.
1.5 Lepidopterans

The limitation of EFAs coming from only certain life stages is not present in all insects. Carnivorous insects like the North American dragonfly (*Anax junius*) for example, may replenish EFAs used during flight through concurrent or post-migratory feeding (May and Matthews, 2008). I focus on Lepidoptera, an order of over 180,000 insects including moths, butterflies and skippers (Powell, 2009). Lepidoptera is a large and diverse Order, but most adult lepidopterans share common characteristics such as 2 pairs of membranous wings, 3 pairs of segmented legs, and the presence of small scales all over the body including on legs and wings (Powell, 2009). Of particular importance to this study, lepidopterans are commonly holometabolous and thus larvae and adults differ greatly in form, function, and feeding (Powell, 2009). One such difference between the larval and the adult stages is that adult lepidopterans typically have a coiled proboscis (Powell, 2009) and thus are only able to consume liquids such as nectar while larvae typically consume solid food (i.e., leaf matter).

As nectivorous insects, migrant lepidopterans will only obtain trace amounts of FAs directly from nectar during the adult stage (Nicolson *et al*., 2007; Krenn, 2010) so the majority of EFAs are derived from larval stage diets. Successful management of fuel requirements with a limited EFA supply requires differential allocation of lipids for various purposes (Angelo and Slansky, 1984; Boggs, 2009). FAs have been significantly less studied in insects compared to other compounds, such as amino acids. However, in an experiment performed by Levin *et al.* (2017) on the hawkmoth *Amphion floridensis*, it was found that adult-generated FAs were primarily used as fuel but not in egg production. Eggs also contain lipid and thus it can be inferred that larval diet-derived FAs could be preferentially shuttled into reproduction instead of fuel. Supporting this, in another study on total carbon source in eggs of hawkmoths it was found that 40-50% of total carbon was derived from the larval diet exclusively (O’Brien *et al*., 2000) which would also lead to the prediction that larval EFAs along with essential amino acids (seen in O’Brien *et al*., 2002) are contributing to egg production and thus are not being used for flight.
1.5.1 Study organisms

As previously mentioned, Lepidoptera is a large order of insects and my study focuses on two migratory species, the monarch butterfly and the true armyworm (TAW) moth. They were used because they both undertake migration as adults in reproductive diapause (Angelo and Slansky, 1984; Zera and Bottsford, 2001; Ramenofsky and Wingfield, 2007). However, they use very different migratory strategies: the monarch migrates within its boundary layer (see below) and takes several months to complete the fall southwards migration layer, while the armyworm does so in several days on upper air masses. These differences could influence how acquired FAs are allocated during migration and the potential tradeoff between flight and subsequent reproduction (Penner 1985; Roff, 1986; Guerra, 2011).

The monarch butterfly is an iconic migrant insect in North America, but Eastern and Western populations have declined considerably over the past 20 years (Semmens et al., 2016). The monarch has also recently been listed as an endangered species by the International Union for Conservation of Nature. Eastern monarchs summer in regions such as southern Ontario and following changes in food quality and habitat conditions, will leave the region in search of more suitable conditions (Wassenaar and Hobson, 1998; Goehring and Oberhauser, 2002; Chapman et al., 2015) to overwinter. The primary overwintering region for eastern monarchs is the central transvolcanic belt of Mexico (Urquhart and Urquhart, 1978; Wassenaar and Hobson, 1998). In spring there is a multigenerational migration north to reproduce (Brower, 1985). In order to successfully complete the fall migration, monarchs must take advantage of key nectaring sites en route to rest and synthesize lipid to refuel. This process is consequential to not just migration, but also to fuel overwintering successfully and subsequently allow northward migration while still reserving some FAs for reproductive purposes (Cenedella, 1971; Alonso-Mejia et al., 1997; Brower et al., 2006; Hobson et al., 2020). Understanding which FAs are used and conserved during flight can provide information on the requirements of nectar and lipid synthesis in monarchs which can be in turn used to help focus land conservation along the monarch migratory and breeding ranges.
In contrast, the TAW moth, also a cross-continental migrant, is a well-known agricultural pest. Although nectivorous in its adult stages, larval TAW feed voraciously on plants such as corn and various cereal crops causing significant damage (Breeland, 1958; Guppy, 1961; McNeil, 1987). This moth is a migrant insect that arrives in southern Ontario in the spring from the southern United States (McNeil, 1987; Hobson et al., 2018). Though TAW moths remain in Ontario during the summer months, a migratory generation emerges towards the fall season as it is unable to overwinter (Guppy, 1961; Fields and McNeil, 1984; McNeil, 1987). Emergent migratory moths maintain some lipid from larval stages, but adults are known to synthesize and store large amounts of lipids fairly quickly (Benoit, 2017; Anparasan et al., 2021) to fuel migration and reproduction. Lipid synthesis and differential allocation of EFAs and NFAs then is vital for the ability of these insects to migrate and produce offspring. Establishing which lipids are used for flight or reproduction and how fuel use can impact future reproductive capacity can allow a greater understanding of the importance of larval and adult nutrition in other EFA limited insects as well.

The pattern of FA allocation and utilization between various insect species can vary based on the strategies these insects have evolved over time to migrate and reproduce. TAW moths and monarchs have similar life histories in the sense that both species must migrate to eventually reproduce but there are also clear differences that can influence how FAs are to be managed. For example, the flight behaviour of these two species varies greatly even though the migratory distance traversed are both cross-continental. The TAW moth is an active, high frequency flapping, nocturnal flier that uses strong upper-air currents to travel large distances and reach overwintering habitats quite rapidly (Guppy, 1961; Taylor, 1974; Fields and McNeil, 1984; McNeil, 1987; Luo et al., 2002). Monarchs contrastingly combine active diurnal flights with periods of gliding/soaring and also fly within the boundary layer (Gibo and Pallet, 1979; Chapman, et al., 2015). Flying within the boundary layer means that monarchs take advantage of atmospheric conditions and winds where they can actively control their flight, thus their wing speed is greater than the ambient wind speed (Chapman, et al., 2015). Boundary layer flight requires more energy, orientation, and time (Gibo and Pallet, 1979; Brower, 1996; Mouritsen and Frost, 2002; Froy et al., 2003; Guerra et al., 2014), thus monarch migrations occur over a much longer period of time compared to the migrations of the TAW moth.
TAW moths also reproduce quickly upon arrival to places such as Texas after their fall migration (Guppy, 1961; McNeil, 1986, 1987). Migratory fall monarchs, however, overwinter in Mexico for months before individuals from the same generation then migrate northward into the southern USA prior to reproduction (Cenedella, 1971; Alonso-Mejia et al., 1997; Brower, 1985; Brower et al., 2006). Thus, lipids gained during the larval phase of monarchs are potentially being conserved over a longer period. However, it is also noted that monarchs have displayed a tendency to stop migration when resources such as non-migratory mates or tropical milkweed (Asclepias curassavica) (Satterfield et al. 2015; Majewska and Altizer, 2019) are present which may be reflected in a less stringent pattern of FA conservation. These differences may affect the patterns of EFA and NFA use between these two species. Due to ease of rearing and the clear differences in migratory behaviors and key life history events between these two species, my project uses a comparative approach to investigate the allocation and source of FAs used by the monarch butterfly and the TAW moth to fuel flight.

1.6 How can we study nutrient allocation strategies in migrant insects?

Nutrient allocation in insects is difficult to assess but some techniques have been successful in determining these patterns. Radio isotopic tracers have been used to determine allocation and turnover of specifically labelled nutrients in laboratory experiments (e.g. Buscarlet, 1974; Showler et al., 1988). While useful, this type of tracer is limited to laboratory experiments and cannot assess a wild population or individual as these tracers are not present typically in the natural environment. Another method by which nutrient allocation can be assessed is chromatographic analysis, such as gas chromatography which allows for mixtures of compounds to be separated based on characteristics such as solubility, polarity, molecular mass, and boiling points (Coskun et al., 2016). The relative concentration of each component can also be extrapolated. The presence of unique compounds and changes in their relative proportions can be used to infer patterns of nutrient sourcing and use. This technique can be applied to FAs as well. Insects that develop on distinct diets as larvae compared to adults may only obtain certain FAs from one life stage or another (Canavoso et al., 2001). Thus, the allocation of these distinct FAs can be assessed through gas or liquid
chromatography to determine if certain FAs are being used for a specific purpose. This is a common technique used on insects that develop in aquatic environments but later become terrestrial because aquatic environments are richer in certain FAs (e.g., EPA) (Taipale et al., 2013; Parmar et al., 2022). Determining the allocation of said FAs can delineate how larval resources are being used by adults (seen in mosquitoes (Diptera: Culicidae; Sushchik et al., 2013). However, if one were to determine not just the stage but the specific geographic origin of nutrients being allocated to a specific tissue or event this technique would be limited to only generalized spatial/temporal origins.

Another means by which nutrient sources and allocation patterns can be established is through the use of naturally occurring stable isotopes (Hobson et al., 2010; Wassenaar, 2019). Stable isotopes are different forms of the same element differing in atomic mass due to differences in the number of neutrons (Hobson, 1999; Peterson and Fry, 1987). This mass difference leads to differences in the rates of physical and chemical reactions of the different isotopes of a particular element. Thus, naturally occurring stable isotopes are distributed among biota in specific patterns due to distinct biogeochemical reactions (Hobson, 1999; Wassenaar, 2019). When organisms uptake nutrients such as lipids, water or carbohydrates from the environment and deposit them in tissues, the isotopic signatures from the environment can be conserved (i.e., they remain traceable). Thus, the allocation of these resources within the body, as well as the potential use of these resources, can be traced through the measurement of stable isotopes. This method has shown great success as there are many studies across taxa (including other nectivorous lepidopterans) that have used isotopic values of bulk tissues to determine the source and allocation of various resources (Evans-Ogden et al., 2004; Morrison and Hobson, 2004; Flockhart et al., 2013; Vander Zanden et al., 2016; Bowen and West, 2019; Anparasan et al., 2021).

The specific isotopic tool I used was the use of $\delta^{13}$C measurements of insect tissues as carbon is a major component of both the lepidopteran diet and the lipids that are generated from that diet. The most abundant isotope of carbon is carbon with an atomic mass of 12 (98.89%) but a heavier isotope, $^{13}$C, is also present (1.11%) (Wassenaar, 2019). The isotopic distribution of carbon in biotic systems is driven by differences in the photosynthetic pathways of C3, C4, and CAM plants (Park and Epstein, 1961; Smith and
All plants use Rubisco (RuBP) in the bundle sheath as the carbon-fixing enzyme for photosynthesis which has been shown to preferentially uptake lighter carbon; a preference which is seen in many biological enzymes (Farquhar et al., 1989, Gannes et al., 1998). What differs between C3, C4 and CAM plants is how each photosynthetic pathway provides RuBP with an environment varying in partial pressures of CO₂ for use in photosynthesis (Smith and Epstein, 1971; Farquhar et al., 1982).

C3 plants integrate CO₂ more passively and directly into the Calvin cycle using a 3-carbon intermediate molecule (3-Phosphoglyceric acid) (O’Leary et al., 1992; Wang et al., 2012). In C4 plants, Phosphoenolpyruvate (PEP) carboxylase converts CO₂ into a C4 intermediate molecule which is then concentrated into the proximate environment of RuBP leading to a higher partial pressure of CO₂ than with C3 plants (Smith and Epstein, 1971; O’Leary, 1989; Wang et al., 2012). In similar fashion CAM plants also aim to increase the partial pressure of CO₂ in the bundle sheath via the temporal closing of their leaf stomata in arid conditions (Farquhar et al., 1989). With a higher partial pressure of CO₂ around RuBP more of the heavier isotopes will eventually be used and thus C4 and CAM plants will typically be isotopically enriched in ¹³C compared to C3 plants (Farquhar et al., 1982; O’Leary et al., 1992; Wang et al., 2012). In particular, C4 plants are typically more isotopically positive (−7 to −15‰) while C3 plants are more negative (−20 to −35‰) (Farquhar et al., 1989; O’Leary, 1989). Thus, plant components such as leaves or carbohydrates found in nectar will also show similar differences.

Differences in the isotopic composition of different diets have been used in many studies to trace the source and allocation of various resources in insects (reviewed by Quinby et al., 2020). In some studies, the isotopic differences in diet are naturally present as with organisms that have an aquatic diet in certain life stages versus terrestrial in others (e.g., Akamatsu et al., 2004). In other cases, diets can be intentionally manipulated to be isotopically distinct to trace the fate of the consumed resources (Quinby et al., 2020). For example, Levin et al. (2017) studied the fate of larval vs adult diet derived amino acids in hawkmoths by providing the moths with differentially commercial ¹³C spiked amino acids during the differing life stages. They inferred the differences in the δ¹³C value of various
tissues (somatic vs reproductive) as indications of larval or adult derived specific amino acids being used for differing purposes.

As previously mentioned, most lepidopterans are nectivorous during their adult stage (Powell, 2009). However, larvae of both the TAW moth and monarch are voracious herbivores. The TAW larvae can be found on the leaves and blades of many wild and crop (C3 or C4) plants such as timothy grass, corn, or pinto bean (Breeland, 1958). The monarch larvae however are limited to feeding on only C3 milkweed (Asclepias spp.) (Erickson, 1978). Lipids that are synthesized or obtained directly from the larval diet then would have a δ\(^{13}\)C value that would reflect the C3 or C4 nature of the diet that was provided. Through obligation or controlled diet manipulations both insects can be given a larval diet that was derived from C3 plants such as the use of pinto beans (synthetic bean-based diet (Shorey and Hale 1965)), and common milkweed (fresh foliage) for the TAW and monarch larvae respectively. In contrast, the adult diet can be derived from a C4 plant such as sugar cane (e.g., used in Min et al., 2006). The carbohydrates the adult insects are given will have an isotopic value that is more positive than that of the C3 larval diet. This would in turn reflect in the lipids that are synthesized from the C4 carbohydrates having a more positive δ\(^{13}\)C value than the lipids that were derived from the larval C3 diet. Due to this isotopic difference larval versus adult diet derived lipids can be traced for use as fuel or reproduction.

Geographical and temporal origins and allocation patterns of EFAs and NFAs in Lepidoptera are difficult to identify, as they can vary based on migration and reproductive strategies, and generally have not been well studied. However, isotopic analyses combined with gas chromatography can provide a tool to determine which/if certain FAs are being conserved, how this allocation pattern arises in both migratory and reproductive morphs, and to determine the dietary source of specific FAs. If certain FAs show consistent fidelity to the larval dietary source over time, these FAs can be used to identify origins of larval nutrients or key stopover sites.
1.7 Prior work

My prior work using stable isotopes found a significant proportion of larval lipid being integrated into the overall adult lipid isotopic composition. TAW moths (Anparasan et al., 2021) and monarchs (Hobson et al., 2020) were fed a C3 larval diet but switched to a C4 sugar water post-eclosion under standard laboratory conditions (25 °C, 65% RH and 16L:8D). Bulk isotopic analysis of 5-day old TAW moths and monarch butterflies indicates a 13% and 48% contribution respectively of larval lipid to the adult lipid δ¹³C value. However, determining if specific FAs are derived from larval stages compared to adult stages and how these FAs are allocated is still a field of research with limited information.

1.8 Overarching objective

My overall goal was to evaluate the source and allocation of FAs used during flight in TAW moths and monarchs under fall (migratory) and summer (reproductive) conditions in a laboratory setting. To accomplish this, I conducted a series of parallel experiments using the TAW moth and the monarch where I explored the similarities and differences in the source and composition of FAs during acquisition and flight of migratory and non-migratory morphs generated based on various environmental conditions. In all experiments the larval diet was C3 while the adult sugar water was C4 allowing for lipids that were synthesized and used in the insects to be isotopically reflective of a specific source (adult vs larval). Based on the results of the primary objective a second objective aimed to explore the potential use of conserved FAs as investment in eggs and spermatophore in the TAW moth after migratory flight. Understanding allocation patterns of FAs, can provide insight into how the mechanisms underlying various ecophysiological, reproductive, and flight strategies differ, and how these strategies influence the ability for these wing monomorphic insects to manage resources. It can also aid in the prediction of how climatic and land use induced changes in nectar availability may affect the migratory success of nectivorous EFA limited migrants.

1.8.1 Sub objectives and predictions
Objective 1

**Evaluate the FA composition and source of stored lipids in newly emerged adults and adults fed for 5 days (to allow adult lipid accumulation) to confirm nutritional origins of EFAs and NFAs in captive monarchs and TAW moths.** In order to determine the use of different FAs in the adult it is necessary to understand which FAs (and the relative amounts of each) are acquired during larval development and which ones they are able to synthesise *de novo* from nectar. To establish this baseline, I conducted experiments to determine the FA composition and isotopic composition of the fat body in both species under different environments using isotopically distinct adult and larval diets.

**Prediction 1**

I predicted that both insect species under summer, fall, or standard laboratory (reproductive) conditions will, at emergence, have lipid comprised of EFAs and NFAs with bulk isotopic values (δ\(^{13}\)C) reflecting the C3 experimental larval diet. The adult diet used to generate NFAs is C4, and thus adult *de novo* synthesized NFAs will isotopically reflect a C4 source. The concentration NFAs was predicted to increase as they are generated in fed adults under all environmental conditions.

Objective 2

**Assess the utilization of FAs acquired during larval and adult stages in monarchs and TAW moths reared under fall and summer conditions during flight.** Environmental conditions can serve as cues that result in significant physiological changes, which could influence energy use in experiments. Thus, 1-, 4- and 6-hour tethered flights were conducted with adults of both species under fall and summer conditions to assess differential fuel use.

**Prediction 2**

I predicted that adult and larval synthesized FAs will be used to fuel flight under all conditions, however, EFAs will be more conserved (thus not as readily used as fuel) in both species under fall conditions compared to summer. I also predicted the δ\(^{13}\)C of
remnant FAs in fall insects would become more negative after flight, reflecting the consumption and loss of primarily C4 derived adult lipid compared to C3 larval lipid.

Objective 2b (TAW ONLY)

Assess the utilization of FAs acquired during larval and adult stages in fall-reared TAW moth during multiple days of flight exercises under various nutritional conditions; fed throughout rest periods between flights, provided only water throughout rest periods between flights and fed only on one rest period between flights. Based off the results of experiment 2, a multi-day fall flight experiment was conducted to explore the persistence of EFA conservation over longer periods of flight. TAW moths migrate over multiple days and thus patterns of FA use could go beyond what was seen in one flight period. Another consideration to FA use is the ability to refuel after flights. Although TAW moths stop during their migration, there is no guarantee that the stopover sites have abundant nectar sources. To address this the multiday flight experiment was conducted under various different regimes of sugar water availability.

Prediction 2b

I predicted that the pattern of EFA conservation and FA use would persist across the fall flight period but only under conditions with nutritionally favorable rest periods. I predicted that under nutritional limitations the pattern of EFA conservation would not be maintained.

Objective 3a (TAW ONLY)

Assess the effect of flight on egg production in female virgin TAW moths after migratory flight under various conditions of nutrient availability. Female TAW moths must produce eggs and thus loss of EFAs under nutritionally unfavorable flight conditions could impact future reproduction. Thus, this experiment explored the impact of flight and loss of EFAs on egg formation by flying virgin fall-reared females for multiple days with or without sugar water being available during rest and then switching the insects to summer conditions (25 °C, 16:8D, 65±5 % RH) to allow egg synthesis.
Prediction 3a

I predicted that eggs would not differ regarding FA composition between fed and fasted females. However, I predicted that females that were fasted when flown and thus experienced a loss of EFAs would have less eggs compared to flown but fed females.

Objective 3b (TAW only)

Assess the role spermatophores may play in donating EFAs during mating to eggs as a potential strategy to reduce cost of migration on reproductive success in TAW moths. Another consideration to reproductive investment in migrants is a possible male contribution of nutrients during mating. Such male nutrient donation during mating (either to the female or directly to eggs) has been seen in previous research and males can increase female reproductive output by contributing ions, peptides, and essential amino acids (Friedel and Gillott, 1977; Engebretson and Mason, 1980; Bowen et al., 1984). However, the role of FAs from the spermatophore in egg investment is unknown (but see Marshall and McNeil, 1989). This objective focused on establishing evidence of male EFA contribution via spermatophore to egg synthesis. Experimental males were provided with $^{13}$C spiked linoleic acid during the larval stages so the EFAs can be traced isotopically to spermatophore and eggs after mating.

Prediction 3b

I predicted that the spermatophores would have a FA composition consisting of both NFAs and EFAs though relative proportions may vary due to adult feeding. I also predicted that males provided with the spiked larval diet would have spermatophores with a higher $\delta^{13}$C value than males who received the control standard diet. I predicted that females would use spermatophore donations in eggs and thus eggs that integrated spiked male donations of EFAs would have a higher $\delta^{13}$C compared to the eggs collected from females mated with unspiked males.
1.9 Thesis structure

This PhD thesis is organized in an integrated article format with three data chapters. The first data chapter focuses on sub-objectives 1 and 2 for the monarch butterfly. The second data chapter also focuses on sub-objectives 1 and 2, as well as 2b but for the TAW moth. The final data chapter focuses on the third sub-objective for the TAW moth as the role of FAs in reproduction deviates from the main objectives of the first two data chapters. The final chapter of my thesis focuses on comparing overall findings of data Chapter 1 and Chapter 2 and reflects on how differing life history between these two insects may play a role in the patterns of FA use. Possible physiological mechanisms of differential FA use are also discussed. Additionally further avenues for my research study topic are suggested.
1.10 Literature cited


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Chapter 2

2 Use of stored fatty acids during flight in Monarch Butterflies (*Danaus plexippus*): Implications for the importance of *en route* migratory nectaring sites

2.1 Introduction

Migration occurs in response to numerous factors but is primarily linked to seasonal changes in the availability of resources and habitat quality (Dingle, 1972, 2014). In insects, this migration is typically undertaken by adults in reproductive diapause (Southwood, 1962; Dixon *et al*., 1993; Dingle, 1972, 2014). Migration is a challenging aspect of the life history of insects and is associated with costs and trade-offs, some of which impact future reproduction (Rankin and Burchsted, 1992; Bonte *et al*., 2012). In North America, there are a number of lepidopteran species that migrate, but the eastern population of the monarch butterfly (*Danaus plexippus*) is undoubtedly one of the most iconic.

As with many other migratory organisms, lipids are a key stored fuel that facilitates the energy requirements of migration in monarchs during both the fall and spring migrations (Alonso-Mejia *et al*., 1997; Brower *et al*., 2006). Lipids are primarily stored in the fat body as triglycerides; the combination of three fatty acids (FA) and a glycerol molecule (Arrese and Soulages, 2010; Fahy *et al*., 2011). Once mobilized, these FAs are transported by lipophorin to working muscles and other tissues (Stanley-Samuelson *et al*., 1988; Van der Horst *et al*., 2002; Roma *et al*., 2010) where they are readily oxidized for energy. Adult monarchs feed primarily on nectar from a variety of flowering plants (Waterbury *et al*., 2019). Although nectar has been shown to contain trace amounts of FAs and amino acids, the majority of FAs obtained directly from the diet in nectivorous insects come from the larval diet (Nicolson and Thornburg, 2007; Krenn, 2010). However, some FAs can also be synthesized from other macronutrients found in nectar, such as carbohydrates. FAs that cannot be synthesized and must be obtained from diet are termed essential FAs (EFAs) (Canavoso *et al*., 2001) while FAs that can be *de novo* synthesized are termed nonessential FAs (NFAs). EFAs, which can only be derived directly from larval diet, can limit the reproductive potential of monarch butterflies as these FAs are also important for egg laying.
and embryonic development (Stanley-Samuelson et al., 1988; Canavoso et al., 2001; Forte et al., 2002). Thus, limited non-replenishable EFAs should be spared and used less for fuel during migration compared to NFAs that can be derived de novo from plants en route to reduce the cost migration can have on future reproductive success. Though monarchs undergo both a fall and multigenerational spring migration (Brower, 1985) this study focuses on the fall migration as the cost of EFA use could be more impactful in the fall migration due to the prolonged period through which one generation of monarchs must undertake energetically expensive flight and eventual overwintering (Urquhart and Urquhart, 1978; Brower, 1985).

Monarchs from the eastern population undergo an extensive fall migration to overwintering grounds in central Mexico (Urquhart and Urquhart, 1978; Brower, 1985, 1995), covering up to 4000 km over an average of 75 days. During the overwintering period monarchs remain in a state of reproductive diapause during which time they generally consume only water (Herman et al., 1989; Calvert and Lawton, 1993; Alonso-Mejía et al., 1997). In the fall migration, stored lipids fuel migration and also supply the butterfly with lipids for the overwintering period (Cenedella, 1971; Alonso-Mejía et al., 1997). In spring, gravid females migrate from the overwinter sites to the southern United States where they finally lay eggs on milkweed (Asclepias spp.) (Brower, 1985). In order to successfully complete the long fall migration, monarchs must take advantage of key nectaring sites en route to rest and synthesize lipids from plant carbohydrates (Beenakkers, 1969; Brower et al., 2006). Thus, a key requirement for successful migration, subsequent overwinter fasting and potential reproduction is the manufacture and use of lipids (Angelo and Slansky, 1984; Boggs, 2009) in such a way that would reduce overlapping nutritional requirements between migration and reproduction. However, it is currently not clear how, or if, monarchs and many other long-distance migratory insects allocate stored FAs differentially for migration and reproduction (but see Anparasan et al., 2023).

Much of monarch conservation has focused on the "milkweed limitation" hypothesis. This hypothesis focuses on the breeding grounds where loss of milkweed can lead to a reduction in the summer population sizes which can influence overwintering populations and future population stability (Pleasants and Oberhauser, 2013; Taylor et al., 2020). For example,
Pleasants, (2017) found that by 2006 71% of milkweed in the Midwest was reduced compared to that seen in the 1990s and this is predicted to have limited the size of the summer breeding population which was paralleled in a decline in the size of the overwintering population. However, little attention has been paid to mortality occurring during migration (but see Inamine et al., 2016; Agrawal and Inamine, 2018; Saunders et al., 2019) or how overwinter lipid dynamics are linked to survival and subsequent reproduction (but see Alonso-Mejia et al., 1997). Determining the source and use of nutrients such as FAs involved in successful migration and subsequent fasting or reproduction is vital for the effective conservation of migratory monarch populations. Unfortunately, to date, it has been difficult to trace the biochemical synthesis and routing of FAs by migratory lepidopterans in general (but see Levin et al., 2017). Determining the origin of different EFAs and NFAs is key to establishing important sources of these nutrients in North America for environmental policy and conservation purposes. In addition, developing a greater understanding of differential fuel use in migrant compared to resident insects will also shed light on potential mechanisms by which the flight-reproduction trade-off (Johnson, 1963; Penner, 1985; Roff, 1986; Guerra, 2011) is managed in EFA limited insects.

Here, I evaluated the source and allocation of EFAs and NFAs used for flight in monarchs under breeding (summer) and migratory (fall) conditions in a laboratory setting using stable-carbon isotope ($\delta^{13}C$) measurements and gas chromatography assays. Environmental conditions can serve as cues that result in significant physiological changes between migrant and non-migrant monarchs (James, 1986; Altizer and Davis, 2010; Schroeder et al., 2020), which could influence FA use in experiments. To evaluate the FA composition and changes in relative concentration of different NFAs and EFAs gas chromatographic analysis of fat body samples was performed. Previous literature has shown that under isotopically distinct diets there is a clear reflection of the isotopic value of the diet in monarch tissue (lipid: Hobson et al., 2020; amino acids: also seen in other butterflies – O’Brien et al., 2005) and thus the differential $\delta^{13}C$ of milkweed (C3) larval and cane sugar (C4) adult diets (as seen in Hobson et al., 2020) in this experiment will lead to lipids also varying in $\delta^{13}C$ based on the dietary source from which they were synthesized which allowed me to determine source of FAs used during flight. I hypothesized that larval-
derived EFAs would be conserved during flight for potential future reproduction in migratory individuals.

2.2 Methods

2.2.1 Rearing

Monarchs were obtained from a colony established in 2021 using immigrating adults captured at the Environmental Sciences Western (ESW) Field Station (43.07°N, 81.34°W). Forty individuals were reared at 25 °C, 65% RH and 16L:8D photoperiod, 70 reared under natural summer conditions in an outdoor insectary in June, and an additional 70 reared under natural fall conditions in August/September. All caterpillars were reared on fresh common (C3) milkweed leaves ($\delta^{13}\text{C}: \sim -26.12 \pm 0.05 \%o, n=5$) collected daily from the ESW. Monarchs were sexed upon emergence, separated by sex, and provided a 15% C4 cane sugar diet (Appendix A1.1) (high $\delta^{13}\text{C}: \sim -12.24 \pm 0.02 \%o, n=5$).

2.2.2 Baseline fat body composition in various rearing conditions

Baseline FA profiles and $\delta^{13}\text{C}$ were obtained from the fat body of 20 (10 male and 10 female) unfed newly emerged adults. Another 20 (10 male and 10 female) adults were fed the C4 cane sugar diet ad lib. for five days to allow for adult-stage derived FAs to accumulate. After 5 days, these insects were sacrificed for isotopic and FA profile analysis. Fat body lipid samples were prepared for GC and isotopic analyses as described below. This was done for monarchs reared under all three conditions. Though monarchs may reach sexual maturity as early as 5 days post-eclosion, the average is approximately 7-9 days to become sexually mature and thus at Day 5 little reproductive development was predicted for most individuals (Barker and Herman, 1976; Goehring and Oberhauser, 2002), however it is noted that age of sexual maturity can vary.

2.2.3 Flight trials

Newly emerged monarchs (n=40/rearing condition) that had been reared under either summer or fall conditions were fed the C4 cane sugar diet ad lib. for five days to allow for adult-stage derived FAs to accumulate. On the fifth day post-emergence, flight tests were performed using a rotational flight mill. The scales on the dorsal surface of the thorax were
removed with a camel-hair brush and a 3 cm piece of clear plastic tubing (2 mm diameter) was attached using EVO–STIK instant contact adhesive (Stafford, UK). The butterfly was attached to a copper wire on one arm of the flight mill while the other arm had a counterbalance (Figure 2.1). The counterbalance was made from small metal disks and masking tape and made to be the average mass of day-five aged monarchs (~0.42 g; Appendix A1.2). The entire process (from scale cleaning to attachment on flight mill) took less than 4 minutes. Flight experiments were conducted for flight durations of 1, 4 and 6 hours (5 males and 5 females for each flight duration). If an insect stopped flying, its back legs were gently prodded with a camel-hair brush but if it did not resume flight after three such stimulations it was not included in subsequent analyses. Five percent were excluded due to this criteria but as 40 were set up at the start, there was always at least 30 (10 per flight duration) for all subsequent analyses (randomly selected 10 insects per flight duration and additional insects were not used for analysis). Afterwards, flown insects were sacrificed and stored at -80°C until fat body samples were taken for isotopic and GC analyses as described below.
Figure 2.1 Monarch being exercised on a rotational flight mill. Attachment of tubing to monarch occurs on the dorsal segment between wings to ensure adhesion does not affect flight performance.
2.2.4 Fat body isotopic analysis

All fat body tissue was dissected out of the abdomen and homogenized by hand using a small spatula. Fat body tissue was separated for isotopic analysis with ~ 5 mg reserved for chromatographic analysis. The remaining fat body tissue was used for isotopic analysis. Lipid was extracted from fat body for isotopic analysis using a solution of 2:1 chloroform: methanol (Folch et al., 1957) with 0.01 % butylated hydroxytoluene (BHT). The sample was left to soak in the solution overnight after which it was filtered and dried overnight in a fume hood (23 °C, 101.3 kPa). Lipid remaining after the solution dried was frozen. For isotopic analysis frozen lipid samples (0.91-0.99 mg) were weighed into tin capsules (8 x 5 mm), crushed, and sent to the Cornell Laboratory by mail courier for isotopic analysis (Ithaca, New York, USA). Crushed capsules were combusted at 1000 °C in a Carlo-Erba NC2500 Elemental Analyser (Carlo Erba, Italy) and CO₂ gas transferred via a ConFlo IV (Thermo Scientific, Bremen Germany) device to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Scientific, Bremen Germany). Machine data were normalized using internal calibrated lab standards (Cayuga brown trout: -25.58 ‰ and corn: -13.02 ‰); instrument linearity was assessed using in-house methionine (-27.2 ‰) and ground deer hair (-20.1 ‰) standards. All values are reported in standard delta (δ) notation relative to the Vienna Pee Dee Belemnite (VPDB) standard in parts per thousand (‰). Measurement error based on within-run standards was estimated as ± 0.1 ‰.

2.2.5 Fatty acid analysis

The sample of fat body tissue was excised (5 mg) and the lipids chemically extracted in 2:1 chloroform: methanol (Folch et al., 1957) with 0.01 % butylated hydroxytoluene (BHT) (2 mL) under N₂ flow, to reduce oxidation of FAs and only for 5 minutes. Twenty μL of an internal standard reference, margaric acid (17:0, 3 mg/mL), was then added to this mixture. One mL of 0.25 % KCl was then added to the solution to separate out aqueous solutes and allowed to react in a 70 °C hot water bath for 5 minutes. The whole solution was then filtered and dried under N₂. The remaining FAs (~100 μg) were then converted into fatty acid methyl esters by adding 200 μL of 0.5 M methanolic hydrogen chloride and reacting at 90 °C for 30 minutes. The solution was then reduced to dryness under nitrogen, and resuspended in hexane (100 μL). These samples were subsequently analysed using
chromatographic techniques. Analysis was performed in a gas chromatography/flame ionization detector (Agilent Technologies® 6890N G1530N, Santa Clara, USA) equipped with a DB23 column (Agilent DB23 122-2332, Santa Clara, USA). The injector temperature was 250 °C and the flame ionization detector temperature was 280 °C. During each run, a 100 µL dichloromethane control was also analysed. The retention times of PUFA standards (Supelco® PUFA and 37 component) were averaged to create a library of known fatty acid peaks. The distinct and clear peaks of each sample chromatograph were compared to the known retention times to identify fatty acids. This, combined with the known concentration of the internal standard, was used to calculate the concentration of each fatty acid (µmol/mL) in the 100 µL aliquot of FAs and hexane that was analyzed where all samples contained a total lipid mass of 100 µg. I quantified the EFAs alpha linolenic acid (ALA) and linoleic (LA) as they cannot be de novo synthesized by Lepidopterans but are essential for development (Canavoso et al., 2001), and the NFAs oleic (OL), palmitic (PA), and stearic acid (ST), as they are found in high levels in Lepidoptera (Subramanyam and Cutkomp, 1987; Canavoso et al., 2001).

2.2.6 Statistical analysis

All statistical tests (ANOVA, Tukey’s post-hoc analysis) were performed using R Studio (Version 3.4.2 (2017-09-28)). δ¹³C values of fat body lipid from unfed, newly emerged, and fed adults were analysed using a single factor one-way ANOVA and Tukey’s post-hoc analysis with rearing condition as the independent variable. A single factor one-way ANOVA and Tukey’s post-hoc analysis was used to compare: (i) fat body δ¹³C values across each treatment of age and rearing condition (i.e. Day 0 fall, Day 0 summer, Day 0 lab, Day 5 fall, Day 5 summer, Day 5 lab), (ii) individual and total FA concentrations across each treatment of age and rearing condition (i.e. Day 0 fall, Day 0 summer, Day 0 lab, Day 5 fall, Day 5 summer, Day 5 lab), and (iii) individual FA, total FA concentrations and fat body δ¹³C across differential flight periods (independent variable) for both summer- and fall-reared insects. Normality of data was confirmed via Skew (-1 to +1) and Kurtosis (-4 to +4) analyses.
2.3 Results

Regardless of rearing conditions the δ\textsuperscript{13}C values of fat body from unfed, newly emerged, adults only provided with larval C3 diet did not differ (-31.2 ± 0.2 ‰; Figure 2.2, ANOVA, df, 2, 59, \( F=2.04, p=0.148 \)) and there was no effect of sex (ANOVA, df, 1, 59, \( F=0.01, p=0.92 \)). After feeding for 5 days on C4 nectar diet the δ\textsuperscript{13}C values of the fat body were significantly different than those of unfed adults, (-22.1 ± 0.3 ‰; Figure 2.2. ANOVA, df, 5, 119, \( F=19.92, p<0.01, \text{ Tukey HSD, } p<0.05 \)). Again, there was no impact of rearing condition (ANOVA, df, 2, 59, \( F=0.16, p=0.85 \)) or sex (ANOVA, df, 1, 59, \( F=0.01, p=0.93 \)) on the values observed in fed adults.
Figure 2.2 The δ¹³C values of fat body lipids of non-flown monarch adults as a function of age and rearing conditions. 0 indicates insects that were newly emerged while 5 indicates insects fed on the adult diet for 5 days post-eclosion. Bar colors indicate feeding status during the adult stage. Significant differences are indicated by different letters (Tukey HSD, p<0.05). N=20 per sample group.
Oleic acid, palmitic acid (both NFA) and alpha-linolenic acid (EFA) made up the majority of the fat body lipid of the 5 FAs sampled at emergence (Table 2.1). Lipid gained during the fed adult stage was through the accumulation of oleic acid (Table 2.1, ANOVA, df, 5, 119, \( F=9.72, p<0.01 \), Tukey HSD, \( p<0.05 \)) and palmitic acid (Table 2.1, ANOVA, df, 5, 119, \( F=7.73, p<0.01 \), Tukey HSD, \( p<0.05 \)). There was no alpha-linolenic acid gained from the adult diet during the adult fed stage compared to the unfed adult stage (ANOVA, df, 5, 119, \( F=0.58, p=0.71 \)) and a slight decline in essential linoleic acid was observed under summer and lab conditions at the fed adult monarch stage (ANOVA, df, 5, 119, \( F=3.92, p=0.04 \), Tukey HSD, \( p<0.05 \)). It is also noted that fall-reared insects had more stearic acid regardless of age (ANOVA, df, 5, 119, \( F=5.19, p<0.05 \), Tukey HSD, \( p<0.05 \)).

**Table 2.1 Concentration (± SE) of non-essential and essential (in bold) fatty acids in the fat body of non-flown adult monarchs as a function of age and rearing conditions.**

0 indicates insects that were newly emerged while 5 indicates insects fed on the adult diet for 5 days post-eclosion. (*) indicate differences in the same age across rearing conditions (Tukey HSD, \( p<0.05 \)) within each fatty acid. Different letters indicate significant differences within each fatty acid within the same rearing condition across age (Tukey HSD, \( p<0.05 \)) in each fatty acid. \( N=20 \) per sample group.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Lab Day 0 (μmol/ml)</th>
<th>Summer Day 0 (μmol/ml)</th>
<th>Fall Day 0 (μmol/ml)</th>
<th>Lab Day 5 (μmol/ml)</th>
<th>Summer Day 5 (μmol/ml)</th>
<th>Fall Day 5 (μmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>75.2 ± 7.1^{(a)}</td>
<td>73.0 ± 6.7^{(a)}</td>
<td>79.4 ± 7.6^{(a)}</td>
<td>90.4 ± 7.7^{(b)}</td>
<td>86.9 ± 8.7^{(b)}</td>
<td>96.7 ± 7.2^{(b)}</td>
</tr>
<tr>
<td>Stearic</td>
<td>22.3 ± 3.6^{(a)}</td>
<td>18.1 ± 4.8^{(a)}</td>
<td>29.1 ± 2.1^{(a)}</td>
<td>16.4 ± 3.8^{(b)}</td>
<td>12.1 ± 2.3^{(b)}</td>
<td>24.2 ± 2.4^{(b)}</td>
</tr>
<tr>
<td>Oleic</td>
<td>76.0 ± 12.6^{(a)}</td>
<td>75.2 ± 6.7^{(a)}</td>
<td>85.6 ± 5.3^{(a)}</td>
<td>112.4 ± 9.6^{(b)}</td>
<td>109.3 ± 9.5^{(b)}</td>
<td>122.9 ± 6.9^{(b)}</td>
</tr>
<tr>
<td>Linoleic</td>
<td>17.1 ± 2.0^{(a)}</td>
<td>15.6 ± 3.5^{(a)}</td>
<td>20.8 ± 1.8^{(a)}</td>
<td>13.3 ± 1.5^{(b)}</td>
<td>10.6 ± 1.5^{(b)}</td>
<td>17.6 ± 1.2^{(a∗)}</td>
</tr>
<tr>
<td>Alpha linolenic</td>
<td>79.9 ± 6.3^{(a)}</td>
<td>77.6 ± 7.1^{(a)}</td>
<td>83.2 ± 5.4^{(a)}</td>
<td>88.6 ± 8.9^{(a)}</td>
<td>77.1 ± 8.0^{(a)}</td>
<td>84.5 ± 5.1^{(a)}</td>
</tr>
<tr>
<td>Total FA</td>
<td>294.9 ± 29.6^{(a)}</td>
<td>285.9 ±24.4^{(a)}</td>
<td>324.9 ± 21.2^{(a)}</td>
<td>347.8 ± 30.3^{(b)}</td>
<td>321.53 ±28.3^{(b)}</td>
<td>377.4 ±21.3^{(b)}</td>
</tr>
</tbody>
</table>
The $\delta^{13}C$ values of the fat body lipid of either summer- (Figure 2.3a. ANOVA, df 3,49, $F=1.10, p=0.36$) or fall-reared (Figure 2.3b. ANOVA, df, 3, 49, $F=0.46, p=0.75$) monarchs did not vary as a function of flight duration or by sex (Summer, ANOVA, df$_{sex}$ =1, 49, $F_{sex}$ = 1.68, $p_{sex}$ = 0.20. Fall, ANOVA, df$_{sex}$, 1, 49, $F_{sex}$=0.75, $p_{sex}$= 0.40).
Figure 2.3 δ¹³C values of fat body lipids from 5-day old monarch adults reared under summer (A) and fall conditions (B) as a function of flight duration. Different letters indicate statistical differences as a function of flight time (Tukey HSD, \( p < 0.05 \)). \( N = 10 \) per sample group except hour 0 (\( N = 20 \))
During flight there was a clear decline in total FA after 4 and 6 hours of flight under both summer (ANOVA, df, 3, 49, $F=11.51$, $p<0.001$, Tukey HSD $p<0.05$) and fall (ANOVA, df, 3, 49, $F=5.01$, $p=0.03$, Tukey HSD, $p<0.05$) conditions. This was mainly driven by a depletion in oleic acid (ANOVA, df, 3, 49, $F_{\text{summer}}=5.53$, $p=0.02$ Tukey HSD, $p<0.05$; $F_{\text{fall}}=14.50$, $p<0.001$ Tukey HSD, $p<0.05$) and palmitic acid (ANOVA, df, 3, 49, $F_{\text{summer}}=13.67$, $p<0.001$ Tukey HSD, $p<0.05$; $F_{\text{fall}}=4.63$, $p=0.03$ Tukey HSD, $p<0.05$) under both summer (Figure 2.4a.) and fall (Figure 2.4b.) conditions.
Figure 2.4 Concentration (± SE) of oleic (OL) palmitic (PA) and stearic (ST) nonessential fatty acids in the fat body of 5-day old monarch adults under summer (A) and fall (B) conditions as a function of flight duration. Different letters indicate statistically significant differences within each fatty acid (Tukey HSD, $p<0.05$). $N=10$ per sample group except hour 0 ($N=20$).
There was also a decline in both EFAs under both summer (Figure 2.5a. ANOVA, df, 3, 49, $F_{\text{summer} \text{LA}}=6.92$, $F_{\text{summerALA}}=4.22$, $p_{\text{summer} \text{LA}}<0.03$, $p_{\text{summerALA}} = 0.03$; Tukey HSD, $p<0.05$) and fall (Figure 2.5b. ANOVA, df, 3, 49, $F_{\text{fall} \text{LA}}=4.03$ $F_{\text{fallALA}}=5.14$ $p_{\text{fall} \text{LA}} = 0.04$, $p_{\text{fallALA}} < 0.03$; Tukey HSD, $p<0.05$) conditions. However, the onset of mobilization occurred sooner (after 4 vs 6 h flight) and the amount used was higher (31 vs. 21%) in summer-reared individuals compared to fall individuals. While the original alpha-linolenic acid concentrations were similar to those of the NFAs (Table 2.1) its overall use was lower than the NFAs in both summer- and fall-reared monarchs (Figure 2.4 and 2.5).
Figure 2.5 Concentration (± SE) of alpha linolenic (ALA) and linoleic (LA) essential fatty acids in the fat body of 5-day old monarch adults reared under summer (A) and fall (B) conditions as a function of flight duration. Different letters indicate statistically significant differences within each category of fatty acid (Tukey HSD, *p*<0.05). N=10 per sample group except hour 0 (N=20).
2.4 Discussion

Monarchs clearly synthesize fats from the nectar carbohydrate source when reared under summer or fall conditions as evidenced by the change in the fat body δ¹³C values after adults reared on the C3 milkweed larval diet source were fed on the C4 cane sugar nectar for 5 days (Figure 2.2). However, the observed increases in concentration of the different FAs are quite small regardless of rearing condition (Table 2.1). Of the 5 FAs selected for this study the majority of the total lipid that was sampled was comprised of PA, OL, and ALA, which is consistent with previous literature on the monarch butterfly (Reisinger and Yurkiewicz, 1969). The decline in the levels of linoleic acid in summer and lab-control monarchs after 5 days post eclosion (Table 2.1) may have reflected incorporation of this FA into the reproductive tissues, as reported in other lepidopterans (Arrese and Soulages, 2010; Martin, 1969). In contrast, under fall conditions adults undergo reproductive diapause and thus little if any resources are allocated to reproduction (Barker and Herman, 1976) and thus no loss of EFA was seen by Day-5 post-eclosion.

These findings support the hypothesis that fall migrant monarch butterflies do not generate large fat reserves prior to the onset of migration and maintain FAs required for fuel through nectaring at stopover sites before significantly increasing lipid accumulation just prior to arriving at the overwintering sites (Brower, 1985; Brower et al., 2006; Hobson et al., 2020). For example, from 1993 to 1994 monarchs leaving for fall migration average ~60 mg of lipid but had an average of 140 mg when they arrived at the overwintering site (Alonso-Mejia et al., 1997). Thus, the availability of nectaring sites along the migratory pathway will be essential for completing migration and as increased fat accumulation occurs later during the migratory period then nectaring sites close to the overwintering sites will be critical for the acquisition of reserves for the overwintering of fall migrating monarchs. Anthropogenic land use changes are predicted to significantly change nectar availability for many insect species (Cane and Tepedino, 2001; Zipkin et al., 2012; Malcolm, 2018). In addition, even if nectaring sites are still available climate change can also affect the quality of the nectar present as events such as drought can reduce nectar availability in plants (Kim et al., 2011; Descamps et al., 2021; Kuppler and Kotowska, 2021). The loss of nectar due to drought was studied in Liatris mucronata flowers by Brower et al. (2015).
and it was found that monarchs that fed on drought stressed plants had significantly less lipid present than monarchs fed on well irrigated plants. This inability to gain NFAs could result in limited EFAs being used as fuel more readily.

As observed in other Lepidoptera (Murata and Tojo, 2002; Sakamoto et al., 2004), the NFAs palmitic acid and oleic acid were the most used to power flight in monarchs (Figure 2.4). The fat body δ¹³C values remained unchanged regardless of flight duration in both summer and fall groups (Figure 2.3) which I infer reflects that both adult and larval resources were used as the energy source. Though EFAs declined under both conditions (Figure 2.5), alpha-linolenic acid was delayed in mobilization in fall monarchs compared to summer monarchs. Fall-reared monarchs also used proportionally less alpha-linolenic acid even though migrant and reproductive monarchs both had similar levels of this FA prior to flight exercise. The difference in fuel use may indicate differences in the fuel use physiology between summer- and fall-reared monarchs. For example, Schroeder et al. (2020) found that fall migratory monarchs had more efficient flight and post-flight metabolism compared to non-migrant monarchs. The more ready mobilization of oleic acid and palmitic acid (NFAs) compared to alpha-linolenic acid (EFA) in fall-reared monarchs, regardless of being found in similar relative concentrations, may indicate that there is still a pattern of greater NFA use over limited EFAs during migratory flight even if EFAs were still used as fuel in this study (also seen in desert locusts (Schistocerca gregaria) (Schneider and Dorn, 1994) and northern armyworm moths (Mythimna separata) (Wang and Ouyang, 1995)). While the mechanism behind this differential use is not known, there are many hypotheses related to storage, mobilization and hormonal regulation in insects that have been proposed (Lok and Van Der Horst, 1980; Zera and Mole, 1994; Tomcala et al., 2010).

The results of this study differ from other recent work on related lepidopteran species such as the true armyworm moth (Anparasan et al., 2023). The true armyworm Mythimna unipuncta accumulates much higher levels of fat, relative to body weight, during the first 5 days post-eclosion than the monarch (Anparasan et al., 2021). Prior to flight TAW moths have lower EFA concentrations in the fat body compared to monarchs as well. Moths also showed a very clear conservation of both EFAs during 6-hour flight periods when reared
under migratory conditions while the monarch displays mobilization of EFAs but to a lesser degree than that of NFAs. The difference in fuel use pattern could correlate to the differences in migratory behaviour (both mode and duration) of the two species. The true armyworm moth flies on upper-air wind currents, flapping at a high frequency throughout, and can fly up to 13 hours without rest (Guppy, 1961; Taylor, 1974; Fields and McNeil, 1984; McNeil, 1987; Luo et al., 2002), and thus has the capacity for continuous flight without reaccumulating fuel. In contrast, the monarch flies within its boundary layer, undergoes bouts of gliding flight, feeds regularly and stops when conditions are unfavorable (Brower, 1996; Urquhart and Urquhart, 1978; Chapman, 2015; Brower et al., 2006; Rudolph et al., 2006). In the field monarchs use a mixture of flapping with long bouts of gliding (Urquhart, 1960; Gibo and Pallett, 1979) and may refuel during a flying bout, neither of which are possible on the flight mill. As a consequence, 4-6 h of forced flapping flight may have drastically depleted the fat body lipid levels (based off some fuel predictions that have been proposed by Gibo and McCurdy, 1993) in a way that would not necessarily occur during natural migration. While the flight pattern used in this experiment was likely more strenuous than what is experienced by wild migrating monarchs, there was still a clear difference in the use of FAs with proportionately less EFAs being used by monarchs during simulated migratory flight compared to NFAs. Clearly additional research comparing other species of moths and butterflies is required to obtain a greater understanding of FA use in EFA limited lepidopteran migrants. For example, the painted lady butterfly (Vanessa cardui) has been shown to take advantage of favorable winds and fly at altitudes over 1000 m (Chapman et al., 2010; Stefanescu et al., 2013).

This study demonstrates the potentially detrimental role that nectar limitation as a result of changes to land use and climate change (Cane and Tepedino, 2001; Kim et al., 2011; Malcolm, 2018) could have on FA allocation in migrant monarchs and thus one approach for conservation would be ensuring suitable foraging sites along the migratory pathway. Future studies using a compound-specific isotopic analysis approach to determine where the individual FAs are acquired and how they are used (Bec et al., 2011; McMahon and Newsome, 2019; Whiteman et al., 2019) could help determine what nectaring regions and plants are being used at different points along the migratory corridor.
2.5 Literature cited


Chapter 3

3 Effect of rearing conditions on fatty acid allocation during flight in nectivorous lepidopteran *Mythimna unipuncta*

3.1 Introduction

Diapause or seasonal migration in response to proximal cues indicating impending habitat deterioration, such as declining temperature, photoperiod, and host plant quality, are key components in the life history of many temperate insect species (Dingle, 1972, 2014; Saunders, 1987). Whether entering a dormant state locally or emigrating to more favorable habitats, there is a need to acquire the appropriate energy reserves, as the failure to do so could result in death or a significant decline in the subsequent reproductive success of survivors.

Acquiring the appropriate energy reserves, predominantly in the form of lipids, is often associated with periods of hyperphagia (Beenakkers, 1969; Schneider and Dorn, 1994; McCue *et al*., 2015). The subsequent allocation of lipids during diapause or migration is important as they are also used for hormone synthesis (e.g. ecdysteroids), neuronal development, membrane integrity (phospholipids), defensive secretions, and reproduction (Stanley-Samuelson *et al*., 1988). Certain fatty acids (FAs) are considered as nonessential (NFAs) as they can be synthesized by the adult insect from other macronutrients, and so could be replaced through feeding post-diapause or migration. However, others cannot be synthesized and are considered essential (EFAs) as they must be obtained directly from the diet. Thus, any EFAs used during migration that cannot be replaced could have negative consequences.

In the case of migratory Lepidoptera, the onset of migration is typically initiated by adults in reproductive diapause (Johnson, 1963; Dingle, 2014) and any subsequent replacement of lipids used during migration would have to be synthesized from nectar (i.e., carbohydrate) sources. However, as nectar only contains trace amounts of FAs (Nicolson and Thornburg, 2007; Krenn, 2010), the majority of EFA acquisition by Lepidoptera occurs during larval development. Thus, any EFAs used during migration would not be replaced.
Previous studies on Lepidoptera have shown that EFAs are not conserved during flight when insects were reared under reproductive conditions (Murata and Tojo, 2002; Sakamoto et al., 2004). However, in their study comparing solitary and gregarious desert locusts (thus nonmigratory and migratory, respectively) (Schistocerca gregaria), Schneider and Dorn (1994) raised the possibility that EFAs may be conserved during migratory flight. If this were the case in migratory Lepidoptera, the conservation of EFAs during migratory flight would limit any negative post-migration effects on reproduction. Therefore, we tested the hypothesis that the physiological changes in response to the environmental cues that induce the onset of migration would also influence the use of EFAs during flight. We predicted that adults reared under fall conditions would conserve larval diet derived EFAs while those under summer conditions would not.

We used the true armyworm, Mythimna unipuncta, a sporadic agricultural pest as a model system as it is a seasonal migrant (Guppy, 1961; Fields and McNeil, 1984; Hobson et al., 2018). Furthermore, previous research has shown that individuals reared under fall conditions have significant physiological differences than those reared under summer conditions, affecting sexual maturation, pheromone communication and lipid accumulation (Turgeon and McNeil, 1983; Delisle and McNeil, 1987; Cusson and McNeil, 1989). We reared insects under several different ecological conditions and then determined the fat body lipid content of newly emerged adults and 5-day old adults using gas chromatography (GC) and isotopically (δ^{13}C) distinct larval and adult diets. In addition, moths were force flown for different lengths of time to determine if rearing conditions, flight duration and resource availability play a role in influencing FA use during flight.

3.2 Methods

3.2.1 Insect colony

All individuals used were from a colony established using adults collected from light and pheromone traps (a minimum of 30 males and 30 females captured during the 2021 spring immigrant flight period) at the Environmental Sciences Western (ESW) Field Station (43.07°N, 81.34°W). The colony was maintained at 25 °C, 16:8D, 70 ± 5% RH, with larvae being reared individually on a (C3: δ^{13}C: ~−25.2 ‰) synthetic pinto bean diet (Shorey and
Hale, 1965) while adults were provided an *ad lib* supply of 25 % (C4: δ¹³C: ~ −12.2 ‰) cane sugar and tap water solution. The adults used for all experiments had not been in rearing for more than two generations. Depending on the specific experiment, the larvae used were reared under the same laboratory conditions as the colony, or in an insectary under natural conditions in June/July and August/September, subsequently referred to as summer and fall conditions, respectively.

### 3.2.2 General protocol for fat body lipid extraction and chromatographic analysis

At the end of each experiment, adults were weighed (to nearest mg, Appendix A2.1) and stored at −80 °C until fat body tissue was analysed. In all experiments, fat body tissue was dissected out of the abdomen and homogenized by hand using a small spatula. Fat body tissue was separated for isotopic analysis with ~ 5 mg reserved for chromatographic analysis. The sample of fat body tissue was excised (5 mg) and the lipids chemically extracted in 2:1 chloroform: methanol (Folch *et al.*, 1957) with 0.01 % butylated hydroxytoluene (BHT) (2 mL) under N₂ flow, to reduce oxidation of FAs and only for 5 minutes. Twenty µL of an internal standard reference, margaric acid (17:0, 3 mg/mL), was then added to this mixture. One mL of 0.25 % KCl was then added to the solution to separate out aqueous solutes and allowed to react in a 70 °C hot water bath for 5 minutes. The whole solution was then filtered and dried under N₂. The remaining FAs (~100 µg) were then converted into fatty acid methyl esters by adding 200 µL of 0.5 M methanolic hydrogen chloride and reacting at 90 °C for 30 minutes. The solution was then reduced to dryness under nitrogen, resuspended in hexane (100 µL). These samples were subsequently analysed using chromatographic techniques.

Analysis was performed in a gas chromatography/flame ionization detector (Agilent Technologies® 6890N G1530N, Santa Clara, USA) equipped with a DB23 column (Agilent DB23 122-2332, Santa Clara, USA). The injector temperature was 250 °C and the flame ionization detector temperature was 280 °C. During each run, a 100 µL dichloromethane control was also analysed. The retention times of PUFA standards (Supelco® PUFA and 37 component) were averaged to create a library of known fatty acid peaks. The distinct and clear peaks of each sample chromatograph were compared to the
known retention times to identify fatty acids. This, combined with the known concentration of the internal standard, was used to calculate the concentration of each fatty acid (µmol/mL) in the 100 µL aliquot of FAs and hexane that was analyzed where all samples contained a total lipid mass of 100 µg. I quantified the EFAs alpha linolenic acid (ALA) and linoleic (LA) as they cannot be de novo synthesized by Lepidopterans but are essential for development (Canavoso et al., 2001), and the NFAs oleic (OL), palmitic (PA), and stearic acid (ST), as they are found in high levels in Lepidoptera (Subramanyam and Cutkomp, 1987; Canavoso et al., 2001).

3.2.3 General protocol for fat body lipid extraction and isotopic analysis

Lipid was extracted from remaining fat body tissue for isotopic analysis using a solution of 2:1 chloroform: methanol (Folch et al., 1957) with 0.01 % butylated hydroxytoluene (BHT). The sample was left to soak in the solution overnight after which it was filtered and dried in a fume hood (23 °C, 101.3 kPa). Lipid remaining after the solution dried was frozen. For δ¹³C analyses 0.91–0.99 mg of frozen lipid was placed into tin capsules (8×5 mm) and crushed. All prepared samples were shipped via expedited courier to the Cornell Isotope Laboratory (COIL, Ithaca, NY, United States) where isotopic measurements were performed. Here, crushed capsules were combusted at 1000 °C in a Carlo-Erba NC2500 Elemental Analyser (Carlo Erba, Italy) and CO₂ gas transferred via a ConFlo IV (Thermo Scientific, Bremen Germany) device to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Scientific, Bremen Germany). Data were normalized using internal calibrated lab standards (Cayuga brown trout: −25.58 ‰ and corn: −13.02 ‰); instrument linearity was assessed using in-house methionine (−27.2 ‰) and ground deer hair (−20.1 ‰) standards. All values are reported in standard delta (δ) notation relative to the Vienna Pee Dee Belemnite (VPDB) standard in parts per 1000 (%). Measurement error based on within-run standards was estimated as ±0.1 ‰.

3.2.4 General protocol for flight assays

The scales on the dorsal surface of the thorax were removed using a camel-hair brush and a 3 cm piece of clear plastic tubing (2 mm diameter) was attached using EVO–STIK
(Stafford, United Kingdom) instant contact adhesive. Each moth was then suspended from a copper wire placed perpendicular to flight apparatus base *via* the thoracic tether and force flown for the required period of time (Figure 3.1). If a moth stopped flying its back legs were gently touched with a camel-hair brush and given the significant flight propensity of TAW moths (Luo *et al.*, 2002) no moths had to be eliminated from the final analyses.
Figure 3.1 Set up used for forced flight experiments of tethered true armyworm moths.
3.2.5 Protocols of specific experiments

To test that the majority of lipid present in the fat body of mature individuals is acquired from adult resources, we reared cohorts under lab, summer, and fall conditions. Under each rearing condition 20 individuals (10 of each sex) were sacrificed at emergence and an equal number fed 25% cane sugar water *ad lib* were sacrificed after 5 days. Fat body samples were taken for both isotopic and chromatographic analysis as described above. The fat body $\delta^{13}$C values of newly emerged individuals would reflect only the C3 larval diet while those of 5-day old individuals would also include the contribution from the C4 cane sugar water diet. EFAs would be limited to only arising from the larval diet.

If lipids used during flight were NFAs and principally of adult (C4) origin, then one would predict that as they were metabolized the $\delta^{13}$C values in the fat body would change, becoming closer and closer to the C3 larval diet value as a function of flight duration. To determine if this was the case, 30 five-day old adults (5 of each sex/duration of flight) were reared under either natural summer or fall conditions, then force flown for 1, 4, or 6h, after which time lipids were extracted from fat body tissue and prepared for $\delta^{13}$C isotopic analysis and chromatographic analysis, as described above.

In light of the differences observed in EFA use between the summer- and fall-reared insects, and the fact that migratory flight generally occurs over more than one night, we conducted additional experiments using a cohort of 120 fall-reared individuals that were fed sugar water *ad lib* for 5 days. Then 20 (10 of each sex) were sacrificed without being flown, while the remaining 100 were flown for 8h, after which 10 (5 of each sex) were sacrificed and fat body samples taken. A subset of 40 moths were flown 8h/day and fed sugar water *ad lib* after each flight period, with a subsample (five of each sex) sacrificed after 1, 2, 3, or 4 days of flight. The same protocol was repeated with 40 moths only provided *ad lib* water after each flight. In addition, a subset of 20 moths receiving only water were provided sugar water *ad lib* once, after the third day of flight. Fat body samples were taken for chromatographic analysis, as described above, to determine the changes in relative FA concentrations of selected FAs as a function of the number of flight periods and the availability of adult resources (summarized in Figure 3.2).
Figure 3.2 Simplified experimental design for multi-day fall-reared armyworm moth flight experiment with differing diet availabilities.
3.2.6 Statistical analyses

All statistical tests (ANOVA, Tukey’s post-hoc analysis, unpaired $t$-tests) were performed using R Studio (Version 3.4.2 (2017-09-28)). A single factor one-way ANOVA and Tukey’s post-hoc analysis was used to (i) compare fat body $\delta^{13}$C values across each treatment of age and rearing condition (i.e. Day 0 fall, Day 0 summer, Day 0 lab, Day 5 fall, Day 5 summer, Day 5 lab), (ii) compare individual and total FA concentrations across each treatment of age and rearing condition (i.e. Day 0 fall, Day 0 summer, Day 0 lab, Day 5 fall, Day 5 summer, Day 5 lab), (iii) compare individual FA, total FA concentrations and fat body $\delta^{13}$C across differential flight periods (independent variable) for both summer- and fall-reared insects (iv) and compare individual FA and total FA concentrations in multi day flown insects across differential flight periods (independent variable) for fasted, fed, and refed moths. Unpaired $t$-tests were used to determine specific differences in FA patterns or $\delta^{13}$C values of the fat body between sexes during 6 hours of flight. Normality of data was confirmed using Skew (−1 to +1) and Kurtosis (−4 to +4) analyses.

3.3 Results

While the $\delta^{13}$C values of the fat body from newly emerged adults reflected the C3 larval diet, after 5 days of *ad lib* feeding on the adult diet, the $\delta^{13}$C values increased regardless of rearing conditions (Figure 3.3., ANOVA, df, 5,112, $F = 936.31$, $p < 0.001$), and clearly reflected the addition of lipids derived from the C4 adult diet (Figure 3.3). In both age groups there were no differences in $\delta^{13}$C values of the fat body due to rearing conditions (Tukey HSD, Day 0 fall versus summer: $p = 0.21$, Day 0 fall versus lab: $p = 0.99$, Day 0 summer versus lab: $p = 0.47$, Day 5 fall versus summer: $p = 0.93$, Day 5 fall versus lab: $p = 0.14$, Day 5 summer versus lab: $p = 0.17$) or sex (ANOVA, df, 1,112, $F = 0.63$, $p = 0.425$). All moths showed mass gain across all rearing conditions by Day 5 post-eclosion (Appendix A2.1). The $\delta^{13}$C value of purely adult diet derived lipid was -14.62‰ (Appendix A2.2).
Figure 3.3 The $\delta^{13}C$ values of lipid in the fat body of non-flown true armyworm adults fed sugar water ad lib as a function of age and rearing conditions. 0 indicates insects that had newly emerged while 5 indicates insects fed on the adult diet for 5 days post-eclosion. Significant differences are indicated by different letters (Tukey HSD, $p < 0.05$). N = 20 per sample.
The concentration of total FAs increased significantly from Day 0 to Day 5 in non-flown adults across all rearing conditions (Table 3.1; ANOVA, df, 5,119, $F = 27.72$, $p < 0.0001$). This was due to the marked changes in the NFAs (Table 3.1; ANOVA, df, 5,119, $F_{PA} = 27.45$, $F_{OL} = 26.52$, $F_{ST} = 8.74$, $p < 0.001$), but there were no significant differences in total FA concentrations between the three rearing conditions at either age (Tukey HSD, Day 0 fall versus summer: $p = 0.99$, Day 0 fall versus lab: $p = 1.0$, Day 0 summer versus lab: $p = 1.0$, Day 5 fall versus summer: $p = 0.69$, Day 5 fall versus lab: $p = 0.25$, Day 5 summer versus lab: $p = 0.97$). In contrast, while the concentration of the two EFAs did not differ between rearing conditions within any age group, both declined with age (Table 3.1; ANOVA, df, 5,119, $F_{LA} = 16.72$, $F_{ALA} = 21.48$, $p < 0.001$).

Table 3.1 Concentration (± SE) of specific essential (bold) and non-essential fatty acids in the fat body of non-flown true armyworm adults as a function of age and rearing conditions. 0 indicates insects that had newly emerged while 5 indicates insects fed on the adult diet for 5 days post-eclosion. Significant differences within each row for individual and total fatty acids are indicated by different letters (Tukey HSD, $p<0.05$). $N= 20$ per sample group.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Lab Day 0 (μmol/ml)</th>
<th>Fall Day 0 (μmol/ml)</th>
<th>Summer Day 0 (μmol/ml)</th>
<th>Lab day 5 (μmol/ml)</th>
<th>Fall Day 5 (μmol/ml)</th>
<th>Summer Day 5 (μmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>39.5 ± 2.5(a)</td>
<td>39.2 ± 2.2(a)</td>
<td>38.9 ± 3.4(a)</td>
<td>86.6 ± 4.7(b)</td>
<td>108.1 ± 6.4(b)</td>
<td>84.1 ± 5.8(b)</td>
</tr>
<tr>
<td>Stearic</td>
<td>3.1 ± 0.5(a)</td>
<td>3.5 ± 0.2(b)</td>
<td>4.7 ± 0.6(a)</td>
<td>7.9 ± 0.6(b)</td>
<td>8.8 ± 0.3(b)</td>
<td>8.4 ± 0.9(b)</td>
</tr>
<tr>
<td>Oleic</td>
<td>58.5 ± 3.3(a)</td>
<td>54.1 ± 3.2(a)</td>
<td>58.1 ± 4.6(a)</td>
<td>153.7 ± 12.5(b)</td>
<td>170.9 ± 11.2(b)</td>
<td>159.9 ± 10.8(b)</td>
</tr>
<tr>
<td>Linoleic</td>
<td>5.0 ± 0.2(a)</td>
<td>5.4 ± 0.4(a)</td>
<td>5.3 ± 0.3(a)</td>
<td>3.9 ± 0.4(b)</td>
<td>4.5 ± 0.2(b)</td>
<td>3.8 ± 0.5(b)</td>
</tr>
<tr>
<td>Alpha linolenic</td>
<td>1.8 ± 0.1(a)</td>
<td>2.0 ± 0.2(a)</td>
<td>1.9 ± 0.2(a)</td>
<td>1.6 ± 0.1(b)</td>
<td>1.5 ± 0.1(b)</td>
<td>1.5 ± 0.1(b)</td>
</tr>
<tr>
<td>Total FA</td>
<td>107.7 ± 8.1(a)</td>
<td>112.7 ± 5.1(a)</td>
<td>118.8 ± 8.4(a)</td>
<td>259.0 ± 18.1(b)</td>
<td>300.3 ± 18.6(b)</td>
<td>266.8 ± 17.4(b)</td>
</tr>
</tbody>
</table>

There was a significant decline in the lipid δ$^{13}$C values of 5-day old individuals as a function of flight duration under both rearing conditions, although the patterns differed. For moths reared under summer conditions, an overall decline was only significant after
6h (Figure 3.4A; ANOVA, df, 3,49, F = 21.24, \( p < 0.001 \), Tukey HSD, \( p < 0.05 \)) and was more pronounced in females than males (Appendix A2.3; Unpaired t-test, df = 1,19, \( t = 9.15, p < 0.05 \)). For fall moths a significant change was observed after 4h (Figure 3.4B; ANOVA, df, 3,49, F = 21.63, \( p < 0.001 \), Tukey HSD, \( p < 0.05 \)), and, as with summer moths, the change was more evident in females than males (Appendix A2.4; Unpaired t-test, df = 1,19, \( t_4 = 3.84, t_6 = 4.72, p < 0.05 \)). In addition, the proportional increase in larval diet derived FAs due to increased loss of adult diet derived FAs was greater in fall-reared moths (Appendix A2.5) compared to summer-reared moths (Appendix A2.6) after 6 hours of flight.
Figure 3.4 $\delta^{13}$C values of the fat body lipid from 5-day old true armyworm moths after different periods of force flight when reared under (A) summer or (B) fall conditions. Significant differences in isotopic values as a function of flight time are indicated by different letters (Tukey HSD, $p < 0.05$). $N = 10$ per sample group ($N = 20$ in 0 hours cohort).
The total concentration of FAs declined as a function of flight duration in both summer (Table 3.2; ANOVA, df, 3,49, $F = 10.32$, $p < 0.001$, Tukey HSD, $p < 0.05$) and fall (Table 3.3; ANOVA, df, 3,49, $F = 10.26$, $p < 0.001$, Tukey HSD, $p < 0.05$) reared 5-day old moths. In summer moths, the decline in NFA was due to decreases in the concentrations of palmitic acid and OA, and both EFAs (Table 3.2; ANOVA, df, 3,49, $F_{PA} = 8.41$ $F_{OL} = 6.30$ $F_{LA} = 5.71$ $F_{ALA} = 4.91$; $p < 0.05$, Tukey HSD, $p < 0.05$, for all). In all cases, the significant declines in FA concentration were observed after 4h of sustained flight (Tukey HSD, $p < 0.05$). In fall-reared moths a significant decline in total FAs was detected after 6h of sustained flight (Tukey HSD, $p < 0.05$), due to lower levels of all three NFA but with no change in the concentration of the two EFAs (Table 3.3; ANOVA, df, 3,49, $F_{PA} = 10.33$, $F_{ST} = 7.65$, $F_{OL} = 9.86$, $p < 0.01$, Tukey HSD, $p < 0.05$; $F_{LA} = 0.67$, $F_{ALA} = 1.80$, $p_{LA} = 0.67$, $p_{ALA} = 0.31$).

Table 3.2 Change in the concentration (± SE) of essential (bold) and nonessential fatty acids in the fat body of 5-day old true armyworm moths as a function of flight duration under summer conditions. Significant differences within each column for individual and total fatty acids are indicated by different letters (Tukey HSD, $p<0.05$). $N= 10$ per sample group ($N=20$ in no flight).

<table>
<thead>
<tr>
<th>Flight</th>
<th>Palmitic</th>
<th>Stearic</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Alpha Linolenic</th>
<th>Total FA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μmol/ml)</td>
<td>(μmol/ml)</td>
<td>(μmol/ml)</td>
<td>(μmol/ml)</td>
<td>(μmol/ml)</td>
<td>(μmol/ml)</td>
</tr>
<tr>
<td>No Flight</td>
<td>84.1 ± 5.8(a)</td>
<td>8.4 ± 0.9[a]</td>
<td>159.9 ± 10.8[a]</td>
<td>3.8 ± 0.5[a]</td>
<td>1.5 ± 0.1[a]</td>
<td>256.8 ± 17.4[a]</td>
</tr>
<tr>
<td>1 Hour Flight</td>
<td>85.9 ± 3.5(a)</td>
<td>8.4 ± 0.5[a]</td>
<td>142.6 ± 8.1[a]</td>
<td>3.4 ± 0.2[a]</td>
<td>1.3 ± 0.1[a]</td>
<td>243.4 ± 11.1[a]</td>
</tr>
<tr>
<td>4 hour Flight</td>
<td>74.2 ± 2.2(b)</td>
<td>7.8 ± 0.3[a]</td>
<td>110.9 ± 6.3[b]</td>
<td>2.8 ± 0.1[b]</td>
<td>1.1 ± 0.05[b]</td>
<td>204.7 ± 9.01[b]</td>
</tr>
<tr>
<td>6 hour Flight</td>
<td>68.3 ± 2.6(c)</td>
<td>7.7 ± 0.2[a]</td>
<td>86.7 ± 2.5[c]</td>
<td>2.4 ± 0.1[c]</td>
<td>1.1 ± 0.06[b]</td>
<td>170.1 ± 4.8[c]</td>
</tr>
</tbody>
</table>
Table 3.3 Change in the concentration (± SE) of essential (bold) and nonessential fatty acids in the fat body of 5-day old true armyworm moths as a function of flight duration under fall conditions. Significant differences within each column for individual and total fatty acids are indicated by different letters (Tukey HSD, *p*<0.05). *N* = 10 per sample group (*N* = 20 in no flight).

<table>
<thead>
<tr>
<th>Flight</th>
<th>Palmitic (μmol/ml)</th>
<th>Stearic (μmol/ml)</th>
<th>Oleic (μmol/ml)</th>
<th>Linoleic (μmol/ml)</th>
<th>Alpha Linolenic (μmol/ml)</th>
<th>Total FA (μmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Flight</td>
<td>108.1 ± 6.4 (a)</td>
<td>8.8 ± 0.3 (a)</td>
<td>170.9 ± 11.2 (b)</td>
<td>4.5 ± 0.2 (a)</td>
<td>1.5 ± 0.1 (a)</td>
<td>300.3 ± 18.6 (a)</td>
</tr>
<tr>
<td>1 Hour Flight</td>
<td>105.1 ± 3.5 (a)</td>
<td>9.2 ± 0.8 (a)</td>
<td>155.3 ± 5.9 (a)</td>
<td>4.4 ± 0.5 (a)</td>
<td>1.6 ± 0.1 (a)</td>
<td>291.3 ± 8.4 (a)</td>
</tr>
<tr>
<td>4 hour Flight</td>
<td>108.9 ± 3.3 (a)</td>
<td>8.8 ± 0.2 (a)</td>
<td>154.0 ± 2.5 (a)</td>
<td>4.8 ± 0.3 (a)</td>
<td>1.6 ± 0.1 (a)</td>
<td>288.5 ± 6.6 (a)</td>
</tr>
<tr>
<td>6 hour Flight</td>
<td>97.0 ± 1.4 (b)</td>
<td>8.3 ± 0.2 (b)</td>
<td>138.5 ± 3.0 (c)</td>
<td>4.5 ± 0.4 (c)</td>
<td>1.6 ± 0.1 (b)</td>
<td>254.9 ± 4.8 (b)</td>
</tr>
</tbody>
</table>

During multiple days of flight, the concentration of total FAs increased after the first day of flight and remained stable when fall moths were fed *ad lib*. This was due to an increase in OL, as the concentrations of the other NFAs and EFAs did not change significantly (Figures 3.5A, 3.6A; ANOVA, df, 4.49, *F*<sub>PA</sub> = 0.96, *F*<sub>ST</sub> = 0.97, *F*<sub>LA</sub> = 0.51, *F*<sub>ALA</sub> = 0.49, *p*<sub>PA</sub> = 0.42, *p*<sub>ST</sub> = 0.41 *p*<sub>LA</sub> = 0.49, *p*<sub>ALA</sub> = 0.51; ANOVA, df, 4.49, *F*<sub>total</sub> = 4.76, *F*<sub>OL</sub> = 5.71, *p* < 0.05, Tukey HSD, *p* < 0.05). In contrast, when moths were only provided water during the rest periods there was an overall decline in the total concentration of FAs. This was due to lower levels of all NFAs and EFAs, with most significant differences being observed after 4 days of forced flight (Figures 3.5B, 3.6B; ANOVA, df, 5.69 *F* = 4.75, *F*<sub>PA</sub> = 6.86, *F*<sub>ST</sub> = 2.53, *F*<sub>OL</sub> = 5.15, *F*<sub>LA</sub> = 3.72 *F*<sub>ALA</sub> = 4.45, *p* < 0.05, Tukey HSD, *p* < 0.05). However, if moths were provided *ad lib* sugar water after the third night of flight, the levels of total FAs remained stable on days 8 and 9 (Figures 3.5C, 3.6C; ANOVA, df, 2.29 *F* = 1.73, *p* = 0.33).
Figure 3.5 Change in the concentration (± SE) of non-essential [palmitic (PA), oleic (OL) and stearic (ST)] fatty acids in fat body of true armyworm moths reared under fall conditions as a function of age and of the number of 8-h flight periods when fed
(A) sugar water *ad lib* daily, (B) provided water only, or (C) provided water on all days except at the end of Day 7 when they were provided sugar water *ad lib* during the rest period. The two values for Day 5 represent samples taken from un-flown moths prior to the flight period and from moths after 8 h of flight. In the other days samples were taken at the end of the flight period. Significant differences within each individual fatty acid are indicated by different letters (Tukey HSD, *p* < 0.05). *N* = 10 per sample group (*N* = 20 in Day 5 no flight).
Figure 3.6 Change in the concentration (± SE) of essential [linoleic (LA) and alpha linolenic (ALA)] fatty acids in the fat body of true armyworm moths reared under fall conditions as a function of age and the number of 8-h flight periods when fed (A) sugar water *ad lib* daily, (B) provided water only, or (C) provided water on all days except at the end of the flight period on Day 7 when they were provided sugar
water ad lib during the rest period. The two values for Day 5 represent samples taken from un-flown moths prior to the flight period and from moths after 8 h of flight. In the other days samples were taken at the end of the flight period. Significant differences within each individual fatty acid are indicated by different letters (Tukey HSD, \( p < 0.05 \)). \( N = 10 \) per sample group (\( N = 20 \) in Day 5 no flight).
3.4 Discussion

Anparasan et al. (2021) reported that non-flown TAW moths accumulated stored lipids under summer like conditions and our current results show this occurs regardless of rearing conditions. This is driven by a rapid accumulation of adult-diet derived NFAs, reflected in the rapid rise in palmitic acid and oleic acid (approximately three times the amount found at emergence; Table 3.1) and the change in δ^{13}C values of the fat body (Figure 3.3). Concentrations of the EFAs in the fat body declined slightly between 0 and 5 days in non-flown individuals under all rearing conditions (Table 3.1) but probably for different reasons. Under summer conditions, most males and females are sexually mature at 5 days post-emergence (Delisle and McNeil, 1987; Cusson and McNeil, 1989; Dumont and McNeil, 1992) and thus some EFAs would be incorporated in different components of the reproductive systems, as reported in other Lepidoptera (Martin, 1969; Arrese and Soulages, 2010). In contrast, under fall conditions that initiate migratory behavior, adults take several weeks to become sexually mature (Delisle and McNeil, 1987; Cusson and McNeil, 1989; Dumont and McNeil, 1992). There is little or no development of the reproductive systems on day five under fall conditions, however as EFAs linoleic acid and alpha-linolenic acid are found at high levels in the thorax muscles of other migratory insects (e.g., Turunen, 1974) the observed decline may be the result of their incorporation in flight muscle.

The decline in the NFAs oleic acid and palmitic acid, as well as the δ^{13}C value of the fat body, as a function of flight duration in 5-day old moths supports the idea that adult-derived FAs are preferentially used by the true armyworm to fuel flight, as reported for other insects (Schneider and Dorn, 1994; Wang and Ouyang, 1995; Murata and Tojo, 2002; Sakamoto et al., 2004; Levin et al., 2017). Furthermore, the fact that following flight the levels of linoleic acid and alpha-linolenic acid in the fat body decline in moths reared under summer conditions but not in those reared under fall conditions (Tables 3.2, 3.3) support our hypothesis of differential FA allocation under different environmental conditions. As these EFAs are important for reproduction (Martin, 1969; Arrese and Soulages, 2010), the fact that they are conserved under the environmental conditions stimulating the onset of migration would ensure more are available once a suitable habitat is located and could reduce the costs of migration on future reproductive success.
The difference in the time taken to see a significant decline in FAs and δ^{13}C value of the fat body in summer- and fall-reared 5-day-old moths as a function of flight duration (Tables 3.2, 3.3; Figure 3.4) may reflect physiological differences related to fuel-use efficiency arising from differences in the physiology of the two seasonal morphs. These could include different levels of muscle FA binding proteins (Haunerland, 1997) and/or increased levels of and responsiveness to lipophorin molecules (Chino et al., 1992). Furthermore, insects reared under migratory conditions may have more developed flight muscles (Boggs, 2009) and/or differential wing loading (Roff and Fairbairn, 1991). The decline in the linoleic acid and alpha-linolenic acid in the fat body of force-flown moths reared under summer conditions may be the result of their mobilization to flight muscles (see above). Under both rearing conditions, there was no significant decline in lipid levels during the first hour of flight and this was likely related to the initial phase of flight being fueled by carbohydrates, as reported in another migratory noctuid, *Agrotis ipsilon* (Sappington et al., 1995). The release of lipids from the fat body of the armyworm is modulated by adipokinetic hormone (AKH) and previous work has shown that it takes about an hour of forced flight for lipid levels to stabilize in the haemolymph (Orchard et al., 1991), a pattern also reported in the tobacco hornworm (*Manduca sexta*; Arrese and Wells, 1997).

As noted, the reduction in the EFAs in summer- but not fall-reared moths supports our hypothesis that physiological changes in response to environmental cues associated with onset of migratory behavior help conserve EFAs during migration. Schneider and Dorn (1994) suggested this may be the case in gregarious migratory locusts and proposed several possible mechanisms to explain such an adaptation. One included the involvement of juvenile hormone (JH), as higher titers may reduce fat body lipid storage in locusts. Previous research on the armyworm has shown that there are lower JH titers in moths reared under fall conditions resulting in the delayed development of the reproductive organs, as well as the production of, and response to, the female sex pheromone (Delisle and McNeil, 1987; Cusson and McNeil, 1989; Dumont and McNeil, 1992). However, additional research is required to determine if JH plays a role in the conservation of EFAs during migration.
In addition to JH, various forms of AKH have been shown to mobilize FAs differentially which may also be a mechanism by which resource limited TAW moths are allocating FAs. The migratory morph of *Locusta migratoria* expresses high levels of AKH II which mobilizes more oleic acid and saturated fatty acids compared to other AKHs (Tomcala *et al.*, 2010). TAW moths may produce similar AKH homologues and merits investigation as the previous study on AKH in TAW did not explore the possible role of different forms of AKH (Orchard *et al.*, 1991).

The results obtained when fall-reared moths were flown for multiple days indicate that EFAs are conserved over prolonged flight durations if there is access to suitable resources. Interestingly, moths flown for only one flight period displayed a sharp decline in oleic acid levels compared with those of non-flown individuals of the same age, while insects fed after each subsequent flight period had oleic acid levels that were similar to the non-flown controls. This is undoubtedly due to adults increasing the amount of resources consumed following forced flight, possibly the result of increased levels of JH, which can affect feeding behaviour (Rankin, 1991; Dingle and Winchell, 1997). Min *et al.* (2004) reported a post flight increase in JH titers for migratory grasshoppers, and Cusson *et al.* (1990) reported increased JH production when TAW adults experienced an increase in ambient temperature. Thus, if increasing body temperature following flight resulted in higher JH levels and led to higher consumption of resources, it would result in greater changes in levels of oleic acid compared with the other FAs, as it is one of the most readily used fuel sources in insects (Schneider and Dorn, 1994; Wang and Ouyang, 1995; Tomcala *et al.*, 2010).

In the absence of resources during multiple days of flight, the concentration of EFAs in the fat body decline significantly (Figure 3.6). However, when water fed moths were provided one *ad lib* meal of sugar water after 3 days of flight they only used NFAs when force flown for two additional days. This shows they only used EFAs when there was no other option and underlines the importance of nectar availability at stopover sites during the migratory process, as any shortages could significantly impact individual reproductive success and whether populations subsequently reach pest densities. Nectar availability could vary significantly at different sites along the migratory path due to different environmental
conditions. For example, extreme weather events associated with climate change could not only affect the density of available flowers but also the quantity and quality of nectar (Takkis *et al*., 2018; Descamps *et al*., 2021). Furthermore, under hot, dry conditions the viscosity of nectar will increase, and many lepidopterans are less effective at acquiring resources when nectar concentrations are high (Pivnick and McNeil, 1985).

We have shown that if adults have access to nectar resources during migration, they are able to conserve the EFAs found in the fat body. However, if some are utilized to sustain flight there is potential for negative impacts on future reproduction as EFAs are a substantial component to the eggs of many lepidopterans (Martin, 1969; Forte *et al*., 2002) and thus a decline in the availability of these FAs can reduce the number of viable offspring. This possibility needs further investigation but when quantifying such potential impacts, one also needs to consider other EFA sources that adults might access following migration. For example, bark beetles break down their flight muscles post migration in response to an increase in JH titers (Borden and Slater, 1968; Sahota and Farris, 1980) and these authors have suggested that these resources are invested in reproduction. As noted above there is a significant increase in JH associated with the onset of reproduction in the true armyworm, and it is possible that this change in titer may stimulate post migratory release of EFA resources from flight muscle. However, unlike bark beetles which do not resume flight after colonizing a tree host, the armyworm and other lepidopterans need functional flight muscles for foraging and reproduction throughout adult life, thus obtaining resources through flight muscle degradation may be limited. Secondly, females of some migratory species are polyandrous (Torres-Vila *et al*., 2004) and thus obtaining several spermatophores from repeated mating could offer them an alternative source of resources. Both eggs and female somatic tissue contain male-derived resources (Boggs and Gilbert, 1979; Boggs, 1981; Wiklund *et al*., 1993), and males of some migratory species transfer JH at the time of mating (e.g., Park *et al*., 1998). As *M. unipuncta* is polyandrous, the spermatophores are potential nutrient sources (Marshall and McNeil, 1989) and repeated mating increases female reproductive output (Svärd and McNeil, 1994), so the potential importance of male derived EFAs merits further attention.
Our study demonstrates the power of using bulk isotopic tracing to examine the evolution of lipid allocation in migrant insects, particularly for insects such as the TAW where larval and adult diets are distinct. The use of carbon isoscape, especially a compound-specific approach using $\delta^{13}\text{C}$ in individual EFAs and NFAs (Whiteman et al., 2019; Pilecky et al., 2022) would allow for a greater understanding about nectaring stopover sites and sources of vital nutrients for migrant species in the wild. In fact, using a similar design setup to this experiment, tracing of isotopically labeled EFAs and NFAs could be used (as seen in studies on amino acids, e.g., Levin et al., 2017) to determine the fate, isotopic changes and confirmed source of FAs from the fat body, as well as the subsequent allocation to reproduction after flight. Clearly, future researchers will need to address all of these possibilities as this will not only contribute to our understanding of basic flight energetics but also how energy use would affect the population of migrants whether they are pests or ones, like the monarch butterfly (*Danaus plexippus*), that we wish to protect.
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Chapter 4

4 Cost of essential fatty acid loss during flight on egg production and fatty acid composition in virgin *Mythimna unipuncta* females and potential post-mating male contributions

4.1 Introduction

Migration, a key component of the life history of many insect species, occurs in response to potential changes in resource availability and habitat quality that are perceived through proximal cues such as photoperiod and temperature (Saunders, 1987; Dixon *et al*., 1993; Dingle, 1972, 2014; Tauber *et al*., 1985). Flight is one the most energetically expensive modes of transport and insect flight muscles are among the most metabolically active of tissues known (Dudley, 2000) with metabolic rates 20–100 times higher during flight than at rest (Ellington, 1985). The cost of developing flight related apparatus has been well documented in wing polymorphic insects (Penner, 1985; Roff, 1986; Guerra, 2011). When comparing winged and wingless morphs the costs may be reflected in longer developmental times (Honek, 1985; Roff and Fairbairn, 2007), reduced mating success and/or fecundity (Penner, 1985; Roff, 1986; Denno *et al*., 1989; Rankin and Burchsted, 1992; Mole and Zera, 1993; Bradshaw *et al*., 1998; Langellotto *et al*., 2000; Saglam *et al*., 2008; An *et al*., 2012; Bonte *et al*., 2012). Thus, there are trade-offs in nutrient allocation between migration and reproduction.

Given the cost of migratory flight (Rankin and Burchsted, 1992; Menz *et al*., 2019) and the potential impact on subsequent reproduction (Dudley, 1995; Dingle, 2014) one would expect that, during the migratory phase, there are adaptations that minimize flight costs. These could include changes in wing shape and wing loading (Dockx, 2007; Altizer and Davis, 2010; Slager and Malcolm, 2015), as well as physiological changes that increase flight muscle efficiency (Haunerland *et al*., 1992; Zhan *et al*., 2014; Jones *et al*., 2015). It has also been noted that adults of most species are sexually immature at the onset of migration so if migratory flight utilizes the same resources required for later reproduction, the cost of the potential trade-off between nutrient allocation used for migration and
reproduction will be determined by the degree to which said resources can be replaced once the migratory phase has terminated (Blanckenhorn et al., 1995; Zera and Brink, 2000). One example would be the use of fatty acids (FAs) by migratory Lepidoptera, as these are important both for flight (Beenakkers et al., 1985; Van der Horst et al., 2002) and different aspects of reproduction (Stanley-Samuelson et al., 1988; Forte et al., 2002). However, as the adults are nectivorous some FAs, termed essential fatty acids (EFAs), are only obtained during the larval stage as nectar contains few or no FAs (Nicolson et al., 2007; Krenn, 2010), so the degree to which EFAs are used to sustain migratory flight will determine the potential impact on subsequent reproductive output. Anparasan et al. (2023) (Chapter 3 of this thesis) recently examined EFA use by adults of true armyworm, *Mythimna unipuncta*, that were reared under different ecological conditions. They chose the true armyworm as it is a seasonal migrant (McNeil, 1987; Hobson et al., 2018) that cannot overwinter in the summer breeding grounds (Guppy, 1961; Fields and McNeil, 1984). A comparison of the North American (migrant) and Azorean (nonmigrant) populations of TAW moths indicate migratory flight comes at a cost to overall reproduction (McNeil, 2011). Anparasan et al. (2023) found that adults reared under summer conditions used both nonessential FAs (NFAs) and EFAs from the fat body when force flown for 6h while those reared under fall conditions conserved the EFAs. Furthermore, fall adults flown for 8h on five consecutive days conserved the EFAs in the fat body, if provided sugar water each day. However, if they were deprived of an adult energy source, which could occur during migration, they did use EFAs from the fat body.

As the majority of lipids incorporated into eggs are transported from the fat body (Ziegler, 1997; Ziegler and Van Antwerpen, 2006; Arrese and Soulages, 2010), the ability to conserve EFAs by fall migrant female TAW moths may be an adaptation to reduce the flight-reproduction trade-off. However, as seen in Anparasan et al. (2023), the ability to conserve EFAs during multiple days of flight is contingent on diet availability. Therefore, we conducted experiments to determine if multiple days of flight with differing diet availability under fall conditions would impact virgin female egg production when transferred to summer conditions. This was quantified by assessing the number of mature eggs and the FA composition of eggs. In addition, as Anparasan et al. (2023) found male and female TAW conserved EFAs, and as males can provide resources that could increase
female reproductive output (Friedel and Gillott, 1977; Engebretson and Mason, 1980; Bowen et al., 1984; Pivnick and McNeil, 1985), we examined if male derived EFAs are found in eggs of mated females.

4.2 Methods and methodology

4.2.1 Insect colony

All individuals came from a colony established in 2022, using adults collected from light and pheromone traps (a minimum of 30 males and females captured during the spring immigrant flight period) at the Environmental Sciences Western (ESW) Field Station (43.07°N, 81.34°W). The colony was maintained at 25 °C, 16:8D, 65±5 % RH, with larvae being reared individually on a pinto bean diet (Shorey and Hale, 1965) while adults were provided an ad lib supply of 8 % cane sugar solution. The adults used for all experiments had not been in rearing for more than two generations. Insects used for the flight experiment were reared as larvae on pinto bean diet under natural conditions in an insectary during August/September 2022 as these conditions induce migratory behaviour and delay reproductive development (Turgeon and McNeil, 1983; Delisle and McNeil, 1987; Cusson et al., 1990; Dumont and McNeil, 1992). The insects used for the male donation experiments were maintained under standard colony rearing conditions.

4.2.2 Female flight experiment

Forty-five female pupae were held in the insectary and upon emergence adults were held for five days in individual cylindrical containers with mesh tops (height: 10 cm, diameter: 4 cm) and provided 25 % cane sugar water daily ad lib. Experimental insects were fed a sugar water diet higher in concentration compared to standard colony to increase lipid accumulation (Benoit, 2017). On Day 5 post-eclosion, scales on the dorsal surface of the thorax were removed and a 3cm piece of clear plastic tubing attached using EVO STIK (Stafford, United Kingdom) instant contact adhesive. Each moth was then suspended from a copper wire attached to a wooden stationary base via the thoracic tether and force flown for the required period of time (following methods outlined in Anparasan et al., 2023). If a moth stopped flying, its back legs were gently touched but if it did not resume flight after three such stimulations it was not included in subsequent analyses. Insects were flown for
5 days (8 hrs/day), with 15 fed cane sugar solution during non-flight rest periods while the other 15 were only provided water. The remaining 15 were fed the 25% cane sugar water \textit{ad lib} during the five-day period but were not flown. After the five-day flight period, all females were held under colony rearing condition with 25 % cane sugar solution provided \textit{ad lib} for 6 days, then sacrificed and stored in -80 °C until further analysis. The entire reproductive tract was removed from 10 females in each treatment, then placed in Grenacher Borax Carmen red solution (Humason, 1972) for 20 min. After this duration the reproductive tract was rinsed twice in saline solution to remove excess stain. The entire reproductive tract was viewed under a dissection microscope (x4 magnification) and the number of chorionated eggs counted. Chorionated (i.e., mature) eggs did not pick up any stain and remained white and un-chorionated eggs had a pink hue (Cusson and McNeil, 1989). The remaining females in each treatment group were used for FA composition analysis (described below). The experimental design is summarized below (Figure 4.1)
Figure 4.1 Experimental design for virgin female armyworm moth egg production in relation to flight and feeding status. Green arrows indicate time spent under fall conditions while red arrows indicate time spent in summer conditions.
4.2.3 Lipid extraction and gas chromatography

A sample of chorionated and immature eggs (~50 mg per female, approximately 555 eggs Appendix A3.1) were dissected from each of the remaining 5 females in each treatment, placed in test tubes with a 2:1 chloroform:methanol solution (Folch et al., 1957) containing 0.01% butylated hydroxytoluene (BHT) and ruptured by agitating with a small scoopula. Samples were left for 5 minutes to extract lipid. The extractions were done under N₂ to reduce oxidation of FAs. Twenty µL of an internal standard reference, margaric acid (17:0, 3 mg/mL), was then added to this mixture. One mL of 0.25 % KCl was then added to the solution to separate out aqueous solutes and allowed to react in a 70 °C hot water bath for 5 minutes. The whole solution was then filtered and dried under N₂. The remaining FAs (~100 µg) were then converted into fatty acid methyl esters by adding 200 µL of 0.5 M methanolic hydrogen chloride and reacting at 90 °C for 30 minutes. The solution was then reduced to dryness under nitrogen, resuspended in hexane (100 µL). These samples were subsequently analysed using chromatographic techniques.

Analysis was performed in a gas chromatography/flame ionization detector (Agilent Technologies® 6890N G1530N, Santa Clara, USA) equipped with a DB23 column (Agilent DB23 122-2332, Santa Clara, USA). The injector temperature was 250 °C and the flame ionization detector temperature was 280 °C. During each run, a 100 µL dichloromethane control was also analysed. The retention times of PUFA standards (Supelco® PUFA and 37 component) were averaged to create a library of known fatty acid peaks. The distinct and clear peaks of each sample chromatograph were compared to the known retention times to identify fatty acids. This, combined with the known concentration of the internal standard, was used to calculate the concentration of each fatty acid (µmol/mL) in the 100 µL aliquot of FAs and hexane that was analyzed where all samples contained a total lipid mass of 100 µg. I quantified the EFAs alpha linolenic acid (ALA) and linoleic (LA) as they cannot be de novo synthesized by Lepidopterans but are essential for development (Canavoso et al., 2001), and the NFAs oleic (OL), palmitic (PA), and stearic acid (ST), as they are found in high levels in Lepidoptera (Subramanyam and Cutkomp, 1987; Canavoso et al., 2001).
4.2.4 Male donations experiment

All male and female insects were reared under colony conditions (25 °C and 16L:8D photoperiod), with the scotophase beginning at 9:00 am and ending at 5:00 pm. Larvae were either reared on the normal C3 pinto bean diet or on the same C3 diet spiked with 7.15 mg of $^{13}$C labelled linoleic acid (99 atom %, Sigma-Aldrich) (Appendix, A3.2). The target atom % enrichment of the linoleic acid in the larval spiked diet was 2 % (Appendix, A3.3). This spiked linoleic acid diet is isotopically distinct from the control diet and thus the isotopic value of the fat body of insects feeding from these diets would differ, reflecting the isotopic difference in linoleic acid. We aimed to have at pupation at least 40 females reared on the control diet, 40 males reared on the control diet and 40 males reared on the EFA spiked diet. Pupae were sexed and held in individual containers (as described above) and the resulting adults then held for three days with an ad lib supply of 25 % cane sugar diet. On Day 4 (average time for reaching sexual maturity; Turgeon and McNeil, 1982) cages were set up with two females and four males (either all EFA spiked or control males) and an ad lib supply of 25% cane sugar water. Observations for mating were made every 10 minutes during scotophase using a flashlight covered with a Kodak red Wratten #29 filter (Turgeon and McNeil, 1982). Whenever a mating pair was observed they were immediately isolated by placing a clear cylindrical container (height: 10 cm, diameter: 4 cm) over them.

Once the insects separated (on average ~60-90 minutes from the start of mating) 10 females from each of the two treatments were immediately frozen at -80 °C. Females were thawed and spermatophores dissected out of the bursa copulatrix and immediately used for sample preparation. Five spermatophores from the control and spiked treatments were prepared and analysed using GC, as described above. The other five spermatophores from each treatment group were processed for stable isotopic analysis (see below). The experimental design for the mating experiment is summarized below (Figure 4.2)
Figure 4.2 Simplified experimental design for reproductive mating experiment in summer-reared armyworm moths. Treatment males were fed a larval diet with 99 atom % $^{13}$C linoleic acid (LA) and so are referred to as spiked. An asterisk (*) indicates that chromatographic analysis was only performed on spermatophores.
Following mating, the remaining 10 females from each treatment were held in smaller cages with sugar water and an oviposition site (folded wax paper) that was replaced every day for six days. Sexually mature females would have no more than ~200 chorionated eggs at the time of mating (Cusson and McNeil, 1989) but over the first five days post-mating lay more than 1500 (Svard and McNeil, 1994) thus this time period for oviposited egg collection allowed us to detect any male donations integrated into newly forming eggs. Due to sample size requirements, eggs from females in each treatment were grouped together by day of oviposition (1-6 days) for isotopic analysis (see below).

4.2.5 Stable isotopic analysis

Spermatophore were freeze dried via Drywinner DW3 benchtop freeze dryer (Heto, Allerod Denmark) transferred to tin capsules (8 x 5 mm), weighed and crushed. For egg samples prior to isotopic analysis eggs were ruptured, soaked in a 2:1 chloroform:methanol solution (Folch et al., 1957) for 24 h, and the lipids recovered following evaporation in a fume hood for >24 hours (23 °C, 101.3 kPa) were frozen. Then 0.91-0.99 mg samples of the extracted lipids from each egg batch were transferred to tin capsules (8 x 5 mm), weighed and crushed. All samples were combusted at 1000 °C in a Carlo-Erba NC2500 Elemental Analyser (Carlo Erba, Italy) and CO₂ gas transferred via a ConFlo IV (Thermo Scientific, Bremen Germany) device to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Scientific, Bremen Germany). Data were normalized using internal calibrated lab standards (Cayuga brown trout: -25.58 ‰ and corn: -13.02 ‰); instrument linearity was assessed using in-house methionine (-27.2 ‰) and ground deer hair (-20.1 ‰) standards. All values are reported in standard delta (δ) notation relative to the Vienna Pee Dee Belemnite (VPDB) standard in parts per thousand (‰). Measurement error based on within-run standards was estimated as ± 0.1‰. All isotopic measurements were performed at the Cornell Isotope Laboratory (COIL).

4.2.6 Statistical analysis

All statistical tests were performed using R Studio (Version 3.4.2 (2017-09-28)). Single factor one-way ANOVAs and Tukey’s post-hoc analyses were used to compare: (i) specific FA and total FA concentrations in eggs based on flight and feeding status (i.e.
fed and flown, fasted and flown, and control non flown) and (ii) the number of chorionated eggs in virgin females based on flight and feeding status (i.e. fed and flown, fasted and flown, and control non flown). An unpaired t-test was used to compare the differences in FA patterns or $\delta^{13}$C values in EFA spiked and control spermatophore. The $\delta^{13}$C values of eggs laid by females mated were analysed using a two factor ANOVA for treatment of male and day oviposited. Normality of data was confirmed using Skew (-1 to +1) and Kurtosis (-4 to +4) analyses.

4.3 Results
4.3.1 Virgin female egg production

Females that were provided sugar water during the five day fall flight period (fed) had significantly more chorionated eggs in the reproductive tract after 6 days under laboratory conditions (to allow for sexual development) that those that had only been provided water (fasted) (Figure 4.3; ANOVA, df, 2.29, $F_{egg} = 6.356, p_{egg} <0.01$; Tukey HSD $p <0.01$), while the non-flown but fed controls did not differ from either of the flown groups (fasted (Tukey HSD, $p= 0.293$) or fed (Tukey HSD, $p=1.29$)).
Figure 4.3 Number of chorionated eggs in virgin TAW moths transferred from fall to summer conditions after being not flown and fed (Control), flown and fasted (Fasted) and flown and fed (Fed) for 5-days. Significant differences among groups are indicated by different letters (Tukey’s HSD, p<0.05). N=10 per treatment group.
The concentrations of total FAs measured did not differ in eggs from different treatments (Table 4.1: ANOVA, df, 2,14, $F_{total} = 0.286, P_{total} = 0.756$), nor for any specific FA: oleic acid (ANOVA, df, 2,14, $F_{OL} = 0.92, p_{OL} = 0.425$), palmitic acid (ANOVA, df, 2,14, $F_{PA} = 0.244, p_{PA} = 0.787$), stearic acid (ANOVA, df, 2,14, $F_{ST} = 0.125, p_{ST} = 0.884$), linoleic acid (ANOVA, df, 2,14, $F_{LA} = 0.154, p_{LA} = 0.859$) and alpha-linolenic acid (ANOVA, df, 2,14, $F_{ALA} = 0.342, p_{ALA} = 0.717$).

Table 4.1 Table 1. FA composition (±SE) of chorionated eggs in virgin TAW moths transferred from fall to summer conditions after being not flown and fed (Control), flown and fasted (Fasted) and flown and fed (Fed) for 5-days. Significant differences within each row for individual and total fatty acids are indicated by different letters (Tukey’s HSD, $p<0.05$). N= 5 per treatment group.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Unfertilized eggs (Control) (µmol/ml)</th>
<th>Unfertilized eggs (Fasted) (µmol/ml)</th>
<th>Unfertilized eggs (Fed) (µmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>77.1 ± 1.7(a)</td>
<td>74.4 ± 3.4(a)</td>
<td>76.1 ± 2.8(a)</td>
</tr>
<tr>
<td>Stearic</td>
<td>7.0 ± 0.5(a)</td>
<td>6.6 ± 0.6(a)</td>
<td>6.8 ± 0.4(a)</td>
</tr>
<tr>
<td>Oleic</td>
<td>101.0 ± 2.9(a)</td>
<td>97.9 ± 5.3(a)</td>
<td>106.1 ± 4.2(a)</td>
</tr>
<tr>
<td>Linoleic</td>
<td>8.3 ± 0.5(a)</td>
<td>7.9 ± 0.7(a)</td>
<td>8.4 ± 0.6(a)</td>
</tr>
<tr>
<td>Alpha linolenic</td>
<td>4.4 ± 0.2(a)</td>
<td>4.0 ± 0.7(a)</td>
<td>4.6 ± 0.7(a)</td>
</tr>
<tr>
<td>Total FA</td>
<td>201.9 ± 5.0(a)</td>
<td>196.6 ± 10.3(a)</td>
<td>205.6 ± 8.7(a)</td>
</tr>
</tbody>
</table>

4.3.2 Spermatophore

Spermatophore from males reared on EFA spiked larval diet had a higher δ^{13}C isotopic value than those on regular diet (Figure 4.4; Unpaired t-test, df = 8, $t_{sperm} = 5.363, p_{sperm} <0.001$). The concentration of total FAs measured was <13 µmol/spermatophore, with EFAs representing 14.14 % ± 0.6, with no difference in the total FA concentration between treatments (Table 4.2, Unpaired t-test, df = 8, $t_{total} = 0.161, p_{total} = 0.438$). The same held true for concentrations of individual FAs; oleic acid (Unpaired t-test, df = 8, $t_{OL} = -0.256, p_{OL} = 0.402$), palmitic acid (Unpaired t-test, df = 8, $t_{PA} = -0.529, p_{PA} = 0.306$), stearic acid
(Unpaired t-test, df = 8, $t_{ST} = 0.102$, $p_{ST} = 0.461$), LA (Unpaired t-test, df = 8, $t_{LA} = 0.536$, $p_{LA} = 0.303$) or alpha-linolenic acid (Unpaired t-test, df = 8, $t_{ALA} = 0.013$, $p_{ALA} = 0.494$).
Figure 4.4 $\delta^{13}C$ value of spermatophore synthesized by male TAW moths fed on either a standard C3 pinto bean larval diet (Unspiked) or a C3 pinto bean larval diet supplemented with $^{13}C$ spiked linoleic acid (Spiked). (*) indicate differences between treatment groups (Unpaired t-test, p<0.05). N=5 per sample group.
Table 4.2 FA concentration (±SE) of spermatophore produced by male TAW moths fed on either a standard C3 pinto bean larval diet (Unspiked) or a C3 pinto bean larval diet supplemented with $^{13}$C spiked linoleic acid (LA) (Spiked). Significant differences within each row for individual and total fatty acids are indicated by different letters (Unpaired t-test, p<0.05). N=5 per sample group.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Spiked Spermatophore (μmol/ml)</th>
<th>Unspiked Spermatophore (μmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>2.6 ± 0.3$^{(a)}$</td>
<td>2.8 ± 0.2$^{(a)}$</td>
</tr>
<tr>
<td>Stearic</td>
<td>1.2 ± 0.1$^{(a)}$</td>
<td>1.2 ± 0.1$^{(a)}$</td>
</tr>
<tr>
<td>Oleic</td>
<td>6.4 ± 0.4$^{(a)}$</td>
<td>6.1 ± 0.9$^{(a)}$</td>
</tr>
<tr>
<td>Linoleic</td>
<td>1.2 ± 0.2$^{(a)}$</td>
<td>1 ± 0.2$^{(a)}$</td>
</tr>
<tr>
<td>Alpha linolenic</td>
<td>0.6 ± 0.09$^{(a)}$</td>
<td>0.6 ± 0.08$^{(a)}$</td>
</tr>
<tr>
<td>Total FA</td>
<td>12.4 ± 1.3$^{(a)}$</td>
<td>12.1 ± 1.4$^{(a)}$</td>
</tr>
</tbody>
</table>

4.3.3 Eggs of mated females

The $\delta^{13}$C value of eggs laid by females mated with EFA spiked males was more positive than those from females mated with control males across all days (Figure 4.5. ANOVA, df =1,59, $F_{mate}= 214.5$, $p_{mate} <0.001$). Furthermore, there was an increase in the isotopic value of the eggs as a function of day since mating regardless of treatment groups (ANOVA, df= 5, 59, $F_{day}= 65.2$, $p_{day}<0.001$). The difference was significant on Days 5-6 in the spiked mating group (Tukey HSD, $p <0.05$) and on Days 4-6 in the control group (Tukey HSD, $p <0.05$). There was also a significant treatment by day of oviposition interaction ($F_{mate+day}= 9.7$, $p_{mate+day} <0.01$).
Figure 4.5 δ^{13}C value of eggs oviposited over 6 days post-mating by female TAW moths mated with male TAW moths fed on either a standard C3 pinto bean larval diet (Unspiked) or a C3 pinto bean larval diet supplemented with ^{13}C spiked linoleic acid (Spiked). (*) indicate differences between treatment groups within each day (Tukey’s post-hoc analysis, p<0.05). N=5 per sample group.
4.4 Discussion

Both flight and diet limitation, either alone or combined, impact reproductive output of insects. Starvation alone can negatively impact egg production in many insects (Boggs and Ross, 1993; Xu et al., 2019; Zhang et al., 2019), while the timing and duration of flight alone can affect reproduction in migrant species. For example, Gunn et al. (1988) reported a low but significant difference in the fecundity of flown and non-flown Spodoptera exempta moths, where flown insects had a decrease in fecundity even though both groups were provided sucrose. Impacts on reproductive output due to flight was also seen by Zhang et al. (2015) when examining the effect of age and the duration of single or repeated bouts of flight on the onset of oviposition and fecundity of female Cnaphalocrocis medinalis. One day old females initiated oviposition at an earlier age after a single bout of 6 or 8 h flight compared to non-flown control females or those flying for 18 h. However, there was a decline in reproductive output for those flown 18 h. When females were flown repeatedly for 4 days there was a delay in the onset of reproduction and a lower lifetime fecundity compared to those flown for 1 or 2 days only. There have been a number of studies examining the combined effects of flight and starvation that have reported quite varied conclusions (e.g., Niitepõld, 2019). This is due to differences in the age at which insects were flown, the duration of single or repeated bouts, the timing and level of starvation and in the life histories of the insects studied.

In the case of the TAW moth, there was no effect of food availability during flight on either the total or proportional composition of the FAs quantified in the eggs produced by virgin females (Table 4.1, Figure 4.3), even though there was a loss of EFAs in the fat body of unfed females after 5 days of flight (Anparasan et al., 2023). This suggests that the composition of eggs is highly conserved to ensure appropriate development. For example, Forte et al. (2002) found a significant decrease in linoleic acid and alpha-linolenic acid in the later stage embryonic development in eggs of the codling moth (Cydia pomonella), most likely due to bioconversion to other EFAs, such as DHAs and EPA, which are important for brain and eye development (Stanley-Samuelson et al., 1988).

There was, however, a significant difference in the number of chorionated eggs in the ovaries of virgin females that had been fed or deprived of food during the flight period, as
reported in other species of noctuid (Willers et al., 1987; Gunn et al., 1988, Mason et al., 1989). However, as starved TAW females only had about 70 fewer eggs than their fed counterparts (180 ± 16 versus 110 ± 12) and one egg contains 0.07 µg of the linoleic acid and alpha-linolenic acid (Appendix A3.4) it is somewhat unlikely that the decline in egg number is related to the lack of EFA availability post-flight in virgin females given the levels of these EFAs in the fat body of unfed females after five days of forced flight (Anparasan et al., 2023) and the number of eggs virgin females can produce (Cusson and McNeil, 1989). However, as mated females lay significantly larger number of eggs (Svard and McNeil, 1994) if female moths are unable to recover EFAs, the loss of EFA during flight may impact egg production to a greater degree later on.

With regards to virgin female egg production when fed or fasted during flight, one must consider alternate explanations for the observed difference. Starvation results in a reduction of adult JH titers in insects from several Orders but once feeding resumes titers increase, although often with a delay of several days (Rankin and Riddiford, 1977; Tobe and Chapman, 1979; Weaver and Pratt, 1981; Perez-Hedo et al., 2014; Nouzova et al., 2018). A similar delay in JH production following starvation in the TAW would account for the difference observed, as it is very similar to the difference in the number of chorionated eggs reported between immature and sexually mature females of a similar age (Cusson and McNeil, 1989). This assumes there was very little ovarian development in either treatment during the five days of flight, something we did not verify in this experiment. However, data from another experiment carried out under natural conditions the previous fall support our assumption as we found no significant difference in the number of chorionated eggs present in starved and fed females flown for five days under fall conditions (Appendix A3.5).

However, if JH titers in previously starved individuals do not return to control levels, as reported in Schistocerca americana (Tobe and Chapman, 1979) there may be a long-term effect. Furthermore, in a closely related species, M. separata, the titers of JH I and JH II in moths starved on day one post emergence differed significantly from controls for several days, even after feeding had resumed (Zhang et al., 2008). As the TAW produces several homologues of JH, that vary as a function of age and differ between migrants and
nonmigrant populations (Cusson et al., 1990; McNeil et al., 1996, 2000) it is possible that long-term changes in the titers of these homologues could negatively impact lifetime fecundity. Conversely, as polyandry is prevalent in the TAW, as with many migratory insects (Oberhauser, 1989; Torres-Vila et al., 2004), donations from repeated mating could increase egg production (Svard and McNeil, 1994).

Mated TAW females have the potential to produce >1500 eggs (Svard and McNeil, 1994) and if the delay in the resumption of JH production was the only effect of starvation during extended flight, then one would not expect a negative impact on lifetime fecundity. This conclusion is particularly true if one considers the possibility that females could potentially recuperate some resources lost during migration through male donations, as seen in Euphydryas editha and Speyeria mormonia (Boggs, 1997). Our results show that male spermatophores contain EFAs (Table 4.2), interestingly at proportions higher than the abdominal fat body (Anparasan et al., 2023). Linoleic acid of male origin is also clearly integrated into eggs (Figure 4.3). Based on the results of Marshall and McNeil, (1989), an average spermatophore would contain about 300 µg of lipid, while an egg about 1.241µg (both values excluding the reported hydrocarbon values). Thus, based on our results, a spermatophore would have ~26.94 µg of linoleic acid and if none was used by the female for somatic maintenance this would be enough for at least 500 eggs.

Clearly, future research should examine the patterns of JH production in virgin females, including examining potential opposing effects of flight and starvation, given that Min et al. (2004) reported an increase in JH titers following long duration flight in the grasshopper, Melanoplus sanguinipes. The isotopic analyses of eggs produced over 6 days post mating showed a general increase in the δ^{13}C value eggs as a function of time (Figure 4.5), regardless of treatment group, indicating an increased use of adult derived (C4–based) resources acquired by females. This has also been reported in the hawk moth, Amphion floridensis, (O’Brien et al., 2000) and certainly merits further investigation. Detailed analysis of changes in JH titers and the composition of eggs should also be carried out throughout the life of mated females, in parallel with studies determining total fecundity. Additionally, as males contribute EFAs and other resource-based donations (Boggs and Gilbert, 1979; Boggs, 1981; Pivnick and McNeil, 1987; Wiklund et al., 1993) males could
also transfer JH directly (Park et al., 1998) and/or increase in vitro JH production by females (Shu et al., 1998) which should also be investigated in TAW moths. It will also be important to determine the effects of both flight and starvation on the number of lifetime mating events a male may acquire, as well as the spermatophore content at each mating. The data from these experiments would provide additional insight into flight reproduction trade-offs and the relative contribution of both sexes to reduce the costs associated with long distance migration.
4.5 Literature cited


Delisle, J. and McNeil, J. N. (1987). Calling behaviour and pheromone titre of the true armyworm *Pseudaletia unipuncta* (Haw.) (Lepidoptera: Noctuidae) under different


Chapter 5

5 General discussion

5.1 Effect of rearing conditions on FA use

The goal of my thesis was to explore if rearing conditions affect source and allocation of FAs used during flight in true armyworm (TAW) moths (*Mythimna unipuncta*) and monarch butterflies (*Danaus plexippus*). Both species were used as TAW moths and monarchs have similar life histories in the sense that both migrate but there are also clear differences that could influence how acquired fatty acids (FAs) are allocated during migration. Both insects have migratory morphs and a major characteristic present in migratory insects is reproductive diapause during the migratory phase or the delay of reproduction until after the migratory period (Angelo and Slansky, 1984; Zera and Bottsford, 2001; Ramenofsky and Wingfield, 2007). This delay in reproduction in favor of migration is associated with a trade-off between these two life history events (Penner 1985; Roff, 1986; Guerra, 2011). I hypothesized that rearing conditions could influence the pattern of FA use during flight in migrant wing-monomorphic lepidopterans. I predicted that adult- and larval-synthesized FAs (both nonessential (NFA) and essential (EFA)) would be used to fuel flight during summer but that EFAs (larval) would be more conserved (and thus not as readily used as fuel) in fall-reared insects due to their limited supply and importance in other physiological functions.

At emergence, adult insects of both species have EFAs and NFAs in the fat body, but monarchs emerge with relatively more of the selected EFAs. At 5-days post-eclosion TAW moths showed a slight decline in EFAs in all rearing conditions (fall, summer, laboratory) compared to the monarchs, where decline was only seen in the summer and laboratory reared insects. Palmitic acid (PA) and oleic acid (OL) were also accumulated in both insects post-eclosion when fed for 5 days. Summer and fall-rearing conditions did not differentially affect FA accumulation in either insect. Under fall conditions exercised TAW moths showed a clear pattern of EFA conservation during flight while monarchs displayed reduced EFA mobilization compared to that of NFAs in both conditions. TAW females have the ability to conserve the EFAs, potentially reducing the impact of EFA loss on
subsequent reproduction. Furthermore, male TAW moths also conserved EFAs thus male contributions of EFAs could further benefit female reproductive output as they were detected in eggs produced by mated females.

There was also a clear difference in the pattern of EFA and NFA proportional loss during exercise in fall-reared compared to summer-reared TAW moths, while monarchs exhibited less variation in FA use based on rearing conditions. However, in both insects there was a clear pattern of differential FA use in fall- versus summer-reared insects with a tendency of fall-reared insects to use EFAs less compared summer-reared insects. TAW moths and monarchs are both nectivorous cross-continental migrants and although their patterns of FA use varied, much of these differences correspond to various components of their life history and migratory strategy as I discuss below.

5.2 Comparative physiology and life history of TAW moths and monarchs in relation to FA profile and use

5.2.1 Role of larval diet and development

The insects were reared on different diets and since FA composition of emergent lepidopteran adults has been shown to reflect larval diets (e.g., Cookman et al., 1984), I expected initial FA composition differences to reflect this. I found that the monarch fat body had a large proportion of alpha linolenic acid (ALA) which was also seen in milkweed (Appendix A4.1). This was also seen in other studies that assessed FA profiles of emergent monarchs relative to the milkweed they were fed at the larval stage (Pilecky et al., 2022b). In contrast, fall-reared TAW moths did not have similar EFA proportions compared to their pinto bean diet (Appendix A4.1). Differences in the FA composition of adult insects at emergence can be caused by changes in FA composition during metamorphosis (Chamberlin, 2004; Wang et al., 2006). Thus, though larval- and pupal-stage development time of both insects was similar (i.e., ~30 days for larval and ~10 days for pupation) with development time being longer in fall conditions, insects may have also altered lipid composition differently in response to environmental conditions during development.
5.2.2 Post-eclosion development

Under summer conditions TAW moths and monarchs become reproductively active faster than under fall conditions. However, age of sexual maturity varies between the two species. Turgeon and McNeil (1982) found that TAW moths reach sexual maturity quickly upon emergence in conditions favoring reproduction (~3–4 days post-eclosion) while monarchs do not become sexually mature until later (~7-9 days post-eclosion) (Barker and Herman, 1976; Goehring and Oberhauser, 2002). By Day-5, summer-reared TAW are likely to have more developed reproductive structures and eggs, which may explain the increased loss of EFAs in the fat body (presumably moved to reproductive structures) (Downer and Matthews, 1976; Stanley-Samuelson et al., 1986; Forte et al., 2002; Arrese and Soulages, 2010; Malcicka et al., 2018), compared to summer-reared monarchs. When examining 5-day old fall-reared TAW moths and monarchs, both were expected to be sexually immature or have reduced reproductive development (Appendix A4.2) thus the age of reproductive development was unlikely to have affected FA allocation. However, FA accumulation patterns during the 5-day feeding period of adults for the fall-reared groups varied. Migrant TAW moths increased mass and fat body NFA concentrations more than fall-reared monarchs and this increase in TAW moths compared to monarchs may have contributed to the reduction in EFA use seen in moths. If TAW moths accumulate more expendable NFAs due to differences in flight strategy and physiology compared to monarchs, then fall-reared TAW moths would use EFAs less readily compared to fall-reared monarchs which have proportionally less expendable NFAs for the same duration of flight (6 hours) in my experiments.

The development of flight muscle related to migratory needs may differ between TAW moths and monarchs. Insect flight muscles are one of the most energetically demanding muscles (Harrison and Lighton, 1998; Marden, 2000) and some insects have lipid stores in the flight muscle to provide more immediate sources of fuel during flight (e.g., Zera et al., 1994). I investigated this possibility by examining right dorsal thorax flight muscle from freshly emerged fall-reared TAW moths and monarchs using FA composition of lipids extracted from equal masses of flight muscle (Appendix A4.1). TAW moth flight muscle had a greater percent proportion of EFAs compared to the fat body, potentially indicating
a shift of more EFAs than NFAs to flight muscle during metamorphosis. In contrast, monarch flight muscle and fat body had similar EFA percent proportions indicating no preferential allocation of specific FAs to flight muscle during metamorphosis. Literature in other taxa has shown EFAs such as linoleic acid and alpha-linolenic acid to be considered excellent sources of fuel due to high reductive potential and mobility (due to unsaturation) (Price et al., 2008; Guglielmo, 2018). TAW moths developed flight muscle with more EFAs than monarchs (per 50 mg of muscle) and thus EFAs may be less may be mobilized from the fat body of moths compared to monarchs.

5.2.3 Migratory strategy

The migratory strategies of these two species differ even though the distances traversed are similar and may have influenced FA use. The TAW moth is an active nocturnal flier that uses high frequency flapping flight in upper-air currents to migrate and has the capacity for sustained flight (Guppy, 1961; Taylor, 1974; Fields and McNeil, 1984; McNeil, 1987; Luo et al., 2002). It is hypothesized that they complete their migration quite rapidly due to use of dominant winds (McNeil, 1987) so long bouts of flight at high altitude may lead to inconsistent feeding opportunities during their migration. Thus, it would be more favorable for these insects to load a large amount of NFAs to use as fuel and limit the use of EFAs unless no other options were available. In support of this, fall-reared moths flown for multiple days displayed a loss of EFAs only when individuals were flown without feeding for at least three days. Monarchs, on the other hand, combine active boundary layer diurnal flights with periods of gliding/soaring (Brower, 1996; Urquhart and Urquhart, 1978; Chapman et al., 2015). In general monarchs also tend to stop frequently along their migratory route to feed on nectar (Brower et al., 2006). Migrating monarchs have been reported to have a low wing loading ratio (wet mass of insect/total wing surface area) (Gibo and Pallet, 1979) and this has been hypothesized to be due to soaring flight being predicted to be more efficient with reduced wing loading (Flockhart et al., 2017) as less fuel is required (Dudley, 1991; Wootton, 1992). To maintain soaring capabilities with reduced fuel use it would be favorable for monarchs to nectar frequently en route rather than gain a large amount of lipid mass during the premigration period. Thus, it is unsurprising that
monarchs proportionally did not gain as much lipid as TAW moths or allocate more lipid to flight muscle.

5.3 Caveats and limitations

My study has demonstrated the capacity of nectivorous migrant lepidopterans to differentially use mostly NFAs vs. EFAs to fuel flight when reared under conditions that favor the induction of migration, which was the main goal of my thesis. This result supports the hypothesis that differential resource use may be a mechanism by which wing monomorphic insects reduce the cost of migration to future reproduction. However, my work involves an important caveat that needs to be recognized.

Six hours of flapping flight by monarchs on the rotational flight mill used in my experiment may reflect a more extreme duration and form of exercise than what these insects may undergo in the wild. This heightened flapping duration may have played a role in the less stringent EFA conservation seen in fall monarchs compared to fall TAW moths. Monarchs were placed on rotational flight mills and although they can have inherent limitations, they have shown remarkable success in exploring the flight capabilities of many insects (reviewed by Minter et al., 2018). However, monarchs spent most of their flight duration continually flapping which is reportedly uncommon in the wild during migration. Using a similar flight mill, Mouritsen and Frost (2002) also found flapping comprised 93% of the flight period for tethered monarchs and the rest was coasting. In other words, as insects were force flown it did not fully reflect the flight behaviour of these insects in nature. In the wild, monarchs use a mixture of flapping with bouts of gliding (Urquhart, 1960; Gibo and Pallett, 1979). In particular, monarchs use several energy saving strategies during migration such as exploiting thermals like birds in addition to flapping flight in the boundary layer (Gibo and Pallett, 1979; Gibo, 1986) which may reduce the amount of FA used as fuel. Monarchs in my experiments used EFAs to fuel a flight during trials even under fall conditions but considering the flight behaviour during forced flight differs from the natural behaviour (i.e., more energetically expensive), EFA use may have been higher than what would be observed in wild migrating monarchs. Improvements in the experimental apparatus used to test FA use by monarchs might involve devices to mimic tail winds to promote more gliding.
5.4 Future directions

5.4.1 Potential mechanisms of differential FA allocation

I found evidence for distinct differences in FA use during flight between reproductive and migratory morphs of wing monomorphic lepidopterans. Much of the literature to date has focused on wing polymorphic insects where reproductive insects are evidently morphologically distinct from the migratory morph (i.e., winged and wingless) and thus the costs associated with migration are more directly focused on the cost of developing flight versus reproductive structures (Angelo and Slansky, 1984; Bonte et al., 2012). In wing monomorphic insects, flight apparatus is developed regardless of morph thus the costs, adaptations, and differences between migrants and residents are less focused on cost of flight structures and more on life history and resource partitioning. More emphasis should be placed on investigating the migration reproduction trade-off in wing monomorphic insects (reviewed by Tigreros and Davidowitz, 2019) as the conclusions seen in polymorphic insects differ from what is seen in my study. Differential FA allocation in fall-reared migrant TAW moths and monarchs indicates that mechanisms must exist that facilitate this pattern of fuel use. These mechanisms are rarely contrasted between migrant and reproductive morphs of insects, and I strongly advocate for future studies to determine the underlying structural, physiological, or hormonal pathway(s) that induce the pattern of EFA conservation suggested here. Some potential mechanisms are discussed below.

5.4.1.1 Hormonal regulation of lipid use

Lipid mobilization during migration and the development of the migratory phenotype is regulated by hormones. One of the hormones responsible for the regulation of the development of migrant and reproductive morphs is juvenile hormone (JH). In adult stages of many migrant insects, juvenile hormone plays a concentration-specific role in transitioning insects between the reproductive and migratory morphs (Rankin and Riddiford, 1977; Wyatt and Davey, 1996) where lower to intermediate levels of JH induce/maintain reproductive diapause. Many insect species produce only one juvenile growth hormone (JH III), but lepidopterans have several forms of juvenile hormone (JH 0, JH I, JH II; Judy et al., 1973). Thus, a possible mechanism by which lepidopterans regulate
FA use could be through the expression of different forms of JH in migrant compared to reproductive morphs. It is also possible that it is related to changes in JH receptors (distribution or binding affinity) and, as noted by Jindra et al. (2021), there is a great deal we still do not understanding about JH receptor activity and composition. There is evidence that receptor binding affinity can vary with different analogs of JH (e.g. Milacek et al., 2021), which could result in differential lipid use by migrant and reproductive morphs. JH may play an indirect role in EFA conservation by regulating feeding behavior (Rankin, 1991; Dingle and Winchell, 1997) and thus influencing the accumulation of NFAs. Detailed studies of JH in winged morphs of adult Gryllus firmus (Zera and Zhao, 2009) showed a cyclic circadian-like rhythm of JH hormone expression in flight selected insects. This fluctuation may be important for increasing feeding behaviour during nonflight periods to allow for the accumulation of more fuel. This hypothesis was supported by Zera and Mole (1994) who found that flight selected crickets increased food acquisition to ensure nutrients to maintain flight capacity came from feeding and did not overlap with nutrients and resources needed for reproduction. Thus, if JH concentration induces changes to feeding in other migrant insects such as lepidopterans, it may ensure the accumulation of more NFAs to be used as fuel and thus EFAs may not be readily used if NFAs are abundant.

Another major hormone that plays a more direct role in FA use is adipokinetic hormone (AKH), a peptide neurohormone produced by the corpora cardiaca (Gade and Auerswald, 2003; Van der Horst, 2003). AKH is typically released in response to flight to mobilize lipid and other fuels from the fat body (Arrese et al., 1996a; Arrese and Wells, 1997; Arrese and Soulages, 2010; Roma et al., 2010). AKH mobilizes lipid from the fat body through the activation of TAG lipases (Ogoyi et al., 1998; Gade and Auerswald 2003). AKH is seen as the regulator of lipid mobilization during more prolonged flights as seen in migratory locusts (Locusta migratoria) (Orchard et al., 1993). The most common AKH identified in lepidopterans was originally elucidated from Manduca sexta and thus is denoted as Manse-AKHR (Ziegler et al., 1985). Orchard et al. (1991) found that it takes about an hour of forced flight for AKH levels to stabilize in the haemolymph of TAW moths. Similar to JH, there are many forms of AKH, and the various forms have been shown to mobilize FAs differentially in other insects which may also be a mechanism by
which resource limited insects allocate FAs. The migratory morph of *L. migratoria* expresses elevated levels of locust AKH II which mobilizes more oleic acid and saturated FAs compared to other AKHs (Tomcala et al., 2010). TAW moths may produce additional forms of AKH that express differently in summer- and fall-reared insects which was not previously studied by Orchard et al. (1991). As previously stated, Manse-AKH is considered the primary AKH in lepidopterans, but Weaver et al. (2012) found the presence of Manse-AKH-II which has been shown to significantly increase lipid mobilization in the tobacco hornworm (*M. sexta*), and other related moths. The possible role of different forms of AKH, that may exist with a given species (as seen in *Bombyx mori*; Sehadova et al., 2020) and merits further investigation. Similarly, further research is needed on AKH receptors, as they can display different binding affinities (Marchal et al., 2018). Individually or in concert these could influence differential FA mobilization in migrant Lepidoptera.

During the shorter first phases of flight the neurohormone octopamine also plays a role in the catabolism of glycogen and TAGs from the insect fat body (Fields and Woodring, 1991; Arrese and Soulages, 2010). The role of octopamine has been studied in migratory locusts where the initial mobilization of DAGs from the fat body of migratory locusts during flight was triggered not by AKH but by octopamine (Orchard et al., 1993). Similar to AKH, octopamine-based lipid mobilization results in changes to intracellular levels of cAMP and/or calcium signaling (Fields and Woodring, 1991; Evan and Maqueira, 2005). In lepidopterans octopamine has also been hypothesized to impact pheromone production (Rafaeli and Gileadi, 1995). There are no homologues of octopamine (Farooqui et al., 2012) and therefore the role of multiple forms of this signaling molecule as discussed for JH and AKH is not applicable. Octopamine does have multiple receptors that could elicit different physiological responses (Roeder et al., 2020) and could vary in the fat body of migrant and reproductive insects but this warrants future exploration as little literature exists on the potential role octopamine or its receptors may have on the mobilization of different FAs.

Serotonin (5-HT) is a monoamine neurotransmitter synthesized from tryptophan (Maddrell et al., 1991; Vleugels et al., 2015) serving as signaling molecule in the communication
between the central nervous system (CNS) and the GI tract (Neckameyer, 2010; Gasque et al., 2013). 5-HT also plays a role in maintaining circadian rhythms, controlling feeding behaviour, and influencing muscle activity and excitation (Evans and Myers, 1986; Yuan et al., 2005; Neckameyer, 2010; Gasque et al., 2013). It has also been suggested that it has a role in the development of the migratory phenotype of some insects (Nelson and Trainer, 2007; Kamhi et al., 2017). In boll-weevils (Anthonomus grandis), Guerra et al. (1991) found that high serotonin concentrations were correlated to increased dispersal flight behavior while more intermediate levels increased saturated FA (energy reserve) concentrations. The role of 5-HT in adult lepidopterans is poorly studied and could vary between migrant and reproductive morphs as a regulator of feeding and fuel use.

5.4.1.2 Structure and storage of lipids

Beyond hormonal regulation, the structure and storage of the FAs in the fat body itself may also contribute to the pattern of differential FA use found between and within species. The fat body can be heterogenous in its composition with different parts of the fat body used for different functions (Haunerland et al., 1990; Haunerland and Shirk, 1995). The fat body can also change in total lipid content and composition during certain life stages, as reported by Anand and Lorenz (2008) when analyzing the compositional (lipid and protein proportions) change of the fat body in Gryllus bimaculatus during larval development. Beyond temporal changes in the fat body, lipid use can also vary based off the sections of fat body that are being activated. In particular, peripheral (under integument) and perivisceral (surrounding internal viscera) fat body tissue have been shown to differ structurally (De Oliveira and Cruz-Landim, 2003; Skowronek et al., 2021) and thus differential storage of FAs between these two divisions may also be a possibility. The fat body is present in both adult and juvenile stages since all life stages require energy (Arrese and Soulages, 2010). When looking at the fat body from larval stages to pupation in the corn earworm (Helicoverpa zea), Wang and Haunerland (1992) found that although the peripheral fat body depletes during the pupal stage, the perivisceral fat body persists. If a similar fat body cell persistence is found in TAW moths and monarchs, EFAs could be stored in fat body cells that were carried over from larval stages and thus distinct from fat body cells in which adult derived resources were stored.
5.4.1.3 Mobilization of lipids

Differences at the level of lipid mobilization could also play a role in the pattern of FA use seen in this study. First, there are different forms of lipases in insects that are activated by different cues. Although Brummer and TAG lipases are the most studied lipases and are discussed below, there are many other additional enzymes and forms of lipases (Toprak et al., 2020) that may differ in expression in fall- and summer-reared insects that have been greatly understudied especially in the lens of comparative rearing conditions. Hormone sensitive TAG lipases are activated by AKH (Van der Horst, 1990, 2003; Auerswald and Gade, 2006; Van der Horst and Rodenburg, 2010) and are expected to be the prominent lipase in migrant insects during longer bouts of flight. Brummer lipase has been shown to be not as sensitive to AKH (as seen in Drosophila; Gronke et al., 2005, 2007) and has also been associated with lipolytic activity outside of flight, such as feeding and starvation (e.g., Gronke et al., 2005, 2007; Zhou et al., 2018). Brummer lipase has been shown by Lu et al. (2018) to be vital for the mobilization of TAGs from the fat body in brown plant hoppers (Nilaparvata lugens) during reproduction and may be sensitive to JH signaling. Differences in the functionality, activity level and lipid mobilization of these two lipases between reproductive and migratory insects may contribute to the differences I found in FA use. In particular, in the TAW moth Day-5 aged summer-reared insects were expected to have developed reproductive structures. Thus if Brummer lipase is more active during this time in addition to AKH based mobilization, and if Brummer lipase differentially mobilizes more EFA, it could explain the differences I detected in EFA mobilization between fall and summer-reared insects. Additionally in TAW moths that were starved over the 5-day period Brummer lipase may have also played a role in FA mobilization during this time as well (seen in plant hoppers; Zhou et al., 2018).

When lipids are mobilized from the fat body for fuel, the TAGs are converted to DAG in a site-specific manner (Ryan and Van der Horst, 2000; Arrese et al., 2001). Some (but not the majority of) DAG can also be produced from the acylation of monoacylglycerol, however less evidence of this is seen in lepidopterans (Arrese et al., 1996b, 2001). Arrese and Wells (1997) found that in the tobacco hornworm, M. sexta, the fat body released primarily sn-1,2-DAG during mobilization via AKH signaling. In a study on the
stereospecific composition of DAG in the hemolymph of flight exercised migratory locusts, Lok and Van Der Horst (1980) found that palmitic acid and oleic acid were found in the highest concentrations on positions sn-1 and sn-2, respectively. Considering that these FAs were highly mobilized in both TAW moths and monarchs, stereospecific mobilization may also play a significant role in FA use in lepidopterans as well. Considering that TAG lipase activity would be required for long bouts of flight in either rearing condition, enzymatic stereospecificity is unlikely to change if the same lipase is being used. As previously mentioned, there are many forms of lipases and hormones that may differentially activate under different conditions, however very little literature is available connecting different lipases/hormones to stereospecific synthesis of specific DAGs in lepidopterans.

5.4.2 Additional avenues of exploring FA allocation

Although the goal of this thesis was to establish if rearing conditions can affect FA mobilization from the fat body, future research should explore the role of larval diet FA composition on the pattern of FA use in the TAW moth. Larval diet affecting flight performance and metrics such as oxygen consumption has been reported in other lepidopterans (Ebada et al., 2022) and thus FA use may differ based on larval diet as well. The flight pattern of the TAW moths on the tethered flight mill was consistent with what has been seen in other studies (Luo et al., 2002). However, the fat body of TAW moths had a lower proportion of EFAs compared to that of monarchs and of the larval pinto bean diet itself. A reduction in fat body EFA in moths may have resulted from the movement of these EFAs to flight muscle during metamorphosis or it may simply be related to the larval diet used in my study. Although the pinto bean diet (Shorey and Hale, 1965) meets the nutritional needs of the TAW moths it is not a true reflection of their natural diet (Breeland 1957). Additionally, seasonal diet quality can affect the development of reproductive and migratory morphs and may act as an additional cue for migration (Southwood, 1962; Dixon et al., 1993). In the natural environment TAW larvae tend to feed voraciously on plants such as corn and various cereal crops causing considerable damage (Breeland, 1957; Guppy, 1961; McNeil 1987). On these plants the TAW larvae commonly feed on foliage (Breeland, 1957) which was not a component of the synthesized diet provided to them in
this study. Different components of plants have been shown to differ in FA quantity and composition (Glew et al., 1997; Malainey et al., 1999; Çakmak et al., 2012) and thus the synthetic diet may not reflect the FAs that TAW larvae may accumulate in the wild. To explore this, wild captured TAW moths could also be compared to lab reared individuals to compare EFA composition. In addition, insects could be reared on natural or synthetic diets and then exercised to assess if larval diet differences influence FA use during flight.

The approach of using bulk stable isotopic analysis allowed me to infer general patterns of larval and adult diet-based FA use in flight. The technique was informative as previous literature has shown a clear correlation between diet and lipid isotopic values (Anparasan et al., 2021). The inferences made using the bulk isotopic data are well supported by the patterns of FA use seen using chromatographic analyses. However, different FAs can undergo synthesis, modifications or bioconversion which can cause varying degrees of isotopic fractionation (DeNiro and Epstein, 1977; Melzer and Schmidt, 1987; Pilecky et al., 2022a,b) which can increase the variability of bulk isotopic analysis (McMahon and Newsome, 2019; Whiteman et al., 2019). For example, Pilecky et al. (2022b) found that there were high C isotopic fractionation differences associated with linoleic acid in neutral vs. polar lipids in female monarch butterflies which bulk isotopic analysis would not be able to distinguish. In my thesis, although bulk stable isotopes provided me with the ability to infer adult- and larval-based lipid use, I could not extrapolate those conclusions at an individual FA level even when coupled with FA profile analysis. For example, while it is clear that oleic acid and palmitic acid are significant fuels for both the TAW moth and monarch, it is unclear how much of each FA that was used as fuel arose from the larval or adult diet.

Recent advances in using isotopic techniques have involved the use of compound specific approaches (McMahon and Newsome, 2019; Whiteman et al., 2019; Twining et al., 2020). Compound-specific isotope analysis (CSIA) can be used to refine current tissue-specific isoscape, identify metabolic differences that can cause bulk isotopic tissue variations, and examine resource utilization with greater detail (McMahon and Newsome, 2019; Whiteman et al., 2019; Twining et al., 2020). The technique is based on the isotopic analysis of individual compounds (e.g., amino acids, FAs) vs. bulk materials. Much of the
CSIA literature has been focused on amino acids, however CSIA can also be applied to FAs (e.g., Pollierer et al., 2012; Twining et al., 2020; Pilecky et al., 2022a). Understanding the pattern of larval- and adult-derived individual FA use can add to the understanding of the importance each stage plays in fuelling insect migration and how changes to larval and adult dietary sources can impact the migratory success of such species in greater detail than what can be assessed using bulk isotopic analysis alone. Additionally, if certain FAs are limited to being obtained from specific life stages or locations, individual FA analysis could function as an additional tool to trace migratory organisms and pinpoint important nectaring sites. The CSIA approach to assessing FAs was applied recently to monarch butterflies. Pilecky et al. (2022b), found that alpha-linolenic acid δ²H values in monarchs correlated with δ²H values of wings indicating that ALA, as seen in my thesis, is limited to being sourced from the larval stage. However, by using the CSIA approach Pilecky et al. (2022b) established that alpha-linolenic acid is not isotopically modified and thus could function as an indicator of individual’s geographic origin. Considering the growing focus on compound-specific approaches to isotopic analysis, I suggest applying such an approach to fuelling energetics studies in migrant organisms to explore the origins, allocation patterns, and metabolic use of lipids with greater detail and resolution.

Another future direction of this research would be to expand the chromatographic analysis performed in my thesis. I assumed that a decrease in the selected FA concentrations in the fat body during flight was an indicator of those FAs being directed to flight muscle to fuel movement. Considering the majority of lipid in many lepidopterans has been shown to be comprised of the 5 I have selected (Subramanyam and Cutkomp, 1987; Canavoso et al., 2001) and the fat body is considered the primary source of FAs for fuelling flight (Beenakkers et al., 1969, 1985; Arrese and Soulages, 2010), the focus on specific FAs within only the fat body was not unreasonable considering the overarching goal of my thesis. In addition, literature on FA mobilization during flight in other lepidopterans has also shown that these 5 FAs were the primary FAs used as fuel as well (Wang and Ouyang, 1995; Murata and Tojo, 2002; Sakamoto et al., 2004). However, future studies assessing the allocation of FAs may want to explore additional analyses. For example, the mobilization of FAs straight to the tissue of interest can be assessed by measuring the FA composition of the fat body and target tissue to confirm that lipid mobilization in the fat
body was reflected at the target tissue (e.g., ovaries, as seen in Ziegler and Ibrahim, 2001). This can be of particular importance if future researchers aim to explore the movement of specific FAs in situations where FAs may be concurrently mobilized to for different purposes. For example, FAs can be mobilized from the fat body to repair membrane damage during starvation, maintain the epicuticular lipid layer to reduce water loss, or for the synthesis of hormones in reproductive insects while also acting as metabolic fuel (Arrese and Soulages, 2010; Wrońska et al., 2023).

5.5 Concluding remarks

Conservation issues are inextricably linked to how potential climate change-induced impacts on nectar availability/quality can affect FAs used by nectivorous insects. Indeed, reduction in nectar sources as a result of events such as shifting agricultural practices and other land-use modifications (Cane and Tepedino, 2001; Zipkin et al., 2012; Malcom, 2018) may significantly reduce the capacity of migrant insects to acquire NFAs, which my research has shown plays a vital role in maintaining patterns of EFA conservation. Even when nectar is available, decreases in quality (e.g., due to changes in viscosity) may also impact the ability of these insects to maintain EFAs for future reproduction. Increases in drought exposure due to climate change can decrease nectar production and increase the viscosity of nectar (Kim et al., 2011; Descamps et al., 2021; Kuppler and Kotowska, 2021). Increased viscosity can decrease the ability of insects to successfully acquire nectar, as lepidopterans have an optimal nectar concentration of 30–40% and increases in viscosity reduce nutrient uptake capacities (Pivnick and McNeil, 1985; Borrell and Krenn, 2006; Kim et al., 2011). Migrant insects, such as the monarch, may be particularly vulnerable due to the potential dependence for refueling on specific locations (stopover sites) for nectar along their migration (Nabhan, 2004). Exploring the predictability of how changing nectar landscapes will impact EFA-limited insects can be key to predicting future migratory success.

Insects represent a group of animals that manifest a range of traits allowing them to cope with changing environmental conditions. Reproduction, growth and development, migration, and overwintering are all events which involve trade-offs because they compete for acquired resources (Angelo and Slansky, 1984; Saglam et al., 2008; Burton et al.,
2010). The costs and adaptations of migratory and reproductive morphs of insects that vary morphologically have been well studied to date (Zera and Bottsford, 2001; Bonte et al., 2012). Conversely, in wing monomorphic insects the costs, adaptations, and differences between migrants and residents can be less distinguishable but just as vital to the success of the respective cohorts. My research indicates that clear fuel use differences exist between migrants and residents of wing monomorphic TAW moths and monarch butterflies. My research has also demonstrated that rearing conditions that induce the development of migrant or reproductive insects also affects FA use in these insects which can play a significant role in the extrapolations that are made when studying these specific insects and phenotypes. I have established that differential FA use, with a preference to conserve limited EFAs, may be a mechanism used by EFA limited insects such as lepidopterans to reduce overlapping nutritional requirements between migration and reproduction. Much of the literature on the flight reproduction trade-off has focused on specific characteristics of fitness (e.g., mating, egg laying, egg size, offspring viability) (Angelo and Slansky, 1984; Rankin and Burchsted, 1992). However, although differential fuel use can play a significant role in the fitness of many organisms, it has been understudied. My research highlights the importance of continued exploration into the detailed role of FAs in migrant insects rather than considering them as a bulk fuel source. The ecophysiology of FA use is clearly complex, and I strongly advocate for the consideration of rearing conditions of wing monomorphic insects in future research on migratory costs and energetics.
5.6 Literature cited


Appendices

Appendix A1: Supplementary data for Chapter 2

A1.1 Composition of adult sugar water diet provided to monarchs in this study. All ingredients were dissolved into approximately 1000 mL of distilled tap water. Diet was developed by Orley Taylor (personal communication) and also used by Hobson et al., 2020.

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<td>Bee pollen</td>
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A1.2 Mass of monarch adults upon eclosion (0) and 5 days (5) post adult feeding under different environmental conditions.

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Appendix B2: Supplementary data for Chapter 3

A2.1 Mass of true armyworm adults upon eclosion (0) and 5 days (5) post adult feeding under different environmental conditions.

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A2.2 Equation for establishing the $\delta^{13}C$ of purely adult diet derived bulk lipid in fat body.

Variables

$A = \delta^{13}C$ of lipid in fat body of Day 5 post-eclosion insects = -16.5‰.

$B = \delta^{13}C$ of lipid in fat body of Day 0 post-eclosion insects = -29.1‰.

$C = \text{proportion of lipid derived from larval diet at Day 5 post-eclosion} = 0.13$
D = proportion of lipid derived from adult diet at Day 5 post-eclosion = 0.87

\( x = \delta^{13}C \) of lipid in fat body of Day 5 post-eclosion insects solely derived from adult diet

\[ A = (C)(B) + D(x) \]

\( x = -14.62\%_o \)
A2.3 (a) δ^{13}C of adult summer-reared male true armyworm fat body during flight on Day 5 post eclosion. (b) δ^{13}C of adult summer-reared female true armyworm fat body during flight on Day 5 post eclosion. (*) indicates isotopic values were statistically lower for females compared to males during same duration of flight (t-test, P<0.05). N=5 per sample group.
A2.4 (a) $\delta^{13}C$ of adult fall-reared male true armyworm fat body during flight on Day 5 post eclosion. (b) $\delta^{13}C$ of adult fall-reared female true armyworm fat body during flight on Day 5 post eclosion. (*) indicates isotopic values were statistically lower for females compared to males during same duration of flight (t test, P<0.05). N=5 per sample group.
A2.5 Proportional change in adult and larval diet derived lipids in the fat body of moths reared under fall conditions after 6 hours of flight.

Variables

A = $\delta^{13}$C of lipid in fat body of Day 5 post-eclosion insects after 6-hour flight = -18.75‰.

B = $\delta^{13}$C of lipid in fat body of Day 0 post-eclosion insects = -29.1‰.

C = $\delta^{13}$C of lipid in fat body of Day 5 post-eclosion insects solely derived from adult diet = -14.62‰ from A2.2

x = proportion of larval diet derived lipid remaining after 6-hour flight

y = proportion of adult diet derived lipid remaining after 6-hour flight

A = x(B) + y(C)

x + y = 1

x = 0.285

y = 0.715

A2.6 Proportional change in adult and larval diet derived lipids in the fat body of moths reared under summer conditions after 6 hours of flight.

Variables

A = $\delta^{13}$C of lipid in fat body of Day 5 post-eclosion insects after 6-hour flight = -17.29‰.

B = $\delta^{13}$C of lipid in fat body of Day 0 post-eclosion insects = -29.1‰.

C = $\delta^{13}$C of lipid in fat body of Day 5 post-eclosion insects solely derived from adult diet = -14.62‰ from A2.2

x = proportion of larval diet derived lipid remaining after 6-hour flight
y = proportion of adult diet derived lipid remaining after 6-hour flight

A = x(B) + y(C)

x + y = 1

x = 0.198

y = 0.802

Appendix C3: Supplementary data for Chapter 4

A3.1 Mass of 1 egg as calculated from a sub-sample of eggs collected post-oviposition from female moths singly mated with either a control larval diet (unspiked) or 99 atom % $^{13}$C linoleic acid supplemented treatment (spiked) male moth.

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<th>Mass of 1 egg (g)</th>
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<td>8.78049E-05</td>
</tr>
<tr>
<td>Spiked</td>
<td>6</td>
<td>0.0073</td>
<td>79</td>
<td>9.24051E-05</td>
</tr>
<tr>
<td>Unspiked</td>
<td>6</td>
<td>0.0065</td>
<td>71</td>
<td>9.15493E-05</td>
</tr>
<tr>
<td>Unspiked</td>
<td>6</td>
<td>0.003</td>
<td>34</td>
<td>8.82353E-05</td>
</tr>
<tr>
<td>Unspiked</td>
<td>6</td>
<td>0.0036</td>
<td>42</td>
<td>8.57143E-05</td>
</tr>
<tr>
<td>Spiked</td>
<td>3</td>
<td>0.0021</td>
<td>26</td>
<td>8.07692E-05</td>
</tr>
<tr>
<td>Spiked</td>
<td>3</td>
<td>0.0026</td>
<td>32</td>
<td>0.00008125</td>
</tr>
<tr>
<td>Spiked</td>
<td>3</td>
<td>0.0045</td>
<td>48</td>
<td>0.00009375</td>
</tr>
</tbody>
</table>
A3.2 Mass of $^{13}$C spiked linoleic acid required to establish a pinto bean diet with linoleic acid enriched to 2 atom %.

Variables

A = proportion of carbon by mass coming from $^{13}$C spiked linoleic acid = 0.0092 (from A3.3)

B = mass of carbon from linoleic acid in 150g of pinto bean = 0.593g

C = proportion of carbon by mass in linoleic acid = 0.77

D = mass of carbon coming from $^{13}$C spiked linoleic acid

E = mass of $^{13}$C spiked linoleic acid needed

$$\frac{D}{B + D} = A$$
D/C = E

D = 0.005506g or 5.506mg

E = 7.151 mg

**A3.3** Proportion of carbon required to be sourced from the $^{13}$C spiked linoleic acid to establish a pinto bean diet with linoleic acid enriched to 2 atom %.

**Variables**

A = larval pinto bean diet atom percent = 1.11% or 0.0111

B = desired pinto bean diet atom percent = 2% or 0.02

C = $^{13}$C spiked linoleic acid atom percent = 98% or 0.98

x = proportion of carbon by mass coming from pinto bean linoleic acid

y = proportion of carbon by mass coming from $^{13}$C spiked linoleic acid

\[ B = (A)(x) + (C)(y) \]

\[ x + y = 1 \]

\[ x = 0.9908 \]

\[ y = 0.0092 \]

**A3.4** Estimation of the amount of linoleic acid (LA) in one true armyworm moth egg. Based off lipid extracted from a sample mass of eggs from virgin females.

<table>
<thead>
<tr>
<th>Sample of egg mass µg</th>
<th>Mass of lipid recovered µg</th>
<th>Number of eggs *</th>
<th>Lipid in one egg µg</th>
<th>Hydrocarbon in egg µg**</th>
<th>Lipid in egg (excluding hydrocarbon) µg</th>
<th>EFA in egg µg</th>
<th>LA in egg µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>50000</td>
<td>810</td>
<td>564.972</td>
<td>1.434</td>
<td>0.120</td>
<td>1.314</td>
<td>0.081</td>
<td>0.054</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>49000</td>
<td>750</td>
<td>553.672</td>
<td>1.355</td>
<td>0.120</td>
<td>1.235</td>
<td>0.077</td>
<td></td>
</tr>
<tr>
<td>48000</td>
<td>720</td>
<td>542.373</td>
<td>1.328</td>
<td>0.120</td>
<td>1.208</td>
<td>0.075</td>
<td></td>
</tr>
<tr>
<td>51000</td>
<td>670</td>
<td>576.271</td>
<td>1.163</td>
<td>0.120</td>
<td>1.043</td>
<td>0.065</td>
<td></td>
</tr>
<tr>
<td>53000</td>
<td>770</td>
<td>598.870</td>
<td>1.286</td>
<td>0.120</td>
<td>1.166</td>
<td>0.072</td>
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</tr>
<tr>
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<td>850</td>
<td>542.373</td>
<td>1.567</td>
<td>0.120</td>
<td>1.447</td>
<td>0.090</td>
<td></td>
</tr>
<tr>
<td>48000</td>
<td>690</td>
<td>542.373</td>
<td>1.272</td>
<td>0.120</td>
<td>1.152</td>
<td>0.071</td>
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</tr>
<tr>
<td>47000</td>
<td>890</td>
<td>531.073</td>
<td>1.676</td>
<td>0.120</td>
<td>1.556</td>
<td>0.096</td>
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</tr>
<tr>
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<td>640</td>
<td>542.373</td>
<td>1.180</td>
<td>0.120</td>
<td>1.060</td>
<td>0.066</td>
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</tr>
<tr>
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<td>553.672</td>
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<td>0.120</td>
<td>1.198</td>
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</tr>
<tr>
<td>49000</td>
<td>800</td>
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<td>1.445</td>
<td>0.120</td>
<td>1.325</td>
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</tr>
<tr>
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<td>650</td>
<td>553.672</td>
<td>1.174</td>
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<td>1.054</td>
<td>0.065</td>
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</tr>
<tr>
<td>47000</td>
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<td>531.073</td>
<td>1.469</td>
<td>0.120</td>
<td>1.349</td>
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<tr>
<td>50000</td>
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<td>564.972</td>
<td>1.540</td>
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<td>1.420</td>
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</tr>
<tr>
<td>51000</td>
<td>700</td>
<td>576.271</td>
<td>1.215</td>
<td>0.120</td>
<td>1.095</td>
<td>0.068</td>
<td></td>
</tr>
</tbody>
</table>

* Determined using average mass of 1 egg from A3.1

** from Marshall and McNeil 1989

A3.5 Number of eggs in the reproductive system of fall-reared, female, 5-day old TAW moths flown for 5-days and provided with either abundant sugar water during every rest period (FED) or only water during every rest period (FASTED). N=5. There were no significant differences between the two groups (Unpaired t-test, df = 8, $t = 1.20$, $p = 0.13$).
Appendix D4: Supplementary data for Chapter 5

A4.1 Proportion of essential and non-essential fatty acids in the fat body, flight muscle and larval diet of non-flown newly emerged fall-reared monarch and true armyworm moth adults. N=5 per sample group except fat body (N=20).

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>True Armyworm Moth</th>
<th>Monarch Butterfly</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pinto Diet (%)</td>
<td>Fat Body (%)</td>
</tr>
<tr>
<td>Palmitic</td>
<td>26.4 ± 3.0(a)</td>
<td>34.8 ± 0.8(b)</td>
</tr>
<tr>
<td>Stearic</td>
<td>9.3 ± 2.8(a)</td>
<td>3.4 ± 0.3(b)</td>
</tr>
<tr>
<td>Oleic</td>
<td>8.1 ± 0.7(a)</td>
<td>47.9 ± 0.9(b)</td>
</tr>
<tr>
<td>Linoleic</td>
<td>29.0 ± 2.9(a)</td>
<td>4.9 ± 0.4(b)</td>
</tr>
<tr>
<td>Alpha linolenic</td>
<td>23.1 ± 2.2(a)</td>
<td>1.8 ± 0.3(b)</td>
</tr>
</tbody>
</table>

Fall-reared TAW moths did not have similar EFA proportions in fat body compared to their pinto bean diet and fall-reared TAW moths had proportionally more EFA in flight muscle compared to fat body (ANOVA, df, 2, 29, F_{LA}=147.4, P_{LA}<0.0001; ANOVA, df, 2, 29, F_{ALA}=306.4, P_{ALA}<0.0001, Tukey HSD p<0.05). Monarch flight muscle and fat body had similar FA compositions (, ANOVA, df, 2, 29, F_{PA}=8.37, P_{PA}<0.01, Tukey HSD, P_{PA}muscle-fatbody = 0.82; ANOVA, df, 2, 29, F_{ST}=45.72, P_{ST}<0.001, Tukey HSD, P_{ST}muscle-fatbody = 0.86; ANOVA, df, 2, 29, F_{OL}=143.1, P_{OL}<0.0001, Tukey HSD, P_{OL}muscle-fatbody = 0.67; ANOVA, df, 2, 29, F_{LA}=7.42, P_{LA}<0.01, Tukey HSD, P_{LA}muscle-fatbody = 0.44; ANOVA, df, 2, 29, F_{ALA}=519.7, P_{ALA}<0.0001, Tukey HSD, P_{ALA}muscle-fatbody < 0.0001).
A4.2 Assessment of reproductive status (number of eggs) of virgin non-flown female true armyworm moths and monarchs reared to day-5 post eclosion under fall conditions. N=10

<table>
<thead>
<tr>
<th>Female Moths</th>
<th>Female Monarchs</th>
</tr>
</thead>
<tbody>
<tr>
<td>No eggs</td>
<td>No eggs</td>
</tr>
<tr>
<td>No eggs</td>
<td>No eggs</td>
</tr>
<tr>
<td>No eggs</td>
<td>No eggs</td>
</tr>
<tr>
<td>No eggs</td>
<td>No eggs</td>
</tr>
<tr>
<td>No eggs</td>
<td>4 eggs</td>
</tr>
<tr>
<td>No eggs</td>
<td>No eggs</td>
</tr>
<tr>
<td>No eggs</td>
<td>N eggs</td>
</tr>
<tr>
<td>10 eggs</td>
<td>No eggs</td>
</tr>
<tr>
<td>No eggs</td>
<td>No eggs</td>
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<tr>
<td>No eggs</td>
<td>3 eggs</td>
</tr>
<tr>
<td>No eggs</td>
<td>No eggs</td>
</tr>
</tbody>
</table>
Curriculum Vitae

Name
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Western University, PhD. Biology, 2019 - current
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Publications


