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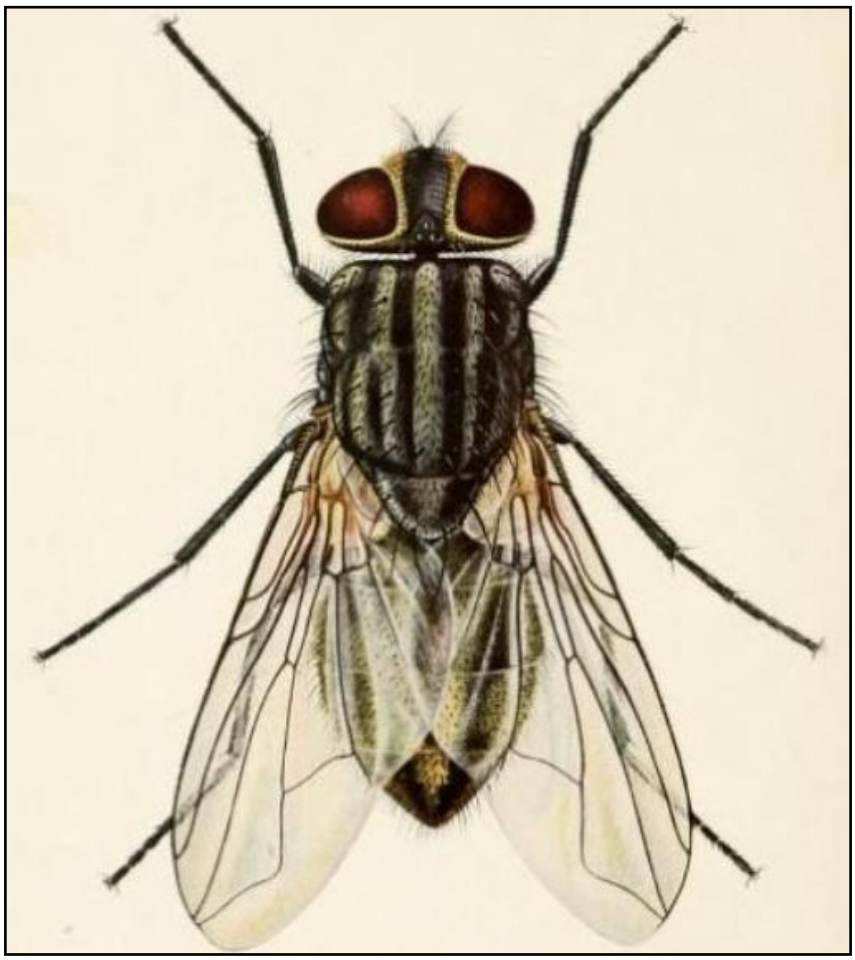

Howard, 1911

Lipid analysis of Musca domestica larvae reared at different temperatures

HBO THESIS PROPOSAL

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AERES UNIVERSITY OF APPLIED SCIENCES

VU VRIJE UNIVERSITEIT AMSTERDAM

Lipid analysis of *Musca domestica* larvae reared at different temperatures

Bachelor thesis of Applied Biology

By Niels Kolder

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Preface

With this research I hope to eliminate part of this knowledge gap by providing insight into the lipid characteristics of the insects under industrial-like breeding conditions. The following work is interesting for possible investors that want to expand their knowledge of insect lipid characteristics. In addition, it might offer a new perspective to people that are working on understanding the interplay between saturation and fatty acid chain length.

This paper has been written in order to fulfil the graduation requirements of the HBO bachelor "Applied Biology". This is part of the last stage of a four-year course at the Aeres University of Applied Sciences. The research itself was conducted at the Vrije Universiteit Amsterdam (Free University Amsterdam) within the Department of Ecological Science. Here the research question was formulated in cooperation with international Doctoral student Francesco Boatta.

I would like to thank mister Boatta for letting me participate within his research and for all his guidance and patience throughout the project. As for other people at the Free University, Professor of Evolutionary Ecology Jacintha Ellers deserves a special mention as head of the department and organiser of the workgroup meetings.

Finally, I would like to thank my coaches from Aeres, Marieke Bos and especially Dinand Ekkel for supervision and help during the final stages of my research.

Niels Kolder,

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Abstract

Soybean and fishmeal are obtained in large quantities to produce animal feed. This puts an immense stress on today's environment. Insect larvae are seen as a possible replacement of soybean and fishmeal but there is still a large knowledge gap preventing large scale production. Many species of insect larvae contain a favourable lipid profile for use in animal feed. These values are usually acquired from insect larvae reared at optimal conditions (25 °C). However, when rearing larvae at mass they are exposed to sub-optimal conditions (35 °C), from the large number of larvae together. The goal of this study is to discover if this change in rearing temperature has an effect on lipid composition. Housefly larvae (*M. domestica*) were used because of their short reproductive cycle and good nutritious value. Eggs were randomly assigned to either the control (25 °C) or treatment group (35 °C). Each group consisted of six jars containing 200 eggs. A portion of the larvae from each group was sampled during the beginning, middle and end of their third (and last) instar. Gas chromatography-mass spectrometry (GC-MS) was also used to observe any differences within the saturation and fatty acid chain length levels. There was an interaction effect found between development stage and temperature. It showed that treatment larvae started with less dry weight and absolute lipid content but ended up with significantly more. The dry weight increase was 22,2 % and the absolute lipid content increase was 20,3 %. This went against expectations as it was believed that the high energy requirements of larvae reared at high temperature combined with the temperature-size rule would limit their maximum growth and lipid storage capabilities. As for the GC-MS results, the two most notable changes were a 34,6 % decrease in Pentadecanoic acid (15:0) and a 19,1 % increase in Oleic acid (18:1). This corresponds roughly with what was expected for fatty acid chain length as long chain fatty acids help the membrane coop with the increased heat. The fact that saturation also went up is odd considering it goes against the expected homeoviscous adaptation of the membrane fluidity.

In conclusion, larvae reared in warmer temperature are surprisingly larger and show an increase in lipid quantity compared to larvae reared under more optimal conditions. As for the lipid profile, it showed small changes on individual lipid level but without causing deterioration of the nutritional value.

Samenvatting (Dutch abstract)

Sojabonen en vismeel worden in grote aantallen verkregen voor de productie van diervoeding. Dit is uiterst belastend voor het milieu. Insectenlarven worden gezien als mogelijke vervanger van sojabonen en vismeel maar er is nog een groot kennistekort wat productie op grote schaal weerhoudt. Veel soorten insectlarven beschikken over een gunstig vetprofiel voor het gebruik in dierenvoeding. Deze waarden worden meestal verkregen van insectenlarven die zijn gekweekt onder optimale omstandigheden (25 °C). Echter, tijdens de massa kweek worden de larven blootgesteld aan suboptimale omstandigheden (35 °C), vooral hoge temperatuur door de grote aantallen larven bij elkaar. Het doel van deze studie is om te ontdekken of deze verandering in kweek temperatuur ook effect heeft op de vetcompositie.

Er is voor de Huisvlieg (*M. domestica*) gekozen om zijn korte reproductie cyclus en geschikte voedingswaarde. Eitjes werden willekeurig toegewezen aan de controle- (25 °C) of treatmentgroep (35 °C). Elke groep bestond uit zes potten met ieder 200 eitjes. Een deel van de larven van elke groep werd bemonsterd tijdens het begin, midden en eind van hun derde (en laatste) stadium. Daarnaast werd Gas chromatography-mass spectrometry (GC-MS) ook ingezet om eventuele verschillen in verzadigdheid en vetzuurlengte waar te nemen.

Er werd een interactie-effect gevonden tussen ontwikkelingsstadium en temperatuur. Het toonde aan dat treatmentlarven beginnen met minder drooggewicht en absoluut vetgehalte, maar aan het einde van het derde stadium aanzienlijk meer drooggewicht (+ 22,2%) en absoluut vetgehalte (+ 20,3%) bevatten. Dit was tegen de verwachtingen in aangezien er van uit werd gegaan dat de hoge energiebehoefte van het kweken bij hoge temperatuur in combinatie met de temperture-size regel hun maximale groei en vet opslagcapaciteit zou beperken. Wat betreft de GC-MS-resultaten, de twee meest opvallende veranderingen waren een afname van 34,6% in pentadecaanzuur (15: 0) en een toename van 19,1% in oliezuur (18:1). Dit kwam ongeveer overeen met de verwachtingen voor vetzuurlengte, aangezien vetzuren met lange ketens het membraan helpen omgaan met de verhoogde warmte. Het feit dat de verzadiging hierdoor ook steeg is vreemd aangezien het indruist tegen de verwachte 'homeoviscous adaptation' van de membraanvloeibaarheid.

In conclusie, larven gekweekt onder warme omstandigheden (35 °C) zijn verassend genoeg groter en vertonen een toename van het absolute vetgehalte in vergelijking met larven die onder meer optimale omstandigheden (25 °C) zijn grootgebracht. Wat betreft het vetprofiel, kleine veranderingen op individueel vetniveau waren zichtbaar, maar dit leidde niet tot verslechtering van de voedingswaarde.

Inhoud

1. Introduction 1
2. Material and methods 4
2.1 Housefly (*M. domestica*) culture keeping 4
2.2 Temperature experiment 4
2.2.1 Control and Treatment setup 4
2.1.1 Sampling and storage 5
2.1.2 Quantitative analysis 5
2.1.3 Qualitative analysis 5
2.3 Processing data and statistical analysis 7
2.3.1 Quantitative statistics 7
2.3.2 Qualitative statistics 7
3. Results 8
3.1 Quantitative results 8
3.1.1 Dry weight 8
3.1.2 Absolute lipid content 9
3.1.3 Relative Lipid Content 9
3.2 Qualitative results 9
3.2.1 Saturation 9
3.2.2 Fatty acid chain length 10
4. Discussion 11
4.1 Reflection on the execution 11
4.2 Changes in Dry weight (Sub question one) 11
4.2.1 Continues growth at 35 °C (Side note) 12
4.3 Changes in Lipid Quantity (Sub question two) 12
4.4 Additional factors within the quantitative experiment 13
4.5 Saturation and fatty acid chain length (Sub questions three and four) 13
4.5.1 Stable palmitoleic acid (16:1) levels (Side note) 14
5. Conclusion 15
Appendix I: Substrate combinations 16
Appendix II: Lipid profile 17
Bibliography 18

1. Introduction

Humans today are able to produce more food than ever before. This is reflected in the number of livestock currently harboured. Chickens are by far the most common livestock with a global stock increase of five times between 1960 and 2010 while simultaneously doubling in average body size ("livestock Systems", n.d.; Robinson et al., 2014). According to the latest data from the Food and Agriculture Organisation of the United States approximately 23 billion chickens are kept as livestock worldwide (Qualman, 2018). This level of food production has made their meat more readily available for a lot of families, however not without its drawbacks (Ritchie & Roser, 2020). The resources and land needed to sustain this production are immense and create a large manure surplus (Veld & Kraan, 2000). To be able to maintain this level of production while at the same time decreasing the waste produced, thorough innovations in the sector are needed. The establishment of a circular approach to manage chicken waste will help decrease the manure surplus and reduce its negative repercussions on the environment (Van Zanten, 2016).

Fishmeal has been an important ingredient for many livestock feed, including chicken, but the price of this commodity increased in time and in order. To keep the production of animal feed viable, manufacturers replaced this ingredient with cheaper and more readily available products like soybean. Next to soybean, the rising popularity of a protein-rich diet based on larvae could also provide an alternative for the stagnating supply of fishmeal and limit its production impact on the already deteriorating aquatic ecosystems (Rumsey,1993). Soybean is, just as fishmeal, considered to be one of the main protein supplements used in animal feed, with a global production of approximately 360.993 million metric tons in 2018 alone (with Brazilian and American farms accounting for over 66%) ("World Soybean Production", 2018). The production of these soybeans is shifting from temperate to more tropical areas as the ground is much cheaper. This poses an immense threat to the biodiversity of mostly Latin American countries (Fearnside, 2001). Both the soybean and fishmeal industries are hard to sustain without pressuring the environment and biodiversity. That is why the research into alternative protein sources is so important. It has already been established that insect larvae contain significantly more crude protein than soybeans, on top of that they have a more balanced amino acid profile which closely resembles that of fishmeal (Makkar, Tran, Heuzé, & Ankers, 2014). Even when looking at micro-mineral levels it shows that the larvae have overall higher manganese, copper and zinc values than soybean and fishmeal (Hussein et al., 2017). There is however one drawback which has to do with their high saturated fatty acid level as unsaturated fatty acids are generally more desired in animal feed (Henfling, 2017). Next to being nutritious they are found almost everywhere in the world and are relatively easy and sustainable to rear (Hogsette, 1992). This makes insect larvae an interesting candidate for the development of a novel eco-friendly protein source.

Further than being an excellent sustainable source of protein, insect larvae are seen as a possible candidate for the creation of a circular approach in livestock waste management, with particular regard to the *Diptera* order which naturally feed and develop on this organic material. Beyond being able to directly feed on manure, insect larvae can also support the decomposition of organic waste by facilitating the aeration, the conditioning and the fragmentation of the substrate which in turn supports the break down action of microorganisms (Yadav & Garg, 2011). This has been tested various times as many works were recently published confirming that larvae are able to convert low-quality organic matter into valuable resources. Housefly larvae (*M. domestica*) reared on cattle manure are able to reduce the manure mass by as much as 5,2% in less than 7 days (Hussein et al., 2017); furthermore when the same larvae are raised on swine manure they significantly reduce the organic carbon and nitrogen content (Zhang, Wang, Zhu, Suneethi, & Zheng, 2012).

Using larvae as an alternative protein source while at the same time reducing waste is not a new concept as it was already recognised as early as in the nineteen sixties and seventies (Calvert, Martin & Morgan, 1969; Miller, 1969; Calvert, 1979), although back then it did not always seem economically viable. However, the rising production demand enhanced the problem of manure management, particularly that of poultry which is difficult to dispose. Coupled with the eco-conscious mentality of nowadays it sparked a renewed interest into the use of insects as animal feed. This study would like to contribute to the realisation of that goal by researching the validity of rearing housefly larvae (*M. domestica*) as animal feed. Housefly larvae (*M. domestica*) were chosen as the focus of this study as they are one of the most widespread and well-known species of *Diptera* (Dahlem, 2009). Another major factor in choosing the housefly was their relatively short reproductive cycle of only 13 days (at 25 °C), compared to the black soldier fly (*Hermetia illucens*) who takes roughly 36 days to reach sexual maturity (Wang, Yang, Zhang, Tao, & Wang, 2018; Ferrarezi, Cannella, Nassef & Bailey, 2016).

Most studies recognise 25-30°C as an optimal range for a fast-larval development, high survival rate and high mean weight and so this is used in many fly rearing projects (Chapman & Goulson, 1999). This is however not representative for an industrial rearing setup as to achieve a large supply of larvae they will need to be stocked together in large quantities causing the temperature to rise. This means that during large scale breeding they will be exposed to more extreme temperatures (35 °C and above). It should be considered that houseflies (*M. domestia*) are ectothermic organisms and therefore their metabolism is strictly related to the environmental temperatures. Beyond this, it has been observed that rearing temperatures have a repercussion on the fatty acid composition/lipid quality. A previous study has explored the performance of the three main glycerophosphatides (phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine) of *M. domestica* larvae under various thermal conditions. It found that if the rearing temperature was increased the amount of unsaturated fatty acids would decrease (Robb, Hammond & Bieber, 1972). Many studies have agreed that this can be attributed to "Homeoviscous adaptation", a process which involves changing the membranal lipid composition in order to maintain the appropriate liquidity (Dooremalen, 2010; Mcelhaney, 1984; Neidleman, 1987). Fluidity of lipids is also bound to the body temperature of the examined organism, meaning that the melting point of the lipids is in turn connected to the temperature at which the organism normally exists. This would indicate that an organism with a high standard body temperature would also possess a high lipid melting point and vice versa (Mcelhaney, 1984). Houseflies are able to survive and reproduce between temperatures of 15 to 40°C (Rozendaal, 1999). As the flies used in this study originate from a Dutch strain it is estimate that their standard body temperature should be aligned more closely to the bottom part of their thermal spectrum (15 to 40°C; Rozendaal, 1999). This is important to keep this in mind as tests with different strains (e.g. strains with a higher melting point) could therefore (among other factors) yield different results.

Saturation is not the only factor playing a part in enhancing membrane fluidity. Fatty acid chain length can also change membrane fluidity due to the intermolecular interactions between the phospholipids. The longer the tails the more interactions between the tails are possible increasing the tenacity and resulting in a less fluid membrane ("What determines fluidity", 2017). Both Fulco & Fujii (1980) and Russell & Sandercock (1980) found this same effect already in 1980 during their studies on microorganism membrane fluidity. This was further expanded upon when it was concluded that cold temperature is a major trigger for shorter chain lengths (Russell, 1983; Dawaliby et al., 2015).

All of the previous mentioned factors could influence the lipid profile of the larvae. This creates an area of uncertainty for anyone who would like to invest in insect-based animal feed. In order to make the investment safer it is necessary to provide insight into the lipid characteristics of larvae under industrial temperature.

Based on this understanding the main research question was drafted:

"Does high temperature (35 °C) have an effect on the lipid composition of larvae (M. domestica) from a qualitative and quantitative point of view?"

This sparked four sub questions that would make it possible to better interpret the main question. **1** Does high temperature influence overall dry weight at different sampling moments? Due to the faster development at high temperature the window in which the larvae can accumulate the nutrients it needs is considerably shorter. It is thus expected that the treatment larvae (reared at 35 °C) will possess a lower dry weight compared to the control (reared at 25 °C). **2** Is there a difference in the overall quantity of lipids among temperatures and between sampling moments? A drop in relative- and absolute lipid quantity in treatment group is expected as it is common for ectotherms to show a reduction in stored lipids when reared at high temperature. This is attributed to the interplay between the nutritional requirements and the high energy requirements of larval stages reared at high temperatures (Lee & Roh, 2010). As for the lipid quantity between sampling moments it is assumed that the larvae will undergo an initial increase in relative- and absolute lipid content for both the control and the treatment animals, followed by a stagnation right before pupation. This is based on a study that found that the most lipids were accumulated during the third (and final) instar of the black blow fly (*Phormia regina*), another prominent member of the *Diptera* family (Wimer & Lumb, 1967). **3** Does the temperature influence the ratio of saturated to unsaturated fatty acids? Expected is to find a substantial difference in the fatty acid composition, more specifically a higher saturated to unsaturated ration. This hypothesis is based on the theory of 'Homeoviscous adaptation' explained earlier. **4** Does the temperature affect the average fatty acid chain length? Following the same theory of "Homeoviscous adaptation" a higher temperature should result in an increase in the overall fatty acid chain length to compensate for the increased fluidity caused by heightened temperature. This would also correspond well with the results found by Russell (1983) and Dawaliby et al. (2015), which both found shorter chain lengths to be more dominant during cold temperatures.

The present work is part of a larger research project (*"Utilizing livestock manure for mass rearing of houseflies to develop a fully-closed production system that can be applied locally, mitigating the need for transport of their end-product."*) whose goal is to take advantage of the ability of housefly larvae to naturally feed on manure to produce a supply of protein-rich feed for chicken and tackle the chicken waste management at the same time. Hopefully this research will be able to deliver valuable information on the development of housefly larvae when reared at temperatures resembling those of industrial settings, while Simultaneously providing a characterization of the lipid profile of these animals for their application as chicken feed. The investigation of their lipid composition will help determine if an industrial setting will produce larvae with lipid content and quality similar to those reared at standard temperatures.

2. Material and methods

The experimental setup was located at the Vrije Universiteit Amsterdam (Free University Amsterdam) which allowed easy access to various high-grade equipment used to aid the research.

2.1 Housefly (*M. domestica*) culture keeping

The experiments were conducted using a Dutch strain of *M. domestica* sampled in Gerkesklooster that has been reared in the lab for 25 generations at 25°C. The colonies were kept in a climate room (25°C) and provided with milk powder, sugar and water *ad libitum*. They were housed in 30x30x33 cm rearing boxes with gauze on three of the sides and glass on the top. The photoperiod was set to 16:8 L:D and the room had a relative humidity (RH) of 65%. To maintain the colony, oviposition was stimulated two or three times a week. The egg laying device was composed of a slightly wet black sock containing hydrated substrate that was placed into a small plastic cup (see appendix I: Substrate combinations). To allow oviposition the device was placed into the adult cage and left there for 5 hours. The laid eggs were removed with a soft brush and weighted. Previous results (Pieterse & Gloy, 2013) have shown that the best seeding ratio for the production of biomass is achieved using 6,32 eggs per gram of wet substrate. Therefore, this ratio was used to rear the larvae destined for colony replenishment. The eggs for fly replenishment were housed in 300 ml jars and placed in the same climate room as the adult colonies.

2.2 Temperature experiment

The temperature experiment was performed using freshy laid *M. domestica* eggs (200 per replicate). The eggs were collected using the egg laying device previously described, individually counted under a stereoscope and transferred to a pre-wet cellulose filter (2 cm diameter). This filter is subsequently placed in a 300 ml jar with mesh on top and filled with 31,65 g of freshly prepared substrate. The jar was than randomly assigned to either the control or the treatment group and placed there as soon as possible.

2.2.1 Control and Treatment setup

The control group was located in a climate room at 25°C, with a photoperiod of 16:8 L:D and a RH of 65 %. The treatment group was reared at 35°C in a Heraeus B 5060 EK CO2 static incubator. A halogen lamp was used to provide a photoperiod (16:8 L:D) while the RH was controlled using a saturated solution of NaCl which at 35 degrees provides a constant RH of approximately 64,6 % (Choudhury, Sahu, & Sharma, 2011). Preliminary tests have shown that this would allow for a relatively stable RH inside the incubator throughout the duration of the experiment.

The test jars maintained a 200 eggs per jar threshold as it has been observed to be an optimal rearing density for maximizing biomass and it makes the results comparable to previous tests (Pieterse & Gloy, 2013).

2.1.1 Sampling and storage

Preliminary tests showed that larvae reared at 25°C started to pupate around five-six days. This is why the samples of the control group were taken 74 (t_1), 98 (t_2) and 122 (t_3) hours after egg laying. These times correspond to the start, middle and end of their third instar (Wang et al., 2018). The larvae were sampled at random after which they were put in a small PCR vial and immediately placed in N_2 (liquid nitrogen). The larvae were then removed from the liquid nitrogen and placed at -20 °C until analysis.

In order to compensate for their faster development, the sampling time of the treatment group was based on Wang et al. (2018) results in which they calculated the development time of housefly larvae reared at 34 °C. This approach was used to compensate for the faster development occurring at higher temperature and to allow the sampling of the larvae at the beginning, middle and end of the third instar for both the control and treatments groups. This meant that the opportunities to sample the treatment group took place 45 (t_1), 67 (t_2) and 85 (t_3) hours after egg laying. Two different experiments took place with larvae from the control and treatment group: the quantitative analysis and the qualitative analysis. For the quantitative analysis five larvae were sampled from every jar during every sample point from both the control and treatment group. For the qualitative analysis also five larvae were sampled from every jar but only during the third sample point (t_3). The five larvae of the qualitative analysis were pooled together in one vial while the larvae from the quantitative analysis were stored separately.

2.1.2 Quantitative analysis

The main goal of the quantitative analysis was to the determine the difference in lipid quantity between larvae reared at 25 and 35 °C. This was achieved via an ether-based extraction. To conduct this analysis, the larvae were (while still frozen) placed into a crucible provided with weighting paper on the bottom. These crucibles were then positioned at 50 °C for 72 hours in a ventilated oven. Half an hour before weighing they were taken out of the oven and placed in a desiccator allowing them to cool off. The weighing was done using a high precision Mettler Toledo UMT2 Ultra-Microbalance (±0,0001 mg). During the weighing sessions silica gel was placed inside the scale to reduce the RH in the microscale chamber and thus the absorption of atmospheric water by the samples. In order to not damage the larvae during handling feather tweezers were standard throughout the whole procedure. After weighing, the larvae were immediately transferred to a test tube with ground glass stopper filled with 5 ml of diethyl-ether. The samples stayed in the vial for 72 hours to allow the solvent enough time to extract the ether-soluble lipids. After the extraction the larvae were removed from the tube, cleaned with fresh diethyl-ether and airdried for another 24 hours before the second weighing session begun. The second weighing session followed the same weighing protocol as the first. For each larvae the lost lipid weight is now known, and they can be compared to each other.

2.1.3 Qualitative analysis

The main goal of the qualitative analysis was to determine fatty acid composition of the larvae. The lipids were extracted using an adaptation of the protocol used by Matyash, Liebisch, Kurzchalia, Shevchenko & Schwudke (2008). Methyl-tert-butyl ether was used as solvent, this allowed for a faster and cleaner lipid extraction than a more standardised chloroform/methanol extraction. The end goal was to prepare the samples for the GC-MS (Gas chromatography–mass spectrometry) analysis. The whole procedure consisted of three main parts: Extraction, saponification and methylation. Both the saponification and the methylation protocols were based upon the MIS Operating Manual (2012) including an adaptation suggested by van Dooremalen & Ellers (2009) to maximize the recovery of poly-unsaturated fatty acids (PUFAs). The five larvae were homogenized in a glass potter homogenizer after being diluted in 5 ml of 0,1% ammonium acetate solution (water/ammonium ac. 99,9:0,1 g/g). Once homogenised a Pyrex tube with Teflon lined cap and 2,5 ml of methanol was prepared. 200 ul of the homogenate was then sampled and poured into the Pyrex tube after which it got vortexed for 10

seconds. The Methyl-tert-butyl ether is now added to the mixture. The incubation took 1 hour and took place at room temperature in a shaker, horizontally placed and with an oscillation frequency of 250 min^{-1}. The shaker made the lipids dissolve in the organic solvent (Vidya-mitra, 2015). To induce phase separation 1,25 ml of MS-grade (Milli-Q) was added and the samples were then centrifuged at 1000 g for 15 minutes at a temperature of 20°C. Now the two phases were clearly visible with the upper phase retaining the lipids in the organic solvent and the lower phase retaining the hydrophilic components and salts (Eggers, 2016). The upper organic phase was collected and placed in a clean tube. This tube was then transferred to a heated water bath (<40°C) under a constant N_2 flux to speed up the evaporation process and minimize lipid oxidation. The samples were then placed back in the shaker for 30 minutes and centrifuged at 1000g for 15 minutes at 20°C. The upper phase of these replicates was sampled and pipetted into the vials already under N_2 Flux (Matyash, 2008).

Once the solvent had evaporated the samples were removed from the N_2 flux and saponified. During the saponification the fatty acids are cleaved from the cell lipids (removal of the glycerol backbone) and are converted to their sodium salts. This was done by adding 1 ml of saponification solution (45 g HaOH, 150 ml CH_3OH, 150 ml Milli-Q H_2O) to each sample. To reduce lipid oxidation during this step, the heads of the tubes were flushed with N_2 and promptly closed before being vortexed for 10 seconds and placed in a water bath of 70°C for 90 minutes (samples were briefly vortexed again after the first 5 minutes). In the end the tubes were removed from the water bath and cooled in cold water. Slow saponification (85 minutes instead of 25 minutes) at a low temperature (70 °C instead of 95 °C) was preferred as it maximizes the quality of the PUFAs retrieved (Dooremalen et al., 2009).

Following this step, the free fatty acids were then converted into their methyl esters (FAMEs) by means of methylation. During methylation the polar carboxyl functional groups are neutralized and chemically converted to a non-polar group in order to make the molecule more volatile, so that it can be analysed in gaseous state (Dasgupta & Wahed, 2014; Řezanka, Pádrová & Sigler, 2016). This was done by adding 2 ml of derivatisation solution (325 ml 6.0N HCl, 275 ml CH_3OH) to each tube. The tubes were then placed once again in the heated bath, this time at 80°C. After 10 minutes the tubes were removed and cooled with cold water (MIDI, 2012). To extract the FAMEs from the acidic aqueous phase and transfer them to an organic phase, a liquid-liquid extraction was performed. This was done by adding 1,25 ml of extraction solvent (Hexane/MTBE 1:1) to the vials that were subsequently placed in the shaker for 10 minutes. After mixing, the solvent (upper) phase was carefully removed and placed into a clean tube, this extraction was repeated twice. The tubes were then placed under N_2 flux to evaporate the solvent. Once evaporated, 3 ml of dilute base solution (Na hydroxide 10.8, 900 ml deionized water) was added to the vials. The base wash will remove the remaining free fatty acids and residue regent that can otherwise damage the chromatographic system (MIDI, 2012). The vials including the base wash were then placed in a shaker for 5 minutes and are subsequently centrifuged at 2000 RPM for 3 minutes. After centrifuging 4 drops of saturated salt solution (NaCl, H_2O) were added to each vial to eliminate the emulsion that will otherwise be present at the interphase of the two liquids. A new Pasteur pipette was used for each sample to extract 2/3 of the upper (organic) phase and transfer it to a GC vial. The solvent was evaporated under N_2 and the lipids were successively diluted again by adding 500 ul of hexane to the vials. At last the vials were capped and send to the Institute for Environmental Studies (IVM) at the VU for the chromatography analysis.

2.3 Processing data and statistical analysis

IBM SPSS Statistics 24 was used to manage the data of both the quantitative and qualitative data.

2.3.1 Quantitative statistics

A two-way ANOVA was executed comparing sample point t_1, t_2 and t_3 of the control (n = 90) and treatment (n = 90) group to one another. The two-way ANOVA is able to tell if there are any main- and simple effects present. What it does not tell is between which groups these effects occur. This was solved by alternating the SPSS syntax in order to compare the effect of temperature (control and treatment) on larvae of sample point t_1, t_2 and t_3 individually. The same method can also be used to observe the difference between t_1, t_2 and t_3 within both temperatures. Both forms of the calculation were present in the statistical analysis. The three variables tested for were dry weight, absolute lipid content and relative lipid content.

2.3.2 Qualitative statistics

The Gas chromatography–mass spectrometry (GC-MS) will provide both the type of lipid and give an indication of the amount present in the sample. It does this by projecting peaks on a chart. Each peak retains a probability value that indicates how confident the program is in linking this peak to a specific type of lipid. By numbering the peaks, it will be possible to compare the control (n = 6) to the treatment (n = 6) as in both tests the same lipids will exit the GC-MS at the same time. Peaks can be merged if multiple peaks are in close proximity and are also of the same kind of lipid. A full list of all the lipids found during this test is included in appendix II: Lipid profile.

To see if the ratio of saturation changed based on temperature each peak (or set of similar peaks) is ranked as either saturated or unsaturated. This was done for both the control and treatment samples. The area of the peaks is used as an indicator of the frequency of the lipids. The peak areas are converted to a relative percentage of their total sample area. These relative peak sizes (not the absolute peak sizes) were used during the statistical analysis. For the test the saturated relative area of the control samples was compared to the saturated relative area of the treatment samples. The same test was repeated but then for the unsaturated relative areas. These comparisons were executed by means of a Mann-Whitney U test as the data was expected to be non-normally distributed as a result of the variable area sizes that were likely appear while testing for many different lipids.

Comparing fatty acid chain length followed roughly the same steps. The relative peaks were again categorised in to one of two groups: Short-chained FA's and long-chained FA's. The short FA group included all the fatty acids with a chain length below sixteen carbons while the long FA group contained all FA above. The overrepresentation of the Palmitic- and Palmitoleic acids (16:0 and 16:1) that was observed in the preliminary test resulted in the use of the 16 carbon fatty acids as dividing factor between the short- and long-chain FA's. The 16 carbon fatty acids were themselves excluded form statistical analyses as it was believed that their large representation would bias the result. The same Mann-Whitney U setup was used to test for differences in chain length. This meant that within the short-chain FA group the control and treatment were tested against each other. This was also done within the long-chain FA group.

3. Results

The result section is divided in to two main chapters. First the data from the quantitative analysis which includes dry bodyweight, absolute lipids and relative lipids. The second part presents the results of the qualitative analysis which includes the saturation comparison and the chain length comparison. During the experiment the fifth jar of the treatment group got compromised which led to the exclusion of these 15 samples from the quantitative experiment. Furthermore, another six samples (five treatment and one control) got removed as they were deemed not trustworthy and representative enough for the experiment. This meant that 89 control samples and 70 treatment samples were available to be used during the quantitative experiment. The exclusion of treatment jar five also effected the number of the qualitative analyses. Eleven GM-MS analysis (six control and five treatment) were now included instead of the planned twelve.

Control temperature and humidity were well regulated by the climate chamber which maintained a steady 25 °C with 65 % relative humidity. Treatment temperature was regulated via the incubator and averaged out at 35,9 °C. Relative humidity proved more of a challenge since the incubator did not include a humidity regulator like the climate room. An average relative humidity of 59,4 % was achieved during the test as a result of the saturated salt solution. As for comparing larval hatch rate between temperatures a one-way ANOVA was used. Treatment jar two was excluded from the test as it proved to be an outlier. The hatch rate turned out to be slightly lower for the treatment group (n = 10; p = 0,003). The control showed on average a hatch rate of 161,3/200 while the treatment showed a hatch rate of 132,5/200.

3.1 Quantitative results

3.1.1 Dry weight

In figure 1, the results of the dry weight analysis are presented. Statistical analysis revealed an interaction effect present between temperature and sample point. Figure 1 clearly shows where the two-way ANOVA found the interaction (n = 159; p = 6,2e-10). Note that, although the figure uses lines, no measurements were done between the sample points. The fact that an interaction was found allows for inspection of the simple effects instead of the main effects.

The first simple effect was found when comparing sample points within the control group as t_1 is both significantly different from t_2 (n = 59; p = 5,8e-11) and t_3 (n = 59; p = 1,1e-9). The difference between t_2 and t_3 was not statistically significant which meant that the dry weight of larvae reared at 25 °C stayed more or less the same between the middle and end of their third instar. When comparing sample points within the treatment group all sample points proved to be significant: t_1 with t_2 (n = 45; p = 6,9e-24), t_1 with t_3 (n = 46; p = 1,1e-31), t_2 with t_3 (n = 49; p = 0,004). This indicates that, unlike the control group, larvae reared at 35 °C would continue to grow larger even during the last phase of their third instar. This is visualised in figure 1.

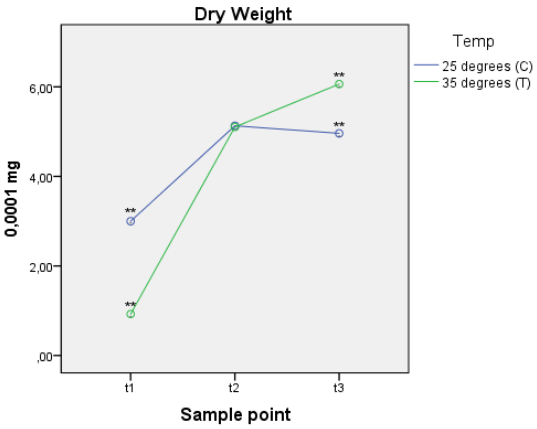

Figure 1: Dry weight content of the two groups of larvae at the beginning (t1), middle (t2) and end (t3) of their third instar.
** means p-value < 0.05*
*** means p-value < 0.01*

3.1.2 Absolute lipid content

In figure 2 the absolute lipid comparison shows (similar to dry weight) a significant interaction effect as the treatment group is faster in its accumulation of absolute lipid content (n = 159; p = 2,4e-4). When compared dry weight this interaction is less strong (See figure 1 & 2).

As for the simple effects, comparing the sample points within the control group resulted in a significant outcome between all the sample points, depicting a continuous increase in absolute lipids; t_1 with t_2 (n = 59; p = 1,5e-5), t_1 with t_3 (n = 59; p = 1,3e-9), t_2 with t_3 (n = 60; p = 0,047). The same is seen within the treatment group as it also shows a significant increase of absolute lipids over time; t_1 with t_2 (n = 45; p = 8,9e-14), t_1 with t_3 (n = 46; p = 4,7e-21), t_2 with t_3 (n = 49; p = 0,006).

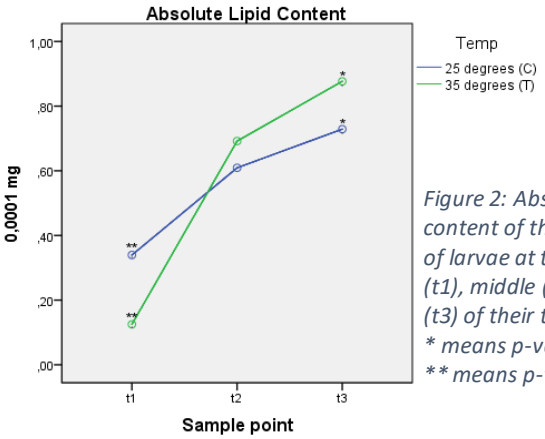

Figure 2: Absolute lipid content of the two groups of larvae at the beginning (t1), middle (t2) and end (t3) of their third instar.
** means p-value < 0.05*
*** means p-value < 0.01*

3.1.3 Relative Lipid Content

As can be seen in figure 3, relative lipid content is the only factor to shows no support for a significant interaction effect. This allows for examination of the main effects, both of which proved to be significant; Temperature (n = 159; p = 0,006) and Sample point (n 159; p = 3,1e-4).

Comparing sample points within the control group reveals that t_1 and t_2 are statistically similar. However, they both differ significantly from t_3 as it shows an increase in relative lipid content; t_1 with t_3 (n = 59; p =4,7e-5), t_2 with t_3 (n = 59; p = 0,002). The treatment shows a different result as here none of the sample points were statistically different from one another. Furthermore, when comparing control to treatment it stands out that during both t_1 (n = 50; p = 0,016) and t_2 (n = 54; p = 0,041) the treatment group contained relatively seen more lipid content then the control group. This difference then disappears when they both reach t_3 (See figure 3).

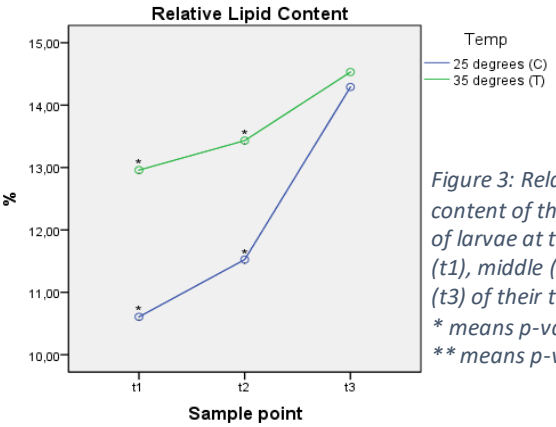

Figure 3: Relative lipid content of the two groups of larvae at the beginning (t1), middle (t2) and end (t3) of their third instar.
** means p-value < 0.05*
*** means p-value < 0.01*

3.2 Qualitative results

The qualitative analysis resulted in 27 different peaks per sample containing 13 different acids (six saturated to seven unsaturated). All the peaks containing the same acid were merged (see appendix II: Lipid profile). Palmitic acid (16:0) proved to be the most abundant acid type with a presence of ± 25 % in both groups closely followed by Palmitoleic acid (16:1) and Oleic acid (18:1).

3.2.1 Saturation

The average level of saturation in the control group (39,6 % saturated and 60,4 % unsaturated) was almost similar to the average level of saturation of the treatment group (38,1 % saturated and 61,9 % unsaturated). Nevertheless, they were analysed by means of a Mann-Whitney U test. Comparing the area of all the saturated fatty acids peaks of the control group (n = 39) to that of the treatment group (n = 32) yielded no significant result with a very large p-value of 0,945. Likewise, comparing the area of the unsaturated fatty acid peaks of the control group (n = 42) to that of the treatment group (n = 35) also shows a large similarity between the two groups with a p-value of 0,878. Therefor it is plausible to assume that during this test no major shift in the level of saturation occurred.

Smaller changes could have been overshadowed during the previous test as it incorporated all lipids together. For this reason, it was decided to run all individual lipids through a separate Mann-Whitney U test. During this analysis four of the thirteen lipids showed a significant difference over the control. The first was pentadecanoic acid (15:0) which turned out to be 34,6% lower in the treatment samples (n = 11; p = 0,006). Pentadecanoic acid made up 4,6 % of the control samples. The second lipid to deviate from the control was margaric acid (17:0) which saw a relative increase of 65,5 % compared to the control (n = 11; p = 0,006). This was the highest relative increase found during this research. The third lipid was called 7,10-hexadecadienoic acid (16:2) and showed a relative decrease of 31,0 % (n = 11; p = 0,006). However, both margaric acid and 7,10-hexadecadienoic acid make up less than 2 % of all the control samples and are therefore not very influential (see table 1). The fourth lipid, called oleic acid (18:1), increased with 19,1 % respectively compared to the control (n = 11; p = 0,006). This was also the only lipid that showed significance which had a realistic chance to alter the overall saturation as it contributed to 18,1 % of all control samples.

Fatty Acid	Saturated	Relative Increase/Decrease Compared to Control	P-value	% of Total Control
Lauric acid (12:0)	Yes	- 28,6 %	,619	0,1 %
Myristic acid (14:0)	Yes	- 9,1 %	,100	7,7 %
Undecenoic acid (11:1)	No	- 2,3 %	,855	0,6 %
Pentadecanoic acid (15:0)	Yes	- 34,6 % *	,006	4,6 %
Palmitic acid (16:0)	Yes	+ 1,7 %	,273	24,9 %
Palmitoleic acid (16:1)	No	- 6,8 %	,100	21,2 %
Margaric acid (17:0)	Yes	+ 65,5 % *	,006	0,3 %
7,10-Hexadecadienoic acid (16:2)	No	- 31,0 % *	,006	0,9 %
Cyclopropaneoctanoic acid, 2-hexyl (17:1)	No	+ 17,1 %	,584	1,4 %
Stearic acid (18:0)	Yes	+ 10,0 %	,584	2,0 %
Oleic acid (18:1)	No	+ 19,1 % *	,006	18,1 %
9,12-Octadecadienoic acid (Z,Z)- (18:2)	No	- 3,4 %	,715	17,1 %
Linolenic acid (18:3)	No	- 5,4 %	,361	1,1 %

Table 1: All fatty acids found within the qualitative test compared to the control. Significant deviations are marked with an asterisk which means p-value < 0.05.

3.2.2 Fatty acid chain length

From the 13 different lipids all lipids with a chain length below 16 carbons (n = 4) were compared to each other and all lipids with a chain length above 16 carbons (n = 6) were compared to each other. This left three lipids each with an exact 16 carbon chain length: Palmitic acid; Palmitoleic acid; 7,10-hexadecadienoic acid. They were taken out of the data set for the calculation. The control group showed an average relative chain length of 12,9/47,0/40,1 % (short/16-carbon/long) which was close to the treatment data of 10,6/45,7/43,7 % (short/16-carbon/long). The Kolmogorov-Smirnov test and the Shapiro-Wilk test showed that for both groups the data was not normally distributed and thus the nonparametric Man-Whitney U test was used. This was expected due to the vastly different peak area sizes per lipid. After comparing the short chain fatty acids from control and treatment to each other no significance was found (n = 44; p = 0,516). Also during the long chain comparison no significance was observed (n = 66; p = 0,425).

To exclude any missed significance the test was repeated but with the three 16 carbon lipids included as short chain fatty acids. This resulted in the control group having a more balanced 59,9/40,1 % (short/long) distribution while the treatment group maintained a similar 56,3/43,7 % (short/long) distribution. The reorganised short chain fatty acid data was implemented into SPSS for the Mann-Whitney U test between control and treatment, again it yielded no significant result (n = 77; p = 0,549).

4. Discussion

The discussion will go along all four sub questions before confronting the main research question. This is done in order to improve readability and to provide maximum support for the final assessment. The sub questions that are the subject of specific paragraphs can be found above these paragraphs. Sub questions three and four (both about lipid quality) are covered in one long section as their outcome proved to be more intertwined than previously thought. In addition, side notes can be found beneath some paragraphs to cover observations that were not necessarily needed for answering the original sub questions. These side notes were included as they might contain interesting information for people willing to delve deeper into the subject matter.

1) Does high temperature influence overall dry weight at different sampling moments?

2) Is there a difference in the overall quantity of lipids among temperatures and between sampling moments?

3) Does the temperature influence the ratio of saturated to unsaturated fatty acids?

4) Does the temperature affect the average fatty acid chain length?

4.1 Reflection on the execution

Overall the research went as expected with the exception of time management. During the research it became clear that even simple tasks needed a more systematic and regulated approach. On top of that it was difficult finding the right place for the treatment group as the normal temperature rooms would not reach all the way till 35 °C. This led to an extension of the preliminary test time which results in a delay of a few weeks. Rearing the cultures also took a little bit more time per day as different jars and holding boxes were experimented with before settling on a good combination.

Weighing the larvae for the quantitative test went well because using the microbalance in combination with handling diethyl ether was practiced a lot prior to the main test. The practical part of the qualitative test also met little resistance. The laboratories at the VU contained all the essential chemicals for the preparation of the GC-MS samples and the manual was easy to follow. Analysing the samples proved to be the most challenging as at first it was unclear which statistical program would be used. IBM SPSS Statistics 24 was ultimately selected but limited knowledge of the program slowed down the progress. By the time all tests were properly executed the time spent had already far exceeded the initial time limit.

4.2 Changes in Dry weight (Sub question one)

Although treatment larvae were initially smaller, they grew very quickly even when taking into account the adjusted sampling times. They caught up with the control larvae amid the third instar and during the last sampling moment (right before pupation) the treatment larvae enjoyed a 22,2 % weight advantage. This was not expected as it seems like a clear violation of the temperature-size rule (TSR) which states that: 'A reduction in environmental temperature causes an increase in adult size in the majority of ectotherms studied' (Atkinson, 1994, cited in Angilletta, Steury & Sears, 2004). However, one of the key words in the previous sentence was 'adult'. During this research only larval weight was examined which is less susceptible to be influenced by the TSR, therefore it is difficult to coin it as 'literal' violation (Forster, Hirst, & Atkinson, 2011; Kingsolver & Huey, 2008). The increase in individual biomass at high temperatures is welcomed as it could act as a counterbalance to the decrease in biomass caused by the lower hatch rate.

4.2.1 Continues growth at 35 °C (Side note)

The research confirms that the dry weigh of larvae reared at 25 °C remains relatively stable during the second part of the third instar. The stagnating growth is explained by the fact that prior to pupation the larvae stop eating and empty out their gut content (gut purging). This makes the growing pattern of the treatment group, which continued to gain in size even before pupating, all the more interesting.

4.3 Changes in Lipid Quantity (Sub question two)

It was predicted that larvae reared at industrial temperatures would contain less lipids than larvae reared in more 'optimal' conditions. This prediction rested on the presumption that the high nutritional requirements of the fast-growing larvae would limit their lipid storage capabilities (Lee & Roh, 2010). However, when comparing both temperatures to one another a pattern similar to that of dry weight is seen, as larvae reared at 35 °C start with less absolute lipid content but end with up with 20,3 % more. This actually makes rearing at high temperature seem more profitable which challenges earlier expectations. Tests between sample points within the same temperature were performed to monitor the lipid characteristics during the larval growth. It was always presumed that the lipid content would stagnate right before pupation (as a result of gut purging) but the result proved to be a bit more complicated. Instead of stagnating near the end of larval development the absolute lipid content displayed a continues increase at both 25 and 35 °C. The initial increase was expected as the third instar is notorious for the accumulation of lipids but for it to continue throughout the whole third instar is uncommon (Wimer & Lumb, 1967). Nevertheless, these results are establishing the end of the third instar as the optimal time for harvesting larvae if maximizing lipid content is the goal.

Relative lipid content is the only variable to shows no significant interaction thus breaking away from the trend seen in dry weight and absolute lipid content. Unusual was the lack of significance within the treatment group as it was expected that relative lipid content of both groups would go up during the third instar (Wimer & Lumb, 1967; Lee & Roh, 2010). This is only seen in the control group where the relative lipid content is raised with 24 % during the second half of their third instar. The jump in relative lipid content within the control group is important as it raises the relative lipid content to the same height as that of the treatment group during the last stage of their instar. This means that there is no difference in relative lipid content at the optimal harvesting time (end of third instar) between both temperatures.

To summarise, the highest lipid content per larvae is achieved at 35 °C as the larvae were bigger and contained more lipids per individual. However, this is not enough to fully mitigate the loss in collective biomass as result of the lower hatch rate. This means that rearing at 25 °C is still preferred for obtaining the highest collective lipid content as the hatch rate around this temperature is better. Still, the fact that the larvae at 35 °C were able to surpass the larvae reared at 25 °C in both dry weight and individual lipid content is astonishing as the predictions were a lot less generous.

4.4 Additional factors within the quantitative experiment

Although most of the research went as planned it should be noted that there were some factors of which it was hard to estimate their overall impact. The first factor had to do with setting up the adjusted sampling times. In order to make the sampling schedule, data from Wang et al., (2018) forensic research was used. Their paper observed the development of *M. domestica* at different temperatures up to 34 °C. This is 1,9 °C lower that the average temperature of 35,9 °C inside of the incubator which makes it possible that the treatment larvae matured faster than scheduled. If this were the case it could provide an alternative explanation for the increased dry weight and absolute lipid content of the treatment group larvae. A second factor was the humidity in in the treatment environment. There were some situational drops in relative humidity as a consequence of opening the incubator every time sampling occurred. These were only brief drops as the saturated salt solution quickly reacted and stabilised the humidity. The third and most important was the difference in hatch rate. The treatment larvae were heavier and had an overall higher lipid content. However, they also possessed a lower hatch rate. It is possible that the eggs containing the lighter larvae with lesser lipid content never hatched as result of the environmental stress thus only showing the larger larvae. This would in turn drastically increase their average weight compared to the larvae that were reared at 25 °C resulting in an observation bias.

4.5 Saturation and fatty acid chain length (Sub questions three and four)

Expected was a high difference between the quality of the lipids, especially to the levels of saturation and fatty acid chain length, based on the theory of 'Homeoviscous adaptation'. The results took an unexpected but nonetheless interesting turn as the larvae did not show a major shift in saturation or chain length. There were however some interesting discoveries made on the individual lipid level. From the thirteen different fatty acids four underwent significant changes in response to the change in temperature. Two of which, margaric- (17:0) and 7,10-Hexadecadienoic acid (16:2), were excluded from the discussion as their contribution to the total lipid content was deemed too low (only around 1,2 %). The discussion will therefore focus on the two remaining significant fatty acids: Pentadecanoic acid (15:0) and Oleic acid (18:1).

Different processes within the phospholipids can alter membrane fluidity. The two most notorious mechanisms responsible for maintaining the appropriate fluidity are: The level of saturation and the length of the phospholipids. Most papers only focus on saturation levels while studying homeoviscous adaptation. This leaves the effect size of phospholipid chain length relatively unexplored. When looking at the data two fatty acids stand out when exposed to a warmer environment: A short-chain saturated fatty acid called Pentadecanoic acid (15:0) which shows a reduction and a long-chain fatty unsaturated fatty acid called oleic acid (18:1n-9) which shows an increase. Saturation-wise these lipids do not obey the existing theories which predicted to see a drop in unsaturated lipids (Russell,1983; Robb et al., 1972). However, when excluding saturation and only looking at chain length they are obedient to the existing theories which state that longer chain lengths can mitigate the effect of the increased temperature (Dawaliby et al., 2015). This could indicate that chain length has the priority over saturation when changing membrane fluidity. If unsaturated long chain fatty acids are easier to acquire than saturated long chain fatty acids, and chain length is prioritised, then it would not be strange to invest in unsaturated long chain fatty acids despite the unfavourable saturation. The long chain unsaturated fatty acids could for example have been acquired from the large presents of oleic acid within the feeding substrate. This is one theory that would explain the increase in oleic acid (18:1) and to a lesser extend the decrease of pentadecanoic acid (15:0).

MUFA's (Mono unsaturated fatty acids) like oleic acid (18:1) are favoured for their ability to improve the retention quality of meat; Unlike linoleic acid (C18:2n-6) which is highly susceptible to meat oxidation if consumed in high amounts by the livestock (O'Neill, Galvin, Morrissey, & Buckley, 1998 cited in Ortiz et al., 2006; Zollitsch, Knaus, Aichinger, & Lettner, 1997 cited in Ortiz et al., 2006). Furthermore, oleic acid is considered 'neutral' in terms of serum cholesterol concentrations; Unlike the stable palmitic acid (16:0) which has the tendency to raise cholesterol levels (Grundy, 2003). In consumer meat 'neutral' or cholesterol lowering fatty acids are sought after as high cholesterol levels are linked to the build-up of fatty deposits inside the blood vessels (NHS Scotland, 2020; Attia, Al-Harthi, Korish & Shiboob, 2017). This can, if unnoticed, lead to coronary heart disease (NHS Scotland, 2020; Wald & Law, 1995). Oleic acid is not considered an essential fatty acid, but the increase still serves as a small improvement of the lipid profile because of its desired qualities. Odd chained fatty acids like pentadecanoic acid (15:0) work a little bit different compared to their even counterparts. For a long time pentadecanoic acid has been believed to be without biological function (Hellgren & Nordby, 2017). This is slowly changing as different studies carefully started to suggest that odd chain fatty acids may be the driving factor in reducing the chance of diabetes type 2 and cardiovascular risk (Khaw, Friesen, Riboli, Luben & Wareham, 2012; Forouhi et al., 2014). Beside its use as internal standard in GC-MS, the research of heath related factors concerning pentadecanoic acid is still in its infancy (Jenkins, West & Koulman, 2015). Therefor it is difficult to make a final judgement on the effect of the pentadecanoic acid.

4.5.1 Stable palmitoleic acid (16:1) levels (Side note)

A noteworthy observation was that all samples contained a large amount of Palmitoleic acid (16:1) that remained unresponsive to the temperature differential. Palmitoleic acid (16:1) was, based upon the work of Robb et al. (1972), the most likely acid to cause a saturation shift, yet none of that was reflected within this study.

5. Conclusion

High temperature (35 °C) does influence both the lipid quantity and quality of *M. domestica* larvae. The larvae reared at industrial temperature (35 °C) start of their third instar relatively small but quickly surpass the larvae reared at 25 °C terms of dry weight and lipid content. This makes, contrary to expectations, higher temperatures more beneficial when selecting for these two traits. As for lipid quality, the overall change of saturation and chain length was so small that it is almost neglectable. Only on an individual lipid level there were some small differences mainly resulting from the increase in oleic acid (18:1) and decrease of pentadecanoic acid (15:0). The quality changes would not form a valid reason to discontinue the research into insect-based animal feed. It only serves as a confirmation that there are no obvious drawbacks of the lipid quality and therefore promotes further exploration.

Interpreting the result was not easy as it challenged both our understanding of the TSR and the theory of homeoviscous adaptation. Recommended is to further explore the influence of saturation and fatty acid chain length on membrane fluidity in relation to each other. When better understood it could perhaps offer some clarification on why fatty acid chain length did obey the theory of homeoviscous adaptation and why saturation did not. Furthermore, after discovering that temperature would not cause a deterioration of lipid quality the next logical step would be to investigate the relationship between dietary lipid intake and the phospholipid composition of *M. domestica*. For example, to see if the lipid quality holds up when the larvae are reared on actual manure. Lastly, it is advised to repeat the temperature experiment with other fly strains. The VU has access to at least a Spanish and Italian strain which may be better able to withstand the industrial breeding temperatures due to their geographical location of origin. If true, this could possibly favour them over the Dutch strain (used during this study) in areas as hatching success.

Various modern techniques, controlled environments and verified sources were used to perform this study. Still a lot of results were unexpected and at times hard to explain. The unpredictable nature of fatty acids as seen in this study could therefore serve as evidence of the many exceptions and asterisks that are intertwined with the contemporary theories of lipid metabolism. It shows that there is still a lot to learn.

Appendix I: Substrate combinations

The recipe used throughout this research derived from an article by Pieterse & Gloy (2013) slightly modified to the research situation. The substrate recipe used for keeping the culture differs slightly from the substrate recipe used for the experimental jars as is shown by table 2 & 3. The reason being that the experimental jars can then be compared to earlier research. Also, there is a difference in the dry substrate/water ratio between the culture and experimental jars as showcased in table 4. The experimental jars (both control and treatment) contained a higher water to dry substrate ratio to compensate for the high experimental temperature.

Preparation manual experimental jars:
1 The yeast water and nipagin are mixed tog with the help of a lab stirrer (low rpm, 40 °C) for one hour.
2 The milk powder, wheat-bran and flour are thrown together and briefly mixed with a spoon.
3 The yeast, water and nipagin mixture is thrown together with the other ingredients and mixed.

Culture	Grams
Wheat bran	1000
Flour	150
Milk powder	120
Inactivated yeast	50
Additive (twilmij)	34

Table 2: Recipe used during culture keeping

Experiment	Grams
Wheat bran	1000
Flour	150
Milk powder	120
Inactivated yeast	50

Table 3: Recipe used during the experiment

Larval diet/wet food mixture	Culture	Experiment
Dry material	200 g	140 g
Tapwater	225 g	180 g
Nipagin solution ($C_8H_8O_3$)	4 ml	4 ml

Table 4: difference in dry substrate/water ratio between culture and experimental jars

Appendix II: Lipid profile

Lipid	Peak_N	Time	Name	Synonym	Formula	Lipid numbers
1	1	11,205	Dodecanoic acid	Lauric acid	C13H26O2	12:0
2	2-3 and 7	11,983 - 12,226	Methyltetradecanoate	Tetradecanoic acid, myristic acid	C15H30O2	14:0
3	4 and 6	12,364 and 12,525	10-undecenoic acid	undecenoic acid, undecyleric acid	C12H22O2	11:1
4	5 and 8-9	12,477 and 12,719 - 12,957	Pentadecanoic acid	same	C16H32O2	15:0
5	10	13,19	Hexadecanoic acid	Palmitic acid	C17H34O2	16:0
6	11-12-13-14	13,327 - 13,492	9-hexadecenoic acid	Palmitoleic acid	C17H32O2	16:1
7	16	13,66	Heptadecanoic acid	Margaric acid	C18H36O2	17:0
8	17	13,702	7,10-hexadecadienoic acid	same	C17H28O2	16:2
9	18	13,892	Cyclopropaneoctanoic acid, 2-hexyl	same	C18H34O2	17:1
10	19	14,117	Octadecanoic acid	Stearic acid	C19H38O2	18:0
11	20-21	14,333 - 14,372	9-Octadecenoic acid (Z)	Oleic acid	C19H36O2	18:1
12	23 and 27	14,671 and 15,181	9,12-Octadecadienoic acid (Z,Z)-	same	C19H34O2	18:2
13	25-26	14,908 -15,085	9,12,15-octadecatrienoic acid	Linolenic acid	C19H32O2	18:3
Removed	15	13,529	Cyclopentaneundecanoic acid	same	C17H32O2	16:1
Removed	22	14,533	3-Methoxymethoxy-2,3-dimethylundec-1-ene	same	C15H30O2	14:0
Removed	24	14,836	9-hexadecenoic acid	Palmitoleinate	C17H32O2	16:1

Table 5: All lipids found during the GC-MS and information about the number of peaks that each lipid contained.

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