The alpha-linolenic acid requirements of developing Heliothines

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von Brent Sørensen (M. Sc.)

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Gutachter:

1. Prof. Dr. David G. Heckel, Max Planck Institute for Chemical Ecology, Jena, Germany

2. Prof. Dr. Klaus H. Hoffmann, Universität Bayreuth, Bayreuth, Germany

3. Prof. Dr. Spencer T. Behmer, Texas A & M University, College Station, Texas, USA

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Abstract

Awareness of the nutritional benefits of alpha-linolenic acid (α-18:3) has increased over the last few decades, although there is less widespread recognition of the importance of this and other polyunsaturated fatty acids to Lepidoptera. Like most insects, Lepidoptera cannot synthesise α-18:3, and it must therefore be obtained from dietary sources. Linolenic acid deficiency typically has little effect on larval growth, but leads to abnormalities in pupation and adult eclosion.

Species may, however, differ in their α-18:3 requirements and in order to compare those of *Heliothis virescens* (a generalist) and *Heliothis subflexa* (a specialist on *Physalis*), two types of artificial diet, with varying concentrations of α-18:3 were prepared. Developmental characteristics (i.e., pupation and eclosion rates) as well as the fatty acid (FA) content of the adults were monitored. Although *H. virescens* and *H. subflexa* responded differently to alterations in α-18:3 content, it remains essential for both species. Thus, in contrast to a previous report, the specialist *H. subflexa* does indeed require dietary α-18:3.

The role of the timing of α-18:3 acquisition in the development of *H. virescens* was further investigated. Larvæ were reared on a range of α-18:3 supplemented diets. A positive correlation was found between the α-18:3 content of the mother and the molar composition of the eggs she produced. This suggests the passive transfer of α-18:3 from females to their eggs. Additionally, larvæ were started on α-18:3 containing diet and transferred to a defi-

cient diet, or transferred to α-18:3 containing diet after being initially raised on deficient diet. Insects obtaining α-18:3 later in development performed better than those obtaining α-18:3 earlier in development. Although the maternally transferred α-18:3 may be sufficient for the growth of neonates, the α-18:3 acquired during later stages appears to be more important to successful development and pupal emergence.

In order to investigate differences in α-18:3 in a natural diet, *Helicoverpa armigera* larvæ were reared on the leaves of wild type tomato plants, jasmonic acid (JA) insensitive plants, and plants with reduced levels of α-18:3 as a result of mutatation in the *leFAD7* gene. On the wild type plants, larvæ had a reduced growth rate, and none survived to pupation. Larvæ on the JA insensitive plants performed better (i.e., higher growth rate, higher pupal weights, and decreased time to pupation and eclosion) than those reared on the α-18:3 deficient plants. While decreased α-18:3 contents may offer the plants some protection against herbivory, the inherent JA induced defences are likely to be more effective.

Finally, as part of a multidisciplinary approach to the investigation of the functions and requirements of α-18:3 in Lepidoptera, we describe the facile synthesis of 2-fluorolinolenic acid using readily available thyme seed oil as a starting material.

Zusammenfassung

Obwohl die ernährungsphysiologische Relevanz der Linolensäure (α-18:3) in den letzten Jahrzehnten an Bedeutung gewonnen hat, gibt es für Lepidopteren deutlich weniger Anerkennung der Relevanz von α-18:3 und anderer mehrfach ungesättigter Fettsäuren. Die meisten Insekten sind nicht imstande α-18:3 herzustellen und müssen sie daher durch die Ernährung aufnehmen. Ein α-Linolensäuremangel hat nur geringe Auswirkung auf das Larvenwachstum, führt jedoch zu Abnormalitäten beim Verpuppen bzw. bei der Imago.

Verschiedene Arten können einen unterschiedlichen Bedarf an Linolensäure besitzen. Um den Linolensäurebedarf von *Heliothis virescens*, ein Generalist, mit dem Bedarf von *Heliothis subflexa*, einem Spezialist auf *Physalis*, zu vergleichen, wurden zwei Arten künstlicher Ernährung mit unterschiedlichen Mengen an α-18:3 hergestellt. Verschiedene entwicklungsbedingte Merkmale, wie die Verpuppungs- und Schlüpfungsanteile der Larven sowie die Fettsäureprofile der Adulten wurden kontrolliert. Obwohl *H. virescens* und *H. subflexa* unterschiedlich auf Änderungen im α-Linolensäuregehalt der Nahrung reagieren, bleibt die Fettsäure trotzdem essentiell für beide Arten. Im Gegensatz zu einem früheren Bericht, ist der Linolensäurebedarf von *H. subflexa* nicht niedriger als der Bedarf von *H. virescens* und kann weiterhin den niedrigeren Anteil der Fettsäure-Aminosäure-Konjugate in den Oralsekrete im Vergleich zu *H. virescens* nicht nachweisen.

Zudem wurde der Einfluss des Zeitpunkts der Linolensäureaufnahme auf die

Entwicklung von *H. virescens* erforscht. Larven wurden auf α-Linolensäure angereicherter Nahrung gezüchtet, die sich in den Gehalten der α-Linolensäure unterschied. Eine positive Korrelation zwischen dem α-Linolensäuregehalt des Muttertiers und des α-Linolensäureprofils der entsprechenden Eier konnte festgestellt werden. Daraus lässt sich schließen, dass α-18:3 passiv von Weibchen zum Ei transferiert wird. Zudem wurden die Larven, welche mit einer α-Linolensäurearmen Ernährung gefüttert wurden auf eine mit α-18:3 angereicherte Ernährung umgestellt oder umgekehrt. Die Larven, die α-18:3 in den späteren Entwicklungsstadien erhielten, waren leistungsfähiger in der weiteren Entwicklung als die Larven die α-18:3 in den früheren Entwicklungsstadien erhielten. Schlussfolgern lässt sich feststellen, dass maternal übertragene α-18:3 für das Anfangswachstum der Neugeborenen ausreicht, jedoch später erhaltene α-18:3, zum erfolgreichen Verpuppen und Schlüpfen der Larven benötigt wird.

Um Unterschiede in Linolensäuregehalten auf natürlicher Ernährung zu untersuchen, wurden Helicoverpa armigera Larven auf Blättern einer Wildtyp Tomate sowie auf Jasmonsäure-unempfindlichen Pflanzen, und Pflanzen mit einem verringerten Linolensäuregehalt, die durch eine Mutation des Genes *leFAD7* entstand, gezüchtet. Auf den Wildtyppflanzen hatten die Larven eine niedrigereWachstumsrate und es fand keine Verpuppung statt. Die Larven auf den Jasmonsäure-unempfindlichen Pflanzen hatten eine bessere Leistungsfähigkeit als die, die auf die Linolensäure-reduzierten Pflanzen gezüchtet wurden. Obwohl verringerte Linolensäuregehalte die Pflanzen etwas gegen Herbivorie geschützt werden, sind die inhärente Jasmonsäure-induzierte Verteidigungen voraussichtlich effektiver.

Schlussendlich, als Teil eines fachübergreifenden Ansatzes um die Funktion und Bedarfs der Linolensäure in Lepidopteren zu erforschen , beschreiben wir die einfache Herstellung des 2-fluorolinolensäure Methylesters, indem man Thymiamsamen als Ausgangsmaterial benutzt.

Chapter 1

A review of fatty acid metabolism and function in Lepidoptera and current and future applications

1.1 Introduction

Awareness of the nutritional benefits of polyunsaturated fatty acids (PUFAs) has increased over the last few decades; however, there is less widespread recognition of the importance of PUFAs, particularly alpha-linolenic acid (α-18:3) (Figure 1.1), to Lepidoptera. Alpha-linolenic acid is essential for successful development; when larvæ consume insufficient amounts, pupæ are deformed and adults that do fully emerge often have malformed wings.

De Moraes and Mescher (2004) published a study comparing two closely related species, *Heliothis virescens* (tobacco budworm), a generalist, and *Heliothis subflexa*, a specialist, and suggested that unlike *H. virescens* and most other Lepidoptera, *H. subflexa* does not have a dietary requirement for α-18:3. If confirmed, these results would make *H. subflexa* unique among

Lepidoptera in being able to synthesise its own α-18:3, and would have considerable implications with respect to the prevailing understanding of lepidopteran FA requirements. This issue will be further explored in Chapter 2.

Although Lepidoptera, especially those from the family Noctuidæ can be serious agricultural pests, the study of FAs and their metabolism in this order remains a relatively small niche and has, at times, stagnated. Recent technological advances have facilitated resurgences in the field and invalidated previous doctrines. For example, it was once thought that there were no long chain PUFAs in Lepidoptera. More sensitive analytical tools (e.g., GC-MS), have allowed for the detection of FAs and derivatives that, although only present in low concentrations, play a substantial role in lepidopteran physiology.

As in other insects and animals, the FA profile of Lepidoptera is largely influenced by diet. Levels of FAs can vary substantially throughout development, and are heavily influenced by environmental factors.

As Parnova (1986) was the last to review insect FA in great depth, an up-to-date review of lepidopteran FAs is overdue. This review intends to fill that gap. Fatty acid metabolism, various metabolites, and their putative roles in Lepidoptera, as well as the factors that affect them, will be described.

While linoleic acid (18:2) (Figure 1.1) is also considered essential to Lepidoptera, discussion will centre on α-18:3 and the absolute requirements of Lepidoptera. Where relevant, knowledge of α-18:3 metabolism and function in other insect orders, as well as in other animals, will be included.

Potential applications of increased knowledge of lepidopteran FAs will be described. The majority of these involve developing more effective pest control strategies, since resistance towards insecticides is a growing concern for many species. FAs derived from Lepidoptera also have implications in industry and health.

Although substantial progress has been made, some fundamental questions remain unresolved, most importantly if there are species that indeed have little or no requirement for α-18:3, or have retained/evolved the genes allowing them to synthesise α-18:3 *de novo*. This review will therefore conclude with a discussion of where FA research in Lepidoptera should head in the decades to come.

1.2 Fatty acid structure and nomenclature

In order to properly discuss their differing properties, the nuances among the structures and nomenclature of FAs, both in their free and esterified form (i.e., glycerolipids, and glycerophospholipids) will first be discussed.

1.2.1 Fatty acids

Fatty acids are long chain carboxylic acids and are named according to the number of carbon atoms, which is generally even. The carbon contained in the carboxyl group is referred to as the delta (Δ)-carbon, while the carbon at the opposite end is referred to as the omega (ω) or the n-carbon. Fatty acids may include one or more double bonds and are referred to as monounsaturated FAs (MUFA) and PUFAs respectively. The double bonds of most naturally occurring FAs are in the *cis*- configuration. The major PUFAs in lepidopteran tissues are 18:2 and α-18:3. Unsaturated FAs are often grouped according to the position of the first ω- bond (i.e., n-3, n-6, or n-9 FAs). Lepidopteran FAs may include further modifications including, but not limited to, branching and hydroxylation.

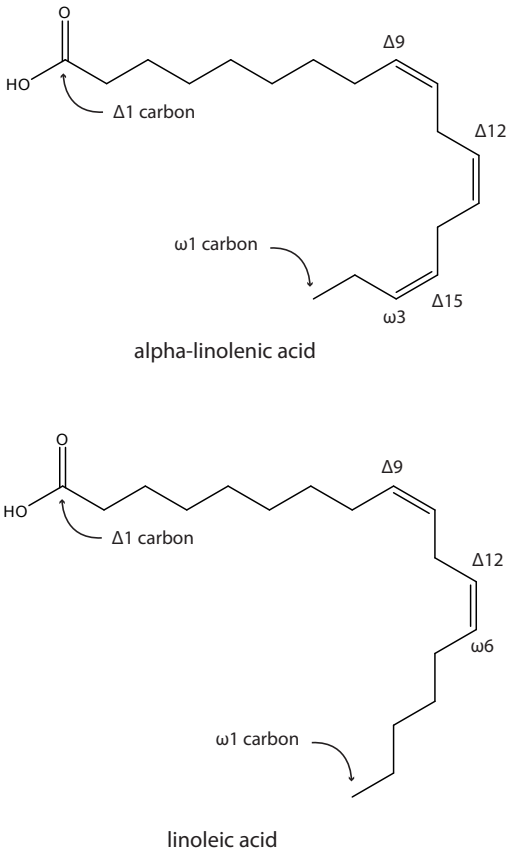


Figure 1.1: Essential fatty acids

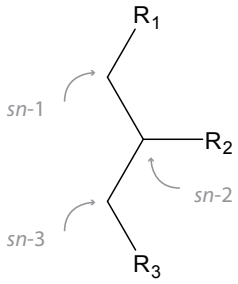


Figure 1.2: Glycerolipids. Monoacylglycerol: R_1=fatty acyl, R_2= R_3=OH Diacylglycerl: R_1= R_2=fatty acyl, R_3=OH Triacylglycerol R_1= R_2= R_3=fatty acyl

1.2.2 Esterified fatty acids

Glycerolipids (Figure 1.2) are FA esters of glycerol. Mono-, di-, and tri-substituted glycerol are referred to respectively as monoacylglycerol (MAG), diacylglycerol (DAG), and triacylglycerol (TAG). Glycerolipids often include stereochemical numbering, or (*sn*) notation to differentiate each of the three carbons in the glycerol backbone.

Phospholipids (PL) (Figure 1.3) are glycerolipids that contain a phosphoric acid derivative at the *sn*-3 position. Phospholipids with only a single FA moiety at either the *sn*-1 or *sn*-2 position are referred to as lysophospholipids.

1.3 Lipid metabolism in Lepidoptera

The fate of dietary FAs, including hydrolysis and mobilisation, will be described. As Lepidoptera are also able to synthesise FA *de novo*, the fundamental differences in Lepidoptera, particularly with respect to desaturase specificity, will be considered.

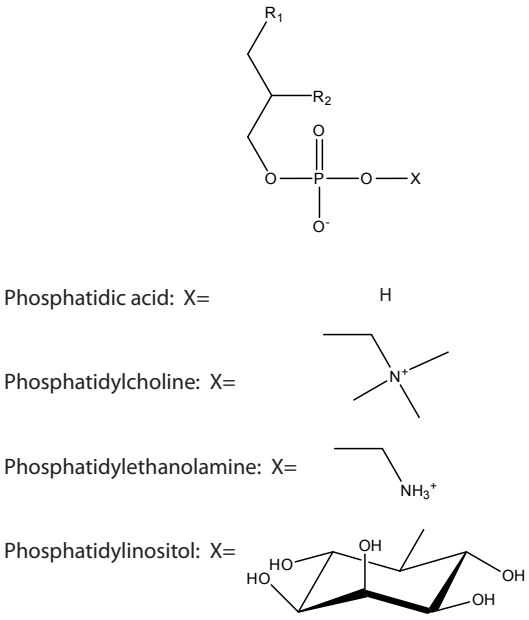


Figure 1.3: Phospholipids. R_1, R_2 = fatty acyl

1.3.1 Metabolism of dietary fatty acids

Most lepidopteran larvæ are phytophagous. Turunen and Chippendale (1989) suggest that insects that feed on photosynthetic tissues (i.e., leaves) are adapted to use FA from polar lipids (i.e., PLs). Triacylglycerols, found in seed oil, tend to be less efficiently hydrolysed. Alpha-linolenic acid is the most abundant FA absorbed (Turunen, 1990), partially because it is the most abundant FA found in plant lipids. Fatty acids are absorbed by midgut cells and converted to DAG and TAG (Canavoso et al., 2004). The acyltransferases responsible for DAG and TAG synthesis tend to be selective, and certain classes of FAs therefore tend to be found at certain positions of the glycerol backbone. For example, the *sn* -2 position of TAG is dominated by 18:2 and α-18:3, while the *sn* -1/3 positions tend to be dominated by saturated FAs (Yeboah and Mitei, 2009).

Fatty acids are stored primarily as TAGs in the fat body. During times

of energy demand (i.e., flight and metamorphosis), FAs are transferred via the hæmolymph as *sn*-1,2-DAG. Several pathways for *sn*-1,2 DAG formation were postulated, but it was elucidated that TAG is first hydrolysed by fat body lipases, which preferentially cleave FAs in the *sn*-1 and 3 positions to form *sn*-2-MAG (Ryan and van der Horst, 2000). An acyl group is then transferred to the *sn*-2-MAG to generate the *sn*-1,2-DAG, a reaction that is catalysed by the enzyme monoacylglycerol acyltransferase (MGAT) (Arrese et al., 1996).

1.3.2 Lipogenesis

Fatty acid synthesis

Starting from acetyl-CoA (2:0), two carbons at a time are added in a sequence of elongation steps that is catalysed by fatty acid synthase (FAS). As a result, lepidopteran tissues contain even chained saturated FAs (SFAs) including palmitic acid (16:0) and stearic acid (18:0). Essentially all insects are thought to have FAs containing up to 20 carbons. Fatty acids containing more than 20 carbons have been detected, although these may only be present in relatively low proportions (Stanley-Samuelson and Dadd, 1983). Propionyl-CoA (3:0) may also serve as a building block for FA biosynthesis, resulting in odd-carbon-chained FAs including 15:0 and 17:0. Propionyl-CoA may result from the oxidation of odd-chained FAs, or from the breakdown of amino acids.

Fatty acid desaturation and elongation

Desaturases catalyse the introduction of double bonds into FAs and are particularly important with respect to pheromone biosynthesis. Both Δ-9 and Δ-11 desaturases have been studied extensively. For example, Rodriguez et al. (2004b) have characterised the genes for both enzymes in the African

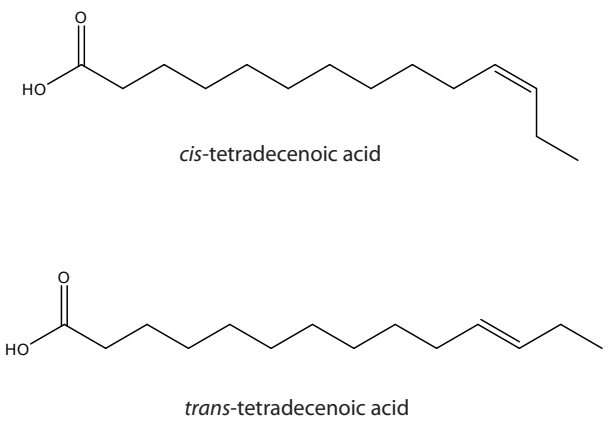


Figure 1.4: Fatty acids with double bonds in the *cis* and *trans* configuration

cotton leafworm (*Spodoptera littoralis*). Some lepidopteran desaturases are capable of generating both the *cis* and *trans* isomers (often referred to as the Z and E isomers, respectively in pheromone related literature). Liu et al. (2002) characterised a desaturase in red-banded leafroller moth (*Argyrotænia velutinana*) that produces both the *cis* and *trans* isomer of 11-tetradecanoic acid (Figure 1.4).

Unlike plant desaturases, lepidopteran desaturases are unable to introduce double bonds at the Δ-12 or Δ-15 carbons. Consequently, Lepidoptera are unable to synthesise 18:2 and α-18:3 *de novo*, which is why they must obtain these FAs from the diet. Some insects, including the cricket *Acheta domesticus* (Cripps et al., 1990) and the cockroach *Periplaneta americana* (de Renobales et al., 1987) do express a Δ-12 desaturase, which allows them to synthesise 18:2(n-6) *de novo* from 18:1. Crickets were grown under axenic conditions, which rules out the possibility of microbial 18:2 biosynthesis (Borgeson and Blomquist, 1993).

To date, Δ-12 desaturase activity has not been reported in Lepidoptera. There have, however, been reports of desaturases with bifunctional activity. Serra et al. (2006) reported a bifunctional Δ-11 desaturase in *S. littoralis*. Palmitic acid (16:0) is converted into *trans* -10, *cis* -12 16:2 by desatura-

tion and migration of the double bonds. In *Thaumetopœa pityocampa*, the processionary moth, Abad et al. (2007) found an enzyme that catalyses the desaturation of both the Δ-11 and Δ-13 carbons. The possibility of a bi-functional lepidopteran enzyme that catalyses the desaturation of the Δ-15 carbon can therefore not be ruled out.

Lepidopteran desaturases do, however, facilitate the introduction of double bonds closer to the Δ-carbon. Most species can therefore elongate and desaturate dietary 18:2 and α-18:3 to form FAs of 20 carbons or more.

1.4 Function of fatty acids in insects

In addition to universal functions such as energy storage, signalling and membrane structure, lepidopteran FAs have several unique functions, which are relevant at all stages of development.

1.4.1 Protection

Fatty acids, either in free form, or as wax esters, are major components of cuticular wax, which prevents moisture loss and protects the insect against fungal and bacterial infection (Gibbs, 1998). Golebiowski et al. (2008) provide evidence that dietary 16:0, 18:0, 18:1, 18:2 were able to protect *Galleria mellonella* (Greater Wax Moth) larvæ from fungal pathogens. These FAs tended to fluctuate during the final instar, and larvæ were more susceptible to infection when the FAs were present at decreased levels. Hoch et al. (2002) found that in *Lymantria dispar* (gypsy moth) larvæ infected by fungi, the hæmolymph levels of the same FAs, plus α-18:3, were significantly reduced.

Unsaturated FAs may also protect insects against other plant derived toxins including 2-undecanone, which is found in the trichomes of wild tomato. Farrar et al. (1992) found that increasing the dietary concentration of several

FAs, including α-18:3, in *Helicoverpa zea*, the tomato fruitworm, was highly effective in reducing pupal mortality normally caused by 2-undecanone. Farrar et al. (1992) suggest that 2-undecanone affects FA transport, particularly that of α-18:3 itself, and is likely not a competing substrate.

A number of insects, including ants and cockroaches, recognise conspecific dead using unsaturated FA (i.e., 18:1 and 18:2) as "necromone" cues. Yao et al. (2009) predicted that Lepidoptera would do the same and subsequently found that both tent caterpillars (*Malacosoma americanum*) and fall webworms (*Hyphandria cunea*) avoided branches that were treated by 18:1 and 18:2.

1.4.2 Phagostimulator

Li and Ishikawa (2004) tested common bluebottle (*Graphium sarpedon nipponum*) larvæ on leaf extracts isolated from its host, the camphor tree (*Cinnamonum camphora*), and found that these stimulated feeding. Alpha-linolenic acid was identified as the bioactive agent. The larvæ also showed increased feeding response when given α-linolenic acid standards compared with controls and γ-linolenic acid (*cis* -6, *cis* -9, *cis* -12 18:3). The feeding activity of the larvæ was also tested on other leaves, including Japanese Orixa (*Orixa japonica*), which does not contain α-18:3. The larvæ could feed on all the plants except for *O. japonica*.

1.4.3 Energy stores

Fatty acids obtained by the larva provide energy for functions later in development. Unsaturated FAs in TAG, including α-18:3, are mainly used as a flight energy source. Murata and Tojo (2002) found that the ratio of α-18:3 in *Spodoptera litura* TAG decreased with increasing flight duration.

In some species, such as *Homona coffearia*, the tea tortrix, both 18:2 and

α-18:3 have been thought to be critical for successful emergence (Sivapalan and Gnanapragasam, 1979). When these FAs were given in suboptimal levels, the moths only emerged partially.

1.4.4 Oviposition deterrent

Females respond to various cues in order to avoid occupied oviposition sites, thus reducing intraspecific competition for limited resources. Gabel and Thiry (1996) found that application of egg extracts from the European grape vine moth (*Lobesia botrana*) resulted in a reduced number of eggs laid by conspecific females. The active compounds were identified as 16:0, 16:1, and 18:1. A similar response was seen when esters of these FA were used. Li et al. (2001) found that when the female *Helicoverpa armigera* (cotton bollworm) contacts the oviposition substrate with her tarsi, a blend of 16:0 and 16:1 are left behind, which deters other females from laying eggs. Similarly, Li and Ishikawa (2004) found that the larval frass of *Ostrinia* species prevented conspecifics from ovipositing. The bioactive extracts consisted mainly of FAs, namely 16:0, 18:0, 18:1, 18:2, and α-18:3. Synthetic mixtures of these FAs also produced significant oviposition deterring effects.

1.5 Fatty acid metabolites in Lepidoptera

Both 18:2 and α-18:3 can be further elongated and desaturated to long chain PUFAs, which in turn may be precursors to eicosanoids and prostaglandins (PG). Fatty acids may also be substrates for FA conjugates, pheromones, or FA esters.

1.5.1 Linoleic acid metabolites

Linoleic acid is important to insects as it is a precursor to dihomo-γ-linolenic acid, 20:3(n-6), and arachidonic acid, 20:4(n-6), which in turn are precursors for biologically active eicosanoids. Stanley-Samuelson et al. (1988) propose the following pathway for n-6 FA biosynthesis in insects:

$$18{:}0 \rightarrow 18{:}1(\text{n-9}) \rightarrow 18{:}2(\text{n-6}) \rightarrow 18{:}3(\text{n-6}) \rightarrow 20{:}3(\text{n-6}) \rightarrow 20{:}4(\text{n-6})$$

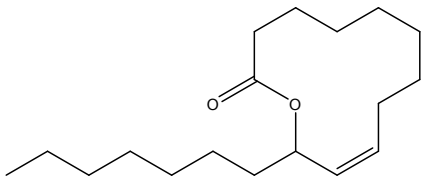
In mammals, 20:4(n-6) may be converted via one of three pathways: 1) the cyclooxygenase pathway, which results in PGs and thromboxanes; 2) the lipoxygenase pathway, which results in hydroperoxyeicosatetraenoic and hydroxyeicosatetraenoic acids (HPETEs and HETEs, respectively); and 3) the epoxidase pathway, where cytochrome P450 enzymes produce epoxyeicosatrienoic acids. The function of PGs has been explored in several species. For example, PGs signal ovarian follicle development in the silkworm, *Bombyx mori* (Machado et al., 2007). Hagan and Brady (1982) suggested that PGs regulate reproductive behaviour in *Trichoplusia ni*. To date, no metabolites of the epoxidase pathway have been described in insects (Stanley, 2011).

1.5.2 Linolenic acid metabolites

In insects, as in animals, α-18:3 is converted into long chain PUFA. The PUFA conversion pathway in animals was outlined by Sprecher et al. (1995):

$$18{:}3(\text{n-3}) \rightarrow 18{:}4(\text{n-3}) \rightarrow 20{:}4(\text{n-3}) \rightarrow 20{:}5(\text{n-3}) \rightarrow 22{:}5(\text{n-3}) \rightarrow \text{C24 PUFA} \rightarrow 22{:}6(\text{n-3})$$

In *G. mellonella*, α-18:3 was elongated and further desaturated to give eicosapentaenoic acid (EPA) (Stanley-Samuelson and Dadd, 1984). Matsuoka et al. (2008) identified metabolites 20:3 (n-3), and 22:3 (n-3) in the wasp moth, *Syntomoides imaon*. Sushchik et al. (2003) investigated the FA compositions of several benthic invertebrates including caddisflies (Trichoptera, which is closely related to Lepidoptera) and found intermediates



(*cis*-9,11S)-octadec-9-en-11-olide

Figure 1.5: A macrolide derived from α-18:3

of the Sprecher pathway.

The compound eyes of *B. mori* also contain considerable amounts of 20:5(n-3) (Eguchi et al., 1994). Eicosapentaeonic acid is found in high amounts in the reproductive tissues of the cabbage white, *Pieris brassicæ*, although the amounts found in the diet are negligible. The majority of this was incorporated into phosphatidylinositol.

An unusual α-18:3 metabolite is the macrolide (*cis* -9,11S)-octadec-9-en-11-olide (Figure 1.5), which has been observed in the scent glands of the Costa Rica longwing butterflies, *Heliconius cydno* and *H. pachinus* (Schulz et al., 2007).

Small white (*Pieris rapæ*) larvæ have glandular hairs that secrete mayolenes (Figure 1.6), compounds derived from 11-hydroxylinolenic acid. Smedley et al. (2002) found that these mayolenes are a potential deterrent towards other insects as shown in bioassays with the ant, *Chrematogaster lineolata.*

Another derivative of α-18:3 is jasmonic acid (JA)(Figure 1.7). This well-known phytohormone can be found in substantial amounts in the eggs and neonates of at least nine species of Lepidoptera (Tooker and de Moraes, 2005). While some of this may have accumulated from the diet, Tooker and de Moraes (2005) suggest that JA may also be synthesised *de novo* as unfed neonates contained significantly higher amounts of JA than the eggs. While JA initiates plant defence responses, the physiological role of JA in Lepdioptera has yet to be determined.

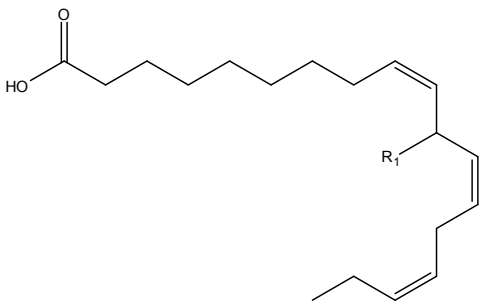


Figure 1.6: Mayolene. R_1 = fatty acyl

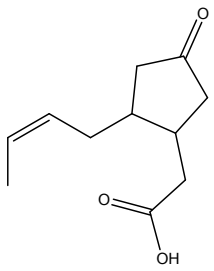


Figure 1.7: Jasmonic acid

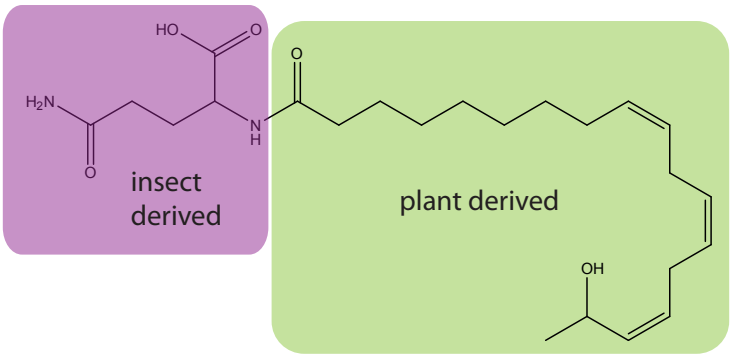


Figure 1.8: Volicitin ((*N*-hydroxylinolenoyl)-L-glutamine)

1.5.3 Fatty acid conjugates

The oral secretions of several species contain FA conjugates (FACs) consisting of a FA (or derivative thereof) and an amino acid, namely glutamine or glutamic acid. One of the best known is volicitin, or (N-hydroxylinolenoyl)-L-glutamine (Figure 1.8) (Alborn et al., 1997). Through labelling studies, Paré and Tumlinson (1999) determined that the FA component was plant derived, although the glutamine originates from the insect. Furthermore, they determined that the hydroxylation and conjugation reactions are carried out in the insect. Volicitin triggers defence responses in plants including the release of volatiles that attract parasitic wasps to attack their larval prey. Another common FAC is N-linolenoyl-L-glutamine. Spiteller et al. (2004) also found FAC-containing phosphorylated FA derivatives.

These FACs, however, are also thought to benefit the insect, acting as a surfactant to aid digestion (Spiteller et al., 2000) or allowing the insect to store glutamine, which is crucial in nitrogen metabolism (Yoshinaga et al., 2010). Although glutamine-containing FACs have been studied in considerable depth, little is known about the physiological function of glutamic acid containing FACs (Mori and Yoshinaga, 2011).

1.5.4 Pheromones

Lepidopteran pheromones either originate from saturated fatty acyl compounds synthesised by the insect *de novo* and are subsequently modified by other reactions (i.e., desaturation, elongation, reduction, and chain shortening), or are derived from dietary FAs (Wei et al., 2004). Most FA-derived pheromones may be classified into various functional groups, which include sex, aggregation, dispersal, alarm, recruitment, and maturation (Tillman et al., 1999).

Several pheromones are derived from α-18:3. In addition, bombykol linolenate (Yamaoka et al., 1985) was found in the hæmolymph of *B. mori*. This ester may function as a trap to prevent the inappropriate release of bombykol. Alpha-linolenic acid may also be reduced to produce linolenal or elongated to give *cis* -11, *cis* -14, *cis* -17 20:3 and *cis* -13, *cis* -16, *cis* -19 22:3 (Tillman et al., 1999). Ding et al. (2011) recently found that in the winter moth (*Operopthera brumata*), *cis* -11, *cis* -14, *cis* -17 20:3 is also further desaturated at the terminal carbon, which is atypical, to give *cis* -11, *cis* -14, *cis* -17, 19 20:4. These in turn are further modified (e.g., decarboxylated, oxidised) to produce other pheromones.

1.5.5 Fatty acid esters

The FA esters found in Lepidoptera generally function as pheromones, although esters are also present at other developmental stages. Bergmann et al. (2007) identified a number of FA derivatives, including docecyl acetate and *cis* -5, *cis* -13 tetradecadienyl acetate in larvæ of the butterworm (*Chilecomadia valdiviana*). None of the compounds were found in the pupæ suggesting that biosynthetic pathways may differ in various developmental stages.

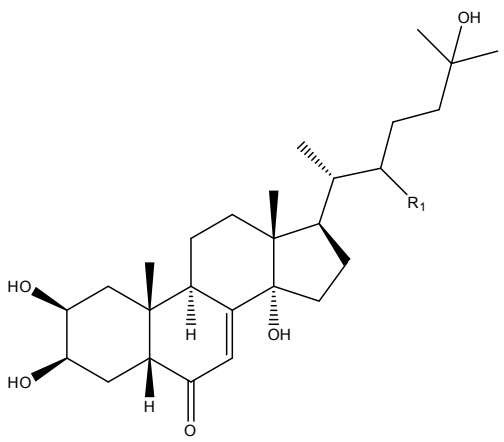


Figure 1.9: Ecdysone-22-fatty acyl ester. R_1 = fatty acyl.

Kayser (1975) identified several FA diesters and monoesters of lutein in the fat body of *P. brassicæ*. In the insects that were reared on natural diets (i.e., plants), linolenate was the predominate ester. During adult life, a strong increase of the diesters was noted.

Ecdysteroids are insect moulting hormones that signal the insect to shed its rigid exoskeleton, enabling further growth. To defend against insect herbivory, many plants produce phytoecdysteroids, which are identical to ecdysteroids normally produced by the insects. The phytoecdysteroids, however, are produced in substantially higher amounts, thereby causing premature moulting. The resistance of *H. virescens* larvæ to phytoecdysteroids (i.e., ecdysone, or 20-hydroxyecdysone) has been attributed to the formation of ecdysteroid acyl esters, which are thought to facilitate the rapid excretion of these hormones (Zhang and Kubo, 1992). The enzyme responsible for their formation, 22-O-acyltransferase, has been located in the gut epithelia (Kubo et al., 1994). Whiting and Dinan (1988) have observed esters of linolenate and ecdysone (Figure 1.9) in *A. domesticus*.

Plants also release large quantities of methanol in response to herbivore attack. To protect against intoxication, FA methyl esters (FAMEs) may be produced by the insect. Guo et al. (2010) found increased levels of FAMES

in the Asian corn borer (*Ostrinia furnacalis*), which had been exposed to methanol-containing diets.

1.6 Factors affecting fatty acid metabolism

The lipid content of Lepidoptera is not fixed, and can be influenced by various factors including the presence of symbionts, developmental stage, environmental conditions, and diet.

1.6.1 Symbionts

The insect gut is inhabited by numerous microorganisms, which may either directly provide FAs to their host (Breznak and Brune, 1994), or produce enzymes that modify lipids already present. Visotto et al. (2009) investigated bacterial contribution to lipase activity in *Anticarsia gemmatalis*, the velvetbean caterpillar. Lipase activity was significantly reduced by tetracycline, although this had little effect on developmental success. Insect microflora are also partly responsible for the synthesis of FACs (Ping et al., 2007).

Parasites may influence insect FA composition. Hoch et al. (2002) investigated the hæmolymph of larvæ of *L. dispar*, that were infected with *Vairimorpha sp.* , which are microsporidian parasites, and found that the levels of 16:0, 18:0, 18:1, 18:2, and 18:3 were decreased. The same effects were not evidenced when *Lymantria* were infected with *Endoreticulatus* (Hoch et al., 2006). Likewise, no effect on FA composition was observed by Nurullahoglu et al. (2004) in their study of the lesser wax moth (*Achoria grisella*) parasitised by *Apanteles galleriae*.

1.6.2 Developmental stage

Lepidopteran eggs necessarily contain a large amount of FAs that are essential for embryonic development. Thiery et al. (1995) investigated FA in codling moth (*Cydia pomonella*) eggs. The amount of FA in the eggs increased until the eggs were four days old, and then decreased prior to hatching. Oocytes are able to synthesise TAG and PL, but the amount of FAs synthesised *de novo* is negligible.

While neutral lipid (i.e., TAG) FA composition remains relatively constant during metamorphosis, that of PL changes (reviewed in Khani et al. (2007)). Wang et al. (2006) investigated the total FA profiles of the Peleides blue morpho, *Morpho peleides*, before and after metamorphosis, and found that there was a "bioenhancement" of PUFA from larvæ to adult. Conversely, Cookman et al. (1984) found that regardless of diet (plant or artificial), the newly eclosed *A. gemmatalis* adults exhibited a decrease in the percentage of α-18:3 when compared to that of the larvæ.

There are differences between FA utilisation in males vs. females. In *P. brassicæ*, males utilised their FA stores more evenly, while females lost unsaturated FAs including α-18:3 during adulthood (Turunen, 1974).

1.6.3 Environmental conditions

Fatty acid composition can change in order to facilitate cold acclimation. In general, a decrease in temperature results in an increase in PUFA content, mainly in membrane PL. For example, Izumi et al. (2009) noted that in last instar larvæ of the Asiatic rice borer *Chilo suppressalis*, the proportion of unsaturated FA in PE (mainly 18:1) increased as temperatures decreased. The increase in membrane PUFA stabilises membrane viscosity and allows for the maintenance of function regardless of temperature.

During extreme conditions, insects enter a physiological state of dormancy,

or diapause, which is often marked by changes in FA composition. Atapour et al. (2007) found that in *C. suppressalis* larvæ 16:1 content increased until the onset of diapause. Shimizu (1992) compared the FA compositions of diapause and non-diapause eggs of *B. mori* and found that, while the composition of TAG had a similar pattern in both eggs, the PC of diapause eggs had a higher proportion of α-18:3.

1.6.4 Diet

Lepidopteran FA composition tends to reflect that of the diet (Cookman et al., 1984), particularly in generalists. Major lipid classes ingested by phytophagous Lepidoptera include triacylglycerols (TAGs), PLs, and glycoglycerolipids. The predominant FAs found in plant lipids include 16:0, 18:0, 18:1, 18:2 and α-18:3 in addition to some longer chain SFAs and MUFAs. Turunen (1974) suggest that some insects can also modify dietary lipids (e.g., elongation or desaturation).

Lepidoptera have different mechanisms for responding to differences in FA availability. *P. brassicæ* larvæ reared on artificial diets deficient in α-18:3 responded with increased synthesis of 16:1, suggesting that the larvæ were attempting to compensate for the lack of essential FAs. "Nutrient self selection" is the ability to select an optimal diet by combining two or more different food sources. Fifth instar *H. zea* larvæ were able to select from both of two experimental diets, one deficient in lipid, and the other in vitamins (Schiff et al., 1988). Similar results were obtained by Stockhoff (1993) in *L. dispar* .

After feeding *H. virescens* a defined diet and analysing the frass, Dikeman et al. (1981) suggested that PUFAs, in particular α-18:3 and 18:2, may be selectively absorbed. Cookman et al. (1984) also found that the α-18:3 composition of *A. gemmatalis* larvæ was four times higher than that of the diet that it was reared on.

It is generally thought that Lepidoptera obtain most of their required FAs during the larval stage in order to establish energy reserves for metamorphosis, flight, and oogenesis. Bauerfeind et al. (2007), however, argue that in some species, adult acquired lipids may also be of great importance to fecundity, although they were not able to detect any beneficial effect of adult-derived PUFAs or cholesterol.

Fatty acids present in the diet can also affect *de novo* FA synthesis. Horie and Nakasone (1971) found that when *Manduca sexta* (tobacco hornworm) larvæ received increased dietary FA, the rate of FA synthesis decreased, suggesting that FA synthesis is regulated via negative feedback.

Other components in the diet can affect the rate of FA synthesis metabolism. Horie and Nakasone (1968) found that *M. sexta* larvæ reared on diets deficient in biotin had reduced levels of 18:1. The levels of 18:2 and 16:1, however increased. One explanation for this effect is that acetyl-CoA carboxylase, an enzyme involved in FA synthesis, requires biotin as part of its prosthetic group. The rate of FA synthesis in *M. sexta* also increased after increasing dietary sucrose (Horie and Nakasone, 1971).

1.7 Absolute linolenic acid requirements

Artificial diets are useful for measuring the FA requirements of Lepidoptera. Vanderzant (1965) outlined the minimal amounts of nutrients and minerals required for successful insect growth. In addition to α-18:3, these included cholesterol (Ritter and Nes, 1981), inositol, and choline.

When rearing Bertha armyworm, *Mamestra configurata,* on an α-18:3 deficient artificial diet, Bracken (1982) noticed that, in comparison to insects reared on leaves, a large proportion developed "pupal syndrome" whereby the pupæ failed to shorten. The occurrence of pupal syndrome was reduced upon addition of α-18:3 to the diet.

Ritter and Nes (1981) found that removal of wheat germ and corn oil and replacement with 18:2 and α-18:3 did not have an effect on maturation or fecundity of *H. zea*, although the growth rate was reduced and pupal weight was decreased.

Rock (1985) investigated dietary FA requirements in *Platynota idaeusalis,* the tufted apple budmoth. Larvæ were reared on artificial diets containing no FAs, corn oil, 18:1, 18:2, α-18:3, or 20:4(n-6). Corn oil, 18:2, and α-18:3 were able to fully alleviate wing deformities. Arachidonic acid was also partially successful in alleviating deformities.

The bioavailability of α-18:3 may have a significant effect on successful insect development. In studies by Bracken (1982), more α-18:3 was sequestered by *M. configurata* when it was fed on leaves than when it fed on the artificial diet (23% vs. 16.3% respectively). A possible explanation for this could be that in the leaves the major form of α-18:3 was MGDG, DGDG, and PL while the artificial diet contained either free FA or α-18:3-containing TAG. Turunen (1979) suggested that PUFAs contained in PL may be more digestible.

1.8 Applications for insect lipids

An increased knowledge of lepidopteran FAs and their metabolism has many potential applications. Fatty acids and derivatives can be directly extracted from insects for industrial purposes. The insect's metabolism can also be manipulated to increase nutraceutical FAs or, conversely, to decrease the biosynthesis of FAs or metabolites in a manner detrimental to survival.

1.8.1 Industrial applications

Insect FAs also have many industrial applications, for example in soap production (Sreekantaswamy and Siddalingaiah, 1981). Chrysalis oil from *M. sexta*, which is rich in α-18:3, is highly valued as a drying oil (Majumder and Sengupta, 1979). Drying oil is an essential component of oil paints and varnishes, allowing them to harden upon exposure to oxygen. α-18:3 is particularly suited to this purpose as it contains a high number of double bonds available for oxygen insertion.

1.8.2 Health and nutrition

In many developing countries, edible insects are an important food source, and their rearing has been considered a strategy for achieving global food security. Although entomophagy has generally been a taboo in Western culture, the practice is gaining increased acceptance. For example, the mophane caterpillar (*Imbrasia belina*) is gaining increased recognition as a food source in southern Africa (Yeboah and Mitei, 2009). Aside from being rich in high quality proteins, phytophagous insects are rich in essential FAs such as 18:2(n-6) and α-18:3.

Park et al. (2006) systematically increased the conjugated linoleic acid (CLA) content of *B. mori* by feeding mulberry leaves sprayed with increasing amounts of the *cis* -9, *trans* -11 isomer, which is known to confer various beneficial properties including anticarcinogenic activity (Ip et al., 1991). Mentang et al. (2011) tested the effects of *B. mori* chrysalis oil, also rich in α-18:3, and suggest that it can prove conditions such as hyperglycermia in rats. These results suggest that silkworm oil can compete with other nutraceutical oils such as fish oil and flax seed oil.

Law and Wells (1989) argued that insects, including *M. sexta*, may be valuable biochemical models, as there are many similarities to mammalian sys-

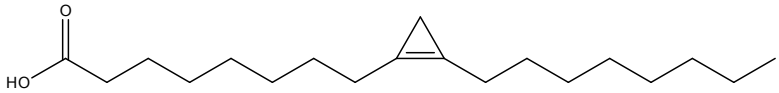


Figure 1.10: Sterculic acid

tems. Insects can be easily reared in large numbers and are generally not subject to legal regulations imposed on other vertebrate test species. Many prevalent human diseases arise from irregularities in FA biosynthetic pathways that are also present in insects.

1.8.3 Pest control

The cyclopropene FA, sterculic acid (Figure 1.10) is an analogue of oleic acid (*cis* -9 octadecenoic acid or 18:1(n-9)) and can competitively inhibit Δ-9 desaturase, an integral enzyme in insect pheromone biosynthesis (Al Dulayymi et al., 1996). Similarly, cyclopropenol analogues inhibited desaturases involved in the pheromone biosynthesis in *S. littoralis*, (Rodriguez et al., 2004a). One isomer of CLA, *trans* -10, *cis* -12 octadecadienoic acid, has also been known to inhibit Δ-9 desaturase (Choi et al., 2000). European corn borer moth (*Ostrinia nubilalis*) larvæ were fed diets supplemented with this isomer (Gereszek et al., 2008). A decrease in unsaturated FAs in pupal and adult tissue along with concurrent decreases in survival rate were noted.

Strategic application of these FAs may potentially aid in pest control, restricting mating and propagation of many pest species. Field testing is, however, required to confirm that they do not interfere with FA desaturation of other species.

1.9 The future of insect lipid research

While the last decades have seen some important contributions to insect lipid research, information about the exact function of α-18:3 remains sparse. It may be prudent to further consider the role of this FA in other animal species, as there are likely to be other parallels to insects. Tinoco (1982) provides a comprehensive review of the function of α-18:3 in a broad range of species.

In humans, EPA is converted into PGs. Pärnänen and Turunen (1987) suggest that the same may occur in *P. brassicæ*, where α-18:3 is a precursor of EPA. Dabrowska et al. (2009) identified an enzyme in *S. littoralis* and *H. armigera* that converts 12-oxophytodienoic acid (*cis* -OPDA) to *iso* -OPDA. This reaction resembles PG transformation in mammals. This suggests that insects may have additional enzymes for PG metabolism. If such transformations are essential, this may provide an additional target for pest control. Büyükguüzel et al. (2011) explored inhibiting eicosanoid synthesis in *G. mellonella* and found that it influenced development to a small extent, although the inhibitors also reduced the production of its parasitoid's (*Bracon hebetor*) eggs.

While α-18:3 can be elongated and further desaturated to produce EPA and DHA, perhaps the major metabolic route of α-18:3 is β-oxidation (Pan and Storlien, 1993). In rats, the greater the proportion of PUFA, the higher the rate of α-18:3 β-oxidation. Another route of α-18:3 metabolism is carbon recycling. When labelled α-18:3 is fed to animals, a substantial portion of the label can be found in SFA and MUFA (Cunnane et al., 1995). Lambremont et al. (1976) reared boll weavils (*Anthonomus grandis*) on diet containing labelled 18:0 and found that the label was incorporated into 16C FAs. This suggests that the same may be possible for α-18:3 in Lepidoptera.

Many plants are abundant in *cis* -7, *cis* -10, *cis* -13 hexadecatrienoic acid, or 16:3(n-3). Cunnane et al. (1995) demonstrated that rats are able to

convert 16:3(n-3) to α-18:3. Insects may be capable of performing the same conversion. It would be worthwhile to assay for such enzyme activity, although this is made difficult by the limited availability of unusual labelled substrates such as 16:3 (n-3).

Some earlier studies (e.g., Municio et al. (1971)) suggest that aphids were able to synthesise α-18:3 from labelled acetate, although it is likely that there was co-migration of labelled 18:1(n-9). The silverleaf whitefly, *Bemisia argentifolii*, appears to be able to convert labelled acetate to α-18:3 (Buckner and Hagen, 2003). This would most likely require Δ-15 desaturase activity. As yet, Δ-15 desaturase has not been observed in insects. In the nematode, *Cænorhabditis elegans*, and some species of *Artemia* (brine shrimp) (Ito and Simpson, 1996), there is also evidence of endogenous α-18:3 biosynthesis.

To a large extent, only the effects of individual dietary FA have been studied in insects. Studies in rats have shown that the ratio of ω-3 to ω-6 FA can also be of importance (Lee et al., 1989). Perhaps this is also the case in insects — the absolute amount of dietary α-18:3 may be less important than the ratio in relation to other FAs.

Insect FAs have only been studied at the ecological/nutritional level. Further insight into the chemical and physical properties of insect FAs is likely to provide a greater understanding of their physiological properties. For example, the position of double bonds can have a substantial effect on the physical properties of FAs. Ehringer et al. (1991) compared the properties of PL bilayers containing α-18:3 or γ-18:3 and found that α-18:3 provided enhanced permeability. With the help of technological advances in a range of scientific fields, a complete and multi-disciplinary understanding of insect FAs is obtainable.

Chapter 2

Nuances in the dietary alpha-linolenic acid requirements of generalist and specialist Heliothinæ

2.1 Introduction

Lepidoptera, like most animals, are neither able to synthesise α-linolenic acid α-18:3) nor linoleic acid (18:2) *de novo*. These essential fatty acids (FAs) must therefore be obtained via their diet. Without sufficient dietary α-18:3, most larvæ are unable to successfully complete development. A low proportion reach the pupal and adult stages, and individuals that eclose are often malformed (Bracken, 1982).

Exceptions may exist. The *de novo* synthesis of 18:2 in cricket (*Acheta domesticus*) via a Δ-12 desaturase has recently been reported (Cripps et al., 1990). Furthermore, labelled α-18:3 was detected in silverleaf whiteflies (*Bemisia argentifolii*) that were fed labelled acetate, thereby suggesting the possibility of *de novo* α-18:3 synthesis in insects (Buckner and Hagen, 2003). In

the nematode (*Cænorhabditis elegans*), an ω-3 desaturase, which is responsible for the conversion of 18:2 to α-18:3, has been characterised (Meesapyodsuk et al., 2000). However, no homologue has been reported in insects. These results suggest that *de novo* synthesis of α-18:3 is theoretically possible in other species. A common ancestor may have had this ability and it was either lost or repressed during evolution.

Heliothis virescens, a generalist, and *Heliothis subflexa*, which specialises on *Physalis* species, are closely related lepidopterans. De Moraes and Mescher (2004) suggested that unlike *H. virescens*, *H. subflexa* does not have typical α-18:3 requirements as they are able to thrive on cutleaf groundcherry (*Physalis angulata*) fruits — despite the apparent absence of dietary α-18:3 in the fruits. *Heliothis virescens* were unable to grow on the fruits unless they were sprayed with α-18:3. Additionally, the regurgitant of *H. subflexa* larvæ fed on *Physalis* fruits did not contain the FA conjugate, volicitin (*N*-(17-hydroxylinolenoyl)-L-glutamine), for which α-18:3 is a precursor. De Moraes and Mescher (2004) suggested that this gives *H. subflexa* another advantage on *Physalis* as they are rendered less vulnerable to the parasitoid, *Cardiochiles nigiceps*, which is attracted to volicitin.

While the claims of De Moraes and Mescher (2004) have serious implications, especially considering that this is the first report of a lepidopteran without dietary α-18:3 requirements, they also provoke additional questions. First, does *P. angulata* really lack α-18:3? Bateman (2006) performed FA analysis on several species of *Physalis* and found substantial amounts of α-18:3 in various tissues. Second, does *H. subflexa* really have a zero requirement for α-18:3? De Moraes and Mescher (2004) neither performed a side-by-side comparison of the two *Heliothis* species on *Physalis* nor on an artificial diet.

It was therefore prudent to follow up on the study of De Moraes and Mescher (2004) with a systematic comparison of the two species. A meridic diet was used, allowing for the manipulation of the α-18:3 available to each species. *Heliothis subflexa* does indeed have dietary α-18:3 requirements, although

the two species appear to respond differently to alterations in dietary α-18:3 composition.

2.2 Materials and methods

2.2.1 Chemicals

Pure FAs (18:1, 18:2, and α-18:3) were obtained from Acros Organics (Geel, Belgium). Fatty acid free casein was from Merck KGaA (Darmstadt, Germany). Vanderzant vitamin mix, Wesson's salt, sucrose, methyl 4-hydroxybenzoate, and sorbic acid were from Bio-Serv (Frenchtown, NJ, USA). Agar and solvents were from Carl Roth GmbH (Karlsruhe, Germany). Boron trifloride and lipid standards, and all remaining compounds were from Sigma-Aldrich (Schnelldorf, Germany).

2.2.2 Insects

Insects were obtained from the Gould laboratory (North Carolina State University, Raleigh). Larvæ were reared on a 16:8 light:dark cycle at 55% relative humidity at 26 °C. *Heliothis virescens* larvæ were normally maintained on a pinto bean diet (Table 2.1), while *H. subflexa* were normally maintained on a corn soy blend diet (Table 2.2).

2.2.3 Minimal diet experiments

The minimal diet used (Table 2.3) was based on one described by Vanderzant (1968) with several modifications. Cholesterol and FA were first dissolved in chloroform adsorbed to the α-cellulose to ensure even distribution of these components. Water soluble β-carotene was prepared as described by Pfitzner et al. (2000). Diets were prepared containing 0, 100, 250, 500, or 1000 μg of

Table 2.1: Composition of standard pinto bean diet

Component	Quantity (for 2400g diet)
ground pinto beans	125 g
wheat germ	100 g
soy protein	50 g
casein	50 g
torula yeast	62.5 g
ascorbic acid	6 g
methyl 4-hydroxybenzoate	5 g
sorbic acid	3 g
chlorotetracycline HCl	250 mg
Vanderzant vitamin mix	10 g
agar	35 g
distilled water	1950 ml

α-18:3 per g of diet (fresh weight). Second instar larvæ were weighed and transferred to individual plastic cups containing a cube of diet approximately 1 cm^3 in size. At two-day intervals, the insects were weighed and supplied with fresh diet. Parameters including pupal weight, time to pupation, time to eclosion, and health of adult were recorded for each insect. For both *H. virescens* and *H. subflexa*, two replicates were performed; one in which the two species were reared concurrently (Trial 1), and one where they were reared at different time points (Trial 2).

2.2.4 Pinto bean diet experiments

Three pinto bean diets were prepared. The PBN diet was the standard diet, on which the *H. virescens* were normally reared. Both PB+ and PB- were made with FA free casein, defatted soy protein and defatted wheat germ (Hafen-Mühlen-Werke GmBH, Bremen). The PB+ diet was supplemented with 1460 μg/g α-18:3, 520 μg/g 18:2, and 105 μg/g 18:1. The PB- diet was only supplemented with 520 μg/g 18:2,and 105 μg/g 18:1. The FAs were adsorbed to α-cellulose as described for the minimal diet. One group

Table 2.2: Composition of corn soy blend diet

Component	Quantity (for 1500g diet)
corn soy blend	350 g
dry milk powder	18.3 g
torula yeast	20 g
methyl 4-hydroxybenzoate	4 g
sorbic acid	2 g
ascorbic acid	30 g
Vitamin mix	7.5 g
agar	30 g
distilled water	1050 ml

Table 2.3: Composition of miminal diet

Component	Quantity (for 800g diet)
α-cellulose	40 g
cholesterol	400 mg
α-18:3	variable
menadione	4 mg
cholecalciferol	4 mg
FA free casein	32 g
Wesson's salt	8 g
sucrose	32 g
cysteine-HCl	800 mg
methyl 4-hydroxybenzoate	2 g
sorbic acid	1.2 g
zinc acetate	400 μg
cobalt chloride	200 μg
sodium molybdate	200 μg
10 M potassium hydroxide	2.4 ml
chlorotetracycline HCl	100 mg
Vanderzant vitamin mix	8 g
water soluble β-carotene complex	200 mg
agar	12 g
distilled water	650 ml

of larvæ was started on the PBN diet. The remaining larvæ were started on the PB- diet. Every three days, a subset of larvæ were transferred to the PB+ diet. Parameters including pupal weight, time to pupation, time to eclosion, and health of adult were recorded for each insect. Adults with no noticeable deformities were classified as "healthy. Partially eclosed adults and fully eclosed adults with crumpled wings were classified as "abnormal . Adults were stored at -20 °C until extraction.

2.2.5 Lipid extraction

Total lipids were extracted from insects as described by Bligh and Dyer (1959). The adult was placed in a 4 ml vial to which 100 μg triheptadecanoin, an internal standard, had been added. Next, 0.8 ml chloroform, 1.6 ml methanol, and 0.8 ml distilled water were added to the vial and the adults were homogenised for 1 min using a Polytron dispersing aggregate (5 mm $\varnothing$, Kinimatica AG, Lucerne, Switzerland). An additional 0.8 ml of chloroform was added to the vial, which was then centrifiged for 2 min at 3000 *g*. The chlorform layer was then transferred to a new vial and the solvent was evaporated under a stream of nitrogen.

2.2.6 GC analysis of insects

The lipid extracts were methylated with 14% boron trifluoride in methanol to produce fatty acid methyl esters (FAMEs) for analysis, and 100 μg of methyl nonadecanoate was added as a second internal standard. Aliquots of 1 μl were injected at 60 °C, with a split ratio of 20:1, into an Agilent 7890 gas chromatograph (GC) equipped with a DB-Wax column (30 m x 0.25 mm diameter x 0.25 μm film thickness) and a flame ionisation detector with helium as a carrier gas. The temperature was held at 60 °C for 4 min, then increased by 15 °C/min to 100 °C, held for 4 min, increased by 15 °C/min to 175 °C and by 1 °C/min to 230 °C and held for 6 min.

2.2.7 Statistical analysis

Differences in proportions of pupated/eclosed/healthy insects across the diets and species were examined using a general linear model (GLM). The significance of the terms in each of our models was tested using a randomisation test where the F values from the above GLM (F_{real}) were compared to those produced when the same GLM was executed but each response variable was shuffled at random (without replacement) across diet and the species (F_{random}) (Manly, 1997). This shuffling procedure was iterated 10 000 times using a Monte Carlo simulation, and the proportion of times (p) that F_{random} exceeded F_{real} was calculated for each term in the model. Two tailed significance values were calculated for each term in the model as 2p if $P < 0.5$ or as 2(1-p) if $P > 0.5$ (Manly, 1997). All randomisation tests were executed in R (R Development Core Team, 2011) using a modified version of the "shuffle" function. For multiple group comparisons, one-way analysis of variance (ANOVA) with LSD post-hoc analysis was performed using SPSS version 17.0 (SPSS, Inc., Chicago IL).

2.3 Results

2.3.1 Performance of *H. virescens* and *H. subflexa* on alpha linolenic acid concentration series

Both *H. virescens* and *H. subflexa* were reared on a series of meridic diets supplemented with increasing amounts of α-18:3 (0-1000 μg/g diet) and developmental parameters were monitored. For Trial 1 (Figure 2.1), *H. subflexa* had a significantly reduced pupation rate compared with *H. virescens* ($F = 204.37$, $P < 0.001$ (Figure 2.1b) on each diet. There was also a significant effect of diet on pupation ($F = 7.69$, $P < 0.001$); for both species, the proportion of larvæ pupating increased with increasing α-18:3. Eclosion was

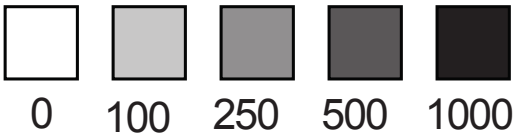
significantly affected by diet (F = 7.14, $P < 0.001$), and the total proportion of healthy insects eclosing increased with increasing α-18:3 in both species (F = 0.43, $P < 0.001$; Figure 2.1c). *Heliothis virescens* had normal eclosion starting at 500 μg/g , while *H. subflexa* began to eclose normally at 250 μg/g α-18:3. Species and diet combined to affect the development of healthy adults (F = 3.81, $P = 0.015$). On diets containing at least 250 μg/g α-18:3, *H. subflexa* larvæ pupated after a longer period . In *H. virescens*, the number of days until pupation decreased with increasing α-18:3 (Figure 2.2b). The time required for *H. virescens* to eclose decreased (Figure 2.2c) and its pupal weight increased (Figure 2.2d) with increasing α-18:3. Similar results were observed for Trial 2 (Figure 2.3 and Figure 2.4).

2.3.2 Performance of *H. virescens* and *H. subflexa* on pinto bean diets

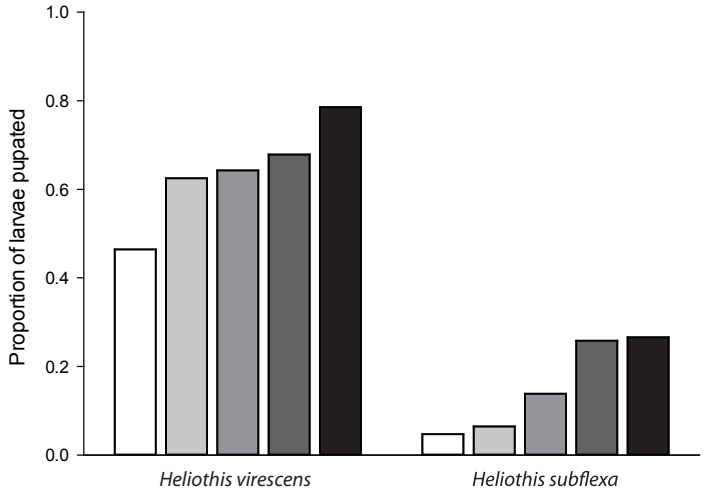
Although the pinto bean diets contained basal amounts of FA, the PB+ diet had significantly higher amounts of α-18:3 ($P < 0.05$)(Figure 2.5). For both *H. virescens* and *H. subflexa*, the rate of pupation and eclosion (Figure 2.6), as well as the pupal weight (Figure 2.7) increased with increasing time feeding on the PB+ diet.

2.3.3 Accumulation of fatty acids in adult *H. virescens* and *H. subflexa*

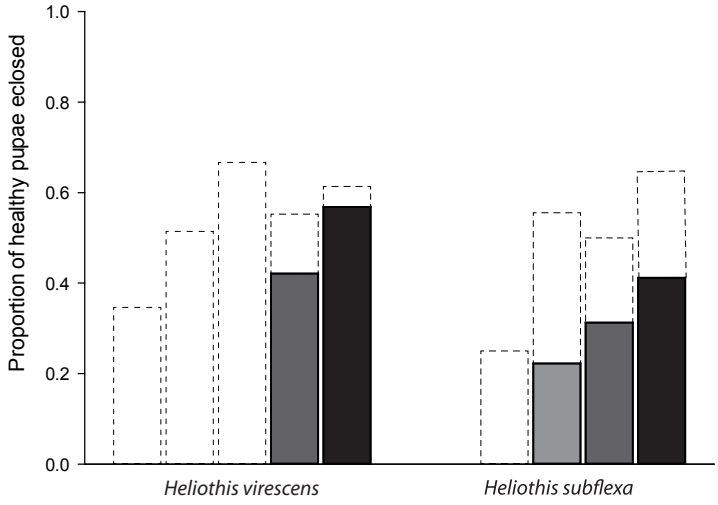
Adults from each treatment were extracted and the FAMEs were analysed via GC. For both *H. virescens* (Figure 2.8) and *H. subflexa* (Figure 2.9), the longer the insects were reared on the PB+ diet, the greater the amount of accumulated α-18:3.



(a) μg α-18:3 per g of diet

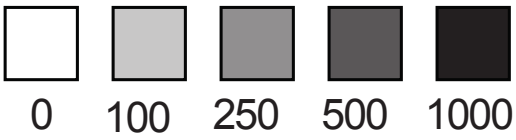


(b) Pupation rate

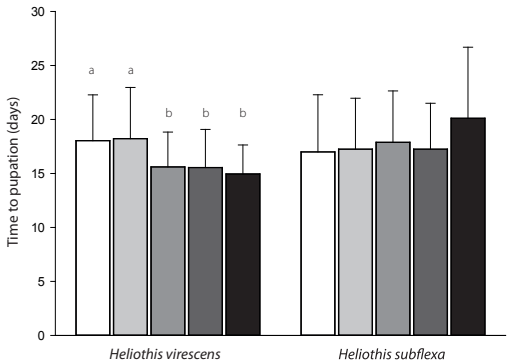


(c) Eclosion rate (healthy and abnormal)

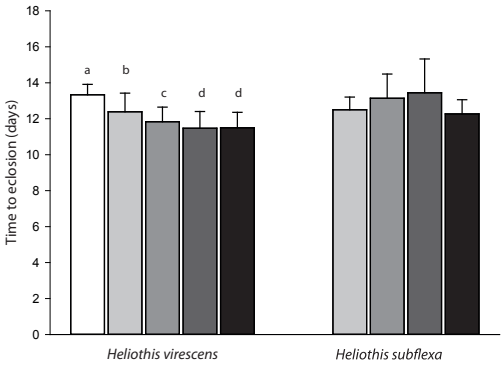
Figure 2.1: Survival rates of *H. virescens* and *H. subflexa* larvæ reared on minimal diet supplemented with α-18:3 (0-1000 μg per g of diet): Trial 1."Abnormal adults represent partially eclosed adults and fully eclosed adults with crumpled wings. "Healthy adults had no noticeable deformities.



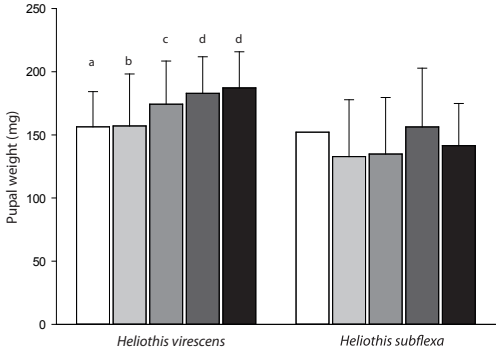
(a) μg α-18:3 per g of diet



(b) Days to pupation. ^{ab} LSD $P < 0.05$

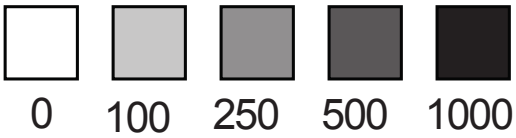


(c) Days to eclosion ^{ac,ad,bd} LSD $P < 0.05$

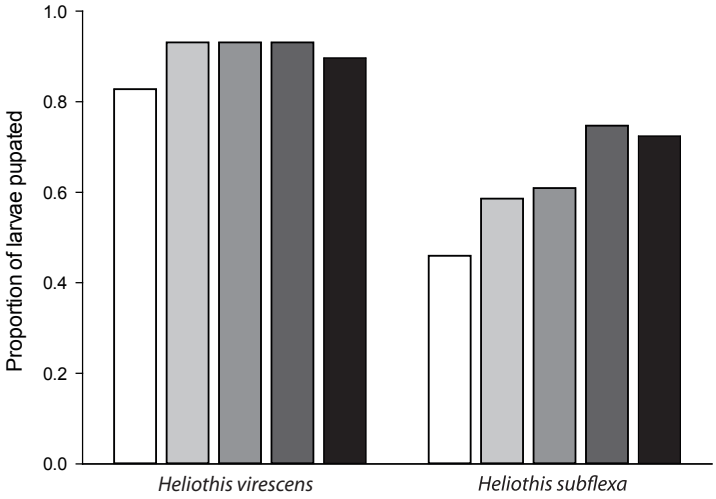


(d) Pupal weight ^{ad,bc,bd} LSD $P < 0.05$

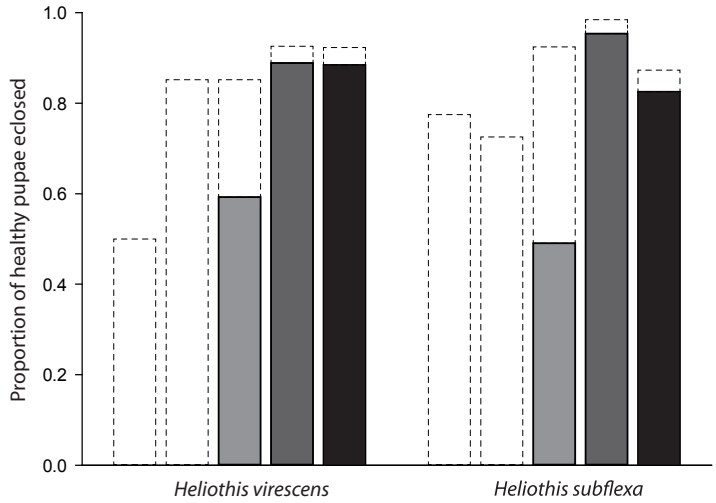
Figure 2.2: Performance of *H. virescens* and *H. subflexa* larvæ reared on minimal diet supplemented with α-18:3 (0-1000 μg per g of diet): Trial 1. Values represent means $\pm$ S.D.



(a) μg α-18:3 per g of diet

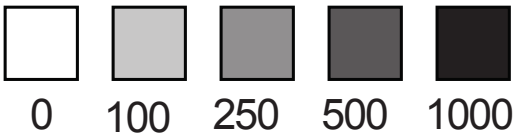


(b) Pupation rate

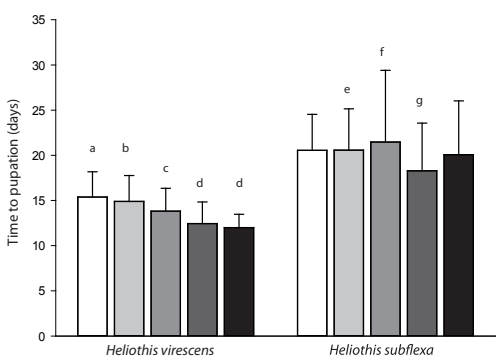


(c) Eclosion rate (healthy and abnormal)

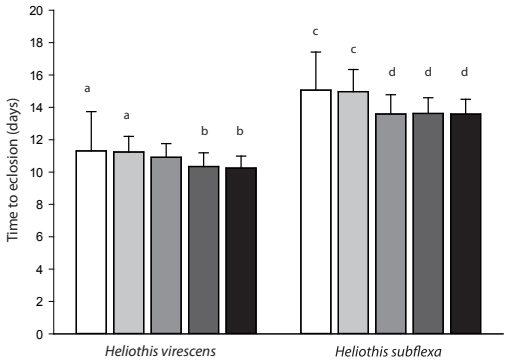
Figure 2.3: Survival rates of *H. virescens* and *H. subflexa* larvæ reared on minimal diet supplemented with α-18:3. (0-1000 μg per g of diet): Trial 2. "Abnormal adults represent partially eclosed adults and fully eclosed adults with crumpled wings. "Healthy adults had no noticeable deformities.



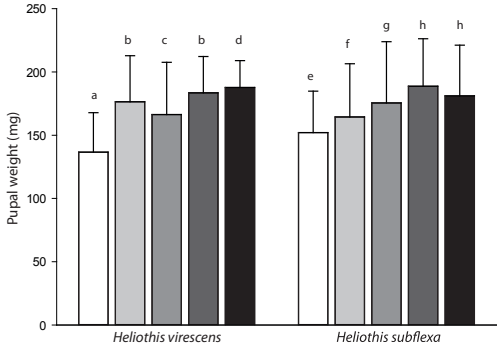
(a) μg α-18:3 per g of diet



(b) Days to pupation ^{ac,ad,bd,cd,eg,fg} LSD $P < 0.05$



(c) Days to eclosion ^{ac,cd} LSD $P < 0.05$



(d) Pupal weight ^{ab,ac,cd,eg,eh,fh} LSD $P < 0.05$

Figure 2.4: Performance of *H. virescens* and *H. subflexa* larvæ reared on minimal diet supplemented with α-18:3 (0-1000 μg per g of diet): Trial 2. Values represent means $\pm$ S.D.

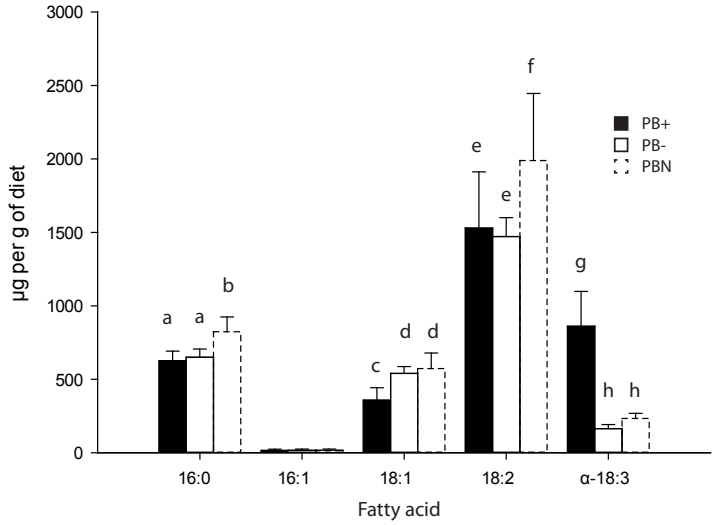
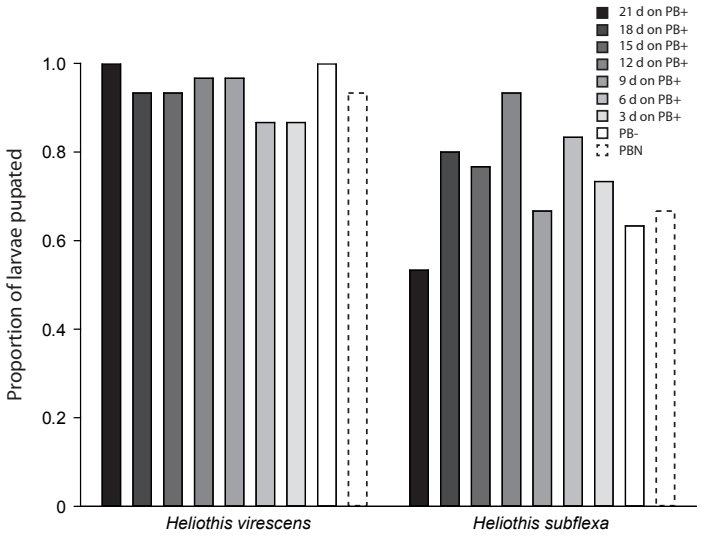


Figure 2.5: Fatty acid composition of pinto bean diets. Values represent means ± S.D.

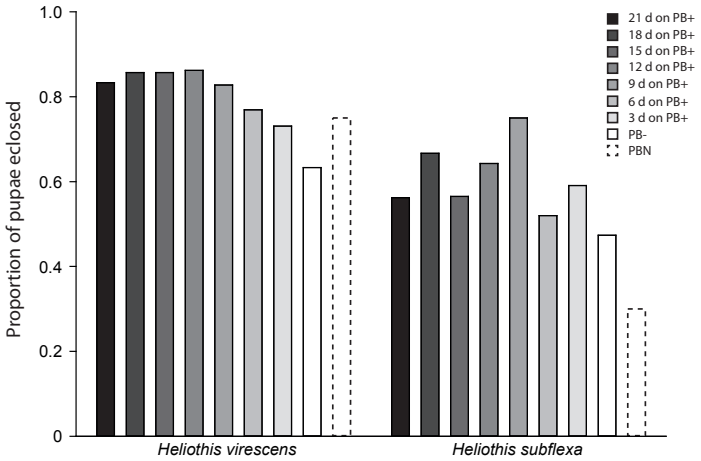
2.4 Discussion

Both *H. virescens* and *H. subflexa* were reared on artificial diets with varying concentrations of α-18:3. For both species, increasing dietary α-18:3 up to 1000 μg/g had a large effect on the rate of healthy eclosion. This is consistent with results obtained by Bracken (1982) who found that similar amounts were able to reduce "wing syndrome" in Bertha armyworm (*Mamestra brassica*). They found that higher levels (approximately 2000 μg/g) were required to affect pupation rates and approximately 5800 μg/g were required to have an effect on eclosion rate. Vanderzant (1968) fed corn earworm (*Heliothis zea*) on a series of minimal diets supplemented with up to 500 μg α-18:3 per gram of diet. Consistent with the current results, increasing α-18:3 had a positive effect on pupation rate. The threshold concentration for healthy eclosion was between 200 and 250 μg /g.

It is unclear why De Moraes and Mescher (2004) were unable to detect α-18:3 in *P. angulata* fruits, but we suspect that the reason lies with their methodology. Using a DB-5 column, 18:2 and α-18:3 can be difficult to



(a) Pupation rate



(b) Eclosion rate

Figure 2.6: Pupation and eclosion rates of *H. virescens* and *H. subflexa* larvæ which had been reared for varying durations on defatted pinto bean diets supplemented with α-18:3 (PB+).

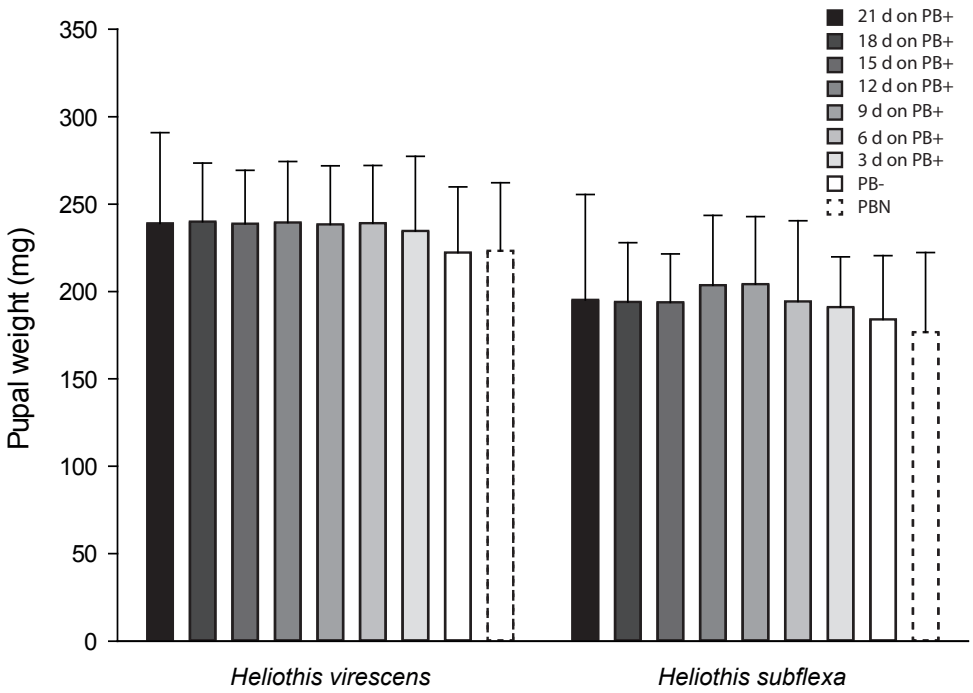


Figure 2.7: Pupal weights of *H. virescens* and *H. subflexa* larvæ which had been reared for varying durations on defatted pinto bean diets supplemented with α-18:3 (PB+). Values represent means ± S.D.

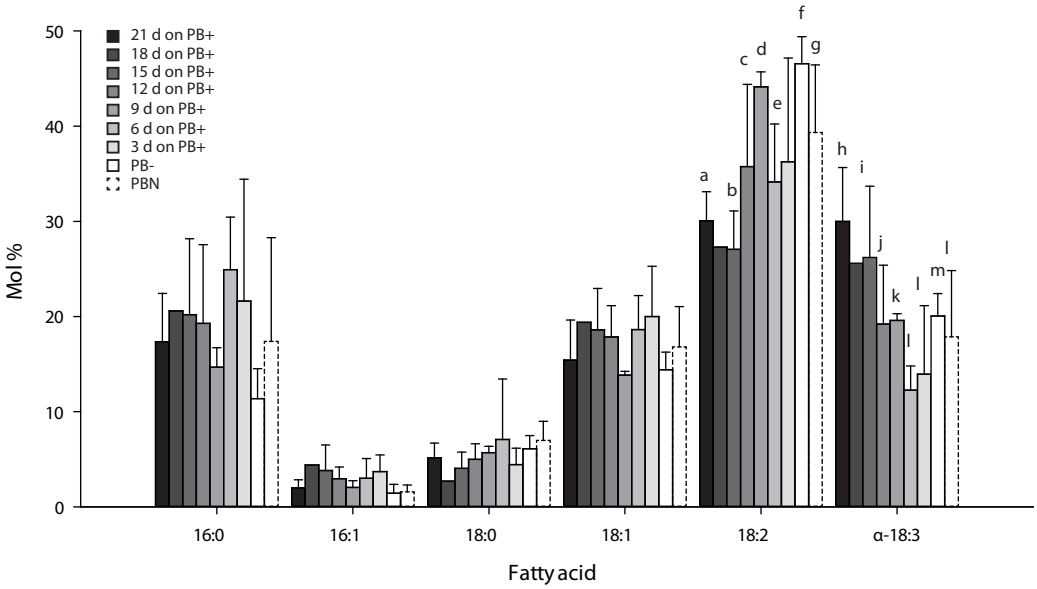


Figure 2.8: Fatty acid composition of *Heliothis virescens* adults that were reared on defatted pinto bean diets supplemented with α-18:3. Larvæ were started on PB- diet and switched to PB+ diet every 3 days. Values represent means $\pm$ S.D. ^{ad,af,ag,bc,bd,bf,cf,de,ef,hj,hk,hl,hm,im} LSD $P < 0.05$

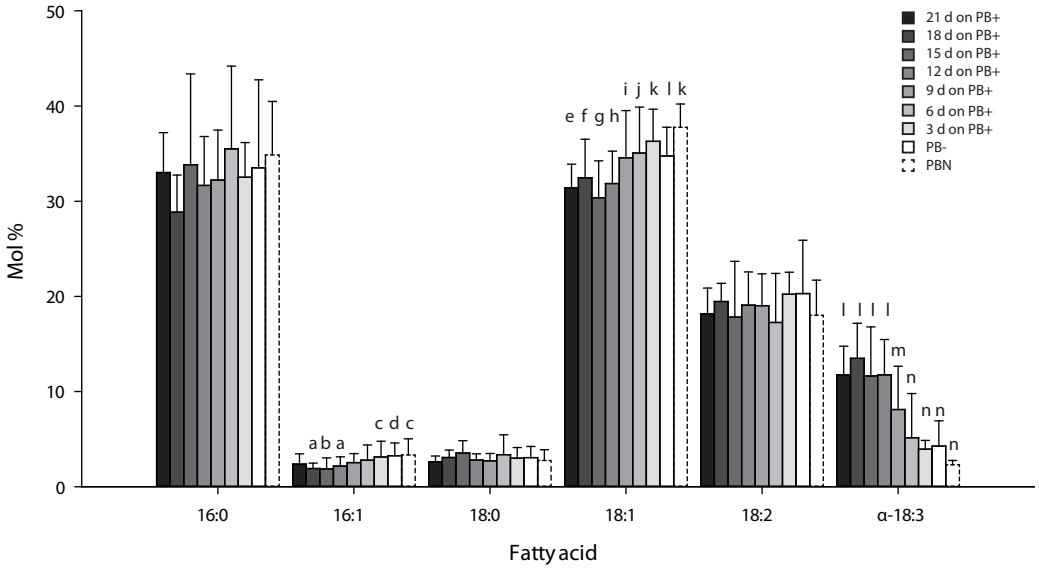


Figure 2.9: Fatty acid composition of *Heliothis subflexa* adults that were reared on defatted pinto bean diets supplemented with α-18:3. Larvæ were started on PB- diet and switched to PB+ diet every 3 days. Values represent means ± S.D. ^{ac,ad,bd,ej,ek,fk,gi,gj,gk,gl,hi,hj,hk,lm,ln,mn} LSD $P < 0.05$

resolve and often overlap. They overcame this difficulty by detecting α-18:3 using GC-MS, by scanning for the characteristic ion (m/z= 292). Using this technique, they reported that α-18:3 was present in the calyx (leafy covering) but absent in the fruits of *P. angulata*. However, the figure presented in support of this statement was based on a single sample, and neither any estimate of the amount actually detected in the calyx nor an estimate of the detection limit of their technique was reported. In all species of *Physalis* tested, Bateman (2006) found levels of α-18:3 equal to or greater than the concentrations found in the diets on which *H. virescens* and *H. subflexa* experienced normal development.

Different species differ in the extent to which the adult and larval FA content reflects that of the diet. In some species, the FA of the larvæ is tightly linked to the FA composition of the diet (referenced in Cookman et al. (1984)). Furthermore, the FA composition undergoes relatively minor changes during metamorphosis. In this respect, the FA composition of the adult can give clues as to the larval diet. Stanley-Samuelson et al. (1985) reared the greater wax moth (*Galleria mellonella*) on diets with increasing levels of α-18:3. Although the α-18:3 content of adult triacylglycerols (TAGs) remained relatively constant, the overall proportion of α-18:3 in adults increased with the diet.

In other cases, however, the FA composition of the diet is not reflected in the FA content of the insect. For example, Wood et al. (1969) found that in *H. virescens* the dietary FAs are not directly reflected in the insect TAGs; 18:1 and 18:2 represented 16% and 53% of dietary FAs respectively and 40-45% and 7-17% of TAGs, respectively. Nelson and Sukkestad (1968) found that the FA composition of *Trichoplusia ni* larvæ substantially differed from the FA composition of the diet; while 18:2 was the major dietary FA (53%), the levels found in the larvæ were only about 11%.

On the pinto bean diets, the FA composition of both *H. virescens* and *H. subflexa* reflected that of the diet in that 16:0 and 18:2 were the predominant

FAs. Even though the diets contained more 18:2, it was the α-18:3 content of the adults that increased in relationship to increased feeding time on the PB+ diet.

It is possible that *H. virescens* and *H. subflexa* differ in the way that they take up and/or retain α-18:3. Dikeman et al. (1981) found that in *H. virescens* polyunsaturated FAs, namely 18:2 and α-18:3, were preferentially absorbed compared with saturated and monounsaturated FAs. Torres-Ruiz et al. (2010) found that caddisflies, which are closely related to Lepidoptera, accumulated 20:5, an essential FA, more readily than any other FA.

The form of a particular FA may also be an important factor in the extent to which it is taken up. Weintraub and Tietz (1973) found that when grasshoppers (*Locusta migratoria*) were fed the TAGs tri-18:1 and tri-16:0, 18:1 was readily absorbed, while 16:0 was not. Palmitic acid, however, was readily absorbed when it was given as free acid. Additionally, Turunen (1973) reported impaired utilisation of α-18:3 in *P. brassicæ* that were reared on a diet supplemented with linseed oil (where α-18:3 is present mainly as TAG). In nature, α-18:3 is mostly available in the forms of phospholipids and glycolipids. Future work could involve preparing minimal diet with differing concentrations of α-18:3 containing glyco- or phospholipids, although these compounds are difficult to obtain in sufficient amounts.

Alpha-linolenic acid is a component of the FA conjugate (FAC) volicitin (*N*-(17-hydroxylinolenoyl)-L-glutamine), found in the regurgitant of larvæ. Aside from being an elicitor of defence responses in plants, FACs may play an integral role in nitrogen metabolism. While the glutamine moiety of FACs is derived mainly from midgut cells, approximately 20% is synthesised from glutamic acid and ammonia. This reaction is catalysed by glutamine synthetase (GS). Yoshinaga et al. (2008) found that enriching the diet of *Spodoptera litura* larvæ with α-18:3 resulted in substantial increases in the assimilation of glutamic acid and ammonia from the diet, and thus increased GS productivity. They propose that the newly formed glutamine is rapidly

coupled to α-18:3 to form FACs. When glutamine is stored in FACs, glutamine in midgut cells is depleted, thereby shifting equilibrium in favour of glutamine synthesis. Furthermore Yoshinaga et al. (2008) propose that FACs also function as the primary stores of glutamine.

De Moraes and Mescher (2004) detected volicitin in the regurgitant of *H. virescens* and *H. subflexa* that had been reared on *Physalis* leaves, but not on the fruit. The amount of FAC in the regurgitant represents an equilibrium between conjugation of the fatty acid and glutamine, and hydrolysis of volicitin. Lait et al. (2010) investigated the rates of biosynthesis and hydrolysis between *H. virescens*, tobacco hornworm (*Manduca sexta*), and *H. zea* and found that both biosynthesis and hydrolysis is fastest in *H. virescens*. The rates have not yet been studies in *H. subflexa*. Kuhns et al. (2012) identified an aminoacylase (L-ACY-1) responsible for hydrolysis of FACs. This enzyme was more abundant and had higher activity in *H. subflexa* than in *H. virescens* resulting in species-specific equilibria between FAC synthesis and hydrolysis, which could explain disparities in the responses to dietary α-18:3.

Chemicals in *Physalis* may serve as deterrents for *H. virescens*, which would account for their poor growth on the fruits. Compounds such as withanole have been shown to have antifeedant activities (Ascher et al., 1980).

Chapter 3

The timing of alpha-linolenic acid acquisition in developing *Heliothis virescens*

3.1 Introduction

Heliothis virescens, the tobacco budworm, is a serious agricultural pest, particularly in the southern United States and Mexico (Molina-Ochoa et al., 2010). Like all Lepidoptera, *H. virescens* requires dietary α-linolenic acid (α-18:3) for successful development. Without sufficient amounts, rates of pupation and eclosion are reduced, and adults that do eclose have malformed wings. A greater understanding of the essential fatty acid (FA) requirements of *H. virescens* may aid in the development of sustainable pest control strategies.

De Moraes and Mescher (2004) had investigated the α-18:3 requirements of *H. virescens* and the related species, *Heliothis subflexa*. Their results raised some important questions, not only about species differences in α-18:3 requirements, but about the requirements for α-18:3 at various developmental stages. Knowing the optimal timing of α-18:3 acquisition would allow one

to target the developmental stage where the insect is most dependent on α-18:3.

While α-18:3 can be obtained from the diet, it may also be transferred from the mother to the eggs, just as a number of other compounds essential to egg survival. Through a series of experiments using a meridic diet supplemented with α-18:3 we show that α-18:3 is transferred maternally to the egg in a dose dependent manner. Furthermore, the timing of α-18:3 acquisition does affect the rate of normal eclosion.

3.2 Materials and methods

3.2.1 Chemicals and insects

The chemicals used were the sames as described in Section 2.2.1. *Heliothis virescens* larvæ were reared as described in Section 2.2.2. A series of minimal diets was prepared as described in Section 2.2.3 except that the diet was supplemented with up to 2000 μg α-18:3 per g.

3.2.2 Maternal effects

Larvæ were reared on the minimal diet series and upon eclosion, adults from corresponding diets were mated and FAs were extracted from the eggs and mothers. Total lipids were extracted from insects as described in Section 2.2.5. Fatty acid methyl esters were prepared and GC analysis was performed as described in Section 2.2.6

3.2.3 Rescue experiment

Second instar larvæ were started on minimal diet either with (L) or without (0) α-18:3. Every two days, a subset of insects was transferred to the opposite diet and maintained on this diet until pupation. The insects were weighed every two days. Pupal weights as well as rates of pupation and eclosion were also recorded.

3.2.4 Statistical analysis

For multiple group comparisons, one-way analysis of variance (ANOVA) with LSD post-hoc analysis was performed using SPSS version 17.0 (SPSS, Inc., Chicago IL).

3.3 Results

3.3.1 Effect of larval diet on adult fatty acid composition

Larvæ were reared on minimal diet supplemented with a range of α-18:3. Between each increasing dietary concentration of α-18:3, there was a significant increase ($P < 0.05$) in the absolute amount of α-18:3 in the total lipid extract of the adult females (Figure 3.1a). Between the 250 and 1000 μ/g α-18:3 diets, there was also a significant increase in the absolute amounts of 16:0, 16:1, 18:0, 18:1, and 18:2 in the lipid extracts of the adult females.

3.3.2 Effect of larval diet on fatty acid composition of the egg

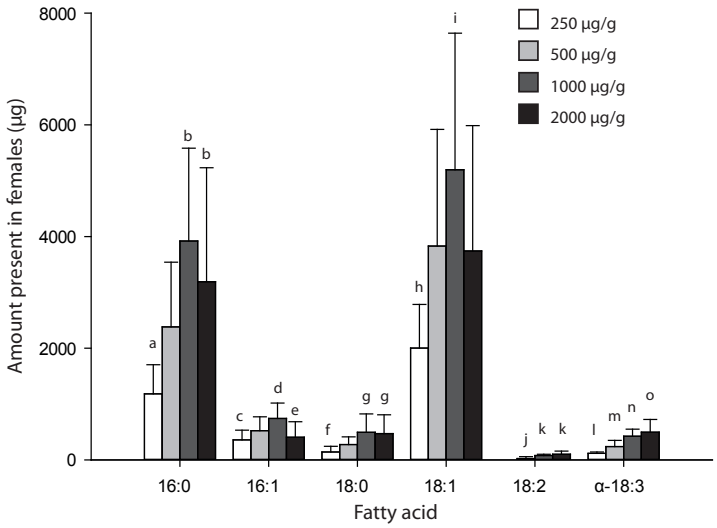
The FA composition of the eggs from the females that were reared on the α-18:3 supplemented diet was also determined (Figure 3.1b). The eggs from females that had fed on diet containing 2000 μ/g α-18:3 had a significantly higher molar ratio of α-18:3 ($P < 0.05$) than those from females that had fed on the diets containing 250 and 500 α-18:3. The eggs from these same mothers had a significantly lower ($P < 0.05$) molar ratio of both 16:1 and 18:1.

3.3.3 Linolenic acid is transferred from the mother to the egg

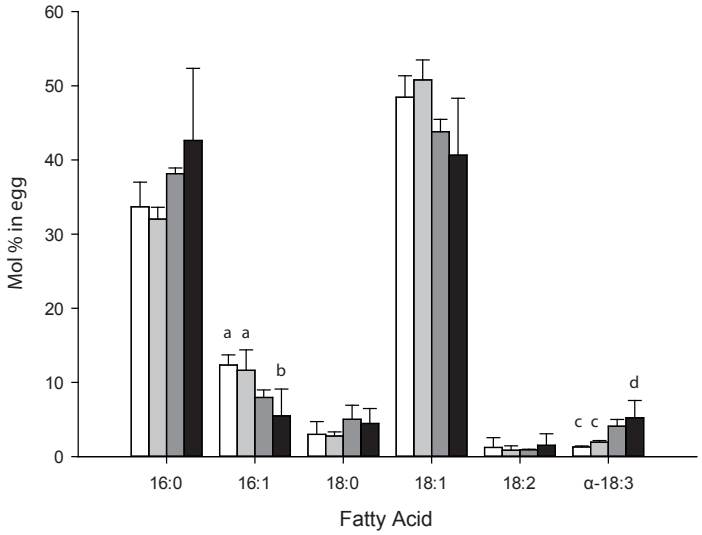
There is a positive correlation between the absolute amount of α-18:3 in the females and the molar ratio of α-18:3 in the eggs (r=.82, $P < 0.01$) (Figure 3.2).

3.3.4 Acquisition of alpha-linolenic acid is essential during later stages of larval development

One group of *H. virescens* larvæ was started on minimal diet containing 500μg of α-18:3 per g (L). Every two days, a subset was transferred to a diet devoid of FA (0) and maintained on this diet until death or pupation. The second group of larvæ were started on diet 0 and subsets were transferred every two days to diet L. In general insects that had been transferred to diet 0, failed to successfully eclose, while a substantial proportion of the insects transferred to diet L successfully eclosed (Figure 3.3). The weight gains for each interval on the α-18:3 diet were summed, the average amount of α-18:3 ingested was assumed to be proportional to these weight gains. For each



(a) Female adults



(b) Eggs

Figure 3.1: Fatty acid composition of female *H. virescens* adults which as larvæ had been reared on minimal diet supplemented with α-18:3 (250-2000 μg per g of diet) and the fatty acid composition of the corresponding eggs. Values represent means $\pm$ S.D.

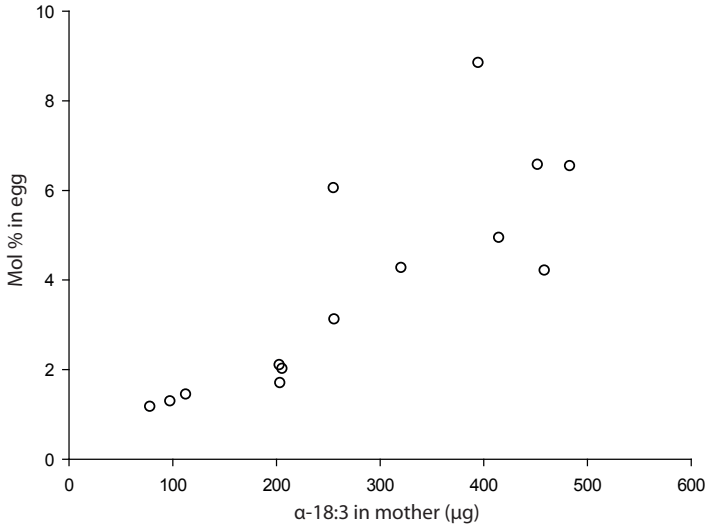


Figure 3.2: Relationship between α-18:3 content in the mother and corresponding eggs

subset, the rate of eclosion was calculated and plotted against the average amount of α-18:3 (Figure 3.4). Approximately 600 μg/g of pupal weight seems to be the threshold amount of α-18:3 that a larva needs to ingest in order to ensure successful eclosion.

3.4 Discussion

At various stages in development, *H. virescens* larvæ were transferred to or from an α-18:3 enriched diet. Insects obtaining α-18:3 later in development performed better than those obtaining α-18:3 earlier in development. The main reason why later acquisition is more successful than early acquisition is that larger larvæ simply eat more and therefore obtain more α-18:3 per unit time than the smaller larvæ. *H. virescens* also typically feeds on leaves in the first or second instar and then moves to fruits in later instars. The linolenic acid content of leaves is higher than that of fruit. If the levels of α-18:3 in fruit are insufficient, this may result in eclosion difficulties.

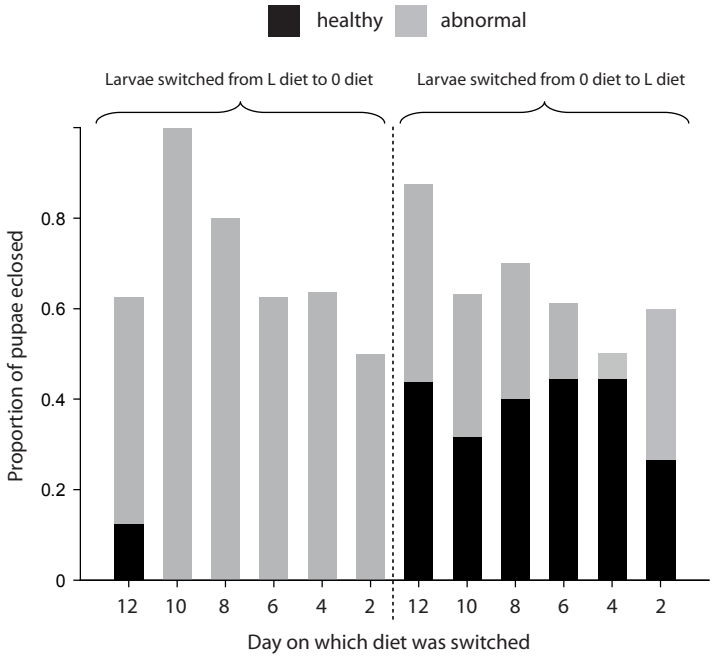


Figure 3.3: Effect of timing of α-18:3 acquisition on the eclosion rates of *H. virescens* larvæupon switching to or from an α-18:3 enriched diet at various intervals during development.

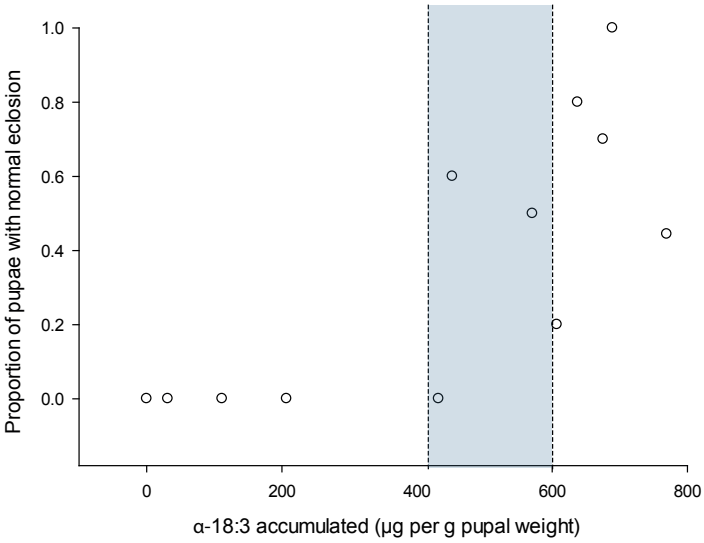


Figure 3.4: The relationship between accumulated dietary α-18:3 and healthy eclosion of *H. virescens*

In addition to increases in α-18:3, there were significant increases in the absolute amounts of palmitic (16:0), palmitoleic (16:1), stearic (18:0), and oleic (18:1) acids in the total lipid fractions when the larvæ were fed diets with increasing amounts of α-18:3 up to 1000g/g (Figure 3.1a). With higher dietary α-18:3 concentrations, however, the levels of all other FA decreased. It is possible that these levels of α-18:3 inhibited *de novo* FA synthesis. A similar effect was observed by Horie and Nakasone (1971) in *Bombyx mori*.

That the adults had increases of other FAs, even though these were not included in the diet, suggests that α-18:3 was involved in carbon recycling, and that its oxidation products were used in the *de novo* biosynthesis of other FAs. This effect was noted by Lambremont et al. (1976). The levels of linoleic acid (18:2), however, did not increase as *H. virescens* is incapable of synthesising this FA.

Earle et al. (1967) found that the FA composition of boll weevil eggs was representative of the maternal FA composition, which in turn reflected that of the diet. There was a significant correlation between the amount of α-18:3 in the *H. virescens* mother and that in the egg. This could represent a form of reproductive investment. Although this was not investigated, it is possible that maternal α-18:3 is divided unevenly among the eggs, thereby giving certain eggs an advantage. It would be prudent to search for differences in the α-18:3 content of the first and last eggs that were laid.

These results suggest that the acquisition of α-18:3 is more crucial during the later stages of larval development. With respect to pest control, the aim should be to deter α-18:3 acquisition during later larval stages. While there is maternal transfer of α-18:3 to the eggs, far less than the 100 μg/mg pupal weight would be transferred, which would require the larvæ to obtain additional α-18:3 from dietary sources. Nevertheless, the α-18:3 transferred to the egg is likely sufficient to support initial larval growth. It would also be interesting to determine whether eggs with a higher ratio of α-18:3 have an advantage on diets deficient in α-18:3. For this, the mothers would need

to be fed on diet with much higher levels of α-18:3, which may exceed the range found in natural diets.

Chapter 4

The performance of *Helicoverpa armigera* larvæ on tomato leaves with normal and reduced levels of alpha-linolenic acid

4.1 Introduction

It is well established that α-linolenic acid (α-18:3) is an essential fatty acid (FA) with respect to the successful development of Lepidoptera. Insects that obtain insufficient amounts of this FA tend to have reduced growth rates, and a lower proportion survive to pupation with even fewer surviving to adulthood. These findings are based on studies in which insects were fed defined amounts of α-18:3 in meridic diets, which did not necessarily reflect the FA content of plants which are the natural foods for these insects. Alpha-linolenic acid is generally only included as a free FA, while in nature, it tends to be found in the form of phospholipids and glycerolipids. Most insects are

generally better able to utilise α-18:3 when it is in this form. While it is theoretically possible to prepare a meridic diet that more closely resembles the FA found in natural diets, this is not often practical, mainly due to the cost factors of including all the α-18:3 in relatively pure form.

Mutagenesis offers the possibility of manipulating the FA composition of living plants. Howe and Ryan (1999) isolated several EMS-induced mutants of tomato that were deficient in the systemin-mediated signal cascade. Li et al. (2003) subsequently showed that one of these mutants, spr2, was caused by a premature stop codon in the *leFAD7* gene which encodes the chloroplastic ω-3 fatty acid desaturase enzyme responsible for production of most of the 18:3 in the plant. *leFAD7* mutant plants have approximately 20% of the α-18:3 as their wild type counterparts (Cañoles et al., 2006). These plants, which also have an inherent defence system, were utilised to test the effects of reduced levels of α-18:3 on *Helicoverpa armigera* (cotton bollworm) larvæInsects that were grown on the *leFAD7* mutants had lower growth rates, as well as lower pupation and eclosion rates.

4.2 Materials and Methods

4.2.1 Plants

The tomato plants were a generous gift from Gregg A. Howe, Michigan State University. The wild type were from the variety Castlemart. The jasmonic acid (JA) insensitive plants (*jai1-1*) were a mutant line derived by mutagenesis of the Castlemart variety, and cannot induce antiherbivory defenses mediated by the JA pathway (Li et al., 2004). The *leFAD7* mutant plants had a reduced α-18:3 content. Since α-18:3 is a precursor of jasmonic acid, these plants are also deficient in antiherbivory defenses. All plants were vegetatively propagated. Total lipids were extracted from leaf material as described in Section 2.2.5 and FAMES were prepared and GC analysis

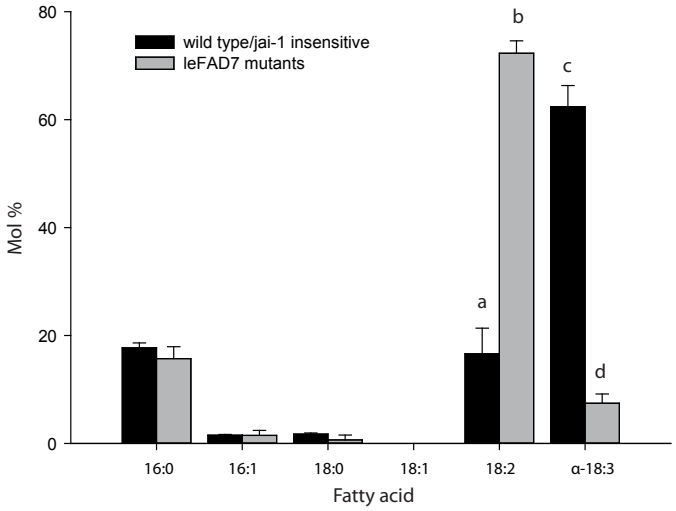


Figure 4.1: Fatty acid composition of tomato leaves with reduced and normal levels of α-18:3

was performed as described in Section 2.2.6.

4.2.2 Insects

H. armigera larvæ were from the TWB strain. Larvæ were normally reared on a pinto bean diet. The insects were propagated via single pair matings. Larvæ were grown directly on the plants to minimise any response that might result from mechanical damage to the leaves (i.e., from cutting the leafs, or removing discs from the leaves) as well as to prevent leaf materal from drying out. Cages were prepared from dome shaped and flat drink cup lids (Solo, USA). A piece of gauze was cut to the dimensions of the opening in the dome lid and glued on. A slit was cut into the lid to allow the cage to be fitted onto a branch. A piece of cotton was wrapped around the stem before the cage was secured in order to reduce any mechanical damage to the stem. A maximum of five cages was placed on any individual plant. The cages were further supported by a scaffold of bamboo sticks.

4.3 Statistics

Statistical analyses were performed using R (R Development Core Team, 2011). For multiple group comparisons, one-way analysis of variance (ANOVA) with LSD post-hoc analysis was performed. Student's t tests were used for comparing means of two groups.

4.4 Results

H. armigera were grown on three lines of tomato plants: wild type, *jai-1* insensitive, and *leFAD7* mutants. Larvæ were weighed every three days until pupation. Larval growth on each of the plants was measured using the mean relative growth rate (MRGR) (Equation 4.1), where W_i is the initial weight, W_f, and d is the time period over which the growth rate was measured.

$$\text{MRGR} = \frac{\ln(W_f) - \ln(W_i)}{d} \tag{4.1}$$

For the first three-day interval, there were significant differences ($P < 0.001$) in the MRGRs of the larvæ grown on the three types of plants (Figure 4.2). The larvæ grown on the *jai-1* insensitive plants had the highest MRGR, while the larvæ grown on the wild type plants had the lowest. The trend continued throughout development, although there were no significant differences for the other intervals. There were also significant differences ($P < 0.001$) in the weights of the larvæ twelve days after being placed on the plants (Figure 4.3). Larvæ reared on the *jai-1* insensitive plants had the highest weights, while those reared on the wild type plants had the lowest.

Only the larvæ that were reared on the *jai-1* insensitive and *leFAD7* mutant plants survived to the pupal stage. Therefore, only these two groups of insects were used for further comparison. A lower proportion of the insects

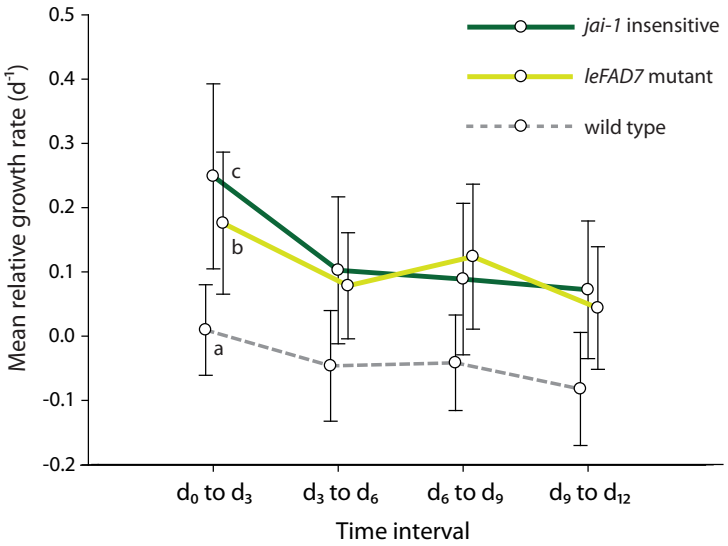


Figure 4.2: Mean relative growth rates of larvæ on tomato plants. Values represent means ± S.D.

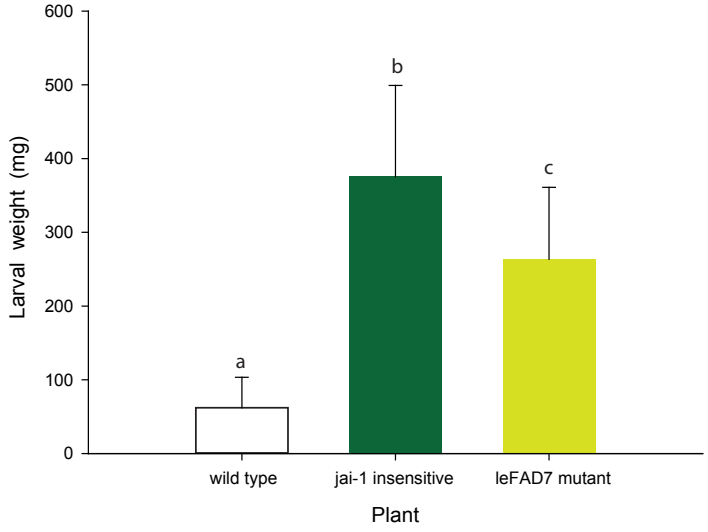


Figure 4.3: Final larval weights (12 days after being placed on tomato plants). Values represent means ± S.D.

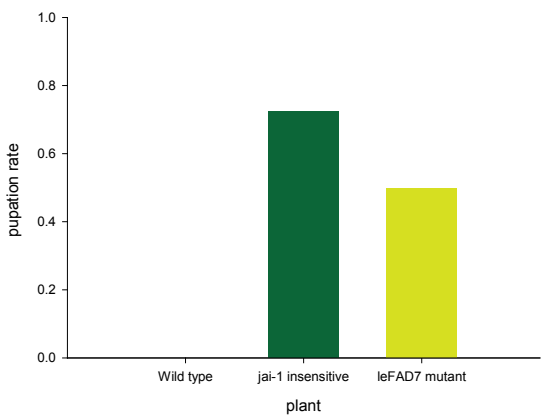
reared on the *leFAD7* mutant plants pupated compared to those on the *jai-1* insensitive plants (Figure 4.4a). Also, a lower proportion of the insects that pupated on the *leFAD7* mutant plants survived to eclosion. (Figure 4.4b) There were, however, no differences in the proportions of normal eclosion between the insects that did eclose on the *jai-1* insensitive and *leFAD7* mutant plants (Figure 4.4c).

The larvæ that fed on the *jai-1* insensitive plants had a shorter time to pupation than those that fed on the *leFAD7* mutant plants (Figure 4.5a), although this difference was only mildy significant ($P < 0.1$). The time to eclosion (Figure 4.5b) of the insects on the *jai-1* insensitive plants was also significantly less ($P < 0.05$). The insects that were grown on the *jai-1* insensitive plants had a significantly higher pupal weight ($P < 0.001$, Figure 4.5c).

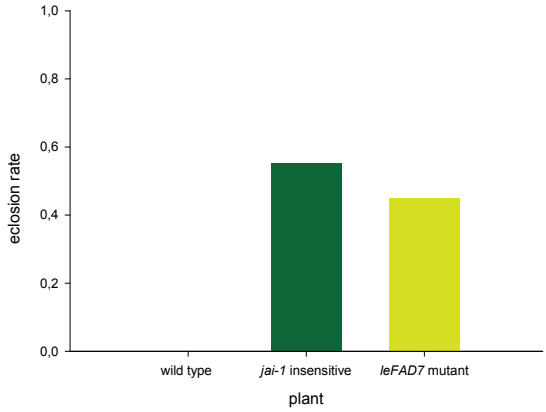
4.5 Discussion

In order to test the effects of α-18:3 on the growth of *H. armigera* on a natural diet, larvæ were grown on three types of tomato plants: wild type; *jai-1* insensitive, which had wild type levels of α-18:3, but reduced responses to jasmonic acid; and *leFAD7* mutant, which had reduced levels of α-18:3. The larvæ had a reduced growth rate on the wild type and *leFAD7* mutant plants, and none of the larvæ grown on the wild type plants survived to pupation. The larvæ grown on the *leFAD7* mutant plants had decreased rates of pupation and eclosion compared to those grown on the *jai-1* insensitive plants.

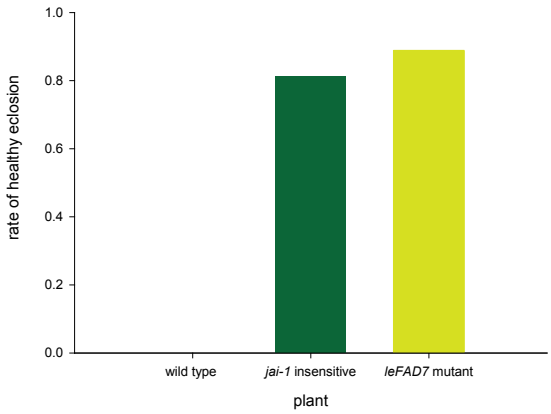
The normal JA induced defences (i.e., those of the wild type plants) are highly effective in protecting against herbivory by *H. armigera*. Therefore, studies of the effects of α-18:3 deficiency can only be done with the *jai-1* insensitive and *leFAD7* mutant plants. The *jai-1* insensitive plants likely have a weaker JA induced response than the *leFAD7* mutant plants and the



(a) Pupation rate

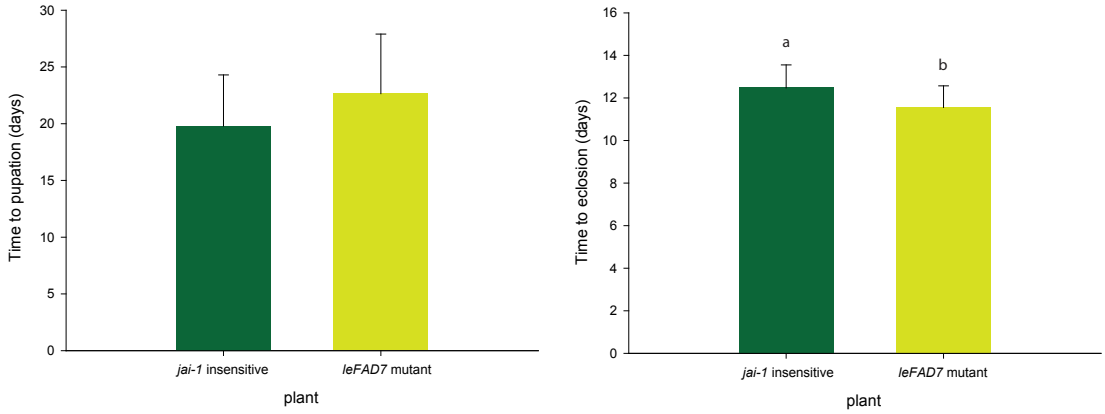


(b) Eclosion rate



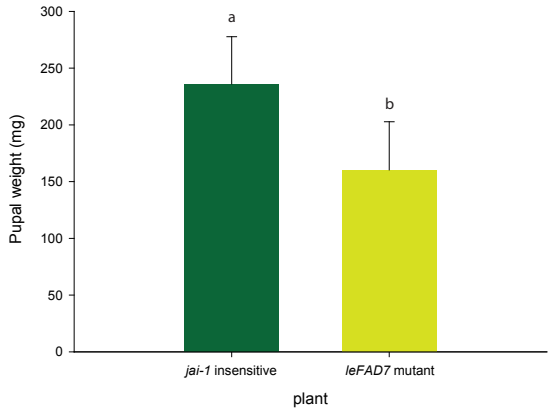
(c) Rate of normal eclosion

Figure 4.4: Rates of pupation, eclosion, and normal eclosion for insects grown on the three types of tomato plants.



(a) Time to pupation

(b) Time to eclosion



(c) Pupal weight

Figure 4.5: Time to pupation and eclosion and pupal weights for insects grown on *jai-1* insensitive and *leFAD7* mutant plants. Values represent means ± S.D.

decreased growth rates, pupal weights, as well as pupation and eclosion rates that were observed for the insects that were grown on the *leFAD7* mutant plants are more likely to be due to α-18:3 than to JA induced responses. In order to fully test this however, it would be necessary to have an additional set of plants — with both decreased levels α-18:3 and decreased JA-induced responses.

These findings may ultimately have use in pest control. If FAD7 tomatoes are planted alongside wild type tomatoes, the larvæ growing on these plants would have a lower chance of survival. Wang et al. (2010) have explored the depletion of ω-3 desaturases with the hope of increasing heat tolearance, which provides additional motivation for utilising tomatoes with reduced α-18:3. Ultimately, however, a high response to JA would be more effective against herbivores than decreased amounts of α-18:3.

Chapter 5

Synthesis of alpha-linolenic acid analogues

5.1 Introduction

It is well established that α-18:3 and metabolites are essential to successful insect development. Inhibition of α-18:3 metabolism may therefore be a useful strategy for pest control. Beta-oxidation is one process that has often been targeted. The products of β-oxidation may be used for pheromone biosynthesis, or recycled back into the synthesis of other FAs.

In recent years, the introduction of fluorine into FA has been explored. The steric influence caused by fluorine substitution is generally small, although fluorine may affect properties such as volatility and stability.

A fluorine atom substituted at the Δ-2 or Δ-3 positon should theoretically block β-oxidation by preventing acyl-CoA dehydrogenase from abstracting a proton. Rosell et al. (1992) describe using both Δ-2 and Δ-3 fluorinated FA to inhibited the chain shortening step in the biosynthesis of *cis*-9, *trans*-11-tetradecadienyl acetate, which is a major sex pheromone component of *Spodoptera littoralis*. These compounds proved to be more effective than

those that had the fluorine at the Δ-4 position. Hernanz et al. (1997) used 2-fluoro derivatives of 16:0 to inhibit pheromone biosynthesis in *S. littoralis*, *Bombyx mori*, and *Thaumotopoea pityocampa*. Bosch et al. (1996) prepared Δ-2,2- , Δ-3,3-, and Δ-4,4- difluoro derivatives of 16:0 and evaluated the efficiency of these derivatives in *S. littoralis*. Only the Δ-2,2- and Δ-3,3- derivatives were active.

Analogues where the fluorine is introduced at a desaturation site have also been used for inhibiting desaturases, which are also integral to pheromone biosynthesis. Abad et al. (2003) used 11-F-14:0 to partly inhibit Δ-11 desaturase in *S. littoralis*. Alpha-linolenic acid has been ignored as a substrate for fluorination although it or its esters are known pheromones (e.g., methyl ester in *Pieris rapæ* or acetate ester in *Triphosahæsitata affirmata*).

A number of syntheses for fluorinated FAs have been (e.g., (Michel and Schlosser, 1996)), although most of these entail multi-step reactions. Diethylaminosulfur trifluoride (DAST) is a useful reagent that converts alchohols to alkylfluorides. Arsequell et al. (1992) used DAST to prepare fluorinated FA from secondary alcohols. The synthesis of these alcohols, however, also involved multiple steps.

Several hydroxylated FA are commercially available, or in some cases make up a considerable fraction of natural oils. For example, thyme (*Thymus vulgaris*) seed oil contains up to 13% 2-OH 18:3 (Smith and Wolff, 1969). Such FAs would provide a quicker route to the synthesis of fluorinated analogues. A facile method for the preparation of fluorinated linolenic acid from thyme seed oil is described (Figure 5.1).

5.2 Experimental

5.2.1 Chemicals

Thyme seeds were from N.L. Chrestensen Erfurter Samen- und Pflanzenzucht GmbH (Erfurt, Germany). Silica gel, boron triflouride, *N*,0-bis(trimethylsilyl) trifluoroacetamide, DAST were from Sigma-Aldrich (Schnelldorf, Germany). Solvents were from Fisher Scientific GmbH (Schwerte, Germany).

5.2.2 Extraction

Thyme seeds were extracted in hexane using a Soxhlet extractor. Fatty acid methyl esters were prepared from the thyme seed oil using BF_3 in MeOH. The hydroxy FAMES were eluted from a silica gel column with hexane/diethyl ether (50:50).

5.2.3 Synthesis of 2-fluorolinolenic acid methyl ester

The fluorination of 2-hydroxylinolenic acid methyl ester was performed according to Bin Omar et al. (2003). 900 mg of 2-hydroxylinolenic acid methyl ester were dissolved in 4.5 ml of *N*,0-bis(trimethylsilyl) trifluoroacetamide and heated for 30 min at 90 °C, after which the excess was evaporated. The residue was dissolved in 90 ml CH_2Cl_2 and cooled to −78 °C in a dry ice/acetone bath. A solution of DAST (0.9 ml in 9 ml CH_2Cl_2) was added and the mixture was allowed to stir for 1 h at −78 °C and for 30 min at ambient temperature. The reaction was then quenched with water, washed with an aqueous solution of of $NaHCO_3$, and dried over $MgSO_4$. The product was purified on a silica column with hexane/ether (95:5). The solvent was removed under a stream of nitrogen, yielding 226 mg (24.9 %) of a yellowish oil. MS: m/z 310 ($C_{19}H_{31}F0_2$) ^{1}H: (500 MHz, $CDCl_3$ δ = 5.36 (6H,

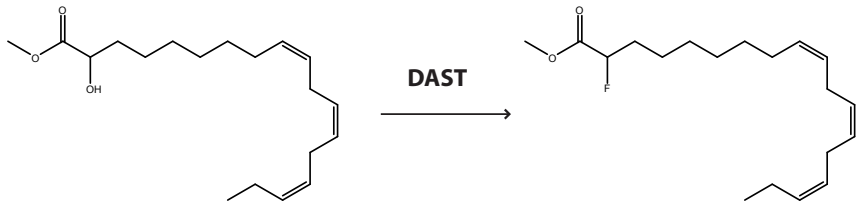


Figure 5.1: Reaction scheme for the synthesis of 2-fluorolinolenic acid methyl ester

m), δ =4.91 (1H, dt, J = 50 Hz, 5.7 Hz), δ =3.80 (3H, s), δ = 2.80 (4H, m), δ = 2.06 (4H, m), δ = 1.87 (2H, m), δ = 1.33 (8H, m), δ = 0.98 (3H, t J = 7.5 Hz).

5.3 Discussion

A fluorinated analogue of α-18:3 was successfully synthesised starting from thyme seed oil. As thyme seeds are in ready supply, this analogue could be used to study the underlying mechanisms of α-18:3 and its derivatives. This facile synthesis could also be applied to other hydroxylated 18:3 derivatives such as 18-hydroxylinolenic acid, which was identified in the cutin of young apple fruits Kolattukudy et al. (1973). Isolation of sufficient amounts may not, however, be feasible. 17-hydroxylinolenic acid is present in the well-known fatty acid conjugate volicitin. Fluorine substution of the hydroxyl group may provide a means of further studying this important FA conjugate.

While hydroxylated linolenic acid derivatives are in short supply, a possible solution may be to employ chemoenzymatic synthesis to introduce a hydroxyl group into commercially available α-18:3. Brodowsky et al. (1992) describe an isomerase from the fungus *Gaeumannomyces graminis*, which is able to introduce a hydroxyl group to the Δ-8 position of α-18:3 without affecting the double bonds. Similarly, Hamberg (1993) found that an

extract of the red algae, *Lithothamnion coralliodes* was able to oxidise 18:3 to produce both 11 and 14 hydroxy 18:3. Weibel et al. (2002) describe a method for the synthesis of 11-OH 18:3, which would serve as a substrate for the synthesis of 11-F 18:3. One possible point of consideration may be stereospecific synthesis. Khrimian et al. (1996) synthesised both S and R 2-F carboxyllic acids and found that only the R- enantiomer was biologically active in *Ostrinia nubilalis*.

Practical studies using this novel α-18:3 analogue are currently being investigated.

Chapter 6

Conclusions and outlook

In recent years, there has been a resurgence of research interest in insects FAs. One area of focus has been the role of α-18:3 in the development of Lepidoptera. The importance of this essential FA to successful development cannot be understated. De Moraes and Mescher (2004) published a rather provocative study in which they suggested that the specialist *Heliothis subflexa* does not have dietary α-18:3 requirements, although the closely related generalist *Heliothis virescens*, not to mention virtually all other species of Lepidoptera, does.

These findings raised an important question — what confers this unique property to *H. subflexa*? One possible hypothesis would be that *H. subflexa* is capable of *de novo* α-18:3 synthesis, which would most likely entail Δ-15 desaturase activity, although this has not yet been reported in Lepidoptera.

Using a well defined artificial diet supplemented with α-18:3 we were able to show that *H. subflexa* does in fact have dietary α-18:3 requirements.

This is not to say that the results of De Moraes and Mescher (2004) are completely invalid. Instead they point to other possible differences between *H. subflexa* and *H. virescens* as well as between other Heliothines and Lepidopterans. This includes differences in the metabolism of FACs such as volicitin which is composed of α-18:3.

While it is perhaps disappointing that *H. subflexa* did not prove to be an example of a Lepidopteran capable of endogenous α-18:3, the possibility remains that such species may exist. Buckner and Hagen (2003) provide evidence that the silverleaf whitefly is capable of this.

Ongoing efforts have led to the identification and characterisation of lepidopteran desaturases, many of which have bifunctional activity. Genes for bifunctional desaturases with Δ-15 desaturase activity may exist, although for whatever reason this activity may have been suppressed, thereby resulting in a dependence on dietary α-18:3.

Changes in dietary α-18:3 not only result in changes in the α-18:3 content of insect tissues, but also of other FAs (i.e., 18:1 and 16:1). The oxidation products of α-18:3 may be recycled back into FA synthesis. As FAs also serve as pheromone precursors, alterations in dietary α-18:3 may have an effect on the pheromone blend of the insect. This remains to be investigated.

Artificial diets have allowed for the careful regulation of essential nutrients, but these may not necessarily represent the composition of the natural diet. *H. armigera* larvæ were reared on tomato plants with decreased levels of α-18:3 as a result of a mutation in the *leFAD7* gene. The insects had decreased growth and survival rates on plants with decreased α-18:3 compared to those grown on plants with wild type levels. However, other factors (i.e., JA-induced defense responses) may contribute to differences in insect performance.

In addition to the *leFAD7* mutants, Domínguez et al. (2010) described several transgenic lines of tomato with increased expression of ω-3 desaturase, which may prove useful in investigating the effects of increased α-18:3 on larval growth. In addition to tomato, other plant species (including those which are threatened by herbivory) may also be conducive to the genetic modification of α-18:3 content.

Finally, the synthesis of an α-18:3 analogue was explored. Using readily

available starting material and simple reaction mechanisms, we were able to synthesise 2-fluorolinolenic acid methyl ester. This analogue could be potentially useful in the further investigation of the requirements and functions of α-18:3 in Heliothines and other insects.

While some of the nuances of the α-18:3 requirements of Heliothines have been revealed, there are many aspects that require clarification. Doing so will require the continued application of techniques from a broad range of disciplines.

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