Manipulation of the nutritional composition of *Tenebrio molitor* larvae (yellow mealworms) for use as a feed ingredient in aquaculture

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Dedicated to my family

and

Edward

I could not have done this without you

"It is not the strongest of the species that survives, not the most intelligent that survives. It is the one that is the most adaptable to change"

Charles Darwin

Abstract

The larvae of *Tenebrio molitor*, a species of coleopteran, have received increasing attention in recent years as a potential sustainable protein source to replace fishmeal (FM) in the feeding of fish. FM is produced using small pelagic fish and is a high price commodity that is considered to be a finite resource. The high price of FM, as a high-quality resource, limits the expansion possible in aquaculture production systems that are reliant on it as the major protein provision in the feed of farmed fish. Provision of a protein source that is sustainable, both environmentally and economically, will enable aquacultural systems to expand and meet the rising demand for healthy animal proteins. Fish have been associated with health benefits to consumers and have a high abundance of long-chain poly-unsaturated fatty acids (LC-PUFA) of the n-3 series, linked to anti-inflammatory effects. Fish require a dietary source of LC-PUFA, which they gain from FM and FM by-product fish oil (FO) in aquacultural systems. Consumption of feeds containing FM and FO helps to meet their nutritional requirements and prevent pathologies in the fish. In addition, FM and FO consumption results in an accumulation of LC-PUFA in the nutritional composition of the fish, from which humans can gain health benefits from consuming them. In order for aquacultural system expansion to help meet an increased meat demand *per capita* and a growing population complete replacement protein sources for FM will be required that provide a source of LC-PUFA in addition to suitable protein contents. If *T. molitor* have the endogenous capacity for LC-PUFA synthesis from dietary precursors, they could be

a suitable FM *and* FO replacement.

The aims of this work were to successfully produce *T. molitor* larvae on a non-competing protein feed source and to determine the capacity to manipulate their nutritional content with feeding inputs. Of particular interest was the manipulation of specific fatty acids (FA) through various oil feeding inputs, as well as determining whether *T. molitor* have the ability to synthesis LC-PUFA endogenously. Further to this, an assessment of gene expression was undertaken in *T. molitor* larvae using RNA-Sequencing and *de novo* transcriptome assembly to establish potential target gene transcripts involved in endogenous lipid metabolism. Finally, techniques for production of *T. molitor* larvae were further assessed and optimised and their suitability as a FM replacement was assessed in *Danio rario* over a 10-week trial period.

The results indicated that *T. molitor* larvae can be as successfully produced on a non-competitor protein source, wheatbran (WB), over sow feed (SF), a conventional production animal feed. Larvae attained the same final weights and had significantly increased crude protein ($P<0.001$) and reduced crude lipid ($P<0.001$) contents, compared to SF treated larvae. Further to this FA composition in *T. molitor* larvae was successfully manipulated by oil inputs in feeds ($P<0.05$). *T. molitor* larvae mimicked feeding inputs of specific oils and achieved a >5-fold accumulation in n-3 alpha-linolenic acid (ALA) through feeding with linseed oil in wheatbran ($P<0.001$).

Feeding with linseed oil at 5% (w/w) in a basal feed of WB resulted in 2-fold and significant differential expression in 41 gene transcripts ($P<0.05$). The up-regulation of genes associated with transcription factor, immunity and lipolysis function were of particular interest and suggested potential for n-3 PUFA modulated effects in larvae gene expression. Furthermore, the potential identification of a delta-12 desaturase enzyme means *T. molitor* have additional FA synthesis capacities above that observed in mammals, birds and fish which is a novel finding

and of particular interest. The presence of this enzyme suggests *T. molitor* have the capacity to elongate to n-6 linoleic acid (LA) from n-9 oleic acid, without the requirement of dietary inputs. Mammals, birds and fish require a dietary source to obtain n-6 LA as they are unable to sythesise LA endogenously.

In comparison to larvae grown in a 13h:11h, light:dark environment, larvae grown in complete darkness had significantly increased growth rates, ($P<0.001$), whilst consuming the same amount of feed and had unaltered gross energy, crude fat, crude protein and FA compositions. These findings will enable production cost savings on both electricity and space, in addition to an increased efficiency and biomass accumulation in *T. molitor*.

Finally, *T. molitor* were found to successfully replace FM in the feeds of *D. rario* at 20% replacement of FM and inclusion of *T. molitor* meal (TM) prepared by feeding WB with an addition of rapeseed oil, which had an overall FA composition that was below the n-3 ALA requirement of the fish. Fish feed containing *T. molitor* larvae supplemented with 5% linseed oil, in a WB basal feed, resulted in significantly reduced deaths in fish compared with TM feeds with additions of rapeseed and/or linseed oil during feed formulations, indicating increased palatability. Replacement of FM in fish with various TM meals had no impact on the FA profiles for LC-PUFAs ARA, EPA and DHA in the fish, which could support the presence of LC-PUFA in *T. molitor* larvae that meet fish requirements or suggests 30% FM that remained in the treatment feeds is enough to meet the LC-PUFA requirements of the fish.

This thesis increased the body of knowledge on the suitability of *T. molitor* larvae as an alternative for FM in the feeding of fish. The novel findings, that *T. molitor* have higher crude protein content when fed on a non-production animal feed indicates that there is a further benefit of producing them on WB, which is a non-competing protein source. In addition, optimisation of rearing methods, such as the increased efficiency of larvae produced in complete darkness, will enable

considerable cost reductions. Furthermore, the ability to manipulate FA content of *T. molitor* with feeding oils, similar to other monogastric animals, have great implications for their use as an n-3 PUFA-enriched protein source in the future. In turn, *T. molitor* successfully replaced 20% FM in a freshwater fish species.

The discovery of a non-mammalian desaturase activity in *T. molitor* is of great interest, as mammals cannot insert unsaturation above delta-9 position. A delta-12 desaturase means that *T. molitor* are not reliant on plant sources for provision of LA which is a n-6 LC-PUFA precursor. Further evaluation of this DN 53206 *T. molitor* transcript is necessary as well as annotation of the 33 *T. molitor* gene transcripts that were not classified. The large variation exhibited within treatment groups suggests biological variation, which could be exploited through selective breeding programs.

There is a requirement for further work to discover the actual capacity of *T. molitor* for endogenous LC-PUFA synthesis, which was inconclusive in this work and further fish trial work replacing FM in fish species consumed by humans, particularly carnivorous species, with a greater requirement for LC-PUFA.

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Now onto the next question: *'Why do we have finger tips, but not toe tips and go up on our tip toes, but not on our tip fingers.'* E. Pickard 2018

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List of abbreviations

AA- amino acid

ALA- alpha-linolenic acid

ARA- arachidonic acid

ATP- adenosine triphosphate

CB- supplementation with cocoa butter

CVD- cardiovascular disease

DHA- docosahexaenoic acid

ELOVL- elongation of very long chain fatty acids protein

ENSO- El Nino Southern Oscillation

EPA- eicosapentaenoic acid

FA- fatty acid

FACoA- fatty acid acyl coenzyme A synthase

FAME- fatty acid methyl esterification

FAS- fatty acid synthase

FCR- feed conversion ratio

FM- fishmeal

FO- fish oil

GC- gas-chromatography

GDP- gross domestic product

GHG- greenhouse gas

LA- linoleic acid

LC-PUFA- long chain polyunsaturated fatty acid

LC-SFA- long chain saturated fatty acid

LS- supplementation with linseed oil

LXR- liver X receptor

MUFA- monounsaturated fatty acid

NEFA- non-esterified fatty acid

P- supplementation with palm oil

PDCAAS- protein digestibility corrected amino acid score

PK- supplementation with palm kernel oil

PPARa- peroxisome proliferator-activated receptor alpha

PUFA- polyunsaturated fatty acid

RS- supplementation with rapeseed oil

SAFF- supplementation with safflower oil

SBM- soybean meal

SREBP-1c- sterol regulatory element-binding transcription factor isoform 1c

SCD- stearoyl CoA desaturase

SFA- saturated fatty acid

TAG- triacylglycerol

VLDL- very low density lipoprotein

WB- wheatbran

Chapter 1

Introduction

1.1 Overview

Global livestock production has grown to accommodate rising meat demand that has accompanied a growth in the human population. Demand for animal protein comes not only from the growing population but a change in consumer preferences. Data for aggregate meat consumption *per capita* (kg/*capita*) supports this and clearly suggests added pressure to the food supply system as a result of a greater demand for processed food, meat, dairy and fish (Henchion et al., 2014). This is being attributed to the steadily increasing economic growth and urbanisation in developing countries. For example meat consumption in developing counties increased three times as much as in developed countries from 1970's to the 1990's (Delgado, 2003).

The ability to produce food will be affected by growing competition for land, water and energy in addition to over-exploitation of natural global resources, such as fish stocks (Premalatha et al., 2011). Although the agricultural and aquacultural industries have grown in an attempt to support demand, they still fall short of meeting the needs of one in seven people who are without access to sufficient protein and energy from their diets and of the many more suffering from micronutrient malnourishment (FAO, 2009).

Soy bean meal (SBM) and fish meal (FM) are arguably the best sources of protein available. They however cost around 60-70% of the total expense of animal food and feed production, limiting potential for the expansion of production animal systems (Steinfeld et al., 2006). Furthermore, there has been a rise in the 'health conscious consumer', and with particular media attention on meat (with the exception of fish) and its high content of saturated fatty acids (SFA) which are associated with risk of disease in humans (Walker et al., 2005).

Dietary provision of long chain-fatty acids of the n-3 series, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been associated with health benefits in humans with evidence they reduce the risk of cardiovascular and neurological diseases (Gillingham, 2013). Although mammals have the ability to synthesis LC-PUFA and they are not regarded an essential component in the diet, their *de novo* biosynthesis capacity is limited. Even in fish, incorporation of EPA and DHA in their natural or farmed diets is essential to avoid pathologies, resulting from impacts on gut microbiota and gut histology, due to a low rate of synthesis, relative to their nutritional requirements (Sánchez-Muros et al., 2014; Merrifield et al., 2011). The gastrointestinal tract is one of the main route for pathogens in fish (Merrifield et al., 2011). Acquisition of LC-PUFA in fish is the result of bioaccumulation from primary producers, which have enzymes that enable them to produce high quantities of EPA and DHA. A dietary provision of LC-PUFA for humans is therefore through the eating fish.

Expansion in aquacultural systems has been rapid in recent years as the over-exploited, wild-caught fish levels plateaued. However, there are economic and ecological concerns over *all* farming practices required to meet global requirements for protein, particularly aquaculture with its reliance on FM taken from finite oceanic resources. The same is the case for SBM, the demand for which, is resulting in rapid deforestation in South American countries, contributing to carbon release and global warming effects, in addition to loss of natural habitats for many species

of flora and fauna.

Insects provide a potential alternative source of protein for the feeds of monogastric production animals. Studies have suggested that compounded insect feeds contain a protein source that is comparable in quality to that present in conventional feed for broiler chickens (Oyegoke et al., 2006; De Marco et al., 2015), pigs (Makkar et al., 2014) and fish (Ng et al., 2001). As a result of high palatability *T. molitor* larvae could replace up to 60% of dietary fishmeal at 26% inclusion in African catfish, (*Clarias gariepinus*) without affecting growth or feed utilisation (Ng et al., 2001). In van Huis et al. (2013), insects have been shown to have favourable nutritional compositions. However, provision of LC-PUFA, EPA and DHA in terrestrial insects is thought to be deficient compared to those in FM and FO sources, which are currently provided to the feeds of farmed fish (Yang et al., 2006; St-Hilaire et al., 2007).

The quality of a fish fillet is dependent on dietary inputs, such as protein quantity/quality and also FA composition of oil provision of the feed (Miller et al., 2008). Currently the digestibility, amino acid balance and fatty acid composition, appear to prevent the inclusion of insects in fish diets at levels higher than 20-30%, highlighting the need for new strategies to enhance the nutritive values of insect-meal that could allow for increased percentages of fishmeal substitution (Sánchez-Muros et al., 2014).

1.2 Background

1.2.1 Increased demand for protein

Food security is defined as "when all people at all times have access to sufficient, safe, nutritious food to maintain a healthy and active life" (World Food Summit, 1996). Over recent years there has been increasing focus and importance on ensuring people have access to adequate supply of food and adequate nutrition from

their food. The contribution of different food products to global food security is primarily reliant on both agriculture and aquaculture industries (Rosegrant & Cline, 2003). However, people living in developing countries tend to be affected by an acute shortage of animal protein, which adversely affects protein intake, leading to pathologies associated with malnutrition (Das et al., 2009).

This problem has been exacerbated by increasing demand for protein sources with an increasing global population, particularly in developing countries where the rate of population growth is at its fastest and there is the greatest risk to food security. More specifically there is an increased demand for protein sourced from animal products both in developed and developing countries where urbanisation is occurring at a relatively fast rate. It had been suggested that world population would increase by 80 million each year, reaching 9 billion by 2050 (Godfray et al., 2010). However by this date global population is now estimated to reach 9.7 billion (UN, 2015). In order to ensure food security, 70% more food will need to be produced (Godfray et al., 2010).

Despite the impacts of climate and culture, human diets have altered relatively consistently as regions or countries become increasingly urbanised with increased incomes (Tilman & Clark, 2014). Increased gross dometic product (GDP) has been associated with nutrient transitions from predominantly legume and grain-based diets to increased *per capita* demand for meat products (Tilman & Clark, 2014). In 2009, the richest 15 countries in the world had a 750% greater *per capita* meat demand from ruminants, seafood, poultry and pork in comparison with the 24 poorest countries and further to this legume protein demand decreased with increasing animal protein consumption (Tilman & Clark, 2014). The shifts in diets, as countries become more urbanise, is clearly observed when comparing data for meat consumption per capita in 1982, at 15 kg when there were 4.5 billion people, with predictions of 37 kg in 2030 (FAO, 2011). The population increase and greater meat demand *per capita* puts further pressure on the animal

production industry's expansion.

1.2.2 Population health

Proteins are a major component of the human body. Animals require exogenous sources of essential amino acids (AA), those that cannot be produced *de novo*, in order to synthesise proteins. Failure to supply balanced AAs in human diets results in protein malnutrition (this is also the case for livestock animals) (Pereira & Vicente, 2013). It has been said that 1 billion people worldwide lack access to adequate food resources, which is a major concern for ensuring global food security (Walker et al., 2005). Animal products are an excellent option for providing nutrient-dense food to millions of people faced with under nutrition, and as a protein source they are far superior to any vegetable sources with respect to human nutrition (de Castro et al., 2012). Animal proteins have high digestibility, with eggs and meat scoring Protein Digestibility-Corrected Amino Acid Scores (PDCAAS) of 1.00 and 0.92, respectively (Pereira & Vicente, 2013). Whereas beans, lentils and peas scored between 0.57-0.71 PDCAAS and wheat gluten scored even lower at 0.25 PDCAAS (Pereira & Vicente, 2013).

However, animal products also provide primary sources of saturated fatty acids (SFAs) (Walker et al., 2005). Over-consumption of SFAs have been associated with health risks, including cardiovascular disease (CVD), type 2 diabetes and some cancers (Walker et al., 2005). Urbanisation tends to result in a nutritional shift in the human diet towards one that is energy dense and high in animal protein and fat, stereotypically associated as a Western diet (Walker et al., 2005). CVD is the main cause of death in most Western countries, and instances of CVD as well as type 2 diabetes and some cancers are increasing in developing countries, where *per capita* meat demand is increasing with urbanisation-linked nutrition trends (Walker et al., 2005).

1.2.3 Food production impacts

Agricultural and aquacultural systems will need to expand in order to meet the global demand for increased animal protein, however the expansion may be limited by availability of finite natural resources, such as land, freshwater, energy from fossil fuels and animal protein from oceanic sources (van Zanten et al., 2015). Currently agriculture is associated as being a major contributor of negative impacts on ecosystems. Agriculture accounts for 20% of total greenhouse gas (GHG) emissions and 30% of global energy consumption, whilst agro-chemicals are the main pollutant to freshwater sources (Tilman & Clark, 2014; Ytrestøyl et al., 2015).

Furthermore, in order to expand animal production systems, an increasing amount of feed will need to be produced. However, the high price of the highest quality protein sources, used as animal feed, limit the expansion possible at 60-70% of the total cost of animal production (Steinfeld et al., 2006). Currently animals, including fish, are fed on high-quality protein sources. These could be consumed directly by humans to alleviate protein malnutrition and help ensure food security in poorer countries. In addition, 30% of global cereal production is currently used as energy sources in feed for livestock (Ytrestøyl et al., 2015). With production of poultry, swine and beef expected to double by 2050, the global capacity for providing enough animal feed is a severe challenge facing the expanding food production industries (Ytrestøyl et al., 2015).

1.2.4 Increasing the health benefits of meat

Animal products provide nutrient-dense food that help to ensure food security, but also provide a high proportion of SFA in their total lipid contents including lauric (12:0), myristic (14:0) and stearic (18:0) acids. Over-consumption of SFAs result in inflammation, which is an associated precursor to diseases such as heart disease, stroke, cancers and auto-immune diseases such as rheumatoid arthitis (Ruxton et

al., 2004). Excessive consumption of SFAs have been shown to directly stimulate inflammatory gene expression via prostaglandins and thromboxanes, with lauric shown to have the largest pro-inflammatory effects over myristic and stearic acids (J. Y. Lee et al., 2001). Further to this, treatment with mono-unsaturated (MUFA) and poly-unsaturated fatty acids (PUFA), were able to block these inflammatory effects (J. Y. Lee et al., 2001).

Omega-3 (n-3) PUFAs, particularly long-chain PUFA (LC-PUFA) such as EPA and DHA, have been shown to have many health benefits to humans, including infant growth and development, reduced risks of CVD, inflammatory and autoimmune diseases (Carragher et al., 2016). However, humans have limited capacity for conversion of essential α-linolenic acid, 18:3n-3 (ALA) to LC-PUFA such as EPA and DHA. Provision of pre-formed EPA and DHA in the diet of humans cannot be matched by providing appropriate levels of ALA in the diet (Gillingham, 2013; Carragher et al., 2016).

The low endogenous synthesis of LC-PUFA of the n-3 series has been attributed to a high majority of ALA being used for β-oxidation (McCloy et al., 2004). In addition, a Western diet contributes a high level of essential FA linoleic acid, 18:2n-6 (LA), which is the precursor for LC-PUFA of the the n-6 series. Despite, enzymes in the LC-PUFA modification pathway having higher levels of affinity to ALA, high dietary levels of LA can saturate rate-limiting delta-6 desaturase enzymes (Liou & Innis, 2009; Orsavova et al., 2015). Futhermore, it has been hypothesised that multiple use of the rate-limiting delta-6 desaturase enzyme for the conversions of ALA to 18:4n-3 and 24:5n-3 to 24:6n-3 may lead to a"bottle-neck" in the metabolic pathway and an associated decrease in the synthesis of DHA (D'andrea et al., 2002; Kitson et al., 2010). High levels of n-6 FA consumption in Western diets has also been associated with formation of arachondonic acid (ARA)-containing (LC-PUFA of the n-6 series) eicosanoid metabolic products that are biologically active in small quantities. In large quantities they can contribute

to formation of thrombus, atheromas, allergic and inflammatory disorders whereas eicosanoid metabolic products derived for n-3 LC-PUFA EPA are associated with anti-inflammatory effects potentially reducing the risk of diseases such as CVD (Simopoulos, 2002a).

In order to improve the perception and health value of meat to consumers, producers are increasingly seeking to manipulate FA compositions within the meat by dietary inputs, using specific vegetable sources and/or their oils. Linseed/flaxseed is a vegetable source with high levels of ALA contents that can be utilised in the feeds of animals to increase the PUFA:SFA ratio value, and reduce the n-6:n-3 PUFA ratio value (Wood et al., 1999). However, majority of studies fail to increase DHA levels (Francois et al., 2003; Gillingham et al., 2011). Alternatively, fish provide a source of LC-PUFA of the n-3 series, which have been obtained as a result of bioaccumulation from algae and phytoplankton in small pelagic fish, consumed in the wild and in aquaculture as FM (Orsavova et al., 2015). Health agencies have recommended the consumption of two meals per week containing fatty fish in order to achieve a combined intake of between 250 and 500 mg/day of EPA and DHA (Kris-Etherton et al., 2002; Carragher et al., 2016). These health recommendations, and a greater reliance on fish for human health, puts further pressures on aquaculture industries to source viable feeding inputs for sustainable expansion to meet demands.

1.2.5 The aquaculture industry

Fish (including crustaceans) are an important food commodity and account for 17% of animal protein intake and 6.5% of total protein consumption, globally (Troell et al., 2014). The global average *per capita* supply of fish has increased dramatically during the last 40 years, from 12.7 kg/year in the 1961 to 21.4 kg/year in 2010 (FAO, 2013). Global fish consumption itself has increased from 9 kg *per capita* per year in 1961 to 16.5 kg in 2003 and is expected to rise to 22.0 kg by 2024

(Delgado et al., 2003; FAO, 2015). However, global marine fish landings reached a peak of 86.3 million tonnes in 1996, which declined to 79.5 million tonnes in 2008 (Ye et al., 2013; Tran et al., 2015). Demands for fish have been increasingly met by aquaculture, which has expanded nine-fold since the 1980s (FAO, 2014; Youn et al., 2014). Fish products themselves comprise one of the most widely traded segments of the world food economy, valued at 129 billion US dollars in 2012 and 362 billion US dollars in 2016, with 232 billion US dollars being contributed from aquaculture (FAO, 2014, 2018).

Aquaculture has been the world's fastest growing food production sector over the past five decades, with an average annual growth rate of 8.9% between 1950-2006 (Tveterås et al., 2012; Silva et al., 2009). Global capture fishery production has plateaued around 90 million tonnes annually and further demands from a growing population and increased *per capita* meat demand must be met by aquaculture (FAO, 2014). The industry is expected to maintain an average annual growth rate of >4% between 2010-2030 (Brugère & Ridler, 2004).

The growth in the aquaculture industry has been further driven by high consumer perceptions of fish, which are associated with having health benefits to humans who consume them and are also considered efficient food converters, with higher edible yields than most terrestrial animals (Naylor et al., 2009; Torrissen et al., 2011). Fish are a rich source of vitamin B12, which is only found in animal products and contributes to healthy functioning of multiple systems such as growth, brain function, and maintenance of the nervous system (Thilsted et al., 2016). In addition, small pelagic fish, which omnivorous and carnivorous fish species feed off, offer high bioavailable sources of calcium, zinc and iron, which are globally considered 'problem micro-nutrients' (Bogard et al., 2015; Hansen et al., 1998; Thilsted et al., 2016). In addition, fish offer a unique source of omega-3 fatty acids which are associated with health benefits in humans (Imhoff-Kunsch et al., 2012; Hibbeln et al., 2007).

The cultivation of fish and crustaceans in terrestrial freshwater and marine aquaculture systems grew at an annual rate of 7.8% worldwide between 1990 and 2010; a rate that substantially exceeded that of poultry (4.6%), pork (2.2%), dairy (1.4%), beef (1.0%), and grains (1.4%) over the same period (FAO, 2013; Tacon & Metian, 2013). This expansion in aquaculture has been considered the last animal domestication, transitioning from hunting to farming techniques, with greater than 600 different animal species now being produced in aquaculture production systems (Troell et al., 2014). These species comprise finfish (catfish, trout, carp, tilapia, salmon), crustaceans (shrimp, prawn, crabs, freshwater crayfish), and molluscs (mussels, oysters and clams) (FAO, 2014).

Production of fish by aquaculture production systems could help to ensure food security. In 2009, a pivatol point in fish production was observed, with aquaculture production almost matching that from wild catch fisheries at 45.7% (Natale et al., 2013). Further to this, in the most recent SOFIA, FAO 2018 report aquacultures contribution to total global fish production in 2016 was 47%, with 80 million tonnes produced from aquaculture systems.

1.2.6 Fishmeal: a major feed source

The world capture fisheries are now stabilised at approximately 90-92 million tonnes per year and this figure is not expected to increase since at least half of the worlds recognised fish stocks are fully exploited and 32% overexploited or depleted (FAO, 2018). It has been suggested that food security can be further ensured with aquaculture compensating for the plateued supply of seafood from fisheries. Aquaculture on the one hand provides an important contribution in feeding the growing human population, with valuable animal proteins and potentially alleviates mounting pressures on wild fish stocks to meet the growing demands for animal protein for food (Natale et al., 2013). On the other hand, aquacultures' reliance on wild fish stocks as a feed input, which is high cost and may be con-

sidered to be a finite resource, is unsustainable and limits key expansion of the industry.

Fishmeal (FM) is a high protein powder used in aquaculture and other animal farming systems as a major source of protein in feed. FM has high nutritional value as a feed source for animals, along with ease of digestibility and excellent composition of AAs (Olsen and Hansen, 2012). In addition, FM contains n-3 LC-PUFA including EPA and DHA, as well as vitamins riboflavin, niacin, vitamins A and D and minerals such as calcium, phosphorus, iron, zinc, selenium and iodine (R. L. Olsen & Hasan, 2012).

FM and fish oil (FO) are produced from small pelagic forage fish species such as anchovy, herring and sardine all of which are suitable for human consumption (Merino et al., 2010; R. L. Olsen & Hasan, 2012). In 2009, around 63% of FM and 81% of FO went into aquaculture feeds the former was equally split between feeds for salmonids, marine fish, crustacean and other species, whereas 66% of the latter was utilised in the feeds for salmonids and 20% in marine fish feeds (Natale et al., 2013). The Peruvian anchovy, *Engraulis ringens*, makes up around 50% of global FM production and as a result less than 1% of the total catch in Peru was used for human consumption in 2006 (Metian, 2009).

Landings used for the production of FM and FO have been in the range of approximately 20-25 million tonnes annually in the period 1980-2005, but recent data suggest this declined to 18 million tonnes from 2006 to 2012, resulting in an annual production of about 5-6 million tonnes of FM and 1 million tonnes of FO (R. L. Olsen & Hasan, 2012; FAO, 2014; Tran et al., 2015). Overexploitation of ocean resources, in addition to variability in capture due to ENSO events, which result in ocean temperature increases and a decline in *E. ringens* stocks, could be contributing to the reduced catches observed (Merino et al., 2010).

Finfish make up the majority of farmed marine fish and are a carnivorous species with a higher dependence on FM and FO provision in the diets to avoid

pathologies in the fish (Naylor et al., 2009; Delgado et al., 2003). FM is also utilised in freshwater fish feed, mainly in carp culturing (Deutsch et al., 2007). In addition, the increased production of carnivorous species, such as shrimp, has exceeded that of total aquaculture production, due to expansion based on economic incentives (Deutsch et al., 2007; Lebel et al., 2002). These developments have increased the international trade of FM and FO, which are essential in their culture (Deutsch et al., 2007; Merino et al., 2010). Salmon, trout and shrimp aquaculture uses almost 50% of the FM used in aquaculture, but provide less than 10% of aquaculture production (Deutsch et al., 2007).

From an ecological standpoint, pelagic forage fish species are extremely important as a primary resource for marine predators and a key transfer of energy in food webs of marine ecosystems from the zooplankton and phytoplankton that they feed on (Pikitch et al., 2014). Continued exploitation of oceanic resources could have catastrophic consequences on species biodiversity. In addition, protein sources are necessary for prevention of malnutrition in the growing population and utilising protein options that are suitable for human consumption for feeding animals is a misuse of already strained resources. Furthermore, the price of FM limits the expansion possible in the aquaculture sector, as a result of variability in the supply and increased demand for the marine resource, which drives price increases for the sought-after commodity. Between 2002 to 2006 the price of FM doubled to 1200 US dollars/tonne (R. L. Olsen & Hasan, 2012). The continued use of FM will prevent, or severely hinder, the viability of the aquaculture industry in the near future. Therefore, alternative protein sources are required to replace FM in aquaculture.

1.2.7 Alternative protein sources for fish

Plants

Plant protein sources have been suggested as potential alternatives for FM in the feeding of fish. Soybean meal (SBM) is often used in literary sources as a replacement for FM, due it having the best crude protein and essential AA profile (Nguyen et al., 2009). However, there are limitations associated with plant-derived protein sources for use in fish feeding, such as palatability issues, presence of anti-nutritional factors, low abundance of sulphur-containing AAs and high proportions of fibre and non-starch polysaccharides (Sánchez-Muros et al., 2014). A further cost of processing is required to denature anti-nutritional factors and to extract unsuitable lipids and a large portion of carbohydrate. Anti-nutritional factors have the potential to cause fish gut inflammation and unsuitable lipids and levels of carbohydrates can reduce digestibility of the feed and the concentrate levels of protein (Xie & Jokumsen, 1997; Day & Gonzalez, 2000). Furthermore, SBM protein source is deficient in methionine and requires synthetic addition of specified essential AAs, dependent on the fish species being reared (Nguyen et al., 2009).

Replacement of 50% FM with SBM protein concentrate, balanced for essential AAs, had no negative affects on growth or nutrient utilisation in salmonid rainbow trout, despite this being a carnivorous species with a high apparent reliance on FM (Mambrini et al., 1999). Furthermore, marine carnivores, such as Atlantic halibut and European seabass, have also had FM replaced by SBM protein concentrate and essential AAs without decreased growth (Berge et al., 1999; Kaushik et al., 2004). Currently, commercial feeds for Atlantic salmon are formulated with around 25% FM, 45% SBM protein concentrate and balanced for essential AAs (R. L. Olsen & Hasan, 2012). Without supplementation with essential AAs, SBM replacement of FM in 30% total protein inclusion resulted in reduced growth and feed efficiency in tilapia, (these effects could also be attributed to presence of trypsin inhibitor)

(Shiau et al., 1989).

Supplementation of FM with increased levels of plant proteins from different sources, such as peas, faba beans, lupins, canola, rapeseed, flaxseed and cottonseed can decrease feed intake and resulted in decreased growth in salmonids at high levels of inclusion (R. L. Olsen & Hasan, 2012; Collins et al., 2013). These plant protein sources are unsuitable for fish feeding as they do not provide a high-energy or nutrient-dense feed for growing fish and instead have relatively low protein, high carbohydrate and lipid contents (Day & Gonzalez, 2000). High carbohydrate containing plant sources used to replace FM in the feeds of salmonids decreased disease resistance in the fish, and increased pathologies, as a result of LC-PUFA deficiencies (Vielma et al., 2003). In addition, European whitefish (a salmonid), have exhibited increased mortality, high blood glucose and increased storage of carbohydrate as glycogen in the liver when fed on high levels of dietary carbohydrate (Vielma et al., 2003).

Furthermore, a large overlap exists between feeding resources used for farmed fish and terrestrial livestock. Currently the livestock industry consumes the greatest share of the world's industrially produced feeds: poultry, 41.5%; porcine, 30%; and ruminant 25%, compared with 4% currently utilised in aquaculture (Troell et al., 2014). Increased replacement of FM with SBM protein concentrate in the feeds of fish will further increase the demand for SBM, resulting in increased price, as other livestock production industries also rely on SBM for a cheaper alternative to FM (Kristofersson & Anderson, 2006). In addition, an increased cultivation of SBM to meet demands for aquaculture and agriculture expansion requires more land, water and energy from fossil fuels, which are all finite resources. Further to this, continued expansion of SBM production is resulting in rapid deforestation in South American countries causing loss of habitat and release of a further GHG emissions (Ytrestøyl et al., 2015).

Micro-organisms

Micro-organisms: yeast and microalgae (eukaryotes) and bacteria (prokaryotes) are one class of alternative options to plants in providing a protein source to replace FM (Matassa et al., 2016). The potential of these micro-organism protein sources will be dependent on their nutritional quality and the cost and ease of their production (Tacon et al., 2006). Yeast, as a by-product of ethanol and beer processing, could provide a low cost source of protein for fish feed. However, current production techniques for microalgae are expensive *and* generate low material yields (Becker, 2007). The costs of bacteria protein meal production are currently prohibitive, due to rising oil prices, however if renewable sources of carbon can be utilised to grow the bacteria, they could become economically viable (Matassa et al., 2016; R. L. Olsen & Hasan, 2012). Micro-organisms may have more of a use in the feeding of fish through provision of specific nutrients like essential AAs and vitamins that are found lacking in the protein source replacements for FM (Toride, 2002; R. L. Olsen & Hasan, 2012).

Traditionally produced livestock animals

By-products of terrestrial animal production, such as meat and bone meal, blood meal and poultry meals are generally considered to have a good nutritional quality and the availability of nutrients is predicted to be two- to three-times higher than that of FM (R. L. Olsen & Hasan, 2012). However, safety concerns surround the use of animal products in the feeding of other production animals, including fish, due to the transmissible spongiform encephalopathy (TSE) neurodegenerative disease, which could be transferred on to humans after consumingd infected meat (Naylor et al., 2009). However, modern processing techniques in compounded fish feed are reported to destroy harmful mammalian prions (the culprit of TSE) and furthermore differences between mammalian and fish physiology are thought to prevent against the absorption of mammalian prions through the fish gut (Ingrosso

et al., 2006; Rivera-Milla et al., 2006; Dalla Valle et al., 2008).

Fishery by-products could be another option for a high-nutritional quality feed provision to the aquaculture industry (R. L. Olsen & Hasan, 2012). Limitations of these by-products are high contents of ash from presence of bone or exoskeletons (crustaceans), the resulting high contents of calcium and phosphorous could interfer with absorption of other trace elements, such as zinc (Hardy & Shearer, 1985).

A further alternative animal protein are zooplankton, which are an unexploited marine resource (Natale et al., 2013; R. L. Olsen & Hasan, 2012). A 10-20% production of marine zooplankton could provide annual landings that match those of pelagic species used for FM production (Natale et al., 2013). Fish feed containing zooplankton was able to replace FM partially and completely in feeds of many fish species (R. Olsen et al., 2006; Suontama et al., 2007; Yoshitomi et al., 2007). However, as a major base in the food webs of ocean ecosystems, attention should not be on utilising these ocean sources, as there is a risk of mass species extinction of the fish, birds and marine predators that rely either directly or indirectly on them (Hewitt et al., 2002; Huntington & Hasan, 2009; Naylor et al., 2009).

1.2.8 Insects as an alternative feed for fish

Terrestrial insects have received increasing attention for their potential as an alternative protein source, reducing and replacing FM in the feeds of farmed fish. Not only have insects played an age-old role in human nutrition, they are still consumed all over the world (DeFoliart et al., 1982; Melo et al., 2011; Moreki et al., 2012). Not only do insects grow and reproduce easiliy, they have been shown to have a high feed conversion efficiency and have even been reared on bio-wastes (van Huis et al., 2013; Makkar et al., 2014).

Invertebrates are included on the European Union *Feed Material Register* for use as a raw material in the feeding of fish and pet species, and their use is likely

to be extended to pigs and poultry feeds in the near future (Marco et al., 2015). To date, a number of authors have reported interesting results about the suitability of different types of insect meal as feed ingredients for pigs, poultry and fish livestock species (Makkar et al., 2014; van Huis et al., 2013; Henry et al., 2015; Veldkamp & Bosch, 2015). Among the species with the greatest potential as food and feed within the European Union are the yellow mealworm (*Tenebrio molitor*), giant mealworm (*Zophobas atratus*), lesser mealworm (*Alphitobus diaperinus*), greater wax moth (*Galleria mellonella*), silkworm (*Bombyx mori*), house cricket (*Acheta domesticus*) and African migratory locust (*Locusta migratora migratorioides*) (Committee, 2015; Adámková et al., 2017).

The viability of insects as a FM replacement has been supported by evidence they can utilise lower grade plant protein sources that cannot be used in fish rearing (Veldkamp et al., 2012). Furthermore, as poikilotherms, insects require no energy from food to maintain body temperature, meaning they have the potential to be highly efficient feed-to-biomass converters (Janssen, 2018). In addition, it has been suggested that insects require much less land and water relative to production of animal feed crops and emit significantly lower amounts of GHG emissions relative to other animal production systems (Janssen, 2018; Oonincx & De Boer, 2012; Oonincx et al., 2010). Insects form a natural dietary component in wild fish species, omnivorous fish species prey on insects found on the bottom of water bodies and juveniles of carnivorous species eat insects before switching to fish-based diets in adulthood (Riddick, 2014).

As a potential alternative protein for the replacement of FM in farmed fish, insects provide a source rich in protein (42-63%), energy, and lipids (8.5-36%), and, contain lower levels of carbohydrate in comparison to plant protein alternatives (<20%) (Tran et al., 2015). In addition, at least some insect species are found to be rich in copper, iron, magnesium, manganese, phosphorous, selenium and zinc, as well as riboflavin, pantothenic acid, biotin and, folic acid and have AA

compositions that meet requirements for humans (van Huis et al., 2013; Rumpold & Schlüter, 2013). Insects are also likely to contain lower fibre contents and anti-nutritional factors compared with plant sources. Chitin, a carbohydrate in insects, could limit their use as fish feeds, but individual fish species' ability to digest chitin is still under debate (Ng et al., 2001; Sánchez-Muros et al., 2014).

Insects could provide 25-30% replacement of FM and with balanced essential AAs have the potential to totally replace FM (Tran et al., 2015). However, in order to do so they must provide a replacement source for FO, to avoid ARA, EPA and DHA deficiencies in farmed fish. van Huis et al. (2013) suggest insects have similar MUFA and PUFA contents to fish, however more research is required into individual insect species' capacity to provide LC-PUFA to farmed fish. This would have benefits of being able to replace total FM and FO in feeds of farmed fish, without pathologies in fish and whilst maintaining the n-3 omega FAs in fish fillets, which are important for consumer health and perceptions'.

Edible insects

Insects belong to the class arthropods and encompass over one million species, which make up between 70-75% of all animal species living on Earth (Katayama et al., 2008). Coleoptera represents the largest of eight orders of insects, the true beetles, which are thought to represent 40% of total insect species and 25% of all known lifeforms on Earth (Capinera, 2008; Stork, 1988). These quantities highlight great opportunities for their use as food and feed in ensuring food security. Globally there are around two thousand species of insects considered edible.

A meeting of the Food and Agriculture Organisation in 2012 discussed the potential of these edible insects and short-listed the black soldier fly (*Hermetia illuscens*) and the yellow mealworm (*Tenebrio molitor*) as the two best candidates for mass-production as food and feed (Figure 1.1). These two species were selected as having a high intrinsic rate of increase, high potential of biomass increase per

day, high conversion rate (kg biomass/kg feedstock), ability to live in high densities and low susceptibility to disease. Furthermore, these species were highlighted as being suitable for large-scale automated rearing, in non-native countries, with additional potential to genetically enhance desirable characteristics through selective breeding (Vantomme et al., 2012).

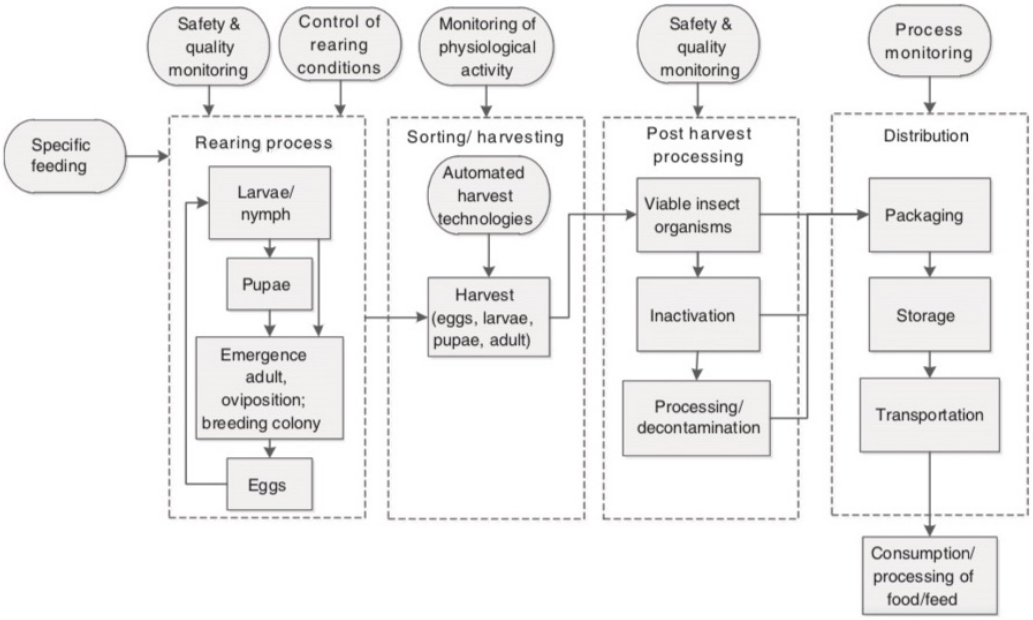


Figure 1.1: Schematic for the mass-rearing of edible insects as feed and food (Rumpold & Schlüter, 2013).

Tenebrio molitor is one of two species of darkling beetle, in the Tenebrionidae family, known more commonly as the yellow mealworm beetle (Makkar et al., 2014). In this particular work, conducted in the United Kingdom, *T. molitor* was considered a more suitable candidate for experimental design, as a European dry store pest, which can be found worldwide. In contrast, *H. illuscens*, which form part of the family Stratiomyidae in the order of Diptera, are typically found in tropical and warmer temperate regions (Diener et al., 2011). They feed in aqueous environments on rotting organic material in particular animal manure and human excreta (Makkar et al., 2014).

Tenebrio molitor life-cycle

The life-cycles of insect species are categorised as either holometabolous that undergo complete metamorphosis from egg, to larva, to pupa and finally emerge as adults, or hemimetabolous that have incomplete metamorphosis from egg, to nymph, to adult (Capinera, 2008).

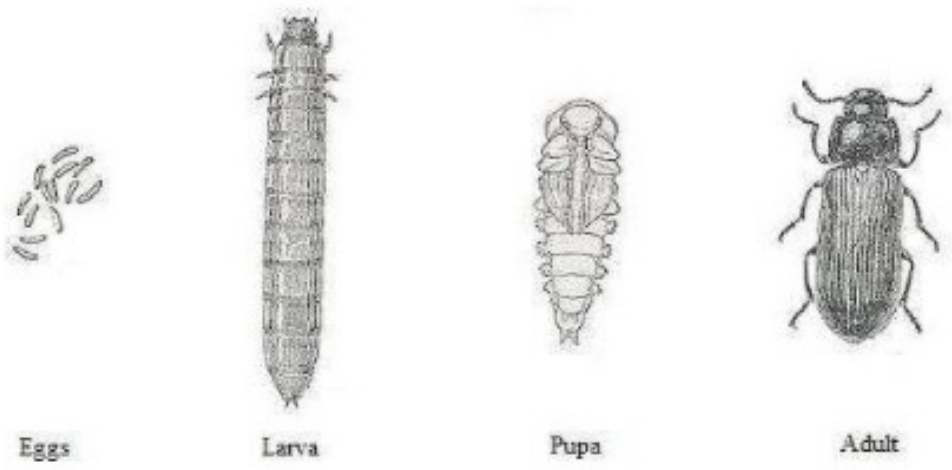


Figure 1.2: The life stages of holometabolous *Tenebrio molitor* (the yellow mealworm) (Metcalf & Flint, 1962).

T. molitor undergo complete metamorphosis (Figure 1.2). Female beetles lay between 250-1000 eggs, with an average of 400-500 eggs and can mate at intervals throughout adulthood, which can range between 37-96 days (Ghaly & Alkoaik, 2009). The bean-shaped, shiny white egg incubates for up to 2 weeks, but typically 7 days, before 2 mm white larvae emerge (Ghaly & Alkoaik, 2009). After a few days the larvae turn yellow as they produce a hard, chitinous exoskeleton (Siemianowska et al., 2013). In optimal temperature and moisture conditions *T. molitor* are thought to remain as larvae for 6-8 months (Ghaly & Alkoaik, 2009; Siemianowska et al., 2013). During this period of development larvae undergo a series of exoskeleton molts, called instars, which allow for increased growth in the larvae. Some researchers have suggested up to 20 molts within the species larval period of development and larvae have been found to reach maximal lengths of

25-35 mm and weights of 200 mg (Park et al., 2014; Cotton & St George, 1929; Siemianowska et al., 2013). At the point of pupation larvae turn white and enter a dormant phase of development, where no food is consumed, their length as pupae is around 10 mm and they emerge as light brown coloured adults after around 6 days. They begin to darken a few days after emergence as the exoskeleton hardens.

Research has shown that it is more beneficial to harvest *T. molitor* during their larval phase, due to loss of weight prior to entering into pupation and the most efficient period for harvesting has been stipulated as between 100-120 mg weight (Ghaly & Alkoaik, 2009).

Tenebrio molitor **meal**

The AA profile of *T. molitor* larvae is close to that of SBM. *T. molitor* larvae meal has been shown to contain higher crude protein than SBM and higher lipid contents close to those of animal by-product meals (De Marco et al., 2015). *Methionine* or *methionine+cystine* are generally the most limiting essential AA when larvae have been fed to production animals. Arginine has also been shown to be limiting in broiler feeds (Veldkamp & Bosch, 2015). For these reasons *T. molitor* meal could be utilised as both a protein and an energy ingredient for fish feeds (De Marco et al., 2015). *T. molitor* have been successfully used as a protein source for catfish (Ng et al., 2001; Su et al., 2017), seabass (Gasco et al., 2016) and chickens (De Marco et al., 2015; Ramos-Elorduy et al., 2002) however, more research into FA compositions and plasticity is required in *T. molitor* and currently little is known about the fat metabolism within the species.

A current limitation of using insects as an alternative protein feed ingredient is the cost of production systems, which currently have high energy consumption and are labour intensive, both due to lack of research into rearing optimisation and automation. Currently, the price of *T. molitor* are around 0.40 euros/kg live weight based on 35% DM content and freeze dried they cost 3.70 Euros/kg (Veldkamp &

Bosch, 2015). Compared with the most frequently used protein-rich feed ingredients, insect protein is the most competitive with FM whose actual price is around 1.24 euro/kg, but expected to increase in the near future (Veldkamp & Bosch, 2015). The current price for SBM is approximately 0.57 Euros/kg (Veldkamp & Bosch, 2015). In addition, future utilisation of insects as sustainable animal feed ingredients require them to be grown on sources of protein that are not likely to be included directly in feeds of fish, pigs, poultry or humans. There is further potential to selectively breed species of insects, including *T. molitor*, for enhanced performance. Only in recent years have fish began to be domesticated and the benefits achieved by genetics and strain selection have resulted in reduced feed conversion ratios (FCR) (Natale et al., 2013). The gains in estimated FCR between 1995 and 2006 for shrimp were from 2.0 to 1.6, for salmon and trout were from 1.5 to 1.3, and for Chinese carp species gains were from 2.0 to 1.7 (Tacon & Metian, 2008). Decreased FCR reduces the overall price of production and increases profits margins meaning the cost of high value aquaculture commodities can be reduced when they reach trading markets, helping to ensure global food security.

1.2.9 Lipids

As previously discussed, CVD has been associated as the major cause of death in Western countries (Orsavova et al., 2015). Coronary heart disease (CHD) is closely linked to atherosclerosis, the narrowing of arteries, which is caused by interactions between lipids, lipoproteins, monocytes, platelets, endothelium, and smooth muscle of arterial walls (Mišurcová et al., 2011). Replacement of dietary SFA and trans-FA in the diet with PUFAs has shown promising results in the reduced risk of CHD (Harika et al., 2013). Lipids are considered one of the most crucial nutrients for humans, as water insoluble organic compounds that play a major role in cells. Lipids form structural components in cell membranes, are

involved in mediating metabolic and hormonal signalling pathways and gene expression (Crook, 2013; Orsavova et al., 2015). They are also the major energy storage molecule in the body and provide substrates for ATP production in muscle, heart and liver through β-oxidation (Lehner & Quiroga, 2015). The four main types of lipid present in plasma are cholesterol, cholesterol ester, phospholipid, and triacylglyceride (TAG) (Crook, 2013).

Lipid classifications

Lipids consist of FAs, which are carboxylic acids, formed of carbon, hydrogen and oxygen. The hydrocarbon chain length in FAs can vary between 4-36 carbons and classification of FA is determined by the length of chain and the presence, number and positioning of double bond (unsaturation) within the hydrocarbon chains (Lehner & Quiroga, 2015). SFA contain no double bonds, MUFA contain one double bond, whereas PUFA, contain two or more double bonds in their hydrocarbon chain.

The configuration of double bonds determines cis or trans-FAs, the former having hydrocarbon chains on one side of the carbon-carbon double bond chemically bonded to either carbon molecules, and both hydrogen groups on the other side of the double bond, and the former having one hyrdogen and one hydrocarbon group on either side of the carbon-carbon double bond (chemically bonded to each carbon molecule). Cis are the naturally occurring configuration of FAs, whereas trans-FAs are the result of either vegetable oil hydrogenation in food processing or biohydrogenation of fats in ruminants by rumen bacteria (Crook, 2013; Palmquist et al., 2005).

PUFA that contain more than one double bond have physiological properties dependent on the position of the first unsaturation relative to the methyl- end and not the carboxyl- end (Lehner & Quiroga, 2015). PUFA are further classified by this first unsaturation as being of the n-6 or n-3 series of PUFA, the former have the

first unsaturation in the sixth position from the methyl- end of the FA, whereas the latter has its first unsaturation in the third position. Mammals, including humans, cannot synthesise n-6 or n-3 PUFAs *de novo*, as they lack delta-12 desaturase and delta-15 desaturase enzymes required for insertion of the n-6 or n-3 unsaturation, respectively (Gillingham, 2013) (Figure 1.3).

Lipogenesis: *de novo* FA synethesis

Within lipogenic tissues, such as the liver and adipose tissue, SFA palmitic acid (16:0), and to a lesser extent myristic (14:0) and stearic (18:0) acids are synthesised *de novo* as a result of excess energy from carbohydrate provided in the diet (Lehner & Quiroga, 2015). Mammals have the enzymes capable of synthesising SFA from acetyl Co-A, otherwise known as acetate, and NADPH, a hydrogen carrier, which are produced as a result of the pentose phosphate pathway (PPP) (Figure 1.4) (Lehner & Quiroga, 2015). Acetyl-CoA is considered the 'building block' for the *de novo* synthesis of FAs, and is converted to malonyl-CoA/malonate via enzyme acetyl-coA carboxylase (ACC). Palmitic acid/palmitate (16:0) is formed from malonate via the enzyme fatty acid synthase (FAS), and via elongase activity stearic acid/stearate (18:0) is formed (Figure 1.3).

When consuming diets low in fat and high carbohydrates (10% calories fat and 75% calories carbohydrate), *de novo* lipogenesis can contribute up to 50% of FA in plasma lipids (mainly TAG) (Lehner & Quiroga, 2015). However, when a Western diet is consumed (30-40% calories from fat and 45-50% calories for carbohydrate), the contribution of *de novo* lipogenesis to plasma lipids is undetectable (Lehner & Quiroga, 2015). In humans, *de novo* lipogenesis would not usually play a major role in lipid metabolism. However, *de novo* lipogenesis, can contribute 25-30% of hepatic and circulating lipids in high fat diets due to insulin resistance (type 2 diabetes), despite the high influx of exogenous FA to the liver (Lehner & Quiroga, 2015).

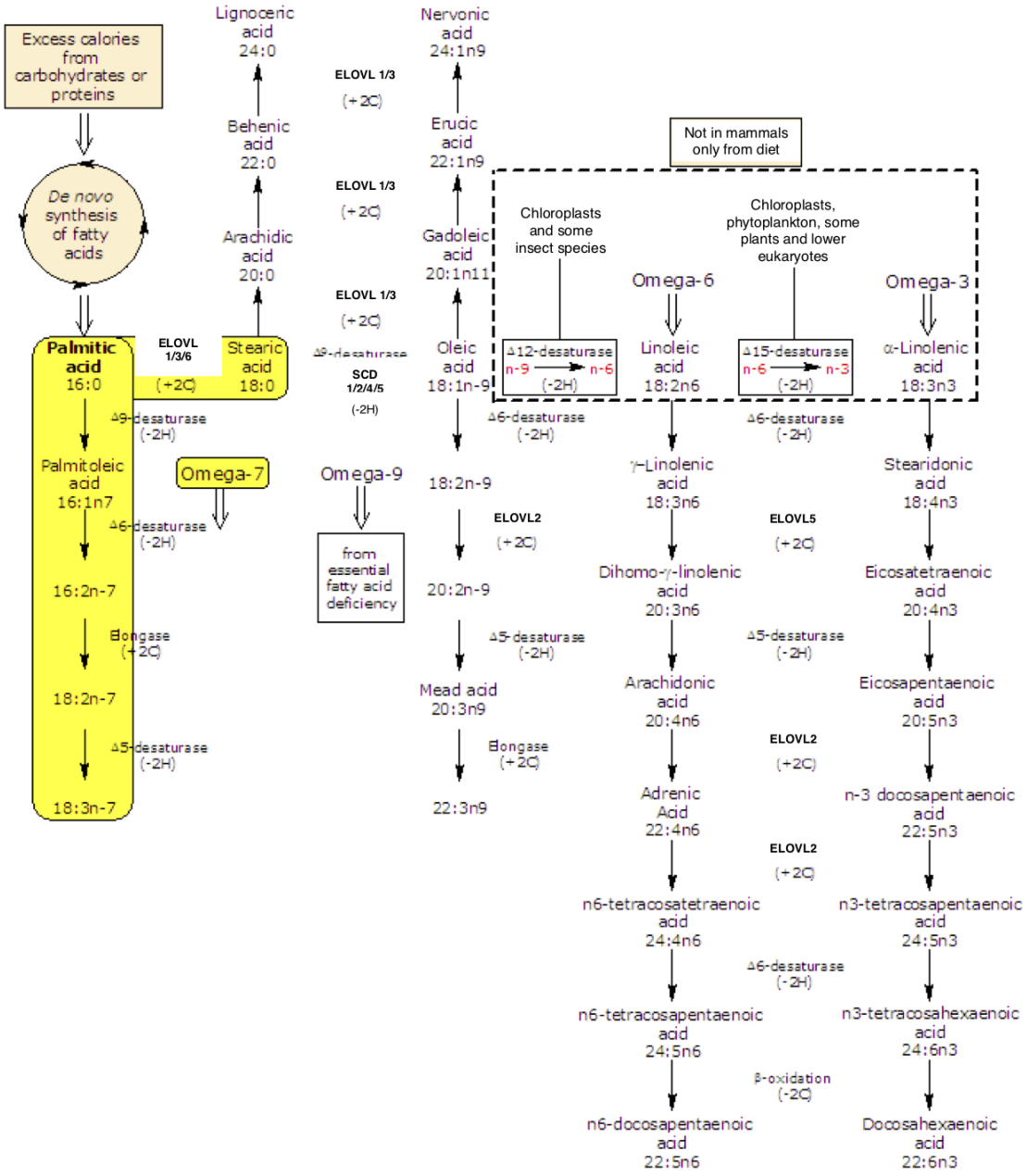


Figure 1.3: ***De novo* biosynthesis of fatty acids.** Edited from `http://www.tuscany-diet.net/2013/02/03/long-chain-fatty-acid-synthesis/` accessed 16.09.2018.

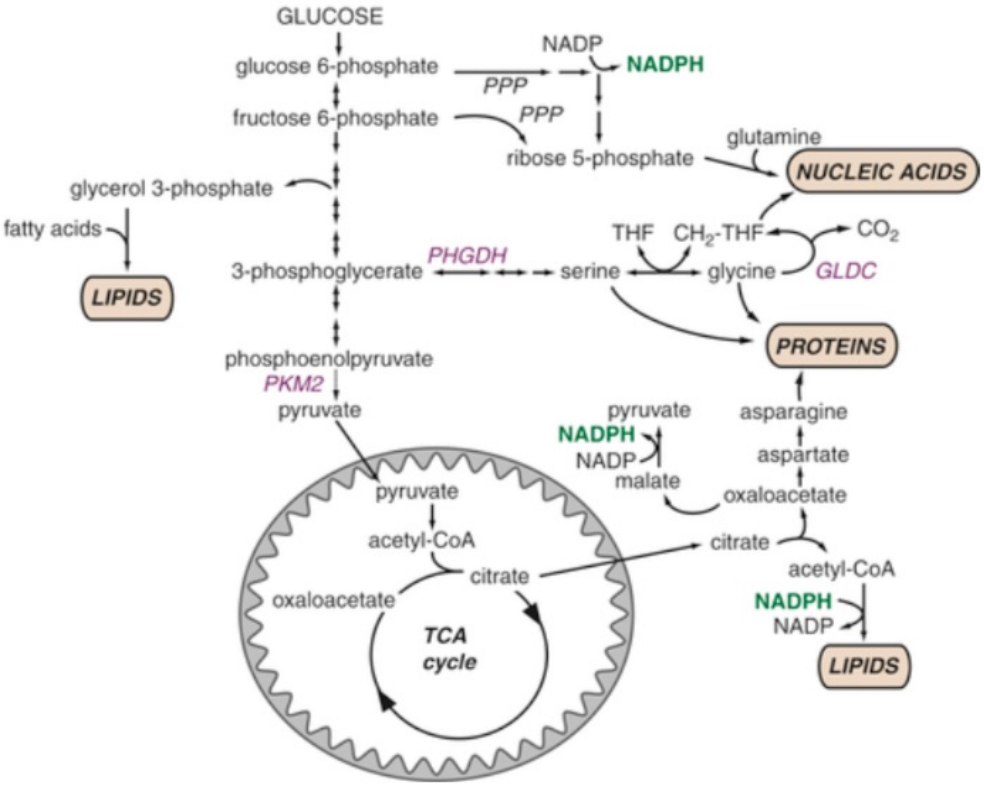


Figure 1.4: **Biosynthesis of biological molecules and their links to the pentose phosphate pathway (PPP).** Obtained from `http://syromonoed.com/metabolism-cell-growth-proliferation/` accessed 16.09.2018. *CH_2-THF*, methylene tetrahydrofolate; *GLDC*, glycine decarboxylase; *PHGDH*, phosphoglycerate dehydrogenase; *PKM2*, pyruvate kinase M2 isoform; *PPP*, pentose phosphate pathway; *THF*, tetrahydrofolate.

FA modification: elongation and desaturation

Both FAs synthesised *de novo* and those obtained from the diet are modified through metabolic pathways that require desaturation and elongation enzymes. These reactions occur primarily in the endoplasmic recticulum (ER) and produce a variety of long-chain-SFA (LC-SFA), MUFA, PUFA, consisting of 16 or more carbons (Lehner & Quiroga, 2015). SFA undergo further modifications, via stearoyl-CoA desaturases (SCD) (a delta-9 desaturases), to form MUFA oleic acid/oleate (18:1) and palmitoleate (16:1) from stearate and palmitate, respectively (Figure 1.3). Elongase enzymatic activity progressively adds two carbons to the FA hydrocarbon chains and desaturases delta-6 desaturase (FADS1 gene) and delta-5 desaturase (FADS2 gene) are involved in adding further unsaturations to the chains of MUFA oleate and palmitoleate and dietary LC-PUFA precursors (Figure 1.3). The roles of MUFA are diverse and crucial in living organisms. FAs 16:1 and 18:1 are the major MUFA of TAG, cholesteroyl esters (CE), wax esters and membrane phospholipids and the ratio of SFA to MUFA contributes to membrane fluidity and changes in the acyl-chain composition of CE and TAG, which affect lipoprotein metabolism (Lehner & Quiroga, 2015).

Dietary derived FA

Dietary fat is an important macronutrient for growth and development of all organisms and changes in the quantity and/or composition of dietary fat ingested impacts cellular processes affecting many physiological systems and cell types (Jump, 2002b). Many of these effects can be linked to changes in membrane-bound phospholipid composition, affecting cell membrane fluidity *or* eicosanoid signalling (Jump, 2002a).

As mammals have no capacity to synthesise unsaturation in the n-6 and n-3 position from the methyl- end of the FA chain, and as a result precursors 18:2 n-6 and 18:3 n-3 are considered essential FAs. Mammals, birds and fish are dependent

on plants for providing double bonds in the delta 12 and 15 positions of the two major precursors of n-6 and n-3 LC-PUFAs, LA and ALA, respectively (Lehner & Quiroga, 2015). The biological activities of individual essential FAs might be derived from the character and three-dimensional configuration of the molecules, as well as their subsequent enzymatic transformation into eicosanoids compounds (Lehner & Quiroga, 2015).

Lipid metabolism

FA are delivered to cells from exogenous (diet) and endogenous (liver) sources as complex lipoproteins: chylomicrons and VLDL, respectively or as non-esterified fatty acids (NEFA) that have been mobilised from storage depots (Jump, 2002b; Crook, 2013). TAG in chylomicrons and VLDL are hydrolysed by the action of lipoprotein lipase (located on capillary walls) to TAG forming FA and glycerol (Lehner & Quiroga, 2015). FA are taken up by adipose tissue, or muscle cells, via fatty acid transporters (Lehner & Quiroga, 2015). Excess lipids that supersede the cell's requirements for processes such as membrane synthesis, mitochondrial β-oxidation and lipoprotein production are stored in lipid droplets within the cell (Crook, 2013). Remnant lipoproteins in plasma are taken up by the liver and hydrolysed by hepatic lipases and glycerol, (the backbone of TAG), enters the hepatic glycolytic pathway (Jump, 2002b).

Once in cells, NEFAs are rapidly converted to fatty acyl coenzyme A (FACoA) thioesters by acyl coenzyme A synthetase, which is a rate limiting step for entry of FAs into β-oxidation (mitochrondrial or peroxisomal), modification by elongation and desaturation or assimilation into complex lipids such as TAG, cholesterol esters or phospholipids (Lehner & Quiroga, 2015; Jump, 2002b). Futhermore, NEFAs are prospective ligands regulating activities of peroxisome proliferator-activated (PPAR) and liver X (LXR) nuclear receptors (Jump, 2002b). FACoA thioesters are thought to regulate the activity of other transcription factors, in-

directly effecting the expression of metabolic pathway genes (Lehner & Quiroga, 2015).

PUFA: associated health benefits

PUFA are the major structural components of cell membranes and are considered pivotal nutrients for preventing non-alcoholic fatty liver disease (Gormaz et al., 2010), autoimmune responses (Simopoulos, 2008), CVD (Superko et al., 2014), colon and breast cancers (Fasano et al., 2017; Cockbain et al., 2011) and diabetes (J.-S. Zheng et al., 2012). PUFA are said to provide their effects through altering composition of the cellular membrane composition and the ability regulate transcription and cellular signalling (Massey & Nicolaou, 2011). EPA and DHA have been shown to reduce inflammatory bowel disease and asthma (Calder, 2013). Furthermore, DHA is necessary for cognition and brain development as well as prevention of neurodegeneration and is a precursor for anti-inflammatory docosanoids (Calder, 2006; Wolfe, 1982). DHA, are proposed to be anti-inflammatory and facilitate cell survival on oxidative stress (Serhan & Petasis, 2011). DHA supplementation is said to protect against many inflammatory diseases such as rheumatoid arthritis, cystic fibrosis, asthma, cancer and CVD (Calder, 2006; Lehner & Quiroga, 2015).

Twenty-carbon n-6 and n-3 PUFAs are precursors that are metabolised to diverse lipid mediators, such as eicosanoids, and docosanoids, such as prostaglandins, prostacyclins and leukotrienes, which have been linked to inflammatory and immune responses (Wolfe, 1982). The effects of n-6 or n-3 eicosanoids are antagonistic, the former are associated with inflammation, whereas the latter are much less inflammatory or even considered anti-inflammatory (Orsavova et al., 2015; J. M. Lee et al., 2016). The concentration of both types of eicosanoids are dependent on the exogenous supply of FA, in addition to the ratio of n-6:n-3 in the diet due to competition between ARA and EPA as substrates for specific enzymes

involved in eicosanoid synthesis: cyclooxygenases and 5-lipoxygenases (Mišurcová et al., 2011; Kinsella et al., 1990; Simopoulos, 2002b). High consumption of plant oils rich in n-6 PUFAs and relatively low consumption of marine fish products could cause this ratio to be too high, as observed in Western countries (Orsavova et al., 2015).

1.2.10 Lipid metabolism in insect species: an overview

The fat body

The fat body of insects is a relatively large tissue which is distributed preferentially underneath the integument, and surrounding the gut and reproductive organs (Dean et al., 1985; Arrese & Soulages, 2010). The fat body in Coleopteran species, as in many other insects, is accumulated mainly in the abdomen of the insect (Crowson, 1981). It is responsible for the synthesis of lipids, glycogen and haemolymph proteins and is the major organ for the nutrient storage and energy metabolism (Arrese & Soulages, 2010; Chapman, 2013). Many insects' tissues have vertebrate analogues; however the fat body is an organ unique to insects (Law & Wells, 1989). In some ways, the insect fat body is functionally and cytologically analogous to vertebrates' liver and adipose tissue, respectively (Liu et al., 2009). However, the liver is a solid structure and the insect fat body is a loose tissue that is arranged in thin lobes that are bathed by hemolymph; thus providing high exposure of the fat body to the haemolymph (Arrese & Soulages, 2010).

Lipids are the main components of the fat body and the basic cell in the fat body is an adipocyte, which is characterised by the presence of numerous lipid droplets (Bailey, 1975; Canavoso et al., 2001; Arrese & Soulages, 2010). At the end of the feeding period, lipid droplets occupy most of the intracellular space along with glycogen and protein granules (Dean et al., 1985). TAG are the

major component of the lipid droplets and are the main group of lipids in the fat body, representing a major source of lipid reserves; these may be supplemented by phospholipids and steroids (Chapman, 2013).

Structure and function

The fat body is the target organ of several hormones that regulated function in respect to physiological requirements that change during different development stages (Arrese & Soulages, 2010; Gäde, 2004). There is further evidence for several other metabolic processes in the fat body that allow it to respond to metabolic requirements of the organ itself and these processes must be tightly coupled to a number of metabolic pathways (Arrese & Soulages, 2010). Fat-body cells usually contain the enzymes required to build up the appropriate reserve substances from simple sugars brought to them by the bloodstream and to breakdown reserve substances to replenish the blood sugars when appropriate (Arrese & Soulages, 2010). This emphasises the importance of the fat body's structure; allowing maximum exposure and ready access to the haemolymph (an insects 'blood/plasma'), aiding in its abilities to adjust concentration of energy precursors in circulation (Arrese & Soulages, 2010).

This hormonal regulation is particularly important in insects that are holometabolous. These insects must accumulate energy reserves during larval feeding periods, which are required for metamorphosis and adult life (Arrese & Soulages, 2010). Development of the fat body does vary according to life-cycle stage and can become depleted in larvae and adult undergoing extended starvation or periods of high activity (Crowson, 1981). These circumstances in turn affect the accumulation of nutrients within the insect, which exhibits effects on growth and the timing of metamorphosis (C. K. Mirth & Riddiford, 2007).

Glycogen, or some very similar polysaccharide, has been detected in fat-body cells of many, if not all, insects studied (Crowson, 1981). Symbiotic micro-

organisms have been identified in the fat body of some insect species; these symbionts are speculated to produce essential components that are not derived from feed inputs, although symbionts have not yet been detected in coleopteran species (Crowson, 1981; Stanley & Nelson, 1993).

Requirements for FA in insects

Requirements for essential FAs, LA and ALA, may differ substantially between insect species; some studies have concluded that either LA or ALA satisfies an insects nutritional requirement for polyunsaturated fatty acids (R. H. Dadd, 1985). This could be because some insects have been shown to contain delta-12 desaturase that can convert oleic acid (MUFA) into LA (PUFA) and or contain delta-15 desaturase that converts LA to ALA (Figure 1.3).

Blomquist et al. (1991), categorised four types of insects based on their requirements for PUFA, which is related to their ability to synthesis n-6/n-3 precursors and further desaturate and elongate to LC-PUFA *de novo*:

1. **Insects that can synthesis PUFA *de novo***. *Periplaneta americana* and *Acheta domesticus* have been shown to possess delta 12 desaturase and elongases to produce ARA *de novo* (De Renobales et al., 1987; Jurenka et al., 1987, 1988).

2. **Insects that require dietary source of n-6 and n-3 precurors, LA and ALA, but can further desaturate and elongate to ARA and EPA, respectively**. *Galleria mellonella* have similar capacity to mammal species' for LC-PUFA modifications (Stanley-Samuelson et al., 1987).

3. **Insects that require ARA and EPA and cannot elongate or add di- and tri- unsaturations to LA or ALA**. Culicidae family require ARA and EPA supplementaitons (R. Dadd, 1983; Rivers et al., 1975).

4. **Insects that do not have a requirement and do not synthesis PUFAs**. *Drosophila melanogaster* have been shown to have evolutionary circumvented requirements for PUFAs (Rapport et al., 1983).

The coleopteran family to which *T. molitor* belong, have shown a requirement for essential PUFA mainly by slow larval growth in their absence (Dadd, 1985). However, there is a lack of knowledge as to those specific requirements as well as the capabilities of *T. molitor* to synthesise FAs *de novo* and further desaturate/elongate to LC-PUFAs. The ability of *T. molitor* to desaturate longer chain SFA or convert SFA to MUFA (18:1); MUFA to LA (18:2); and/or LA to ALA (18:3), would be of interest (Figure 1.3). Furthermore, the capabilities of *T. molitor* to desaturate and elongate dietary PUFAs to ARA, EPA and DHA, would be encouraging in continued investigations into their use in the feeding of fish and ultimately the complete replacement of FM and FO.

1.3 The problem

There is an increased pressure on ensuring a growing human population has access to sufficient, safe, nutritious food to maintain a healthy and active life. A good option for helping to ensure food security is to increase livestock (mammals, birds and fish) production systems to provide nutrient-dense animal proteins to people. However, this puts strains on finite resources such as water, land, energy and primary marine protein sources. A further burden on food production systems is as a result of increased meat demand *per capita*, which is attributed to nutrient-shift in developing countries as they become increasingly urbanised.

CVD is now the number one cause of mortality in Western countries and its cause is associated with over-consumption, particularly of SFAs, which are a prominent lipid in animal food sources. Inflammatory affects of SFA excess consumption could be reduced through increased dietary consumption of n-3 PUFA,

which subsequently reduce ratios of SFA:PUFA and n-6:n-3, to a favourable level of <5, promoting anti-inflammatory effects in humans and reduce the risk of CVD (Wood et al., 2004).

A readily available source of protein and n-3 series LC-PUFA are found in fish. Global fish production from fisheries has plateaued at around 90 million tonnes annually and aquaculture, as the fastest growing industry in the past half a century is increasingly subsidising the global demand for fish. Further expansion of the aquaculture industry, however will be affected by its reliance on marine fish for the production of FM and FO, which form the main protein source in fish feeds. Alternative protein sources are therefore required, that provide high-quality and highly digestible protein. Furthermore, if FM and FO are to be replaced altogether, an alternative for FO and its high contents of n-3 LC-PUFA EPA and DHA will also be required in order to avoid pathologies in fish and provide unaltered fish fillet FA profiles, which are sort after by consumers.

1.4 Insects to provide the solution

Having highlighted in Section 1.2.7 the need for FM replacements that are not from soybean plant sources, *T. molitor*, with an AA profile similar to *that* of SBM, but a higher crude protein content could provide a suitable alternative protein source for feeding farmed fish species. In addition, *T. molitor* contains less fibre to SBM and anti-nutritional factors, resulting in the potential for reduced feed processing of *T. molitor* meals, so long as the chitin does not require removal. *T. molitor* also contain higher lipid contents compared to SBM and could therefore provide not only a protein feed replacement for FM, but also an energy ingredient in the feed of fish. Lipid-rich *T. molitor* also have the potential to fully replace FM and FO, if specific FAs can be manipulated in their composition and a capacity for increased n-3 series LC-PUFA synthesis is discovered. This would enable the

aquaculture industry to move away from using finite oceanic resources and continue industry expansion to meet growing demands for fish protein, particularly carnivorous species that have the highest reliance on FM and FO.

1.5 The work conducted in this thesis

Although the number of literary sources for the use of insect species as feed has increased in recent years (reviewed by van Huis 2017), there are many gaps in the knowledge of optimal conditions for production, species-specific nutritional requirements and even robust methodology to determine FCR for basic cost-analyses. Furthermore, species-specific information on lipid metabolism in insects and their ability to modify dietary lipid source to LC-PUFA, is also lacking. The work in this thesis aims to fill in some of the gaps in that knowledge and aims to assess whether *T. molitor* is a viable prospective feed for fish.

Work carried out in this thesis utilised *T. molitor* species during the larval stage of their development cycle. Chitin, the fibrous carbohydrate in the exoskeleton of insects, increases after metamorphosis into adult beetle form. The insect trials featured in this thesis were carried out over a three year period. After a detailed explanation as to the methods utilised for calculating growth characteristics; analysing nutritional composition; and assessing the apparent gene expression within *T. molitor* larvae, I progress onto the main body of work, which features nine experiments reported over 4 chapters. Individual trial methodologies will be given in each Chapter separately.

I begin with two experiments utilised to determine whether growth and nutritional composition of the larvae are affected by different feeding ingredients and whether *T. molitor* could be viably produced as a feed for fish, on a non-competing protein source. During these experiments, husbandry methods were being developed for consistency in the future experimental work conducted in later chapters

of the thesis. For instance, in the first experiment (Chapter 3) larvae were measured using total biomass and number of individuals were not recorded, which prevented determinations of mortality and also biomass gain normalisations on a per larvae basis. In the second experiment, (Chapter 3) although number of larvae was assessed, the amount of biomass produced was not sufficient for nutritional analysis.

Following on from this I begin to assess the ability to manipulate the FA content in *T. molitor* larvae, similar to what has been seen in production animal species (Chapter 4). Analysis of FA profiles, will enable observation of potential capacity for further elongation of unsaturated FAs to LC-PUFA and any affects of SFA on *T. molitor* larvae. The manipulation of FA profiles within larvae continues into Chapter 5, where the impacts of different feeds on apparent gene expression within *T. molitor* larvae are assessed.

Finally, a freshwater fish trial utilising *T. molitor* larvae meal and *T. molitor* meal that has been manipulated for increased ALA will be compared against each other *and* FM, for its potential effects on growth and nutritional composition in fish. This final experimental chapter (Chapter 6) also features two additional experiments that intend on optimising techniques for *T. molitor* larvae rearing, enabling a potential reduction in their current production costs. The final discussions and conclusions are made in Chapter 7.

1.5.1 Thesis Aims

1. To develop growing techniques for production of *T. molitor* larvae as a viable feed ingredient.

2. To assess how different feed ingredients alter proximate nutrient content of *T. molitor*. In addition to determining the ability to manipulate specific FA abundance in *T. molior*, by feeding input.

3. To assemble whole transcriptome for *T. molitor* larvae and determine the effects of different oil supplementation in feed ingredients on the transcriptome of *T. molitor* larvae.

4. To identify genes that are associated with lipogenesis and lipolysis within *T. molitor* larvae.

5. To optimise husbandry techniques involved in rearing *T. molitor* larvae to maximise efficiency and further provide evidence for the viability of *T. molitor* larvae as a 'sustainable' feed source for animals.

6. To assess the replacement of FM with *T. molitor* larvae in the feeding of freshwater fish and whether FA profile manipulation within larvae is a future option as a fish feed ingredient.

1.5.2 Hypothesis

1. *Tenebrio molitor* larvae can be manipulated for increased specific fatty acids of the n-3 series and provide a viable alternative protein source to FM in the feeding of fish.

2. Manipulation of specific FAs within *Tenebrio molitor* nutritional composition will affect apparent expression of genes associated with FA metabolism.

3. *Tenebrio molitor* larvae will provide the benefit of an economically and environmentally viable feed ingredient for production animals that gives increased quantities of n-3 FA in the diets of humans, promoting human health.

Chapter 2

Methods

2.1 Materials

Tenebrio molitor larvae were purchased for growth trials from two suppliers; Monkfield Nutrition Ltd., Royston, Cambridgeshire, UK and CJ WildBird Foods Ltd., Shropshire, UK. These suppliers obtained their *T. molitor* larvae from the Netherlands and maintained them on *ad libitum* access to wheatbran (WB) and carrot. Once these larvae arrived at the University of Nottingham they were maintained on the same feed until this was replaced with growth-trial specific feeds. Two distinct sizes of larvae were purchased from Monkfield (standard and mini mealworms) as appropriate for experimental designs and one size was purchased from CJ WildBird Foods (mini mealworms). The standard mealworms, ("large larva"), 18-26 mm in length and >6 weeks old, were found to be suitable for pupation trials (Chapter 3), pupating within a 14-day growth period. Whereas the mini mealworms, ("small larva"), 15-18 mm in length and <6 weeks old, proved suitable for larval growth trials (Chapters 3, 4, 5 and 6) with the aim of providing a nutrient-dense production animal feedstuff.

Table 2.1 shows the equipment used for production of feed for growth trials and the rearing of *T. molior* in these trials. Table 2.2 shows all feed ingredients used for larvae feeding trials throughout this body of work and the suppliers of

the feedstuffs sourced.

Specific conditions for *T. molitor* larvae and *D. rario* juvenile growth trial' are described in individual chapters.

Table 2.1: **Animal handling and feed production equipment used in *T. molitor* rearing.**

Equipment	**Product**	**Company**
Disposable respirator	9320+ FFP2[1] (APF[2] = 10)	3M Aura
Sieve	2mm slots, d: 200mm, h:50mm	Retsch
Analytical scale	4 decimal places A1205	Sartorius
ImageJ	Computer software used for counting	NHI
Mill	Cutting mill (1999), 0.5-3.0 mm inserts	Fritsch
Platform scales	max: 60 kg, precision: 20 g	Kern IXS

[1] *Filtering face piece, level 2*
[2] *Assigned protective level*

Table 2.2: **Compiled list of feed ingredients utilised in *T. molitor* trials and company sourcing information.**

Product (abbrev.)	**Company**	**Sourcing**	**Featured**
Wheatbran (*WB*)	Buy Whole Foods Online	www.buywholefoodsonline.co.uk	Chapters 3, 5, 6
Breeding sow rolls (*SF*)	Heygates Ltd.	www.heygatesfeeds.co.uk	Chapter 3
Country chick crumb (*chick crumb*)	Heygates Ltd.	www.heygatesfeeds.co.uk	Chapter 4
Palm kernel oil	Sigma Aldrich	www.sigmaaldrich.com/United-Kingdom	Chapter 4
Palm oil	Sigma Aldrich	www.sigmaaldrich.com/United-Kingdom	Chapters 4, 5
Cocoa butter	Naissance	www.amazon.co.uk	Chapter 4
Organic safflower oil	Naissance	www.amazon.co.uk	Chapter 4
Biona organic rapeseed oil	Quasaro	www.amazon.co.uk	Chapters 4, 5
Raw linseed oil	Amazon EU	www.amazon.co.uk	Chapters 4, 5, 6

2.2 Equations for growth measurements

2.2.1 Biomass weight gain

Total biomass weight gain was a performance characteristic used consistently throughout this thesis work. Biomass gain over the total trial course was calculated using the equation 2.1, where larvae were weighed with full gut contents for initial and end weight recordings. In this thesis, biomass weight gain value is presented as an average of treatment replicates.

$$\text{Biomass weight gain} = \text{End weight} - \text{Initial weight} \tag{2.1}$$

2.2.2 Average growth rate

The average growth rate was calculated in both *T. molitor* and *D. rario* trials using equation 2.2. The calculation utilised values of weights per individual per replicate, recorded over at least four time points during larvae and fish growth trials. Values were plotted on a scatter graph, where growth (y) was plotted over time (x). Assuming constant growth, equation 2.2 was obtained where m represented the growth over time. For larvae this growth over time (m) represented average growth per individual, per replicate, per day (where possible), whereas for fish the gradient represented the average growth per individual, per replicate, per week.

$$y = mx + c \tag{2.2}$$

Where individual number of animals per replicate was not determined on individual weigh days, biomass growth rates were calculated using equation 2.2 however, were described as biomass weight gain per replicate on a per day/per week basis, seen in Chapter 3 and 5.

2.2.3 Total proportion dead

Mortality was used as another performance characteristic and was represented as a proportion, equation 2.3.

$$\text{Proportion dead} = \frac{\text{Total number dead}}{\text{Starting number of individuals}} \tag{2.3}$$

For *T. molitor* larvae trials, values were obtained from photographs; taken (on weigh days) during growth trials and counted using ImageJ computer software, which was used to count larvae present in replicates, by manually selecting larvae in the image. For *D. rario* trials, fish were monitored on a daily basis and the number of deaths were recorded as they were observed, for each replicate tank.

2.2.4 Total average consumption

As the protocols for *T. molitor* larvae growth trials developed, they began to include a measure of estimated total consumption recordings, of feed (which varied across trials) and water source (carrot). Total consumption calculations were likely underestimations of consumption in larvae, as frass (insect excrement) could not be separated from the remaining feed present in husbandry containers (Chapters 4, 5, 6). These values were determined either as total consumption using equation 2.4 or as a total consumption per individual using equation 2.5, where appropriate. For *D. rario* trials waste feed collection from the tanks was not possible; therefore, the amount of feed given was used as total feed intake (Chapter 6). A feed conversion ratio was estimated as total feed input (feed and water source) per biomass gained using equation 2.6 for *T. molitor* trials.

$$\text{Total consumption} = \text{Feed In} - \text{Feed Out} \tag{2.4}$$

$$\sum_{i=1}^{4} \frac{\mathrm{I}_i - \mathrm{O}_{(i+1)}}{\mathrm{N}_i} = \text{Total consumption per individual} \tag{2.5}$$

Time points, $\mathrm{t}_i = \{\text{Day } 0, \text{Day } 4, \text{Day } 7, \text{Day } 11, \text{Day } 14\}$

$\mathrm{i} = \{1, 2, 3, 4, 5\}$

Food In, I_i

Food Out, O_i

Total larvae, N_i

$$\text{FCR} = \frac{\text{Total feed input}}{\text{Total biomass gained}} \tag{2.6}$$

2.3 Nutritional analysis

2.3.1 Drying methods

Methods for proximate analysis of crude protein, crude fat, and gross energy used in this thesis required samples to be fully dehydrated. Freeze-drying and oven-drying are the two most commonly used methods for preserving food by dehydration (Shadung et al., 2012). Smith (1973) indicated the most acceptable heat drying results were obtained through drying between 70-100°C, providing a temperature low enough to limit thermochemical degradation and a drying rate fast enough to reduce chemical changes and loss of dry matter (DM). Freeze-drying is a process used to dehydrate materials using freezing and sublimation, which is the transfer of solid water to gaseous water vapour phase. Frozen material is heated to above 0°C (the freezing temperature of water), whilst a vacuum pump lowers chamber pressure to below 0.06 ATM, allowing for the water to thaw,

but preventing it entering the liquid phase. Freeze-drying has been associated with the least effects on sample chemical composition compared with other drying methods and produces compositional results that correlate with those of the fresh sample (Kaldy et al., 1979). However, it is neither a cost, nor a time-effective method for drying large numbers of samples (Kaldy et al., 1979). It has also been suggested that oven-drying at a temperature of 65°C is the maximum to avoid adverse thermal reactions in samples (Shadung et al., 2012). Indeed, oven drying of insects at 66°C for 24 hours resulted in increased essential and non-essential AA in a coleopteran species, compared with freeze drying methods at a pressure of 085 mtorrat -55C for 24 hours (Shadung et al., 2012). Another possible consideration is the oxidation of UFAs, the rate of which can be affected by degree of unsaturation as well as exposure to oxygen, light and heat (Ismail et al., 2016).

For these reasons a mixture of methods were utilised throughout the course of this developing body of work and these are specified in the relevant method sections of chapters. Drying methods included in this thesis work were: oven-drying (Gallenkamp) at 100°C; oven-drying at 55-65°C; and freeze-drying (E-C apparatus).

Oven drying at 100°C was conducted dependent on the size of the sample, either in small porcelain crucibles, medium aluminium containers or large metal baking trays using an oven (Gallenkamp). Dehydrated sample containers were dried for an hour at 100°C in the preheated oven. Dried containers were then cooled in a desiccator for 15 minutes. Containers were weighed (Tw) and then the sample added. Total weight was recorded (Tw+Sw) before samples were oven dried for 24 hours or until no more weight was lost (Tw+dSw). The DM% was calculated for three replicates per sample and averaged, using equation 2.7.

$$\mathrm{DM\%} = \frac{(\mathrm{Tw} + \mathrm{dSw}) - \mathrm{Tw}}{(\mathrm{Tw} + \mathrm{Sw}) - \mathrm{Tw}} \times 100 \tag{2.7}$$

Oven drying was conducted between 55-65°C prior to the nutritional analysis of larvae produced for fish feed in Chapter 6, using a similar protocol as described above using an oven (Gallenkamp). This temperature was selected as samples were subsequently to be analysed for their amino acid composition and temperatures above this range would influence this analysis. In Chapter 6, *D. rario* samples were dried using freeze- drying (E-C apparatus) methods, to minimise the risk of oxidation of volatile constituents, as fish and their feed contained a high content of unsaturated fatty acids (UFAs), which are susceptible to oxidation.

2.3.2 Crude protein analysis

Organic elemental analysis is a technique that determines the amount of a specific element within a compound, including nitrogen (N). Nitrogen is a key element in an amine group, which is one of the two functional groups found in all amino acids, the other being a carboxyl group. Amino acids are monomer units of polypeptides, or proteins and almost all nitrogen within organic compounds is found within proteins. Proteins have been found to contain, on average, 16% nitrogen by weight of total protein. Therefore, total protein content in feed, was estimated by assuming that protein contains this proportion of nitrogen and using the multiplier 6.25 to estimate total protein weight (equations 2.8). Percentage nitrogen was normalised by sample weight and equations 2.9 and 2.10 were utilised to determine the total protein in sample in grams per kilogram.

$$\text{Proportion of nitrogen in protein} : 100\% \div 16\% = 6.25 \tag{2.8}$$

$$\text{Nitrogen per mg sample} \times 6.25 = \text{Total protein in 50mg sample} \tag{2.9}$$

$$\frac{\text{Total protein in 50mg sample}}{\text{Sample weight}} \times 1000 = \text{Protein g/kg} \tag{2.10}$$

Within the elemental analyser samples react completely with oxygen in a combustion reactor that operates at 1000°C. Products of this combustion were then carried by helium through reagents that produce CO_2, H_2O, N_2 and NO_x (Krotz et al., 2017). Copper was then used to reduce NO_x to N_2 and samples pass through H_2O and CO_2 traps, before entering a GC column, before passing through a Thermal Conductivity Detector (Krotz et al., 2017). Although more modern equipment and software calculates the protein content of samples, the machine used in this thesis work was only able to give an amount of nitrogen element within the sample weight. Hence the requirement of the nitrogen multiplier in determining crude protein content of samples.

Aspartic acid (10.52 (w/w) N%) (D5055, Elemental Microanalysis), was a nitrogen standard and positive control used to calibrate the Flash EA 1117 series N/protein analyser (Thermo Scientific, UK) and to ensure measurements being determined by the thermal conductivity detector were correct, by adjusting a time peak on the Eager 300 computer software (Thermo Scientific, UK) for N_2 in the aspartate sample. Following an initial aspartic acid sample used to calibrate, two negative controls (sucrose: a monosaccharide with 0% N) (Fisher Bioreagents), were recorded as *blanks* in the computer software and analysed. Following these, a further 4 aspartate samples were weighed and analysed, two were recorded as *N standard*, two recorded as *unknowns*. All trial samples were analysed following this and recorded as *unknown* on the software. A negative sucrose control was analysed as an *unknown* after every 10 samples, in the trial undergoing analysis. In order to determine crude protein content in the samples, N was multiplied by 6.25 using equation 2.11 and the crude protein in the sample was determined using equation 2.12.

$$\text{Crude protein in sample} = \text{N in sample} \times 6.25 \tag{2.11}$$

$$\text{Crude protein g/kg} = \frac{\text{Protein in sample}}{\text{Sample weight}} \times 1000 \tag{2.12}$$

2.3.3 Amino acid analysis

Proteins, polypeptides and peptides are so called as they are formed of various combinations of amino acid monomer units. Amino acids (AAs) are joined together via condensation reaction forming peptide linkage bonds between individual monomer units. For analysis of AAs within linear peptide chains, monomer unit's cleavage, via hydrolysis, is required. The determination of specific AAs is reliant on liquid chromatograph and detector techniques, using an amino acid analyser.

Internal standards were used during hydrolysis as free and protein-bound AAs in samples behave differently (EuropeanPharmacopoeia, 2005). Reagents used for standard preparations were: amino acid standard, 2.5 μmol/ml (Cat. No. AA-S-18, Sigma); cysteic acid (Cat. No. C7630); methionine sulfone (Cat. No, M0751, Sigma); and norleucine (Cat. No. N1398, Sigma).

Hydrolysis was conducted using 30% hydrogen peroxide (Cat. No. H/1800, Fisher), diluted 1:10 into 100 ml with formic acid/phenol solution. Formic acid (90-100%, Cat. No. F/1900) and phenol (Cat. No. P/2320, Fisher) solution was made up using 735 ml of the former mixed with 111 ml of the latter. Samples were hydrolysed *in vacuo* for 24 hours at 110$\pm$1°C.

This analysis was conducted by Calum Greenhalgh, a Nutritional Science technician at the University of Nottingham (2014 - 2017).

2.3.4 Gross energy analysis

Bomb calorimetry is a technique that measures enthalpy change of combustion of a substance within a high-pressure, constant-volume reaction chamber (6300 calorimeter, Parr), using equation 2.13 (Parr, 2010). The standard used in this procedure was a calorific grade benzoic acid (1 g pellets, 65-85-0, Parr), which has

a known energy value of 26.454 MJ/kg.

$$W = \frac{Hm + e1 + e2 + e3}{T} \tag{2.13}$$

W = Energy equivalent of the calorimeter in °C

H = Heat of combustion of standard benzoic acid sample in calories per gram

m = mass of the sample in grams

T = Temperature rise in °C

e1 = Correction for heat of formation of nitric acid in calories

e2 = Correction for sulphur

e3 = Correction for heating wire and combustion of cotton thread

The sample was weighed out into the capsule (crucible), at roughly 1 g of material (the weight of the material was recorded to 4 decimal places). If not pelleted, the material was flattened and pushed down into the capsule, avoiding contamination. The sample plus the crucible was reweighed and was then placed into the capsule holder (which was attached to the bomb head).

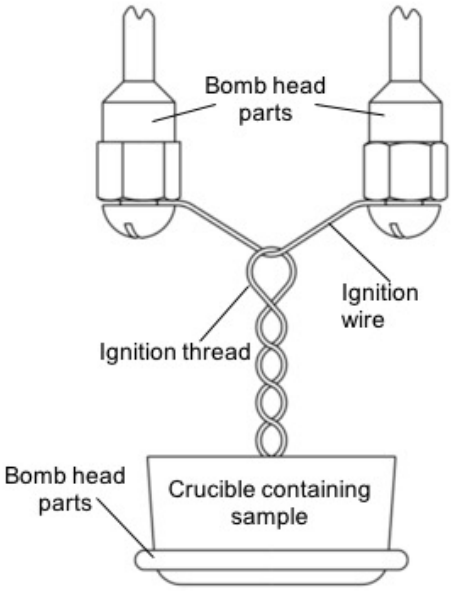


Figure 2.1: **Bomb calorimetry: bomb head and ignition set up.** Adapted from Parr (2010).

A 10 cm cotton ignition thread (845DD2, Parr) was then attached, linking the ignition wire of the bomb head to the capsule, containing the sample (Figure 2.1). The bomb head was then installed into the calorimeter (6300 calorimeter (Parr Instrument Company, Illinois, USA) and the lid was closed, and the latch engaged. From the display menu "*determination*" was selected from the *Calorimeter Operations Page, Operating Mode*. Individual ID information and the weight of the sample was entered to 0.1 mg. The units for energy content in samples was MJ/kg.

2.3.5 Crude lipid extraction

Gerhadt's Soxtherm machine was utilised in the extraction of crude fat from all samples in this thesis work. This fully automated system allows for increased efficiency of the semi-continuous Soxhlet method, with up to five programmable

stages, in order to determine total fat content in samples. This method of lipid extraction utilises an organic solvent to separate lipid-soluble components, from water-soluble components within a sample i.e. fat portion of the sample is separated from carbohydrate, protein and mineral portions. The type of solvent utilised in this machine effects the efficacy of the lipid extraction, in particular the polarity of the solvent will affect the solvents absorbed. A polar solvent, such as alcohol, dissolves polar lipids, such as glycolipids and phospholipids, whereas a non-polar solvent, such as hexane, dissolves non-polar lipids, such as triacylglycerol(s) (TAG). The non-polar organic solvent petroleum ether (an alkane fraction of petroleum) was used for triacylglycerol extraction from samples. The solvent has a low boiling point (60-80°C) and is relatively inexpensive however, extraction must take place in a fume hood due to toxicity of the solvent and care must be taken due to its highly flammable nature.

Prior to extraction, samples were dried and finely ground, allowing for maximal penetration of the organic solvent (a result of absence of water and increased surface area-to-volume ratio). Whilst the Soxtherm machine (Gerhadts Soxtherm apparatus) rose to a temperature of 150°C the samples were weighed to four decimal places (0.6-1.0 g) onto two layered, circular filter papers (diameter: 11 cm). Filter papers (11425208 Fisherbrand), were folded, labelled and placed within a porous extraction thimble, then sealed with compact cotton wool (01515, Synergy Health). Three boiling stones (1000774, Gerhardt), were added to glass extraction beakers, which are then weighed to four decimal places on the weighing balance. Beakers were then filled with 164 ml of petroleum ether (40-60°C, Fisher Chemical), within a fume hood (also the location of the Soxtherm machinery). The thimble, containing a sample, was suspended above and within the beaker containing the solvent, using a metal claw. The beakers were attached securely to the Soxtherm extraction chamber and the pre-programmed settings started. At this point the sample was positioned within a porous thimble, suspended in the

solvent containing beaker (positioned on a 150°C hot plate), below a condenser.

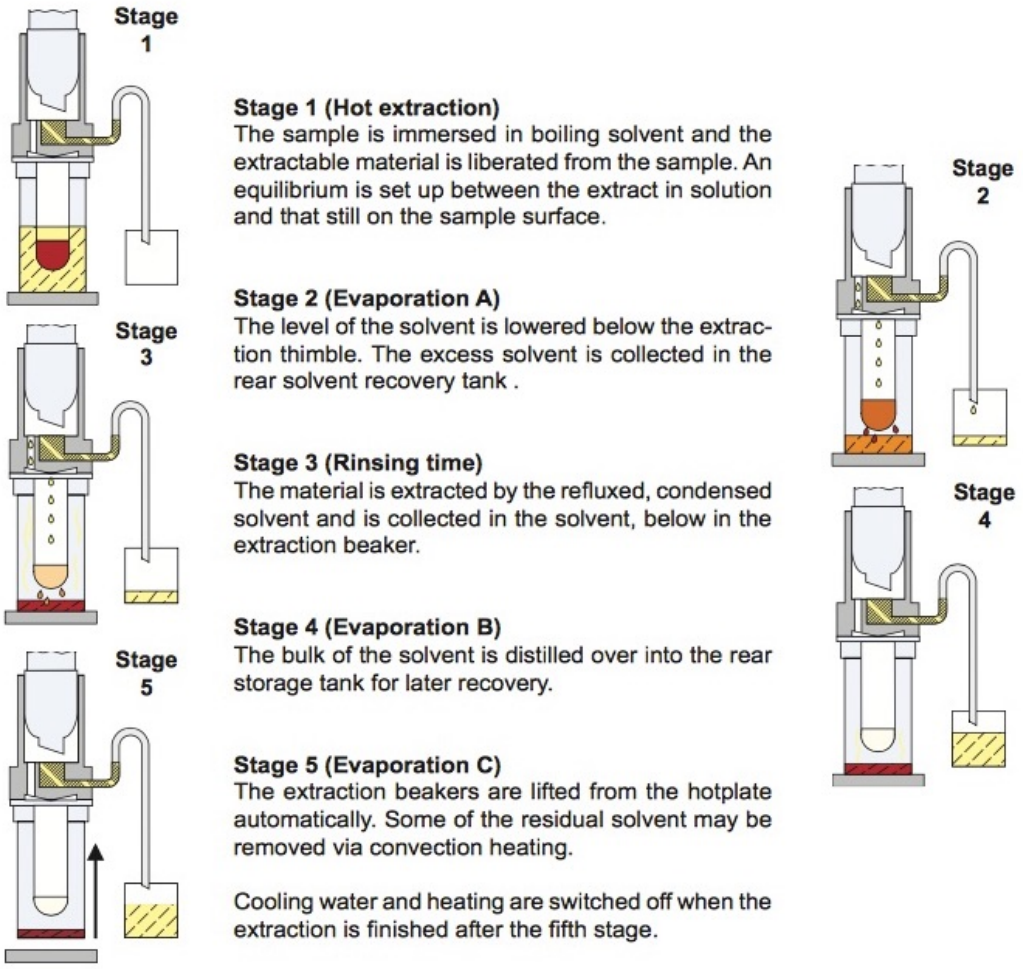


Figure 2.2: **The Soxhlet method of lipid extraction, using Soxtherm, Gerhardt machinery** from Gerhardt (2009).

The flask, containing the solvent, was heated to the solvent's boiling point and the machine removed excess solvent (which was collected and recycled), lowering the solvent to below the porous thimble (Stage 1 and 2, Figure 2.2). The solvent that remained in the beaker then underwent refluxing (Stage 3, Figure 2.2). The condenser converted evaporated solvent back into a liquid, which passed over the sample, dissolving fat from the material and carrying it through the filter paper and porous thimble into the flask. Extracted lipid remained in flasks due to its low volatility. The solvent was refluxed over the sample for around 110 minutes,

before the condenser automatically turned off and the solvent was distilled from the beaker (stored in the back of the machinery and recycled) (Stage 4, Figure 2.2).

$$\text{Crude fat (g/kg)} = \frac{(\text{Flask + fat wt}) - (\text{Flask wt})}{\text{Sample wt}} \times 1000 \qquad (2.14)$$

On completion of the cycle, de-fatted sample, thimble and metal claw were removed and placed within a fume hood to dry. The beaker containing the crude fat product was placed within an oven set to 100°C for 1 hour in order to evaporate any remaining petroleum ether. After that time the beakers were placed into a glass desiccator to cool, before the beaker was weighed. Total crude fat, displayed as g/kg, was calculated using equation 2.14. Once petroleum ether evaporated from thimbles, the de-fatted samples were stored in a desiccator for use in sample fibre analysis (Section 2.3.8).

2.3.6 Fatty acid methyl esterification

As previously mentioned (Section 2.3.5), the crude fat extraction process utilised petroleum ether solvent, resulting in the majority of fat contents within the sample collected being TAG biomolecules. TAG is the main store of fat within lipid droplets (a lipid storage organelle) and consists of three FA joined to one glycerol (propane-1,2,3-triol) molecule by ester bonds, forming the "glycerol backbone" of TAG. FA are biological molecules known as carboxylic acids. TAG molecules can consist of the same three FA, called a simple TAG, or three different FA, known as a complex TAG. In order to analysis the type of FA present in samples, a breakdown of TAG molecules is first required. The process of fatty acid methyl esterification (FAME) combines the breakdown of TAG molecules into it's individual components and further to this, the esterification of the FA constiuents to esters. The FAME process comprises two main steps, which involve two reaction catalysts; a base (KOH) and an acid (H_2SO_4). The base catalyses the breakdown

of the TAG molecule and the acid catalyses the formation of an ester from a carboxylic acid (in this instance the FA component) and an alcohol. Esters are volatile molecules, which enable FA detection by gas-chromatography (GC) to be achieved at lower temperatures <250°C (James & Martin, 1956).

To carry out the FAME process alcohol (methanol) and a base (KOH) are added to samples. Hydroxide ions from the base remove a proton from the alcohol (CH_3OH) leaving $3CH_3O^- + 3H_2O$ and catalyse trans-esterification of the TAG molecule by nucleophilic attack of ionised methanol (CH_3O^-). This nucleophile attack breaks the three ester bonds between the three FA molecule and the glycerol backbone. Following this, an acid is added to the samples (H_2SO_4) and catalyses the formation of an ester bond between the individual FA molecules and the ionised methanol. This lipid faction is soluble in hexane and is separated from other test tube constituents using centrifugation and pipetting off the top layer, containing FAs, into individual sample tubes.

Individual crude fat samples were removed from glass beakers (Section 2.3.5), by pipetting 2 ml hexane into the beaker. Swirling the mixture released the boiling stones, which allowed maximal crude fat to be dissolved, in hexane solvent, prior to FAME. The hexane and crude fat were then removed using a glass pipette and placed into a 10 ml glass, screw top, methylation tube. Once this was completed for all samples to be analysed, 0.7 ml 10 M potassium hydroxide (KOH) (MW: 56.11, P/5640/60, Fisher Reagents), followed by 5.3 ml methanol were added to each sample and all tubes were placed in the water bath (preheated to 55°C). Samples remained in the water bath for 90 minutes, with a 5 second vortex (Whirlmixer, Fisherbrand) of each solution at 20-minute intervals (four vortexes in total). All samples were placed in a cold-water bath at approximately 5°C (made by adding ice to water in a container and placing in a refrigerator until required), for 10 minutes in the refrigerator. The water bath was then removed from the refrigerator and 0.58 ml 12 M sulfuric acid (H_2SO_4) (M= 98.08 g/mol, 7664-93-9, Merck) to

samples, whilst in cold water bath. Samples were then put back into the hot water bath at 55°C and incubated, mixed and then cooled as before. Then 3 ml hexane was added to each sample, followed by a 30 second vortex to dissolve lipid soluble test-tube constituents. Samples were then centrifuged (GS-6R, Beckman) at 2000 rpm for 5 minutes to separate lipid soluble (top layer) from non-lipid soluble components. Using a glass pipette (230 mm, VWR) the top solvent layer was transferred into an LP4 tube, and the remaining mixture was discarded. LP4 tubes were stored at -40°C until preparation for GC analysis (Section 2.3.7).

2.3.7 Gas-liquid chromatography

Gas-chromatography (GC) and mass spectrometry are methods used in this thesis for the separation and detection of separate molecules from a mixture of FAME (produced in Section 2.3.6), determined utilising a standard (100 mg, 18919-1AMP, Supelco), with known FAME content, diluted 10 mg/ml in hexane. A small amount of sample was injected (mechanically) via syringe into an oven mechanically and the contents of the sample vaporised. Helium, acting as a carrier gas, moves the vaporised sample through the column, where it begins to separate out into individual compounds. These compounds emerge at the end of the column and are detected by a mass-spectrometer, forming a peak on a chromatogram that can be analysed, for each sample with the exception of hexane controls. The final chromatogram displays multiple peaks corresponding with the mixture of substances present in the sample.

The FAME samples and standard solution were removed from storage at -40°C and allowed to reach room temperature before use. Samples were mixed by vortex to re-suspend the lipids in hexane. Samples were used in this form for GC analysis, or alternatively were dried down under nitrogen and re-suspended in 1 ml hexane (increasing concentration of the solution). FAME samples, produced from the fish trial (Chapter 6), contained high concentrations of lipid, which exceeded the peaks

on the chromotogram charts and required further drying down under nitrogen, as well as a 1 in 10 dilution with hexane.

The GC vials (2 ml crimp top vials, clear, Thermo Scientific) were labelled with sample ID and 1 ml of each sample was pipetted into each vial, followed by a tight sealing of the lid. For fish samples 10 μl was added to 90 μl hexane in 100 μl glass spring inserts (401BS-530, WP), placed within GC vials and then sealed. All samples were then loaded in a specified order onto the machine's sample carousel.

The GC method was optimised, and all machine maintenance was carried out by Nutritional Sciences technician Calum Greenhalgh, based at the University of Nottingham, UK (2014-2017). The total GC run time was 33.5 min, and the setup of Clarus 580 (PerkinElmer) was as follows:

1. Initial: 50°C, hold for 0 minutes (min)
2. Ramp 1: +20°C/min to 190°C, hold for 2 min
3. Ramp 2: +0.5°C/min to 192°C, hold for 0 min
4. Ramp 3: +45°C/min to 215°C, hold for 20 min

Results from GC analysis were accessible using TotalChrom Workstation (PerkinElmer). Area% was utilised to determine mol per FAME percentage using equations 2.15 and 2.16.

$$\text{mol (\%)} = \frac{\text{Area (\%)}}{\text{fatty acid methyl ester Mr}} \times 100 \tag{2.15}$$

$$\text{mol per FAME (\%)} = \frac{\text{mol (\%)}}{\text{Total mol (\%) of all FAME}} \times 100 \tag{2.16}$$

The molecular weight (Mr) of FAMEs were calculated by addition of a methylene group of one carbon and two hydrogen atoms, with atomic weights of 12 and 1, respectively. Therefore, Mr of 14 was added onto the Mr of each FA identified using GC methods (equation 2.15). The FAME Mr values were then used to calculate mol per FAME (%) (equation 2.16) and these values were used in statistical

analysis of FA data (Section 2.5) and displayed in graphs produced in this body of work.

2.3.8 Fibre analysis

The method used for crude fibre analysis, was a method designed using Gerhardt equipment that aimed to maximise accuracy, precision and economy of fibre extraction. Starch and sugars are removed by digestion in acid and protein is removed by digestion in alkali, leaving fibre as remains.

ADF fibre bags (10-0127, Gerhardt) were dried in an oven for 4 hours at 100°C, before being cooled in a desiccator for 5 minutes and weighed to 4 decimal places. All fibre bags are labelled with independent identification, using a waterproof marker pen. De-fatted samples produced in Section 2.3.5 were weighed to 4 decimal places. Sample were then carefully transferred into their corresponding, labelled fibre bag. A spacer was inserted into the fibre bag which was then placed onto a carousel. A carousel holds six fibre bags with spacers. Gerhardts fibre analysis equipment allows for three carousels per extraction, resulting in up to 36 samples being conducted per extraction.

For each carousel, 360 ml Sulphuric acid (0.128 M) (Fisher Reagents) was placed in a beaker (one per carousel). A carousel handling tool was attached to the carousel and then each carousel was lowered into the beaker. Thorough mixing was achieved through rotation and raising the carousel up and down in the acid beaker. The carousel handling tool was then removed and acid beakers containing the carousels were placed onto a cold hotplate and condensers were fitted to the top of the beakers. Acid within beakers was brought to boil over 5-minute intervals for 30 minutes. Care was taken at around 90°C, as acid could bubble up through and out of the fibre bags, carrying with it, the sample. Throughout the 30 minutes temperature was regularly adjusted to achieve a simmering acid. Any loss of sample from fibre bag, as a result of vigorous boiling, would result in a negative

value for crude fibre. Crude fibre content was calculated using equation 2.17.

The hotplates were then turned off and the carousel handler tool was attached to remove the carousels from the acid beaker. Carousels were placed into a clean beaker, and the acid containing dissolved substrates were discarded. To the beaker was added 300 ml of boiling water and the carousel tool was used to vigorously rinse the fibre bags of the acid. This process was repeated three times, at which point the final wash was a neutral pH (tested with litmus paper).

To digest protein from the samples, to a clean beaker (one per carousel) 360 ml sodium hydroxide (0.313 M) (Fisher Reagents) was added and the carousel lowered into the beaker using the carousel handling tool. The beaker was placed back on the hotplate, and the condenser replaced. The alkaline was brought to the boil, reducing the temperature setting when alkaline reached around 90°C, to avoid alkaline boiling sample out of fibre bag. This was repeated at 5-minute intervals for 30 minutes. The alkaline solution and solutes was then discarded. The carousel was then lowered into a clean beaker and washed with 300 ml boiling once, then a 300 ml wash with hydrochloric acid (0.1 M) (Fisher Reagents) and twice more with 300 ml boiling water. In order to remove the spacer whilst leaving sample within the fibre bag a jet of distilled water was directed from a wash-bottle onto each spacer. Excess water was removed from fibre bags by lying the bags on absorbent blue paper for 5 minutes. The fibre bags were then oven dried at 100°C for four hours, desiccated for 15 minutes then weighed to determine the residue contents. Fibre bags were rolled and placed in labelled crucibles. Crucibles with fibre bag were weighed to four decimal places. The samples ashed in these crucibles were placed in a muffle furnace (CWF 1100, Carbolite) at 600°C, for 4 hours. Contents of the crucibles were brushed out and the empty beaker was weighed. Crude fibre was calculated using equation 2.17.

$$\text{Crude fibre (\%)} = \frac{\text{Fibre residue} - \text{Ash content}}{\text{Initial sample weight}} \times 100 \tag{2.17}$$

2.3.9 Ash analysis

The mineral content in a sample can be analysed through ashing. Ashing is a process by which powdered, dry sample is heated to temperatures over 500°C. Ash constituents consist of potassium, sodium, calcium and magnesium in addition to aluminium, iron, copper, manganese, zinc, arsenic, iodine, fluorine and other mineral content.

Porcelain crucibles were labelled, and individual weights were recorded. Each crucible was tared on scales and an oven-dried sample was accurately weighed out into the crucible, at around 3 g, with individual weights recorded to 4 decimal places. Crucibles containing samples were transferred to the muffle furnace (CWF 1100, Carbolite) and were ashed at >500°C for 8 hours. The individual ashed samples and crucibles were weighed together, before ashed material was removed and content discarded using a paint brush and the crucible weight was recorded. Crude ash was then calculated using equation 2.18.

$$\text{Crude ash (\%)} = \frac{(\text{Crucible + sample wt}) - (\text{Crucible wt})}{\text{Sample wt}} \tag{2.18}$$

2.4 Gene analysis

2.4.1 Larval killing and storage methods

A trial was designed to determine the most appropriate way in which to kill and store larvae for RNA extraction and future gene analysis work. Previously for determination of nutritional information larvae had been killed and stored in a freezer at -20°C. However, in order to keep the RNA intact it was necessary to assess different methods for killing and storing larvae that best preserved the RNA. Four parameters were measured in this methodology trial: RNA quality (using 260 nm/280 nm values); RNA nucleic acid quantity (ng/μl); qRT-PCR crossing

point-PCR cycle, (C_P value); and apparent mRNA concentration, using qRT-PCR techniques and standard curve methodology (Section 2.4.6). Oligonucleotide pairs for TML27a transcript were used for this trial, a known internal standard (D. H. Kim et al., 2013; Jo et al., 2016; "TmCactin plays an important role in Gram-negative and-positive bacterial infection by regulating expression of 7 AMP genes in *TENEBRIO MOLITOR*, author=Jo, Yong Hun and Kim, Yu Jung and Park, Ki Beom and Seong, Jeong Hwan and Kim, Soo Gon and Park, Soyi and Noh, Mi Young and Lee, Yong Seok and Han, Yeon Soo, journal=Scientific Reports, volume=7, pages=46459, year=2017, publisher=Nature Publishing Group", n.d.).

The trial assessed four treatment groups, n=4, comprised of alternative killing and storage methods; kill/store at -20°C; kill/store at -40 °C; kill using dry ice and store at -80°C; and kill using liquid nitrogen and store at -80°C. All larvae used in the trial had been acclimatised in the same container and fed on the same feed and water source for 6 days prior to trial. Larvae were randomly selected for the trial, 50 individuals per replicate and weights of the treatment groups were determined as not significantly different before proceeding with the trial. Larvae were starved for 24 hours and then killed and stored in accordance with their corresponding treatment groups.

There were no statistically significant differences observed between the treatment groups for any of the four parameters measured (Figure 2.3). However, killing with liquid nitrogen and storage at -80°C was selected as the most appropriate method in order to carry out analyses in gene expression work, with a higher apparent mean for RNA quantity compared with dry ice and similar standard error of mean values.

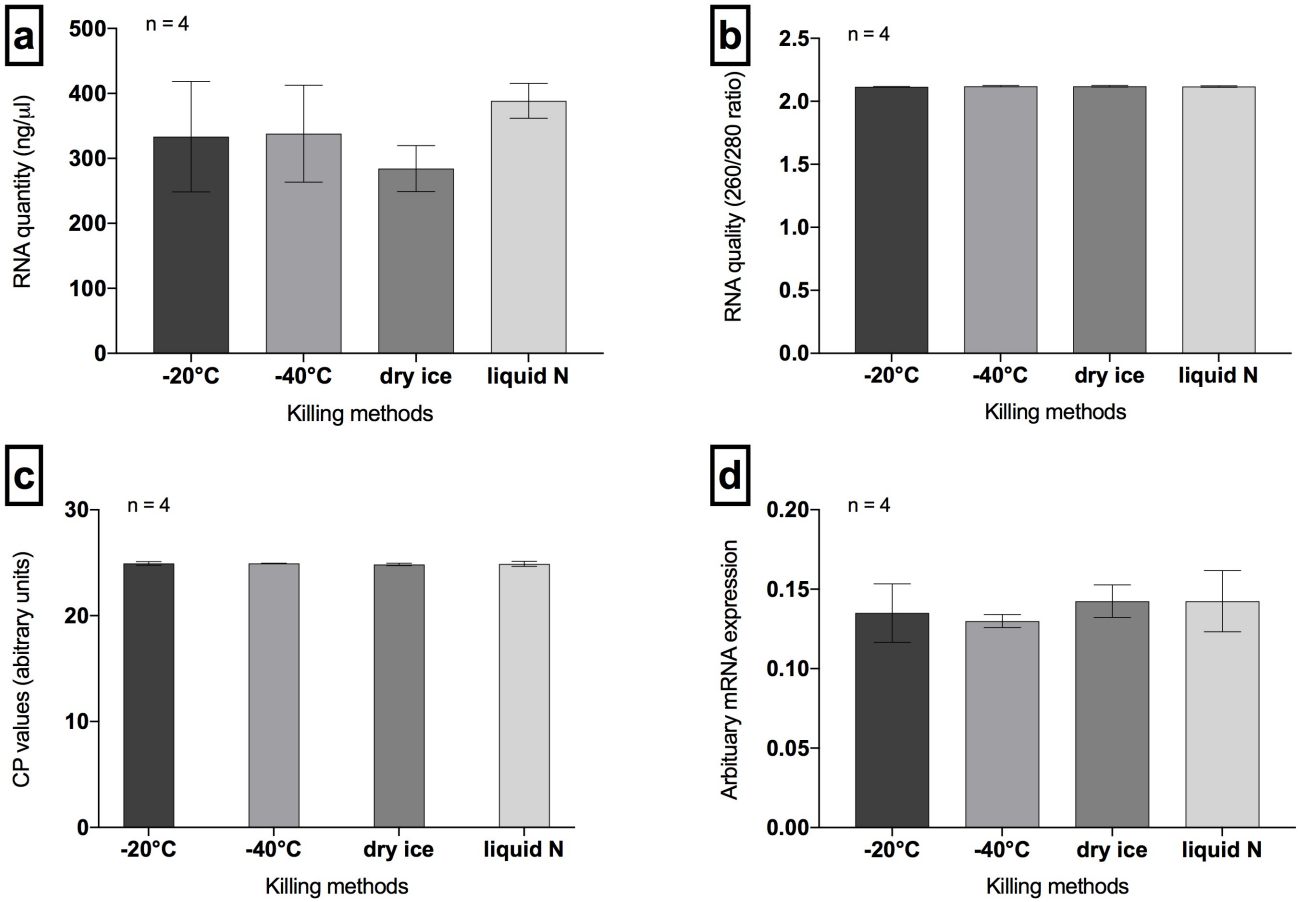


Figure 2.3: **The effect of kill and storage methods on the RNA quality and quantity and qRT-PCR outputs using oligonucleotide set for TmL27a transcript.** Larvae were acclimatised for 6 days, before being separated into one of four treatment groups, n=4: kill/store at either -20°C (-20°C); or at -40 °C (-40°C); or kill on dry ice and store at -80°C, (*dry ice*); or kill using liquid nitrogen and store at -80°C (*liquid N*). Each replicate was starved for 24 hours prior to killing and storing. Values in [d] are expressed as mRNA expression arbituary unit per weight equivalent total RNA. TmL27a is a proven internal gene regulator for qRT-PCR in *T. molitor* and primer pairs were used from "TmCactin plays an important role in Gram-negative and-positive bacterial infection by regulating expression of 7 AMP genes in *TENEBRIO MOLITOR*, author=Jo, Yong Hun and Kim, Yu Jung and Park, Ki Beom and Seong, Jeong Hwan and Kim, Soo Gon and Park, Soyi and Noh, Mi Young and Lee, Yong Seok and Han,

2.4.2 RNA extraction

The RNA extraction protocol technology combined the binding properties of lysed and homogenised RNA mixed with ethanol, to silica membranes. When the sample was applied to a spin column the contaminants are washed through into the collection tube with the use of various buffers. Finally, RNA was then eluted in 100 μl of RNase-free water.

Samples were stored at -80°C in a freezer prior to RNA extraction. Qiagen RNeasy Mini Kit (Cat. No. 74104), was used for purification of total RNA from animal cells and animal tissues, in addition to clean-up of RNA from crude RNA preps and enzymatic reactions. Sample was weighed into a 1.5 ml micro-centrifuge tube, which was pre-cooled by immersion in liquid nitrogen for 5 seconds. To this was added 600 μl of the prepared Buffer RLT, β-ME (lysis buffer). Using a syringe and RNase-free syringe needle (28 gauge), the solution containing the sample was passed through the needle 10×, in order to shear DNA in the solution. The lysate was mirco-centrifuged (1-15, Sigma) for 3 minutes at full speed (14,000 rpm) at room temperature, after which the supernatant was carefully removed into a new micro-centrifuge tube and the volume recorded. To this was added 1 volume of 70% (w/w) ethanol (usually 500 μl) and the solution then mixed by repeat pipetting 5 times. Into RNeasy spin column, placed within a 2 ml collection tube, was transferred 500 μl of sample (including any precipitates formed) and this was micro-centrifuged (Sigma) for 15 s at >10,000 rpm, at room temperature. The through-flow was discarded, and the process repeated adding the remaining 500 μl of sample. To the RNeasy column was added 350 μl Buffer RW1 and the column was micro-centrifuged for 15 s at >10,000 rpm, at room temperature, with the through-flow discarded. To column was added 80 μl DNase I incubation mix. After leaving to incubate on the bench top (20-30°C) for 15 min, 350 μl Buffer RW1 was added to the RNeasy column and this was micro-centrifuged (Sigma) for 15 s at >10,000 rpm, room temperature and through-flow discarded. This was

followed by adding 500 μl Buffer RPE to the RNeasy spin column, which was then micro-centrifuged for 15 s at >10,000 rpm, room temperature and then the through-flow discarded. Then 500 μl Buffer RPE was added to the RNeasy spin column, which was micro-centrifuged (Sigma) for 2 min at >10,000 rpm, room temperature and the through-flow was discarded. After placing the RNeasy spin column into a new 2 ml collection tube it was micro-centrifuged (Sigma) at 14,000 rpm for 2 min, room temperature, to dry the column membrane. The RNeasy spin column was then placed into a new 1.5 ml micro-centrifuge tube and 50 μl RNase-free water added directly onto the column membrane. The RNeasy column was micro-centrifuged (Sigma) for 1 min at 10,000 rpm, room temperature, to elute the RNA, and the elution then repeated with an additional 50 μl RNase-free water and centrifugation.

The concentration and purity of RNA samples was determined using a Nanodrop ND-100 (Thermo Scientific, Wilmington, USA). A 260 nm/280 nm ratio was used to determine purity, with 2.0 suggesting minimal contaminants and pure RNA. Samples were diluted to 100 ng/l and run on a non-denaturing 1% (w/w) agarose gel (Fisher BioReagents) to check integrity of ribosomal RNA 18S and 28S bands (Section 2.4.3). Absence of DNA in samples and therefore success of the DNA digestion was determined using quantitative real-time polymerase chain reaction (qRT-PCR) (Section 2.4.6).

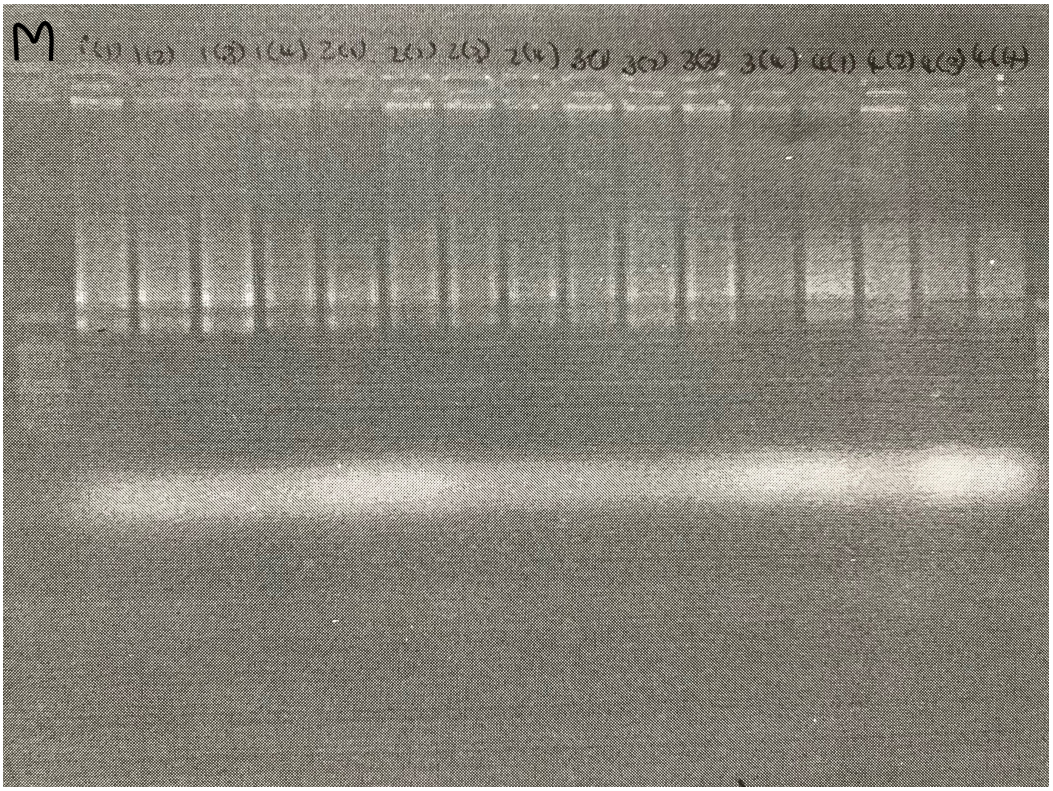


Figure 2.4: **Non-denaturing gel electrophoresis: RNA integrity check on kill and storage trial samples featured in Section 2.4.1**.

2.4.3 Non-denaturing gel electrophoresis

Non-denaturing gel electrophoresis was carried out using agarose (BP1356-500, Fisher Scientific) gel or Metaphor (50180, Lonza, Rockland, USA) gel where appropriate, utilising either 1 × TAE or 1 × TBE buffer. Agarose and metaphor preparations were conducted using Cambrex Bio Science Rockland, Inc instructions.

Agarose preparation: Firstly, the size of gel appropriate for the number of samples to be run, was selected, (volume is roughly 60 cm^3 for the smallest mould and increases by 60 cm^3 as sizing increases). Then 50×TAE buffer (EC-872, National Diagnostics) was diluted to a working concentration of 1×TAE using elix water. The agarose solution was prepared in a clean conical flask by adding

the appropriate volume of 1×TAE buffer to agarose powder (BP1356-500, Fisher Scientific) at a final concentration of 1% (w/v) agarose. The buffer soaked the agarose for 15 min. The conical flask was then placed in a microwave oven at full power for 2 min and gently but frequently swirled to mix and check the progress for the agarose dissolving without over-boiling. When completely dissolved, boiling distilled water was added to the conical flask, to make the solution up to initial weight, and mixed thoroughly. The flask was cooled under a cold tap, until able to touch the surface. The appropriate comb was added to the gel mould and the gel was slowly cast from one position. The comb was used to gently rid the cast of any bubbles. The gel was left to set, then placed in 4°C for a minimum of 20 min for optimal resolution.

Metaphor agarose preparation: For the heating of metaphor (50180, Lonza) gels >4% (w/v) was begun by heating on a medium power for 1 min and then placed on the bench top for a further 15 min to stand before progressing with a further 1 min medium power heating, followed by bringing to the boil once or twice on high power.

RNA and cDNA checks were run on 1% (w/v) agarose gels with 1 × TAE buffer, utilising sample volumes of 10μl at 100ng/μl concentration and 5μl at 50ng/μl. These samples were mixed (by pipetting) with 2μl loading dye (Promega) on a PM-992 film. Following this, the combined sample and dye were pipetted into gel wells. Samples were run against a 100 bp DNA ladder.

PCR products using TmL27a housekeeping primer set were tested using 4% (w/v) Metaphor gel with 1×TBE buffer. Product volume of 10 μl was mixed by pipetting with loading dye and pipetted against a 100 bp and 25 bp DNA ladders (Promega). In addition, primer sets; SCD, ELOVL1 and ELVOL2 were also analysed using end-point PCR and prepared for non-denaturing gel electrophoresis after successfully passing qRT-PCR testing of amplification, and melt curves,

using pooled samples. The products of these primer pairs were further tested through a run on concentrations between 4.0-5.0% (w/v) Metaphor gel, (dependent on the predicted amplicon size), with a 1×TBE buffer; 4% (w/v): 20-130 bp and 5% (w/v): <80 bp, amplicon product lengths. After confirmation of single amplicon products of roughly the expected sizes (bp), PCR product methods were repeated for PCR product sequencing. PCR products for sequencing were run on % (w/v) Metaphor gels appropriate for size of amplicon determined through gel electrophoresis testing. PCR reactions were conducted to produce 50 μl product per reaction. Samples were mixed pipette with loading dye as well as a fluorescent gel (SYBR Green I nucleic acid gel stain) (Section 2.4.8).

All gels were run at a potential difference of 100 V. Agarose gels were run for roughly 30 min, metaphor gels were run for up 1.5 hours. On completion of a run, the gels were placed in ethidium bromide for staining, using appropriate safety protocols and left for 20 mins and photographs were taken using Gel Doc UV radiation imaging and computer software (Gel Doc 2000 Bio Rad). For sequencing gels that used fluorescent dye SYBR Green I nucleic acid gel stain (S9430, Sigma), PCR product bands were visualised on a DarkReader (DR-88M, Clare Chemical Research), (Section 2.4.8).

2.4.4 cDNA synthesis

This protocol was used to synthesise a complementary DNA strand from single-stranded RNA, utilising reverse transcriptase and DNA polymerase enzymes. DNA, double-stranded material is required for amplification utilising PCR protocols which are catalysed by specific DNA enzymes.

Two master mixes were required for first strand cDNA synthesis utilising RevertAid RT kit (Thermo Scientific), these were both be prepared on ice, prior to starting the protocol (Table 2.3). The cDNA kit was thoroughly thawed on ice, mixed and briefly centrifuge before starting. The components in Table 2.3 were

added in the order listed into sterile, nuclease-free tubes on ice. The RNA solutions were diluted to 100 ng/μl utilising information from Nanodrop technology for non-denaturing gel electrophoresis RNA integrity check. For first strand cDNA synthesis 500 ng of RNA was required. Each RNA diluted sample was placed, at volumes of 10 μl, into a sterile, nuclease-free well on a 96 well plate, on ice.

Table 2.3: **Components for two master mixes required for first strand cDNA synthesis.** Each component added to the appropriate master mix in the order indicated.

Components 1st Master Mix	**Volume**
Random Hexamer Primer	1μl × rxn[1]
PCR-grade water	1μl × rxn
Final volume	**2μl × rxn**

Components 2nd Master Mix	**Volume**
5 × Reaction Buffer	4μl × rxn
RiboLock RNase Inhibitor (20 U/μl)	1μl × rxn
10mM dNTP Mix	2 μl × rxn
RevertAid RT (200U/μl)	1μl × rxn
Final volume	**8μl × rxn**

[1] *Reaction*

To this 2 μl of 1st Master Mix (Table 2.3) was added to each well containing RNA diluted sample. The wells were sealed with lids on the appropriate rows. The plate was then incubated in a thermo cycler (GeneAmp PCR system-9700, Applied Biosystems) at 65°C for 5 min. The samples were then chilled on ice, spun down in a mini plate spinner (MPS1000, Labnet) and on ice 8 μl 2nd Master Mix (Table 2.3), was added to each reaction well. Contents of reaction wells were mixed gently and then spun down briefly. A thermo cycler program was set up with the following temperatures and timings: 25°C for 5 min; 42°C for 60 min; 70°C for 5 min; 4°C at ∞ until samples were retrieved. Following this incubation samples were removed from the thermo cycler and 80 μl RNase-free water was added to each cDNA well. Samples were checked for successful cDNA synthesis,

using qRT-PCR methods (Section 2.4.6). Samples were then stored at -40°C until further use.

2.4.5 Primer Design

The *T. molitor* genome is currently unsequencesd and unannotated, and to date only a minimal number of specified mRNA sequences have been determined. In order to design oligonucleotide sets for amplification of genes involved in FA metabolism, specific genes of interest were selected for either their enzymatic involvement in FA metabolism or genes for transcription factors associated with FA metabolism pathways that have previously been shown to be affected by PUFA supplementation in animals (Sampath & Ntambi, 2005). The online resources used for primer design were as follows:

- UniProt: used for searching accession numbers, of mouse genes of interest, for AA sequences (https://www.uniprot.org)
- NCBI BLAST: BLASTp suite was used for comparing mouse AA sequences with Organisms in database under "Coleoptera (taxid: 7041)". tblastn was utilised for converting protein sequences to RNA sequences.

  (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
- Clustal Omega, EBI: was used for direct comparison and visualisation of gene conservation of RNA sequences between mouse, *Drosophila melanogaster* and coleopteran species (found using the pBLAST search (https://www.ebi.ac.uk/Tools/msa/clustalo/). nBLAST was then used to determine similar RNA sequences in specific coleopteran RNA collections.

Primer pairs selected for investigation are displayed in Chapter 5 methods and the full details for working oligonucleotides can be found in Appendix A. The process of primer design from the initial genes of interest, were as follows:

1. Firstly, genes of interest in the mouse, (a well characterised mammal species genome), were searched for using UniProt database.

2. Using pBLAST suite (NCBI) the accession numbers of genes of interest were compared against sequences for *Drosophila melanogaster* (a well-characterised insect species), as well as coleopteran species.

3. The corresponding mRNA to proteins matched across mouse, *D. melanogaster* and coleopteran species were sourced using nBLAST suite (NCBI).

4. A cross-species comparison of mRNA sequences was then conducted using a multiple alignment tool (Clustal Omega, EBI) in order to visualise the conservation of genes across species. These sequences were then copied into Microsoft Word for additional annotations, including potential exon-exon boundaries.

5. Species' *D. melanogaster* and *Triboleum castaneum* were further compared against each other usign sequence alignment tool (Clustal Omega, EBI).

6. The exon-exon boundaries were mapped out onto *T. castaneum* mRNA sequence using both *D. melanogaster* gene information (Uniprot) and the sequence alignment output of both species mRNA sequences, assuming there was a relatively high level of conservation. If genes were not well conserved exon-exon boundaries were left unannotated in Primer Express software (Applied Biosystems).

7. Finally *T. castaneum* mRNA sequence for gene of interest were inserted into primer design software and any predicted exon boundaries (if applicable) were added. The most appropriate oligonucleotide pairs were selected, taking into account predicted exon boundaries (if applicable) and/or penalty scoring of primer pairs (Primer Express software, Applied Biosystems).

2.4.6 Quantitative Real-Time Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a method for amplification of a small section of cDNA exponentially. This is achieved using an appropriate Master Mix (Table 2.4) and thermal cycling (Table 2.5) using a range of temperatures. The Master Mix for qRT-PCR requires an appropriate buffer for the reaction, along with deoxyribonucleotide triphosphates (dNTPs), a specific primer pair (forward in the 5'-3' strand; and reverse in the 3'-5' strand), in addition to DNA polymerase. The specific primer pair will anneal to single stranded DNA at a specific sequence and allow for elongation of that section of the DNA strand. Real-time PCR (RT-PCR) allows for quantitative output for the amount of DNA produced per PCR cycle and is so termed qRT-PCR in this body of work. This qRT-PCR technique is achieved using fluorescent dyes that produce a fluorescent signal that is directly proportional to the number of amplicons (PCR products produced).

Using Applied Biosystems Power SYBR Green Master Mix (Thermo Scientific, UK) a separate qRT-PCR Master Mix was prepared, on ice, for each primer being tested using components in Table 2.4. On ice, standard curve solutions were prepared using pooled neat cDNA from all samples being tested, with a dilution factor of 2, ranging from neat to $\frac{1}{256}$. Each dilution analysis was done in triplicate.

Table 2.4: **Components for Master Mix required for quantitative real-time PCR (qRT-PCR) on cDNA samples, using Roche Light Cycler 480.** Each component was added to master mix in the order indicated. Master Mix can also be utilised on RNA samples to ensure absence of DNA.

Components Master Mix	**Volume**
SYBR Green Master Mix	7.50μl × rxn[1]
Forward primer	0.45μl × rxn
Reverse primer	0.45μl × rxn
RNase-free water	1.60μl × rxn
Final volume	**10μl × rxn**

[1] *Reaction*

Table 2.5: **Cycling instructions for Roche Light Cycler 480 using Applied Biosystems Power SYBR Green Master Mix for qRT-PCR.**

Cycle step	Cycle no.	Temperature	Time
Pre-incubation		95°C	5 minutes
Amplification	45	95°C	10 seconds
		60°C	15 seconds
		72°C	15 seconds
Melt curve	1	95°C	5 seconds
		65°C	1 minute
		97°C	∞
Cooling	1	40°C	10 seconds

Using a multi-dispenser pipette, 10 μl Master Mix was placed into a 384 well plate, so each sample was analysed in triplicate.

Testing absence of DNA in RNA extracted samples: During RNA extraction, the inclusion of a DNA digestion step (Section 2.4.2), should destroy all DNA in samples, leaving only a remaining RNA sample. However, if there is any DNA remaining in samples this could affect qRT-PCR results. Therefore, as an additional RNA quality check samples were run through the Light Cyler-480 (Roche, Burgess Hill, UK) using the same protocol for cDNA tests, minus a standard curve of pooled samples. One working primer pair was used for this assessment (Table 2.4), usually the housekeeping gene, in this case TmL27a gene for a *T. molitor* ribosomal protein ("TmCactin plays an important role in Gram-negative and-positive bacterial infection by regulating expression of 7 AMP genes in *TENEBRIO MOLITOR*, author=Jo, Yong Hun and Kim, Yu Jung and Park, Ki Beom and Seong, Jeong Hwan and Kim, Soo Gon and Park, Soyi and Noh, Mi Young and Lee, Yong Seok and Han, Yeon Soo, journal=Scientific Reports, volume=7, pages=46459, year=2017, publisher=Nature Publishing Group", n.d.). RNA and cDNA sample testing were conducted on the same plate. No amplification should occur in RNA samples as DNA polymerase in SYBR green in Master Mix (Table 2.4), is unable to amplify single stranded RNA. If there was a positive

result this would suggest that the RNA sample has been contaminated during/ after the extraction processes or the DNA digestion step in the RNA extraction procedure was unsuccessful or not conducted. In these cases where a positive result was seen for RNA samples, RNA extraction samples were discarded, and RNA extractions were repeated.

Procedure for optimising cDNA concentration for qRT-PCR: Samples of cDNA were fully thawed on ice prior to optimisation work necessary to determine a suitable standard concentration for cDNA samples. A pooled sample was generated by taking an aliquot from each cDNA sample (ensuring there was enough for 40 μl of neat sample per primer pair). The standard concentration curve was made using pooled cDNA samples 1 in 2 serial dilutions from neat (1). Using 5 μl of cDNA sample from each dilution per reaction (in triplicate) qRT-PCR was carried out. Amplification techniques using qRT-PCR produced crossing point, C_P values (the cycle number where fluorescence reaches a certain threshold point) on the Light Cycler-480 software program. Using these C_P values (y-axis) and the log transformation of known concentration values (x axis) a linear, standard curve graph was formed. The optimal gradient of the slope was -3.3 with exponential efficiency of 2.0 (PCR reaction 100% efficient). This was calculated as a 10-fold increase in PCR amplicon being produced every 3.32 cycles (log(2), 10 = 3.3219). If the slope efficiency is >-3.3 (i.e. -3.1), it is indicative of sample quality or pipetting issues. If slope efficiency is <-3.3 (i.e. -3.5), it is indicative of a reaction less than 100% efficient. If optimal conditions of the standard curve were met ($\geqslant$3.3 gradient), then a concentration within the standard curve was selected, by which to dilute all cDNA trial samples, (usually 1 in 8 dilution). Any results (C_P values) produced by qRT-PCR assessment of diluted samples that were the same as or exceed the neat sample C_P value (from the standard curve) were disregarded.

2.4.7 PCR methods

End-point PCR involves the same PCR process as Section 2.4.6, but does not allow for robust quantification. Instead it is a technique utilised for amplification of a specific section of DNA, and results of amplification are analysed at the end of the experiment. It is the method utilised in this thesis to amplify PCR products of designed oligonucleotide pairs for sequencing.

An initial high temperature activates Phusion Hot Start II DNA Polymerase (Thermo Scientific). Once activated the thermal cycling enters the denaturation, annealing and elongation cycling stage that is repeated between 25-35 cycles and will yield $\times 2^{25-35}$ increase in DNA material. Denaturation at a high temperature causes the double-stranded DNA to break apart, which is achieved through disruption of hydrogen bonding between specific bases in the sense and anti-sense strand of DNA, which were bonded through complementary base pairing laws. During annealing, primers attach to their respective single-stranded DNA and dNTPs begin to attract to the exposed bases on each strand, again using the laws of complementary base pairing. Elongation temperature is an optimised temperature for the enzyme DNA polymerase, which acts by catalysing the condensation reaction that results in a phosphodiester bond forming between adjacent nucleotides. The phosphodiester bond forms between the phosphate groups attached to the 5' carbon of the deoxyribose sugar and an adjacent 3' carbon in the adjacent nucleotide forming what is commonly known as the sugar phosphate backbone. Finally, after completion of the denaturation/annealing/elongation cycling stage a final elongation allows for any remaining single-stranded DNA is fully elongated.

Table 2.6: **Components for Master Mix required for end-point PCR on cDNA samples.** Each component is added to Master Mix in the order indicated. Pipette appropriate amount of thawed cDNA sample (recommend pooled samples) in triplicate DNase-free tubes and add the appropriate amount of Master Mix to each tube. For cDNA check 20 μl reaction would be appropriate for gel electrophoresis. For sequencing 50 μl reaction is recommended. Master Mix can also be utilised as a technique to ensure absence of DNA in RNA samples.

Components Master Mix	**20 μl rxn**[1]	**50μl rxn**	**Final Conc**
DNase-free water	8.4μl	28.5μl	
5 × Phusion HF Buffer	4.0μl	10.0μl	1 ×
10mM dNTPs[2]	0.4μl	1.0μl	200μM
Forward primer	1μl*	2.5μl*	0.5μM
Reverse primer	1μl*	2.5μl*	0.5μM
Phusion DNA Polymerase	0.2μl	0.5μl	0.02 U/μl
Master Mix volume	**15μl × rxn**	**37.5μl × rxn**	
DNA template	5μl	12.5μl	
Final volume	**20μl**	**50μl**	

[1] *Reaction*
[2] *Deoxyribonucleotide triphosphates*
***** All primers are prepared for use in a dilution of 10 μM, therefore the appropriate volumes (V_2) of primers have been calculated using equation $C_1V_1 = C_2V_2$.

Table 2.7: **Cycling instructions for Thermo Scientific Phusion Hot Start II High-Fidelity DNA Polymerase.** For three-step thermo cycling, *X°C is determined as follows using Thermo Scientific Tm calculator: for primers >20 nucleotides temperature should be +3°C onto the lower Tm primer, for primers ≤20 nucleotides use an annealing temperature equal to the Tm of the primer with the lowest Tm. Use the two-step protocol for when primer Tm values are 69°C and lengths are >20 nucleotides, or when primer Tm values are 72°C and lengths are≤20 nucleotides.

Cycle step	**Two-step**	**Three-step**	**Cycles**
Initial denaturation	98°C, 30 s[1]	98°C 30 s	1
Denaturation	98°C, 10 s	98°C, 10 s	35
Annealing		*X°C, 30 s	35
Elongation	72°C, 30 s	72°C, 30 s	35
Final elongation	72°C, 10 min[2]	72°C, 10 min	1
Hold	4°C	4°C	

[1] *Seconds*
[2] Minutes

This protocol was used in this thesis work mainly to test what DNA product was amplified by primers, during both PCR and qRT-PCR methods. PCR end-point product was stored in a refrigerator between 4-10°C indefinitely. Products were run on a non-denaturing agarose gel to determine whether primers work and whether PCR had been successful, and to determine size of amplicon produced. If successful the amplicons were run again on a non-denaturing gel with fluorescent stain and the band produced was removed and sequenced (Section 2.4.8).

2.4.8 Sequencing of PCR products

As *T. molitor* species was not available on genome resource websites, it was necessary to determine the PCR product that was formed using designed primers. Sanger Sequencing of purified gel products was conducted by Source BioScience, Nottingham, UK. Requirements were 10 ng/μl of PCR product, which were determined using Nanodrop. In addition, 5 μl of each primer pair was prepared at 3.2 μM. Primer pair products for TmL27a (4% (w/v) gel), SCD (5% (w/v) gel), ELOVL1 (5% (w/v) gel) and ELOVL2 (5% (W/v) gel) were run on again on non-denaturing Metaphor gels, in triplicate with additional fluorescent dye SYBR Green I nucleic acid gel stain (S9430, Sigma), visible under LED light.

MinElute Gel Extraction Kit, Qiagen (28604) was used to isolate PCR amplicons from non-denaturing Metaphor gels. PCR products were cut from the gel under LED light (DarkReader transilluminator, Clare Chemical Research), using a fresh scalpel for each product. Each product was placed in a separate pre-weighed 1.5 ml micro-centrifuge tube and then re-weighed to determine the weight of gel and product. For gels with >2% agarose gels, 6 volumes of Buffer QG was added to the gel (300 μl Buffer QG to 100 mg gel). In order to completely dissolve gels micro-centrifuge tubes, containing gel and buffer, were incubated for 10 min at 50°C, with vortex mixing every 2-3 min during the incubation. The colour yellow indicates correct pH for proceeding, a violet or orange colour indicates incorrect

pH and 10 μl of 3 M sodium acetate (pH 5.0) was added and mixed. Isopropanol was then added to the sample at 1 gel volume (100 mg gel requires 100 μl isopropanol) and mixed by inverting the tube several times. The sample was applied to the MinElute column and micro-centrifuged (1-15, Sigma) for 1 min, for volumes >800 μl, columns were loaded and spun again. Through-flow was discarded and 500 μl Buffer QG was added to the spin column and micro-centrifuged for 1 min. Again, through-flow was discarded and 750 μl Buffer PE was added to wash the MinElute column, micro-centrifuged for 1 min and discarded in through-flow. The column was centrifuge for additional 1 min at 10,000 rpm. The columns were placed into a clean 1.5 ml micro-centrifuge tube and the DNA eluted by adding 10 μl of Buffer EB to the membrane. The column was allowed to stand for 1 min and then micro-centrifuged for 1 min, an average volume of 9 μl was eluted. PCR product concentrations of DNA were evaluated using the Nanodrop and concentrations were adjusted with RNase-free water to meet Source BioScience's concentration requirements for Sanger Sequencing.

2.5 Statistical analysis

Data was analysed using SPSS (version 24, IBM) and Genstat (18th edition, VSNI). Firstly, data was analysed using the *Explore* function as individual samples (irrespective of treatment groups) to assess outliers, and where appropriate substantial outliers that were indicative of an issue arising were removed from further analysis. Normality and homogeneity of variance was then assessed as a whole dataset population.

Normally distributed data were analysed either using a t-test (2 groups) or one-way analysis of variance (ANOVA) (>2 groups), dependent of number of treatment groups. In addition, all FA were analysed using ANOVA, with a Bonferroni adjustment of the P value. This adjustment was used in order to manage the Type

I error rate when multiple hypothesises tests were carried out. Where ANOVA was found to be significant, a Tukey HSD *post-hoc* comparison was used to determine specific between-group differences. The Tukey HSD test was selected over a Dunnett t-test as it allowed for multiple comparisons between treatment groups in experiments, rather than limiting result comparisons of individual treatment group with only the control groups.

For non-normally distributed data, (determined by Shapiro-Wilk testing) and/or data that did not meet assumptions for homogeneity, (Levene test) or robust equality of the mean (Brown-Forsythe test), either Mann-Whitney U (2 groups) or Kruskal-Wallis (>2 groups) non-parametric tests were used, dependent on the number of treatment groups. *Post-hoc* comparisons were made for significant Kruskal-Wallis results using the SPSS output generated by pairwise comparisons.

Proportions of individuals dying were assessed in Genstat, using a generalised linear model, assuming modelling of binomially distributed errors (logistic regression) and a logit link function. Total starting number of individuals (per replicate) were represented as *Number of Subject(S)* (i.e. binomial totals), total deaths (per replicate) were represented as *Number of Successes* (i.e. response variate) and treatment group was specified as the explanatory variable (*Model to be Fitted*). All data was reported as back transformed means ± SEM.

Specific details of statistical analysis and method of displaying results are provided in methods section of each chapter.

Chapter 3

Growth and proximate nutrition of *T. molitor* larvae grown on feeds of varying nutritional composition

3.1 Introduction

Animal products provide a major source of protein to humans. Protein deposition in animals is limited by the quality of the protein source provided in feed. If energy requirements for maintenance are met, protein deposition within an animal can be increased by feeding on sources of protein that are similar to those of their body protein. Monogastric animals such as pigs, poultry and fish tend to be limited by the supply of essential amino acids (AAs), such as methionine and lysine. The main protein sources utilised in the feeding of production animals are soybean meal (SBM) and fishmeal (FM). These sources are considered to have AA compositions that meet the requirements of monogastric production animal species.

Due to the economic and environmental limitations associated with these two major protein feed sources, potential alternatives have been identified. Edible insect species have received particular attention in recent years and have been generalised as having nutritional profiles that are protein-rich, with 'good fats,

and high levels of calcium, iron and zinc (van Huis et al., 2013). Herein lies a major assumption about insects that (unlike mammals, birds and fish) their nutritional composition is constant, irrespecitve of dietary inputs and rearing conditions. Lack of evaluation of this assumption is a knowledge gap of considerable potential importance. As a specific example the majority of studies aiming to assess replacement of FM with *T. molitor* larvae in the feeding of fish, state no knowledge of the feeds of larvae produced by external sources, and have neither attempted to control the feeds provided to larvae nor acknowledged the impact the feed of larvae could have on their composition. It is likely that diet affects the biochemical status of *T. molitor* larvae as varying nutritional compositions are reported (Ng et al., 2001; Song et al., 2018; Iaconisi et al., 2018).

A further assumption still being made about edible insects is that they are sustainable. However this assumption has not been evaluated based on ascertaining the nutritional requirements of specific species of insects or the optimal husbandry methods for their growth. Instead sustainability evaluation has focussed on production of edible insect species on waste stream sources that are not only likely to be unsuitable on a nutritional basis but could be considered unsafe for entry into feed and food systems. Furthermore, insects previously examined for sustainability benefits through feeding of a variety of waste-stream sources and conventional production animal feeds were shown to be less sustainable than hypothesised (Smetana et al., 2016).

The sustainability benefits from utilising insects as feed could be a result of reduced land per tonne feed produced or indeed the high prolificacy of insects, or the reduced energy inputs for poikilotherm production, or the ability to manipulatable aspects of their nutritional composition for benefits to livestock animal health and nutritional composition, which are yet to be fully assessed. For instance, an increased n-3 PUFA composition in edible insects could improve the PUFA status in the meat animals that consume them and increase the SFA:PUFA

content in meat, improving their consumer acceptability and reduce the n-6:n-3 ratio, with beneficial anti-inflammatory effects.

A third issue encountered when reading literary sources surrounding edible insects is the production of insects on either SBM or FM or fish oil, which could be a result of confusion over their potential use as feed and/or food (Finke, 2015). Firstly, if the focus of edible insect production is for food, then similar research to those investigations conducted in domesticated agricultural species should be a priority for research and notably insects cannot be produced on waste sources. If, however the focus is on edible insects for replacement of SBM and FM as an animal feed then it makes no sense to produce them on SBM, FM or FO, which is a by-product from the FM reduction processing.

Success of animal production (in this case *T. molitor* larvae) is dependent on many factors, most importantly a feed provision that meets the nutritional requirements of the given species. Feeding standards have been well defined for the production of mainstream animals, but not in different insect species or at different development stages. This is the case for *T. molitor* larvae. Feeding standards for domesticated livestock species have been determined through growth trial studies that assess the effects of feeds chemical composition and energy value on the live weight gain and nutritional deposition in the animals. Effects of different feed inputs on nutritional composition in *T. molitor* larvae have yet to be studied. In addition, larvae must be produced sustainably. Smetana et al. (2016), states that identification of insects as a more sustainable source of protein than traditional and other emerging sources will be the only force capable of shifting to the use of insects as feed or food.

Trials described in this Chapter compare two distinctively different feeds used to produce *T. molitor* larvae. A production animal feed (sow feed, SF) and a cheap, abundant source of dietary fibre that has low protein digestibility in mammals, birds and fish species (wheatbran, WB) (Onipe et al., 2015; Harika et al.,

2013). The first could be used directly to produce monogastric production animals, the latter does not have the potential to provide for the AA requirements to monogastric production animals. The trials featured in this chapter were designed to gain insight into the affect of vastly different feed on growth and nutritional deposition in larvae. Furthermore, trials aimed to develop methodologies for repeat production of *T. molitor* larvae in the continued investigation into their potential use as a sustainable feed protein source replacement.

Aims

1. To assess whether growth and nutritional characteristics of larvae are affected by varying protein quantity and quality of feed composition.

2. To determine whether feeding composition affects the weight at which larvae start to pupate.

3. To assess the effect of feed composition on total mortality in larvae.

4. To determine if feed composition affects the growth characteristics of younger larvae in a similar way to the larger larvae.

Hypothesise

It is hypothesised that growth characteristics and nutritional content of *T. molitor* larvae will be affected by feeding a production animal feed versus a poorer fibrous basal feed.

3.2 Methods

3.2.1 Large larvae trial

Animal husbandry

A kilogram of standard *T. molitor* larvae, obtained from Monkfield Nutrition, Ltd. UK, were aged ⩾6 weeks and are herein referred to as large larvae. Large larvae were separated into 50 g replicate groups, 10 per treatment group (10 × 20 × 15 cm). Feeding groups were as follows: wheat bran (WB) (167 g/kg crude protein, 30 g/kg crude fat, 19.03 MJ/kg gross energy), provided by Buy Whole Foods Online, or sow feed (SF) (175 g/kg crude protein, 19 g/kg crude fat, 18.30 MJ/kg gross energy), provided by BOCM Pauls Farmgate Ltd. UK. Larvae were fed *ad libitum* throughout the trial. Feed intake was not recorded in this trial.

Experimental design

Total weights of larvae within replicates were recorded on days 0, 5, 11, 15 and 21, or until individuals within the replicate showed the first signs of batch pupation. First signs of batch pupation were defined as a minimum of one larva entering its pupal stage of development, at which point the batch was weighed and the weight at pupation was recorded, before all individuals in the batch were killed by cold exposure at -20°C.

Proximate nutritional analysis

All samples were prepared via oven drying at 100°C and subsequently ground using a coffee grinder for gross nutritional analysis of crude protein, crude fat and gross energy, following methods are outlined fully in Chapter 2.

3.2.2 Small larvae trial

Animal husbandry

Mini *Tenebio. molitor* larvae, obtained from Monkfield Nutrition, Ltd. UK were aged $\leqslant$6 weeks and are herein referred to as small larvae. Small larvae were acclimatised for 3 days prior to trial start date on WB and carrot, the latter being provided as a water source. On the start day of trial, 20 larvae were randomly allocated to each replicate petri dish (diameter: 150mm), of one of two treatment groups, WB or SF.

Experimental design

Larvae were weighed prior to start of trial and initial weights were checked for lack of statistically significant differences between treatment groups before trial proceeded. Larvae were then grown over a 41-day period. Weights and number of larvae were assessed on days 4, 7, 21 and 40, before being killed by cold exposure at -20°C. No gross nutritional analysis was conducted, due to the low weight of replicate groups. Feed intake was not recorded in this trial.

3.2.3 Statistical testing

Data were analysed using SPSS (version 24 IBM). Continuous response variable data that met assumption of normality and homogeneity of variance (determined using Shapiro-Wilk and Levene tests, respectively), were analysed by an independent sample, 2-tailed t-test. Any continuous data that did not meet the assumptions of normality or homogeneity of variance, were assessed using a non-parametric Mann-Whitney U test. Statistical significance was taken as $P<0.05$. Data was presented as means $\pm$SD. The proportion of small larvae dying were analysed using a generalised linear model (logistic regression) conducted in Genstat (version 16 VSN), which assumed binomially distributed errors and presented

as back transformed means ±SEM. Kaplan-Meir survival analysis using Log Rank Mantel-Cox test was used in SPSS to test the time larval development time to pupation and are presented as means ±SEM.

3.3 Results

3.3.1 Large larvae trial

Feeding input had a significant effect on the growth rates of total larvae over a 21-day period, ($P<0.01$) with SF fed larvae growing at a faster rate compared with WB fed larvae (Table 3.1). Between days 13 and 22, replicates of both groups began to pupate, at this point final weights (pupation weights) were recorded. There were no statistical differences observed in time to pupation or final weights and total biomass weight gain, between treatment groups (Table 3.1).

Larvae fed on SF had significantly greater dry matter ($P<0.01$), gross energy ($P<0.05$) and crude fat ($P<0.001$) contents in comparison with WB fed larvae (Table 3.1). However, WB fed larvae contained significantly greater crude protein contents in comparison with SF fed larvae ($P<0.001$), (Table 3.1).

Table 3.1: **The effect of different basal feeds on the performance and nutritional content in large *T. molitor* larvae grown to pupation.** Larvae were fed up until the point of pupation. Performance and nutrition were assessed per replicate. Results are displayed as means ±SD. Days to pupation are displayed as means ±SEM.

Performance	WB[1]	SF[2]	*Sig. (P)*
Initial weight (g)	50.0±0.4	50.3±0.3	NS
End weight (g)	106.2±15	114.4±16	NS
Growth rate (g/day)	2.8±0.4	3.5±0.4	<0.01
Total biomass gain (g)	56.2±15	64.1±16	NS
Days to pupation	18.6±1.1	16.8±1.1	NS
Nutritional composition			
Dry matter (g/kg)	342±11	359±10	<0.01
Crude protein (g/kg)	528±20	490±19	<0.001
Crude fat (g/kg)	271±11	309±27	<0.001
Gross energy (MJ/kg)	26.26±0.3	26.74±0.6	<0.05

[1] *Wheatbran*
[2] *Sow feed*

3.3.2 Smaller larvae trial

Feeding group had a significant effect on the growth rates of larvae, SF fed larvae achieved faster growth rates in comparison to WB fed larvae (P<0.001), (Table 3.2). Interestingly however, in the small larvae trial the larvae fed on SF achieved significantly greater 'end weights' and the biomass accumulated was greater in the SF fed larvae (Table 3.2). In the small larvae trial, it was possible to assess the effect of feeding group on the overall mortality in *T. molitor* larvae. Treatment group had no significant impact on the proportion of larvae dying (Table 3.2).

Table 3.2: **The effect of different basal feeds on the performance of small *T. molitor* larvae grown over a 41 day period.** 20 starting larvae. Growth rates, which were determined using a linear equation. All values are presented as means ±SD. Death proportion are reported as back-transformed binomial proportions ±SEM.

Performance	WB[1]	SF[2]	*Sig. (P)*
Initial weight (mg/larvae)	3.7±0.6	3.4±0.5	NS
End weight (mg/larvae)	6.3 ±0.9	7.7±1.0	<0.01
Growth rate (mg/larvae/day)	0.06±0.02	0.10±0.10	<0.001
Total biomass gain (mg)	48.7±5.5	81.4±13.7	<0.001
Death proportion	0.04±0.01	0.04±0.02	NS

[1] *Wheatbran*
[2] *Sow feed*

3.4 Discussion

The weights at pupation were not different in larvae fed on varying feed inputs, despite differences in growth rates measured over a 21-day period in large larvae. These results suggest weight is the major determinant of metamorphosis in *T. molitor* and has been previously reported in other species (C. K. Mirth & Riddiford, 2007). It has been suggested that larval species must surpass three weight checkpoints during instar development, in order to progress toward metamorphosis (C. K. Mirth & Riddiford, 2007). In addition to this, during the final instar both *Drosophila melanogaster* and *Manduca sexta* species have been described as having to surpass a minimum viable weight, where the fat body contains adequate nutrient deposition to survive metamorphosis and meet a critical weight in order to enter into metamorphosis (C. K. Mirth & Riddiford, 2007; Nijhout & Williams, 1974; Nijhout, 1975).

Regulation of growth rates in the larval stage of metabolobous insect species is dependent on a number of exoskeleton molts (instars), which are affected by various factors including feed quality (Morales-Ramos et al., 2010). In unfavourable

nutritional conditions, the number of instars would increase, and growth rate of larvae would decrease (Cotton & St George, 1929; Morales-Ramos et al., 2010). The number of instar molts was not recorded during the large larvae trial, however time to pupation was unaffected by treatment. A measure of feed intake could indicate whether differences in growth rate were a result of varying feed intake levels between treatment groups, however this was not measured during the trial.

High dietary fibre content in WB feed (33.4-63.0%) has been associated with reduced appetite, which has been attributed to satation and reduced feed intakes (Samra & Anderson, 2007; Ötles & Ozgoz, 2014). In comparison to SF, the increased feed viscosity and reduced energy density of WB, could have affected dietary intake in larvae. Crude fibre content of feed in *T. molitor* larvae >5% has been suggested as having a detrimental effect on palatability (Li et al., 2016). Reduced feed intakes could have affected growth rates of WB fed larvae, however there were no differences in mortality between treatment groups. This suggests that individuals within replicates met threshold weight checkpoints during instar development and a minimum viable weight during the final instar, which has otherwise resulted in death in *D. melanogaster* and *M. sexta* species' (Nijhout, 1975).

Negative regulators of growth in larval species have been described as ecdysteroids and juvenile hormone (Truman et al., 2006; C. Mirth et al., 2005). Positive regulators of growth rates have been described within *D. melanogaster* larvae as insulin-like proteins, which mediate cell growth via the insulin signalling pathway (Oldham & Hafen, 2003). It has been suggested that the fat body of larval species regulates growth rate by nutrient-sensing mechanisms, potentially by controlling suppression/inducement of ecdysone molting hormone as well as inducement/suppression of insulin-like proteins (Colombani et al., 2003). However, another evaluation is that the fat body induces normal growth rates, independent of ecdysone levels, unless under suboptimal conditions, when ecdysteroids will

suppress growth in larvae (C. K. Mirth & Riddiford, 2007). Insulin-like proteins were down-regulated in *D. melanogaster* amino-acid starved larvae, highlighting that the fat body may regulate based on intracellular concentrations of free amino acids (Colombani et al., 2003). If intracellular concentrations of amino acids are affected by quantity and/or quality of crude protein available in the feed, then in WB fed larvae less insulin-like proteins will be humorally regulated and there will be less of an effect on cell growth mechanisms within the fat body (Colombani et al., 2003) .

The increased crude protein and reduced crude fat deposition observed in WB fed larvae could be explained by an overestimation of metabolisable energy in WB feed due to high levels of non-starch carbohydrates (Brouns et al., 2012). The bomb calorimetry methods for gross energy analysis does not distinguish between available and non-digestible sources of energy, meaning that WB feed is likely to have provided lower metabolisable energy in comparison to SF. The increased fat deposition in SF fed larvae is likely the result of over-provision of energy resulting in increased conversion to storage lipid TAG within lipid droplets in the fat body of the larvae, similar to fattening in grower finisher pigs.

A major limitation in the large larvae trial was the lack of literature on robust techniques for culturing *T. molitor* larvae (as of September 2014). The small larvae trial was a progression on the original methodology from the large larvae trial and enabled mortality analysis. However the small larvae trial did not provide enough material for proximate nutritional analysis. On the one hand, the initial large larvae produced enough material for proximate analysis however, mortality could not be assessed as treatment groups were defined by initial weight and not initial number of individuals.

3.4.1 Conclusion

In this Chapter the effect of feed composition on growth rates, nutritional deposition and mortality was assessed in *T. molitor* larvae, on two nutritionally distinct feeds, in two independent trials.

The hypothesis for this Chapter, that basal feed will affect growth rates and gross nutritional composition in *T. molitor* larvae, is supported. However the proportion of dead larvae was found to be unaffected by feeding inputs in *T. molitor* larvae.

Chapter 4

The effect of feed oil content on the fatty acid profile of *T. molitor* larvae

4.1 Introduction

Human populations of less economically developed countries are experiencing a nutrient shift towards that found in a typically 'Western' meat-eating diet. Meanwhile in Western countries there has been an increase in the 'health-conscious consumer', and in particular there has been growing interest in dietary fatty acids (FA) and their effects on human health (Hargis & van Elswyk, 1993). Monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) are FA categories that have been associated with health benefits for humans (Fernandez & West, 2005). Of particular interest are FAs of the n-3 series which have been associated with reduced inflammatory responses and cardiovascular benefits (J. H. Lee et al., 2009; Swiatkiewicz et al., 2015). An essential FA precursor to longer chain-PUFAs (LC-PUFA) of the n-3 series, is α-linolenic acid (ALA)which is chiely provided in human diets through the consumption of a number of plant species and some can also be obtained from consumption of animals (Astorg et al., 2004). However, with a high content of saturated FA (SFA), animal products, and in particular red meats, have been associated with negative health implications in humans; these

include increased inflammation, which is a strong predictor of cardiovascular diseases (Baer et al., 2004; Valsta et al., 2005; Morgan et al., 1992). A growing area of research interest has been to manipulate specific FA profiles of animal products, through feeding, in an attempt to increase the levels of 'healthier' fatty acids within the fat deposition (Gibbs et al., 2010).

Animal tissues are capable of biosynthesis of LC-PUFA from essential dietary FA precursors linoleic acid (LA) and ALA, via elongation and desaturation reactions. Many researchers have investigated the ability to manipulate n-3 and n-6 LC-PUFA biosynthesis within animals, through the provision of dietary essential precursors LA and ALA (Ibrahim et al., 2017; Nuernberg et al., 2005; Hargis & van Elswyk, 1993; Wood et al., 1999). By supplementing with LA or ALA it is hoped that further desaturation and elongation will result in increased LC-PUFAs: arachidonic acid (ARA) (n-6) or docosapentaenoic acid (DPA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (n-3), respectively, in the FA composition of meat and eggs. However, it is also acknowledged, that production animals and indeed humans have little ability to elongate dietary ALA into DHA (Wood et al., 2004; Brenna et al., 2009). It has been shown humans have 5% efficiency in converting ALA to EPA and only a 0.5% efficiency in converting ALA to DHA (Gillingham, 2013). Therefore, to gain the health benefits associated with increased DHA status, this FA must be obtained from the diet.

Obtaining DHA from the diet is achieved typically through the consumption of fish (Mantzioris et al., 2000). Fish contain these rich sources as a result of marine food chains that bioaccumulate n-3 LC-PUFA from primary producers such as micro-algae. Over-exploitation of wild fish stocks is no longer sustainable, this includes the removal of fish in order to produce fish meal for aquaculture feeding (Miller et al., 2008).

Whilst attempting to reduce the cost and increase the sustainability of aquaculture production, researchers have successfully substituted SFAs in fishmeal (FM)

and fish oil (FO) of reared fish. Hydrogenated SFA-rich oils were found to increase the bioavailability of DHA in fish in comparison to C18 UFA oil sources (Rombenso et al., 2015, 2016).

This Chapter explores the impact of altering dietary oils in the feeding of *T. molitor* larvae. In particular, the impact of supplemented oils on growth, gross nutrition and FA profiles of whole larvae. Any evidence for *T. molitor* larval capabilities to synthesise LC-PUFAs from essential FAs of the n-6 and n-3 series was assessed. If found to be efficient synthesisers of DHA, *T. molitor* larvae would be an extremely useful alternative feed source for production animals, providing human consumers, indirectly, with a potential sustainable dietary source of DHA.

Prior work has shown increased growth rates and reduced mortality in the larvae fed on SF (Chapter 3). In addition to this, larvae appeared to exhibit fattening characteristics, with increased deposition of fat as well as an increase in energy content and a decreased protein deposition compared to WB fed larvae. A production animal feed was selected, in which to add oil supplementations, in order to promote fat deposition and best observe any potential influences on specific FA composition within the larvae. A production feed of chick crumb provides a higher crude protein content compared with SF and further ensures the most commonly limiting amino acids lysine and methionine (at least in production animal species), were provided.

Aims

1. To begin to design a robust method for assessing the consumption of both feed and carrot by larvae throughout trials.

2. To determine whether supplementation with 5% fat resulted in altered growth and mortality characteristics in larvae.

3. To determine whether supplementation with additional lipid results in al-

tered proximate nutritional content.

4. To assess whether the supplementation with SFA and UFA oils has specific effects on altering the FA profiles of whole larvae.

5. To evaluate whether larvae are capable of further elongating and desaturating n-3 and n-6 series FAs to EPA, DHA and ARA.

Hypothesis

It is hypothesised that specific fatty acids can be altered within *T. molitor* larvae based on supplementation of the basal feed. It is also hypothesised that *T. molitor* larvae will have the ability to convert dietary LA and ALA into LC-PUFAs; ARA (n-6) and EPA, DPA and DHA (n-3), respectively.

4.2 Methods

Two separate experiments were conducted to determine the effects of specific FA supplementation on a number of characteristics in *Tenebrio molitor*. The methods outlined below were the same for both trials, unless stated otherwise. For a detailed overview of methods please see Chapter 2.

4.2.1 Animal husbandry

Tenebrio molitor larvae were supplied by Monkfield Nutrition Ltd. (Royston, Cambridgeshire, UK) for both trials. Prior to each trial larvae were fed on WB and carrot; the latter was provided as a water source. Before the trials (Day 0), larvae were randomly assigned to replicates within different feed treatment groups. Treatment feeds and water source were provided *ad libitum* throughout all larvae trials. All trials were conducted over a 14-day period. Trials were conducted at the University of Nottingham, Sutton Bonington Campus, UK.

4.2.2 Feed preparation

Specified oils for SFA and UFA trials were incorporated into a basal control feed of chick crumb (C) (crude protein 19%, crude fat 4%, crude fibre 4%, crude ash 6%, lysine 1%, methionine 0.35% (w/w) supplied by Heygates Limited (Northampton, UK), at an inclusion of 5%(w/w) of the chick crumb, using a bag mixing technique (Table 4.1). Feeds for both trials were designed to contain increased concentration of specific fatty acids, which was achieved using specific oils high in the desired fatty acid(s). Oils selected to provide increased SFA were palm kernel oil, palm oil, and cocoa butter, which provided increases in lauric and myristic acid (12:0 and 14:0), palmitic acid (16:0), and stearic acid (18:0), respectively, above that of the control (C) feed (Table 4.2). Oils selected for increases in the UFA content of the feed above that of the C feed were rapeseed oil, safflower oil, and linseed oil. These oils were supplemented to provide an increase in oleic acid (C18:1), linoleic acid (C18:2), and α-linolenic acid (C18:3), respectively, above that of the C feed (Table 4.2).

Table 4.1: **The formulation of *T. molitor* larvae feeds enriched in fats, g/kg displayed.**

	SFA trial				UFA trial			
Components (g/kg)	C[1]	PK[2]	P[3]	CB[4]	C	RS[5]	SAFF[6]	LS[7]
Chick crumb	1000	950	950	950	1000	950	950	950
Palm oil	-	50	-	-	-	-	-	-
Palm kernel oil	-	-	50	-	-	-	-	-
Cocoa butter	-	-	-	50	-	-	-	-
Rapeseed oil	-	-	-	-	-	50	-	-
Safflower oil	-	-	-	-	-	-	50	-
Linseed oil	-	-	-	-	-	-	-	50
Total	1000	1000	1000	1000	1000	1000	1000	1000

[1] *Wheatbran control*
[2] *palm kernel oil* 5% (w/w)
[3] *palm oil* 5% (w/w)
[4] *cocoa butter* 5% (w/w)
[5] *rapeseed oil* 5% (w/w)
[6] *safflower oil* 5% (w/w)
[7] *linseed oil* 5% (w/w)

Table 4.2: **The fatty acid composition (moles%) of *T. molitor* larvae feeds enriched in fats containing either saturated fatty acid (SFA) or unsaturated fatty acid (UFA).**

	SFA trial feeds				UFA trial feeds			
Fatty acid	C[1]	PK[2]	P[3]	CB[4]	C	RS[5]	SAFF[6]	LS[7]
Caprylic 8:0	-	1.6	-		-	-	-	-
Capric 10:0	-	2.3	-	-	-	-	-	-
Lauric 12:0	1.0	35.2	-	-	1.6	-	-	-
Myristic 14:0	1.0	11.7	1.3	-	1.1	-	-	-
Palmitic 16:0	32.3	13.4	42.4	27.2	29.7	5.1	7.3	6.4
Stearic 18:0	6.0	2.3	4.2	24.4	3.8	1.8	2.2	3.7
Oleic 18:1 (n-9)	35.5	19.4	35.8	32.5	32.3	64.1	15.0	19.1
Linoleic 18:2 (n-6)	23.8	13.1	14.2	13.5	28.0	20.8	72.5	15.3
Linolenic 18:3 (n-3)	1.0	1.1	1.0	1.2	2.9	6.5	2.4	54.9
Unknown	0.4	0.0	1.2	1.3	0.7	1.7	3.1	0.5

[1] *Wheatbran control*
[2] *palm kernel oil* 5% (w/w): *lauric*
[3] *palm oil* 5% (w/w): *myristic + palmitic*
[4] *cocoa butter* 5% (w/w): *stearic*
[5] *rapeseed oil* 5% (w/w): *oleic*
[6] *safflower oil* 5% (w/w): *linoleic*
[7] *linseed oil* 5% (w/w): *linolenic*

4.2.3 Experimental design

On day 0, replicates, of one hundred larvae, were randomly allocated to a petri dish (diameter: 150 mm). There were eight replicates per feed treatment group. There was no significant difference in initial total biomass between treatment groups (Table 4.3). On day 7, 11 and 14 total biomasses of larvae (dead and alive) were recorded. Dead larvae were then removed and the total number of larvae remaining were recorded. Remaining feed and water sources were weighed and then replaced on days 7 and 11 of the trial and weighed on day 14. On day 14, larvae from each replicate were killed by cold exposure at -20°C, then the replicate batches were stored at -20°C separately, before being prepared for proximate nutritional analysis.

4.2.4 Proximate nutritional analysis

Tenebrio molitor larvae samples were oven dried at 100°C, before nutrient composition analysis. Proximate nutritional analysis was conducted on samples from the total pooled individuals for each replicate. The proximate analysis included crude fat assessment via Soxhlet ether extraction method (Soxtherm, Gerhardt), crude protein assessment via combustion analysis by nitrogen analyser (FlashEA 1112 Series, Thermo Fisher Scientific), and gross energy estimation via bomb calorimetry (6300 Calorimeter, Parr) (Chapter 2). FA composition was also determined for all replicates following crude fat extraction via FAME methods (Chapter 2) and analysis was conducted using Gas-Chromatography (Clarus 580, Perkin Elmer) and detection software (TotalChrom Workstation, Perkin Elmer) (Chapter 2).

4.2.5 Statistical testing

Data was analysed using SPSS (version 24 IBM). Continuous response variable data that met assumption of normality and homogeneity of variance (determined using Shapiro-Wilk and Levene tests, respectively), were analysed using one-way analysis of variance (ANOVA). Specific between-group differences in measured variables were determined using Tukey HSD *post-hoc* comparisons. Any continuous data that did not meet the assumptions of normality or homogeneity of variance, were assessed using Kruskal-Wallis one-way analysis of variance, between-group differences were determined by Dunn-Bonferroni post-hoc testing. Statistical significance was taken as $P<0.05$. All individual FAs were analysed using ANOVA, with a Bonferroni adjustment (0.05 ÷ no. of dependent FAs) of the 0.05 alpha level of acceptance, in order to avoid type I errors in results. Data is presented as means ±SD.

Mortality (the proportion of larvae dying) were analysed using a generalised linear model (logistic analysis) conducted in Genstat (version 16 VSN), which

assumed binomially distributed errors. Data were presented as back-transformed means ±SEM.

4.3 Results

4.3.1 SFA supplementation

Supplementation of the *T. molitor* larvae feed with fats containing high levels of specific SFA, at 5% (w/w) for 14 days, resulted in no differences in the average weight gain per day per larvae, nor on the total average consumption of feed or carrot per larvae (Table 4.3). Mortality was found to be significantly affected by supplementation of specific SFA sources, with CB and PK fed groups having a lower mortality compared with C and P groups (P<0.001), (Table 4.3).

The exposure to high SFA resulted in significant differences in larval nutrient composition. Larvae supplemented with oils containing high levels of SFA had significantly increased crude fat content (P<0.001), (Table 1.3). Gross energy was also affected by treatment group (P=0.011). Larvae in the control group had significantly less gross energy content compared with P (P=0.018) and CB (P=0.026) groups (Table 4.3). In addition, a trend for higher crude energy was observed when comparing PK supplemented groups with controls (P=0.056), (Table 4.3). Crude protein content was unaffected by treatment group (Table 4.3).

The FA profiles of larvae were affected by SFA supplementation, with differences in the contents of C12:0 (P<0.001), C14:0 (P<0.001), C16:0 (P<0.001), and C18:0 (P<0.001) between treatment groups (Table 4.4). Larvae supplemented with PK had significantly greater C12:0 (P<0.001) and C14:0 (P<0.001) content compared with all other feeding groups (Table 4.4). The lowest C16:0 content was observed in PK fed larvae, who had significantly less C16:0 compared with C groups (P=0.004). P fed larvae had significantly greater contents of C16:0 SFA compared with all other treatment groups (P<0.001) and a higher level of C14:0

in comparison to C (P = 0.038) and CB groups (P= 0.023), (Table 4.4). Larvae supplemented with CB had increased levels of C18:0 SFA in comparison to all other treatment groups (P<0.001), and larvae also had a significantly greater content of C16:0 compared with PK fed larvae (P<0.001), (Table 4.4).

Table 4.3: **The effect of feed enriched in fats containing saturated fatty acid (SFA) on the performance and nutritional content of *T. molitor* larvae following a 14-day treatment.** n = 8, growth rate per larvae was determined using a linear regression. All values are presented as means ±SD in units mol% FAME. Mortality reports back-transformed means ±SEM.

Performance	C[1]	PK[2]	P[3]	CB[4]	*Sig. (P)*
Initial weight (mg/larvae)	61.1±10	61.9±10	60.9±7	65.9±9	NS
End weight (mg/larvae)	115.9±13	110.4±12	117.2±8	111.0±14	NS
Growth rate (mg/larvae/day)	3.8±1	3.6±1	4.2±1	3.3±1	NS
Total feed intake (mg/larvae)	64.6±9	62.1±5	44.4±28	59.3±5	NS
Total carrot intake (mg/larvae)	205±26	212±17	213±23	216±22	NS
Mortality	0.40 ± 0.03^{a}	0.35 ± 0.03^{b}	0.42 ± 0.03^{a}	0.32 ± 0.03^{b}	<0.001
Estimated feed conversion ratio	5.70±1.9	6.23±2.0	5.13±1.2	5.41±102	NS
Nutritional composition					
Crude protein (g/kg)	501±20	490±11	489±13	487±13	NS
Crude fat (g/kg)	386 ± 7^{b}	405 ± 12^{a}	405 ± 8^{a}	406 ± 11^{a}	<0.001
Gross energy (MJ/kg)	28.42 ± 0.3^{b}	28.76 ± 0.2^{ab}	28.82 ± 0.3^{a}	28.80 ± 0.2^{a}	<0.05

[1] *Wheatbran control*
[2] *Palm Kernel oil* 5% (w/w)
[3] *Palm oil* 5% (w/w)
[4] *Cocoa butter* 5% (w/w)
[a,b] change in letter indicates significant difference

Table 4.4: **The effect of feeds enriched in fats containing saturated fatty acid (SFA) for 14 days on fatty acid composition (moles%) in *T. molitor* larvae.** n = 8, all values are presented as means ±SD. Significance level of acceptance with Bonferroni adjustment was, $P<0.005$.

Fatty acid	C[1]	PK[2]	P[3]	CB[4]	*Sig. (P)*
12:0	-^{b}	1.4±0.1^a	-^{b}	-^{b}	<0.001
14:0	3.9±0.2^c	5.8±0.2^a	4.3±0.2^b	3.9±0.2^c	<0.001
16:0	18.8±0.3^b	17.9±0.2^c	20.1±0.6^a	19.1±0.3^b	<0.001
16:1	2.3±0.1	2.3±0.1	2.3±0.0	2.2±0.1	NS
18:0	2.8±0.1^b	2.7±0.1^b	2.6±0.1^b	3.5±0.2^a	<0.001
18:1 (n-9)	48.8±0.5	48.2±0.8	47.5±1.7	49.5±0.9	<0.05
18:2 (n-6)	19.5±0.9	18.2±0.7	18.8±0.7	18.1±1.0	<0.05
18:3 (n-3)	0.8±0.1	0.7±0.1	0.6±0.1	0.8±0.1	0.058
Unknown	3.0±1.0	2.7±1.7	3.8±2.5	3.0±1.6	NS

[1] *Wheatbran control*
[2] *Palm Kernel oil* 5% (w/w)
[3] *Palm oil* 5% (w/w)
[4] *Cocoa butter* 5% (w/w)
[a,b] change in letter indicates significant difference

4.3.2 UFA supplementation

When *T. molitor* larval feed was supplemented for 14 days with fats high in specific UFA at 5% (w/w) the average weight gain per larvae per day and average total carrot consumption per larvae were unaffected. However average total feed consumption per larvae was significantly different in UFA treatment groups ($P<0.05$), with the RS fed group consuming significantly greater amounts feed compared with the C group P ($P<0.05$), (Table 4.5). Mortality was also significantly affected by feeding group, ($P<0.001$), UFA fed larvae had a greater total mortality in comparison to C groups (Table 4.5).

Following 14 days exposure to feed high in UFA there was a significant effect on crude protein, crude fat and gross energy levels in larvae 4.5). However only the RS fed group was significantly different to the C group, having significantly greater energy ($P<0.05$) and fat ($P<0.05$) as well as significantly less crude protein

($P<0.05$), (Table 4.5).

There were highly significant differences in both the SFA and UFA content between the feed groups for the larval content of C16:0 ($P<0.001$), C16:1 ($P<0.01$), C18:1 ($P<0.001$), C18:2 ($P<0.001$), and C18:3 ($P<0.001$) content (Table 4.6). Compared to group C, there was significantly lower C16:0 in the RS and LS fed groups ($P<0.001$) and significantly less C16:1 in RS ($P<0.01$) and SAFF ($P<0.01$) groups (Table 4.6). RS groups had significantly greater C18:1 compared with all other treatment groups ($P<0.001$) with the SAFF ($P<0.001$) and LS ($P<0.001$) fed groups being the lower than the C treatment group. As might have been expected, the SAFF fed *T. molitor* larvae had significantly greater C18:2 to all other treatment groups, ($P<0.001$) whilst feeding LS resulted in significantly greater C18:3 content in comparison with all other groups ($P<0.001$). The 18:2 content of LS fed *T. molitor* larvae and the 18:3 content of SAFF group were both significantly lower than C group ($P<0.001$).

Table 4.5: **The effect of feed enriched in fats containing unsaturated fatty acid (UFA) on the performance and nutritional content of *T. molitor* larvae following a 14-day treatment.** n = 8, growth rate per larvae was determined using a linear regression. All values are presented as means ±SD. Mortality reports back-transformed means ±SEM.

Performance	C[1]	RS[2]	SAFF[3]	LS[4]	*Sig. (P)*
Initial weight (mg/larvae)	48.0±2	49.3±1	49.8±2	49.3±2	NS
End weight (mg/larvae)	82.4±8	86.5±6	82.9±3	82.5±6	NS
Growth rate (mg/larvae/day)	2.4±0.5	2.5±0.5	2.2±0.3	2.3±0.4	NS
Total feed intake (mg/larvae)	44.2 ± 5^{b}	52.5 ± 5^{a}	47.3 ± 6^{ab}	45.1 ± 5^{ab}	<0.05
Total carrot intake (mg/larvae)	233±17	244±22	231±15	228±9	NS
Mortality	0.08 ± 0.01^{b}	0.16 ± 0.02^{a}	0.17 ± 0.02^{a}	0.15 ± 0.002^{a}	<0.001
Estimated feed conversion ratio	8.10±1.4	7.70±1.2	8.10±1.1	8.04±1.3	NS
Nutritional composition					
Crude protein (g/kg)	548 ± 24^{a}	517 ± 19^{b}	531 ± 21^{ab}	527 ± 18^{ab}	<0.05
Crude fat (g/kg)	309 ± 21^{b}	348 ± 25^{a}	340 ± 31^{ab}	334 ± 22^{ab}	<0.05
Gross energy (MJ/kg)	26.99 ± 0.2^{b}	27.49 ± 0.3^{a}	27.29 ± 0.4^{ab}	27.37 ± 0.3^{ab}	<0.05

[1] *Wheatbran control*
[2] *Rapeseed oil* 5% (w/w)
[3] *Safflower oil* 5% (w/w)
[4] *Linseed butter* 5% (w/w)
[a,b] change in letter indicates significant difference

Table 4.6: **The effect of feeds enriched in fats containing unsaturated fatty acid (UFA) for 14 days on fatty acid composition (moles%) in *T. molitor* larvae.** n = 8, all values are presented as means ±SD, in units mol% FAME. Significance level of acceptance, with Bonferroni adjustment was, $P<0.006$.

Fatty acid	C[1]	RS[2]	SAFF[3]	LS[4]	*Sig. (P)*
14:0	3.4±0.3	3.0±0.2	3.4±0.3	3.2±0.2	NS
16:0	18.0±0.6^{a}	15.9±0.2^{b}	17.2±0.6^{ab}	16.4±0.6^{b}	<0.001
16:1	2.6±0.3^{a}	2.2±0.1^{b}	2.2±0.2^{b}	2.4±0.3^{ab}	<0.01
18:0	2.7±0.5	2.6±0.5	2.8±0.4	2.6±0.2	NS
18:1 (n-9)	45.3±3.0^{b}	49.1±1.0^{a}	39.3±2.0^{d}	42.7±1.0^{c}	<0.001
18:2 (n-6)	23.6±0.9^{b}	23.6±1.0^{b}	31.5±2.0^{a}	21.3±1.0^{c}	<0.001
18:3 (n-3)	1.1±0.1^{c}	1.6±0.1^{b}	0.7±0.1^{c}	9.2±0.6^{a}	<0.001
Unknown	3.4±2.4	2.0±0.5	2.9±0.8	2.2±0.5	NS

[1] *Wheatbran control*
[2] *rapeseed oil* 5% (w/w)
[3] *safflower oil* 5% (w/w)
[4] *linseed butter* 5% (w/w)
[a,b] change in letter indicates significant difference

4.4 Discussion

Supplementation of basal animal production feed, chick crumb, with various SFA and UFA resulted in no differences in phenotypic growth characteristics and protein content in larvae. The only exception to this was RS fed larvae that were found to consume increased feed in comparison and have reduced protein contents in comparison with C groups. Studies testing type of oil supplementation and varying level (non-lipid, non-isoenergetic) of oil supplementation in grower-finisher pigs, found similar results that supplementation with palm kernel oil or fish oil had no differential effects on daily weight gain, feed intake or lean meat content (Hallenstvedt et al., 2010). Consumption of increased feed may have led to reduced protein deposition and increased fat deposition in RS fed larvae, in addition to an increased energy content in larval composition compared with C

groups.

In contrast to the majority of Prior studies, which have focussed on testing of different oil supplementations in isolipid/isoenergetic feed, both the SFA and UFA trial compared various oil types against a control with different levels of lipid and energy content. Oil supplemented groups consisted of 95% chick crumb basal feed at 4.5% crude lipid and 5% oil, supplying 8.8% total lipid contents to treatment feeds. However, literature supported the findings that various oil supplementation have no effects on growth and feed intake characteristics in animals. In *T. molitor* larvae produced on feeds varying in FA composition, no differences were observed between groups for biomass weight gain, over a 9-day trial period (Fasel et al., 2017). In pigs, various SFA and UFA supplementations had no impact on growth rate, feed intake or biomass weight gained; for animal fat versus linseed oil (J. Kim et al., 2014); or supplementation with linseed oil, rapeseed oil, fish oil or lard (Sobol et al., 2015); or supplementations with tallow, soybean, linseed or fish oils (Morel et al., 2013).

In chicken supplemented with oils, results appear to further support findings of this Chapters' trials. Comparisons of chickens supplemented at 4.5% (w/w), with either linseed or sunflower oil (similar to safflower oil with high LA contents), resulted in no differences observed between growth characteristics (Ibrahim et al., 2018). In contrast, combinations of oils; linseed and sunflower (1:1) and sunflower and fish oils (1:1) resulted in increased feed efficiency and final body weights in chickens, compared with separate supplementations (Ibrahim et al., 2018). Further to this, boiler chickens fed on 2.5% linseed oil over tallow oil exhibited a lower feed conversion ratio and increased growth (Carragher et al., 2016).

Mortality was reduced by feeding of PK and CB in larvae, whereas feeding with UFA resulted in greater mortality in comparison with C groups. However, SFA trial larvae exhibited higher average mortality, than those observed in UFA trial. Even comparisons of SFA control groups with UFA control groups, which

were both chick crumb feed, show large differences in overall mortality in larvae. There is limited information in the literature on mortality in animals treated with oils. Previously, broiler chickens supplemented with animal fat (relatively high SFA) versus linseed oil at 2.5% (w/w), resulted in no differences in bird mortality (Carragher et al., 2016). This source appears to contradict the observations between SFA and UFA trial groups.

The level of fat supplementation within treatment groups, in comparison with controls would have likely resulted in increased fat deposition and overall energy content in larvae, however this was only the case in the SFA trial. In the UFA trial, SAFF and LS fed larvae had the same fat and energy contents as controls, despite containing additional oil supplementations. Larvae fed on RS were the only UFA supplemented group to have an increased fat and energy content in comparison with controls and this could be the result of the increased feed consumption observed in larvae. Tests of isolipid feeds in pigs found no differences in total lipid content, of adipose or mucle tissue, between isolipid feeding groups (De Tonnac et al., 2017). These results support observations that showed no differences between the crude fat deposition between RS, SAFF, and LS or P, PK, and CB treated larvae in the separate trials. Supplementation of feed with oils of similar FA composition to safflower oil resulted in increased fat content in salmon fillets and pig carcass when compared with palm, rapeseed and linseed oil supplementation or tallow oil (relatively high in SFA), respectively (Rosenlund et al., 2001; Realini et al., 2010). In addition, pigs exhibited lower protein contents in 10% sunflower oil supplemented versus 10% tallow oil supplemented (Realini et al., 2010). These results are interesting as SAFF fed larvae did not exhibit differences in fat content compared with controls, whereas all SFA treated larvae achieved higher fat content compared with controls and there were no differences observed between SAFF, RS and LS fed larvae, as previously mentioned.

Feeding trials using *T. molitor* larvae have successful alteration of FA com-

position over a 9-day growth period (Fasel et al., 2017). In this Chapter, results showed that specific SFA and UFA provided in the feed compositions were increased in the overall body composition of the larvae. There were no increases in larvae 18:1 FA content in those fed on CB, which might have been expected due to increased dietary provision of 18:0, which can be further converted to 18:1 by desaturation by stearoyl-CoA desaturase (SCD) (Valsta et al., 2005).

Results support findings in other monogastric species. In the muscle of Atlantic salmon supplementation with feed containing 16.2% palm oil resulted in significantly increased 16:0, which supports Chapter findings (J. Kim et al., 2014). Animal fat containing 14:0 at 1.28%, 16:0 at 17.38% and 18:0 at 12.79%, supplemented at 5%, resulted in increased 16:0 in finisher pigs (J. Kim et al., 2014). However, only a trend was observed for increased 18:0 contents in adipose tissue, compared to linseed oil supplementation which successfully increased LA and ALA in adipose tissues (J. Kim et al., 2014). Further to this, 18:1 was not increased in pigs supplemented with 5% animal fat, similar to SFA trial results, where 18:1 content was not different between larval groups (J. Kim et al., 2014).

The content of 18:1 was the highest in UFA larvae fed RS, larvae also contained the second highest content of LA after larvae fed SAFF. Findings for the FA content in larvae fed RS were supported by trials in fish, pigs and chickens. Atlantic salmon fed rapeseed oil supplementation, to replace fish oil, had increased 18:1 content at 28.1% and LA at 12.4% (Rosenlund et al., 2001). Pigs supplemented at 5% (w/w) olive oil (18:1: 66.4% FAME versus rapeseed at 63.3% FAME (Orsavova et al., 2015), in a basal pig feed containing 4.2% lipids, had increased 18:1 to other treatment groups, including those supplemented linseed oil (Nuernberg et al., 2005). Furthermore, a correlation was found between rapeseed oil supplementation and increased 18:1 and LA content in broilers (Scaife et al., 1994). In comparison with other UFA treatment groups, RS fed larvae had significantly different gross nutritional composition to C groups. Gross nutrition

was likely affected by increased feed intake, as previously mentioned. It has previously been shown that *ad libitum* versus restricted feeding did not affect the FA compositions in pigs fed various oils (J. Kim et al., 2014). This can help to rule out an effect of increased feed intakes on FA profiles observed in RS fed larvae.

There are few prior studies of animals supplemented specifically with safflower oil, however soybean and sunflower oil (66.3% FAME) supply high levels of LA, similar to safflower oil at 79% FAME (Orsavova et al., 2015). In salmon 50-60% replacement of fish oil with soybean oil resulted in increased LA content in fish (Rosenlund et al., 2001). In calves 4.5% supplementation of soybean or sunflower oils increased LA and decreased ALA, in subcataneous fat compared with controls (González et al., 2014).

Supplementation with LC-PUFA precursors, LA and ALA resulted in no apparent increases in larvae LC-PUFA overall fat composition, despite LS fed larvae containing 8-fold increases in ALA above that in C groups. Previous work feeding linseeds at either 76% and 96% of *T. molitor* feed against a wheat flour control, resulted in no evidence for ability to further elongate to LC-PUFA of the n-3 series (Fasel et al., 2017). Supplementation of linseed oil in pigs at 3.5% (w/w) resulted in a 3-fold increase in ALA (Sobol et al., 2015). Further it has been shown that 1.5% (w/w) linseed oil supplementation resulted in a 2-fold increase in pigs compared with a soybean oil (high LA) control, in addition to 3-fold and 4-fold increases in EPA and contrary to many literary resources, DHA (Jiang et al., 2017). It has been stated that adipose tissue of pigs has a high capacity to retroconvert DHA, in comparison to the ability to convert ALA to DHA (De Tonnac et al., 2017). For instance, in pigs 5% linseed oil supplementation results in no differences in LC-PUFA content (J. Kim et al., 2014). The same may be true for *T. molitor* larvae.

4.4.1 Conclusion

The trials in this Chapter assessed the performance, nutritional composition and fatty acid composition of *T. molitor* larvae fed on the same basal feed supplemented with various oils. Growth rates, feed intake and protein deposition were unaffected by feed composition in *T. molitor* larvae, with the exception of RS fed larvae, which had increased feed intake, reduced crude protein and increased gross energy and crude fat depositions. Altered nutrient content in RS larvae is attributed to increased feed intake. Overall, the results from the UFA trial suggest that certain UFA supplementation can reduce fat deposition in larvae, whilst increasing specific essential FAs within larvae composition such as LA (n-6) and ALA (n-3).

Mortality was affected by feeding group and those larvae supplemented with UFA had increased deaths compared with controls. In contrast, feeding with certain SFA oils (PK and CB) reduced the mortality in larvae compared with controls, whilst still increasing the crude fat deposition in larvae.

In conclusion, findings support the hypothesis that specific FAs in feed can alter specific FA in the fats deposited in *T. molitor* larvae. Methods for detection of LC-PUFA *de novo* synthesis within *T. molitor* larvae require improvement as it was not possible to determine LC-PUFA presence in the samples tested in these trials. However, the quantity of unknown FAs in samples suggest they are present but uncharacterised using current methods.

Further investigations are required into cellular mechanism for fat metabolism within *T. molitor* larvae, including the apparent ability of n-6 and n-3 UFA to reduce lipid deposition and, further, whether there is any evidence for capacity to desaturate and elongate essential FA to LC-PUFAs ARA, EPA and DHA.

Chapter 5

Effects of feed FA manipulation on gene expression within *T. molitor* larvae

5.1 Introduction

In Chapter 4 it was concluded that dietary FA had an impact on specific FA content of whole *T. molitor* larvae and that the supplementation of UFA in some cases had no affect on increasing overall crude fat contents. These findings reflected the results from trials on other animal species, whereby the dietary FA composition of the feed impacted the FA composition of monogastric animals (Li et al., 2016; J. Kim et al., 2014). FA obtained from the diet has also been associated as having regulatory function on enzymes involved in lipid metabolism in mammals (Menoyo et al., 2003). Dietary PUFA in mammals are thought to affect fat metabolism within the liver, adipose tissue as well other tissues, whereby they down-regulate the expression of fat synthesising genes and up-regulate the expression of genes involved in oxidation controlled at either the transcriptional level or by post-transcriptional mechanisms (Salter & Tarling, 2007). In particular, genes of the adipose tissue in mammals have been shown to respond to n-3 series PUFA, with the exception of stearoyl-CoA desaturase 1 (SCD1), which is regulated by n-6 series PUFA (Raclot et al., 1997). PUFAs have been shown to elicit effects

on gene expression through a number of nuclear receptors including peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR) as well as transcription factors such as sterol regulatory element-binding transcription factor 1c (SREBP-1c) (Sampath & Ntambi, 2005; Salter & Tarling, 2007).

The mechanisms for fat metabolism in *T. molitor* are currently unknown. Part of this lack of knowledge is because the genome of *T. molitor* has yet to be determined, which makes designing working oligonucleotides for gene expression investigations challenging. Interestingly, due to the high conservation of genes associated with fat metabolism found in mammals, birds and fish species, insects are also expected to share at least some similarities with mammals in their fat synthesis and degradation regulation mechanisms. However, being invertebrates, these systems may have significant variation compared to other well characterised species. Examining the abundance of *T. molitor* mRNA may provide an insight into the physiology of an organism (Liang, 2013). The abundance of mRNA in time and space is referred to as the transcriptome (Liang, 2013).

RNA transcriptomics is one of many methods that can be used to better understand organisms and identify phenotypes of interest within a species. Deep RNA-Sequencing (RNA-Seq) technology is a method for high throughput of DNA sequencing methods, which allows sequences to be mapped and transcriptomes to be quantified (Wang et al., 2009). Transcriptome analysis provides information on gene expression as well as alternative splice variants, by using data analysis methods such as filtering, mapping, expression and counting, in addition to identification of novel sequences (Merican et al., 2019).

The feeding trials described in this chapter were a novel investigation of both the effects of dietary fat sources and effects of the increasing supplementation with specifically linseed oil in the feed on performance, the overall FA composition profile, and mRNA expression of genes associated with lipid metabolsim in *T. molitor* species. Two trials were designed to assess impact of dietary fat or linseed

oil supplementation on expression of genes associated with fat metabolism as well as the effect of linseed oil on the transcriptome within *T. molitor* larvae. The first trial assessed the effects of supplementing basal feed (consisiting of WB) with different oils at 5% (w/w), (similar to the trial described in Chapter 4). The oils used were palm, rapeseed, and linseed oils and the linseed oil treated group were then utilised in a deep RNA-Seq investigation and *de novo* tanscriptome assembly and analysis to determine whether and which genes were differentially expressed, within the transcriptome of *T. molitor* larvae, in response to treatment. The second trial assessed the effects of increasing inclusion of linseed oil in the feeds of *T. molitor* larvae (LS dose trial).

Aims

1. To alter the crude fat content and FA profiles within *T. molitor* larvae treated with different oils or with different inclusions of linseed oil supplementation.

2. To assess the effects of fat type or feeding level of linseed oil supplementation on the performance characteristics in *T. molitor* larvae.

3. To assess mRNA expression of genes associated with lipid metabolism within *T. molitor* larvae, supplemented with various oils or different volumes of linseed oil.

4. To gain the first insights into differentially expressed gene transcripts in *T. molitor* larvae transcriptome, after 5% (w/w) linseed oil supplemented feeding, from RNA-Seq investigation output and *de novo* transcriptome assembly analysis.

5. To determine differential gene expression of *T. molitor* larvae supplemented with various oils providing different FA feed contents, using novel transcripts

from differentially expressed genes from *de novo* assembled *T. molitor* transcriptome output.

Hypothesis

It is hypothesised that feed contents of specific types of fatty acid, will result in differential gene expression within *T. molitor*. This will result in up-regulation of lipolytic genes and down-regulation in lipogenic genes. In addition, it is also expected that feeds supplemented with an increasing quantities of linseed oil, as a source of n-3 FA ALA, will result in regulation of expression of genes within *T. molitor* larvae. These genes are expected to be similar to those shown to have increased/decreased expression in a dose-dependent manner in other species.

5.2 Methods

5.2.1 Animal husbandry

Six-week-old *T. molitor* larvae were supplied by CJ WildBird Foods Ltd (Shropshire, United Kingdom) for both trials. Prior to each trial larvae were acclimatised on WB and carrot, the latter was provided as a water source. At, Day 0, larvae were randomly assigned to replicates within different treatment groups. Larvae were fed with treatment feeds corresponding to each individual trial set-up. Feeds and water source were provided ad libitum throughout all larvae trials. Both trials grew larvae over a 14-day period.

5.2.2 Feed preparation

Trial 1 was set up to assess type of fat source supplementation on differential gene expression in *T. molitor* larvae was set up similar to Chapter 4 (fat type trial). Feeds were supplemented with oils at 5% (w/w) in the control feed of WB, which

was ground to 3.0 mm uniform particle size. The control feed was WB whilst the three treatment feeds contained palm oil (P), rapeseed oil (RS) or linseed oil (LS) in order to increase the concentration of lauric as well as myristic acid, oleic acid or ALA, respectively, above that of the control.

Trial 2 was designed to assess the dose-dependent effects of increasing linseed oil on differential gene expression in *T. molitor* larvae utilised LS to provide 3 levels of dose supplementation at 5%, 7.5% and 10% (w/w) into a control feed of WB (0%) (LS dose trial).

Table 5.1: **The formulation of *T. molitor* larval feeds enriched in different types of fats or various doses of linseed oil.** Fat type trial, n=10. ALA-dose trial, n=4.

	Trial 1: fat type				**Trial 2: LS dose**			
Components (g/kg)	C	P	RS	LS	0%	5%	7.5%	10%
Wheatbran	1000	950	950	950	1000	950	925	900
Palm oil	-	50	-	-	-	-	-	-
Rapeseed oil	-	-	50	-	-	-	-	-
Linseed oil	-	-	-	50	-	50	75	100
Total	1000	1000	1000	1000	1000	1000	1000	1000

Feed for both trials were mixed in entirety before the start of trials. Mixing was conducted using a plastic bag, one for each treatment group. The respective oils were added first to their respective bags and WB feed was gradually incorporated and mixed by shaking, until oil was evenly distributed.

5.2.3 Experimental design

Both trials' 1 and 2 were conducted separately. For both trials larvae were acclimatised for 3 days in one container on an *ad libitum* supply of WB and carrot, which provided a water source. On day 0 of the trial, individual larvae were randomly allocated by hand to each replicate box (17.4 × 14.8 × 12 cm). Trial 1 contained four hundred larvae per replicate and each treatment contained 10

replicates. Trial 2 contained three hundred larvae per replicate and each treatment contained 4 replicates. On day 0 of both trials, total biomass (g) of each replicate was recorded and checked for non-significant initial differences between groups before proceeding with the trial. Feed, containing the appropriate treatment, plus carrot, was weighed and placed into each of the replicate boxes. Larvae were photographed and counted on day 0 and replicate boxes were then placed in complete darkness for the 14-day growth period. In contrast to Chapter 3 and 4, only initial and final weights and number of larvae were recorded for both trials. Replacement WB and carrot were weighed and added to replicates on days 4, 7, and 11. On day 14 final weights were recorded for total biomass (g), remaining feed and carrot (g) and number of larvae surviving. Larvae were then transferred into two 50 ml falcon tubes (with a pierced hole in the lid) and killed by immersion in liquid nitrogen. Samples were stored at -80°C until crushing.

5.2.4 Proximate nutritional analysis

Larvae were crushed under liquid nitrogen and three aliquots of each replicate sample were taken by filling three 1.5 ml screw cap microtubes and samples stored at -80°C before analysis. The remaining sample material from each replicate was then oven dried at 100°C this was then used for to assess crude fat content and fatty acid profiles, as described in Chapter 2.

5.2.5 Gene expression analysis

Total RNA was extracted from each replicate sample from both trials and DNase treated and the quantity and quality of the nucleic acid extracted was assessed using a Nano-drop (Chapter 2.4.2). Quantity accepted was >100 ng/μl and a 260 nm/280 nm value close to 2.0 was accepted as good quality. All samples were diluted to 100 ng/μl and run by non-degenerative gel electrophoresis to check

for integrity of *T. molitor* ribosomes 18S and 28S (Chapter 2.4.3). Insects, like other eukaryotes, have four cytoplasmic ribosomal RNAs (rRNA), two large (18S and 28S) and two small 5.8S and 5S (Winnebeck et al., 2010). The two bands observed using non-degenerative gel electrophoresis methods are formed by post-transcriptional folding of mature 18S, which makes up the small ribosomal subunit and the other three rRNAs which mature to form the large ribosomal subunit (Winnebeck et al., 2010).

To ensure that DNase treatment was effective, RNA samples were tested using qRT-PCR, which should result in no amplification due to lack of double strands in single-stranded RNA (Chapter 2.4.6). Following this cDNA was synthesised, and checked (Chapter 2.4.4 and 2.4.6). A primer for *T. molitor* 60S subunit ribosomal protein L27a (TmL27a) was used for the qRT-PCR checks for cDNA synthesis (successful complimentary strand synthesis results in amplification curves and one melt curve peak). TmL27a has previously been shown to be a successful internal control for qRT-PCR experiments in *T. molitor* larvae (D. H. Kim et al., 2013; Jo et al., 2016; "TmCactin plays an important role in Gram-negative and-positive bacterial infection by regulating expression of 7 AMP genes in *TENEBRIO MOLITOR*, author=Jo, Yong Hun and Kim, Yu Jung and Park, Ki Beom and Seong, Jeong Hwan and Kim, Soo Gon and Park, Soyi and Noh, Mi Young and Lee, Yong Seok and Han, Yeon Soo, journal=Scientific Reports, volume=7, pages=46459, year=2017, publisher=Nature Publishing Group", n.d.).

Following cDNA checks, oligonucleotide pairs for genes associated with lipid metabolism were designed for *T. molitor* larvae, utilising NCBI BLAST database (more detailed description Chapter 2.4.5). Genes of interest were fatty acid synthase (FAS), stearoyl-Co-A desaturase-1 (SCD), elongation of very long chain fatty acids 1 (ELOVL1); elongation of very long chain fatty acids 2 (ELOVL2); fatty acid desaturase 1 (FADS1); fatty acid desaturase 2 (FADS2); and sterol regulatory element-binding protein 1c (SREBP-1c).

- *Fasn* gene that encodes FAS is the enzyme involved in the the conversion of malonyl-coA (converted from acetyl co-A provided by the pentose phosphate pathway and glycolysis) to palmitate (16:0).

- *SCD* is involved in *de novo* synthesis of oleate from stearate (18:0 - 18:1) desaturating at delta 9 to form the precursor of the n-9 series FA oleic acid.

- *ELOVL1* is associated with elongation of endogenous or exogenous palmitate to stearate, and then the further SFA elongation (through progressive addition of two carbons) to 24 carbon chain length (Lignocerate). Further to this *ELOVL1* is involved in the elongation of n-9 series oleate (18:1) to 24:1 (nervonate).

- *FADS2* is a gene that encodes delta 6 desaturase enzyme, which is involved in the further desaturation of either *de novo* synthesised or dietary oleate to 18:2n-9 (octarecadienate). Further to this, the desaturase is involved in desaturating dietary n-6 and n-3 essential precursors LA and ALA to 18:3n-6 (γ-linolenic) and 18:4 n-3 (octadecatetraenoic acid) and desaturation of 24:4n-6 and 24:5n-3 to 24:5n-6 and 24:6n-3, respectively.

- *ELOVL2* is associatedwith the elongation 18:2 n-9 (octarecadienate) to 20:2 n-9 (eicosadientate). In addition, *ELOVL2* is involved in the elongation of n-6 series and n-3 series FA. Firstly ELOVL2 elongates 18:3n-6 to 20:3n-6 and 18:4n-3 to 20:4n-3. Then *ELOVL2* elongates AA (20:4 n-6) to 22:4 n-6 (docosatetrenoic) and then 24:4 n-6 (tetracosatetraenoic) adding two carbons each step. Furthermore, *ELOVL2* in two steps adds four carbons from EPA (20:5 n-3) to 24:5 n-3 (tetracosapentaenoic), this is then converted by *FADS2* delta 6 desaturase to 24:6 n-3 and finally partially oxidised to 22:6n-3 (DHA).

- *FADS1* is a gene that encodes delta 5 desaturase, which not only desaturates

20:2 n-9 to 20:3n-9 in the n-9 series, it is also involved in the addition of another double bond into 20:3n-6 to form 20:4n-6 and 20:4n-3 to form 20:5 n-3 in the n-6 and n-3 pathways, respectively.

- *SREBP-1c*- is synthesised as a precursor transmembrane protein associated with the ER, however in order to become an active nuclear transcription factor *SREBP-1c* requires transport from the ER, by SCAP transport protein to the Golgi apparatus (Lehner & Quiroga, 2015). *SREBP-1c* regulates the increased expression of FAS and desaturases delta 5, 6, and 9 (Lehner & Quiroga, 2015).

Primer pairs were tested for successful, one product amplification, using qRT-PCR (Chapter 2.4.6). Working primer pairs were then amplified using PCR (Chapter 2.4.7), run on a non-denaturing gel electrophoresis (Chapter 2.4.3) with an LED fluorescent dye, and sequenced (Chapter 2.4.8), following which a nBLAST of amplicon sequence was conducted (Appendix A). Successful oligonucleotide pairs were used for qRT-PCR on each sample from trial 1 and trial 2, qRT-PCR was run in triplicate per sample and concentrations were determined by the standard curve method (Chapter 2.4.6).

5.2.6 Deep RNA-Seq and *de novo* transcriptome assembly

Sample preparation and RNA-Sequencing

Deep RNA-Seq techniques and data analysis were conducted at Queens Medical Centre, Nottingham, UK by Nadine Holmes, Matthew Carlile and Dr. Daniel Zadik. The investigation compared RNA samples, n=3 from the trial 1, utilising samples from the controls (fed WB) and LS treated larval groups, that contained the highest levels of specific FAs and required a RNA concentration >200 ng/μl as well as the highest nucleic acid content. Controls were selected for top three

highest abundance of LA, whereas LS were selected for highest abundance of ALA in their lipid contents.

RNA concentrations were measured using the Qubit Fluorometer and the Qubit RNA BR Assay Kit (ThermoFisher Scientific; Q10211). RNA integrity (RIN) was assessed using the Agilent Bioanalyzer 2100 and the Agilent RNA 6000 Nano Kit (Agilent; 5067-1529). Due to the unusual ribosomal RNA profiles of these samples, the Bionalyzer thresholds had to be manually adjusted in order to calculate the RIN. Visual comparison of all RNA traces confirmed that all samples had similar profiles and there appeared to be no detectable degradation or DNA contamination in the samples.

The Biomek 4000 Automated Workstation (Beckman Coulter) was used to prepare sequencing libraries. mRNA was purified from 1 μg of total RNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490). Indexed sequencing libraries were then prepared using the NEBNext Ultra Directional RNA Library Preparation Kit for Illumina (NEB; E7420) and NEBNext Multiplex Oligos for Illumina, Index Primers Sets 1 and 2 (NEBNext; E7335 and E7500). Libraries were quantified using the Qubit Fluorometer and the Qubit dsDNA HS Kit (ThermoFisher Scientific; Q32854). Library fragment-length distributions were assessed using the Agilent Bionalyzer 2100 and the Agilent High Sensitivity DNA Kit (Agilent; 5067-4627). Libraries were pooled in equimolar amounts and final cDNA library quantification performed using the KAPA Library Quantification Kit for Illumina (Roche; KK4824). The library pool was sequenced on the Illlumina NextSeq500 on a NextSeq500 High Output 300 cycle kit (Illumina; FC-404-2004), to generate over 20 million pairs of 150-bp paired-end reads per sample.

Bioinformatics: *de novo* assembly of *T. molitor* transcriptome

The following work was conducted by Dr. Daniel Zadik: Adaptor sequence was trimmed off using Skewer (`https://github.com/relipmoc/skewer`). The fol-

lowing settings were used: m pe (paired-end mode), q 20 (quality threshold for end-trimming) and Q 20 (quality threshold for mean-trimming). This generated two fastq files from each paired set of reads representing the 2 reads in each pair. Transcriptomic reads from four publicly available *Tenebrio molitor* studies were downloaded from the SRA: DRX001789, SRX546209, SRX372068 and SRX371958. The trimmed, concatenated reads from both the investigation data and the downloaded projects were then assessed using the FastQC read analysis tool (`http://www.bioinformatics.babraham.ac.uk/projects/fastqc/`). The number and quality of the reads was assessed.

A transcriptome was then assembled from the investigations trimmed reads and the trimmed public reads. This was done using Trinity (`https://github.com/trinityrnaseq/trinityrnaseq/wiki`) with a min_contig_length of 200. Candidate coding regions were identified with Transdecoder (`https://github.com/TransDecoder/TransDecoder/wiki`) using both Blast and Pfam. Trinotate (`https://trinotate.github.io/`) was then used to annotate the the candidate coding regions with predicted functions. A t-and-rRNA-only FASTA file was extracted from the transcriptome assembly. To remove noisy t- and rRNA from the dataset, the investigations trimmed reads (and not the public reads) were then mapped onto this t-and-rRNA-only file using HISAT2 (`https://ccb.jhu.edu/software/hisat2/index.shtml`). These unmapped reads were then mapped against the Transriptome Assembly, using HISAT2. Reads that mapped to multiple positions in the genome and those that were not mapped to correct positions relative to their partner read were then filtered out using AWK (`https://en.wikipedia.org/wiki/AWK`), based on the mapping flags assigned by HISAT2. The number of reads with a mapping quality of >= 20 mapped to each gene from the filtered BAM file were counted using SAMtools view htseq-count (`http://samtools.sourceforge.net/`), with the following settings: q, s reverse, i gene_name and a 20. Reads Per Kilobase of transcript per Million mapped reads (RPKM) files were gener-

ated from the counts file with a bespoke Perl script. The read count files for all samples were merged in to matrix using a bespoke Perl script. Differential Expression analysis was done in R (https://www.r-project.org/) using DESeq2 (`https://bioconductor.org/packages/release/bioc/html/DESeq2.html`).

Transcriptome verification

The following work was conducted by Dr. Zoe Daniels: It is essential for transcriptome profiles generated from RNA-Seq data to be validated due to potential influence by variability resulting from multi-process technical procedures such as RNA extraction, cDNA synthesis and modification for generation of fragments for libraries construction (Fang & Cui, 2011; Wang et al., 2009). Using qRT-PCR techniques, transcript expression can be assessed against a reference gene, with increased number of replicates. Primers were designed for eight differential transcripts and transcripts were verified using qPCR reaction techniques on all trial 1 treatment groups and replicates, n=10.

5.2.7 Statistical testing

Data were analysed using SPSS (version 24 IBM). Continuous response variable data that met assumption of normality and homogeneity of variance, determined using Shapiro-Wilk and Levene tests, respectively, were analysed using one-way analysis of variance (ANOVA). Specific between-group differences in measured variables were determined using Tukey HSD *post-hoc* comparisons. Any continuous data that did not meet the assumptions of normality or homogeneity of variance, were assessed using Kruskal-Wallis one-way analysis of variance, between-group differences were determined by Dunn-Bonferroni *post-hoc* testing. Statistical significance was taken as $P<0.05$. All individual FAs were analysed using ANOVA, with a Bonferroni adjustment ($0.05 \div$ no. of dependent FAs) of the 0.05 alpha level of acceptance, in order to reduce type I errors. Data are presented as

the means ±SD.

Mortality was analysed using a generalised linear model (logistic analysis) conducted in Genstat (version 16 VSN), which assumed binomially-distributed errors. Data on proportions were presented as back transformed means ±SEM.

Outliers were determined from qRT-PCR analysis of trial 1 and 2 samples (diluted $\frac{1}{8}$), if their crossing point (C_P) values were lower than those observed in the averaged neat samples from the standard curve. Due to outlier removal some treatment groups were unequal, in these cases total replicates of the trial were expressed, N and individual treatment group n values were described in the corresponding figure legend. All differences between apparent mRNA expression were determined using ANOVA and all values are displayed in figures as means ±SEM.

5.3 Results

5.3.1 Growth

Similar to findings in Chapter 4, supplementation with different types of oils containing either SFA or UFA, resulted in no effects on performance characteristics of the larvae, with the exception of overall mortality (Table 5.2). All supplemented larvae had significantly greater mortality compared with controls, ($P<0.01$) (Table 5.2). Furthermore, the performance characteristics including biomass weight gain, total feed and carrot consumption were similarly unaffected by linseed oil supplementation dosage over a 14-day period (Table 5.3). There was a trend for different mortalities in larvae treated in trial 2 with varying levels of linseed oil, 0-10%, however the apparent overall death proportions appeared to be higher in trial 2 than those observed in trial 1, which could suggest stocking density effects on mortality.

Table 5.2: **The effect of feed enriched in fats containing saturated fatty acid (SFA) and unsaturated fatty acid (UFA) on the performance and crude fat content of *T. molitor* larvae.** n = 10, 400 starting number of larvae per replicate. All values are presented as means ±SD, except for mortality which is given as back-transformed means ±SEM.

Performance	C[1]	P[2]	RS[3]	LS[4]	*Sig. (P)*
Initial weight (mg/larvae)	47.9±2	47.5±2	46.5±1	47.9±2	NS
End weight (mg/larvae)	100.2±7	105.3±10	103.3±8	103.1±7	NS
Total biomass gain (g)	11.5±2	12.3±2	11.9±2	11.9±2	NS
Total feed intake (mg/larvae)	115.3±1.6	115.9±4.7	115.6±4.2	115.6±2.5	NS
Total carrot intake (mg/larvae)	146±4.9	150.3±9.7	151.4±10	148.9±4.7	NS
Mortality	0.29 ± 0.01^{b}	0.35 ± 0.01^{a}	0.34 ± 0.01^{a}	0.32 ± 0.01^{a}	<0.01
Estimated feed conversion ratio	3.57±0.3	3.32±0.4	3.51±0.5	3.59±0.5	NS
Nutritional composition					
Crude fat (g/kg)	341 ± 24^{b}	375 ± 15^{a}	374 ± 11^{a}	367 ± 14^{ab}	<0.01

[1] *Wheatbran control*
[2] *Palm oil* 5% (w/w)
[3] *Rapeseed oil* 5% (w/w)
[4] *Linseed oil* 5% (w/w)
^{a,b} change in letter indicates significant difference

Table 5.3: **Linseed oil dose-dependent effects on the performance and crude fat content of *T. molitor* larvae.** n = 4, 300 starting number of larvae per replicate. All values are presented as means ±SD, except for mortality which is given as back-transformed means ±SEM.

Performance	0%[1]	5%[2]	7.5%[3]	10%[4]	*Sig. (P)*
Initial weight (mg/larvae)	53.1±1	53.6±1	53.4±2	52.1±1	NS
End weight (mg/larvae)	127.8±13	126.3±4	124.5±7	127.9±8	NS
Total biomass gain (g)	9.9±2	9.9±1	10.0±2	9.3±1	NS
Total feed intake (mg/larvae)	147.3±7.3	144.4±4.5	143.6±6.0	150.1±6.7	NS
Total carrot intake (mg/larvae)	271.9±9.3	268.1±8.1	271.8±11.7	267.0±5.9	NS
Mortality	0.45±0.03	0.44±0.03	0.41±0.03	0.52±0.3	0.074
Estimated feed conversion ration	5.73±0.9	5.68±0.2	5.86±0.3	5.57±0.7	NS
Nutritional composition					
Crude fat (g/kg)	308±6^{b}	358±19^{a}	353±14^{a}	368±11^{a}	<0.001

[1] *Wheatbran control*
[2] *Linseed oil* 5% (w/w)
[3] *Linseed oil* 7.5% (w/w)
[4] *Linseed oil* 10% (w/w)
^{a,b} change in letter indicates significant difference

5.3.2 Crude lipid content

Crude fat composition of *T. molitor* larvae was affected by supplementation with SFA or UFA oils at 5% w/w ($P<0.01$), (Table 5.2). Findings mimicked the results observed in Chapter 4, when chick crumb was supplemented with various SFA or UFA oils. Larvae fed P or RS had significantly greater crude fat contents compared with C, ($P<0.01$, $P<0.05$, respectively). Whereas, no difference was observed between the crude fat contents of LS fed larvae and C groups (Table 5.2). Trial 2 results contradicted these findings and all linseed oil fed larvae had increased crude fat contents compared with C groups ($P<0.001$) (Table 5.3) despite LS larvae having previously shown no significant increases in crude fat content above controls, in Chapter 4 and trial 1. Supplementations of 5%, 7.5% and 10% (w/w) linseed oil resulted higher levels of crude fat above 0% controls ($P<0.001$, $P<0.01$, and $P<0.001$, respectively), (Table 5.3). However, crude fat content was not increased by increasing supplementation of linseed oil above 5%.

5.3.3 FA profiles

Fatty acid composition profiles of *T. molitor* larvae were altered by supplementation of feed with SFA or UFA oils, results are displayed in Table 5.4. As observed in Chapter 4 supplementation with specific oils influenced specific increases in FAs. P fed groups contained the highest levels of 16:0 ($P<0.001$), RS fed larvae had the highest levels of 18:1 (P<0.001), and LS fed groups had the highest abundance of 18:3 (P<0.001), (Table 5.4). Slight differences observed between trial 1 and Chapter 4 are attributed to a change in basal feed supplied to the growing larvae over a 14-day period.

Table 5.4: **The effect of feed enriched in fats containing saturated fatty acids (SFA) and unsaturated fatty acids (UFA) on the fatty acid composition in *T. molitor* larvae.** n = 10 per treatment level, all values are presented as means ±SD in units mol% FAME. Significance level of acceptance, with Bonferroni adjustment was, $P<0.006$.

Fatty acid	C[1]	P[2]	RS[3]	LS[4]	*Sig. (P)*
14:0	3.3 ± 0.2^{b}	3.7 ± 0.2^{a}	3.0 ± 0.1^{c}	3.0 ± 0.2^{c}	<0.001
16:0	18.3 ± 1.1^{b}	20.8 ± 0.5^{a}	16.3 ± 0.9^{c}	16.3 ± 0.6^{c}	<0.001
16:1	1.8 ± 0.1^{a}	1.7 ± 0.1^{a}	1.4 ± 0.2^{b}	1.4 ± 0.1^{b}	<0.001
18:0	2.7±0.1	2.7±0.1	2.6±0.1	2.7±0.1	NS
18:1 (n-9)	39.3 ± 0.2^{b}	39.6 ± 1.0^{b}	42.5 ± 1.8^{a}	35.4 ± 1.0^{c}	<0.001
18:2 (n-6)	25.0 ± 1.4^{a}	23.0 ± 0.8^{b}	23.6 ± 1.2^{a}	22.6 ± 1.0^{b}	<0.001
18:3 (n-3)	1.4 ± 0.2^{bc}	1.1 ± 0.1^{c}	1.8 ± 0.2^{b}	10.3 ± 0.5^{a}	<0.001
Unknown	8.3±2.4	7.4±2.2	8.8±2.4	8.4±2.7	NS

[1] *Wheatbran control*
[2] *Palm oil* 5% (w/w)
[3] *Rapeseed oil* 5% (w/w)
[4] *Linseed oil* 5% (w/w)
[a,b] change in letter indicates significant difference

Increasing dosage of linseed oil supplementation resulted in significantly altered FA composition profiles in larvae (Table 5.5). Levels of 16:0 and 16:1 decreased in a dose-dependent manner, from 0% to 7.5% (w/w) linseed oil inclusion, where 0% contained the highest amounts of both FA compared to all other groups (P<0.001), however there were no further decreases between 7.5% and 10% supplementation (Table 5.5). The content of 16:0 was significantly higher in 5% LS supplemented versus 7.5% (P<0.05) and 10% (P<0.01). Similarly, for 16:1, the FA contents of 5% linseed oil supplemented were higher than 10% (P<0.05) there were no differences between 5% and 7.5% supplementations. Supplementation with linseed oil increased the amount of 18:3 in larvae in a dose-dependent manner, whereby 5% supplementation had significantly higher contents compared to 0% (P<0.001), 7.5% had higher content above 5% (P<0.001) and 10% had the highest ALA content above all other groups (P<0.001), (Table 5.6). The assessment of linseed oil supplementation at 5% showed similar results to those observed in Chapter 4

and in the trial 1 with highly significant increases in 18:3 above control groups (Table 5.4).

Table 5.5: **Dose-dependent effects of linseed oil supplementation on the fatty acid composition in *T. molitor* larvae.** n=4 per treatment level, all oils were supplemented in a basal feed of chick crumb, at 5% (w/w). All values are presented as means ±SD in units mol% FAME. Significance level of acceptance, with Bonferroni adjustment was $P<0.006$.

Fatty acid	0%[1]	5%[2]	7.5%[3]	10%[4]	*Sig. (P)*
14:0	3.0±0.1	2.6±0.2	2.5±0.2	2.5±0.1	NS
16:0	20.2±0.5^a	17.1±0.4^b	15.9±0.3^c	15.0±0.2^c	<0.001
16:1	1.6±0.0^a	1.2±0.1^b	1.2±0.1^{bc}	1.1±0.0^c	<0.001
18:0	2.6±0.1	2.8±0.1	2.7±0.1	2.8±0.0	NS
18:1 (n-9)	36.8±1.0	35.1±1.3	34.1±0.1	34.1±0.1	NS
18:2 (n-6)	27.3±1.0^a	22.5±0.6^b	21.3±0.3^b	21.3±0.1^b	<0.001
18:3 (n-3)	1.7±0.1^d	11.9±0.7^c	14.9±0.8^b	17.1±0.6^a	<0.001
Unknown	6.9±1.1	6.8±1.0	7.3±1.1	6.3±0.5	NS

[1] *Wheatbran control*
[2] *Linseed oil* 5% (w/w)
[3] *Linseed oil* 7.5% (w/w)
[4] *Linseed oil* 10% (w/w)
^{a,b} change in letter indicates significant difference

5.3.4 Gene expression

RNA extractions

RNA extraction from *T. molitor* samples was successful in all samples from both trial 1 and trial 2. Unfortunately, a 5% treated sample from trial 2, was destroyed prior to RNA extraction. Concentration of nucleic acid was high and quality was consistent within and between treatment groups and across both trials, the data obtained from Nanodrop are displayed in Tables 5.6 and 5.7.

qRT-PCR on lipid metabolism genes

House-keeper qRT-PCR showed non-significant differences for all samples tested in trials 1 and 2 (Figure 5.1) and was utilised as an internal control for all primer pairs assessed.

In order to initially test the primer designs, the pooled cDNA samples from trial 1 were used and standard dilutions were made, ranging from neat (1) to $\frac{1}{256}$. These results showed working primer pair designs, and an appropriate dilution concentration for samples was determined. All primer designs tested are displayed in Table 5.8, with only three working primer pairs, individual cDNA samples were diluted to $\frac{1}{8}$ for testing of SCD, ELOVL1 and ELOVL2 primer pairs. No significant differences were observed between treatment groups in trial 1 or 2 for SCD, ELOVL1 or ELOVL2 apparent gene expression (Figures 5.2 and 5.3).

Table 5.6: **Trial 1: concentration of RNA extracted from pooled *T.molitor* in replicate samples (nucleic acid, ng/μl) and quality value of RNA extracted (260 nm/280 nm ratio).**

Treatments:	RNA (ng/μl)	260/280			
C[1]	113.0	2.11			
	*447.0	2.12			
	178.6	2.12			
	253.7	2.12			
	153.9	2.15			
	*479.9	2.13			
	*288.2	2.14			
	210.4	2.13			
	255.0	2.15			
	362.2	2.14	Mean:	**274.2**	**2.13**
P[2]	218.3	2.14			
	361.0	2.12			
	208.2	2.13			
	151.8	2.14			
	343.2	2.13			
	142.8	2.11			
	346.3	2.14			
	322.4	2.15			
	180.0	2.14			
	152.0	2.12	Means:	**242.6**	**2.13**
RS[3]	160.4	2.15			
	255.9	2.15			
	163.3	2.13			
	228.8	2.13			
	325.3	2.16			
	226.0	2.13			
	178.2	2.14			
	320.9	2.14			
	251.6	2.14			
	203.9	2.13	Means:	**231.4**	**2.14**
LS[4]	227.3	2.14			
	339.7	2.11			
	233.7	2.13			
	149.0	2.12			
	*272.1	2.15			
	130.5	2.12			
	*312.3	2.15			
	289.9	2.15			
	215.5	2.15			
	*254.9	2.15	Means:	**242.5**	**2.14**

[1] *Wheatbran control*
[2] *Palm oil* 5% (w/w)
[3] *Rapeseed oil* 5% (w/w)
[4] *Linseed oil* 5% (w/w)
* samples selected for Deep RNA-Seq investigation

Table 5.7: **Trial 2: concentration of RNA extracted from pooled *T.molitor* in replicate samples (nucleic acid, ng/μl) and quality value of RNA extracted (260 nm/280 nm ratio).**

Treatments:	RNA (ng/μl)	260/280			
0% [1]	122.1	2.12			
	190.9	2.14			
	278.5	2.16			
	299.4	2.16	Means:	**222.7**	**2.15**
5% [2]	152.9	2.14			
	-	-			
	292.4	2.17			
	376.8	2.15	Means:	**274.0**	**2.15**
7.5% [3]	330.5	2.15			
	282.5	2.14			
	333.3	2.14			
	225	2.18	Means:	**292.8**	**2.15**
10% [4]	195.6	2.13			
	188.2	2.14			
	347.8	2.16			
	331.7	2.18	Means:	**265.8**	**2.15**

[1] *Wheatbran control*
[2] *Linseed oil* 5% (w/w)
[3] *Linseed oil* 7.5% (w/w)
[4] *Linseed oil* 7.5% (w/w)
- raw sample perished

Table 5.8: **Designed oligonucleotide pairs for genes associated with lipid metabolism within *T. molitor* larvae utilising https://blast.ncbi.nlm.nih.gov/Blast.cgi.**

Gene name	Accession no.	Forward primer *5'-3'*	Reverse primer *5'-3'*	Success
SCD	NM_001193649.1	CGGACGACCGCAAACTG	TAGAGACGTTGCGACTTTTGCA	×
		ACTTTGAACGCCACCTGGTT	CGAAATTCTCAGCTGGATTGATAA	✓
FAS	XM_008202063.2	TGGGTCGCCCACTTACGTA	CTCGGTCACAGCCTTTTGTTG	×
		CGGCAATTGGACGGATTTAC	GGAAGCTTACAGGGTGGTACAAA	×
ELOVL1	MF279188.1	CTAACTACCTTCCAGATGGTGCAA	TCCGTGAAGAGAAGCTGGAACT	✓
ELOVL2	MF279189.1	GTTGCTGGTGGTACTACTTTTCCA	CGCAAAACGAAGAAGATCGTATC	✓
FADS1	XM_961451.3	AGACTGCACAAAAGCCTTCGA	TTTGTACTGTTTGAGCTCTTTTTTCG	×
		TCCAGCAACCCCAAAGAAGA	GCACAGACCGCATGTCAAGT	×
FADS2	XM_969201.3	AGGCTGGGAAAGACGGAAGT	GTTCACGTGCGTCACTGGAA	×
		TCCCAGTCGTCTTCGTCACA	CCGAGAGTGACGGGCAAA	×
SREBP-1c	XM_969102.3	TCCCTTGTCCTTCGCTATGAA	CGGGAGTCGTTAAAGTTTGTAGTG	×
		TCGCTGTGCTGGCAAGTG	CACCGCCCGATCCATAAC	×

[1]

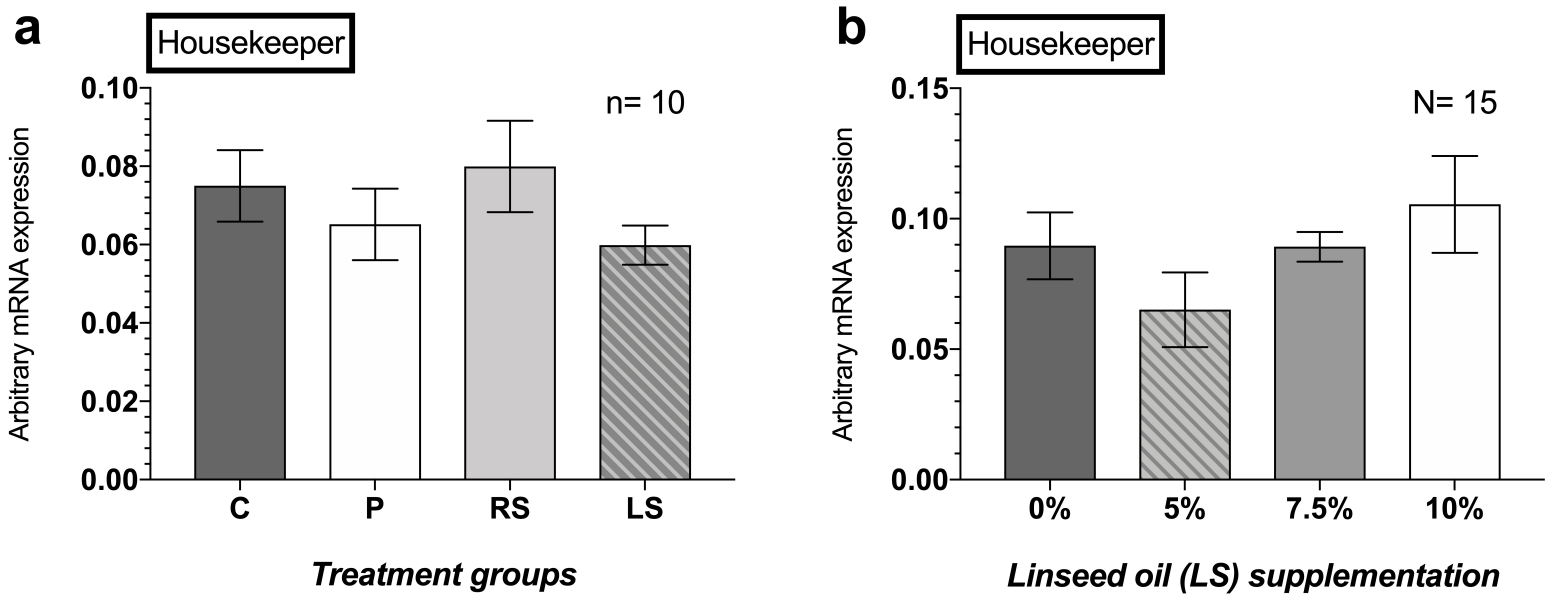


Figure 5.1: **The assessment of potential housekeeper *T. molitor*, TmL27a, a 60 S ribosomal protein for use in gene expression analysis.** All larvae were grown over a 14-day period. [**a**] Four feeding groups were assessed: *C*: wheat bran control; *P*: 5% palm oil; *RS*: 5% rapeseed oil; *LS*: 5% linseed oil. [**b**] Four feeding groups were assessed: *C*: wheat bran control (n=4); *5%*: 5% linseed oil (n=3); *7.5%*: 7.5% linseed oil (n=4); *10%*: 10% linseed oil (n=4). Values are expressed as mRNA expression arbituary unit per weight equivalent total RNA.

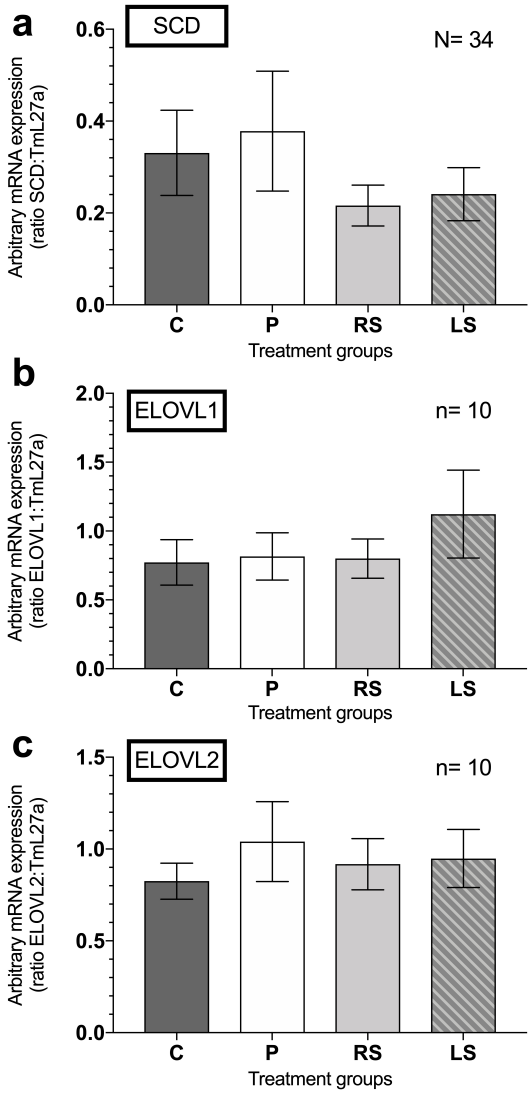


Figure 5.2: **The effect of specific fat supplementation on *T. molitor* larvae apparent expression in genes associated with lipid metabolism.** Larvae were grown over a 14 days period on wheatbran basal feed with various oils added at 5%: control (C), palm oil (P), rapeseed oil (RS), and linseed oil (LS). Apparent expression of 3 genes were assessed [**a**] *SCD*, [**b**] *ELOVL1* and [**c**] *ELOVL2*. Due to outliers being removed SCD, N= 34, where C: n=9, P: n=8, RS: n=9, LS: n=8. Values are expressed as ratio of ng total RNA equivalent used for first strand synthesis SCD/ELOVL1/ELOVL2 mRNA to TmL27a mRNA (internal standard).

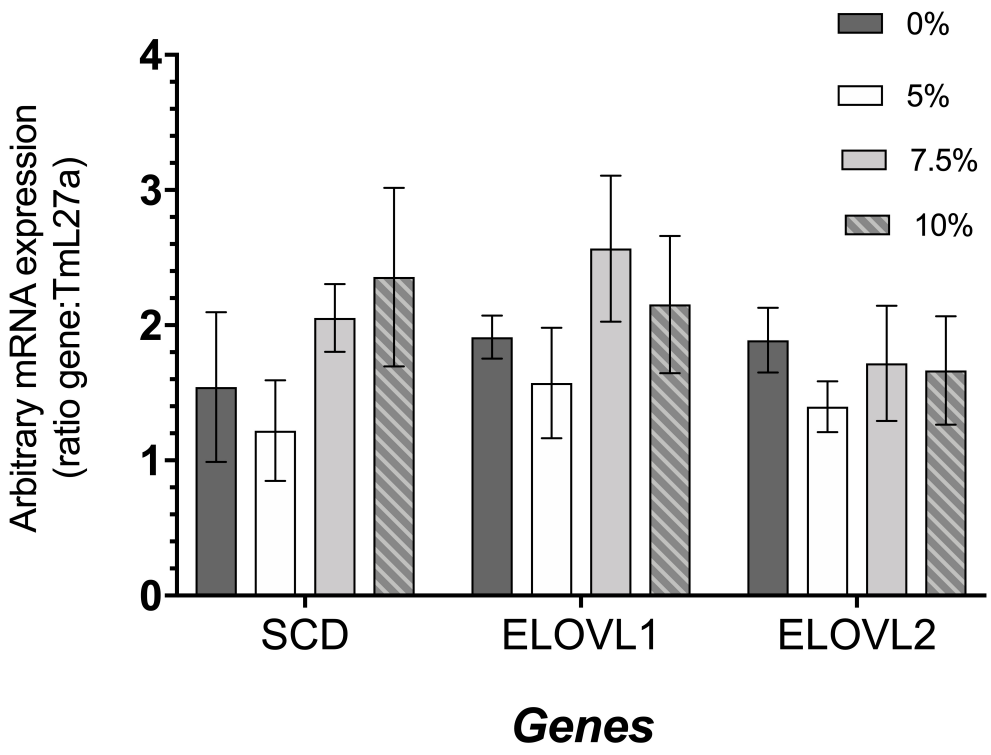


Figure 5.3: **The dose-dependent effects of linseed oil supplementation on *T. molitor* larvae apparent expression in genes associated with lipid metabolism.** Larvae were grown for 14 days on a wheat bran basal feed supplemented with linseed oil (LS) at 0, 5, 7.5 and 10%. Larvae were then killed and assessed for apparent expression of *SCD*, n=3, 3, 3, 2, respectively; *ELOVL1*, n= 4, 4, 3, 4, respectively; *ELOVL2*, n= 4, 4, 3, 4, respectively. Values are expressed as ratio of ng total RNA equivalent used for first strand synthesis SCD/ELOVL1/ELOVL2 mRNA to TmL27a mRNA (internal standard).

Deep RNA-Seq investigation using *de novo* transcriptome assembly

From trial 1, six samples were selected based on highest levels of specific FAs, with >200 ng/μl RNA concentration. For C groups n=3 contained an 18:2 (LA) abundance of 26.4 mol%/FAME (RNA: 447.0 ng/μl); 26.4 mol%/FAME (RNA: 288.2 ng/μl); 26.2 mol%/FAME (RNA: 479.9 ng/μl), (Table 5.6). For LS groups n=3 contained an 18:3 (ALA) abundance of 10.9 mol%/FAME (RNA: 312.3 ng/μl); 10.8 mol%/FAME (RNA: 272.1 ng/μl); 10.4 mol%/FAME (RNA: 254.9 ng/μl), (Table 5.6). Outputs from transcriptome work are displayed in Table 5.9.

Table 5.9: **Outputs for RNA-Seq and *de novo* transcriptome assembly of *T. molitor* larvae.**

Output	**Bases**	**Reads**	**Longest seq length**	**Shortest seq length**	**Mean seq length**	**N50**
Reads from trial	75,010245,779	521,838,576	151	81	143.7	150
Public reads	23,819,639,731	245,705,541	639	18	96.94	100

Output	**Bases**	**Contigs**	**Longest seq length**	**Shortest seq length**	**Mean seq length**	**N50**
Trinity assembly	310,389,985	373,984	28,646	201	830	1,637

Output	**Amino acids**	**Peptides**	**Longest seq length**	**Shortest seq length**	**Mean seq length**	**N50**
Transdecoder predictions	35,548,937	115,770	9,468	85	307.1	417

Analysis of *de novo* assembled transcriptome sequences, showed that feeding *T. molitor* larvae with a feed containing 5% (w/w) linseed oil resulted in significant differences in their transcriptomes' relative to larvae fed on control feed WB. There were, 41 gene transcripts which had a >2-fold differential (adjusted $P<0.05$) in concentration compared to the control, with there being 21 up- regulated and 20 down-regulated genes relative to the control (Table 5.10). Of these top genes, RPKM data values for individual samples (n=3) were obtained for 14 of the differentially expressed genes, these values have been displayed in a heatmap (Figure 5.4).

Further nBLAST investigation against NCBIs Nucleotide collection (nr/nt) database located annotated coleopteran gene analogues for 8 of the differentially expressed genes from the *de novo* assembled *T. molitor* genes transcripts, including annotations for *T. molitor* genes. Furthermore, at least some of these genes can be attributed as having a function in lipolysis and lipogenesis (Table 5.11). Supplementation with linseed oil resulted in up-regulation of genes associated with lipid oxidation and down-regulation of genes involved in lipid synthesis. One of the genes found to have been down-regulated by linseed oil supplementation was a potential melanisaton-related protein and the specific *T. molitor* protein (UniProt: Q9NDN7) has previously been associated with lipid transporter activity. Genes found to be up-regulated within the larvae as a result of linseed oil supplementation were: acetyl-coA - 11 desaturase, carboxylesterase and a forkhead domain containing protein. Acetyl-coA -11 desaturase in the Argyrotaenia velutinana (UniProt: Q95VE7) has been associated with involvement in lipid metabolic processes in particular FA biosynthesis processes via oxidoreductase activity. The gene has a histidine box domain which could contain an active site and/or be involved in metal binding of its cofactor iron (Fe). Carboxylesterase (gene: Ces1d) in mice (UniProt: Q8VCT4) is a major lipase within white adipose tissue. Its associated function is as a carboxylic ester hydrolase; TAG lipase; and

fatty acyl/ethyl/ester synthase. The enzyme has been associated with biological processes such as lipid catabolism including acylglycerol, VLDL lipoprotein particle assembly, cholesterol biosynthesis and the acetyl coA metabolic process, with fatty acyl coenzyme A products being a rate-limiting step in β-oxidation of FA (Jump, 2002b).

Table 5.10: **Differential 2-fold change expression results obtained from RNA-Seq output in *T. molitor* larvae, treated with 5% linseed oil over wheatbran controls, fed over a 14-day period.** n=3, all genes displayed in table exhibited a minimum significance value of $P<0.05$.

Down-regulation			**Up-regulation**		
Transcript ID	Log[1]	Adj.[2]*P.*	Transcript ID	Log	Adj.*P.*
DN49678*	-5.5	<0.001	DN48154	2.1	<0.05
DN45560β	-4.7	<0.001	**DN45607**β	2.4	<0.001
DN44582	-4.6	<0.01	DN45778Δ β	2.4	<0.05
DN44801	-4.5	<0.001	DN53206β	2.6	<0.05
DN42680	-4.3	<0.01	**DN53206**	2.7	<0.01
DN56618	-3.9	<0.05	DN46668Δ	2.8	<0.05
DN43747	-3.9	<0.05	DN41307β	3.0	<0.05
DN43918	-3.6	<0.01	DN46439β	3.1	<0.05
DN42963	-3.6	<0.01	DN52375Δ	3.1	<0.05
DN46899	-3.5	<0.05	DN49246	3.2	<0.05
DN40015	-3.4	<0.05	DN43059Δ	3.3	<0.05
DN46811	-3.4	<0.05	DN44492	3.6	<0.05
DN53813	-3.3	<0.05	DN75057	3.7	<0.05
DN53256	-3.2	<0.01	DN44021	3.9	<0.05
DN43826Δ	-3.2	<0.05	DN42755Δ	4.1	<0.05
DN49225	-3.1	<0.05	DN6298Δ	4.1	<0.05
DN52346Δ	-2.7	<0.05	**DN54771**Δ	4.1	<0.001
DN46437β	-2.0	<0.01	DN42694Δ	4.2	<0.05
DN54019Δ	-1.9	<0.05	DN47078Δ	4.2	<0.05
DN46769β	-1.8	<0.01	DN54646(g4)Δ	4.2	<0.05
			DN54646(g6)*Δ	5.5	<0.001

[1] *Loglinear change in gene expression*
[2] *Adjusted*
Bold *genes randomly selected for qRT-PCR testing of Trial 1 samples.*
* *Failed oligonucleotide pair design for qRT-PCR analysis of whole trial.*
β *Sequences where analogues of genes have been located using NCBI nblast.*
Δ *The genes that were located in RPKM value lists and were further visualised in a heatmap.*

Transcript ID	Control 1	Control 2	Control 3	LS1	LS2	LS3
DN54646(g6)	0	0	0.0103	0.6180	0.2814	2.8946
DN54771	0.4243	0.6843	0.4799	6.7168	6.9025	23.3051
DN54646(g4)	0	0	0	0.2806	0.2811	1.0468
DN45778	0.9292	3.4345	2.6276	12.9584	11.4807	13.7900
DN47078	0	0	0	0.5290	0.1806	1.6442
DN42694	0.1105	0.3340	0	8.1178	0.2274	30.2291
DN42755	0	0	0	0.3666	0.1669	1.3676
DN46668	7.6350	10.1791	2.5072	4.8735	8.6218	10.5035
DN52346	64.3371	19.8461	29.1615	12.0582	90.1319	3.5149
DN54019	4.3349	8.2029	10.2365	1.9778	1.2528	1.0692
DN6298	0	0	0	0.4743	0.1993	1.4518
DN43059	0.2399	0.4030	0.1809	0.8136	6.0915	4.7210
DN52375	0.4402	0.0887	0.1992	2.4887	1.4503	2.6406
DN43826	48.1005	50.1697	9.0963	3.7746	6.5616	0.7111

Figure 5.4: **The effect of feed supplemented with 5% linseed oil in wheat-bran control, on the RPKM values of differentially expressed transcripts in *T. molitor* larvae with a 2-fold change in expression.** Results generated by RNA-Seq output and *de novo* assembled transcriptome analysis and normalisation of transcript reads. Transcripts are ordered by P value according to RNA-Seq analysis of LS versus control, with most significant differentials listed first. Transcript ID names in '*Italic*' are representative of transcripts for which oligonucleotide pairs were successfully designed and tested during verification of transcripts on all Trial 1 data, from which RNA-Seq samples (n=3) were sourced.

Table 5.11: **Gene analogues for *T. molitor* transcripts regulated by 5% linseed oil supplementation in wheatbran.** Listed in order for differential, 2-fold expression of genes from *de novo* trasnscriptome sequence assembly using Trinity. Gene analogue accession numbers, descriptions and identities found using NCBI *nBLAST* database.

Transcript ID	Accession no. (top blast hit)	Species (top blast hit)	Description	Identity (%)	Log change	LS oil regulation
DN45560	DQ356022.1	*T. molitor*	Putative serine proteinase	99	-4.7	↓
DN46437	XM_015982302.1	*T. castaneum*	Myosin heavy chain 1	92	-2.0	↓
DN46769	AB037697.1	*T. molitor*	Melanisation-related protein	96	-1.8	↓
DN45607	KP859367.1	*T. molitor*	Carboxylesterase	89	2.4	↑
DN45778	XM_008200752.2	*T. castaneum*	Fork-head domain containing protein	84	2.4	↑
DN53206	XM_020016030.1	*A. tumida/T. castaneum*	Acyl Co-A delta(11) desaturase/delta(12) desaturase	76	2.6	↑
DN41307	JX888167.1	*T. molitor*	Cadherin 1	89	3.0	↑
DN46439	KP859367.1	*T. molitor*	Carboxylesterase	91	3.1	↑

Verification of transcript expression

From the 41 gene transcripts shown to have differential expression as a result of LS feeding, four up-regulated transcripts and four down-regulated transcripts were randomly selected. Primer designs were carried out by Dr Zoe Daniel, using Primer Express software (Thermo Fisher Scientific). The genes selected for primer design are shown in Table 5.12. All genes selected exhibited a 2-fold differential expression with an adjusted alpha level of $P<0.01$.

Analysis of RNA-Seq generated transcripts using qRT-PCR methods on all trial 1 samples was conducted by Dr. Zoe Daniel and showed no differential expression between treatment groups, with the exception of DN53206 (Figure 5.5). Supplementation of feed with 5% oils resulted in an apparent increase in relative mRNA expression of the DN53206 gene, ($P<0.01$), which from nBLAST results suggests that crude fat supplementation results in up-regulation of *T. molitor* acyl-coA δ desaturase gene (Figure 5.5).

Table 5.12: **Oligonucleotide pairs designed using significantly different gene transcripts obtained from RNA-Seq analysis of *T. molitor* larvae fed 5% linseed oil in a basal control feed of wheatbran.** Gene names in *italics* represent oligonucleotide designed sets that failed qRT-PCR testing. Amplicon lengths are displayed as a prediction from Primer Express software: PCR products are yet to be sequenced.

Transcript ID	Forward primer *5'-3'*	Reverse primer *5'-3'*	Predicted amplicon length (*bp*)
DN54646(g6)	CGGACCTCTCCAGAAGGTAATTT	GCGGGTGCTGTAGCGAAA	74
DN54771	AGCTCGGCATTGCTCTAAGTG	AAATCTGAACACAAATCCGCTATACA	145
DN53206	TCTCCGCAGGATTGATAAACCT	GCCACCTGGTTGGTGAACTC	76
DN45607	CAACTGCCACTCTTTTTCAGTCA	CGGTTTTGATCCTTTTTCAACAA	78
DN42963	GCAAACAAACACCATGTACAAAAAC	TCTGCATCATCGATCAAATCAGA	96
DN44801	CGGGATTCGCATATTTCAAAG	TGGACTCTTGGAGCGAGATAGAT	69
DN45560	GCGCGATCAAACTGAACTTG	GGGCGTGTCATGGAGACTCT	69
DN49678	GGGCGTTGCAAGTTTTATTAGC	ACTGCAGCTGTAACATTATTAATCCAA	108

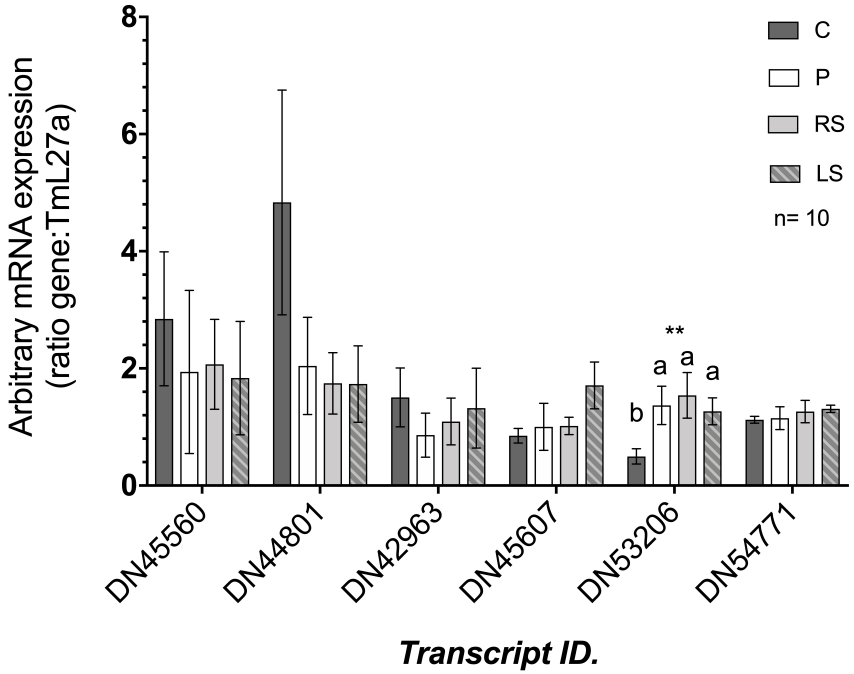


Figure 5.5: **Effects of specific FA supplementation on gene expression, using primers designed from differentially expressed *de novo* assembled *T. molitor* larval transcripts.** Treatment groups: *C*: wheat bran control; *P*: 5% palm oil; *RS*: 5% rapeseed oil; *LS*: 5% linseed oil. Larvae were grown over 14 days. Primers were designed from RNAseq analysis of n=3; C and LS groups (selected for highest quantity of RNA and highest levels of 18:2 or 18:3 fatty acids, respectively) transcriptome assembly counts that were first normalised for RPKM. Values are expressed as ratio of ng total RNA equivalent used for first strand synthesis transcript mRNA to TmL27a mRNA (internal standard).Analysis was conducted using ANOVA, '**'<0.01 and Tukey HSD *post-hoc* testing; *'a, b'*, where a change in letter represents a significant difference.

5.4 Discussion

5.4.1 Growth characteristics

Trial 1 was a hybrid trial design based on a combination of dietary oil sources from the SFA and UFA trials featured in Chapter 4. The combination of both SFA source palm oil and UFA sources rapeseed and linseed oils were just one alteration in the trial design. In contrast to Chapter 4, trial 1 utilised WB as a basal feed ingredient instead of chick crumb. The use of WB as a basal feed was to provide further support for the viability of *T. molitor* production as an alternative animal feed ingredient.

Phenotypic data for growth and feed/carrot intakes were found to be unaffected by feeding groups, including RS fed larvae that were previously found to consume increased feed in Chapter 4. The only phenotypic characteristic that was affected by the type of oil supplementation in WB was the overall mortality in replicates. Feeding larvae RS and LS had previously been shown to increase mortality compared to controls, however P fed larvae in Chapter 4 exhibited the same mortality as controls. Differences observed between this Chapter and Chapter 4 could be a result of WB basal feed switch from chick crumb.

Similar results were obtained from trial 2, in regard to phenotypic characteristics that were found to be unaffected by the level of ALA content in feeds. In addition, mortality was also unaffected in larvae supplemented at 5% with linseed oil, which contrasts with results for LS fed larvae in trial 1. Further to this, it has been found that increasing levels of ALA content in the feed of *T. molitor* larvae had no effect on overall mortality. These apparent differences could stem from uncontrolled ambient or husbandry factors in growth rooms. Environmental effects on the phenotypic data for *T. molitor* require further evaluation and control in order to improve repeatability of results. The apparent differences between the separate trial results could highlight an effect of stocking density on mortality

in *T. molitor* larvae: larvae for the two trials had a different number of starting individuals, trial 1 started with 400, whereas 'trial 2 started with 300 individuals.

5.4.2 Crude lipid and FA compositions

Apparent differences between trials 1 and 2 were also observed in crude fat content in the larvae fed on 5% linseed oil. Trial 1's findings appear to support those in Chapter 4, irrespective of basal feed ingredient. Larvae fed P and RS had increased crude fat content compared to C groups, whereas LS fed larvae contained the same amounts compared to all feeding groups. Trial 2's results however, contradict these findings for crude fat content of larvae fed at 5% linseed oil supplementation and crude fat was increased in these feeding groups above the control. However, interestingly levels of linseed oil supplementation in trial 2 had no further effects on increasing total lipid content of larvae.

The FA profiles of larvae in trial 1 reflected the majority of observations seen in Chapter 4 trials that also utilised P, RS and LS feeding groups and are discussed in Chapter 4. Differences observed are thought to be a result of the different FA contents of the basal feed ingredients. Results of this Chapter may however, further support the use of WB rather than chick crumb for the production of *T. molitor* larvae. Controls fed WB, contained similar amounts of ALA to RS fed larvae, whereas controls fed chick crumb in Chapter 4 contained reduced amounts compared to RS fed larvae. WB feed could therefore provide a more appropriate feed not only in terms of sustainability, but also for maximum promotion of ALA in growing larvae.

Trial 2 resulted in some dose-dependent effects of linseed oil supplementation on the relative abundance of certain FAs. As linseed oil supplementation increased from 0% to 10%, there was a reduced abundance of 16:0 and 16:1 in larvae. Linseed oil supplementation at 5% and 7.5% led to decreased 16:0 compared with controls, there were no further decreases in 16:0 abundance between 7.5 and 10%. Similarly,

5% supplementation resulted in reduced abundance of 16:1 to controls, this was further reduced at 10%, however 7.5% was not different from 5 or 10%. Increasing levels of supplementation of linseed oil resulted in increased ALA abundance in larvae in a dose-dependent manner, with 10% (w/w) supplemented larvae having the highest content and 0% controls having the lowest.

The LA content of larvae was affected by supplementation with linseed oil, but not in a dose-dependent manner. Despite WB containing LA as its second most abundant FA after 18:1, it was progressively replaced with linseed oil, which has around 20% LA and 20% 18:1, as well as its high ALA content (>50%) (Orsavova et al., 2015). Therefore, progressive replacement of WB with increasing levels of linseed oil, results in the LA lost in WB being replaced by the progressive oil supplementation. In contrast, the dose-dependent effects observed in increasing ALA contents with increasing levels of WB supplementation with linseed oil, is due to WB's low ALA content.

There was no specific evidence of LC-PUFA within the larvae. However, in recent trials with *T. molitor* larvae, LC-PUFA have been reported when feeding WB and cabbage water source, although these were not ARA, EPA, DPA or DHA (T. Zheng et al., 2017). FA 20:1, 20:2, and 22:1 were only present in small amounts (<1% of total FA each), which could highlight that current GC methods for FA detection require further precision for LC-PUFA detection (T. Zheng et al., 2017). The amount of unknown FA further highlights that detection methods could be improved in future investigations.

5.4.3 Novel gene expression work conducted in *T. molitor*

Feed supplemented with various oils as in Chapter 4 had significant impacts on specific alterations in the FA profiles of larvae. Types and quanitiy of specific FAs are thought to regulate the expression of enzymes involved in lipid metabolism pathways, via activation or interaction with transcription factors such as, peroxi-

some proliferator-activated receptor alpha (PPARα) (Beck et al., 1992; Keller et al., 1993) and sterol response element-binding protein-1c (SREBP-1c) (Brown & Goldstein, 1997).

Working oligonucleotides for SCD, ELOVL1 and ELOVL2 were tested on trial 1 and 2 samples, as key enzymes known to be involved in mammalian lipogenesis and biosynthesis of LC-PUFAs. SCD is involved in the desaturation of stearic acid to oleic acid, through insertion of a carbon-carbon double bond at position delta-9, endogenously synthesising the n-9 family of FAs. ELOVL1 is involved in the elongation (addition of two carbons to the hydrocarbon chain) of palmitic acid to form stearic acid and through further elongation steps forms long chain SFA 24:0 lignocerate. ELOVL2 in contrast, is involved in the elongation of LC-PUFA precursors LA and ALA to ARA and EPA that are required for the synthesis of eicosanoids, including prostaglandins. EPA is further elongated to 22:5 n-3 docosapentaenoic acid (DPA) and then n-3 24:5 tetracosapentaenoic acid, which undergoes further desaturation and partial β-oxidation to form DHA, which is required for incorporation into docosanoids (Wolfe, 1982).

Normalised qRT-PCR reactions found no significant differences between treatment groups of varying SFA, MUFA or PUFA supplementations or differences in expression as a result of varying inclusion of linseed oil in the feeds of larvae. These results were different to what was expected.

SCD as a lipogenic enzyme, is thought to be down-regulated by PUFA and was expected to be down-regulated in larvae as a result of ALA dietary supplementation (Igarashi et al., 2007; Duran-Montgé et al., 2009). ALA has previously been shown to suppress SCD activity (Clarke & Jump, 1993) and both lean and obese rats fed high PUFA diets had a 75% decrease in SCD1 mRNA expression (Jones et al., 1996). N-6 PUFA have also been shown to reduce SCD activity in the 3T3-L1 adipocyte cell line, ARA decreased SCD1 mRNA expression in a dose-dependent manner (80% maximum repression), as did LA, ALA, and EPA

(Ntambi, 1999). De Tonnac et al. (2016), showed high dietary ALA (provided by extruded linseed) versus high oleic acid (provided by RS) in pigs fed *ad libitum*, had no effects on expression of *de novo* lipogenesis genes, including FAS. Although De Tonnac et al. (2016), did not test SCD expression both FAS and SCD are said to be linked to SREBP-1c activation and FAS expression was unaltered by feeding groups. These observations are supported in *T. molitor* larvae with no differences in SCD expression observed between RS and LS treated groups. Furthermore, it was thought that high MUFA-containing diets fed to larvae in RS treated groups, would negatively regulate their biosynthesis in a similar way to PUFA, through reduced expression of the delta-9 desaturase (SCD) required for their endogenous synthesis. Interestingly, both PUFAs and oleic acid have been shown to reduce the expression of promoters with sterol regulatory elements by inhibiting the proteolytic maturation of SREBP (Worgall et al., 1998; Thewke et al., 1998).

Contrastingly SFA are thought to up-regulate *de novo* lipogenesis and as a result, larvae supplemented with palm oil had increased palmitic acid, which was expected to increase the expression of ELOVL1 for its *de novo* conversion to stearic acid and further elongation activity to long chain SFA. In addition, palm oil supplemented larvae were expected to have increased SCD gene expression for increased endogenous production of MUFA 18:1 (Raz et al., 1998; Garg et al., 1988; Landau et al., 1997). Activation of SCD by SFA is thought to be the result of liver X receptor (LXR) controlling SREBP-1c, which then enters the nucleus and effects transcription of certain genes (Ntambi, 1999). A study in rats, showed ELOVL1 to be up-regulated by SREBP-1c, same as lipogenic genes (Moon et al., 2001). This supports the prediction that P treated larvae were expected to have higher levels of ELOVL1 expression compared with other treatment groups and that larvae treated with LS would have resulted in significantly decreased ELOVL1 gene expression.

High levels of PUFA in the diet of rats have been shown to down-regulate

lipogenic enzymes and enzymes responsible for LC-PUFA synthesis, such as delta-6 desaturase (Tu et al., 2010). Therefore, linseed oil supplementation was expected to have a down-regulatory effect on SCD, ELOVL1 and potentially even ELOVL2 expression, particularly in trial 2 with increasing levels of linseed oil inclusion up to 10% (w/w). Reducing the LA:ALA ratio with use of linseed oil over 4 diets in rats, from a value of 7.4 to 0.4, resulted in no differences in hepatic gene expression or FADS1, FADS2, ELOVL2 or ELOVL5 genes (Tu et al., 2010). In contrast, high versus low PUFA did show effects in FADS2 and ELOVL2 mRNA expression in rats, which contrasted results in *T. molitor* larvae of trial 2 where no differences were observed between control and linseed oil supplemented groups (Tu et al., 2010). Both FADS2 and ELOVL2 were significantly higher in the low PUFA diets, which interestingly had twice the total MUFA contents compared with the high PUFA diet (Tu et al., 2010). These observations contrast results observed in trial 1 *T. molitor* larvae, where no difference was found in ELOVL2 between RS and LS treated groups.

In results observed the lack of significance could be down to lack of effect of dietary oils on gene expression, which supports J. M. Lee et al. (2016) who suggests that dietary inclusion of FA has more of an effect on FA composition over gene regulated control. However, another reason for lack of significance could be a large in-treatment variability, resulting in lack of significance between treatment groups. This is optimistic for future trials aiming to select for individuals with higher expression of particular genes involved in lipid metabolism, however the resulting lack of significance in this trial is misleading if this is the case. An increased number of replicates, may enable significant results to be achieved. A further limitation for this work was the difficulty in designing working primers for mammalian analogue enzymes, thought to be involved in insect fat metabolism, due to lack of a reference genome. With the complete *de novo* assembled transcriptome it may now be possible to redesign primers for FAS, FADS1, FADS2

and SREBP-1c as well as other transcription factors that may be used to infer regulation for lipid biosynthesis within *T. molitor*.

5.4.4 Novel assessment of the whole *T. molitor de novo* assembled transcriptome

The determination of 41 gene transcripts that were differentially expressed by *de novo* transcriptome analysis, as a result of 5% linseed oil supplementation in *T. molitor*, is a novel finding. Furthermore, the determination of 8 annotated gene analogues in other coleopteran species gives an insight into function of these transcripts that appear to be regulated by dietary linseed oil supplementation.

Down-regulated genes that were characterised included a *T. molitor* transcript for putative serine protease, and melanisation-related protein. Insects use melanins for exoskeletal pigmentation, cuticular hardening, wound healing and innate immune responses (Sugumaran & Barek, 2016). Reduced expression in linseed oil treated larvae may explain increased mortality as a result of reduced gene activity for sclerotisation, which is a process for the hardening of the exoskeleton and critical for larvae survival (Sugumaran & Barek, 2016). Serine proteases are known to dominate the larval gut of Lepidoptera and contribute to 95% of total digestive activity (Srinivasan et al., 2006). It has been suggested that serine proteases in larvae of Lepidotera undergo modulation throughout larvae development, in order to achieve optimal digestion of protein required for normal growth and development (Srinivasan et al., 2006). A mechanism for linseed oil regulation is unknown.

The nBLAST results suggest the identification of two carboxylestase transcripts in *T. molitor* that are up-regulated by linseed oil supplementation. These both matched a previously annotated *T. molitor* carboxylesterase, available on the NCBI database, (accession no. KP859367.1). Both transcripts had 89% identities with this transcript, however DN45607 and DN46439 transcripts are not identical,

as can be seen from their alignment (Figure 5.6). Mammalian carboxylesterases belong to a multigene superfamily encoding enzymes that catalyse hydrolysis of ester- and thiester- bond containing endogenous compounds, including lipids as well as amide-bond containing endogenous compounds (Lian et al., 2017). Genes encoding six human carboxylesterases and twenty mouse carboxylesterases have been classified and these enzymes are said to have an important physiological function in lipid metabolism and energy homeostasis (Lian et al., 2017). Interestingly, it has previously been suggested that carboxylesterase have been highly conserved during evolution, which is reflected in intra- and interspecies similarities (Williams et al., 2010). Humans have been found to share 39-46% amino acid sequence identity and mouse Ces1d and human CES1 proteins share 78% identity and 88% similarity at the amino acid level (Holmes et al., 2010; Lian et al., 2017). Furthermore, mice Ces1d and Ces1g share 76% amino acid sequence identity. However, the α-helix 1 domains are distinct, and the two enzymes exhibit very different biological functions (Lian et al., 2017). Ces1g is thought to exhibit beneficial effects on lipid and carbohydrate metabolism when their activities are increased (Lian et al., 2017). In contrast, Ces1d inhibitors to high-fat diet fed mice or db/db mice were protected from weight gain, reduced plasma lipids, ameliorated liver steatosis, and improved glucose tolerance (Dominguez et al., 2014). Transcripts DN45607 and DN46439 are likely similar in function to Ces1g and Ces2c mouse genes, as both genes have specificity for PUFA-containing TAG (Ko et al., 2009). Furthermore, Ces2c is thought to release FA by hydrolysis from TAG, increase FA β-oxidation and inhibit SREBP-1c, decreasing *de novo* lipogenesis (Lian et al., 2016).

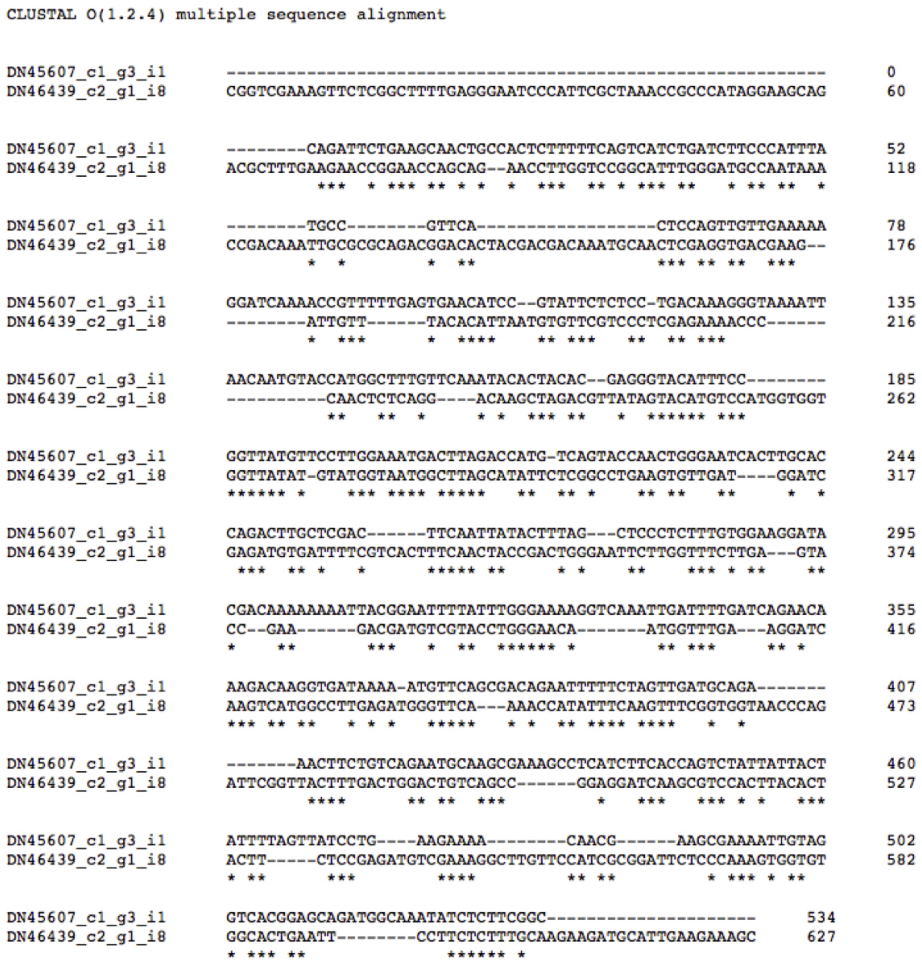


Figure 5.6: **Clustal omega alignment of two *T. molitor* gene transcripts found to be analogous to a gene encoding carboxylesterase isoenzyme in nBLAST Coleopteran RNA database search.** Image generated using `https://www.ebi.ac.uk/Tools/msa/clustalo/` software.

The fork-head domain containing protein was also found to be up-regulated as a result of linseed oil supplementation. Fork-head box transcription factor

FoxA2 has been associated with oxidation of FAs and has been shown to increase β-oxidation in FAs (Bose et al., 2014). FoxA2 has previously been identified as a potential regulator for gene expression in cattle supplemented with n-3 PUFA in their feeds (Waters et al., 2014). *T. molitor* gene transcript DN45778 could be an analogue for FoxA2 transcription factor, and PUFA n-3 could mediate its regulation of gene expression.

Another up-regulated transcript was found to be analogous to cadherin 1 gene in *T. molitor* species. E-cadherin is one of the most important molecules in cell to cell adhesion in epithelial tissues (Gumbiner, 1996). Suppression of E-cadherin is associated with disruption to this adhesion, which has correlated with increased invasiveness and metastasis of tumors (Vleminckx et al., 1991). PUFA has previously been described in this thesis as beneficial to human health and its positive impacts on disease, including some cancers. Up-regulation of a tumour suppressor gene through increased linseed oil in *T. molitor* is of interest and could suggest a potential ALA-mediated effect on disease repression.

Linseed supplementation also resulted in increased expression in a desaturase, which contradicts literature that suggest PUFA reduces the expression of SCD (delta-9 desaturase), FADS1 (delta-6 desaturase), and FADS2 (delta-5 desaturase) genes (Tu et al., 2010). nBLAST results suggested that *T. molitor* have the capacity to unsaturate FA at either position 11 or 12, capacity for which is not present in mammalian species. In insects, delta-11 desaturase is associated with sex pheromone production in adult Lepidoptera (Knipple et al., 2002). However, it seems unusual that *T. molitor* would produce this desaturase in the larval stage of their development cycle.

The delta-12 desaturase gene, was first identified in *Arabidopsis thaliana* (Okuley et al., 1994), and in a fungus *Mortierella* (Sakuradani et al., 1999). Delta-12 desaturase has now however, also been characterised in insect species, having been located in both cockroaches and house crickets (Cripps et al., 1990; Borgeson et

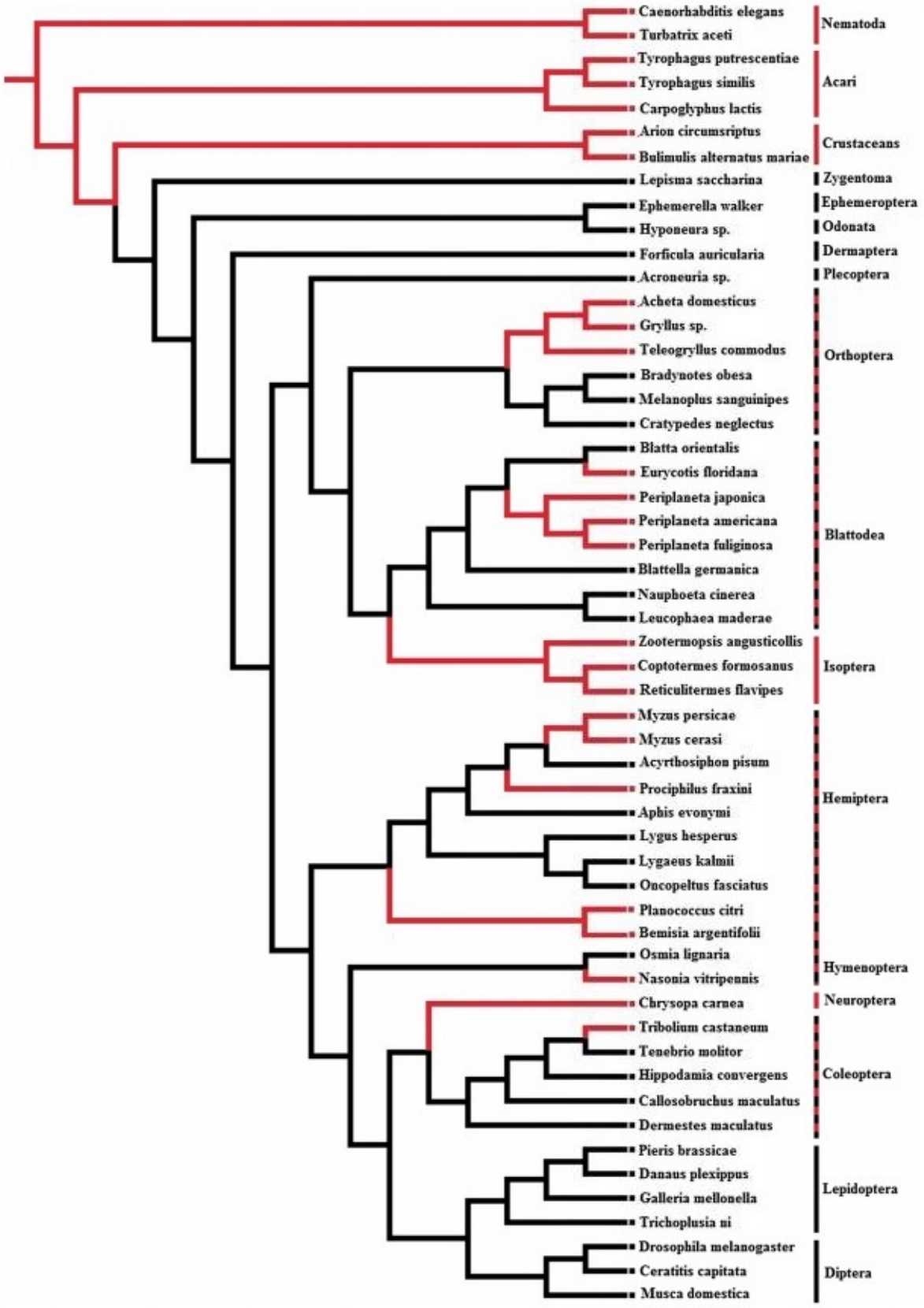


Figure 5.7: **Phylogenetic tree depicting species with the ability to synthesise linoleic acid *de novo* (red branches) and those unable (black branches) (Malcicka et al., 2018).**

al., 1990). Furthermore, delta-12 desaturase has also been characterised in the red flour beetle, a coleopteran, which is phenotypically very similar to *T. molitor* (Zhou et al., 2008) (Figure 5.7). Characterisation of this transcript in *T. molitor* larvae is novel and is in contrast to previous data that suggest inability to produce LA *de novo* (Malcicka et al., 2018). Of further interest was that during transcript verification using internal control TmL27a and qPCR reaction techniques, this desaturase was found to be expressed in all *T. molitor* larvae that were supplemented with oil.

Other transcripts were matched with analogues of *A. thaliana* chloroplast genes, which were found to be downregulated by linseed oil supplementation. *Arabidopsis* leaves have been associated as having the capacity for delta-15 desaturase activity (Qi et al., 2004; Spychalla & Kinney, 1997). Delta-15 desaturase although not present in mammals has been identified as the FAT-1 gene in lower eukaryotes *Caenorhabditis elegans* and recognises 18 to 20 carbon substrates (Spychalla & Kinney, 1997). Further work should be conducted to determine specific characterisation for these gene transcripts in *T. molitor* and whether *T. molitor* larvae have the ability to endogenously produce essential FAs independently of plants.

5.4.5 Conclusion

This Chapter's work led on from previous Chapter(s)' discoveries that feed input altered gross nutritional composition of larvae (Chapter 3) and further to this that feed supplemented with various oils affected specific FA within *T. molitor* (Chapter 4). The main objective of this Chapters' experiments was to identify differentially expressed genes within the *T. molitor* larval transcriptome that could be involved in FA metabolism.

The transcriptome of *T. molitor* larvae was significantly affected by linseed oil supplementation in feed and 41 differentially expressed genes were determined. Of those transcripts, 8 analogous were annotated, providing further insight into

mechanisms for n-3 UFA regulation of fat metabolism within *T. molitor* larvae and similarities with those in mammals with n-3 UFA potentially down-regulating genes associated with lipogenesis in larvae. Findings supported in part this Chapter's hypothesis concerning the effects of n-3 ALA supplementation on gene regulation. *De novo* transcriptome assembly and analysis have also provided promising gene transcripts for further investigation into lipid metabolism mechanisms within *T. molitor* species.

Future work should look to assess gene function of those transcripts identified by sequence specific gene suppression using RNAi techniques. In addition, further work should aim to isolate FADs 1 and 2 genes within *T. molitor* larvae with the aim of determining whether firstly larvae are capable of converting LA and ALA to LC-PUFAs and secondly whether delta-5 and -6 genes can be further manipulated for increased LC-PUFA synthesis.

There was large generic variation exhibited in SCD, ELOVL1 and ELOVL2 genes in *T. molitor* between treatment groups, further work should aim to selectively breed the species to reduce within group variation and successfully manipulate genes by feeding input. Further work should aim to develop selection indexes for the production of individual larvae with specific phenotypic qualities, similar to selection of phenotypes for production animals from wild type genetic population. Larvae could for instance be selected as having increased capacity to synthesise LC-PUFA.

Chapter 6

Optimising production of *T. molitor* larvae for use as a potential fishmeal replacement.

6.1 Introduction

Systems for the production of animals need to expand to provide protein sources in the diets of humans. As the production of animals increases so to will the competition for global resources. This is particularly the case for the farming of fish. Fishing remains the only large-scale hunting practice that is required for provision of fish as a feed source for both aquaculture and terrestrial livestock feed in the form of fishmeal (FM). Not surprisingly, FM provides the best suited source of protein for fish, in terms of its amino acid quality, and additionally provides fish oil (FO), which is a by-product of the reduction processing (Turchini et al., 2010). Despite an increased growth in the aquaculture industry to meet the growing demands for fish, further growth of production systems remains limited by the supply and increasing cost of sourcing FM (Tacon & Metian, 2008).

The FO from FM, provides a major source of LC-PUFA of the n-3 series to fish whose rate of biosynthesis is insufficient to meet requirements (Monroig et

al., 2013). Farmed carnivorous fish, such as salmonoids, are provided with their requirements for essential FAs and LC-PUFAs, by being fed FM/FO made from caught and processed wild small oily fish that have grazed on LC-PUFA-rich marine algae (Monroig et al., 2013; Miller et al., 2008). With a growing interest in provision of LC-PUFA in the diets of humans, there is mounting pressure to increase FM/FO provision in the feeds of farmed fish, to further encourage deposition of desired LC-PUFAs in the fillet of the fish, which will be consumed by humans (Miller et al., 2008).

Wild marine fisheries on the other hand, are being regarded more commonly now as a finite resource (Carson, 1962). Global catch is decreasing annually due to short supply, which is subsequently reflected in the escalating costs for the resource (De Silva & Soto, 2009). Protein provision in the feeds of production animals costs up to 70% of the entire production costs (Steinfeld et al., 2006). FM and FO are unsustainable from both an economic and environmental stand point.

Alternative vegetable sources of oil have been used in the feeds of aqua- culture species, to replace FM/FO , however these oil sources provide high levels of n-6 UFA and they lack LCPUFAs (Miller et al., 2008). There is also concern over the already high n-6:n-3 FA ratio in the Western diet, which could increase susceptibility to inflammation, CVD, and cancer in humans (Simopoulos, 2002b). Complete replacement of FO with 100% vegetable oil in salmon resulted in pathologies in the fish such as, severe heart lesions, thinning of ventricular walls, muscle necrosis and arteriosclerotic development changes (Bell et al., 1991; Seierstad et al., 2005). In addition, replacement of FM with vegetable sources of protein provide a less ideal amino acid (AA) profile for the fish, potentially reducing their growth rate, as well as providing complex carbohydrates and high anti-nutritional factors that can result in inflammation of the fish gut (Vielma et al., 2003).

Ideally an alternative feeding source to FM/FO should be prepared by a more

sustainable procedure. Indeed, Smetana et al. (2016), believe that identification of insects as a more sustainable source of protein than traditional and other emerging sources will be the *only* force capable of shifts to use insects as feed. As described previously, part of the objective of the work described in this thesis was to produce *T. molitor* larvae on a non-competing protein source such as WB, as described in Chapter 3 and Chapter 5. Literature has not determined basic husbandry techniques for mass-rearing of *T. molitor* larvae and optimisation of husbandry techniques is required in order to reduce fixed costs in larvae production as a feed. Currently production is associated with high costs and one of those costs is electricity. Optimal temperature and lighting conditions are some of the most important information required if *T. molitor* larvae are to be determined both economical and sustainable as a feed ingredient. In this Chapter I report research carried out to determine the impact of lighting conditions on *T. molitor* larvae growth.

A further consideration for larvae trials, where vegetable oils are being mixed into the basal feed ingredient, is uniformity of the feed particle size. This Chapter also aims to assess whether particle size of the basal feed influenced growth characteristics of *T. molitor* larvae. A smaller particle size requires more intensive processing than a larger particle size and increases the time taken to prepare basal feed and the wear-and-tear on the milling machinery.

Although in the previous Chapters' investigations LC-PUFA biosynthesis within *T. molitor* larvae was inconclusive, it was shown that larvae have plasticity to rapidly integrate specific FA from feed compositions into triglycerides, which are thought to make up approximately 90% of all lipids stored in insects (Arrese & Soulages, 2010; Bailey, 1975; Canavoso et al., 2001). In addition, supplementation with linseed oil has been shown to effect apparent mRNA expression of genes within *T. molitor* transcriptome, some most-likely involved in fat metabolism. Insects form the natural diet of many wild fish and could provide a partial re-

placement to FM due to the lack of certain LC-PUFA. Furthermore, it would be interesting to not only evaluate the practicality of using *T. molitor* larvae for the feeding of fish, but to assess the potential benefits of supplementing them with linseed oil for increased ALA within their fat deposition.

Aims

1. To determine if milled basal feed particle size has effects on the growth characteristics of *T. molitor larvae.*

2. To assess the impact of light environment on growth, gross nutrition and FA profiles of *T. molitor* larvae.

3. To determine if *T. molitor* larvae can replace FM 20% w/w replacement/inclusion, without adverse effects to the growth rates, overall mortality and proximate nutritional composition in fish.

4. To assess whether it is beneficial for fish fillet nutritional composition to supplement insect meal with increased ALA content through larval feeding, prior to formulation of fish feed.

Hypothesis

Particle size trial: It is hypothesised that a larger particle size, will result in reduced feed intakes and growth rates in *T. molitor* larvae, due to reduced surface area.

Light conditions trial: It is hypothesised that husbandry conditions can affect growth and nutritional composition of *T. molitor* larvae.

Replacement of FM in aquaculture: It is hypothesised that *T. molitor* larvae can replace 20% portion of fishmeal in the feeding of fish and through fatty

acid manipulation of larvae (in the production process), will provide adequate ALA levels to meet fish requirements, without the need for added linseed oil to fish feed.

6.2 Methods

6.2.1 Part 1: Optimising husbandry techniques

Animal husbandry

Six-week-old *T. molitor* larvae were supplied by Monkfield Nutrition Ltd. (Royston, Cambridgeshire, UK) for both trials. Prior to each trial larvae were fed on WB and carrot, (the latter was provided as a water source) and acclimatised for 3 days. Before the trials (Day 0), larvae were randomly assigned to replicates within different treatment groups. For both trials, feed was kept constant. Larvae were provided WB and carrot as a water source, *ad libitum* throughout the trials. All trials were conducted over a 14- day period. Both trials were conducted at the University of Nottingham, Sutton Bonington Campus, UK.

Experimental designs

***Particle size trial*:** Three treatment groups were made utilising a feed mill (Fritsch) and inserts of 0.5 mm, 1.0 mm; and 3.0 mm, which were used to grind a basal feed of WB to those corresponding uniform particle sizes. Each treatment group consisted of four replicate petri dishes (diameter: 150 mm), with two hundred individual larvae per replicate. On day 0 of trial, larvae were randomly allocated by hand to each replicate, and petri dishes were placed within a transparent tray (31.2 × 42.7 × 7.5 cm). Total initial biomass (grams) of larvae within each replicate was recorded and these were checked for non-significance before proceeding. Total biomass of larvae (dead and alive) and total number of living

larvae were recorded on days 4, 7, 11 and 14. Feed and water sources were weighed and replaced on days 4, 7, and 11 and weighed on start and end day of trial, which was day 14. On day 14 of trial all individuals were killed within replicates by cold exposure at -20°C.

***Lighting conditions trial*:** Two treatment groups consisted of different housing systems, designed in order to expose *T. molitor* larvae to either natural light/dark cycles (13:11 hours, in a 24-hour period) or complete darkness. Both housing systems consisted of a transparent petri dish (diameter: 150 mm), placed within a large transparent tray (31.2 × 42.7 × 7.5 cm), which were housed in a 3×4 tray stack. Replicates consisted of two hundred larvae, and each treatment group was allocated six replicates. Randomly selected larvae, for the natural light:dark cycle exposure, were placed within petri dishes within the transparent trays. Those selected at random for exposure to complete darkness were placed in a petri dish which was then placed inside an opaque box (24 × 35 × 7 cm), within the large transparent tray. Shelving design was equally spread between the two treatment groups.

On day 0 of the trial, two hundred larvae were randomly allocated by hand to each replicate petri dish, total biomass (grams) of larvae within each replicate was recorded and these were checked for non-significance before proceeding. On day 4, 7, 11 and 14 total biomass (g) of larvae both dead and alive were recorded. Dead larvae were removed, and the total number of remaining larvae were recorded. Feed and water source were weighed and replaced on days 4, 7 and 11 of trial, and weighed on days 0 and 14. On day 14 of trial all individuals were killed within replicates by cold exposure at -20°C.

6.2.2 Part 2: Replacement of FM in aquaculture

Animal husbandry

***T. molitor larvae*:** To produce the larvae meals that would be used to replace FM, 2 kg of six-week-old *T. molitor* larvae were supplied by Monkfield Nutrition Ltd. (Royston, Cambridgeshire, UK). Larvae were acclimatised in the same container and fed WB and carrot (as a water source), for 3 days. Following acclimatisation, larvae were separated into two distinct groups consisting of 1 kg larvae each. Production of *T. molitor* larvae was conducted at the University of Nottingham, Sutton Bonington Campus.

***D. rario fish*:** Fish were bred 'in-house' at the University of Liverpool, UK. All fish work was carried out at the University of Liverpool, under an Establishment Licence for the University of Liverpool (X70548BEB). Therefore, meeting all the standards required under the Animals (Scientific Procedures) Act 1986. The work was thoroughly reviewed, ethical approval was granted (ref number AWC0082) via the University of Liverpool's AWERB (Animal Welfare and Ethical Review Body), and the work was deemed below threshold for regulation. Throughout the trial welfare was monitored independently by the facilities named animal and care welfare officer (NACWO).

Fish were raised on ZM000, ZM100 and ZM200 (ZM fry foods), live newly hatched *Artemia nauplii* and subsequently standard flake food (Tetramin). Once allocated to the trial tanks fish were allowed to acclimatise to replicate tanks for one week, before the trial commenced. A total of 20 identical 9 L tanks (Aquatic Habitats) were connected to a 300 L centrally filtered system. Fish were exposed to a 12:12 (light:dark) light cycle and kept at 28±1°C. Waste from tanks was easily removed from the filter units with minimal disturbance to the fish. Water quality in the tank system was maintained at Ammonia (NH_4) 0 mg/L; Nitrite (NO_2) 0 mg/ L; Nitrate (NO_3) <20 mg/L; and pH; 7.0. During the trial fish were

fed a growth ration of 4% bodyweight in feed daily. This ration was split into two feeds per day, one feed was given between 8:30-10:30am and the other between 13:00-15:00pm.

Fish were euthanised using 100 mg/L benzocaine dissolved in a stock solution with ethanol. Benzocaine remains a neutral pH when added to water and therefore does not cause skin irritation to fish unlike MS222.

Feed preparation

***T. molitor* larvae meal production:** Larvae were housed in two separate large, opaque containers (70×45×35 cm). *T. molitor meal* (TM) was produced by feeding one group of larvae in complete darkness with 3 mm ground WB feed. ALA enriched *T. molitor* meal (lsTM) were produced by feeding WB enriched with 5% (w/w) replacement with linseed oil. After a 14-day growth period both larvae groups were killed, dried (at 50°C for 72 hours) and analysed for crude protein, crude fat, gross energy, fatty acid composition, AA composition, fibre and ash content (Table 6.1).

***Fish feed production*:** A control feed containing 51% FM was compared with three other fish feeds formulated with a blend of 1:4 *T. molitor* larvae meal:FM, at 51% total inclusion in the feeds of fish. Three larvae meals were designed, two containing *T. molitor* fed on WB forming TM and one meal containing *T. molitor* fed on WB supplemented 5% (w/w) with linseed oil (WB5ls) forming lsTM. One of two fish feed blends containing TM contained addition of rapeseed and linseed oils (TMrsls) and the other failed to meet the proposed ALA requirements for the fish, with only rapeseed oil added to the fish feed blend mixture (TMrs). Fish feed blends formulated with lsTM contained no further vegetable oil supplementations. All feeds were designed to be isonitrogenous, isolipid and isoenergy. Feeds were designed to enable direct comparisons between FM and a TM meals both

containing added rapeseed and linseed oil on phenotypic and nutritional effects in the fish. Further to this, lsTM could be compared with TMrsls in provision of oils from lipid of larvae supplemented first with linseed oil. Finally, the importance of ALA in the feeds of fish was assessed with comparisons of TMrsls and TMrs, which contained suboptimal contents of ALA for *D. rario*.

Four nutritionally balanced experimental feeds were formulated, utilising the nutritional information of FM and *T. molitor* meal, (either made by feeding WB to larvae or by feeding WB5lsTM to larvae), in a best fit modeller programme, Diet Modelling System AF, created in-house at the University of Liverpool. The control feed of FM contained 51% (w/w) FM (Table 6.3). The treatment feeds replaced 20% (w/w) FM with either TM plus added FA sources using oils or WB5lsTM. Rapeseed and linseed oils were added in combination with TM, in fish feed formulation of TMrsls, to provide requirements for LA and ALA (Tables 6.2 and 6.3). WB5lsTM was utilised to produce lsTM fish feed. The feed provided slightly lower LA than the stated *D. rario* requirement, but WB5ls provided lsTM with ALA contents above requirements and similar to those of FM and TMrsls fish feed, without the need for added linseed oil (Tables 6.2 and 6.3). Further to this, TMrs fish feed was formulated, with added rapeseed oil only, to meet LA requirements of fish. However, without added linseed oil TMrs provided inadequate levels of ALA to the fish (Tables 6.2 and 6.3).

The ingredients required for each feed (Table 6.3) were thoroughly mixed using Hobart food mixer and water was added to achieve a 'dough-like' consistency. Dough was spread thinly on trays and dried for 24 hours at 50°C in a food dehydrator (Parallexx Excalibur). Once dry the feeds were crushed by hand and processed through two sieves with apertures of 850 μm followed by 425 μm. With the desired pellet size falling between the two sieves. All diets were stored at 4°C before the trial start date and throughout the trial.

Table 6.1: **The proximate nutritional contents of feed used to produce *T. molitor* larvae and the larvae produced for use in fish feed blends for *D. rario*.**

Nutritional	WB[1]	TM[2]	WB5ls[3]	WB5lsTM[4]
Dry matter (%)	71.9	31.4	72.3	35.0
Crude fat (%)	**1.0 ± 0.0**	**23.7 ± 0.4**	**5.3 ± 0.1**	**27.1 ± 1.0**
Fatty acids (% moles)				
14:0	-	2.5 ± 0.1	-	1.94 ± 0.1
16:0	-	16.9 ± 0.5	-	13.9 ± 0.6
16:1	-	1.4 ± 0.0	-	1.12 ± 0.7
18:0	-	2.7 ± 0.1	-	2.5 ± 0.1
18:1 n-9	-	32.2 ± 0.6	-	28.3 ± 0.9
18:2 n-6	-	28.2 ± 0.8	-	23.2 ± 1.0
18:3 n-3	-	**1.6 ± 0.1**	-	**10.4 ± 0.6**
20:5 n-3 (EPA)	-	1.0± 0.6	-	1.2 ± 0.4
22:6 n-3 (DHA)	-	**1.1 ± 0.6**	-	**1.8 ± 0.7**
Crude protein (%)	16.0 ± 0.1	58.5 ± 0.6	15.5 ± 0.2	56.5 ± 0.4
Essential AAs[5] (%)				
Methionine	-	1.6	-	1.6
Histine	-	3.4	-	3.4
Tyrosine	-	4.3	-	4.6
Threonine	-	4.7	-	4.6
Valine	-	8.4	-	8.3
Isoleucine	-	6.7	-	6.6
Phenylalanine	-	4.1	-	4.2
Cysteine	-	1.1	-	1.1
Aspartate	-	9.1	-	8.8
Leucine	-	9.2	-	9.2
Lysine	-	6.7	-	6.6
Arginine	-	3.9	-	4.4
Non-essential AAs (%)				
Serine	-	5.2	-	5.3
Glutamine	-	13.6	-	13.5
Glycine	-	5.6	-	5.6
Alanine	-	12.3	-	12.1
Gross energy (MJ/kg)	**18.6 ± 0.2**	**24.9 ± 0.4**	**19.7 ± 0.2**	**25.7 ± 0.1**
Crude fibre (%)	10.4 ± 0.7	8.7 ± 0.3	12.1 ± 2.0	10.1 ± 1.6
Crude ash (%)	4.8 ± 0.0	4.7 ± 0.1	4.7 ± 0.0	4.3 ± 0.0

[1] *Wheatbran*; [2] *T. molitor fed* [1]; [3] 5% (w/w) *Linseed oil in wheabran*; [4] *T. molitor fed* [3]; [5] Amino acids.

Table 6.2: **The predicted nutritional contents of four balanced feed formulations for *D. rario*, produced using a best fit modeller programme, *Diet Modelling System AF*.**

Nutrient (%)	Requirement	FM[1]	TMrsls[2]	TMrs[3]	lsTM[4]
Crude protein	37-47	46.57	46.55	46.55	46.12
Crude fat	8-16	11.67	11.96	11.96	11.17
Carbohydrate	15-35	35.62	34.17	34.17	35.12
Crude ash	8	9.11	8.31	8.31	8.24
Crude fibre	1	0.07	1.79	1.79	2.06
Gross energy	16.19	22.26	22.20	22.19	22.09
LA[5]	2.00	1.55	2.05	2.08	1.71
ALA[6]	0.47	0.59	0.59	0.23	0.58
ARA[7]	2	0	0	0	0
EPA[8]	0.47	0	0	0	0
DHA[9]	0.47	1.07	0.88	0.88	0.96
Arginine	2.00	2.50	2.15	2.15	2.20
Histidine	0.84	0.51	0.95	0.95	0.96
Isoleucine	1.40	1.00	1.77	1.77	1.79
Leucine	2.48	1.82	2.96	2.96	2.99
Lysine	2.56	3.21	2.74	2.74	2.76
Methionine	0.58	2.95	0.58	0.58	0.58
Cysteine	0.58	0.58	0.42	0.42	0.42
Phenylalanine	1.22	1.22	1.22	1.22	1.22
Tyrosine	1.22	1.22	1.22	1.22	1.22
Threonine	1.36	1.36	1.36	1.36	1.36
Valine	1.60	1.60	1.60	1.60	1.60
Tryptophan	-	-	-	-	-
Total	100	100	100	100	100

[1] *Fishmeal (51%) blended fish feed*
[2] 20% (w/w) *T. molitor meal* [1] *replacement + rapeseed oil + linseed oil*
[3] 20% (w/w) *T. molitor meal* [1] *replacement + rapeseed oil*
[4] 20% (w/w) *Linseed oil supplemented T. molitor meal* [1] *replacement , no added oils*
[5] *Linoleic acid*
[6] *Alpha-linolenic acid*
[7] *Arachidonic acid*
[8] *Eicosapentaenoic acid*
[9] *Docosahexaenoic acid*

Table 6.3: **The ingredients added in formations of four nutritionally balanced feeds for *D. rario* 10-week trial.** Feeds were formulated using a best fit modeller programme, *Diet Modelling System AF.*

Nutrient (%)	FM[2]	TMrsls[3]	TMrs[4]	lsTM[5]
Fishmeal	51	41	41	41
Wheat gluten	12	6	6	6
Corn starch	31.3	30.3	30.3	31.8
Vitamins	0.3	0.3	0.3	0.3
Minerals	0.4	0.4	0.4	0.4
Binder	0.5	0.5	0.5	0.5
WB5lsTM[1]	-	-	-	20
TM	-	20	20	-
Rapeseed oil	4.2	0.7	1.5	-
Linseed oil	0.3	0.8	-	-
Total	100	100	100	100

[1] *T. molitor larvae fed* 5% (w/w) *Linseed oil in wheatbran*
[2] *Fishmeal* - 51% in a blended fish feed
[3] 20% (w/w) *T. molitor meal* [2] *replacement + rapeseed oil + linseed oil*
[4] 20% (w/w) *T. molitor meal* [2] *replacement + rapeseed oil*
[5] 20% (w/w) [1] *T. molitor meal* [2] *replacement , no added oils*

Experimental design

One hundred *D. rario* were randomly assigned to one of four treatment groups, the tank was the experimental replicate (n=5). Individual tanks were fed on a daily basis, at 4% (w/w) of the total fish biomass per tank, using the weekly assessment of biomass to determine the following weeks feed requirements. Tank biomass measurements were recorded by removing all fish in each replicate, using a net, discarding any excess water, before transferring fish into a 1.5 L weighing tank (by net inversion). Weigh tank was filled with approximately 2 cm depth of water that was previously tared on scales. During fish weighing the experimental tanks were cleaned thoroughly and refilled with fresh water before fish were returned. Final biomass weights were recorded after week 10 and fish were killed (Schedule 1 procedure), counted and placed on dry ice. Fish were stored at -80°C prior to

nutritional analysis.

6.2.3 Proximate nutritional analysis

Tenebrio molitor larvae from the light condition trial were oven dried at 100°C, larvae for the fish feeding trial were oven dried at 50°C and *D. rario* fish were dried by freeze drying, before nutrient composition analysis. Proximate nutritional analysis of the trial larvae and fish was conducted on samples from the total pooled individuals for each replicate. Analysis included: crude fat assessment via Soxhlet ether extraction method (*Soxtherm, Gerhardt*); crude protein assessment via combustion analysis by nitrogen analyser (*FlashEA 1112 Series, Thermo Fisher Scientific*); AA analysis (conducted by Calum Greenhalgh) and gross energy estimation via bomb calorimetry (*6300 Calorimeter, Parr*). FA compositions were also determined for all replicates following crude fat extraction via FAME methods and FAME analysis was conducted using Gas-Chromatography (*Clarus 580, Perkin Elmer*) and detection software TotalChrom Workstation (*Perkin Elmer*). For full methods descriptions refer to Chapter 2.

6.2.4 Statistical testing

Data were analysed using SPSS (version 24 IBM). Continuous response variable data that met assumption of normality and homogeneity of variance, determined using Shapiro-Wilk and Levene tests, respectively, were analysed using one-way analysis of variance (ANOVA). Specific between-group differences in measured variables were determined using Tukey HSD *post-hoc* comparisons. Any continuous data that did not meet the assumptions of normality or homogeneity of variance, were assessed using Kruskal-Wallis one-way analysis of variance, between-group differences were determined by Dunn-Bonferroni *post-hoc* testing. Statistical significance was taken as $P<0.05$. All individual FAs were analysed using

ANOVA, with a Bonferroni adjustment (0.05 ÷ no. of dependent FAs) of the 0.05 alpha level of acceptance, in order to reduce type I errors in results. Data is presented as means ± SD.

Mortality was analysed using a generalised linear model (logistic regression) conducted in Genstat (version 16 VSN), which assumed binomial proportions. Data was presented as back transformed binomial proportions means ± SEM.

6.3 Results

6.3.1 Part 1: Optimising husbandry techniques

Particle size trial

Particle size of basal feed WB over a 14-day period had no significant effect on the growth rates, total feed consumption, total carrot consumption or overall mortality of the *Tenebrio molitor* larvae (Table 6.4). In addition, estimates for feed conversion ratios between treatment groups were unchanged (Table 6.4).

Table 6.4: **The effect of basal feed uniform particle size on the performance and nutritional content of *T. molitor* larvae following a 14-day treatment.** n=4, 200 starting larvae. Growth rate per larvae was determined using a linear regression. All values are presented as means ±SD. Mortality reports back-transformed means ±SEM.

	Particle size (mm)			
Performance	0.5	1.0	3.0	*Sig. (P)*
Initial weight (mg/larvae)	71.2±2	73.5±5	73.4±5	NS
End weight (mg/larvae)	103.7±6	104.1±7	105.3±5	NS
Growth rate (mg/larvae/day)	2.2±0.4	1.9±0.1	2.0±0.1	NS
Total weight gain (g)	5.1±1	6.0±0.3	5.2±1	NS
Total feed intake (mg/larvae)	23.6±2	25.8±2	25.3±1	NS
Total carrot intake (mg/larvae)	238±10	243±3	242±13	NS
Mortality	0.05±0.03	0.05±0.03	0.03±0.01	NS
Estimate feed conversion ratio	8.19±1.1	8.81±0.5	8.39±0.3	NS

Lighting conditions trial

Exposure to different lighting conditions over a 14-day growth period, had significant effects on *T. molitor* larval performance. Larvae exposed to complete darkness consumed the same feed and reduced carrot as those exposed to natural light/dark conditions, however larvae achieved significantly greater growth rates ($P<0.001$), heavier end weights ($P<0.01$) and a higher total replicate biomass accumulation ($P<0.001$), in comparison to larvae exposed to natural light/dark cycles (Table 6.5). Lighting condition had no effect on total death proportion of larvae (Table 6.5). The composition of the larvae exposure to the two different lighting conditions had no effects on gross nutritional composition nor alterations in FA content and profiles of the larvae (Tables 6.5 and 6.6). Estimations of feed conversion ratio were shown to be significantly different between treatment groups, with those larvae grown in complete darkness appearing to have increased efficiency (Tables 6.5).

Table 6.5: **The effect of different light exposures on the performance and nutritional content of *T. molitor* larvae following a 14-day treatment.** n=6, 200 starting larvae. Growth rate per larvae was determined using a linear equation. All values are presented as means ±SD. Mortality reports back-transformed means ±SEM.

Performance	Light[1]	Dark[2]	*Sig. (P)*
Initial weight (mg/larvae)	68.1±7	71.7±3	NS
End weight (mg/larvae)	112.0±7	123.9±3	<0.01
Growth rate (mg/larvae/day)	3.3±0.1	4.1±0.2	<0.001
Total biomass gain (g)	6.9±0.4	8.8±1	<0.001
Total feed intake (mg/larvae)	38.1±3	38.8±2	NS
Total carrot intake (mg/larvae)	329±16	298±17	<0.01
Mortality	0.07±0.02	0.06±0.02	NS
Estimate feed conversion ratio	10.19±0.3	7.82±0.6	<0.001
Nutritional composition			
Crude protein (g/kg)	532±9	530±14	NS
Crude fat (g/kg)	295±11	289±12	NS
Gross energy (MJ/kg)	26.75±0.2	26.71±0.2	NS

[1] *13:11 hour light:dark photoperiod*
[2] *Complete darkness*

Table 6.6: **The effect of different light exposures on the fatty acid profiles of *T. molitor* larvae following a 14-day treatment.** n=6, growth rate per larvae which was determined using a linear equation. All values are presented as means ±SD, in units mol% FAME.

Performance	Light[1]	Dark[2]	*Sig. (P)*
14:0	3.9±0.3	4.2±0.4	NS
16:0	30.8±2.3	33.4±3.0	NS
16:1	1.9±0.1	1.7±0.2	NS
18:0	5.0±0.5	5.8±0.9	NS
18:1 n-9	36.9±1.6	35.6±1.8	NS
18:2 n-6	6.1±2.6	4.5±2.5	NS
18:3 n-3	1.4±0.5	0.9±0.4	NS
Unknown	10.3±1.5	10.7±1.7	NS

[1] *13:11 hour light:dark photoperiod*
[2] *Complete darkness*

6.3.2 Part 2: Replacement of FM in aquaculture

Fish feeding trial

As demonstrated in Chapters 4 and 5, feeding *T. molitor* larvae with feeds supplemented with sources rich in specific FAs increased the concentration of these FAs in the larvae. In addition, linseed oil supplementation at 5% into a basal feed of WB resulted differential gene expression within the transcriptome of *T. molitor* larvae. Using this information, a batch of *T. molitor* larvae meal (WB5lsTM) were produced with enriched ALA (18:3) content, for use in fish feed lsTM. Larvae were produced on a basal feed of WB ground to 3 mm particle size and grown in complete darkness for 14 days. As the particle trial demonstrated that larvae grow the same on the larger particle size, which saves time and energy required to produce the WB feed (Section 6.3.1). In addition, it was also demonstrated in the light conditions trial that larvae grow in darkness more effectively (Section 6.3.1). Interestingly, there was also the first evidence for the presence of EPA as well as presence and even potential enrichment of DHA in WB5ls fed larvae (Table 6.1).

The lsTM fish feed was generated through incorporating WB5lsTM into the *D. rario* feed at 20% (w/w), replacing 20% (w/w) of the FM. The other fish feeds prepared, utilised TM added at 20% (w/w), replacing 20% (w/w) of the FM. The TM feeds had either added rapeseed oil (TMrs) or rapeseed and linseed oils (TMrsls) added to the fish feed formulations (Table 6.3). These were compared to a control, 51% FM-blended feed (FM).

Table 6.7: **The effect of feeds containing *T. molitor* larvae meal on performance and gross nutritional contents of *D. rario* after a 10 week feeding trial.** n=5, growth rate per fish was determined using a linear regression. Level of significance accepted at $P<0.05$.

Performance	FM[1]	TMrsls[2]	TMrs[3]	lsTM[4]	*Sig. (P)*
Initial weight (mg/fish)	57.2±4	59.6±5	61.6±6	59.6±1	NS
End weight (mg/fish)	187.0±13^{a}	175.5±10^{ab}	173.1±9^{ab}	168.6±6^{b}	<0.05
Growth rate (mg/fish/day)	12.9±1^{a}	11.0±1^{b}	11.6±1^{ab}	10.9±1^{b}	<0.01
Total biomass gain (g)	12.4±1^{a}	8.7±2^{b}	9.8±1^{b}	9.9±1^{b}	<0.01
Total feed intake (mg/fish)	51.1±4	49.9±1	50.4±4	48.1±1	NS
Mortality	0.02±0.01^{c}	0.09±0.01^{a}	0.08±0.01^{a}	0.05±0.01^{b}	<0.001
Estimated feed conversion ratio	0.39±0.02^{a}	0.43±0.03^{ab}	0.45±0.05^{b}	0.44±0.02^{ab}	<0.05
Nutritional composition					
Crude protein (g/kg)	464±7	450±16	461±15	473±23	NS[7]
Crude fat (g/kg)	383±13^{ab}	386±21^{a}	377±22^{ab}	350±19^{b}	<0.05
Gross energy (MJ/kg)	26.61±0.3	26.49±0.3	26.01±0.8	25.87±0.4	0.058

[1] *Fishmeal (51%) blended fish feed*
[2] 20% (w/w) *T. molitor meal* [1] *replacement + rapeseed oil + linseed oil*
[3] 20% (w/w) *T. molitor meal* [1] *replacement + rapeseed oil*
[4] 20% (w/w) *Linseed oil supplemented T. molitor meal* [1] *replacement , no added oils*
[a,b] change in letter indicates significant difference

There was a significant effect of treatment group on performance in *D. rario* fish. Biomass weight gain was the greatest in replicates fed FM control feed compared to all other feeding groups that incorporated TM and WB5lsTM feed sources ($P<0.01$), and there were lower average weekly growth rates in TMrsls ($P<0.01$) and lsTM ($P<0.01$) groups in comparison with FM feeding groups. However, TMrs groups, with sub-optimal provision of ALA resulted in the same average growth rates per fish per week (Table 6.7). Furthermore, the end weights of fish were the same between FM and TM incorporated feeding groups, whereas lsTM had significantly lower end weights compared with FM ($P<0.05$), (Table 6.7). Total feed intake was the same across treatment groups, however due to significant differences in total biomass gain between groups estimated feed conversion ratios were significantly different between FM and TMrs groups, with FM exhibiting increased efficiency ($P<0.05$), (Table 6.7).

Feeding group had effects on the overall death proportions in replicate treatment groups ($P<0.001$), (Table 6.7). FM control groups achieved the lowest death proportion of all of the groups at 2%, whereas feeds formulated with TM feed resulted in the highest total mortality in fish, TMrs at 8% and TMrsls at 9% (Table 6.7). Fish fed lsTM had a significantly lower total average death proportion than fish feeds that incorporated TM, however lsTM still had significantly higher death proportions compared to FM controls, at 5% (Table 6.7).

When examining the composition of the fish at the end of the trial no significant differences were observed in crude protein content between feeding groups but there was a trend ($P=0.058$) for gross energy which was highest in FM and lowest in lsTM replicates (Table 6.7). FM, TMrsls and TMrs had similar crude fat contents, however interestingly lsTM had significantly lower crude fat contents compared with TMrsls replicates $P<0.05$) (Table 6.7).

Feed treatment had an effect on specific FAs within the FA composition of the fish, there were differences observed for their contents of 18:1 ($P<0.01$),

18:2 ($P<0.01$), and 18:3 ($P<0.001$), (Table 6.8). The lsTM treated group had the lowest 18:1 content being significantly lower than TMrs ($P<0.001$) and FM ($P<0.05$). There was significantly greater 18:2 in the lsTM ($P<0.01$) and TMrs groups ($P<0.01$) compared with the FM group. The lsTM group had a significantly lower 18:3 compared to TMrsls ($P<0.01$) and FM ($P<0.05$), whilst the TMrs had a significantly lower content than lsTM ($P<0.001$) (Table 6.8).

Table 6.8: **The effect of feeds containing *T. molitor* larvae meal on FA composition of *D. rario* after a 10 week feeding trial.** n=5, significance level of acceptance with Bonferroni adjustment is, $P<0.005$. Units mol% FAME.

Fatty acids	FM[1]	TMrsls[2]	TMrs[3]	lsTM[4]	*Sig. (P)*
14:0	2.3±0.1	2.3±0.1	2.3±0.1	2.6±0.1	NS
16:0	18.4±0.7	18.4±1.4	18.6±1.0	19.3±0.5	NS
16:1	2.4±1.4	2.8±0.2	2.8±0.1	3.1±0.1	NS
18:0	3.3±0.1	3.6±0.1	3.5±0.2	3.6±0.2	NS
18:1 (n-9)	30.8 ± 1.4^{a}	30.2 ± 1.5^{ab}	31.7 ± 1.0^{a}	28.0 ± 0.5^{b}	0.002
18:2 (n-6)	13.0 ± 2.3^{b}	15.7 ± 2.1^{ab}	17.0 ± 0.7^{a}	17.6 ± 0.5^{a}	0.003
18:3 (n-3)	6.2 ± 0.2^{a}	6.3 ± 0.2^{a}	4.1 ± 0.1^{c}	5.8 ± 0.2^{b}	<0.001
20:4 ARA (n-6)	1.2±0.1	1.1±0.1	1.0±0.0	1.2±0.0	NS
20:5 EPA (n-3)	2.1±0.2	1.8±0.3	1.6±0.1	2.0±0.1	NS
22:6 DHA (n-3)	3.9±0.6	3.3±0.5	3.3±0.2	3.8±0.3	NS
Unknown	16.3±3.9	14.6±1.8	14.0±2.5	13.0±1.7	NS

[1] *Fishmeal (51%) blended fish feed*
[2] 20% (w/w) *T. molitor meal* [1] *replacement + rapeseed oil + linseed oil*
[3] 20% (w/w) *T. molitor meal* [1] *replacement + rapeseed oil*
[4] 20% (w/w) *Linseed oil supplemented T. molitor meal* [1] *replacement , no added oils*
[a,b] change in letter indicates significant difference

6.4 Discussion

6.4.1 Part 1: Optimising husbandry techniques

There is minimal literary sources to date for the optimisation of husbandry methods in the growing of specific insect species and is an ongoing area of research. Despite this, sources have reported differences in growth outputs in *T. molitor* larvae grown under different conditions. Temperature (Bjørge et al., 2018), density (Weaver & McFarlane, 1990) and moisture (Urs & Hopkins, 1973), which have all been shown to affect growth and nutritional deposition in *T. molitor* larvae.

Particle size trial

The findings of the particle size trial make it possible to save on energy expenditure, general wear-and-tear costs of a feed mill and labour time required to mill WB to a uniform size of 3.0 mm, instead of 0.5mm, for use as a basal feed in TM production. Milling at 0.5 mm caused the mill to over-heat, putting a delay on processing WB. There was also a longer through-put time as a result of increased surface area of the end product, in comparison to 3.0 mm particle size.

Light conditions trial

The results of the light conditions trial will enable *T. molitor* larvae to be produced whilst cutting the cost of electricity, which has previously been demonstrated as attributing between 15-19% of the total cost of insect mass rearing systems (Smetana et al., 2016). In addition, space savings can be made through the stacking of boxes, containing growing larvae. This will not only reduce the area of land required for *T. molitor* production but also could reduce labour required, as production systems can be more compact in vertical systems. Furthermore, profits are maximised from growing *T. molitor* larvae in the dark versus a natural light:dark environment, with increased growth rates, on the same amount of feed

and reduced carrot intakes. In addition, the nutritional compositions of larvae were unaffected. The importance of these novel findings is clear, particularly with literary sources showing no consistency in lighting conditions for optimal growth of *T. molitor* species. Recent sources have produced larvae in natural lighting conditions (Bjørge et al., 2018; McConnell & Judge, 2018). Findings in this Chapter have shown these conditions to be unfavourable for growth of *T. molitor* larvae. Alternatively, sources have adopted other specific photoperiod conditions such as: 8:16, L:D, (T. Zheng et al., 2017), without first investigating, the impacts on *T. molitor* growth.

Differences observed for the growth of *T. molitor* in different light conditions, could be a result of water content of larvae. It has previously been described that a free-choice water source increased weights in *T. molitor* larvae, with a reduced number of molts and a reduced mortality (Urs & Hopkins, 1973). During this experiment water was provided *ad libitum* and it is worth noting there were no effects on mortality in larvae. The presence of an opaque container in the dark condition group could have however, reduced the concentration gradient between haemolymph and atmosphere relative humidity, reducing water loss in larvae. Whereas, a lack of additional (opaque) box, could have resulted in a higher concentration gradient between the haemolymph of larvae and atmospheric relative humidity, thereby increasing transfer of water to the surroundings (Machin, 1976). This theory is supported, by *T. molitor* larvae consuming increased carrot water source, when exposed to natural light/dark conditions. An alternative explanation could be differences in temperatures for growth (Bjørge et al., 2018), which could have been increased in larvae placed in an opaque, black box which is likely to have better absorbed heat radiation from the surroundings, compared with those in a transparent container.

The husbandry research in this Chapter is novel and will have great implications for the future consistency of *T. molitor* larvae production for feed and food

purposes. The findings of this Chapter build evidence that *T. molitor* larvae, as an alternative feed ingredient for production animals, can be "justified from an environmental point of view with consideration of insect diet, processing conditions and product properties" (Smetana et al., 2016). There is an apparent need for further research into husbandry methods of rearing *T. molitor* larvae, in relative humidity and temperature optimised conditions for maximum growth efficiency in *T. molitor* larvae.

6.4.2 Part 2: Replacement of FM in aquaculture

T. molitor larvae meal production

Although larvae were grown for use in fish feed and not prepared in an experimental setup, their apparent FA contents showed WB5lsTM had an apparent 10-fold increase in ALA, similar to observations in Chapters 4 and 5, however in contrast to those Chapters' findings, DHA was found to be present in the *T. molitor* larvae grown. There was also an apparent 63% increase in DHA content compared to TM. These observations may further be confirmed by the evaluation of *D. rario* FA contents. Replacement of FM with TM/lsTM had no effect on the contents of LC-PUFA, with abundance of ARA, EPA and DHA specifically being unaffected. No fish oil was added to the feeds, so all LC-PUFA came from either FM and/or *T. molitor* meal FM replacement.

The presence of LC-PUFA in the *T. molitor* larvae grown for this feeding trial highlights potential previous errors in the accuracy and determination of LC-PUFA within larvae whilst using gas-chromatography (GC) and mass spectrometry techniques. Any other potential differences in growing environment between these larvae and previous trials were in the mass-rearing of larvae in high temperature and humidity, due to them being grown in summer within a large, matt black, opaque boxes (relatively high absorbent material for heat radiation).

Up-regulation of rate-limiting delta-6 desaturase enzyme has been associated with changing temperatures and salinities in some fish species for example (X. Zheng et al., 2005).

General observations made in the growing of these *T. molitor* larvae would be a substantially increased stocking density. In addition, there was a very high temperatures in the large box larvae were grown in, due to high summer temperature and a matt black opaque box (a relatively high absorbent material for heat radiation). In addition, there was high humidity due to relatively high amounts of condensation and closed plastic lids on containers, which has not previously been the case in growing of larvae.

Further work is required to calculate accurate feed intake measurements in *T. molitor* larvae for FCR evaluations and cost-analysis to be conducted for future experiments, particularly when assessing *T. molitor* larvae as a potential feed for fish. In addition, further work is also required to determine the *actual* capacity of *T. molitor* larvae to synthesis ARA, EPA and DHA from dietary LA and ALA provisions and whether LC-PUFA *de novo* synthesis can be further manipulated in the species. This work will be required to determine if *T. molitor* larvae meal can act as a total replacement alternative to FM and FO. Feeding not only freshwater fish, but domesticated carnivorous marine species also, whose requirements for LCPUFAs are more substantial and essential for not only the health of fish but the benefit to human consumers.

Growth

The highest biomass weight gain was observed in FM groups, which could be the result of higher quality protein provision or the reduced death proportions observed in FM fish compared with the other feeding groups. Average growth rate per fish, per tank per week, which eliminates the 'death effect' shows TMrs had the same growth rates as FM, despite the feed being formulated with ALA

below *D. rario* recommendations. In addition, TMrsls fed fish had the same end weight as fish fed TMrs and FM. Growth rates and end weights were significantly lower than FM fish when fish were fed lsTM. However, there were no differences in total biomass weight gain, end weights or growth rates between fish fed *T. molitor* FM replacements, potentially due to the significantly reduced mortality in fish fed lsTM over those fed TM incorporated feed.

In the past few years the use of *T. molitor* larvae in the feeding of a number of fish species has been a growing area for research. Replacement of 35% FM with 20-40% TM has been shown to have no effects on the final mean weights, percentage weight gain, or specific growth rates in African catfish (Ng et al., 2001). The same was true in rainbow trout, where replacement of FM with TM at 25% and 50% of the total feed and 34% and 60% of FM, respectively, resulted in no difference in weight gain in the fish (Belforti et al., 2015). These findings were further supported in rainbow trout showing that 25% and 50% replacement of FM resulted in no effects on body weight (Iaconisi et al., 2018). Sea bream fed increasingly levels of TM at 35% and 71% replacement of FM resulted in no differences to the daily feed intake, weight gain, final body weights or growth rates, compared with FM controls (Piccolo et al., 2017). In addition, research in the blackspot sea bream had the same daily intake ratio, feed conversion ratio and specific growth rate compared with FM controls, when substituted with 25 and 50% TM (Iaconisi et al., 2017). However, over 60% supplementation of FM with TM in African catfish, and 26% total inclusion in the feed, resulted in the reduced growth performances and at complete replacement at 43% of total feed, the growth performance in fish was shown to be the lowest (Ng et al., 2001). In contrast, in juvenile yellow catfish FM replacement with TM up to 75%, and therefore 27% total inclusion in the feed, resulted in no negative performance effects, suggesting species and/or age effects of FM replacement in fish (Su et al., 2017). Juvenile European sea bass can be fed on FM replaced with 35% TM at a 25% total inclusion without negative affects

to growth, however inclusion at 75% and replacement at 71.4% of FM resulted in lowered final weights, weight gain and specific growth rate in addition to feeding rate compared to FM control (Gasco et al., 2016). Juvenile pearl gentian grouper achieved higher final weights at 12.5, 18.75, 25 and 31.25% inclusion of TM, in comparison with 0 and 6.25% feeds, however this could be a result of isolipidic and isonitrogenous feeds being formulated with increasing amounts of vital gluten meal and fish oil with reducing FM (Song et al., 2018).

Results support literature that *T. molitor* larvae can be used to replace FM in the feeding of fish, however contradict sources that showed same final weights and biomass gains in fish fed *T. molitor* larvae versus FM. Limited sources were found that described mortality effects in fish fed on *T. molitor* larvae FM replacement. Findings for mortality in *D. rario* suggest a potential effect of protein quality on mortality, with regards to FM having the more ideal profile of AAs and less overall mortality (Figure 6.1). Results contradicted literary sources in catfish species, that have found that TM substitution of FM from 20-100% resulted in no diet dependent effects on mortality in African catfish (Ng et al., 2001), and that supplementation of 75% FM with TM (TM75) resulted in increased survival rates in yellow catfish and that 50% replacement of FM resulted in no difference in survival compared with FM control or TM75 (Su et al., 2017). These sources suggest that potentially an increased TM/lsTM inclusion could have resulted in more beneficial survival outcomes in the fish. Results for mortality in this Chapter also highlighted differences between TM incorporated feeds and lsTM, which could be a result of a lower palatability of TM feeds formulated with added vegetable oils, in comparison to feeds formulated with WB5lsTM only.

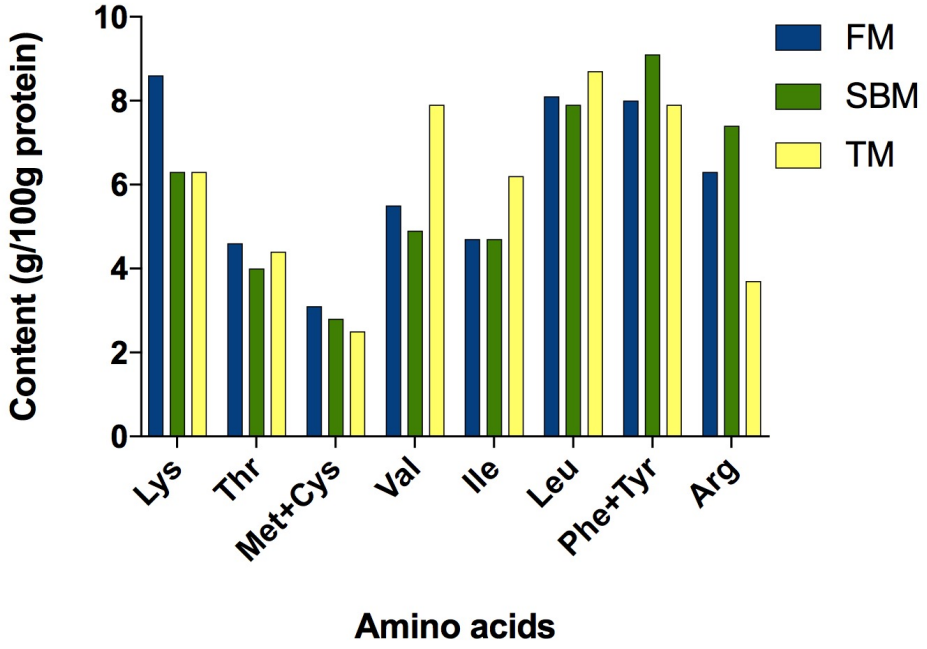


Figure 6.1: **The apparent essential amino acid of *T. molitor* larvae compared to the two major protein sources used in production animal feeding: fishmeal and soybean meal.** *T. molitor* were produced for 7-days on a feed of wheatbran. **Lys**: lysine; **Thr**: threonine; **Met+Cys**: methionine and cysteine; **Val**: valine; **Ile**: isoleucine; **Leu**: leucine; **Phe+Tyr**: phenylalanine and tyrosine; **Arg**: arginine; **FM**: fishmeal; **SBM**: soybean meal; **TM** *T. molitor* larvae (fed on wheatbran).

Proximate nutrient content

Balancing of the macro-nutrients in the feeds was necessary but challenging and formulations were the closest match to meet fish requirements and provide feeds to fish that were iso-lipid/protein/energy. Despite the view that FM provides a higher quality of protein to fish species (Figure 6.1), crude protein content in fish was unaffected by feeding group.

There was a trend for feeding group and gross energy content in fish proximate nutrient contents, but no statistical difference was observed, which could have been expected based off predicted feed formulations (Table 6.2). The gross energy content for lsTM appeared to be much lower than the other treatment feeds as no additional oils were added to the formulation unlike TM formulated feeds. A higher

apparent gross energy content in WB5lsTM in comparison to TM could explain the lack of significance in gross energy in fish between treatment groups (Table 6.1). Linseed supplementation in WB5lsTM would have resulted in increased apparent gross energy contents in that feed supplement (Table 6.1).

The lowered crude fat content in lsTM fed fish reflects the formulations of fish feeds, where no additional oils were added to lsTM formulation for fish (Table 6.3) or potentially highlights the beneficial effects previously observed in LS larvae in Chapter 5. Larvae had lowered fat deposition, potentially a result of up-regulated genes associated with β-oxidation of FAs. Alternatively, results could suggest feed formulations achieved isonitrogenous provisions across all treatment groups, however did not attain isolipid contents. Results were similar to literary sources, which showed mostly that crude protein composition of fillets in rainbow trout (Iaconisi et al., 2018), blackspot sea bream (Iaconisi et al., 2017), and juvenile European seabass (Gasco et al., 2016) were unaffected by TM substitution of FM. In addition, 25-50% replacement of FM with TM had no effects on total lipid content (Iaconisi et al., 2018). In contrast with results, crude protein was found to be significantly increased in rainbow trout through 25 and 50% inclusion of TM in replacement of 34 and 60% FM, respectively, although this was specifically in the dorsal muscle (Belforti et al., 2015).

Fatty acid composition

Previously, literature found 25-50% FM replacement with TM increased 18:1 in rainbow trout (Iaconisi et al., 2018) and decreased 18:1 in juvenile European seabass (Gasco et al., 2016), however lack of treatment effect could be a result of added RS oils to FM/TMrs and TMrsls. The lowest 18:1 acid content was observed in lsTM fish that had no added RS supplementation to fish feed.

Sources found that TM substitution of FM tended to result in increased LA content in fish, which supported findings that LA content was highest in fish

supplemented with lsTM and TMrs in comparison with controls (Iaconisi et al., 2018, 2017; Gasco et al., 2016; Belforti et al., 2015). However, TMrsls had the same LA content compared with the controls.

Content of ALA was found to be the highest in fish fed on either FM or TMrsls, above that observed in lsTM. These results appear to reflect the predicted feed compositions (Table 6.2). TMrs, as expected, had the lowest observed ALA, due to the lowest provision of ALA in the feed, below that of the requirements for *D. rario*. It is therefore, interesting that lowered ALA in the fish fed TMrs, had no effects on the growth rates of fish meeting those observed in FM. In literature, ALA content was most commonly found to be decreased through TM inclusion in replacement for FM in fish, without affecting growth in fish (Iaconisi et al., 2018; Gasco et al., 2016; Belforti et al., 2015). The addition of linseed oil to the fish feed formulation of TMrsls appears to have successfully counteracted these effects on reduced ALA content that have been observed in literary sources. Supplementation either by WB5ls to form lsTM or through adding linseed oil to TM resulted in significantly increased ALA above that observed in TMrs fish.

Replacement of 20% FM with TM/lsTM had no effect on the LC-PUFAs in fish, which could further support the presence of these FAs in *T. molitor* larvae and therefore a capacity to elongate and desaturate ALA (provided in the feed WB5ls) to EPA and DHA. Trials in rainbow trout, blackspot seabream and juvenile European sea bass showed that TM replacement of FM resulted in reduced LC-PUFA and increased 14:0 and 16:1 acids in fish compositions (Iaconisi et al., 2018; Belforti et al., 2015; Iaconisi et al., 2017; Gasco et al., 2016). As both LC-PUFA and SFA contents in *D. rario* were found to be unaffected, results could highlight the differential requirements of freshwater and marine fish. Further work should be carried out in marine fish species.

6.4.3 Conclusion

Optimising husbandry techniques: The husbandry trials conducted in this Chapter, highlight the importance of determining the optimum systems for production, with proof that they can be used to reduce costs and maximise profits in *T. molitor* production. Another experiment should be designed to determine whether there are any growth impacts when larvae are feed on unprocessed WB, as this would eliminate the need to further process WB to a 3.0 mm particle size. Further work is also required to determine the exact ambient environments for promotion of efficiency in the mass-production of *T. molitor* for animal feed. Although growing of larvae in complete darkness increased efficiency, further work is necessary to remove potential effects of an additional box used in their culture to provide the absence of light. Future work should look to examine more varied lighting conditions to establish an exact ambient environment for promoting *T. molitor* larvae growth.

Replacement of FM in aquaculture: The results of the 10-week fish trial show it is possible to include *T. molitor* larvae as an insect meal with added rapeseed oil (TMrs) in replacement of 20% FM used in a diet at 51% total inclusion, without negative impacts on growth rates per fish. In addition, the end weights of fish grown on FM, TMrs and TMrsls were similar, however total biomass gain in fish was significantly reduced in fish fed with *T. molitor* supplementations compared with FM.

Mortality was highest in fish fed on TM incorporated feeds, with added vegetable oil (TMrs and TMrsls), and lowest in FM. Fish fed lsTM had significantly greater deaths than FM, but lower overall deaths compared with TM formulated feeds. Results could indicate increased palatability of feeding *T. molitor* larvae supplemented with linseed oil, over the addition of pure vegetable oils to a TM feed. This could suggest that linseed oil manipulation of *T. molitor* larvae, allows

for proportions of FM/FO to be replaced indirectly by vegetable oils, with lowered risk of pathologies such as inflammation of the gut. This would require further experimental analysis. Furthermore, sources suggested that higher inclusion levels of ALA could be beneficial for growth and ALA content in fish. In Table 5.3 higher levels of ALA were achieved with dosages of 7.5% and 10% w/w linseed oil, without significant mortality effects (Chapter 5).

The FA composition in fish corresponded to feeding inputs that were predicted. Sub-optimal requirements of ALA, resulted in no issues with growth per fish, nor increased deaths in comparison with TMrsls feed. In addition, 10% presence of various TMs as a 20% replacement of FM, resulted in the same levels of LC-PUFA, ARA, EPA and DHA in fish.

In conclusion the hypothesis for particle size trial was not supported and particle size had no effects on *T. molitor* performance characteristics. The light condition trial hypothesis was partially rejected with lighting conditions not found to effect gross nutritional composition of *T. molitor*, however darkness increased growth rate and reduced feed intake in *T. molitor* larvae. In aquaculture trial, the hypothesis was supported with TMrs successfully replacing FM in feeds of fish, however lsTM did not provide the same levels of ALA as FM fed larvae, which was likely a limitation of feed formulations and a lower ALA content provided in the feed.

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Daily feeding of fish during 10-week trial: Technician staff at the University of Liverpool.

Weekly weighing of fish and 7-days worth of feed per tank: Ellen Tetlow, PhD candidate, University of Nottingham.

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Chapter 7

Discussion

7.1 Development of the growing techniques used for rearing *T. molitor* as a viable feed ingredient

In the first growth trials of this thesis *T. molitor* larvae were assessed for their ability to grow on different feed ingredients. Wheatbran (WB) tends to provide a laxative ingredient in the feeds of animals to which it is supplied, however does not contribute a protein feed source that could be utilised efficiently by production animals.

The overall findings of Chapter 3 were that *T. molitor* larvae could be successfully grown on WB feed with increased protein and reduced fat deposition, albeit at a reduced growth rate. Treatment effects on growth rates were consistent across younger and older larval groups tested. A reduced growth rate observed in larvae fed on WB could have been a result of reduced feed intake, due to high dietary fibre content of feed causing satiation. A major limitation for both trials in Chapter 3, was the lack of recorded information as to the amount of feed supplied and total feed intakes in the larvae. Feed intakes were crudely assessed moving forward through Chapter 4-6 in *T. molitor* larvae and apparent FCR values were

determined (Table 7.1).

Table 7.1: **Summary of estimated feed conversion ratios (FCR) in *T. molitor* larvae grown on different feeds across chapters.** Averages are displayed across replicates and in some cases across treatment groups where specified.

Chapter	Basal feed	Treatment	FCR (feed + carrot)	FCR (w/o carrot)
4	Chick crumb	Controls	6.90	1.67
		SFA[1]	5.59	1.12
		UFA[2]	8.0	1.34
5	WB[3]	Controls	4.65	2.10
		P[4]	3.32	2.01
		RS[5]	3.51	2.03
		LS[6] (5-10%)	5.18	2.02
6	WB	Particle/light	8.68	0.81

[1]*Saturated fatty acid* [2]*Unsaturated fatty acid* [3]*Wheatbran* [4]*Palm oil supplemented 5% (w/w)* [5]*Rapeseed oil supplemented 5% (w/w)* [6]*Linseed oil supplemented 5-10% (w/w)*

A robust methodology for feed intake measurement in growth trials is required in order for reliable FCR calculations. These are necessary for determining the economic viability of *T. molitor* as an alternative feed source through cost-analysis. The FCR calculations across chapters varies greatly and are extremely high suggesting that larvae are economically unsustainable. Further work should first aim to remove carrot as a water source from the culture of *T. molitor*, because carrot is an additional feedstuff as much as it acts as the main water source in experiments. Soaked cotton wool could be utilised to remove carrot feedstuff and provide solely water for larvae. Removal of carrot from experiments would aim to reduce the apparent high FCR values to more sustainable levels, between 0.1-1.9 and to increase consistency between trials. A further reason for extremely high FCR values is the removal of dead larvae from biomass weights. It is unlikely that processing of *T. molitor* larvae for use in animal feed will remove dead individuals from the mix. It is recommended that methodology for future trials should include dead larvae as part of the total biomass recorded for replicates during experiments.

FCR without (w/o) carrot suggest *T. molitor* larvae, when fed on a chick crumb feed source, have an FCR similar to salmonids (Table 7.2). In Chapter 5, *T. molitor* larvae FCR w/o carrot appears to more resemble that for chicken when fed on WB (Table 7.2). Whereas, Chapter 6 FCR w/o carrot values appear to be better than salmonids, which seems unbelievable (Table 7.2).

Table 7.2: **A comparison of common FCR among different animal species.** Information obtained from `www.aqua-techna.com`. All rights reserved and strictly limited (Techna, 2018).

Animal	Average FCR[1]
Tropical shrimps	1.6 - 2.0
Domesticated carnivorous marine	1.3 - 1.6
Omnivorous fish	1.4 - 1.8
Salmonids	1.0 - 1.2
Broiler	1.8 - 2.1
Fattening pig	2.5 - 3.0

[1] *Feed conversion ratio*

van Huis et al. (2013) reports values for *Acheta domesticus* between 0.9-1.7, which are similar to those evaluations obtained for *T. molitor* throughout this thesis work if FCR w/o carrot are considered (Nakagaki & Defoliart, 1991; Stanley & Nelson, 2005). Further to this, taking into account the edible proportion of the insect compared with terrestrial production animals it is reported that *A. domesticus* are twice as efficient as chickens, four times as efficient as pigs and 12 times more efficient than cattle (van Huis et al., 2013).

Specific comparisons with *T. molitor* FCR reported in Oonincx et al. (2015) show similar values to this thesis work when considering FCR feed + carrot, with high variability and high overall values. The highest FCR value recorded in *T. molitor* fed on a low protein high fat diet with carrot was 19.1 and a low protein low fat diet with carrot resulted in an FCR value of 10.9 (Oonincx et al., 2015). These values are higher than the high values stated in this work and could be in part accounted for by Oonincx et al. (2015), assuming all *ad libitum* feed provided

was consumed by *T. molitor*. Further work will need to assess the impact of utilising carrot as a water source on larval growth, larval nutritional composition and evaluate other methods for water provision. Apparent differences between FCR values in this thesis work and literature highlight that a robust methodology for assessing FCR in *T. molitor* is still required (van Broekhoven et al., 2015; Oonincx et al., 2015).

A further consideration for developing growth trials in *T. molitor* could potentially be a control of stocking density. In Chapter 5, trial 1 and trial 2 apparent differences highlighted a potential effect of stocking density on overall mortality and crude fat composition in larvae. Future work must also determine an accurate method for feed intake measurement for reliable FCR calculation. In addition, further work could also aim to assess the available energy to *T. molitor* larvae (metabolisable energy), as it was highlighted that analysis of feeds gross energy likely provided an overestimation of useful energy to the larvae.

7.2 Optimisation of husbandry techniques for rearing of *T. molitor* larvae to maximise efficiency as a viable feed

Taking into account FCR values without carrot, the current price of chick crumb is £0.62 per kg and the average estimated FCR (w/o carrot) that was observed across trials in Chapter 4 was 1.24. Therefore an estimated cost to produce 1 kg of live weight *T. molitor* larvae on chick crumb would be £0.77. Current price of WB is £0.41 per kg and in Chapter 5 the average estimated FCR without carrot was 2.04 across the two trials, resulting in a cost of £0.83 per kg *T. molitor* larvae live weight produced. The average estimate of FCR recorded across two larval trials in Chapter 6 was 0.81 resulting in a cost of £0.31 per kg *T. molitor* larvae

produced. This is more competitive than both FM (£1.11 per kg) and SBM (£0.51 per kg) and previous estimates of cost for *T. molitor* larvae live weight production at £0.40 per kg (Veldkamp & Bosch, 2015). However, with dry matter recorded at 34% for WB fed larvae (Chapter 3), an average FCR (without carrot) value of 0.81, results in an estimated dry mass cost of £0.89 per kg. However, other fixed and variable cost have not been taken into consideration in these basic cost analyses.

The cost of animal protein feed has previously been discussed as a limiting factor in their ability to further expand (Chapter 1). Therefore costs effects *T. molitor* viability as competition for finite resources increases over the coming years. As a result, optimisation of animal production systems is crucial for sustainability. In Chapter 6, environmental factors associated with growing *T. molitor* larvae were assessed in an attempt to further optimise the efficiency of their production. Increased efficiency in production systems, reduces the costs of production. A high cost of production currently limits *T. molitor* species as a viable protein source for the feeding of fish due to it being comparable to the cost of FM (Veldkamp & Bosch, 2015).

Smetana et al. (2016) previously stated that electricity consumption accounts for 15-19% of the overall impact of insect rearing systems. Findings in Chapter 6, are crucial for progressive development and optimisation of *T. molitor* production systems. The fact that apparent FCR was reduced to 0.77 in dark fed larvae, meant cost of feed WB inputs was reduced and more live weight was produced. Furthermore, results support the suitability for vertical rearing systems and the minimised space required for growth. This is extremely relevant in today's climate with land being a finite resource that limits cultivation of plant animal feed production. Electricity and maintenance costs on the feed mill would also be saved as a result of outputs from the particle size trial. Further research is required into optimised conditions for rearing *T. molitor* species, which have been highlighted

in this thesis work with apparent differences in phenotypic data collected from *T. molitor* larvae across trials. Stocking density was highlighted as having the potential to effect mortality and crude fat nutritional composition of larvae (Chapter 5) and therefore, requires further evaluation in future experiments. Research into automation in insect rearing systems will also be able to cut down on requirements for labour intensive systems.

7.3 Assessment of the plasticity of *T. molitor* nutritional composition for specific accumulation of FA in feed

Generally the results from FA manipulation of feed with oils, in Chapters 4 and 5, highlighted the ability to manipulate specific FA content within *T. molitor* larvae. The impact of dietary FA input has been said to be the main determinant of FA deposition in monogastric animal species. However, in contrast with monogastric animal trials that supplemented n-6 and n-3 LC-PUFA precursors into feeds, there was no evidence for *T. molitor* larvae ability to synthesis ARA or DPA and EPA *de novo*. In terms of DHA *de novo* synthesis, FA manipulation trials in *T. molitor* larvae showed either no DHA abundance in larvae or an undetectable amount. Results for *T. molitor* larvae support those in humans and monogastric species supplemented with ALA, which showed no effect of dietary ALA on ability to synthesis DHA (Gillingham, 2013; J. Kim et al., 2014).

Methods for detection of FA in *T. molitor* larvae may require improvement. There were unknown FAs that were detected at a later time then ALA in GC method, suggesting a potential presence of LC-PUFAs in the samples that could not be accurately characterised, using the current method. Interestingly, under extreme heat conditions >30°C, *T. molitor* larvae produced for *D. rario* fish trial

appeared to have an apparent LC-PUFA abundance (Chapter 6). Suggesting *T. molitor* might have the capacity under certain temperature and humidity conditions to synthesise LC-PUFA. In Atlantic salmon, gene expression of delta-6 desaturase, a rate-limiting enzyme in the LC-PUFA biosynthetic pathway, is up-regulated in cold temperatures and low salinities and is at its highest around the point of seawater transfer and lowest during the seawater phase (X. Zheng et al., 2005). Further research into *T. molitor* capacity to synthesis LC-PUFA may be required.

7.4 Novel *de novo* transcritpome analysis in *T. molitor* larvae and assessment of lipid metabolism genes

Novel research findings showed that supplementation with linseed oil resulted in the differential expression of 41 gene transcripts within *T. molitor* larvae, 20 down-regulated and 21 up-regulated. To my knowledge this work is novel. Furthermore, characterisation of these genes may have highlighted delta-11 or delta-12 desaturases in *T. molitor* larvae, which are of great interest because they are not present in mammals, birds or fish. Further assessment of *T. molitor* transcripts that were found to be different with linseed oil treatment alligned with chloroplasts in *Arabidopsis thaliana*, which have the delta-12 and delta-15 desaturase genes capable of *de novo* synthesis of LC-PUFA precursors LA and ALA, respectively (J. M. Lee et al., 2016). Further characterisation of these gene transcripts are required to establish whether *T. molitor* transcript is delta-11 or delta-12 desaturase and whether *T. molitor* have specific genes analogues to chloroplasts or if these are contaminations of RNA samples.

Working oligonucleotides designed to isolate SCD, ELOVL1 and ELOVL2

showed that supplementation with different oils and with linseed oil at increasing levels of inclusion resulted in no significant gene expression within *T. molitor* larvae assessed. These results contrasted with what was expected from mammalian studies as previously described in Chapter 5.4.4. In addition, difficulties in designing primers for gene transcripts in *T. molitor*, without a reference genome, was highlighted with only 25% of designs found to actually work. Now with a reference *T. molitor* transcriptome, primer design may become easier. However, the lack of annotated Coleopteran species available on RNA collection databases is a limitation. RNA interference work could be used to help determine some of the functions of the differentially expressed genes located in *T. molitor* transcriptome treated with linseed oil.

A potential limitation resulting in the lack of differences exhibited in the gene expression in both the lipid metabolism gene investigation and the verification of *de novo* transcriptome, is due to the high variability of gene expression within replicates. However, this indicates that genetic variation is high, which suggests that some individuals with treatment exhibit higher expression than their counterparts and could be selected for. Future work could look to introduce selection parameters for genetic enhancement of *T. molitor* larvae, particularly with regards to enhancing the LC-PUFA *de novo* FA modification, potentially by genetic enhancement of ELOVL2 activity.

7.5 The use of *T. molitor* as a fishmeal replacement in feed for fish

Preliminary fish studies on *D. rario* successfully showed TM meal can replace FM 20%, without adverse effects to growth rate, gross nutrition, or FA composition. Supplementation of *T. molitor* larvae with linseed oil, prior to their use as a fish feed, may be of interest as it appeared to result in fish having increased surviv-

ability to TM fed fish. Limitations in dietary formulation meant that perhaps WB5lsTM required greater than 5% linseed oil supplementation to increase the amount of ALA provided to fish. Previous work shows increasing supplementations of linseed oil increased ALA in a dose-dependent manner, without affecting mortality in larvae (Chapter 5).

A robust cost-analysis is required for *T. molitor* larvae production systems to be able to further assess the species suitability as a FM replacement and highlight areas for optimisation that could be further exploited to increase efficiency. These trials were conducted in zebra fish as a preliminary model for larger species consumed by humans. Further evaluation using these fish species is required.

7.6 Conclusions

1. *T. molitor* larvae can be produced on an alternative protein source WB with favourable nutritional outcomes and without major affects to instar development. Whereas feeding of an arguably unsustainble feed source, such as SF, resulted in fattening of larvae. This is likely as a result of surplus metabolisable energy provision.

2. The overall fatty acid profiles of *T. molitor* larvae appeared to mimic the feeding inputs of oils rich in specific fatty acids. There did not appear to be any presence of LC-PUFAs in the samples and therefore no evidence of ability to further elongate and desaturate LA and ALA. This suggests low capacity for synthesis pathways, similar to that identified in mammals and birds.

3. Supplementation with 5% (w/w) oils in WB consisting of high abundance in SFA, MUFA and PUFA had no significant impact on the gene expression of SCD, ELOVL1 or ELOVL2 in *T. molitor* larvae. However, treatment

resulted in large within group variation which may have prevented significance. This work highlights further opportunity for gene manipulation in *T. molitor* and a requirement for selective breeding.

4. Supplementation of *T. molitor* larvae with 5% (w/w) linseed oil, resulted in differential gene expression of 41 transcripts. These included 20 down-regulated gene transcripts and 21 up-regulated transcripts. Characterisation of 8 of these genes was possible using nBLAST. Apparent transcript expression was enhanced in gene transcripts associated with lipolysis, transcription mediation, desaturation and immunity. Furthermore, apparent transcript expression was down-regulated in gene trasncripts associated with protease activity and sclerotisation.

5. *De novo* transcriptome analysis validation found that supplementation with 5% oil, either palm, rapeseed or linseed oil, resulted in increased expression of either delta-11 or delta-12 desaturase gene transcript within *T. molitor* larvae. As these are desaturases that are not known to be present in mammals, birds or fish species, they are of great interest. Delta- 11 would enable insects to insert a desaturation for a 14:1 FA, which is a sex pheromone precursor in insects. Delta-12 desaturase activity would enable *T. molitor* to synthesis LA from oleate *de novo*, providing a source of essential FA of the n-6 series. The likelihood of discovering a sex pheromone in larvae is unconvincing, however delta-12 activity has previously been identified in *T. castaneum* which are closely related to *T. molitor*. However, previously the phylogenetic relationship has been thought to show differences in the ability to synthesise LA *de novo* (Malcicka et al., 2018). A delta-12 desaturase activity within *T. molitor* is in contrast to literature and a novel finding.

6. *T. molitor* larvae grow better in the dark than a natural light:dark environment, whilst consuming the same amount of feed and had not impacts on

proximate or FA composition. Furthermore, basal feed particle size of WB between a range of 0.5 mm to 3.0 mm, had no effects on growth.

7. The results of the 10-week fish trial show it is possible to include *T. molitor* larvae, as insect meal TMrs, in replacement of 20% FM used in a diet at 51% total inclusion, without negative impacts on growth rates per fish. However, TMrs fed fish had reduced efficiency with higher FCR in comparison to FM fed fish. In addition the total biomass gain in fish was significantly lower in all fish treated with *T. molitor* feed replacements. Supplementing *T. molitor* larvae with linseed oil (lsTM) appeared to result in reduced mortality in fish compared with TMrs and TMrsls feeds which contained *T. molitor* larvae with added vegetable oils. However, growth rate, end weights, total biomass gain and mortality were all negatively impacted in fish fed lsTM compared with FM treatment groups. Overall, the FA compositions for LC-PUFA, ARA, EPA and DHA were unaffected in *D. rario* fed TM and lsTM alternatives as partial FM replacements. This suggests the presence of LC-PUFA in *T. molitor* was adequate to meet fish requirements or that the remaining FM was able to cater to the fish requirements for LC-PUFA.

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APPENDIX A

Figure A1- Available SCD sequences.

1). NM_009127.4
- Mouse

2). NM_144474.1
- *Drosophila melanogaster*

3). NM_001193649.1
- Red_flour_beetle

Figure B1- Multiple alignment of sequences for SCD. Target location for PCR: primer locations highlighted with rectangle and arrows.

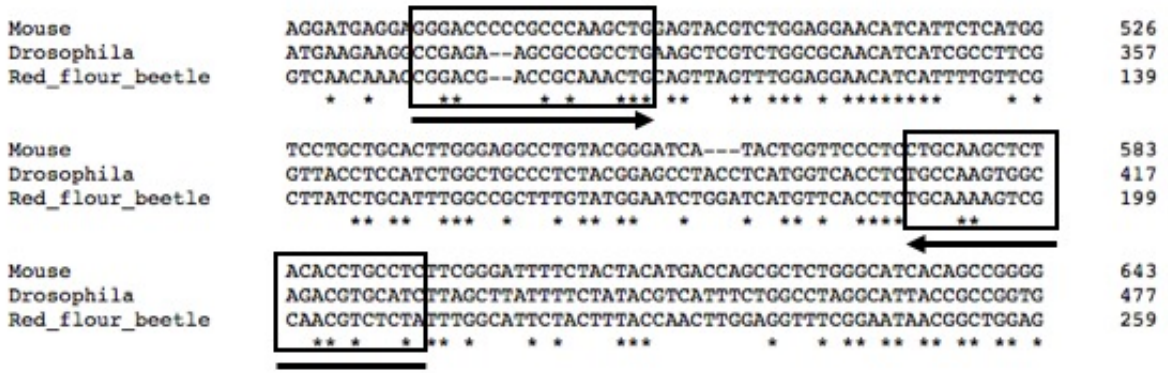


Figure C1- PCR conditions.

Fwd. primer- 17bp
(CGGACGACCGCAAACTG)

Start- 92 of 1053 bp
(Red_flour_beetle sequence)

Rev. primer- 22bp
(TAGAGACGTTGCGACTTTTGCA)

Start- 210 of 1053 bp
(Red_flour_beetle sequence)

Amplicon size: 119 bp
Annealing temp: 63.7oC
Cycles: 35

Phusion Hot Start II DNA Polymerase

Figure D1- SCD PCR product run on 5% metaphor gel alongside 25bp and 100bp markers, respectively.

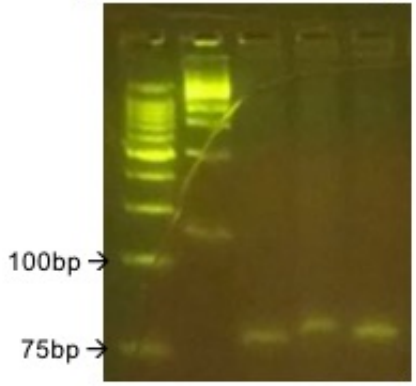


Figure E1- DNA sequencing cloned cDNA insert.

TCCATGTGGGGCGACAACCCTACGACAGG
TTTATCAATCCAGCTGAGAATTTCGAGTT
CACCAACCAGGTGGCGTTCAA - 79 bp

Figure A2- Available ELOVL1 sequences.

1). NM_141217.3
-*Drosophila melanogaster*

2). MF279188.1
-*Tenebrio molitor*

Figure B2- Multiple alignment of sequences for ELOVL1. Target location for PCR: primer locations highlighted with rectangle and arrows.



Figure C2- PCR conditions.

Fwd. primer- 24bp
(CTAACTACCTTCCAGATGGTGCAA)

Start- 701 of 1243 bp
(T. molitor sequence)

Rev. primer- 22bp
(TCCGTGAAGAGAAGCTGGAACT)

Start- 768 of 1243 bp
(T. molitor sequence)

Amplicon size: 68 bp
Annealing temp: 65.2°C
Cycles: 35

Phusion Hot Start II DNA Polymerase

Figure D2- ELOVL1 PCR product run on 5% metaphor gel alongside 25bp marker.

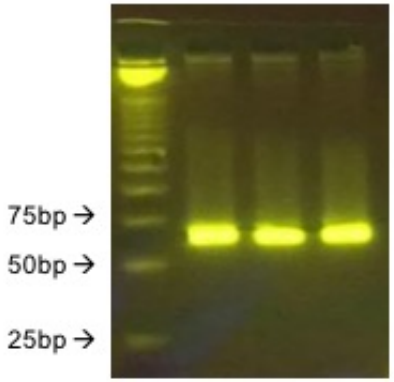


Figure E2- DNA sequencing cloned cDNA insert.

CTGATGTACTCTCAGTTCAGCTTCTCTTC
ACGAGAGGTATCGCTACGATGCACCATCT
GGAGGTAGTTAGA - 71 bp

Figure A3- Available ELOVL2 sequences.

1). NM_169001.4
-*Drosophila melanogaster*

2). MF279189.1
-*Tenebrio molitor*

Figure B3- Multiple alignment of sequences for ELOVL2. Target location for PCR: primer locations highlighted with rectangle and arrows.

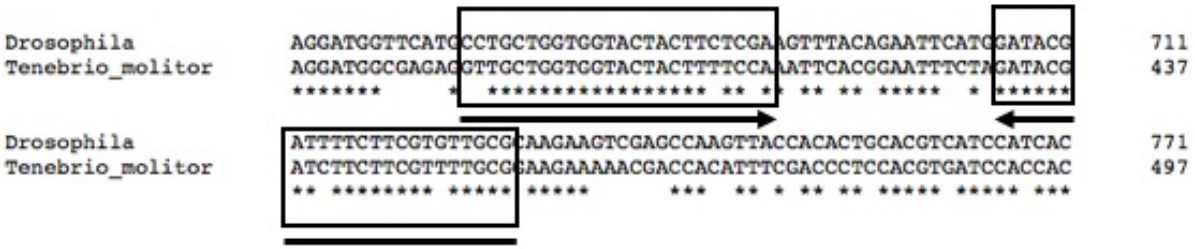


Figure C3- PCR conditions.

Fwd. primer- 24bp
(GTTGCTGGTGGTACTACTTTTCCA)

Start- 391 of 1026 bp
(T. molitor sequence)

Rev. primer- 23bp
(CGCAAAACGAAGAAGATCGTATC)

Start- 454 of 1026 bp
(T. molitor sequence)

Amplicon size: 64 bp
Annealing temp: 62.9°C
Cycles: 35

Phusion Hot Start II DNA Polymerase

Figure D3- ELOVL2 PCR product run on 5% metaphor gel alongside 25bp marker.

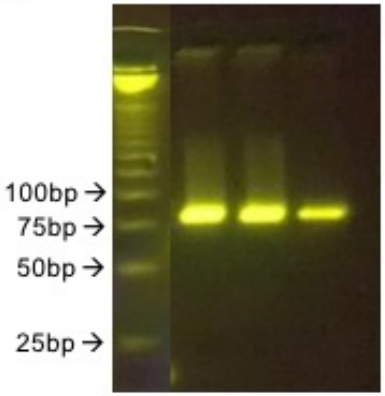


Figure E3- DNA sequencing cloned cDNA insert.

Inconclusive.