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Temperature and Polyunsaturated Fatty Acid's Effect on Daphnia magna Reproduction

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Temperature and Polyunsaturated Fatty Acid's Effect on *Daphnia magna* Reproduction

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by

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ABSTRACT

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Organisms adapt to their environments by adjusting their biochemistry and physiology; such adaptation is limited by resource availability and physiological constraints. The freshwater crustacean *Daphnia magna* inhabits a wide range of environments and must survive and reproduce within a range of temperatures. One limit to low-temperature adaptation is thought to be the availability of unsaturated fatty acids necessary to maintain proper fluidity of cellular membranes. *D. magna* maintained at 10 ºC on a diet poor in unsaturated fatty acids have been observed to produce clutches that fail to develop. However, this has not been observed on a diet rich in unsaturated fatty acids or at a higher temperature regardless of diet. Clonal variation is commonly seen in *D. magna* life history traits, including heat tolerance, and was also investigated. *D. magna* were kept at two temperatures and fed two algal diets that differ in unsaturated fatty acid content. To investigate the role of fatty acid composition on the reproductive success of *D. magna*, fatty acids were extracted from adults and eggs. Of the twenty-one clones studied, no clonal variation was seen in the ability to produce successful clutches at 10 °C on a diet poor in unsaturated fatty acids. Gas chromatography revealed significant differences in 20-carbon fatty acids and suggest a parent-offspring conflict over a limited resource.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

Homeoviscous Adaptation

An organism's ability to maintain cellular functions essential to life is contingent on its environment and its capacity to adapt to that environment. Poikilotherms in particular can face a notable challenge of widely changing internal temperatures and the effect that has on cellular conditions, such as membrane viscosity (Angilletta et al. 2004). When internal temperature is below the physiological range of an organism, the temperature at which it has adapted or acclimated to, acyl chains in the lipids that form cell membranes adopt trans conformations, which straighten the chains (Hazel 1995). In this conformation, the lipids are more compact, and the membrane becomes viscous and gel-like as a result (Hazel 1995). If not corrected, this viscosity can have detrimental effects on the cell, such as reducing the activity of membrane-bound proteins (Aho and Vornanen 1998), slowing the rate of lateral protein diffusion (Padrón-Pérez 2000), and reducing cellular respiration rate (Atkin and Tjoelker 2003). However, thermal adaptation can help cells defend against the effects of unfavorable temperatures by altering the lipid composition of their membranes (Hazel 1995). An almost universal response to temperature change is a decrease in the proportion of saturated fatty acid and an increase in the proportion of unsaturated fatty acids or polyunsaturated fatty acids (PUFAs) in the membrane (Hazel and Williams 1990). PUFAs resist straightening at lower temperatures more than saturated fatty acids, due to PUFAs having multiple double covalent bonds between carbon atoms and thus have a lower melting point (Hazel and Williams 1990). This temperature-induced remodeling of the membrane's lipid composition is also known as homeoviscous adaptation (Hazel 1995).

Although prokaryotes do not have membrane-bound organelles, it is still necessary that they regulate the fluidity of their cell membrane (Cronan 2006). A paper by Sinensky (1974) examined membrane lipids in *Escherichia coli* using electron spin resonance spectroscopy to determine that the lipid composition of *E. coli*'s membrane can be dramatically altered by temperature. *E. coli* lack PUFAs, but they do produce two unsaturated fatty acids, palmitoleic acid and cis-vaccenic acid, that they can incorporate into their cell membranes (Scheuerbrandt and Bloch 1962). Due to their limited ability to move to other environments or thermoregulate, it stands to reason that prokaryotes would need robust regulation of membrane fluidity.

Plant cells also show homeoviscous adaptation. Plant biosynthesis of membrane glycolipids follow one of two pathways, either beginning with C16:0 or C18:1 fatty acids (Shimakata and Stumpf 1982). Fad2 mutant *Arabidopsis* have deficient C18:1 desaturase that is responsible for synthesizing polyunsaturated glycolipids for the cell membrane (Miquel et al. 1993). These mutants were unable to grow in cold temperatures, while the wild-type was only slowed (Miquel et al. 1993).

Polyunsaturated Fatty Acid Biosynthesis

PUFAs are known as essential fatty acids for animals because of their integral role in development and growth. For example, eicosapentaenoic acid (C20:5n-3, EPA) and arachidonic acid (C20:4, ARA) are precursors to prostaglandins, which regulate molting and reproduction in decapods (Harrison 1990). Decosahexaenoic acid (C22:6n-3, DHA) has roles in human neural development and function (Crawford 1993). Despite the integral role of PUFAs, animals have a very limited capacity to synthesize them and must rely on their diet for PUFAs (Monroig et al. 2013). Certain photosynthetic algae, as well as some heterotrophic protists and bacteria, can synthesize PUFA by the sequential addition of double bonds on saturated fatty acids (Guschina

and Harwood 2006). The pathway for PUFA synthesis begins with the saturated fatty acid stearic acid (C18:0), which is desaturated by Δ9 and Δ12 desaturase enzymes producing linoleic acid (C18:2n-6, LA) (Guschina and Harwood 2006). LA can be further desaturated with Δ15 desaturase creating α-linolenic acid (C18:3n-3, ALA) (Guschina and Harwood 2006). Either LA or ALA can be further converted, through a series of Δ6, Δ5, and Δ4 desaturases, along with elongases, to insert double bonds and add carbons to convert them to ARA and EPA, respectively (Monroig et al. 2013).

While most animals can produce saturated fatty acids via elongases adding two carbons together at a time, they cannot synthesize PUFAs from saturated fatty acids or monounsaturated fatty acids as they lack the necessary desaturases Δ12 and Δ15 (Tocher 2003). However, if the animal obtains LA or ALA through its diet, they can be desaturated and elongated to form the crucial EPA and DHA (Tocher 2003). Therefore, omega-3 and omega-6 fatty acids are called essential fatty acids for humans. Certain invertebrates, such as some crustaceans, do have some capacity to synthesize PUFAs. For example, the copepod *Eucyclops serrulatu*s has shown the ability to endogenously produce DHA when raised on a diet lacking it (Desvilettes et al. 1997). *Daphnia magna* (Crustacea, Cladocera) has also been shown to have a limited capacity to biosynthesize EPA when provided the omega-3 fatty acid, ALA (Schlechtriem et al. 2006). The rate-limiting step to this reaction is believed to be the creation of an additional double bond by Δ6 desaturase (Vannice and Rasmussen 2014).

Lipid Analysis

Study of an organism's lipid composition requires a rapid and efficient method for extraction and isolation of lipid molecules. The Bligh and Dyer's method for lipid extraction has long been called the "gold standard" for lipid extraction methods (Bligh and Dyer 1959). It

begins with a solution of chloroform, methanol, and water in ratio of 1:2:0.8, respectively (Bligh and Dyer 1959). This forms a monophasic solution, however, with the addition of a sufficient amount of water and chloroform, becomes biphasic (Breil et al. 2017). The water-methanol inorganic layer contains the proteins, carbohydrates, and phospholipids, while the chloroform organic layer, contains the lipids (Breil et al 2017). These layers organize by density, with the denser settling lower, after proper mixing and centrifuging. Potassium chloride (KCl) can be added to further improve the separation of lipids into the organic phase. Acidic lipids are present in the water-methanol aqueous phase as disassociated salt and in the organic phase as their undissociated salts (Folch et al. 1957). Adding KCl forms salts with the acidic lipids and consequently shifts them to the organic phase (Breil et al. 2017). Further preparation for gas chromatography include transesterification of fatty acids. Transesterification is the chemical process by which an ester group is converted to another by means of cleavage by an alcohol. If the cleaving alcohol is methanol, the process is called transmethylation (Liu 1994). Fatty acid methyl esters are created when the ester of a fatty acid (usually glycerol) is replaced with a methyl group through transmethylation. Transmethylation is commonly done to prepare a fatty acid sample for gas chromatography, as methyl esters are stable and separate well in the column (Christie 1993).

Gas chromatography with flame ionization detector (GC-FID) provides a rapid technique for identifying and quantifying compounds, such as fatty acids based on their boiling point, size, polarity, and other identifying factors. GC-FID works by first dissolving the sample in a neutral solvent, such as hexane, which is injected into the column through the injector port via syringe (Restek n.d.). The column is a long thin tube which is heated within the column oven. The solvent is carried through the column by an inert carrier gas, such as helium, known as the

mobile phase (Restek n.d.). Within the column is the stationary phase, and how the sample interacts with the stationary phase is a primary way to separate compounds (Restek n.d.). At the end of the column, the compounds combust over a flame sustained by a flow of air and nitrogen. The carbon from the sample is oxidized to form carbon dioxide or CHO^+, the latter of which produces a charge that is detected by the FID (Sobrado et al. 2016). The intensity of the charge is related to the quantity of molecules combusting and will result in a higher peak (Sobrado et al. 2016). The retention time is how long it takes for the molecule to reach the end of the column to combust and informs us of the size of the molecule (Sobrado et al. 2016). The boiling point of a molecule is the primary factor in influencing its retention time, but size and interactions with the stationary phase are also important (Christie and Han 2010). Longer fatty acid chains generally have higher boiling points, and greater unsaturation of the fatty acid also leads to longer retention (Sobrado et al. 2016).

The polarity of the stationary phase within the column is the next greatest influence on retention time, after differences in the samples' boiling points (Restek n.d.). A variety of stationary phases specialize in improving the retention times, and therefore the resolution, of specific compounds. Nonpolar stationary phases, such as dimethyl polysiloxane or diphenyl, are well-suited for separating nonpolar compounds, such as lipids (Restek n.d.). The polyethylene glycol stationary phase in the Zebron ZB-Wax column (Phenomenex, Torrance, California) used in this study, has a medium to high polarity and can separate alcohols and glycols well (Christie and Han 2010; Phenomenex 2018). While not ideal for fatty acid methyl esters, it is recommended for routine tests and general purposes (Christie and Han 2010).

Clonal Variation in *Daphnia magna*

D. magna is a planktonic freshwater crustacean which serves as a key primary consumer in standing bodies of freshwater around the world (Ebert 2005). It has been a model species in many studies in part due to its major role in trophic webs, ease of culture, and short lifespan (Glazier and Calow 1991). Cyclic parthenogenic reproduction of *Daphnia* provides the ability to maintain clones of genetically identical individuals without inbreeding, which is critical for the studies of the interactions between genetics and the environment. *Daphnia* lay a clutch of diploid parthenogenic eggs after every molt, which develop into genetically identical females (Ebert 2005). If environmental conditions are stressful, *Daphnia* may instead produce diploid parthenogenetic male offspring or haploid resting eggs, called ephippia, which require fertilization by a male to develop (Ebert 2005). Genetically identical lineages of *Daphnia*, referred to simply as clones, give researchers a unique tool to study differences in genotype responses to the environmental changes. Inter-clonal variation in Daphnia has been observed in temperature adaptation (Hietala et al. 1997; Yampolsky et al. 2014), energy allocation (Glazier and Calow 1991) and reproduction rate (Young 1979; De Coninck et al. 2013). However, literature is lacking on possible clonal variation in reproduction at cold temperatures, where reproduction may be limited by the availability of PUFAs. Given the additional challenges faced by *D. magna* exposed to cold temperatures, such as a greater abundance of unsaturated fatty acids required, phenotypes that could reproduce more successfully in this condition would be strongly selected for.

Daphnia Reproduction in Cold Temperatures

Fatty acids are essential for an organism's growth, reproduction, and membrane integrity (Ravet and Brett 2006). However, most animals lack the desaturase enzymes necessary to

synthesize certain fatty acids themselves, and thus must rely on obtaining them through their diet (Abrusán et al. 2007). Diets low in PUFAs, particularly eicosapentaenoic acid (EPA), cause reduced growth rates and egg production in *D. magna* (Becker and Boersma 2005). Egg production is believed to require a significant amount of fatty acids from the mother (Becker and Boersma 2005). This is exacerbated further in cold temperatures at which *Daphnia* require greater amounts of PUFAs to maintain membrane fluidity (Martin-Creuzburg et al. 2012). This is shown by Martin-Creuzburg et al. (2012), who observed lower production of viable offspring in *D. magna* fed *Scenedesmus obliquus* than those fed *Nannochloropsis limnetica* when held at 10 °C. *S. obliquus* has a significantly lower total PUFA content than *N. limnetica* and no detectable amount of EPA (Martin-Creuzburg et al. 2012). Additionally, diets low in fatty acids will reduce the total amount of lipids in a *Daphnia*'s eggs and somatic tissue with cholesterol being an exception (Putman et al. 2015).

Parent-Offspring Conflict

In organisms that supply eggs with a significant amount of nutrients or provides additional care to its young during embryo development, parent-offspring conflict is a strong selective force (Schrader 2009). This is because for the mother it is equally advantageous that each of her offspring survive, while for each offspring it is most advantageous that they survive (Schrader 2009). This creates a conflict between the amount of resources that the mother commits to each offspring and the resources that each offspring demands. This is most apparent in species where the phenotype of the offspring can be expressed during maternal care, and the offspring has some control over maternal investment, as it can be selected for maximizing self-gain (Parker et al. 2002). In a lecithotrophic organisms in which maternal care is not given to its young beyond providing the egg, and parent-offspring conflict is believed to play a lesser, but

non-zero evolutionary role (Schrader 2009). Nevertheless, variability in the size of offspring at birth and the number of offspring in a clutch are likely indicators of the level of parental investment even in lecithotrophic organisms (Gasperin and Kilner 2016). Furthermore, shifting environmental conditions may favor a paradigm of fewer, larger offspring over more, smaller offspring or vice versa (Gasperin and Kilner 2016). This creates a balance needed between parental fitness and offspring fitness, where optimal parental fitness is directly reliant on offspring number and fitness, but offspring fitness is highest when offspring number is lowest.

D. magna show limited influence on their offspring size but can greatly vary the number of offspring produced depending on resource availability. Larger offspring have been shown to have increased triacylglycerides investment from the mother compared to smaller counterparts, giving them an advantage if put into limited food conditions (Tessier and Consolatti 1989). However, more of a conflict may arise if fatty acids are limited for the mother, not only in the trade-off of clutch size and offspring fitness, but also between parental and offspring fitness. If investing resources poorly into a clutch allows the mother to survive longer, then limiting offspring fitness may result in higher parental fitness over her life. Since the mother and her parthenogenetic offspring share identical genotypes, selection could benefit reduced provisioning a limited resource into eggs if retaining them in the maternal tissues would increase the mother's survival and subsequent reproduction.

Hypotheses

While the relationship between the availability of fatty acids and viable egg production in *D. magna* at low temperatures has been studied, clonal variation of this relationship has largely not been investigated. Clonal variation in *D. magna* has been seen in many attributes, including tolerance to high temperatures (Yampolsky et al. 2014). One of the challenges faced by clones

with a higher heat tolerance would be efficient restructuring of the lipid composition in cell membranes. If clones with a higher heat tolerance exhibit a preference for saturated fatty acids, it could aid them in higher temperatures, but hinder them in lower temperatures. Therefore, it is hypothesized that *D. magna* clones with a low heat tolerance will be able to reproduce more successfully at cold temperatures than clones with a high heat tolerance. This can be rationalized as a possible trade-off for heat tolerance and could suggest genetic differences among clones in the expression of membrane lipid restructuring. Furthermore, eggs produced in cold temperatures which are part of a clutch that developed successfully are predicted to have a greater PUFA investment from the mother when compared to equivalent eggs that are part of a clutch which failed to develop. Meanwhile, adult *D. magna* that laid a failed clutch are predicted to have a greater amount of PUFA than adults that laid a successful clutch, and eggs that failed to develop will have less PUFA than eggs that successfully develop. These predictions on PUFA content will support the hypothesis of a parent-offspring conflict over an important limited resource.

These hypotheses were tested by first observing the reproductive success of several *D. magna* genotypes kept at cold temperatures and fed a diet limited in PUFAs. The objective of this experiment is to elucidate which clones can reproduce under these conditions and how heat tolerance plays a role in it. Secondly, a fatty acid analysis was performed on *D. magna* and their eggs when reared in two different temperatures and diets. In this second experiment, PUFA investment from mother to egg was measured and the differences in fatty acids between a successful clutch and a failed clutch were quantified.

CHAPTER 2

MATERIALS AND METHODS

Algae Cultures

The green alga, *S. obliquus*, was chosen as the PUFA-poor diet due to its ability to subsist *D. magna* while lacking ARA and EPA entirely. *S. obliquus* was grown on site in 1 L Erlenmeyer flasks filled with COMBO medium adjusted for algae (Table 1, Kilham et al. 1998). Essential vitamins, including B-vitamins, were supplemented to provide complete nutrition for *D. magna* (Mehdipour et al. 2011). An air stone was provided, and the flasks were placed in front of three 30-watt, 30 cm long white light fluorescent bulbs. Cultures were started from previously grown stock and allowed to grow until deep green, or about one week. While growing, algae were kept at a room temperature of about 22-24 ºC but were provided with a fan for air flow to dissipate heat from the lights. The heterokont alga, *N. limnetica*, was chosen for the PUFA-rich diet due to its high quantities of ARA and EPA, as well as its presence in similar studies (Martin-Creuzburg et al. 2012). *N. limnetica* was provided by AquaAlgae (Brunswick, Ohio). Algae were stored in plastic or glass containers and kept in an incubator at 10 ºC which was kept from light to prevent further growth. Algae concentration was determined by using a Qubit 2.0 fluorometer (Thermofisher Sci., Waltham, Massachusetts) via delayed fluorescence excitation spectroscopy. Fluorometer readings were calibrated to direct cell counts of diluted samples using a hemocytometer.

Table 1: *COMBO Medium*. From Kilham et al. (1998): compounds and their respective concentrations in both animal and algal versions of COMBO medium presented in μmol of each compound in 1L of medium.

Compound	**μmol/L**
$CaCl_2$	250
$MgSO_4$	150
K_2HPO_4	50
$NaNO_3$	1000
$NaHCO_3$	150
Na_2SiO_3	100
H_3BO_3	388
KCl	100
Algal Trace Elements	
Na_2EDTA	11.7
$FeCl_3$	3.7
$MnCl_2$	0.9
$CuSO_4$	0.004
$ZnSO_4$	0.08
$CoCl_2$	0.05
$NaMoO_4$	0.09
H_2ScO_3	0.012
Na_3VO_4	0.01
Animal Trace Elements	
LiCl	7.3
RbCl	0.6
SrCl	0.57
NaBr	0.16
KI	0.02

Animal Acclimation

Twenty-one different clones of *D. magna* were used for this study, which are maintained in a laboratory at East Tennessee State University and originated from geographically separated populations (Table 2; see Yampolsky et al. 2014). Five replicants of each clone (n = 105) were

cultured in 100 mL glass jars filled with 100 mL of COMBO water medium and stored in an incubator at 20 °C (Kilham et al. 1998). These clones were designated as having either a high heat tolerance or low heat tolerance. Clones that have high heat tolerance can survive at 33 °C, and clones with low heat tolerance are not able to survive at 31 °C or higher (Yampolsky 2018, unpublished data; unreferenced). Since males cannot produce eggs, only females were used in this study, and all males were discarded. *D. magna* were fed every two days with live *S. obliquus* at a concentration of approximately 100,000 cells per 100 mL of medium. A complete water change was performed every four days by transferring animals to a new container with new medium via pipet. Feeding took place after water changes on days that both occurred. *D. magna* show phenotypic plasticity in the young based on the environment that the mother was exposed to (Agrawal et al. 1999). To avoid this maternal effect based on stock conditions, the animals were acclimated to experimental conditions for three generations (G2). Offspring from these G2 clones (G3) were used for the experiment, but only if they were hatched within six days of each other, the approximate time between clutches in *D. magna*, to synchronize ages in the experiment. Clones that did not hatch within six days of the previous group of clones were used in a staggered simultaneous experiment after they hatched.

Table 2: *The Twenty-One Daphnia magna Clones Used*. Clonal designation along with their country of origin is shown. Clones that have high heat tolerance can survive at 33 °C, and clones with low heat tolerance are not able to survive at 31 °C or higher (Yampolsky 2018, unpublished data; unreferenced). Clones indicated in bold were used for lipid analysis.

Clone designation	**Country of origin**	**Heat tolerance**
CN-W1-1	China	High
DE-S3-3	Germany	High
ES-HT-1	**Spain**	**High**
FR-SA-1	France	High
GB-EL75-96	United Kingdom	High
HU-HO-2	Hungary	Low
HU-K-6	**Hungary**	**High**
IL-BN-1	Israel	High
IL-M1-1	Israel	High
IR-GG1-1	Iran	Low
IT-PER-2	Italy	High
MN-DM1-1	Mongolia	High
NO-AA-1	Norway	Low
PL-1-1	Poland	High
RU-BAI1-2	Russia	Low
RU-BOL1-1	Russia	Low
RU-HA1-1	Russia	Low
RU-R2-1	Russia	Low
RU-YAK-1	Russia	High
SE-G4-20	Sweden	Low
TN-RA-2	**Tunisia**	**Low**

Clonal Variation in Cold-Temperature Fecundity Experiment

To determine which clones can produce viable eggs at low temperature on a diet low in PUFAs, a single G3 individual from each replicant of each clone (n = 105) was kept in a 100 mL French glass square jar (The Cary Company, Addison, Illinois) filled with 50 mL of COMBO water medium. Due to mortality, not all G2 mothers produced five offspring, and the final

number of G3 individuals was n = 87. *Daphnia* were kept at 10 °C and fed approximately 100,000 cells of *S. obliquus* per 100 mL of medium every two days. Water was changed every four days by moving the animals to new jars with new medium via pipet. The new medium was chilled to approximately 10 °C before moving the animals. Positions of jars in the 10 ºC incubator were randomly assigned.

For two months, *Daphnia* were observed daily under a dissecting scope to check for egg development. If eggs were present in the brood chamber, the time and date they were first seen was recorded as the time of oviposition. Over the next two days, the deposited eggs were visually inspected for signs of development. Eggs that developed completely were considered successful, and eggs that failed to develop completely were considered failed.

Fatty Acid Analysis

Using the previous experiment's results, three *D. magna* clones that showed a mix of successful and unsuccessful clutches were chosen for fatty acid analysis: ES-HT-1, HU-K-6, and TN-RA-2. Throughout this experiment, *Daphnia* were kept in 100 mL glass jars filled with 100 mL of COMBO water medium (Kilham et al. 1998). To avoid maternal effects, *D. magna* were kept for two generations before being used for the experiment. The first generation was kept with one animal per jar, fed *S. obliquus*, and kept at a room temperature of about 22-24 °C. A single female from the first clutch (G2) of these *Daphnia* was transferred to each of the following experimental conditions: 10 °C and fed *S. obliquus,* 10 °C and fed *N. limnetica*, 25 °C and fed *S. obliquus*, and 25 °C and fed *N. limnetica*. The three latter treatments will serve as controls. Five females from the first clutch (G3) of each of these *Daphnia* were transferred to new jars in the same conditions and were used for this experiment. All feeding was at a concentration of approximately 100,000 cells per 100 mL of medium every two days. All animals were

transferred to new jars with clean water every four days. There were five replicants of the three clones in these four conditions (n = 60). As soon as the third generation *Daphnia* laid eggs, the eggs were extracted. The eggs and the mother were stored separately in 2 mL Eppendorfs (Biotech, Hamburg, Germany) at -80 °C to prevent develop while samples were accumulated. To determine the success of the clutch in the 10 °C *S. obliquus* treatment (the only treatment known to produce failed clutches) before freezing, 1-2 eggs from each clutch were stored in a 96-well plate, kept at 10 °C, and observed over the next 2-5 days for signs of development. Since eggs in clutches generally all develop or all fail to develop, the success of the clutch was inferred from the success of these 1-2 eggs (Yampolsky 2018, personal observation; unreferenced). After gathering eggs in this manner, it was discovered that a greater amount of sample material from the 10 ºC *S. obliquus* population was needed. This was due to a higher mortality of G3 individuals in this treatment as well as the later division of samples into those that developed successfully and those that failed to develop. To help with this, a separate population of HU-K-6 was started from five 10 ºC stock *D. magna* fed *S. obliquus*. These *Daphnia* were kept in a single 100 mL glass jar filled with 100 mL of COMBO medium (Kilham et al. 1998). An additional 145 G3 individuals were prepared this way (n = 205). Animals were fed twice as much at a concentration of approximately 200,000 cells of *S. obliquus* every two days to encourage greater egg-laying. Although this did help alleviate sample size problems, differing feeding concentrations were confounded in the 10 °C *S. obliquus* treatment group. All other procedures were performed identically to other treatment groups. The fatty acid profile of the frozen eggs and adult *Daphnia* were analyzed using gas chromatography.

Gas Chromatography

Lipids were extracted from the animal tissue using Bligh and Dyer's chloroform-methanol method (Bligh and Dyer 1959). Only glass tubes, Teflon-lined caps, and glass Pasture pipets were used throughout lipid preparation to avoid plastic contamination. The samples were homogenized via crushing with mortar and pestle and suspended in 0.2 mL of deionized water. Chloroform, methanol, and water were then added in a 1:2:0.8 ratio. After thoroughly mixing and centrifuging at 10,000 rpm for 7.5 minutes, three chloroform washes were performed to ensure complete separation of lipids from the inorganic phase. The organic, chloroform layers were removed and combined before being washed with potassium chloride and water to further improve separation of acidic lipids and associated salts.

Methylation of fatty acids through transesterification is needed to analyze samples with gas chromatography, as methyl esters are more stable and separate well in the column (Christie 1993; Carvalho and Malcata 2005). To do this, potassium methoxide and hexane was added to the lipid extract. The alkaline reaction takes place over roughly two minutes at room temperature and then is stopped with 2M hydrochloric acid. The solution was centrifuged at 10,000 rpm for 7.5 minutes and the organic, hexane layer was removed and stored in a separate container. Two more hexane washes were performed to ensure thorough separation of the methyl esters. The combined hexane layers were dried using nitrogen gas under low flow and then resuspended in 20 µL of hexane to achieve a comparable final concentration of lipids. 5 uL of a C17:0, heptadecanoic acid, solution in hexane at a concentration of approximately 0.56 mg/uL was added to each sample as an internal standard for comparing fatty acid time signatures. The samples were kept in 125 mL polypropylene autosampler inserts (Thermo Scientific Waltham,

Massachusetts) inside 8 mm glass amber autosampler vials with screw thread caps (ThermoFisher Sci., Waltham, Massachusetts).

The gas chromatograph used for this study was a Shimadzu GC-2010 (Shimadzu Corp., Kyoto, Japan) provided by Dr. William A. Clark at East Tennessee State University's Valleybrook campus. The column used was a 30 m length x 0.25 mm internal diameter x 0.25 µm film thickness Zebron ZB-Wax capillary column (Phenomenex, Torrance, California). Helium was used for the mobile phase at a flow rate of 30 mL/min. Hydrogen at a flow rate of 40 mL/min and air at 400 mL/min supplied the flame. The column oven's temperature program started at an initial temperature of 160 ºC for 5 minutes, which was raised at a rate of 2 °C/min to 170 ºC for 8 minutes, 180 °C for 10 minutes, 190 °C for 15 minutes, 200 °C for 15 minutes, and a final temperature of 210 °C for 20 minutes. The total run time was 120 minutes. The flame ionization detector temperature was 255 °C, and the injector port temperature was 250 °C. Samples were manually injected by syringe at a volume of 1 µL.

After the run is finished the chromatograms were integrated using the software LabSolutions to remove the solvent peaks and clean up improperly identified peaks (Shimadzu Corp., Kyoto, Japan). Fatty acid peaks were compared to the GLC-782 fatty acid analytical standard (Nu-Chek Prep, Inc., Elysian, Minnesota) based on retention time and proximity to the internal standard. See Appendix for specific compounds present in this standard (faculty.etsu.edu/yampolsk/data/GC/Albright.zip). Fatty acids were quantified as a percent of total area under the peak.

Analysis

Peaks in chromatograms were manually matched by retention times with the cut-off value to differentiation between neighboring peaks chosen as the midpoint between the lowest retention time of the later peak and the highest retention time of the earlier peak. A catalog of peaks was created based on the two algae samples, the fatty acid external standard, and three adult *D. magna* samples. The matched peaks were then examined by eye to eliminate any apparent mismatches. Peaks that were observed in only one sample were discarded. Statistical analyses used the percent area under each peak out of the total area under the peak for identified fatty acid methyl esters.

Statistical analyses for this study were performed in the software JMP (SAS Institute, Cary, North Carolina). In the clonal variation in cold-temperature fecundity experiment, a nested Analysis of Variance (ANOVA) was performed to study the effect that heat tolerance and clone has on clutch size and portion of eggs developing. A Principle Component Analysis was performed to identify relationships between fatty acids and treatment groups. Two-way ANOVA's were performed throughout the study to determine significant correlation between categorical variables, such as temperature and diet, and a continuous variable, percent area of a fatty acid's peak in the chromatogram. An Analysis of Covariance was used to compare the percent area of C18:3n-3 to the covariate C18:2 with the success of the sample. The significance level for these analyses was $\alpha = 0.05$. The normality of residuals was verified by a Shapiro-Wilk test. Due to the robustness of ANOVA to normality assumptions, a significance level of $\alpha = 0.03$ was used in Shapiro-Wilk tests. No significant deviations from normality were found unless otherwise stated.

CHAPTER 3

RESULTS

Clonal Variation in Cold-Temperature Fecundity Experiment

Of the twenty-one *D. magna* clones studied, three clones, DE-S3-3, RU-YAK-16, and SE-G4-20, did not produce more than one clutch and were excluded from statistical analyses. Of the remaining eighteen clones, no clone produced only successful clutches, three clones produced only failed clutches, and the rest produced failed and successful clutches intermittently (Table 3). A positive relationship between clutch size and the portion of eggs completing development was found (Figure 1A). Further investigation revealed that heat tolerant clones showed a significantly greater mean clutch size compared to heat intolerant clones ($p = 0.005$, Figure 1B, Table 4). Although they generally produced larger clutches, heat tolerant clones did not have a significantly greater portion of clutches completing development ($p = 0.087$, Table 4A). When examining individual clones' effect on clutch size, it was seen that clones do produce differently-sized clutches, but all clones have the same ability to produce successful clutches in these conditions ($p = 0.001$, $p = 0.13$, Table 4A). Although eggs were observed together with their clutches, if treated as separate, individual observations, sample size is increased greatly. If treated this way, a nested contingency table shows that clones, but not heat tolerance, did have a significant effect on the success of eggs developing ($p < 0.0001$, Table 4B).

Table 3. *Clutches Produced And Percent Developed.* N clutches refers to the number of clutches produced by the clone. SE is the standard error. % dev is the percent of clutches that developed successfully. Three clones, DE-S3-3, RU-YAK-16, and SE-G4-20, did not produce more than one clutch and were excluded from statistical analyses.

Clone	N clutches	Mean clutch size	SE (clutch size)	% dev	SE (%dev)
CN-W1-1	20	8.000	0.913	40.833	0.167
ES-HT-1	24	13.462	1.930	70.074	0.126
FR-SA-1	12	9.375	1.051	0.000	0.000
GB-EL75-69	20	6.750	1.359	64.701	0.167
HU-HO-2	16	5.250	2.750	54.487	0.231
HU-K-6	20	10.200	1.116	56.000	0.100
IL-BN-1	16	8.692	1.322	63.810	0.124
IL-M1-8	20	12.000	1.106	53.255	0.093
IR-GG1-1	12	8.750	1.548	43.875	0.230
IT-PER-2	24	5.143	0.986	29.677	0.145
MN-DM1-1	20	6.000	3.000	16.667	0.056
NO-AA-1	12	1.500	1.003	86.607	0.009
PL-W1-1	4	13.750	3.198	67.982	0.223
RU-BAI1-2	20	1.400	1.400	0.000	
RU-BOL1-1	8	1.500	0.982	83.333	0.167
RU-HA1-1	20	0.769	0.690	33.333	0.333
RU-R2-1	12	3.000	3.000	0.000	
TN-RA-2	24	8.400	0.779	59.524	0.127

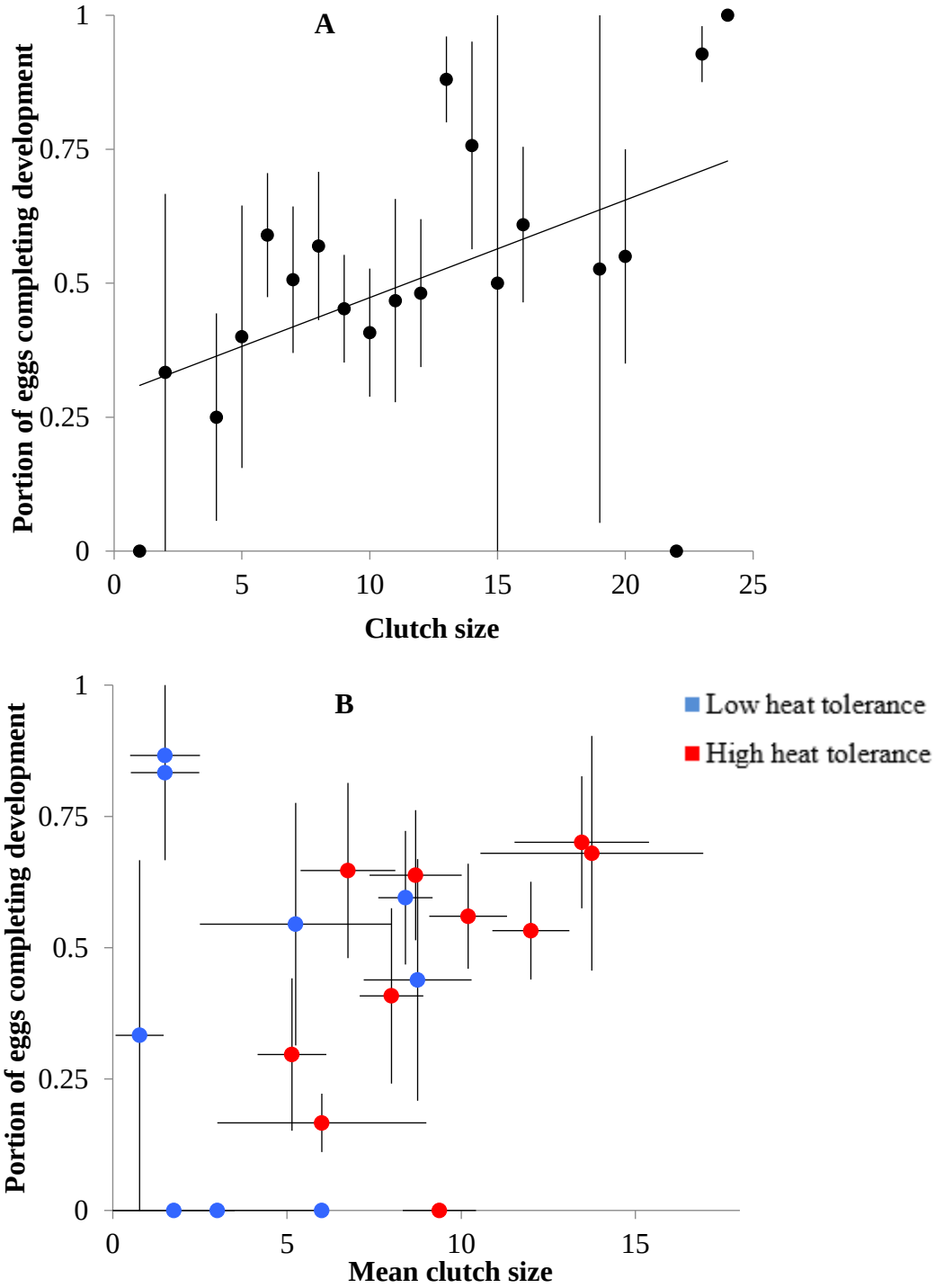

Figure 1. *Relationship between clutch size and portion of eggs completing development*. *A.* The correlation between the size of a clutch and the success of that clutch. A trendline is provided to clarify the correlation between the variables. *B.* The mean clutch size for each clone and the mean portion of eggs completing development are compared.

Table 4. *Analysis Of Clonal And Heat Tolerance Effects On Clutch Size And Portion Of Eggs Developed.* Three clones, DE-S3-3, RU-YAK-16, and SE-G4-20, did not produce more than one clutch and were excluded from statistical analyses. *A.* A nested ANOVA between heat tolerance groups and among clones was performed on clutches laid. DF refers to degrees of freedom, SS refers to sum of squares, F Ratio is ratio of variances, and the significance is shown as a probability of the F ratio being greater than 1. *B.* A nested contingency table was performed on the success of eggs developing as if eggs were observed separately from the clutches they were in. DF refers to degrees on freedom and the significance is shown as a probability of the log-ratio being greater than Chi^2.

A **Nested ANOVA for clutches**

Response: clutch size

Source	**DF**	**SS**	**F Ratio**	**P > F**
Heat tolerance	1	204.22	9.82	0.005
Clone (heat tolerance)	17	424.58	4.1	0.001
Error	25	152.16		

Response: portion of clutches developed

Heat tolerance	1	0.003	0.03	0.87
Clone (heat tolerance)	17	2.264	1.69	0.13
Error	20	1.58		

B **Nested contingency table for eggs**

Source	**DF**	**Log-ratio Chi^2**	**P > Chi^2**
Heat tolerance	1	1.17E-06	> 0.9
Clone (heat tolerance)	17	248.78	< 0.0001
All observations	1222		

Fatty Acid Analysis and Gas Chromatography

Fatty acid peaks in chromatograms were arranged in a predictable manner based on chain length and unsaturation. See Appendix for this comparison as well as all chromatogram files (faculty.etsu.edu/yampolsk/data/GC/Albright.zip). These peaks were identified by comparison of retention times between samples, the external standard, and algal samples (Figure 2). The mean percentages of each identified fatty acid in the different treatments are shown (Table 5). ARA (C20:4) and EPA (C20:5n-6) were completely absent from the adults that laid successful

clutches in the 10 °C *S. obliquus* treatment, but present in adults that laid a failed clutch. This supports the idea that the parent must make an investment of these PUFA into their eggs for the egg to develop. The clutch of failed adults may have failed because of a lack of this investment, evidenced by ARA and EPA remaining in the adult. However, while eggs that failed to develop in the 10 °C *S. obliquus* treatment indeed lack ARA and EPA, eggs which successfully developed also lack them. While the expected pattern for a parent-offspring conflict is seen in adults and failed eggs in the 10 °C *S. obliquus* treatment, it is not supported by successful eggs.

The ratios of the total amount of unsaturated fatty acids to saturated fatty acids, as well as PUFA to saturated fatty acids is shown (Figure 3). The ratio of unsaturated fatty acids, specifically EPA, to saturated fatty acids in the 25 °C treatments appears abundantly greater than other treatments. Comparing 10 °C *S. obliquus* treatments, failed adults and eggs both showed greater ratios of unsaturated fatty acids than successful samples (Figure 3B). This pattern supports the hypothesis of parent-offspring conflict in adult samples but not in egg samples.

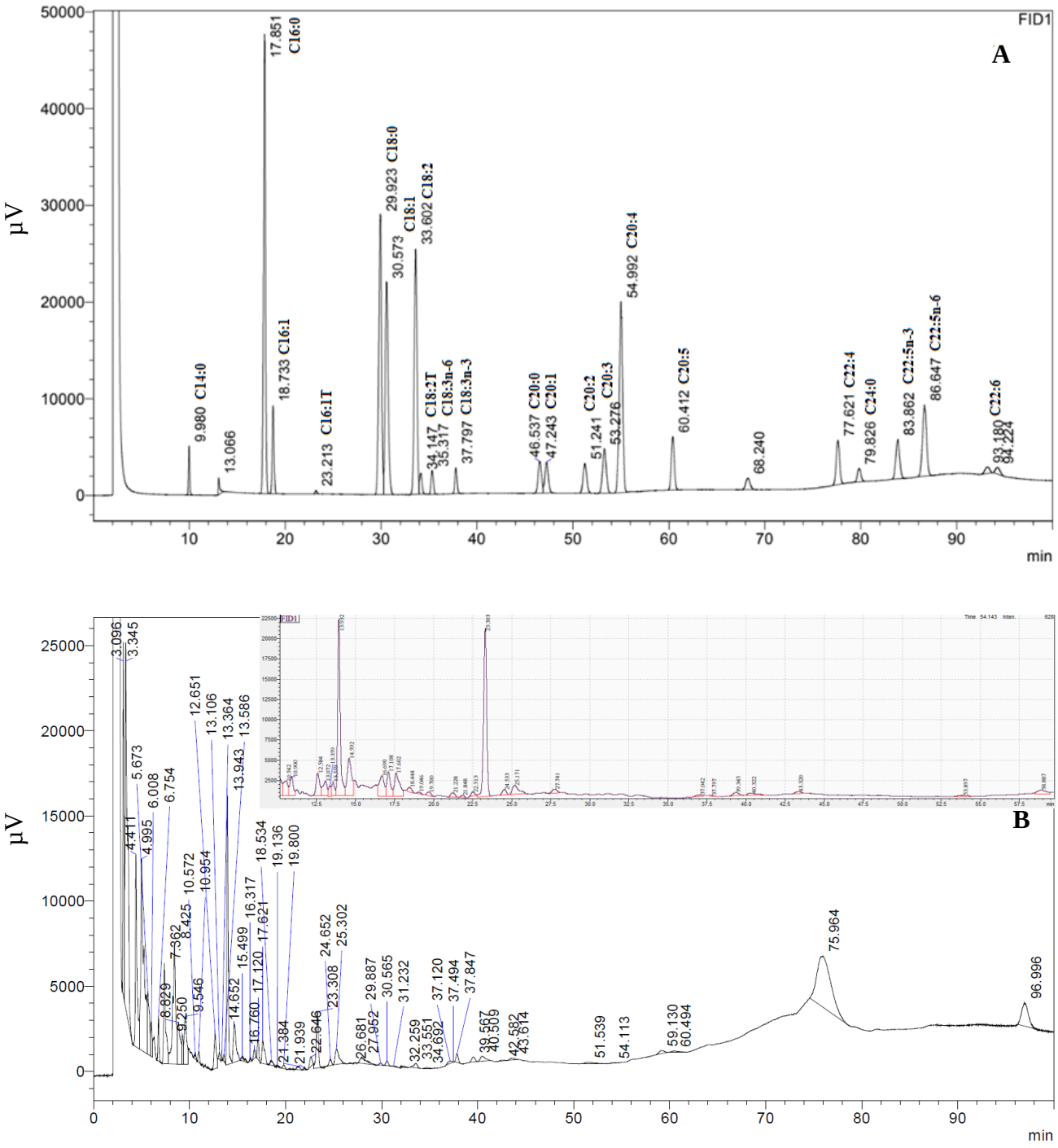

Figure 2. *GLC-782 external standard chromatogram and one selected sample*. Retention times, in minutes, are labeled above each peak, along with the fatty acid that is believed to cause the peak. The first large peak is the solvent, hexane, and its retention time is omitted. *A.* The GLC-782 fatty acid analytical standard (Nu-Chek Prep, Inc., Elysian, Minnesota). *B.* An adult sample from the 10 °C *S. obliquus* treatment which produced eggs that failed to develop. Inset shows peaks between 10 minutes and 55 minutes for better visibility.

Table 5. *Mean Percent Of Identified Fatty Acids In Samples*. Percent of fatty acids were calculated by the mean percent area under the chromatogram peak out of the total area under identified peaks. N and S refer to the algae, *Nannochloropsis limnetica* or *Scenedesmus obliquus*, fed to adults or fed to the mother, in the case of eggs. Failed or successful refers to adults that laid a clutch that either failed to develop or was successful in developing or eggs that were part of a clutch that either failed to develop or was successful in developing. T in the name of fatty acids refers to the trans configuration of that fatty acid. The sums of saturated, unsaturated, and polyunsaturated fatty acids are provided. The alga included here is *S. obliquus.*

	Algae	**Adults**		**Eggs**				
		10 °C S failed	**10 °C S successful**	**10 °C S failed**	**10 °C S successful**	**10 °C N successful**	**25 °C S successful**	**25 °C N successful**
C14:0	17.662	8.255	25.460	6.160	23.029	21.615	8.660	9.039
C16:0	53.608	38.778	43.760	35.809	41.581	33.034	28.759	20.227
C16:1	14.176	2.477	0.000	12.769	0.000	2.659	9.641	3.519
C18:0	0.000	4.616	1.357	8.833	6.136	3.831	4.045	2.134
C18:1	2.766	12.378	4.115	6.636	14.993	6.904	9.375	1.399
C18:1T	0.000	1.869	3.584	0.000	0.000	1.340	0.000	22.812
C18:2	1.036	9.824	13.433	19.856	10.159	11.319	18.303	1.901
C18:2T	1.887	0.556	0.000	0.000	0.000	0.000	0.000	2.828
C18:3n-3	4.252	8.184	4.145	4.969	2.051	3.205	6.957	0.294
C20:4	4.614	1.351	0.000	0.000	0.000	3.397	7.304	3.903
C20:5n-6	0.000	3.529	0.000	0.000	0.000	9.490	0.000	31.651
Σsat	71.270	51.648	70.577	50.802	70.747	58.480	41.464	31.400
Σunsat	28.730	40.168	25.278	44.229	27.202	38.315	51.579	68.307
ΣPUFA	11.788	23.444	17.579	24.825	12.210	27.412	32.563	40.577

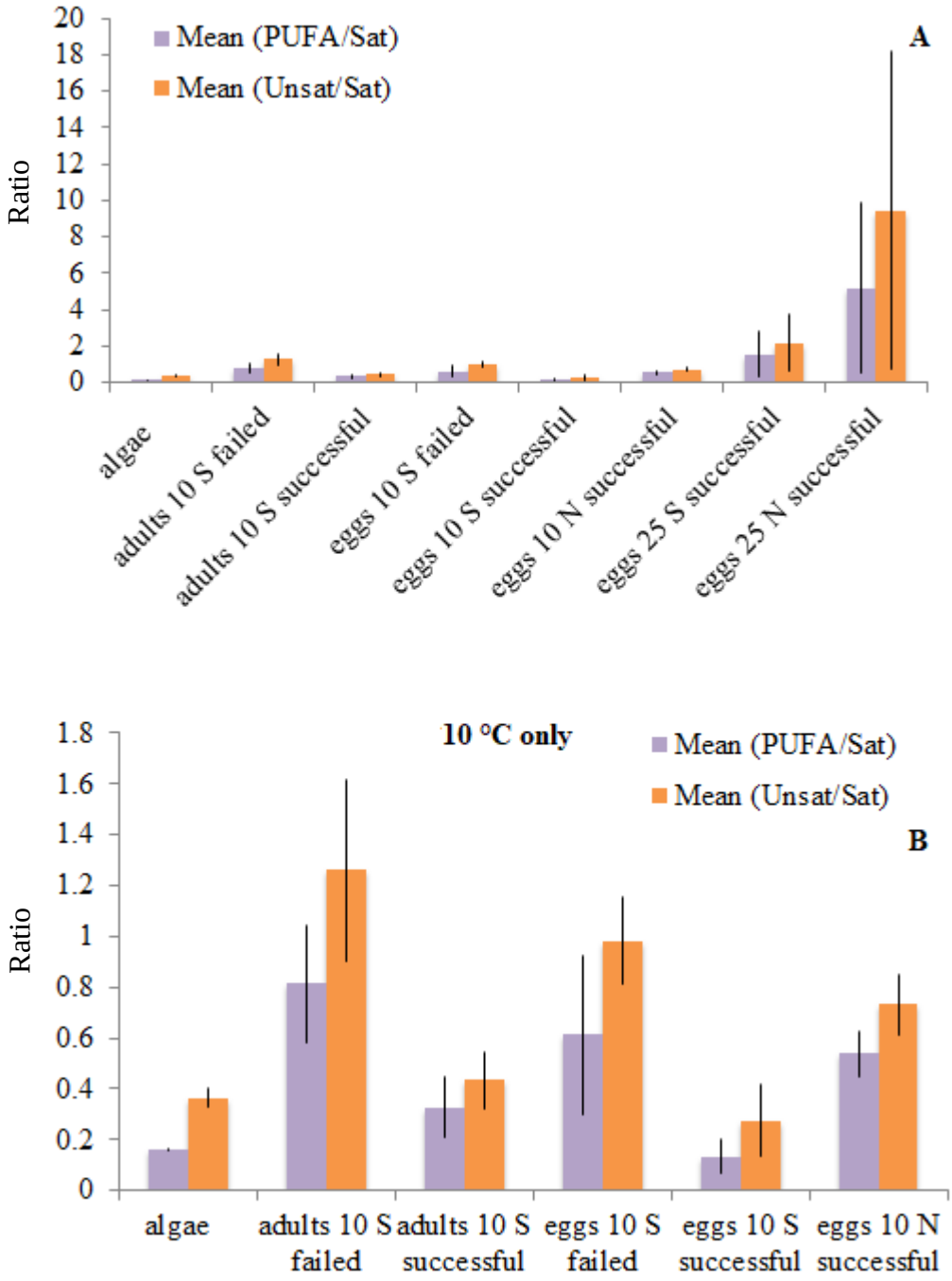

Figure 3. *The ratio of unsaturated fatty acids and polyunsaturated fatty acids to saturated fatty acids in four treatments.* The ratios of the mean of total unsaturated fatty acids to saturated fatty acids are shown in orange, and the ratios of the mean of polyunsaturated fatty acids (PUFA) are shown in purple. Standard error bars are provided in black. N and S refers to the alga, *Nannochloropsis limnetica* or *Scendesmus obliquus*, fed to adults or fed to the mother, in the case of eggs. Failed or successful refers to adults that laid a clutch that either failed to develop or was successful in developing or eggs that were part of a clutch that either failed to develop or was successful in developing. *A.* All treatment groups are shown. *B.* Only 10 °C treatment groups are included for visibility.

A principle component analysis was performed to visualize differences in identified fatty acids among samples (Figure 4). No compelling separation of samples based on sample type, adult or egg, or treatment temperature, 10 °C or 25 °C, was seen. Failed samples, particularly adults, showed moderate grouping and separation from successful samples, mainly along principle component 1. Principle component 1 showed strong separation of 18-carbon fatty acids (Table 6). This is suggestive that 18-carbon fatty acids are related to sample success, and this is investigated further below.

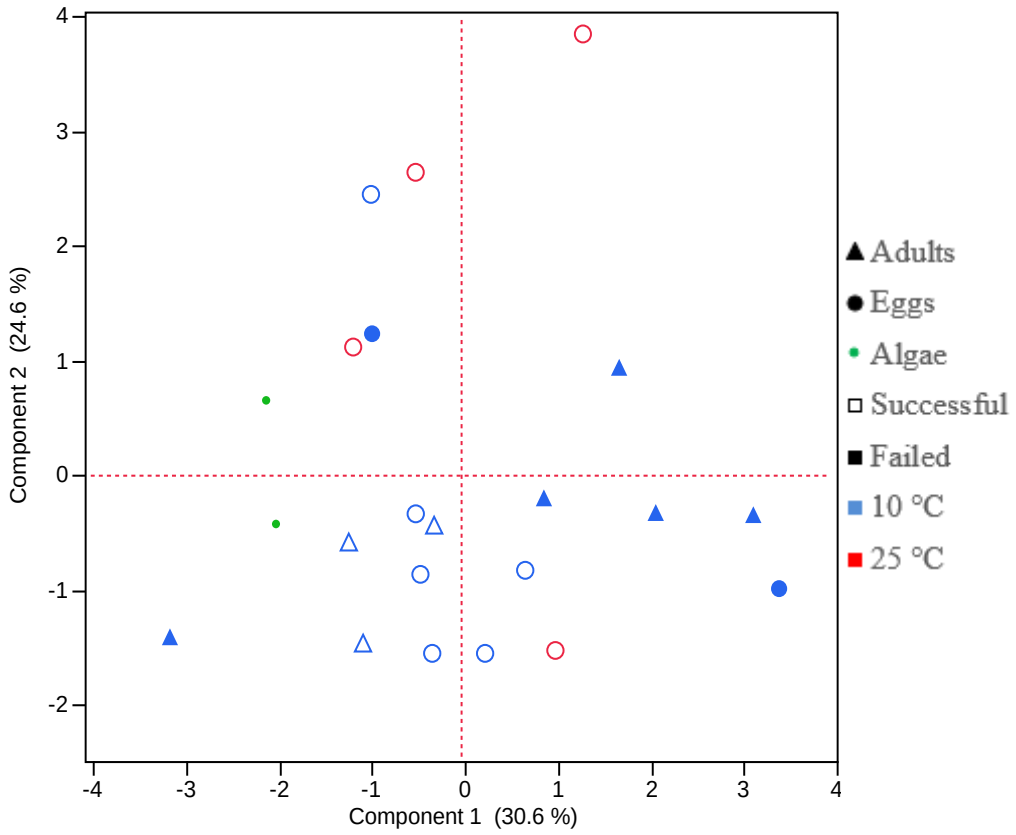

Figure 4. *Distribution of samples in first two principle components.* Percent peak area of total identified fatty acid methyl esters are investigated by Principle Component Analysis. C18:3n-6 was present in only one sample and is therefore omitted. Principle component 1 explains 30.6% of the variation, and principle component 2 explains 24.6% of the variation.

Table 6. *Principle Component Loading For Identified Fatty Acids*. The first three principle components (PC1, PC2, and PC3) and the correlation to selected percent area of total identified fatty acid methyl ester peaks. Fatty acids present in less than half of samples were omitted. Values higher than 0.65 or lower than -0.65 are bolded for emphasis.

	PC1	PC2	PC3
C14:0	**-0.73817**	-0.49475	0.10939
C16:0	-0.55509	-0.55922	0.47436
C16:1	-0.16546	**0.66648**	0.45811
C18:0	**0.68962**	-0.38861	-0.14435
C18:1	**0.65091**	-0.50608	-0.15062
C18:2	**0.68042**	-0.06323	0.42078
C18:3n-3	**0.71072**	0.15046	0.42053
C20:4	-0.01714	**0.76551**	0.30847
C20:5n-6	-0.04242	0.44184	**-0.88507**

A similar pattern of separation is seen when looking at the correlation between mean unsaturation of fatty acids and mean chain length (Figure 5). The positive relationship between these two variables was expected due to the longer fatty acids, ARA and EPA, also having greater unsaturation. Greater mean unsaturation and chain length in failed adult samples may also support the parent-offspring conflict hypothesis, but this pattern cannot be discerned in other sample groups.

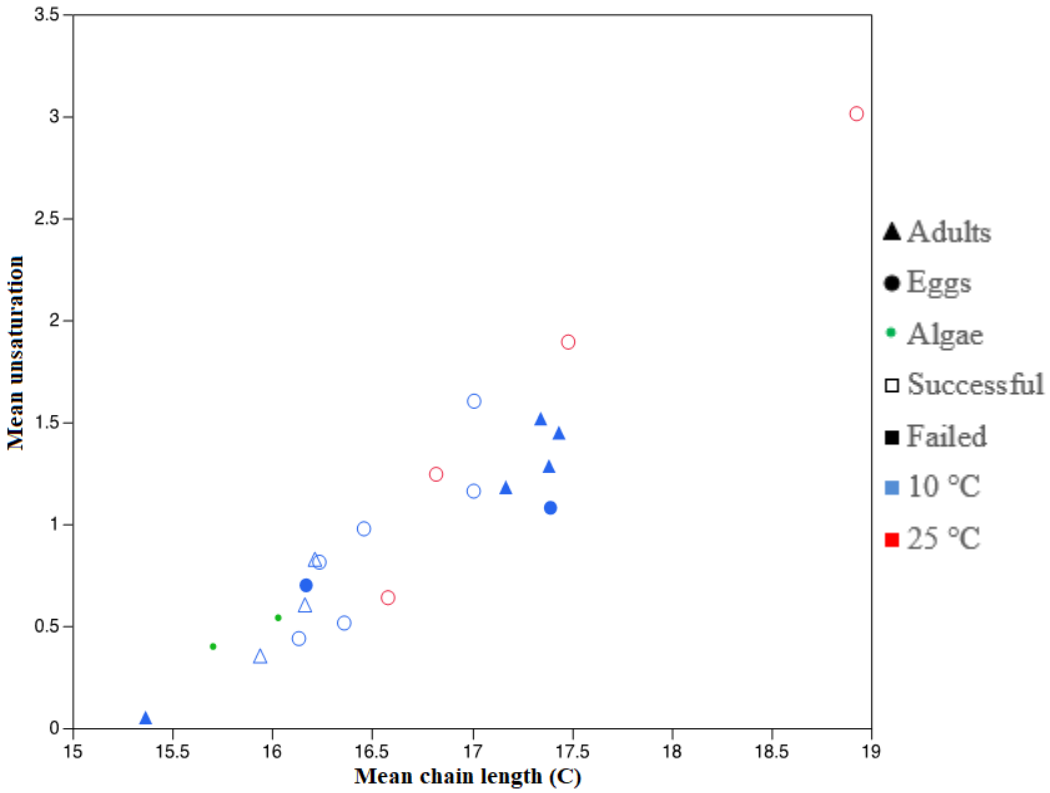

Figure 5. *Mean chain lengths correlation with mean unsaturation in adults and eggs.* The correlation between the mean chain length of fatty acids found in adults and eggs in four treatment groups.

To compare algal treatments and temperature treatments, the ratio of the sum of 20-carbon PUFA to the sum of 18-carbon PUFA was compared between eggs from different treatments (Figure 6). Differences in these fatty acids are the major distinctions between the algae, and success at low temperatures is believed to be contingent on the ability of the animals to convert 18-carbon fatty acids to 20-carbon fatty acids (Schlechtriem et al. 2006). A two-way ANOVA showed that temperature and diet both had a significant effect on this ratio ($p = 0.018$, $p = 0.002$, Table 7). A Shapiro-Wilk test showed that residuals in this comparison were not normal, making these results suspect. However, a square root transformation of the data produced normal residuals and similar results, except that the interaction of temperature and diet was no longer significant. The significant difference between diets supports that the *N. limnetica* diet increased

the amount of ARA and EPA in *D. magna* eggs. However, a higher portion of 20-carbon fatty acids was seen in 25 °C eggs compared to 10 °C eggs. This is contrary to the expectation that ARA and EPA would be a greater concentration at 10 °C due to a greater need of unsaturated fatty acids for maintaining proper membrane fluidity.

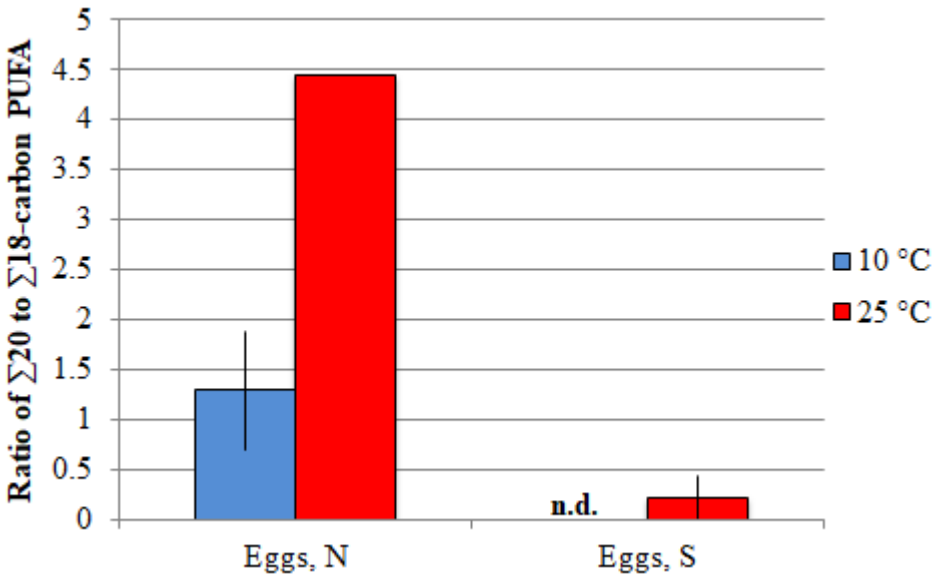

Figure 6. *The ratio of Σ20-carbon polyunsaturated fatty acids to Σ18-carbon polyunsaturated fatty acids in eggs with two diets and two temperatures*. The ratio of the sums of the percent areas under the peaks of 20-carbon polyunsaturated fatty acids (PUFA) (C20:4 and C20:5n-6) to 18-carbon PUFA (C18:2 and C18:3n-3) is shown in eggs with two diets, *Nannochloropsis limnetica* (N) and *Scenedesmus obliquus* (S), and two temperatures, 10 ºC (blue) and 25 ºC (red). Standard error bars are given in black. The 10 ºC and *S. obliquus* treatment had no detectable 20-carbon unsaturated FA.

Table 7. *Two-Way Analysis Of Variance Of The Ratio Of Σ20-Carbon Polyunsaturated Fatty Acids To Σ18-Carbon Polyunsaturated Fatty Acids In Eggs*. Temperature, 10 °C or 25 °C, was tested, along with diet, *Nannochloropsis limnetica* or *Scenedesmus obliquus*, for correlation with the ratio of Σ20-carbon polyunsaturated fatty acids (PUFA) to Σ18-carbon PUFA. The interaction of temperature and diet was also tested. DF refers to degrees of freedom, SS refers to sum of squares, F Ratio is ratio of variances, and the significance is shown as a probability of the F ratio being greater than 1.

Source	DF	SS	F Ratio	P > F
Temperature	1	5.665	9.465	0.018
Diet	1	15.195	25.385	0.002
Temperature*diet	1	4.315	7.209	0.031
Error	7	4.190		

To determine what effect the ratio of the sum of 20-carbon PUFA to the sum of 18-carbon PUFA may have on sample success, the correlation between this ratio and the success of adults and eggs was investigated (Figure 7). Although levels of 20-carbon PUFA were neither detectable in adults that laid a successful clutch nor in eggs from a failed clutch, a significant effect of sample type on sample success was still found between adults and eggs ($p = 0.009$, Table 8). The ratio of 20-carbon to 18-carbon fatty acids was not significantly correlated with sample success but was within each sample type ($p = 0.999$, $p = 0.019$, Table 8). When all treatments are considered, differences in this ratio support the hypothesis of parent-offspring conflict.

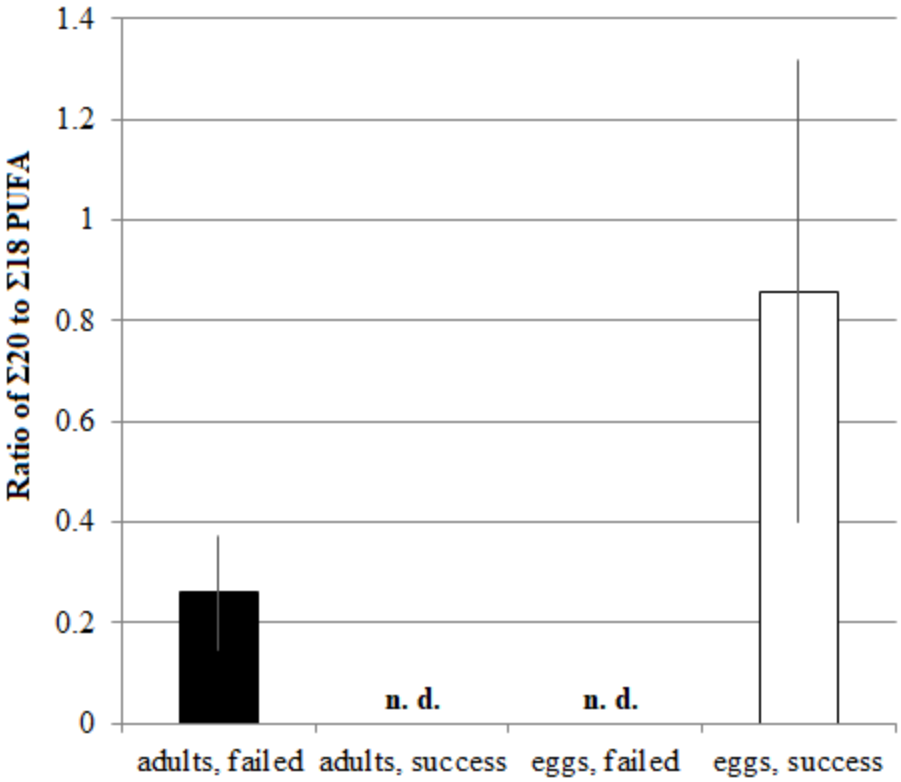

Figure 7. *The ratio of Σ20-carbon polyunsaturated fatty acids to Σ18-carbon polyunsaturated fatty acids in adults and eggs by success or fail.* The ratio of the sums of the percent areas under the peaks of 20-carbon polyunsaturated fatty acids (PUFA) to 18-carbon PUFA is shown in adults and eggs. Hollow bars represent either adults that laid a clutch that successfully developed or eggs from a clutch that successfully developed, filled bar represent either adults that laid a clutch that failed to develop or eggs from a clutch that failed to develop. Standard error bars are provided in black. Adults successfully laid a clutch and eggs that failed to develop had undetectable levels of 20-carbon PUFA.

Table 8. *Logistic Regression Analysis For The Ratio Of Σ20-Carbon Polyunsaturated Fatty Acids To Σ18-Carbon Polyunsaturated Fatty Acids In Adults And Eggs By Success Or Fail*. Sample type, adult or egg, and the ratio of the sum of 20-carbon PUFA to the sum of 18-carbon PUFA was tested, for correlation with sample success. The interaction between sample type and chain length was also tested. DF refers to degrees on freedom and the significance is shown as a probability of the log-ratio being greater than Chi^2.

Source	DF	Log-ratio Chi^2	P > Chi^2
Sample type	1	6.868	0.009
C20:C18 ratio	1	2.43E-06	0.999
Sample type*C20:C18 ratio	1	5.466	0.019
Observations	19		

The portion of C18:2 and C18:3n-3 present in samples was believed to be related to sample success, so diet and temperature and the percentage of C18:2 and C18:3n-3 was examined for significant differences between treatments (Figure 8). No significant effects of temperature or diet was seen in C18:2, although it may appear as if C18:2 is different between the two algae at 25 °C (Table 9A). When looking at C18:3n-3, however, a significant interaction between temperature and diet is seen (p = 0.026, Table 9B).

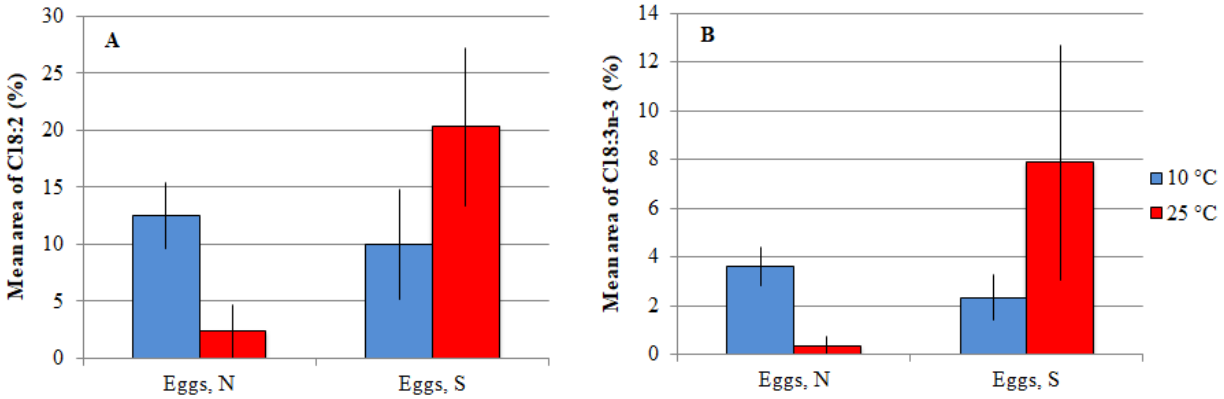

Figure 8. *Mean percent areas of 18-carbon polyunsaturated fatty acid peaks in eggs with two diets and two temperatures.* The standard error is given as black lines. *Nannochloropsis limnetica* (N) and *Scenedesmus obliquus* (S) are the two algal diets used.

Table 9. *Two-Way ANOVA Of The Percent Area Of C18:2 And C18:3n-3 Peaks In Eggs.* DF refers to degrees of freedom, SS refers to sum of squares, F Ratio is ratio of variances, and the significance is shown as a probability of the F ratio being greater than 1. *A.* Percent area of C18:2 peaks in eggs *B.* Percent area of C18:3n-3 peaks in eggs.

A

Source	DF	SS	F Ratio	P > F
Temperature	1	0.008	0.0001	0.993
Diet	1	167.182	1.876	0.201
Temperature*diet	1	295.804	3.318	0.099
Error	10	891.418		

B

Source	DF	SS	F Ratio	P > F
Temperature	1	3.725	0.467	0.510
Diet	1	27.550	3.454	0.093
Temperature*diet	1	54.551	6.839	0.026
Error	10	79.770		

To see if these fatty acids were related to sample success, the ratio C18:3n-3 to C18:2 was studied in adults and eggs for all treatment groups (Figure 9). Algae were included in the figure for comparison purposes and was not included in statistics. The sample type, adult or egg, as well as success, whether a sample failed or was successful, had a significant effect on this ratio ($p = 0.018$, $p = 0.010$, Table 10). Samples that failed were only present in the treatment group kept at 10 °C and fed *S. obliquus*, so only that group was further investigated by comparing the ratios of various 18-carbon unsaturated fatty acids to success in eggs and adults (Figure 10). An analysis of covariance was performed to see how C18:3n-3 and the covariate C18:2 correlate with each other and sample success. In adults, the percentage of C18:2 does correlate with the percentage of C18:3n-3, as does the success of the adult ($p = 0.047$, $p = 0.032$, Table 11A). In eggs, nearly opposite relationships are seen. C18:3n-3 is significantly correlated with C18:2 in eggs, but success and C18:2 with success are not ($p = 0.014$, $p = 0.137$, $p = 0.056$, Table 11B). All these results suggest that the ratio of C18:3n-3 to C18:2 is a good indicator of success in adults, but not eggs. Greater unsaturation is thought to be needed at 10 °C due to maintaining membrane viscosity (Martin-Creuzburg et al. 2012). The higher ratio of the more unsaturated C18:3n-3 seen in adults support the hypothesis of parent-offspring conflict, but the opposite pattern is not seen in eggs.

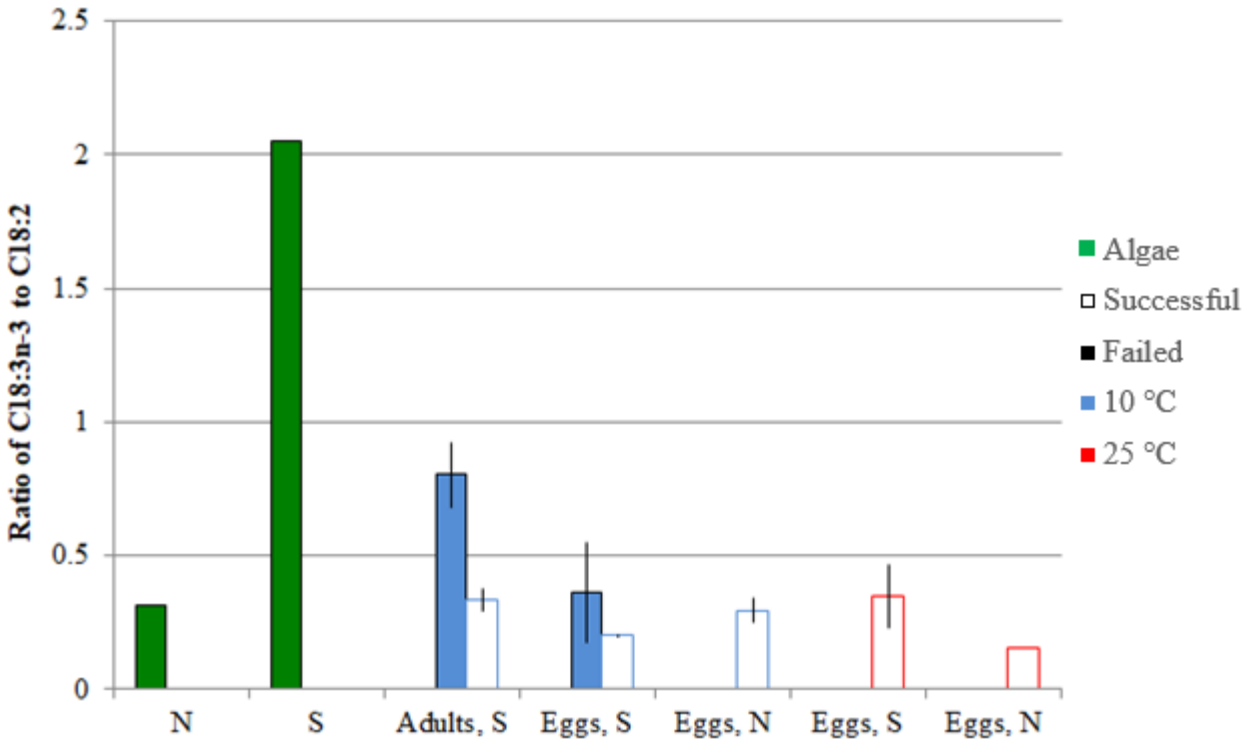

Figure 9. *The ratio of mean peak areas of C18:3n-3 to C18:2 in algae, adults, and eggs.* N and S on the x-axis refer to the genus of algae, *Nannochloropsis limnetica* or *Scenedesmus obliquus*, they were fed, or their mothers were fed, in the case of eggs. Standard error bars are given in black.

Table 10. *Two-Way ANOVA For The Ratio Of Mean Peak Areas Of C18:3n-3 To C18:2 In Adults And Eggs.* Sample type, adult or egg, was tested, along with success, whether an adult laid a clutch that successfully developed vs. failed to develop or if the eggs were from a clutch that successfully developed vs. failed to develop, for correlation with the ratio of the mean peak areas of C18:3n-3 to C18:2. The interaction of sample type and success was also tested. DF refers to degrees of freedom, SS refers to sum of squares, F Ratio is ratio of variances, and the significance is shown as a probability of the F ratio being greater than 1.

Source	DF	SS	F Ratio	P > F
Sample type	1	0.204	7.668	0.018
Success	1	0.259	9.707	0.010
Sample type*success	1	0.111	4.170	0.066
Error	11	0.293		

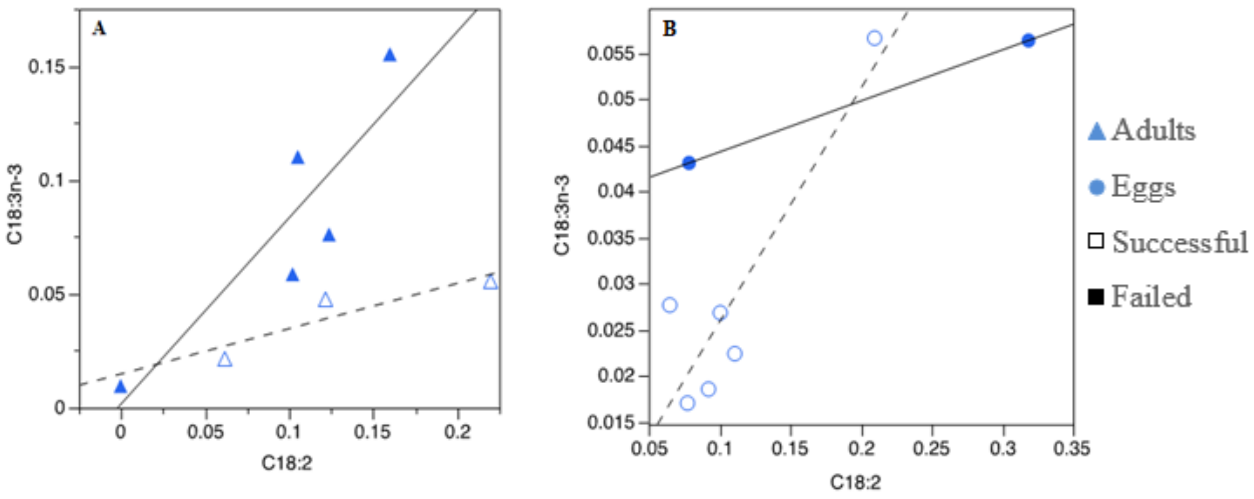

Figure 10. *Ratio of C18:3n-3 to C18:2 fatty acids in adults and eggs*. Ratio of 18-carbon polyunsaturated fatty acids are shown as ratios. All samples were kept at 10 °C and were fed *Scenedesmus obliquus*, solid trendlines show the trend of successful samples, and dashed trendlines show the trend of failed samples.

Table 11. *Analysis Of Covariance Tests For 18-Carbon Unsaturated Fatty Acids In Adults And Eggs*. An analysis of covariance was performed on C18:3n-3 to C18:2 fatty acids in adults and eggs. All samples were kept at 10 °C and were fed *Scenedesmus obliquus*. DF refers to degrees of freedom, SS refers to sum of squares, F Ratio is ratio of variances, and the significance is shown as a probability of the F ratio being greater than 1. *A.* In adults, the correlation of percent area of C18:3n-3 with C18:2, success of the sample, and the interaction of C18:2 and success. *B.* In eggs, the correlation of percent area of C18:3n-3 with C18:2, success of the sample, and the interaction of C18:2 and success.

A	Source	DF	SS	F Ratio	P > F
	Success	1	0.005	8.103	0.047
	C18:2	1	0.007	10.497	0.032
	Success*C18:2	1	0.003	3.889	0.120
	Error	4	0.003		

B	Source	DF	SS	F Ratio	P > F
	Success	1	0.0002	3.442	0.137
	C18:2	1	0.0009	17.363	0.014
	C18:2*success	1	0.0004	7.139	0.056
	Error	4	0.0002		

CHAPTER 4

DISCUSSION

Daphnia Reproduction in Cold Temperatures

Maintaining membrane fluidity in believed to be one of the predominant constraints on an organism's ability to acclimate to temperatures below its' physiological range (Hazel 1995). The mechanism by which an organism achieves proper homeviscous adaptation is thought to be the allocation of an increased ratio of unsaturated fatty acids to saturated fatty acids in its' cellular membranes (Hazel and Williams 1990). This study sought to examine possible inter-clonal variation in the ability of *D. magna* to reproduce in cold temperatures when limited in PUFA by diet. After studying twenty-one *D. magna* clones kept at 10 °C and fed *S. obliquus*, no significant differences were seen in the clones' ability to lay a clutch that successfully develops (Table 4A). Furthermore, the heat tolerance of the clone did not make a significant difference (Table 4A). This lack of clonal variation is contrary to a multitude of other attributes of *D. magna* that have been shown to have variation among clones (Young 1979; Glazier and Calow 1991; Hietala et al. 1997; De Coninck et al. 2013; Christian et al. 2018). The observations in this study could be evidence of an insurmountable constraint in the treatment. *D. magna* have been shown to not produce failed clutches at 10 °C when fed *N. limnetica* or at 25 °C when fed *S. obliquus* (Yampolsky 2018, unpublished data; unreferenced). Therefore, this study suggests that the interaction between 10 °C and *S. obliquus* produces a combination that the studied clone-types are unable to overcome. However, the contrary is suggested when the data were studied by the success of individual eggs rather than clutches (Table 4B). Although observations of eggs are confounded with observations of clutches, keeping in mind that clones did differ in mean clutch size (Table 4A), this could represent a fault in the sample size of the study. Besides this, it could

be that *S. obliquus* simply does not provide adequate unsaturated fatty acids for consistent reproduction at 10 °C. This prospect was explored, in part, by gas chromatography of fatty acid methyl esters.

Parent-Offspring Conflict

Greater unsaturation and great chain length of fatty acids were shown to correlate with adults that laid a failed clutch in the 10 °C *S. obliquus* treatment (Figure 5). This distinction is mostly driven by the greater amount of ARA and EPA in failed adults (Table 5). Although fed a diet lacking EPA entirely, *D. magna* are believed to have the capacity to convert 18-carbon fatty acids to ARA and EPA, although it is believed to be limited (Schlechtriem et al. 2006). These PUFA are believed to be crucial at low temperatures, likely due to their use in maintaining membrane viscosity (Schlechtriem et al. 2006; Martin-Creuzburg et al. 2012). The presence of these fatty acids in adults that produced failed clutches, but not those that produced successful ones, could support the hypothesis of a parent-offspring conflict, where adults withhold an important limited resource from their eggs in favor of their own survival (Table 5). This is further supported by the lack of ARA and EPA in eggs that failed to develop (Table 5). However, eggs in the 10 °C *S. obliquus* that successfully developed did so without detectable amounts of these PUFA, despite the PUFA being missing from the adults that laid these eggs (Table 5). Although successful eggs from the 10 °C *S. obliquus* treatment do not support the hypothesis of parent-offspring conflict, nor the hypothesis of highly unsaturated PUFA being essential for egg development in these conditions, successful eggs from other treatments do contain some 20-carbon PUFA (Figure 6).

Differences between sample success at 10 °C were also seen in the ratio of ALA (C18:3n-3) to LA (C18:2). A greater portion of ALA was correlated to the success of adults, but

not eggs (Table 11). ALA is more unsaturated than LA and therefore may provide more efficient membrane restructuring at 10 °C (Hazel 1995). Since a greater portion of the more unsaturated fatty acid was seen in failed adults compared to successful adults, this could support the parent-offspring conflict hypothesis (Figure 10). However, the trend does not continue in eggs, and the portion of ALA to LA more closely matches that of the mothers (Figure 9). Therefore, it is not believed that this ratio supports the parent-offspring conflict. A more applicable relationship between ALA and LA may be seen in the biosynthesis pathway of EPA. Both ALA and LA can be converted to EPA using Δ5, Δ6, and Δ17 desaturases along with elongases (Guschina and Harwood 2006). Preference for one of these substrates has been seen in other organisms, including *Nannochloropsis* algae, and the differences in the ratio of ALA to LA in samples could be evidence of a preference in the animals, although this has not been studied in *D. magna* (Guschina and Harwood 2006). Additionally, ARA is a precursor to many important compounds, such as prostaglandins and other signaling molecules (Sargent et al. 2002). ARA can also not be synthesized from ALA, but can be synthesized from LA, due to ARA being an omega-6 fatty acid. This could create a difference in demand for the 18-carbon fatty acids and explain some of the differences in the ratio that was seen in the samples.

Unsaturation of Fatty Acids at Two Temperatures

The level of 18 and 20-carbon PUFA was also compared between treatment groups (Figure 6). In eggs, significant difference was seen between the diets, with *N. limnetica* diets showing greater portions of 20-carbon fatty acids (Table 7). *N. limnetica* has been shown to produce greater portions of ARA and EPA compared to *S. obliquus*, and this is supported by both temperature treatments. However, a greater portion of 20-carbon fatty acids was seen in 25 °C treatments than at 10 °C in both algal treatments. This is contrary to the hypothesis of highly

unsaturated fatty acids being more conserved at lower temperatures (Schlechtriem et al. 2006). However, it should be noted that sample size for eggs in the 25 °C *S. obliquus* and *N. limnetica* treatments was poor, only two representing each, and it is therefore difficult to draw meaningful conclusions from these data. Nevertheless, an alternative explanation could lie in the increased metabolism of *Daphnia* at higher temperatures (Martin-Creuzburg et al. 2012). *D. magna* has been shown to have increased growth rate and earlier clutch-laying at higher temperatures, and increased growth rate will demand greater amounts of metabolically-important 20-carbon PUFA (Giebelhausen and Lampert 2001). This suggestion could have implications on temperature's role in trophic upgrading of fatty acids by *D. magna* in the ecosystem, but a more conclusive study with greater sample size would need to be performed.

Conclusions

This study examined *D. magna* inter-clonal variation in fecundity at 10 °C when fed *S. obliquus* and found no difference in the ability to produce successful clutches among clones. The heat tolerance of the clone also did not significantly influence this ability, rejecting the hypothesis of clonal variation at cold temperatures when limited in PUFA, and suggesting a lack of a trade-off for high heat tolerance. This lack of variation may suggest a critical restriction to consistent production of successful clutches in these conditions. It is likely that this restriction would be in the availability of unsaturated fatty acids. Investigation of the long-chain fatty acids of *D. magna* and their eggs in these conditions showed that a greater ratio of 20-carbon fatty acids to 18-carbon fatty acids correlate with adults that laid a failed clutch and eggs from a successful clutch. No detectable levels of 20-carbon fatty acids were found in adults that laid a successful clutch nor eggs from a failed clutch. This may suggest a parent-offspring conflict over the essential ARA (C20:4) and EPA (C20:5n-3) when this resource is limited. Higher levels of

unsaturation of 18-carbon fatty acids also loosely correlated with sample success, although sample size was low. The hypothesis of parent-offspring conflict over a limited resource was partially supported.

Future Research

Clonal variation studies on *D. magna* have been used to shed light on genetic differences in a variety of life history traits. The absence of clonal variation in this study may suggest that the experimental variables of temperature and diet were too extreme for any clone to perform well. Therefore, a follow-up study should be performed using a range of temperatures between 10 °C and 20 °C. The latter of which has been suggested to be the optimal temperature for *D. magna* fitness (Giebelhausen and Lampert 2001). Alternatively, since the limiting factor to cold-temperature reproduction is believed to be the limited ability to convert 18-carbon PUFA to 20-carbon PUFA, direct supplementation of 20-carbon PUFA, such as EPA or ARA, could relax this limiting factor. Supplementation of EPA and ARA to a *S. obliquus* diet has shown to increase population growth of *D. magna* kept at 10 °C up to a level equal to *N. limnetica* diet (Martin-Creuzburg et al. 2012). Varying dosage level of these PUFA could reveal a concentration at which certain phenotypes have an advantage in reproduction at 10 °C and could be analogous to PUFA levels in a polyculture of algae that a *Daphnia* might encounter in nature.

Support in this study for a parent-offspring conflict over PUFA fell short in the eggs of the 10 °C *S. obliquus* treatment that successfully developed. Adult *D. magna* that laid eggs that successfully developed in this treatment were found to not contain either ARA or EPA, and these PUFA were not found in the eggs they laid. A more direct measurement of fatty acids as they are taken up by *D. magna* from the diet, possibly converted into other fatty acids, and supplied to the eggs could be labelling the algal diet using carbon-13 isotopes. A metabolic flux analysis could

then be used to map where those carbon atoms are being metabolized in the animal and if they are invested in eggs (Wiechert 2001). ^{13}C may be able to be incorporated into algae in the form of CO_2 (Yang et al. 2005). Although ^{13}C metabolic flux analysis has not been widely used to track the metabolic pathways of *D. magna*, it has been used to track fatty acid synthesis in other organisms (Alonso et al. 2010). Mass spectrometry of labelled animals at different life stages, including before and after laying a clutch, would provide valuable detail on fatty acid investment into eggs.

REFERENCES

Abrusán G, Fink P, Lampert W. 2007. Biochemical limitation of resting egg production in *Daphnia*. Limnol. Oceanogr. 52(4):1724-1728.

Agrawal AA, Laforsch C, Tollrian R. 1999. Transgenerational induction of defences in animals and plants. Nature. 401:60-63.

Alonso AP, Dale VL, Shachar-Hill Y. 2010. Understanding fatty acid synthesis in developing maize embryos using metabolic flux analysis. Metabolic Engineering. 12:488-497.

Angilletta MJ, Steury TD, Sears MW. 2004. Temperature, growth rate, and body size in ectotherms: fitting pieces of a life-history puzzle. Integr. Comp. Biol. 44:498-509.

Atkin OK, Tjoelker MG. 2003. Thermal acclimation and the dynamic response of plant respiration to temperature. Trends in Plant Science. 8(7):343-351.

Becker C, Boersma M. 2005. Differential effects of phosphorus and fatty acids on *Daphnia magna* growth and reproduction. Limnol. Oceanogr. 50(1):388-397.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. 57(1):289-300.

Bligh EG, Dyer WJ. 1959 A rapid method of total lipid extraction and purification. J. Biochem. Physiol. 37: 911–917.

Breil C, Vian MA, Zemb T, Kunz W, Chemat F. 2017. “Bligh and Dyer” and Folch Methods for Solid–Liquid–Liquid Extraction of Lipids from Microorganisms. Comprehension of Solvatation Mechanisms and towards Substitution with Alternative Solvents. Int. J. Mol. Sci. 18(4):708.

Carvalho AP, Malcata FX. 2005. Preparation of fatty acid methyl esters for gas-chromatographic analysis of marine lipids: insight studies. J. Agric. Food Chem. 53:5049-5059.

Christian W, Maja I, Elert E. 2018. Differences in heat tolerance within a *Daphnia magna* population: the significance of body PUFA content. Hydrobiologia. DOI: 10.1007/s10750-018-3769-7. 1-10.

Christie WW. 1993. Advances in lipid methodology II. Oily Press. Bridgewater, UK. 342 p.

Christie WW, Han X. 2010. Lipid analysis – Isolation, separation, identification and lipidomics analysis (4th edition). Oily Press. Bridgewater, UK. 446 p.

Crawford MA. 1993. The role of essential fatty acids in neural development: implications for perinatal nutrition. The American Journal of Clinical Nutrition. 57(5):703S-710S.

Cronan JE. 2006. A bacterium that has three pathways to regulate membrane lipid fluidity. Molecular Microbiology. 60(2):256-259.

De Coninck DIM, Janssen CR, De Schamphelaere KAC. 2013. An investigation of the inter-clonal variation of the interactive effects of cadmium and *Microcystis aeruginosa* on the reproductive performance of *Daphnia magna*. Aquatic Toxicology. 140-141:425-431.

Desvilettes C, Bourdier G, Breton JC. 1997. On the occurrence of a possible bioconverstion of linolenic acid to docosahexaenoic acid by the copepod Eucyclopds serrulatus fed on microalgae. Journal of Plankton Research. 19:273-278.

Ebert D. 2005. Ecology, epidemiology, and evolution of parasitism in *Daphnia*. Bethesda (MD): National Library of Medicine 139 p.

Evangelou E, Ioannidis JPA. 2013. Meta-analysis methods for genome-wide association studies and beyond. Nature Reviews Genetics. 14:379-389.

Gasperin OD, Kilner RM. 2016. Interspecific interactions and the scope for parent-offspring conflict: high mite density temporarily changes the trade-off between offspring size and number in the burying beetle, *Nicrophorus vespilloides*. PLoS ONE. 11(3): e0150969. doi:10.1371/journal.pone.0150969

Giebelhausen B, Lampert W. 2001. Temperature reaction norms of *Daphnia magna*: the effect of food concentration. Freshwater Biology. 46:281-289.

Glazier DS, Calow P. 1991. Energy allocation rules in *Daphnia magna*: clonal and age differences in the effects of food limitation. Oecologia. 90(4):540-549.

Guschina IA, Harwood JL. 2006. Lipids and lipid metabolism in eukaryotic algae. Progress in Lipid Research. 45:160-186.

Harrison KE. 1990. The role of nutrition in maturation, reproduction and embryonic development of decapod crustaceans: a review. Journal of Shellfish Research. 9:1-28.

Hazel JR, Williams EE. 1990. The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. Prog. Lipid Res. 29:167-227.

Hazel JR. 1995. Thermal adaptation in biological membranes: Is homeoviscous adaptation the explanation?. Annu. Rev. Physiol. 57:19-42.

Hietala J, Laurén-Määttä C, Walls M. 1997. Sensitivity of *Daphnia* to toxic cyanobacteria: effects of genotype and temperature. Freshwater Biology. 37(2):299-306.

Kilham SS, Kreeger DA, Lynn SG, Goulden CE, Herrera L. 1998. COMBO: a defined freshwater culture medium for algae and zooplankton. Hydrobiologia. 377:147-159.

Liu KS. 1994. Preparation of fatty acid methyl esters for gas-chromatographic analysis of lipids in biological materials. J. Am. Oil. Chem. Soc. 71:1179-1187.

Martin-Creuzburg D, Wacker A, Ziese C, Kainz MJ. 2012. Dietary lipid quality affects temperature-mediated reaction norms of a freshwater key herbivore. Oecologia. 168:901-912.

Mehdipour N, Fallahi M, Azari-Takami G, Vossoughi G, Mashinchian A. 2011. Freshwater green algae Chlorella sp. and Scenedesmus obliquus enriched with B group of vitamins can enhance fecundity of *Daphnia magna*. Iranian Journal of Science & Technology. A2:157–163.

Miquel M, James D Jr., Dooner H, Browse J. 1993. Arabidopsis requires polyunsaturated lipids for low-temperature survival. Proc. Natl. Acad. Sci. 90:6208-6212.

Monroig Ó, Tocher DR, Navarro JC. 2013. Biosynthesis of polyunsaturated fatty acids in marine invertebrates: recent advances in molecular mechanisms. Marine Drugs. 11.

Tessier AJ, Consolatti NL. 1989. Variation in offspring size in Daphnia and consequences for individual fitness. Oikos. 56(2):269-276.

Padrón-Pérez D. 2000. Role of membrane lipid restructuring in thermal compensation of endocytosis [dissertation]. Arizona State University. 139 p.

Parker GA, Royle NJ, Hartley IR. 2002. Intrafamilial conflict and parental investment: a synthesis. The Royal Society. 357:295-307.

Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MAR, Bender D, Maller K, Sklar P, de Bakker PIW, Daly MJ, Sham PC. 2007. PLINK: a toolset for whole-genome association and population-based linkage analysis. American Journal of Human Genetics. 81.

Putman A, Martin-Creuzburg D, Panis B, Meester LD. 2015. A comparative analysis of the fatty acid composition of sexual and asexual eggs of *Daphnia magna* and its plasticity as a function of food quality. Journal of Planktonic Research. 37(4):752-763.

Ravet JL, Brett MT. 2006. Phytoplankton essential fatty acid and phosphorus content constraints on *Daphnia* somatic growth and reproduction. Limnol. Oceanogr. 51(5):2438-2452.

Restek. Guide to GC column selection and optimizing separations [online]. Restek. Available at: https://www.restek.com/pdfs/GNBR1724-UNV.pdf

Sargent JR, Tocher DR, Bell JG. 2002. Fish Nutrition. San Diego (CA): Academic Press 824 p.

Schlechtriem C, Arts MT, Zellmer ID. 2006. Effects of temperature on the fatty acids composition and temporal trajectories of fatty acids in fasting *Daphnia pulex* (Crustacea, Cladocera). Lipids. 41(4):397-400.

Scheuerbrandt G, Bloch K. 1962. Unsaturated fatty acids in microorganisms. J. Biol. Chem. 237:2064-2068.

Schrader MS. 2009. The expression and impact of parent-offspring conflict in natural populations of the least killifish, *Heterandria formosa* [dissertation]. Ann Arbor: Florida State University. 111 p.

Shimakata T, Stumpf PK. 1982. Purification and characterizations of beta-Ketoacyl-[acyl-carrier-protein] reductase, beta-hydroxyacyl-[acyl-carrier-protein] dehydrase, and enoyl-

[acyl-carrier-protein] reductase from Spinacia oleracea leaves. Proc. Natl. Acad. Sci. 79:5808-5812.

Sinensky M. 1974. Homeoviscous adaptation – A homeostatic process that regulates the viscosity of membrane lipids in Escherichia coli. Proc Natl Acad Sci. 71(2):522-525.

Sobrado LA, Freije-Carrelo L, Moldovan M, Encinar JR, Alonso JIG. 2016. Comparison of gas chromatography-combustion-mass spectrometry and gas chromatography-flame ionization detector for the determination of fatty acid methyl esters in biodiesel without specific standards. Journal of Chromatography A. 1457:134-143.

Tocher DR. 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Reviews in Fisheries Science. 11(2):107-184.

Vannice G, Rasmussen H. 2014. Position of the Academy of Nutrition and Dietetics: dietary fatty acids for healthy adults. J Acad Nutr Diet.114(1):136-153.

Wiechert W. 2001. 13C Metabolic Flux Analysis. Metabolic Engineering. 3:195-206.

Yang TH, Heinzle E, Wittmann C. Theoretical aspects of 13C metabolic flux analysis with sole quantification of carbon dioxide labeling. Comput. Biol. Chem. 29(2): 121-133.

Young JPW. 1979. Enzyme polymorphism and cyclic parthenogenesis in *Daphnia magna*. I. Selection and clonal diversity. Genetics. 92(3):953-970.

Yampolsky LY, Schaer TMM, Ebert D. 2014. Adaptive phenotypic plasticity and local adaptation for temperature tolerance in freshwater zooplankton. Proc. R. Soc. B. 281: 20132744. http://dx.doi.org/10.1098/rspb.2013.2744

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