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Investigation of Lipid Profile Patterns Along Latitude in an Eastern Australian Cline and After Stress Selection in the Laboratory, and Experiments on Stress Tolerance with RNAi Knockdown of Lipid Biosynthesis Genes

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Investigation of Lipid Profile Patterns Along Latitude in an Eastern Australian Cline and After Stress Selection in the Laboratory, and Experiments on Stress Tolerance with RNAi Knockdown of Lipid Biosynthesis Genes

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Investigation of Lipid Profile Patterns Along Latitude in an Eastern Australian Cline and After Stress Selection in the Laboratory, and Experiments on Stress Tolerance with RNAi Knockdown of Lipid Biosynthesis Genes

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Homeoviscous adaptation in membranes and changes in energy storage lipids such as triacylglycerols (TAGs) are the two main themes of adaptation to environmental conditions. Basal patterns of lipid compositional profile of *Drosophila melanogaster* along a latitudinal gradient in the eastern coast of Australia indicate variations of lipidome associated with climatic patterns. Lipidome data were collected for eight locations along the eastern Australian cline for both sexes in *D. melanogaster* and *D. simulans*. To further investigate the relationship between lipidome patterns and environmental factors, we utilized laboratory selection studies using stress conditions including high temperature tolerance, desiccation survival, and chill-coma recovery. Furthermore, to gain insights into possible underlying mechanisms between lipid biosynthesis and stress tolerance, knockdowns using RNA interference (RNAi) for various lipid biosynthesis enzymes were investigated. Observations in geographic patterns in the eastern Australian cline include a higher degree of acclimation in membrane lipids in phosphatidylcholine and phosphatidylethanolamine (PE/PC) ratio for higher latitude populations. There are increases of abundance for polyunsaturated and monounsaturated phospholipids as latitude increased Additionally, *D. melanogaster* populations originated from the only urban population consist of higher abundances for

most lipid classes when compared to all other locations along the latitude. In selection studies, PE/PC ratio did not have any noticeable variations under any of the stress selections. There is an increase of diacylglycerols (DAGs) in heat tolerance selected lines, this finding is consistent with the previous study of physiological response to heat stress. Furthermore, there is the unexpected finding of few responses to selection for chill-coma recovery in the laboratory. For RNAi knocked down studies, there is the surprising finding of chill-coma recovery patterns differ significantly between two isoforms of stearoyl-CoA desaturases knockdowns. Moreover, there are decreases in heat tolerance when TAG synthesis was disrupted. The cline study generates patterns of variation in lipids that suggests adaptation in wild populations. Insight into the evolution of lipid composition was obtained from stress-based laboratory selection experiments. Disruptions of lipid biosynthesis pathways provided insight into the relationship between lipids and stress tolerance.

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LIST OF ABBREVIATIONS

BHT	butylated hydroxytoluene
°C	degree Celsius
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
Ct	cytidylyltransferase
DAG	diacylglycerol
Desat	Δ9 desaturase
DGAT	diglyceride acyltransferase
GPAT	glycerol-3-phosphate acyltransferase
H_2O	dihydrogen monoxide
HPLC	high-performance liquid chromatography
KCL	potassium chloride
KD	knocked-down
lysoPE	lysophosphatidylethanolamine
lysoPC	lysophosphatidylcholine
mg	milligram
ml	milliliter
min	minute
µl	microliter
nmol	nanomole
PA	phosphatidic acid
PAP	phosphatidate phosphatase
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RT	room temperature
TAG	Triacylglycerol

Chapter 1.

Introduction

1.1 Overview

Climatic shift and geographic patterns of stress resistance

Climatic shifts have been observed globally. Surface temperature has been increased by about 0.2°C per decade over the last three decades (Hansen et al. 2006) , with projected temperature increase of up to 5.8°C by the end of this century (IPCC 2013). Due to the warmer surface temperatures, hotter summer, and colder winters are more common and there are longer warm seasons due to global warming (Hansen et al. 2012). Weather is predicted to become more extreme with global warming. There will be more rainfall during wet seasons, and dry seasons will be longer as well as drier (Suppiah 2007). These climatic changes, such as temperature and water resources shift, will impact ecosystems and biodiversity. In aquatic environments, it has been shown that there are variations in biomass even under minor temperature alterations (Brodersen et al 2011). Recent studies have shown the climatic shifts may have contributed to the expansion of ectotherms (temperate fish) from lower latitude to higher latitude, as well as retracting from lower latitude locations causing a shift in dwelling range (Franco et al. 2006, Farmer et al. 2015, Hickling et al. 2006, Parmesan 1996, Parmesan and Yohe 2003).

Ectotherms are important for terrestrial biodiversity (Wilson 1992) and are vulnerable to temperature changes (Cossins and Bowler 1987). It was suggested that insects in lower latitude are living in their optimum temperatures and will be impacted more by warmer global surface temperatures, while high latitude dwelling insects have greater thermal

plasticity as they are currently living in temperatures lower than optimal conditions (Bonebrake and Deutsch 2012, Deutsch et al. 2008, Dillon et al. 2010). The plasticity and tolerances with climatic changes are essential for population survival, especially with increasing anomalies in seasonal conditions (van Heerwaarden and Sgro 2014). The increasing warmer and drier conditions may contribute to shifting in clinal patterns. In a study of geographical genetic patterns of Adh^{s}, In(2L)t, IN(3R)Payne and α-$Gpdh^{f}$ along the eastern cline of Australia, it is shown that lower latitude populations now exhibit genetic patterns that were historically in higher latitude dwelling populations (Umina et al. 2005).

A study regarding tropical rainforest restricted *Drosophila* and widespread *Drosophila* in Australia revealed a lower genetic variance for rainforest restricted *Drosophila* species and high levels of genetic variance for desiccation stress resistance associated genes (Kellermann et al. 2009). The rainforest restricted *Drosophila* populations have a lower potential to response to low humidity conditions when under desiccation tolerance selections (Hoffmann et al. 2003). In van Heerwaarden et al. (2014), life-history traits of closely related *Drosophila* species, of rainforest and widespread distribution, were investigated. It was shown that while life-history traits such as fecundity, developmental success and development time, as well as heat resistance are similar between rainforest and widespread species, cold tolerances are higher for widespread species (van Heerwaarden et al. 2014). It was also shown that climatic limits for evolving for cold and desiccation resistance were limited in *Drosophila* species from a tropical environment, but heat resistance reached a plateau when setting at a stressful upper limit (Hoffmann 2010). This particular study also provided evidence that there are more variations

between thermal limits between different species of *Drosophila* compared to populations of same species from different climate environments. Thus *Drosophila* from lower latitude tropical areas may not be more vulnerable to climate shift compare to high latitude temperate species (Hoffmann 2010).

Drosophila as a model organism for geographic patterns and stress response studies

D. melanogaster has been used as a model organism for many years. *D. melanogaster* is a powerful tool for genetic study, as a large amount of genomic data are available. Developmental biology has been highly investigated using *Drosophila* genetic tools. *Drosophila* has also been used for environmental and adaptation studies. Geographic patterns have been studied regarding life history traits including fecundity, developmental time and reproduction diapause and other aspects of overwintering. Selection on specific genes, temperature adaptation and acclimation, climate change and shifts in species distribution, stress traits, heritable variation, behavior, population dynamics, bacterial symbionts, population genetics and physiology have all been investigated. Furthermore, stress selections and tolerance assays have been conducted in thermal stress, desiccation, chill-coma recovery and starvation conditions.

D. melanogaster has been used to assess the plasticity of heritable traits (Hoffmann et al. 2005). These adaptive patterns for various condition including high and low temperatures, desiccation, as well as starvation had been investigated. *D. melanogaster* has been used to investigate geographical evolution for *D. melanogaster* and adaptations in various ways including relevant laboratory selection studies.

Roles of lipids in insects

The fat body has an essential role in not only energy storage but is also an organ for biosynthetic and metabolism activity. Fat body in insects often carries out similar functions as adipose tissue and liver in mammals. In insects, about 90% of the lipid stored in the fat body is triacylglycerol (TAG), stored in the anhydrous form. Another key nutrient stored in the fat body includes glycogen, which is stored in a bulky hydrated form in the fat body. These glycogen storages are used as an energy reserve and for the synthesis of chitin and sugar alcohols. Some fatty acids are also stored in the fat body and can be utilized for phospholipid, eicosanoids and pheromone synthesis (Padmanabha and Baker 2014).

Insects often acquire lipids in forms of TAGs from dietary sources. TAGs are later digested in the midgut with a lipase to form diacylglycerols (DAGs) and free fatty acids. Then these dietary lipids are trafficked from the gut by Lpp which is an ApoB lipoprotein lipophorin, as lipoprotein particles. Typically, Lpp will transport DAGs in a 15:1 ratio to TAGs and these cargos of lipoprotein particle will be transported to the fat body for storage. Midgut localized lipid droplets with TAGs can also be formed and stored. Theses droplets will be mobilized when the insect is under starvation conditions (Padmanabha and Baker 2014).

Phospholipids are important components in membrane structure. In *Drosophila*, cellular membrane lipids are constructed primarily with phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (Jones et al. 1992). PC composed of about 40% and PE about 28% of all polar lipids in *Drosophila* (Scheitz et al. 2013). Where phosphatidylserine (PS) comprised about 3-5% of membrane lipids (Dobrosotskaya et al. 2002), as

phosphatidate (PA) influences curvature of the membrane, it is membrane component for ER and mitochondrial membranes (van Meer et al. 2008). PE is also a regulator for releasing sterol regulatory element-binding protein (SREBP) as a feedback control for the synthesis of fatty acid and phospholipids in *Drosophila* (Dobrosotskaya et al. 2002). PS is essential for recognizing and clearing apoptotic cells as well as important signaling factors for activation of many enzymes (Vance 2003). PA can recruit cytosolic proteins as a signaling lipid (Fernandis and Wenk 2007). Phosphatidylinositol (PI) is important in cell growth and survival (Cully et al. 2006). Functions of phosphatidylglycerol (PG) are not well understood in insects, as the only few functions identified are coating of lung alveoli in mammals (Okano and Akino 1979) and formation of cardiolipin in mitochondrial membranes (Claypool and Koehler 2012).

Free fatty acids are important precursors for many lipids including TAG, phospholipids, as well as cuticular lipids. Cuticular lipids have profound impacts on ecological and behavioral aspects of the insect, such as being a barrier to desiccation and potential microbial pathogens. Surface hydrocarbons also contribute to numerous biochemical, physiological, and behavior functions. Hydrocarbons, mainly n-alkanes, alkenes and methyl-branched chains, are the most common cuticular lipids (Blomquist 1987). In insects, the hydrocarbon is synthesized from fatty acids via an elongation-decarboxylation pathway as proposed by Dallerac et al. 2000.

Lipid and stress response

An important stress response is homeoviscous adaptation, where modifications of lipid composition occur during thermal acclimation to maintain membrane structure and fluidity (Sinensky 1974). The degree of homeoviscous adaptation has been a focus of

lipid adaptation in response to environments and can influence cellular aspects including the membrane-localized enzymatic reaction and membrane-protein functions (Hochachka and Somero 2002). Studies of homeoviscous adaptations have been conducted using *D. melanogaster* populations originated from low to high latitude locations on the eastern coast of the United States by Cooper et al. (2013). Where *D. melanogaster* from a high latitude location showed greater degrees of homeoviscous adaptation in PE and PC ratio of membrane lipids when reared in warmer temperature with greater decreases of PE and PC ratio from higher latitude populations when there is an increase of developmemntal temperature (Cooper et al. 2013). This indicated higher plasticity of membrane lipid adaptation from a high latitude environment. Thermal limits of animals show a strong correlation with structures of the membrane in *Drosophila*. When exposed to cold temperature, both long and short term, lipid compositions in *Drosophila* will be modified as described immediately following. An increase of saturation to membrane lipid from C16:0 to C16:1 and C18:1 to C18:2 will occur (Cooper et al. 2011, Overgaard et al. 2006). Thermal stress also influences the concentrations of phospholipids, when developed at various temperatures, *Drosophila* exhibited strong correlations between PE/PC ratio and development temperatures, as warmer development temperatures result in lower PE/PC ratio (Cooper et al. 2014).

In response to heat stress, lipid storage decreases with the number of double bonds. This trend is consistent with humidity increases (Parkash et al. 2014). When under heat stress, it is shown that small heat shock protein (sHSP) can regulate membrane lipid polymorphism and influence none membrane lipids by altering saturations.

Membrane fluidity is related to the degree of unsaturation in lipids and lipid chain length. As the degree of unsaturation increases, the fluidity of phospholipid membranes increases as well (Hazel 1995). Chain length and membrane is based on motions of acyl chains in glycerophospholipids in the membrane, as the chain length increased, the degree of fluidity increases (Morrow et al. 1995).

1.2 Objectives

For better understanding of geographic patterns with a lipidome perspective, our first objective was to establish a baseline of lipid composition along latitude. In chapter two, we utilized laboratory populations of *D. melanogaster* and *D. simulans* originated from various locations along the eastern coast of Australia to generate a detailed dataset of lipid profile including about 350 lipid species within two neutral lipid classes, seven polar lipid classes, and free fatty acids. The analysis was conducted for relative abundance of lipid species within classes along with a latitude gradient. In this dataset, there was an intriguing suggestion that in male and female *D. melanogaster* of a trend for PE/PC adaptation to elevated rearing temperature decreasing from north to south along the cline. This pattern was similar to Cooper et al. (2011). One observation was that lipid compositional patterns varied along the latitudinal gradient with gender as well as between species. A major observation was that almost all classes and species of lipid were higher in the Melbourne sample.

The next objective was to investigate patterns for lipid composition from various selected populations using stress including heat, desiccation, and chill coma recovery. The

selection studies described in chapter three could be related to clinal variation in the first objective. In the laboratory selection experiments, it was shown that there are no significant changes in PE/PC ratio under any of the stress selections. There was a decrease of TAG in desiccation selected lines, which is consistent with findings in (Gibbs et al. 1997) and (Chippindale et al. 1998) but not in (Djawdan et al. 1998). There was a pronounced increase in DAG abundance in heat selected lines. This can result from a carried-over effect of generations of adaptive response to heat stress selection inferred from the fact that heat stress reduces the ability for *D. melanogaster* to store TAG by irreversible damage to fat cells (Klepsatel et al. 2016). Additionally, there was little significant variation between chill-coma recovery selected lines and control. This lack of difference in lipid composition is potentially consistent with previously proposed mechanisms of induction of chill coma as only one mechanism is related to membrane fluidity under low temperature (Macmillan and Sinclair 2011). Another observation that desiccation survival selected line showed greater significance in variations of lipid classes. This can be resulted from differences in selection pressure, as survival selection experiments are stronger than tolerance selection experiments (Stearns et al. 2000).

To understand lipid biosynthesis interference and stress tolerance, as relevant to the cline and selection data, in chapter four, a set of knock-downs of lipid biosynthesis enzymes using RNAi constructs were employed. In this work, there were delays in chill-coma recovery time when stearoyl-CoA desaturase genes, Desat1 or Desat2 were knocked down. This indicated possible important relationships between unsaturation in lipid structures and chill-coma recovery. There were significant differences between chill-coma recovery patterns between Desat1 and Desat2, which indicated they may play

different roles in lipid biosynthesis pathway. Our previous work indicated that Desat1 and Desat2 behaved similarly for various life-history features including longevity, fecundity, and larval survival under minimal fat diet, as well as lipid composition under different diets (Ko et al. 2013). This was the first time that a significant difference in a phenotypic response has been seen between Desat1 and Desat2. When synthesis of TAG was disrupted, there was a decrease in heat tolerance. This may have resulted from an increase of metabolic rate, which then depleted energy reserves faster, and when the fly ran out of energy reservoir, it became immobile.

Overall, our work established an understanding of geographic patterns in lipidome along latitude in the eastern coast of Australia which motivates future studies on urban versus rural population and homeoviscous adaptation along clines. Laboratory stress selection indicated the important of understanding the role of DAG as a TAG precursor heat adaptation evolution studies and in relationship to elevated heat phenotypic studies. It is important to understand genetic mechanisms of desiccation survival evolution. Moreover, the nature of chill-coma recovery as a low-temperature trait needs investigations to understanding if is associated with cellular membranes. Our lipidome analysis of the response to selection suggests it is not. Our RNAi knockdown data suggest that chill-coma recovery is correlated with unsaturation level of lipids which is novel and suggests avenues of future research.

1.3 Literature cited

Blomquist G. J., Nelson D. R., Derenobales M. 1987. 'Chemistry, biochemistry, and physiology of insect cuticular lipids.', *Arch. Insect Biochem. Physiol.*, 6: 227-65.

Bonebrake, T. C., and C. A. Deutsch. 2012. 'Climate heterogeneity modulates impact of warming on tropical insects', *Ecology*, 93: 449-55.

Brodersen, J., J. L. Rodriguez-Gil, M. Jonsson, L. A. Hansson, C. Bronmark, P. A. Nilsson, A. Nicolle, and O. Berglund. 2011. 'Temperature and resource availability may interactively affect over-wintering success of juvenile fish in a changing climate', *PLoS One*, 6: e24022.

Chippindale, A. K., A. G. Gibbs, M. Sheik, K. J. Yee, M. Djawdan, T. J. Bradley, and M. R. Rose. 1998. 'Resource acquisition and the evolution of stress resistance in Drosophila melanogaster', *Evolution*, 52: 1342-52.

Claypool, S. M., and C. M. Koehler. 2012. 'The complexity of cardiolipin in health and disease', *Trends Biochem Sci*, 37: 32-41.

Cooper, B. S., L. A. Hammad, J. A. Karty, and K. L. Montooth. 2011. 'Evolution of membrane acclimation in experimentally evolved populations of Drosophila melanogaster', *Integrative and Comparative Biology*, 51: E177-E77.

Cooper, B. S., L. A. Hammad, and K. L. Montooth. 2013. 'The evolution of cellular generalization and specialization in natural populations of Drosophila melanogaster', *Integrative and Comparative Biology*, 53: E40-E40.

Cooper, B. S., L. A. Hammad, and K. L. Montooth. 2014. 'Thermal adaptation of cellular membranes in natural populations of Drosophila melanogaster', *Funct Ecol*, 28: 886-94.

Cossins, Andrew R., and K. Bowler. 1987. *Temperature biology of animals* (Chapman and Hall: London ; New York).

Cully, M., H. You, A. J. Levine, and T. W. Mak. 2006. 'Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis', *Nat Rev Cancer*, 6: 184-92.

Dallerac, R., C. Labeur, J. M. Jallon, D. C. Knipple, W. L. Roelofs, and C. Wicker-Thomas. 2000. 'A delta 9 desaturase gene with a different substrate specificity is responsible for the cuticular diene hydrocarbon polymorphism in Drosophila melanogaster', *Proc Natl Acad Sci U S A*, 97: 9449-54.

Deutsch, C. A., J. J. Tewksbury, R. B. Huey, K. S. Sheldon, C. K. Ghalambor, D. C. Haak, and P. R. Martin. 2008. 'Impacts of climate warming on terrestrial ectotherms across latitude', *Proc Natl Acad Sci U S A*, 105: 6668-72.

Dillon, M. E., G. Wang, and R. B. Huey. 2010. 'Global metabolic impacts of recent climate warming', *Nature*, 467: 704-U88.

Djawdan, M., A. K. Chippindale, M. R. Rose, and T. J. Bradley. 1998. 'Metabolic reserves and evolved stress resistance in Drosophila melanogaster', *Physiol Zool*, 71: 584-94.

Dobrosotskaya, I. Y., A. C. Seegmiller, M. S. Brown, J. L. Goldstein, and R. B. Rawson. 2002. 'Regulation of SREBP processing and membrane lipid production by phospholipids in Drosophila', *Science*, 296: 879-83.

Farmer, T. M., E. A. Marschall, K. Dabrowski, and S. A. Ludsin. 2015. 'Short winters threaten temperate fish populations', *Nat Commun*, 6: 7724.

Fernandis, A. Z., and M. R. Wenk. 2007. 'Membrane lipids as signaling molecules', *Curr Opin Lipidol*, 18: 121-8.

Franco, A. M. A., J. K. Hill, C. Kitschke, Y. C. Collingham, D. B. Roy, R. Fox, B. Huntley, and C. D. Thomas. 2006. 'Impacts of climate warming and habitat loss on extinctions at species' low-latitude range boundaries', *Global Change Biology*, 12: 1545-53.

Gibbs, A. G., A. K. Chippindale, and M. R. Rose. 1997. 'Physiological mechanisms of evolved desiccation resistance in Drosophila melanogaster', *J Exp Biol*, 200: 1821-32.

Hansen, J., M. Sato, and R. Ruedy. 2012. 'Perception of climate change', *Proc Natl Acad Sci U S A*, 109: E2415-23.

Hansen, J., M. Sato, R. Ruedy, K. Lo, D. W. Lea, and M. Medina-Elizade. 2006. 'Global temperature change', *Proc Natl Acad Sci U S A*, 103: 14288-93.

Hazel, J. R. 1995. 'Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation?', *Annu Rev Physiol*, 57: 19-42.

Hickling, R., D. B. Roy, J. K. Hill, R. Fox, and C. D. Thomas. 2006. 'The distributions of a wide range of taxonomic groups are expanding polewards', *Global Change Biology*, 12: 450-55.

Hochachka, Peter W., and George N. Somero. 2002. *Biochemical adaptation : mechanism and process in physiological evolution* (Oxford University Press: New York).

Hoffmann, A. A. 2010. 'Physiological climatic limits in Drosophila: patterns and implications', *J Exp Biol*, 213: 870-80.

Hoffmann, A. A., R. J. Hallas, J. A. Dean, and M. Schiffer. 2003. 'Low potential for climatic stress adaptation in a rainforest Drosophila species', *Science*, 301: 100-2.

Hoffmann, A. A., J. Shirriffs, and M. Scott. 2005. 'Relative importance of plastic vs genetic factors in adaptive differentiation: geographical variation for stress resistance in Drosophila melanogaster from eastern Australia', *Functional Ecology*, 19: 222-27.

IPCC. 2013. 'Summary for Policymakers.' in T.F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.), *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (Cambridge University Press: Cambridge, United Kingdom and New York, NY, USA).

Jones, H. E., J. L. Harwood, I. D. Bowen, and G. Griffiths. 1992. 'Lipid composition of subcellular membranes from larvae and prepupae of Drosophila melanogaster', *Lipids*, 27: 984-7.

Kellermann, V., B. van Heerwaarden, C. M. Sgro, and A. A. Hoffmann. 2009. 'Fundamental evolutionary limits in ecological traits drive Drosophila species distributions', *Science*, 325: 1244-6.

Klepsatel, P., M. Galikova, Y. Xu, and R. P. Kuhnlein. 2016. 'Thermal stress depletes energy reserves in Drosophila', *Sci Rep*, 6: 33667.

Ko, L., Black, P. N., Harshman, L. G. 2013. 'Investigation of the life history and lipid content consequences of reduced abundance of Δ9 Desaturases in *Drosophila melanogaster*.'

Macmillan, H. A., and B. J. Sinclair. 2011. 'Mechanisms underlying insect chill-coma', *J Insect Physiol*, 57: 12-20.

Morrow, M. R., Whitehead, J. P., and Lu, D. (1992). Chain-length dependence of lipid bilayer properties near the liquid crystal to gel phase transition. *Biophysical Journal*, *63*(1), 18–27.

Okano, G., and T. Akino. 1979. 'Variations in the molecular species of lung phosphatidylglycerol', *Lipids*, 14: 541-6.

Overgaard, J., J. G. Sorensen, S. O. Petersen, V. Loeschcke, and M. Holmstrup. 2006. 'Reorganization of membrane lipids during fast and slow cold hardening in Drosophila melanogaster', *Physiological Entomology*, 31: 328-35.

Padmanabha, D., and K. D. Baker. 2014. 'Drosophila gains traction as a repurposed tool to investigate metabolism', *Trends Endocrinol Metab*, 25: 518-27.

Parkash, R., P. Ranga, and D. D. Aggarwal. 2014. 'Developmental acclimation to low or high humidity conditions affect starvation and heat resistance of Drosophila melanogaster', *Comp Biochem Physiol A Mol Integr Physiol*, 175: 46-56.

Parmesan, C. 1996. 'Climate and species' range', *Nature*, 382: 765-66.

Parmesan, C., and G. Yohe. 2003. 'A globally coherent fingerprint of climate change impacts across natural systems', *Nature*, 421: 37-42.

R. Suppiah, K.J. Hennessy, P.H. Whetton, K. McInnes, I. Macadam, J. Bathols, J. Ricketts and C.M. Page. 2007. 'Australian climate change projections derived from simulations performed for the IPCC 4th Assessment Report.', *Australian Meteorological Magazine*, 56: 131-52.

Scheitz, C. J. F., Y. Guo, A. M. Early, L. G. Harshman, and A. G. Clark. 2013. 'Heritability and Inter-Population Differences in Lipid Profiles of Drosophila melanogaster', *PLoS One*, 8.

Sinensky, M. 1974. 'Homeoviscous adaptation--a homeostatic process that regulates the viscosity of membrane lipids in Escherichia coli', *Proc Natl Acad Sci U S A*, 71: 522-5.

Stearns, S. C., M. Ackermann, M. Doebeli, and M. Kaiser. 2000. 'Experimental evolution of aging, growth, and reproduction in fruitflies', *Proceedings of the National Academy of Sciences of the United States of America*, 97: 3309-13.

Terblanche, J. S., A. A. Hoffmann, K. A. Mitchell, L. Rako, P. C. le Roux, and S. L. Chown. 2011. 'Ecologically relevant measures of tolerance to potentially lethal temperatures', *J Exp Biol*, 214: 3713-25.

Umina, P. A., A. R. Weeks, M. R. Kearney, S. W. McKechnie, and A. A. Hoffmann. 2005. 'A rapid shift in a classic clinal pattern in Drosophila reflecting climate change', *Science*, 308: 691-3.

van Heerwaarden, B., R. F. Lee, J. Overgaard, and C. M. Sgro. 2014. 'No patterns in thermal plasticity along a latitudinal gradient in Drosophila simulans from eastern Australia', *J Evol Biol*, 27: 2541-53.

van Heerwaarden, B., and C. M. Sgro. 2014. 'Is adaptation to climate change really constrained in niche specialists?', *Proc Biol Sci*, 281.

van Meer, G., D. R. Voelker, and G. W. Feigenson. 2008. 'Membrane lipids: where they are and how they behave', *Nat Rev Mol Cell Biol*, 9: 112-24.

Vance, J. E. 2003. 'Molecular and cell biology of phosphatidylserine and phosphatidylethanolamine metabolism', *Prog Nucleic Acid Res Mol Biol*, 75: 69-111.

Wilson, E. O. 1992. 'The diversity of life.′ Harvard Univ Press, Cambridge, MA.

Chapter 2.

Geographic patterns of *Drosophila melanogaster* lipidome along the eastern coast of Australia.

2.1 Introduction

Latitudinal clines of *D. melanogaster* are found on multiple continents, and phenotypic traits including life-history and stress traits, are known to varied geographically (Bochdanovits and de Jong 2004). *D. melanogaster* was originally an inhabitant of Africa having colonized Australia and North America relatively recently (Schmidt and Paaby 2008). The North American and Australian populations have responded with reproductive diapause; diapause generally results in modulation of energy compound (glycogen, lipids) stores allowing for increased energy to be available during periods where feeding is reduced or food is not available (Denlinger 2012).

Lipids are essential for membrane structure and energy storage in organisms, and exhibit a variety of signaling functions. Two primary foci in studies of the evolutionary biology of lipids are differences in abundance of energy storage compounds and homeoviscous adaptations in membranes. The energy storage compounds such as TAGs, support life history adaptation and stress survival. Homeoviscous adaptations in terms of membrane fluidity alteration typically are in response to temperature differences. Adaptation and geographic variation in lipids have been investigated in various organisms. In this chapter, we focus on the lipidome of *D. melanogaster* in samples collected from locations along the latitude of the eastern coast of Australia.

Studies of cricket dispersal polymorphism have become a model for investigation of the mechanisms of adaptive lipid storage. The relationship between these studies and the present research is an emphasis on the perspective that focuses on the possible mechanisms in relationship to lipids and adaptation. Wing polymorphism is a manifestation of a life history tradeoff in crickets between high levels of reproduction early in life versus dispersal capability (Bell 1986, Zera and Denno 1997). Studies of flightless morphs versus long-winged forms of *Gryllus* have determined that relatively large ovaries are associated with an absence of flight, whereas large wing muscles for dispersal are associated with approximately 30% greater triglyceride storage (Mole and Zera 1993, Zera and Larsen 2001, Zera et al. 1994). High levels of reproduction are associated with phospholipid synthesis for egg production, whereas enhanced biosynthesis of triglyceride as a flight fuel is a characteristic of the dispersal-capable long-winged crickets (Zhao and Zera 2002). In comparison to flightless crickets, intermediary metabolism enzyme activities associated with lipid biosynthesis and enzymes that directly synthesize triglyceride are elevated in the long-winged crickets (Schilder et al. 2011, Zera and Harshman 2011, Zera and Zhao 2003). Although there is an amplified difference in lipid biosynthesis between wing morphs on high carbohydrate diets, a nutritional geometry study has revealed that lipid biosynthesis is consistently greater in the long-winged morph from across a wide-range of environments (Zera, et al. 2016). It can be argued that lipid differentiation is well-canalized in terms of stability in response to diet. The stability of genetic-based biosynthesis across environments is possibility relevant to the maintenance of geographic variation in lipid compositions after long-term culture in the laboratory environment in our study.

D. melanogaster has been used as a model organism to study geographic variation and adaptation in clines found on the eastern coast of North America and eastern coast of Australia (Hoffmann and Weeks 2007, Schmidt et al. 2005). There is evidence that the Australian and North American clines are similar in terms of the influence of latitude on genetic adaptation. As an example of specific genetic correspondence, an inversion (*In(3R)Payne*) is present along the east coast of Australia, the east coast of North America, and other clines elsewhere in the world (Kapun et al. 2016). Typically, this inversion is at a mid-range frequency at low latitudes and uncommon or absent at high latitudes. The inversion is associated with larger body size variation in Australia and North America but is not correlated with development time, cold resistance, egg-to-adult survival, oxidative stress resistance, or lipid content.

Given that the North American and Australian clines are at least similar in genetic adaptation, it stands to reason that biochemical lipid-related adaptations associated with the North American cline will be relevant to the Australian cline and implicate geographic lipid adaptation in Australia. Thus, the evolution of intermediary metabolism genes in *Drosophila* along a temperature gradient in eastern North America is relevant to the present study. In these studies, there has been a long-standing focus on polymorphism and evidence for selection on the *G6PD* gene which encodes a key pentose shunt enzyme that plays a key role in the biochemistry of lipid synthesis (Eanes 1999). Strong inference about the effect of allelic variation at the *G6PD locus* on triglyceride pools was obtained by generating a series of P-element excision alleles crossed to a common genetic background (Merritt et al. 2006). In general, there is evidence for selection on intermediary metabolism genes (Eanes 1999, Flowers et al. 2007, Sezgin et al. 2004)

often relevant to lipid biosynthesis. One of these genes, the *glucose-6-phosphate dehydrogenase gene* exhibits evidence for selection within species by latitudinal variation and by the pattern of amino acid substitution between species (Eanes 2011). Genetic evidence for lipid-related adaptations in *D. melanogaster* and in relationship to a related species is relevant to the present study based on the geographic variation of lipids in *D. melanogaster* and closely related *D. simulans*.

The eastern North America cline of *D. melanogaster* showed a female reproductive diapause phenotype (Schmidt et al. 2005). The significance of this phenomenon for the present study is that adult *D. melanogaster* is mostly post-mitotic, but reproductive diapause is a result of active cell division potentially related to various types of cellular signaling that could be related to geographic variation in signaling polar lipids. Diapause responds to laboratory selections using environmental stress, and thus there is a clear genetic basis to it (Schmidt and Conde 2006), an underlying genetic basis is the couch potato gene (Schmidt et al. 2008). Along the eastern North America cline, populations in higher latitude exhibit higher abundances of TAG compared to lower latitudinal populations (Schmidt et al. 2005) this can result from selection by seasonal cold and starvation conditions. When diapause phenotype is expressed, there is a slight decrease in lipid content although reproductive diapause is associated with stress resistance, such as survival under cold and starvation conditions (Schmidt et al. 2005). In general, the diapause phenotype is related to lipid adaptation associated with latitude which is relevant to reproductive diapause along the Australian cline.

Highly relevant to the present study is a geographic variation lipidomics study in *D. melanogaster* based on 92 inbred lines derived from five location around the world

(Scheitz et al. 2013). The dataset included eight classes of polar lipids including PE and PC which are the most abundant and second major polar lipids in *D. melanogaster* and their precursors lyso PEs and lyso PCs (Fast 1966, Hammad et al. 2011, Jones et al. 1992). Neutral lipids in this dataset included 61 species of TAG and 33 species of DAG. Using a biochemical pathway software framework (Cytoscape) the abundance covariation of lipid classes was analyzed and correspondence to clusters of known pathways and novel connections between lipids was identified. Measurements of lipid abundance in inbred lines allowed for quantification of lipid heritability. Also, in this paper variation in membrane phospholipids, suggested a role they play in modulating membrane fluidity in the Netherlands population as these flies were more resistant to ethanol stress (Scheitz et al. 2013). The precedent for the test of membrane fluidity by ethanol stress is described in Montooth et al. (2006).

In relationship to the present research, another highly relevant study of geographic variation was conducted to examine homeoviscous adaptation (Cooper et al. 2014). The degree of homeoviscous adaptation has been an important focus in environmental lipid adaptations (Hochachka and Somero 2002). Structurally, PE is a cone shaped phospholipid which increases membrane fluidity when presented in membranes. While PC is a rod shaped phospholipid which decreases membrane fluidity when presented in membrane structures. Typically, membrane fluidity can be calculated with PE to PC ratio. A higher PE compares to PC ratio results in membranes, which are less rigid and subject to dysfunction at high temperature. When compared fly samples collected from along the latitude in eastern North America and subjected the lines to various temperatures during development and adult stages, the population collected at higher

latitude was more thermally variable in the natural environment, they have the strongest response to elevation of temperature as shown by a greatest decrease in PE/PC ratio (Cooper et al. 2014).

Numerous studies have been conducted on *Drosophila* species along the latitude in eastern Australia for research in genetics, life history and stress resistance of climatic adaptation-disciplines (Hangartner et al. 2015, Hoffmann and Weeks 2007, Kennington and Hoffmann 2013, Weeks et al. 2006). An overarching goal of this work has been to investigate climatic adaptation (Hoffmann and Weeks 2007). Many studies have been conducted on species distributed along this latitudinal cline in eastern Australia, and many datasets exist from this research (Hangartner et al. 2015). Research on *D. melanogaster* is highly represented, but other cosmopolitan species and many Australian species are represented. The datasets include morphological features such as thorax length and wing morphology and pigmentation. Life-history traits include datasets on developmental time, survivorship data, and reproductive traits. Another category of datasets is based on stress including cold, desiccation, heat, and starvation resistance. Genetic-related datasets are also present including DNA polymorphism, genetic markers, gene expression and inversion polymorphisms. There is a lipid dataset on *D. melanogaster* females that were total lipid amount extracted by ether. Our research greatly extends the information on lipids in two cosmopolitan species; *D. melanogaster* and *D. simulans*. The thermal and life history adaptation focus of research on the Australian cline (Hoffmann and Weeks 2006) provides a compelling framework for the lipid profile investigation reported in the present study. We here report the abundance of

approximately 350 lipids belonging to two neutral lipid classes, seven polar lipid classes, and free fatty acids.

2.2 Methods

Fly collections and isofemale line maintenance

Flies were collected using a net-sweeping method. The environmental conditions of collection, the locations (Fig. 2.1), and latitudes in parenthesis: fallen citrus fruits in Innisfail (-17.5), a bucket of mixed fruits in Bowen (-20), roadside fruit stall bin in both Rockhampton (-23.4), and Maryborough (-25.5), a banana plantation shed in Coffs Harbour (-30.2), a citrus farm bin in Gosford (-33.4), a domestic compost bin in Melbourne (-37) and a winery grape pile in North Tasmania (-41.2). Once flies reached the laboratory they were identified and *D. melanogaster* females were set up in individual vials to form isofemale lines. On the emergence of F1 flies, the identification was confirmed. Flies were cultured on a standard *Drosophila* diet consisting of 7.3% yellow cornmeal, 3.5% dried yeast, 2% soy flour, 7.5% dextrose, 0.6% agar, 1.4% acid mix and 1.65% Nipagin at 19°C. Flies from all populations were reared in the laboratory at 19°C for 29 generations before they were collected for the present study except for Melbourne which was reared for 30 generations (provided by Dr. Sandra Hangartner from Hoffmann laboratory at University of Melbourne).

The populations used for lipid assays are as follow: eight *Drosophila* populations were collected during a similar time frame as mentioned aboved. Female *D. melanogaster* from eight of these populations were used for extracts for lipid profiles. Resources to analyze samples were limited and fewer than eight populations were used as a source of

male samples. Two northern and two southern populations were used for males. A minimum of three samples was perceived to compare *D. simulans* with *D. melanogaster*. Three populations were used for male and female for samples to be extracted representing *D. simulans*.

Lipid extractions and analysis

Eggs were collected and reared at 25°C for adult lipid extractions. When adults emerged they were transferred to fresh food after the first day of adult life and held at 25°C. This temperature is approximately the average daily summer (December) temperature in Melbourne (Hoffmann et al. 2005). The lines were maintained at 19°C with is approximately the average daily winter (May) conditions in Melbourne (Hoffmann et al. 2005). Therefore, the sample lines used for lipid prospectively experienced a seasonal shift from winter to summer. Mixed sex vials with these flies were allowed to mate until through day 4 of adult life. At this time females and males were separated under CO_2 and then flash frozen in liquid nitrogen. Five males or five females were frozen per vial which was stored at -20°C.

These flies were subjected to a standard lipid extraction protocol (Bligh and Dyer. 1959) which generally extracts the range of lipids present in animals. Each extraction was from 5 mated flies of only either males or females. Each tube with aqueous (HPLC grade water) chloroform (BHT) - methanol solution and 5 flies was inverted gently five times and left at room temperature (approximately 24°C) for 1 hour. The tubes were then held overnight at -20°C. The extraction tubes were removed from -20°C and placed at RT for one hour. An internal standard (15:0 TAG) was added to each vial based on the weight of 5 comparable flies and the approximate expected triglyceride content of the flies

estimated; the internal standard was present at approximately 20-30% of the total triglyceride. Equal parts of chloroform and H_2O were added to the extraction solution. The samples were back-extracted three times and washed with a KCL solution. Once extractions were made completed, the samples were dried under a gentle stream of nitrogen in 2 ml glass vials with Teflon caps. The dried samples were transported to the University of Nebraska-Lincoln and sent to Kansas State University for lipid analysis.

The analysis of the lipid extraction was performed at the Kansas Lipidomics Research Center (KLRC). *Drosophila* samples with standards were introduced into a tandem mass spectrometer (Applied Biosystems API 4000) by continuous infusion insolvent into the electrospray ionization source. The ion fragments of the lipids were separated in an electric field and sequentially scanned to identify lipids by class with peaks within individual lipid classes corresponding to different lipid species. Quantification of each lipid species occurred by comparison to internal standards. The KLRC estimated the ratios of TAGs to polar lipids, DAGs to polar lipids and free fatty acids to polar lipids. For example, the normalized signal for total TAGs (nmol) was divided by the total nmol for polar lipids. This parameter was not an exact measurement of concentration, as the estimate of TAGs was not precise due to an inability to determine each of the three fatty acids present on each triglyceride molecule. The estimated ratios were not used for statistical analyses.

Statistical analysis

Statistical analyses were conducted using generalized linear mixed models (GLIMMX) in SAS 9.4 unless otherwise notated. To determine statistical significance between

normalization based on per fly or lean mass, two-way ANOVA was performed using GraphPad Prism V6.0.

2.3 Results

Geographical lipid patterns of abundances

As shown in Tables 2.1A-F, there are significant trends in geographic patterns for lipid composition presented for the various lipid classes. In male *D. melanogaster*, as latitude increase, there are significant increases in total abundances of free fatty acids and decreases in PA, PI, PS, lysoPE and lysoPC as shown in (Table 2.1A). For female *D. melanogaster*, there are significant increases in free fatty acids and decreases in total polar lipids, PE, PC and PA in abundance along the latitude (Table 2.1B). Slight elevation of TAG was also observed in both sexes of *D. melanogaster* (Table 2.1A and B).

D. simulans tended to exhibit different trends in abundance of each lipid class compared with *D. melanogaster*. In general, the slope of change in lipid abundance for *D. simulans* males and females were insignificant (Table 2.1C and D). While examining the variation of lipid abundance between *D. melanogaster* and *D. simulans*, there were significant differences in free fatty acids, PA, and PS for males and total polar lipids, free fatty acids, PE, PC and PA in females (Table 2.1E and F).

Trends of lipid patterns in abundances are consistent between normalization to per fly or body mass.

Since geographical adaptations have also been shown in body size variations, it is important to examine if there are deviations of lipid trends when correcting for body mass. Here we looked at several lipid classes when they are calculated based on nmol per fly or nmol per mg of lean mass in *D. melanogaster*. As results showed, when the abundances of total lipids are calculated per fly (Fig. 2.2A) compared to calculated per lean mass per fly (Fig. 2.2B) the trend of the two remain the same. Using two-way ANOVA, the p-values between male and female slopes based on per fly and per lean mass are 0.67 and 0.8 respectively (data not shown) which are statistically insignificant. These trends are consistent in total polar lipids as well, when the concentrations of lipid are normalized to per fly in total polar lipids, the general trends of abundance for total polar lipids remained the same and the slopes between normalizations were insignificantly different with p-values of 0.16 for males and 0.31 for females with two-way ANOVA (Fig. 2.3A and B).

Additionally, when total neutral lipids concentrations are calculated based on per fly and normalized to lean mass the p-values for comparing the slopes using two-way ANOVA are 0.98 for males and 0.85 for females (Fig. 2.4A and B). While TAG being the major component of neutral lipids, the trends for normalization based on per fly or per lean mass had no significant deviations with p-values of 0.79 for males and 0.96 for females (Fig. 2.5A and B).

Geographical patterns in membrane lipid compositions

Membrane lipids composition is another indication of local adaptation to climate, as PE/PC ratio for *D. melanogaster*, decreased along latitude from 5.5 to 4.3 (Fig. 2.6A). This trend was not significant, but it was observed for females and males and the slopes

were similar. Moreover, the response to elevated rearing temperature is similar to what was observed in Cooper et al. (2014). For *D. simulans*, PE/PC ratio increases from 5.3 to 6.3 as latitude increases (Fig. 2.6B). As is often the case, the two species differed in lipid geographic variation pattern.

There was a change in abundance for polyunsaturated and monounsaturated lipids and saturated PE and PC in *D. melanogaster*. In male *D. melanogaster*, there were increases in monounsaturated and polyunsaturated PE whether calculated the concentration based on per fly or lean mass per fly (Fig. 2.7A and B). Monounsaturated PE increased with a change of about 0.5 nmol per fly between northern location to southern location and polyunsaturated PE increased about 2 nmol per fly between northern and southern locations (Fig. 2.7A). Female *D. melanogaster* had an increase of about 1 nmol per fly for both monounsaturated and polyunsaturated PE (Fig. 2.7C). These trends are consistent when normalizing the concentration of lipids to lean mass (Fig. 2.7D), with a general trend of increase in monounsaturated and polyunsaturated PE with a spike of the concentration of both at Melbourne location.

The abundance of various PC species showed a similar trend to PE in both male and female *D. melanogaster*. There is a slight elevation of 0.02 nmol per fly in monounsaturated PC and a 0.5 nmol per fly in polyunsaturated PC (Fig. 2.8A). For *D. melanogaster* females, there is a 0.5 nmol per fly increase in abundances of monounsaturated PC and 1nmol per fly increase in polyunsaturated PC as latitude increased (Fig. 2.8C).

D. simulans presented different trends for abundances of various PEs, there is a general decrease of monounsaturated and polyunsaturated PE as latitude increase. In males, the

decrease of monounsaturated and polyunsaturated PE is about 0.5 nmol per fly from north to south (Fig. 2.7E). Females showed a decrease about 1 nmol per fly from north to south geographically (Fig. 2.7F), and both *D. simulans* males and females exhibited a spike of a decrease in lipid concentrations at Coffs Harbor (Fig. 2.7E and F). *D. simulans* showed an opposite trend compare to *D. melanogaster* in both males and females, as there is a general decrease in monounsaturated PC and polyunsaturated PC for *D. simulans* (Fig. 2.8E and F).

Lysophospholipids are typically in low abundance as they are intermediates and are usually further processed into other lipid species. As showed in Fig. 2.8A and B, as the concentrations of PE or PC increase, there are decreases of lysoPE and lysoPC respectively as latitude increases (Fig. 2.9A and B).

In general, for the lipid geographic trends are the same for data calculated per fly or per lean mass. Other than geographic trends of total lipids, total polar lipids, total neutral lipids and TAG mentioned above, other examples include abundances of polyunsaturated, monounsaturated, saturated PE or PC (Fig. 2.7A-D and Fig. 2.8A-D).

Trends of predominant species of various lipid classes in the eastern coast of Australia.

Generally, the predominant lipid species in each lipid class behaves the same in terms of abundance along latitude. When examining phospholipids in *D. melanogaster* across latitude, the dominated PE species in both male and female are 34:2, 34:1 and 36:3 (where the first number indicates the number of carbon and the second number indicates the number of double bonds). These PE species showed a general trend of large increase

in Melbourne, but the concentrations of these PEs are greater in southern locations (Fig. 2.10A and B). While the most abundant PC species in the male are 32:1, 34:2 and 34:1, and 34:3, 34:1 and 36:3 in females, they exhibited a trend of increase concentrations in southern locations as well (Fig. 2.10C and D). PA, PI, PG and PS are in smaller concentration, although similar trends of increase of concentrations in southern locations are presented in all phospholipids except PG (Fig. 2.10E-L). PG fluctuated geographically with a non-linear trend (Fig. 2.10I and J).

Polar lipids exhibited trends of increasing concentration in polyunsaturated and monounsaturated species, in both *D. melanogaster* male and female (Fig. 2.11A and B). Where neutral lipids such as DAG and TAG, in male *D. melanogaster*, fluctuated geographically (Fig. 2.12A and C). In female *D. melanogaster*, DAG varied throughout latitude and TAG showed an increase of concentration in Melbourne (Fig. 2.12 B and D).

Structural patterns of lipids in different classes

Latitudinal trends for lipid structures were also observed in different lipid classes. Structure refers to the degree of unsaturation and chain length. In *D. melanogaster* males, as latitude increase, there are significant increases in chain lengths of DAG and lysoPE (Table 2.2A). In *D. melanogaster* females, significant increases were seen in total lipids, total neutral lipids, and lysoPE, and decreases of chain length were shown in total polar lipids and PI as latitude increased (Table 2.2B). Furthermore, *D. simulans* males exhibited significant increase trends in chain lengths for TAG, and in PG for females (Table 2.2C and D). There were significant differences in DAG and lysoPE chain lengths for males, and PG and lysoPE in females (Table 2.2E and F).

Another trend of lipid structures is unsaturation level in *D. melanogaster* males as latitude increases, is a significant increase in the number of double bonds in DAG and decrease in PG, lysoPE, and lysoPC (Table 2.3A). In *D. melanogaster* females, a significant increase in the numbers of double bonds as latitude increases was seen in free fatty acids only. While significant decreases were observed in total lipids, total polar lipids, total neutral lipids and TAG as latitude increased (Table 2.3B).

D. simulans males exhibited significant increase in the numbers of the double bond in total neutral lipids and decrease in lysoPE (Table 2.3C). *D. simulans* females showed a significant increase of double bonds in PG (Table 2.3D). While examining the variation of degrees of unsaturation between species, there were significant differences in free fatty acids, DAG and PG for male, and TAG and PG in females (Table 2.3E and F).

Free fatty acids are an important component in biological pathways, as they are often precursors for circular hydrocarbons. The degree of saturation of free fatty acids can provide informative patterns of geographic variation. In this study, it is shown that free fatty acids in *D. melanogaster* male and females decrease in abundance of monounsaturated, polyunsaturated and saturated lipids (Fig. 2.13A and B). However, in *D. simulans*, a trend of increase in free fatty acids for all degrees of unsaturation was observed (Fig. 2.13C and D).

Analysis of lipid adaptation when excluding Melbourne.

Throughout our data on *D. melanogaster* there is a consistent trend of increased lipid abundance at Melbourne. A statistical analysis was conducted, excluding the Melbourne sample, to test if this location has an undue effect on geographic trends. As our results on

the abundance of each lipid class along latitude, the general trend is of variation in *D. melanogaster* females remained the same (Table 2.4A). For chain lengths across latitude, there is an opposite slope in PA, where when excluding Melbourne the latitudinal trend changed from decreasing to increasing, with a negative impact on p-values for all other lipid classes (Table 2.4B). An apparent increase in DAG saturation levels was lost when the Melbourne data was removed for analysis (Table 2.4C).

2.4 Discussion

A notable and interesting feature of our data is the pronounced increase in the content of most lipids in the Melbourne population compared to other populations for both sexes of *D. melanogaster* (Fig. 2.8A and B, 2.10A-F, and 2.10H-L, 2.11C, and 2.11D). This phenomenon is observed on the basis of lipid per fly or lean mass. This is the case for all classes of lipids except for PGs and free fatty acids (polyunsaturated, monounsaturated and saturated) in females and males. In addition, DAGs did not increase in males in the Melbourne populations. Most lipid abundances were elevated in both sexes in the Melbourne sample, but there were a substantial of lipid classes that did not increase in abundance in this sample. Although it is not clear why this striking increase in most lipids was present in this sample, we do note that Melbourne is the only large city urban environment for fly collection. The peak of lipid abundance could be a result of dietary differences between large urban environments and relatively rural habitats. Toledo et al. (2016) showed differences in fatty acid composition in *Parus major* eggs. Birds in urban habitats have access to supplemental feeding and perhaps an unusual abundance of energy-rich food sources (carbohydrate and fat). This observation motivates future studies to determine if *Drosophila* from the city are more lipid-rich than those found in

rural habitats. There may be an opportunity for future ecology and micro-evolution studies comparing urban and rural *Drosophila* populations.

In the context of adaptive membrane lipid research, an intriguing trend in our study is the decline in the PE/PC ratio going from northern to southern populations. Our lipid extractions were taken from flies reared at 25°C after 29 or 30 generations in the laboratory at 19°C. This represents a thermal shift from winter to summer conditions as described by Hoffmann et al. (2005). Therefore, there is an opportunity to investigate a temperature acclimation response of the populations in the context of a shift from a typical winter-level temperature to a summer-level temperature. PE and PC are major components membrane structures, where PE is a cone shaped phospholipid, an increase of PE destabilized membrane structures, PC being a rod shaped phospholipid increases the stability of membrane structures. Thus, an increase of PE over PC can increase membrane fluidity (Hazel 1995, Hochachka and Somero 2002). Cooper et al. (2012) investigated three latitudinal-differentiated eastern United States *D. melanogaster* populations from Vermont, Indiana, and North Carolina. The samples were maintained as isofemales lines in the laboratory at 20.5°C. Eggs from laboratory lines maintained at 20.5°C were collected at this temperature and reared at either 16°C or 26°C followed by holding adults at the same temperature. The objective was to test the degree of acclimation of membrane lipids to temperature. The Vermont population was characterized by greater seasonal temperature variation and it was predicted that flies from Vermont would exhibit a greater plasticity in membrane lipid composition as “high variance in temperature across-generations favored greater developmental response of PE to PC ratio as a response to warmer developmental temperatures”. This prediction was

met as the PE/PC for all populations was similar at 16°C, but the ratio dropped to a greater degree at 26°C. In our study, all populations were shifted from long-standing maintenance at 19°C, to one-generation of rearing and maintenance of adults at 25°C. In our results, PE/PC tended to drop in a consistent manner in females and males from lower to higher latitude under temperature shift conditions (Fig. 2.5A). In terms of the general trend, our result is consistent with the results of Cooper et al. (2012).

A greater number of double bonds in the predominant membrane polar lipids (PC and PE) is an indicator of higher levels of membrane fluidity. In our data, the PE, PC, and total polar lipid increases, from north to south in Australia. This trend is greater for polyunsaturated polar lipids than monounsaturated or saturated lipids (Fig. 2.7A and B, and 2.8A and B, and 2.11A and B). This pattern of change in lipid characteristics is in the opposite direction of the decreasing PE/PC change in response to a shift to an elevated temperature (Figures 2.6A and B). PE/PC is generally thought to be the stronger determinant of overall membrane structure. Perhaps, the PE/PC is more responsive to a temperature shift than in the level of unsaturation. A consideration is the degrees of heritability measured in Scheitz et al. (2013) which indicated that unsaturated lipids are more heritable than saturated. Assuming this is the case in flies investigated in our study, then it might be that unsaturated lipids are genetically influenced to match the environment in which laboratory temperature selection has produced an adaptation to maintain fluidity and prevent something like a gel state of membranes at a relatively cold temperature for reproductively active *D. melanogaster*. Therefore the notable positive slope from north to south in unsaturated reflects the previous historical and laboratory culture conditions rather than the assay temperature employed in this study. In general,

there is a strikingly consistent trend in the female and male *D. melanogaster* data for the polyunsaturated fatty acids to increase to a greater degree than monounsaturated compared to the stasis in saturated lipids going from north to south in Australia.

Free fatty acids show an opposite pattern of abundance for different unsaturation species (Fig. 2.13A and B) when compared to membrane phospholipids (Fig. 2.7A and C, Fig. 2.8A and C). The ratio of the geographically-defined slopes of polyunsaturated to saturated acyl chains in polar lipids was approximately 3.5:1. The saturated and polyunsaturated free fatty acids were decreasing in parallel with a negative slope of approximately 3.5:1. The consistency in geographic change between free fatty acid and membrane phospholipids suggests an adaptive role for changes in the degree of unsaturation on the cline. Free fatty acids are important precursors of cuticular hydrocarbons and wax esters that underlie mechanisms to prevent water loss, which would be expected to vary geographically with desiccation stress.

However, there is no significant geographic variation in *D. melanogaster* for desiccation resistance from north to south along the East Coast cline in Australia (Hoffman et al. 2005). This is the case whether flies were reared at summer-like or winter-like conditions. However, there are reciprocal clines for elevated temperature tolerance (northern populations) and faster chill-coma recovery (southern populations), the latter cline is steeper (Hoffmann et al. 2002, Hoffman et al. 2005). As a baseline general response, there may be more unsaturated free fatty acids incorporated into membrane phospholipids in southern populations which would be expected as a response to prevent membrane lipids from transitioning to a gel state in the colder southern climates.

There is a range of differences in lipid composition between female and male *D. melanogaster.* Per fly or per lean mass per fly, there is a trend for females to have an increasing abundance of total neutral lipids from north to south (Fig. 2.4A and B). In general, females exhibit a steeper slope for geographic change in lipid abundance than do males. Females have a greater abundance of total neutral lipids than males (P = 0.006); this difference may reflect the storage lipids that are associated with egg production in females. Females exhibit a statistically significant trend, whereby the degree of unsaturation of free fatty acids increases from north to south (Table 2.2B). Males show a similar trend that was only marginally statistically significant (Table 2.2A).

There were differences between *D. melanogaste*r and *D. simulans* lipid profiles. A potential feature of the data was that there was a statistically significant difference between closely related species (Table 2.1E and 2.1F) in free fatty acids for both males and females, respectively. Both females and males of *D. melanogaster* exhibited a trend in which free fatty acids increased from north to south, whereas *D. simulans* exhibited the opposite trend that showed a decrease in abundance from north to south. Another notable difference is that the tendency for PE/PC to decrease in *D. melanogaster* is not found in *D. simulans* which demonstrated an adaptive response in this ratio. More information about PE and PC is that the geographic pattern of abundance of polyunsaturated, monounsaturated and saturated fatty acids that characterize these lipids in both sexes was opposite in *D. simulans* which showed shallow negative slopes of these categories of PE and PC. In *D. melanogaster* PE decreased in both sexes and PC decreased with a shallow slope; in *D. simulans* there were little geographic changes in PE or PC. In general, *D.*

simulans presented little evidence for differences between males and females for the abundance of most lipid classes, which was not the case for *D. melanogaster*.

An aspect of our data that warrants comment concerns the precursor forms of two lipids: lysoPE and lysoPC in *D. melanogaster*. The precursor lipids are present at low abundance. An interesting observation in our data is that when there is a large spike in most lipid classes, including PE and PC in the Melbourne population, lysoPE and lysoPC decrease. This implies internal consistency in our data such that PE and PC precursors are depleted in the course of production of an abundance of these two most abundant membrane lipids.

Many studies have documented changes in lipids stored as TAG as an adaptive response. In the Introduction to this study we recount the model work on crickets and *Drosophila*, the latter in the context of research on the *D. melanogaster* North American cline which documents increased levels of TAG in higher latitudes (Schmidt et al. 2005). In the present study, in *D. melanogaster* females and males we do not find a statistically significant increase in TAGs at higher latitudes. However, there is a shallow positive slope in TAG abundance in *D. melanogaster* females (Figure 2.5A) and the same is observed for total neutral lipids overall in *D. melanogaster* in females and males (Figure 2.4A). As a potentially relevant point, there is no clearly defined geographic trend for lean mass geographic variation in *D. melanogaster* females (data not shown).

2.5 Literature cited

Bell, G. and V. Koufopanou. 1986. *The cost of reproduction.* (Oxford University Press: Oxford UK.).

Bligh, E. G., and W. J. Dyer. 1959. 'A rapid method of total lipid extraction and purification', *Can J Biochem Physiol*, 37: 911-7.

Bochdanovits, Z., and G. de Jong. 2004. 'Antagonistic pleiotropy for life-history traits at the gene expression level', *Proc Biol Sci*, 271 Suppl 3: S75-8.

Cooper, B. S., L. A. Hammad, N. P. Fisher, J. A. Karty, and K. L. Montooth. 2012. 'In a variable thermal environment selection favors greater plasticity of cell membranes in Drosophila melanogaster', *Evolution*, 66: 1976-84.

Cooper, B. S., L. A. Hammad, and K. L. Montooth. 2014. 'Thermal adaptation of cellular membranes in natural populations of Drosophila melanogaster', *Funct Ecol*, 28: 886-94.

Denlinger, D.L., G.D. Yocum, J.P. Reinhardt. 2012. 'Hormonal Control of Diapause', *Insect Endocrinology*: 430-63.

Eanes, W. F. 2011. 'Molecular population genetics and selection in the glycolytic pathway', *J Exp Biol*, 214: 165-71.

Eanes, W.F. 1999. 'Analysis of selection on enzyme polymorphisms. ', *Annu. Rev. Ecol. Syst.*, 30: 301-26.

Fast, P. G. 1966. 'A comparative study of the phospholipids and fatty acids of some insects', *Lipids*, 1: 209-15.

Flowers, J. M., E. Sezgin, S. Kumagai, D. D. Duvernell, L. M. Matzkin, P. S. Schmidt, and W. F. Eanes. 2007. 'Adaptive evolution of metabolic pathways in Drosophila', *Mol Biol Evol*, 24: 1347-54.

Hammad, L. A., B. S. Cooper, N. P. Fisher, K. L. Montooth, and J. A. Karty. 2011. 'Profiling and quantification of Drosophila melanogaster lipids using liquid chromatography/mass spectrometry', *Rapid Commun Mass Spectrom*, 25: 2959-68.

Hangartner, S. B., A. A. Hoffmann, A. Smith, and P. C. Griffin. 2015. 'A collection of Australian Drosophila datasets on climate adaptation and species distributions', *Sci Data*, 2: 150067.

Hazel, J. R. 1995. 'Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation?', *Annu Rev Physiol*, 57: 19-42.

Hochachka, Peter W., and George N. Somero. 2002. *Biochemical adaptation : mechanism and process in physiological evolution* (Oxford University Press: New York).

Hoffmann, A. A., J. Shirriffs, and M. Scott. 2005. 'Relative importance of plastic vs genetic factors in adaptive differentiation: geographical variation for stress resistance in Drosophila melanogaster from eastern Australia', *Functional Ecology*, 19: 222-27.

Hoffmann, A. A., and A. R. Weeks. 2007. 'Climatic selection on genes and traits after a 100 year-old invasion: a critical look at the temperate-tropical clines in Drosophila melanogaster from eastern Australia', *Genetica*, 129: 133-47.

Jones, H. E., J. L. Harwood, I. D. Bowen, and G. Griffiths. 1992. 'Lipid composition of subcellular membranes from larvae and prepupae of Drosophila melanogaster', *Lipids*, 27: 984-7.

Kapun, M., C. Schmidt, E. Durmaz, P. S. Schmidt, and T. Flatt. 2016. 'Parallel effects of the inversion In(3R)Payne on body size across the North American and Australian clines in Drosophila melanogaster', *J Evol Biol*, 29: 1059-72.

Kennington, W. J., and A. A. Hoffmann. 2013. 'Patterns of genetic variation across inversions: geographic variation in the In(2L)t inversion in populations of Drosophila melanogaster from eastern Australia', *BMC Evol Biol*, 13: 100.

Merritt, T. J., E. Sezgin, C. T. Zhu, and W. F. Eanes. 2006. 'Triglyceride pools, flight and activity variation at the Gpdh locus in Drosophila melanogaster', *Genetics*, 172: 293-304.

Mole, S., and A. J. Zera. 1993. 'Differential Allocation of Resources Underlies the Dispersal-Reproduction Trade-Off in the Wing-Dimorphic Cricket, Gryllus-Rubens', *Oecologia*, 93: 121-27.

Montooth, K. L., K.T. Siebenthall, A.G. Clark. 2006. ' Membrane lipid physiology and toxin catabolism underlie ethanol and acetic acid tolerance in Drosophila melanogaster', Journal of Experimental Biology 2006 209: 3837-3850; doi: 10.1242/jeb.02448

Parisi, Michael, Renhua Li, and Brian Oliver. 2011. 'Lipid profiles of female and male Drosophila', *BMC Research Notes*, 4: 198.

Scheitz, C. J., Y. Guo, A. M. Early, L. G. Harshman, and A. G. Clark. 2013. 'Heritability and inter-population differences in lipid profiles of Drosophila melanogaster', *PLoS One*, 8: e72726.

Schilder, R. J., A. J. Zera, C. Black, M. Hoidal, and C. Wehrkamp. 2011. 'The biochemical basis of life history adaptation: molecular and enzymological causes of NADP(+)-isocitrate dehydrogenase activity differences between morphs of Gryllus firmus that differ in lipid biosynthesis and life history', *Mol Biol Evol*, 28: 3381-93.

Schmidt, P. S., and D. R. Conde. 2006. 'Environmental heterogeneity and the maintenance of genetic variation for reproductive diapause in Drosophila melanogaster', *Evolution*, 60: 1602-11.

Schmidt, P. S., L. Matzkin, M. Ippolito, and W. F. Eanes. 2005. 'Geographic variation in diapause incidence, life-history traits, and climatic adaptation in Drosophila melanogaster', *Evolution*, 59: 1721-32.

Schmidt, P. S., and A. B. Paaby. 2008. 'Reproductive diapause and life-history clines in North American populations of Drosophila melanogaster', *Evolution*, 62: 1204-15.

Schmidt, P. S., A. B. Paaby, and M. S. Heschel. 2005. 'Genetic variance for diapause expression and associated life histories in Drosophila melanogaster', *Evolution*, 59: 2616-25.

Schmidt, P. S., C. T. Zhu, J. Das, M. Batavia, L. Yang, and W. F. Eanes. 2008. 'An amino acid polymorphism in the couch potato gene forms the basis for climatic adaptation in Drosophila melanogaster', *Proc Natl Acad Sci U S A*, 105: 16207-11.

Sezgin, E., D. D. Duvernell, L. M. Matzkin, Y. Duan, C. T. Zhu, B. C. Verrelli, and W. F. Eanes. 2004. 'Single-locus latitudinal clines and their relationship to temperate adaptation in metabolic genes and derived alleles in Drosophila melanogaster', *Genetics*, 168: 923-31.

Toledo, A., M. N. Andersson, H. L. Wang, P. Salmon, H. Watson, G. C. Burdge, and C. Isaksson. 2016. 'Fatty acid profiles of great tit (Parus major) eggs differ between urban and rural habitats, but not between coniferous and deciduous forests', *Naturwissenschaften*, 103: 55.

Weeks, A. R., S. W. McKechnie, and A. A. Hoffmann. 2006. 'In search of clinal variation in the period and clock timing genes in Australian Drosophila melanogaster populations', *J Evol Biol*, 19: 551-7.

Zera, A. J., R. Clark, and S. Behmer. 2016. 'Lipogenesis in a wing-polymorphic cricket: canalization versus morph-specific plasticity as a function of nutritional heterogeneity', *J Insect Physiol*.

Zera, A. J., and R. F. Denno. 1997. 'Physiology and ecology of dispersal polymorphism in insects', *Annu Rev Entomol*, 42: 207-30.

Zera, A. J., and L. G. Harshman. 2011. 'Intermediary metabolism and the biochemical-molecular basis of life history variation and trade-offs in two insect models', *Mechanisms of Life History Evolution: The Genetics and Physiology of Life History Traits and Trade-Offs*: 311-28.

Zera, A. J., and A. Larsen. 2001. 'The metabolic basis of life history variation: genetic and phenotypic differences in lipid reserves among life history morphs of the wing-polymorphic cricket, Gryllus firmus', *J Insect Physiol*, 47: 1147-60.

Zera, A. J., S. Mole, and K. Rokke. 1994. 'Lipid, Carbohydrate and Nitrogen-Content of Long-Winged and Short-Winged Gryllus-Firmus - Implications for the Physiological Cost of Flight Capability', *Journal of Insect Physiology*, 40: 1037-44.

Zera, A. J., and Z. Zhao. 2003. 'Life-history evolution and the microevolution of intermediary metabolism: activities of lipid-metabolizing enzymes in life-history morphs of a wing-dimorphic cricket', *Evolution*, 57: 586-96.

Zhao, Z., and A. J. Zera. 2002. 'Differential lipid biosynthesis underlies a tradeoff between reproduction and flight capability in a wing-polymorphic cricket', *Proc Natl Acad Sci U S A*, 99: 16829-34.

2.6 Tables

Table 2.1A. Latitudinal trends in relative abundance of different lipid classes in male *D. melanogaster*. A positive slope indicates an increase of abundances along latitude and a negative slope indicates a decrease of abundances along the latitude. Std error indicates standard errors with n=4. P-values less than 0.05 were notated with *, less than 0.01 with **, and less than 0.001 with ***.

Species	Sex	Lipid class	Slope	Std error	P-values
D. melanogaster	Male	Total lipids	-0.5040	1.3275	0.7087
		Total polar lipids	-1.0140	0.6380	0.1294
		Total neutral lipids	0.5100	1.1297	0.6571
		Free Fatty Acids	3.4480	1.4339	0.0272*
		TAG	0.5720	1.0280	0.5848
		DAG	-0.06194	0.2126	0.7741
		PE	-0.7100	0.5186	0.1878
		PC	-0.1610	0.08137	0.0633
		PA	-0.00584	0.001475	0.0009***
		PI	-0.00903	0.003484	0.0184*
		PS	-0.00446	0.001049	0.0005***
		PG	-0.00002	0.02922	0.9995
		lyso PE	-0.1005	0.03237	0.0061*
		lyso PC	-0.02314	0.007975	0.0095**

Table 2.1B. Latitudinal trends for relative abundances of different lipid classes in female *D. melanogaster*. A positive slope indicates an increase of abundances along latitude and a negative slope indicates a decrease of abundances along the latitude. Std error indicates standard errors with n=4. P-values less than 0.05 were notated with *.

Species	Sex	Lipid class	Slope	Std error	P-values
D. melanogaster	Female	Total lipids	1.5345	2.1062	0.4719
		Total polar lipids	-0.8177	0.3267	0.0180*
		Total neutral lipids	2.3545	2.0790	0.2664
		Free Fatty Acids	2.7231	1.0589	0.0153*
		TAG	2.5925	2.0111	0.2072
		DAG	-0.2422	0.3229	0.4591
		PE	-0.5921	0.2315	0.0158*
		PC	-0.1490	0.05593	0.0123*
		PA	-0.00488	0.002059	0.0245*
		PI	-0.00421	0.005274	0.4312
		PS	-0.00400	0.002719	0.1515
		PG	0.007825	0.01996	0.6979
		lysoPE	-0.06526	0.05626	0.2552
		lysoPC	-0.02150	0.01941	0.2768

Table 2.1C. Latitudinal trends in relative abundance of different lipid classes in male *D. simulans*. A positive slope indicates an increase of abundances along latitude and a negative slope indicates a decrease of abundances along the latitude. Std error indicates standard errors with n=4.

Species	Sex	Lipid class	Slope	Std error	P-values
D. simulans	Male	Total lipids	1.4372	1.4311	0.3286
		Total polar lipids	0.2864	0.6878	0.6820
		Total neutral lipids	1.1508	1.2179	0.3572
		Free Fatty Acids	-1.6687	1.5458	0.2946
		TAG	0.6915	1.1082	0.5405
		DAG	0.4594	0.2292	0.0603
		PE	0.2907	0.5591	0.6094
		PC	0.06347	0.08772	0.4786
		PA	0.001530	0.001590	0.3486
		PI	-0.00181	0.003756	0.6349
		PS	0.002007	0.001131	0.0929
		PG	0.02003	0.03150	0.5329
		lysoPE	-0.07327	0.03489	0.0501
		lysoPC	-0.01624	0.008597	0.0751

Table 2.1D. Latitudinal trends in relative abundance of different lipid classes *D. simulans* female. A positive slope indicates an increase of abundances along latitude and a negative slope indicates a decrease of abundances along the latitude. Std error indicates standard errors with n=4.

Species	Sex	Lipid class	Slope	Std error	P-values
D. simulans	Female	Total lipids	1.5778	2.6338	0.5536
		Total polar lipids	0.2800	0.4154	0.5054
		Total neutral lipids	1.2978	2.6052	0.6220
		Free Fatty Acids	-0.8954	1.3338	0.5072
		TAG	1.0018	2.5310	0.6950
		DAG	0.2960	0.4032	0.4685
		PE	0.2850	0.2952	0.3420
		PC	0.06256	0.07179	0.3904
		PA	0.002125	0.002605	0.4210
		PI	0.002564	0.006652	0.7026
		PS	0.003578	0.003427	0.3049
		PG	-0.01243	0.02499	0.6224
		lysoPE	-0.05057	0.06946	0.4722
		lysoPC	-0.01282	0.02428	0.6014

Table 2.1E. Comparison of latitudinal trends for relative abundance of different lipid classes in male *D. melanogaster* and *D. simulans*. P-values less than 0.05 were notated with *, and less than 0.001 with ***.

Species	Sex	Lipid class	P-values
D. mel versus *D. sim*	Male	Total lipids	0.3332
		Total polar lipids	0.1826
		Total neutral lipids	0.7042
		Free Fatty Acids	0.0260
		TAG	0.9379
		DAG	0.1127
		PE	0.2059
		PC	0.0769
		PA	0.0032*
		PI	0.1761
		PS	0.0006***
		PG	0.6464
		lysoPE	0.5743
		lysoPC	0.5636

Table 2.1F. Comparison of latitudinal trends for relative abundance of different lipid classes in *D. melanogaster* and *D. simulans* females. P-values less than 0.05 were notated with *, and less than 0.001 with ***.

Species	Sex	Lipid class	P-values
D. mel versus *D. sim*	Female	Total lipids	0.9898
		Total polar lipids	0.0465*
		Total neutral lipids	0.7534
		Free Fatty Acids	0.0420*
		TAG	0.6263
		DAG	0.3057
		PE	0.0262*
		PC	0.0270*
		PA	0.0434*
		PI	0.4312
		PS	0.0935
		PG	0.5313
		lysoPE	0.8706
		lysoPC	0.7819

Table 2.2A. The latitudinal trend of chain length (number of carbons) in male *D. melanogaster*. A positive slope indicates an increase of chain lengths along latitude and a negative slope indicates a decrease of chain length along latitude. Std error indicates standard errors with n=4. P-values less than 0.05 were notated with * and less than 0.001 with ***.

Species	Sex	Lipid class	Slope	Std error	P-values
D. melanogaster	Male	Total lipids	0.3534	0.1750	0.0586
		Total polar lipids	-0.2756	0.1749	0.1325
		Total neutral lipids	0.1082	0.08319	0.2097
		Free Fatty Acids	-0.01385	0.01541	0.3806
		TAG	0.02421	0.01403	0.1017
		DAG	0.07831	0.02850	0.0132*
		PE	-0.02118	0.01207	0.0964
		PC	0.000402	0.01794	0.9824
		PA	0.06326	0.1368	0.6494
		PI	-0.04330	0.2365	0.8568
		PS	-0.8014	0.4547	0.0950
		PG	-0.2143	0.1253	0.1046
		lysoPE	0.02260	0.005690	0.0009***
		lysoPC	-0.00738	0.02301	0.7520

Table 2.2B. The latitudinal trend of chain length (number of carbons) in female *D. melanogaster*. A positive slope indicates an increase of chain lengths along latitude and a negative slope indicates a decrease of chain length along latitude. Std error indicates standard errors with n=4. P-values less than 0.05 were notated with *, less than 0.01 with ** and less than 0.001 with ***.

Species	Sex	Lipid class	Slope	Std error	P-values
D. melanogaster	Female	Total lipids	0.2369	0.09180	0.0150*
		Total polar lipids	-0.2356	0.1151	0.0495*
		Total neutral lipids	0.1008	0.04237	0.0239*
		Free Fatty Acids	-0.01010	0.009884	0.3151
		TAG	0.005060	0.02475	0.8394
		DAG	0.02217	0.03523	0.5338
		PE	-0.02843	0.01674	0.0998
		PC	-0.00612	0.01595	0.7037
		PA	-0.00551	0.08998	0.9516
		PI	-0.1819	0.05260	0.0017**
		PS	0.04435	0.2067	0.8316
		PG	-0.1112	0.06420	0.0935
		lysoPE	0.01043	0.002306	<0.0001***
		lysoPC	-0.01006	0.01116	0.3745

Table 2.2C. The latitudinal trend of chain length (number of carbons) in male *D. simulans*. A positive slope indicates an increase of chain lengths along latitude and a negative slope indicates a decrease of chain length along latitude. Std error indicates standard errors with n=4. P-values less than 0.05 were notated with *.

Species	Sex	Lipid class	Slope	Std error	P-values
D. simulans	Male	Total lipids	0.04929	0.1886	0.7968
		Total polar lipids	0.1655	0.1886	0.3916
		Total neutral lipids	-0.00941	0.08969	0.9176
		Free Fatty Acids	-0.00129	0.01661	0.9388
		TAG	0.04305	0.01513	0.0107*
		DAG	-0.02678	0.03072	0.3948
		PE	-0.01093	0.01302	0.4122
		PC	-0.00155	0.01934	0.9370
		PA	-0.1574	0.1475	0.3000
		PI	-0.00116	0.2549	0.9964
		PS	-0.2374	0.4902	0.6340
		PG	0.04701	0.1351	0.7320
		lysoPE	-0.00390	0.006134	0.5330
		lysoPC	0.02568	0.02481	0.3143

Table 2.2D. The latitudinal trend of chain length (number of carbons) in female *D. simulans*. A positive slope indicates an increase of chain lengths along latitude and a negative slope indicates a decrease of chain length along latitude. Std error indicates standard errors with n=4. P-values less than 0.05 were notated with *.

Species	Sex	Lipid class	Slope	Std error	P-values
D. simulans	Female	Total lipids	0.03099	0.1169	0.7927
		Total polar lipids	0.08706	0.1444	0.5509
		Total neutral lipids	0.02438	0.05338	0.6512
		Free Fatty Acids	0.01483	0.01222	0.2344
		TAG	0.02597	0.03101	0.4090
		DAG	-0.01438	0.04438	0.7481
		PE	0.000568	0.02134	0.9790
		PC	0.007316	0.02013	0.7189
		PA	0.01900	0.1101	0.8641
		PI	-0.01987	0.06433	0.7596
		PS	-0.08058	0.2555	0.7546
		PG	0.1656	0.08012	0.0475*
		lysoPE	-0.00228	0.002818	0.4240
		lysoPC	-0.02013	0.01387	0.1570

Table 2.2E. Comparison of latitudinal trends between male *D. melanogaster* and *D. simulans*. P-values less than 0.05 were notated with *, less than 0.01 with **.

Species	Sex	Lipid class	P-values
D. mel versus *D. sim*	Male	Total lipids	0.2526
		Total polar lipids	0.1035
		Total neutral lipids	0.3490
		Free Fatty Acids	0.5863
		TAG	0.3734
		DAG	0.0219*
		PE	0.5708
		PC	0.9418
		PA	0.2872
		PI	0.9049
		PS	0.4100
		PG	0.1734
		lysoPE	0.0053**
		lysoPC	0.3414

Table 2.2F. Comparison of latitudinal trends between female *D. melanogaster* and *D. simulans*. P-values less than 0.05 were notated with *, less than 0.01 with ** .

Species	Sex	Lipid class	P-values
D. mel versus *D. sim*	Female	Total lipids	0.1761
		Total polar lipids	0.0908
		Total neutral lipids	0.2711
		Free Fatty Acids	0.1232
		TAG	0.6021
		DAG	0.5237
		PE	0.2935
		PC	0.6046
		PA	0.8643
		PI	0.0606
		PS	0.7065
		PG	0.0114*
		lysoPE	0.0015**
		lysoPC	0.5760

Table 2.3A. Latitudinal trends for degrees of unsaturation (numbers of the double bond) in *D. melanogaster* males. A positive slope indicates an increase of unsaturation level along latitude and a negative slope indicates a decrease of unsaturation level along latitude. The Std error indicates standard errors with n=4.P-values less than 0.01 with ** and less than 0.001 with ***.

Species	Sex	Lipid class	Slope	Std error	P-values
D. melanogaster	Male	Total lipids	-0.01221	0.006591	0.0803
		Total polar lipids	-0.03474	0.01722	0.0588
		Total neutral lipids	-0.00002	0.002087	0.9929
		Free Fatty Acids	0.01584	0.007944	0.0615
		TAG	-0.00170	0.003624	0.6450
		DAG	0.009005	0.002188	0.0006***
		PE	-0.01262	0.006801	0.0799
		PC	-0.03864	0.02046	0.0752
		PA	-0.01956	0.01699	0.2647
		PI	-0.1037	0.04465	0.0321
		PS	-0.07477	0.06781	0.2847
		PG	-0.08936	0.03037	0.0087**
		lysoPE	-0.00519	0.001409	0.0017**
		lysoPC	-0.01819	0.006730	0.0146**

Table 2.3B. Latitudinal trends for degrees of unsaturation (numbers of the double bond) in female *D. melanogaster*. A positive slope indicates an increase of unsaturation level along latitude and a negative slope indicates a decrease of unsaturation level along latitude. Std error indicates standard errors with n=4. P-values less than 0.05 were notated with *.

Species	Sex	Lipid class	Slope	Std error	P-values
D. melanogaster	Female	Total lipids	-0.01281	0.004764	0.0116*
		Total polar lipids	-0.03052	0.01367	0.0332*
		Total neutral lipids	-0.00948	0.003930	0.0222*
		Free Fatty Acids	0.01204	0.005516	0.0370*
		TAG	-0.00932	0.004060	0.0288*
		DAG	-0.00051	0.005587	0.9285
		PE	-0.01291	0.009476	0.1834
		PC	-0.01887	0.01725	0.2827
		PA	-0.01423	0.007873	0.0808
		PI	-0.04105	0.02810	0.1544
		PS	-0.03136	0.06226	0.6182
		PG	-0.02636	0.01823	0.1586
		lysoPE	-0.00610	0.003521	0.0933
		lysoPC	-0.02048	0.01049	0.0603

Table 2.3C. Latitudinal trends for degrees of unsaturation (numbers of the double bond) in male *D. simulans*. A positive slope indicates an increase of unsaturation level along latitude and a negative slope indicates a decrease of unsaturation level along latitude. The Std error indicates a standard error with n=4. P-values less than 0.05 were notated with *, less than 0.01 with **.

Species	Sex	Lipid class	Slope	Std error	P-values
D. simulans	Male	Total lipids	0.003366	0.007105	0.6414
		Total polar lipids	0.001943	0.01856	0.9178
		Total neutral lipids	0.005070	0.002250	0.0370*
		Free Fatty Acids	-0.01114	0.008564	0.2098
		TAG	0.007683	0.003907	0.0649
		DAG	-0.00159	0.002359	0.5100
		PE	-0.00931	0.007331	0.2203
		PC	0.007047	0.02206	0.7531
		PA	-0.00950	0.01831	0.6103
		PI	-0.01706	0.04813	0.7271
		PS	-0.03546	0.07310	0.6335
		PG	0.01587	0.03274	0.6337
		lysoPE	-0.00554	0.001519	0.0018**
		lysoPC	-0.00004	0.007255	0.9958

Table 2.3D. Latitudinal trends for degrees of unsaturation (numbers of the double bond) in female *D. simulans*. A positive slope indicates an increase of unsaturation level along latitude and a negative slope indicates a decrease of unsaturation level along latitude. Std error indicates standard errors with n=4. P-values less than 0.05 were notated with *.

Species	Sex	Lipid class	Slope	Std error	P-values
D. simulans	Female	Total lipids	0.002936	0.006126	0.6352
		Total polar lipids	0.006053	0.01755	0.7326
		Total neutral lipids	0.002290	0.005014	0.6512
		Free Fatty Acids	-0.00554	0.006939	0.4311
		TAG	0.004696	0.005161	0.3701
		DAG	-0.00324	0.007049	0.6491
		PE	-0.00075	0.01217	0.9511
		PC	0.008100	0.02227	0.7186
		PA	0.008552	0.009639	0.3820
		PI	-0.00591	0.03581	0.8699
		PS	-0.1093	0.07794	0.1712
		PG	0.04700	0.02278	0.0479*
		lysoPE	-0.00303	0.004508	0.5059
		lysoPC	-0.00739	0.01357	0.5900

Table 2.3E. Comparison of latitudinal trends in degrees of unsaturation (numbers of the double bond) between male *D. melanogaster* and *D. simulans*. P-values less than 0.05 were notated with *, less than 0.01 with **.

Species	Sex	Lipid class	P-values
D. mel versus *D. sim*	Male	Total lipids	0.1253
		Total polar lipids	0.1646
		Total neutral lipids	0.1147
		Free Fatty Acids	0.0330*
		TAG	0.0953
		DAG	0.0041**
		PE	0.7442
		PC	0.1463
		PA	0.6920
		PI	0.2034
		PS	0.6980
		PG	0.0300*
		lysoPE	0.8690
		lysoPC	0.0832

Table 2.3F. Comparison of latitudinal trends in degrees of unsaturation (numbers of the double bond) between female *D. melanogaster* and *D. simulans*. P-values less than 0.05 were notated with *.

Species	Sex	Lipid class	P-values
D. mel versus *D. sim*	Female	Total lipids	0.0513
		Total polar lipids	0.1106
		Total neutral lipids	0.0746
		Free Fatty Acids	0.0566
		TAG	0.0410*
		DAG	0.7632
		PE	0.4370
		PC	0.3460
		PA	0.0772
		PI	0.4461
		PS	0.4409
		PG	0.0175*
		lyso PE	0.5956
		lyso PC	0.4514

Table 2.4A. Latitudinal trends of relative abundance excluding Melbourne populations for different lipid classes in female *D. melanogaster*. A positive slope indicates an increase of abundances along latitude and a negative slope indicates a decrease in abundance along the latitude. Std error indicates standard errors with n=4. P-values less than 0.05 were notated with *.

Species	Sex	Lipid class	Slope	Std error	P-values
D. melanogaster	Female	Total lipids	1.6356	2.3157	0.4854
		Total polar lipids	-0.8483	0.3691	0.0287*
		Total neutral lipids	2.4840	2.2929	0.2873
		Free Fatty Acids	2.9214	1.1643	0.0177*
		TAG	2.6879	2.2367	0.2389
		DAG	-0.2039	0.3523	0.5671
		PE	-0.5757	0.2633	0.0367*
		PC	-0.1544	0.06425	0.0227*
		PA	-0.00540	0.002254	0.0231*
		PI	-0.00670	0.005352	0.2203
		PS	-0.00504	0.002855	0.0876
		PG	0.008807	0.02198	0.6915
		lysoPE	-0.08485	0.05728	0.1489
		lysoPC	-0.02493	0.02110	0.2467

Table 2.4B. Latitudinal trends of chain length (number of carbons) excluding Melbourne populations for different lipid classes in female *D. melanogaster*. A positive slope indicates an increase of chain length along latitude and a negative slope indicates a decrease in chain length along latitude. Std error indicates standard errors with n=4. P-values less than 0.05 were notated with *, less than 0.01 with ** and less than 0.001 with ***.

Species	Sex	Lipid class	Slope	Std error	P-values
D. melanogaster	Female	Total lipids	0.2555	0.1027	0.0186*
		Total polar lipids	-0.1858	0.1174	0.1241
		Total neutral lipids	0.1016	0.04725	0.0397*
		Free Fatty Acids	-0.00972	0.01064	0.3685
		TAG	0.002751	0.02724	0.9202
		DAG	0.04579	0.03158	0.1575
		PE	-0.01832	0.01622	0.2678
		PC	-0.00541	0.01784	0.7639
		PA	0.02136	0.09136	0.8167
		PI	-0.1895	0.05505	0.0017**
		PS	0.06367	0.2218	0.7760
		PG	-0.1133	0.07036	0.1178
		lyso PE	0.01156	0.002260	<.0001***
		lyso PC	-0.00703	0.01176	0.5544

Table 2.4C. Latitudinal trends of the degree of unsaturation (numbers of the double bond) excluding Melbourne populations for different lipid classes in female *D. melanogaster*. Std error indicates standard errors with n=4. P-values less than 0.05 were notated with *.

Species	Sex	Lipid class	Slope	Std error	P-values
D. melanogaster	Female	Total lipids	-0.01301	0.005504	0.0248*
		Total polar lipids	-0.02811	0.01558	0.0813
		Total neutral lipids	-0.00972	0.004476	0.0379*
		Free Fatty Acids	0.01345	0.005974	0.0318*
		TAG	-0.01037	0.004483	0.0277*
		DAG	0.002929	0.005241	0.5803
		PE	-0.01278	0.01093	0.2518
		PC	-0.02205	0.01983	0.2752
		PA	-0.01385	0.008324	0.1067
		PI	-0.06053	0.02551	0.0243*
		PS	-0.03824	0.06830	0.5797
		PG	-0.02872	0.01990	0.1593
		lysoPE	-0.00521	0.003943	0.1960
		lysoPC	-0.01646	0.01159	0.1660

2.7 Figures

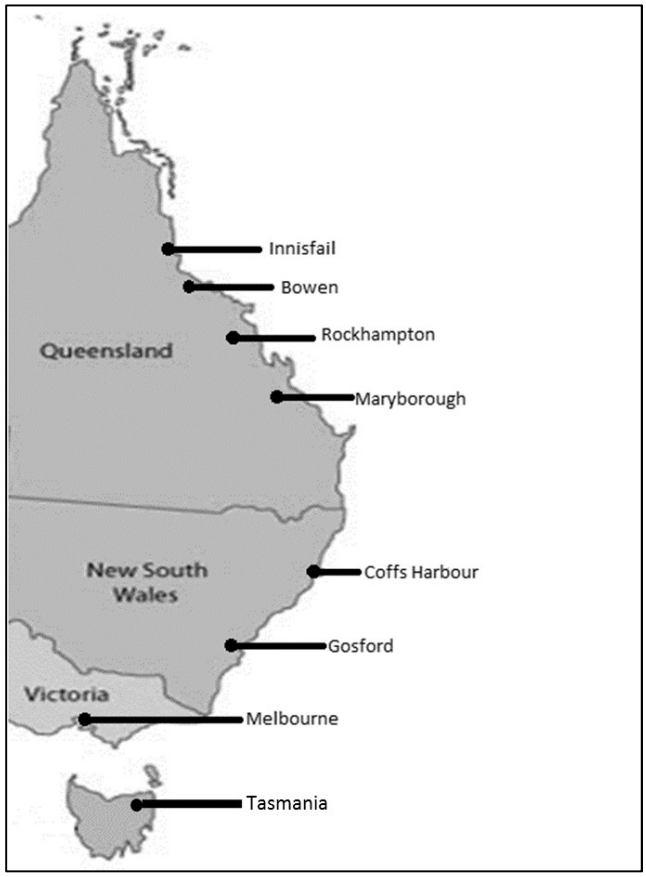

Figure 2.1. Map of fly collection locations along the eastern coast of Australian latitudinal cline.

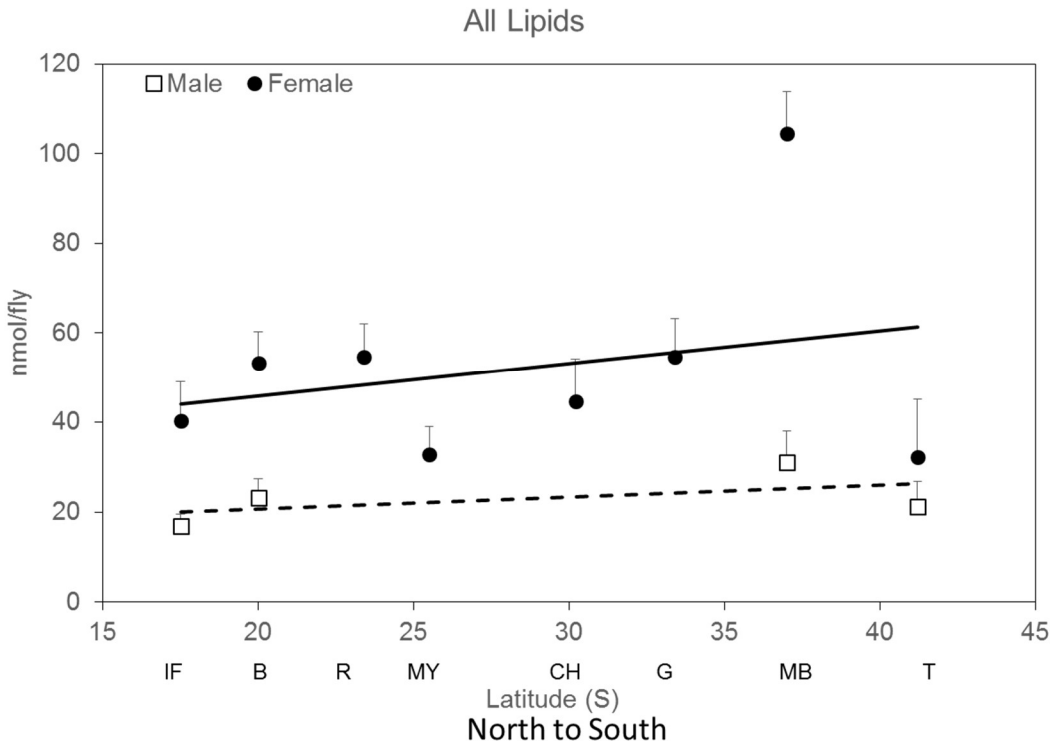

Figure. 2.2A. The latitudinal trend for relative abundance for total lipid in *D. melanogaster* normalized to nmol per fly. Dashed line indicates slope for male, and the solid line indicates slope for female, error bars represent the SE (n = 4).

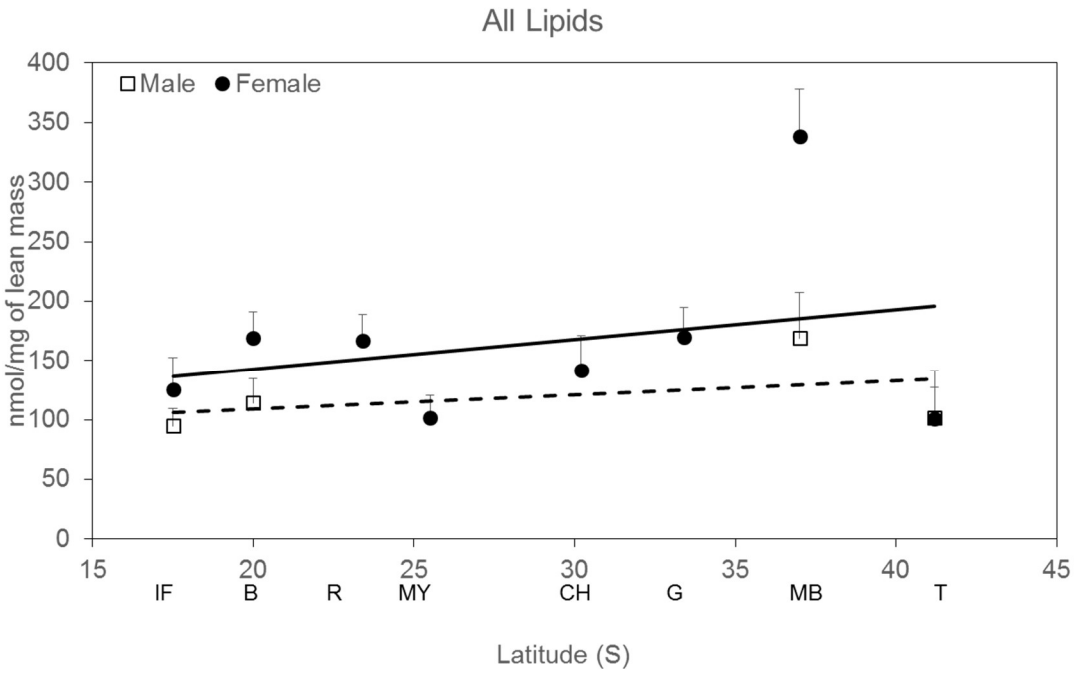

Figure 2.2B. The latitudinal trend for relative abundance of total lipid in *D. melanogaster* normalized to nmol per mg of lean mass. Dashed line indicates slope for male, and the solid line indicates slope for female, error bars represent the SE (n = 4).

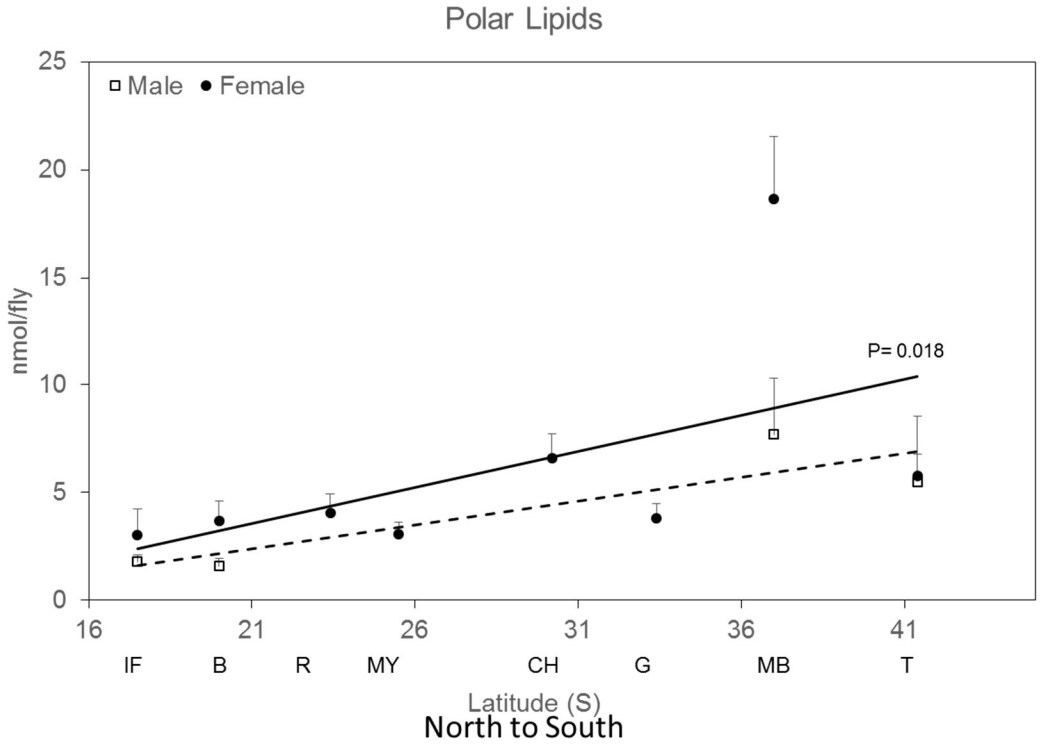

Figure 2.3A. The latitudinal trend for relative abundance of total polar lipids in *D. melanogaster* normalized to nmol per fly. Dashed line indicates slope for male, and the solid line indicates slope for female (p=0.018), error bars represent the SE (n = 4).

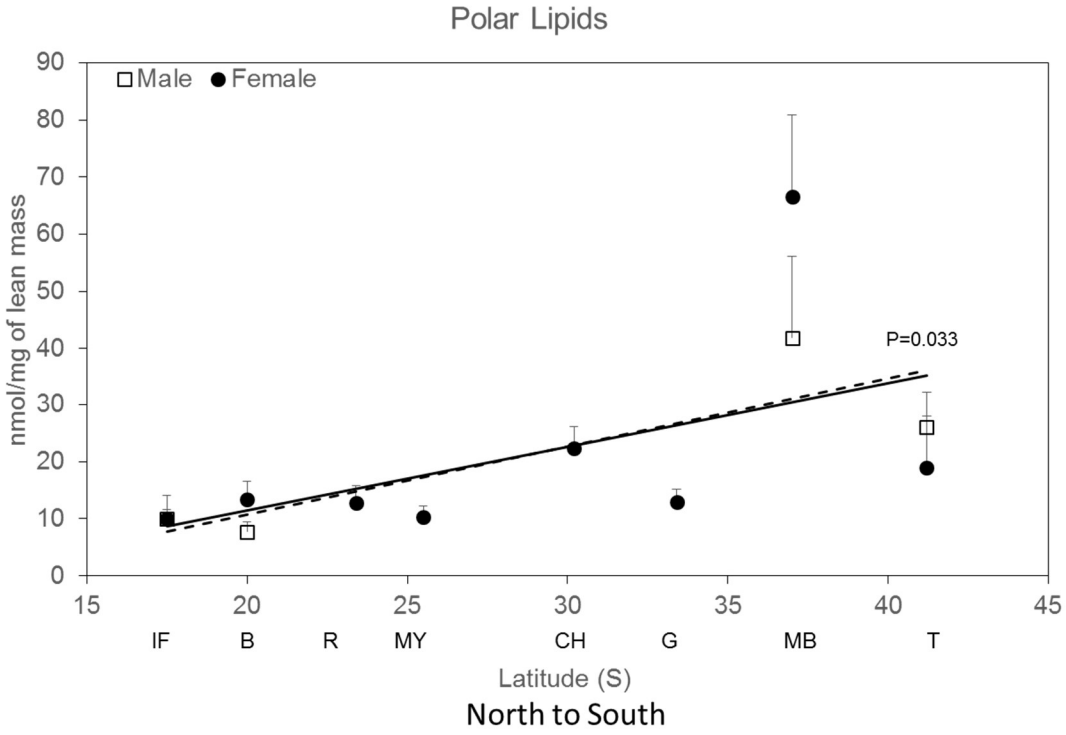

Figure 2.3B. The latitudinal trend for relative abundance of total polar lipids in *D. melanogaster* normalized to nmol per mg of lean mass. Dashed line indicates slope for male, and the solid line indicates slope for female (p= 0.033), error bars represent the SE (n = 4).

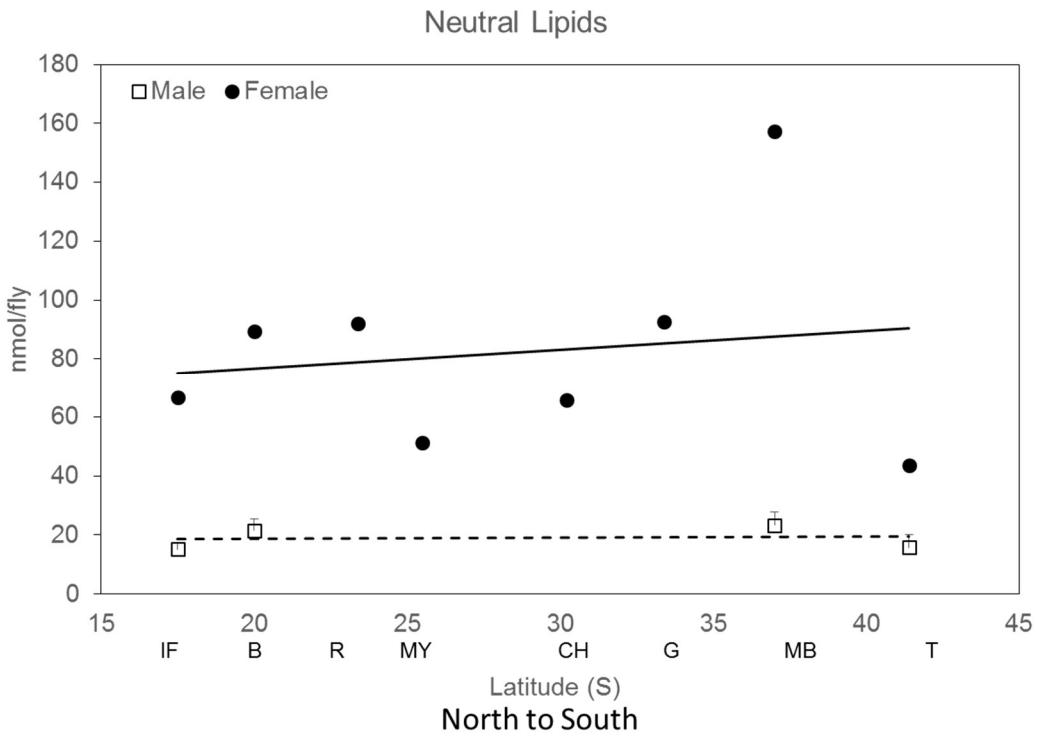

Figure 2.4A. The latitudinal trend for relative abundance of total neutral lipids in *D. melanogaster* normalized to nmol per fly. Dashed line indicates slope for male, and the solid line indicates slope for female, error bars represent the SE (n = 4).

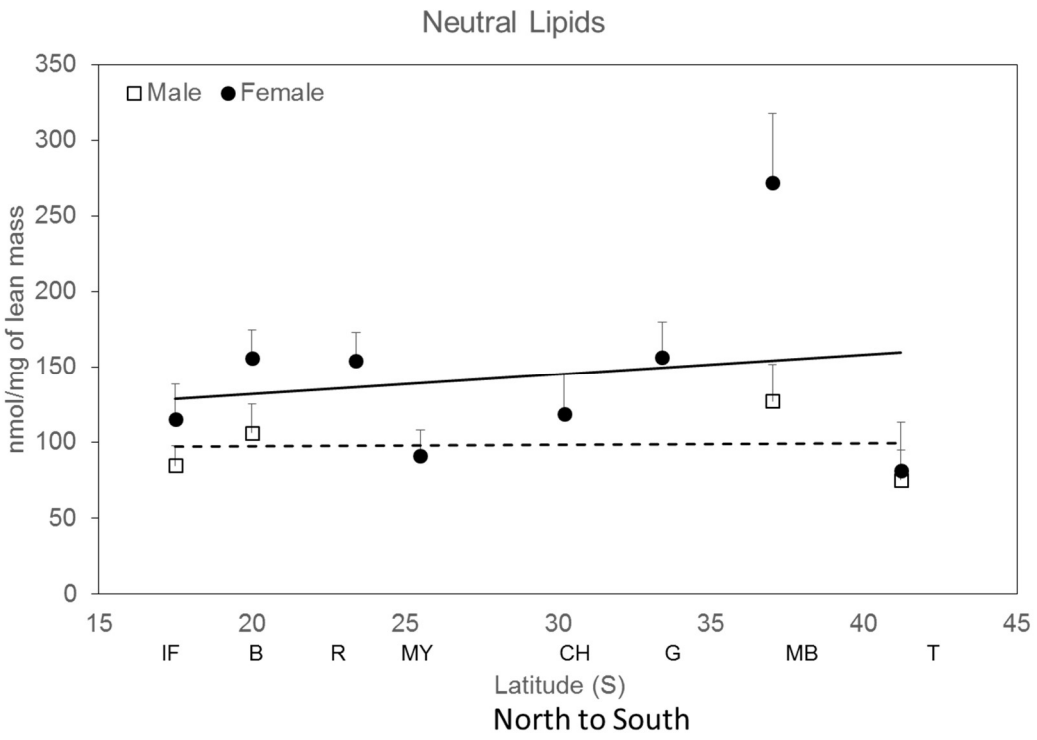

Figure 2.4B. The latitudinal trend for the relative abundance of total neutral lipids in *D. melanogaster* normalized to nmol per mg of lean mass. Dashed line indicates slope for male, and the solid line indicates slope for female, error bars represent the SE (n = 4).

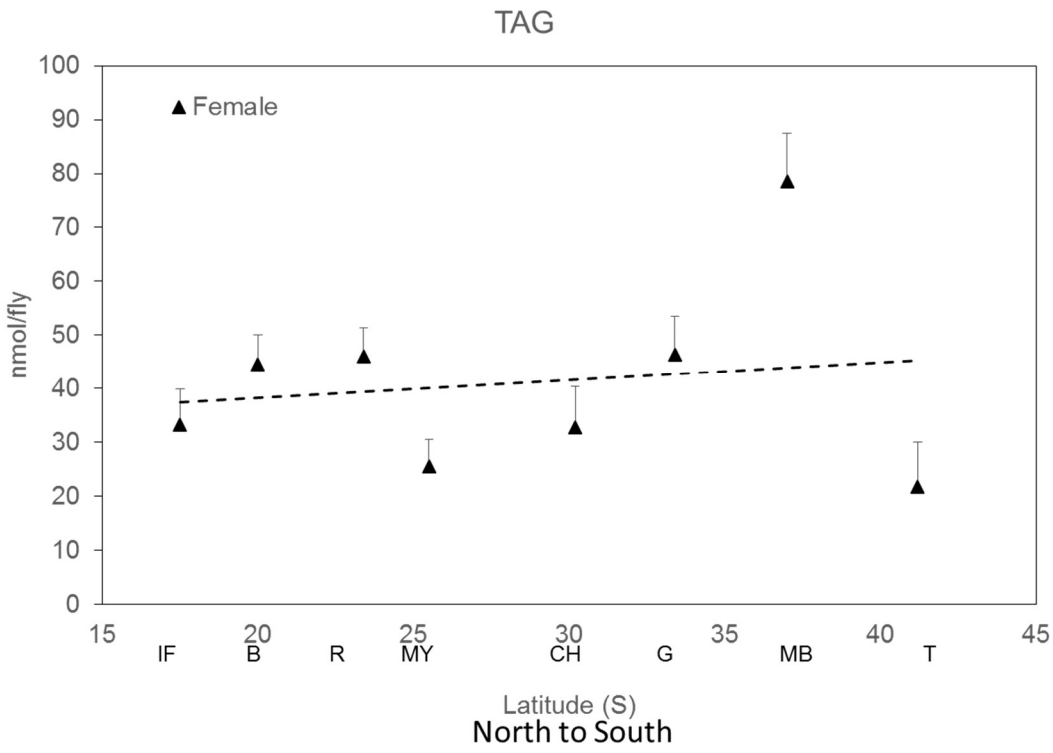

Figure 2.5A. Latitudinal trends of relative abundance for TAG in *D. melanogaster* females, calculated based on per fly. Dashed line indicates the slope of changes along latitude, error bars represent the SE (n = 4).

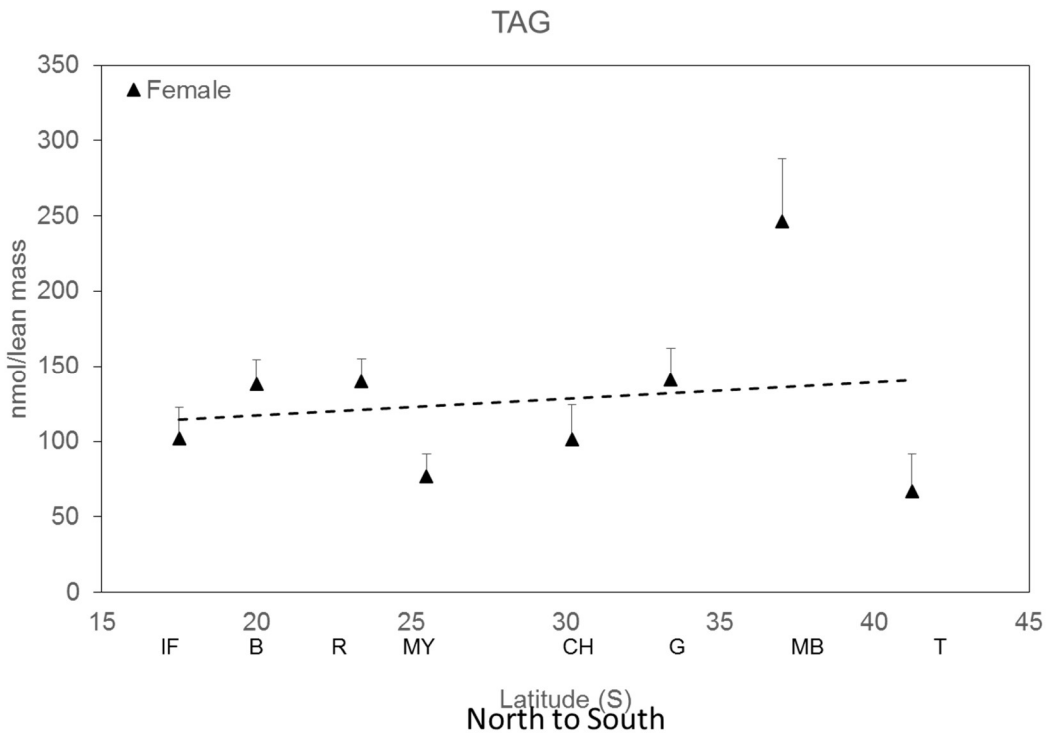

Figure 2.5B. Latitudinal trends of relative abundance for TAG in *D. melanogaster* females, calculated based on per mg of lean mass. Dashed line indicates the slope of changes along latitude, error bars represent the SE (n = 4).

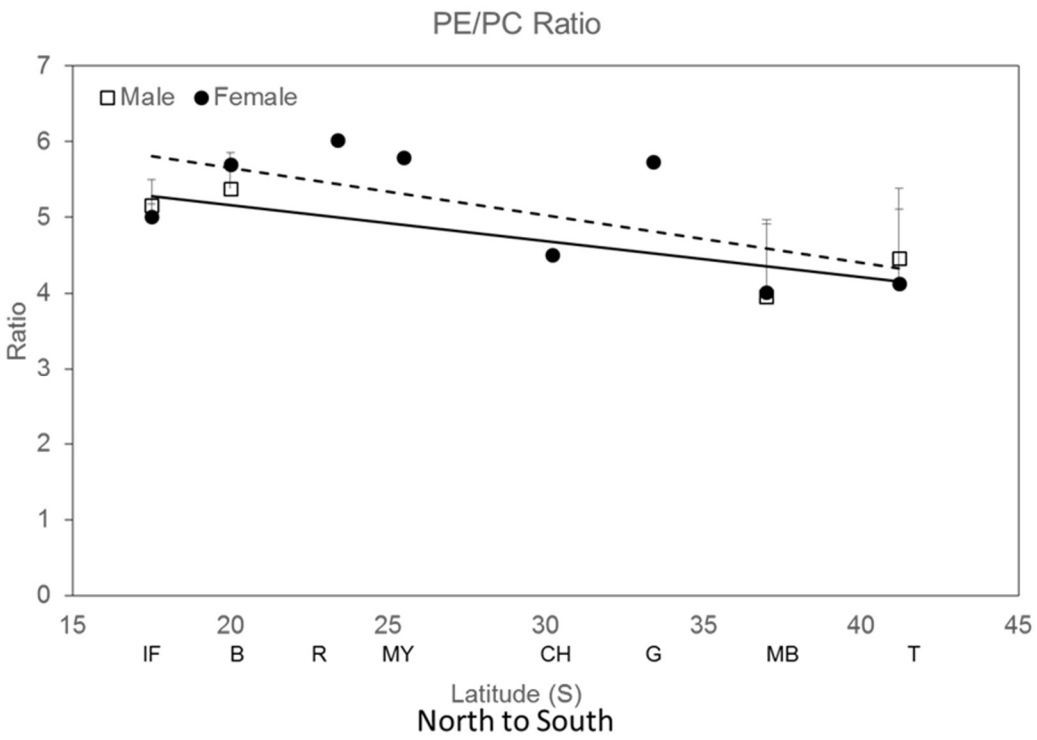

Figure 2.6A. Latitudinal trends PE/PC ratio in *D. melanogaster* reared at 25°C, calculated based on per fly. Dashed line indicates slope for male, and the solid line indicates slope for female, error bars represent the SE (n = 4).

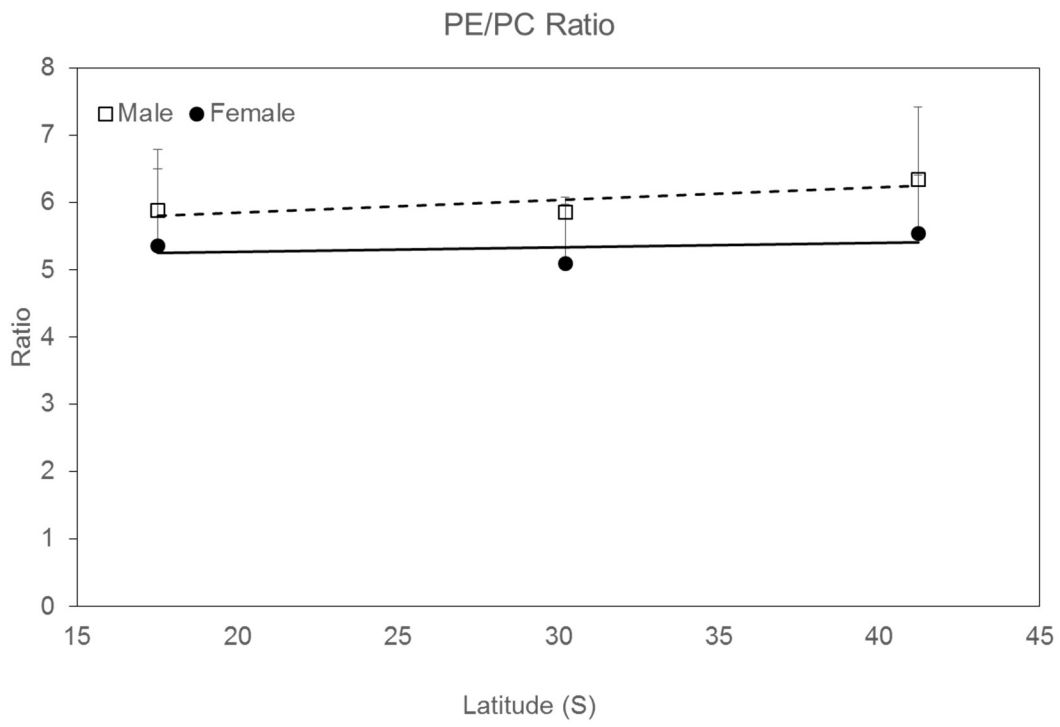

Figure 2.6B. Latitudinal trends PE/PC ratio in *D. simulans* reared at 25°C, calculated based on per fly. Dashed line indicates slope for male, and the solid line indicates slope for female. Dashed line indicates slope for male, and the solid line indicates slope for female, error bars represent the SE (n = 4).

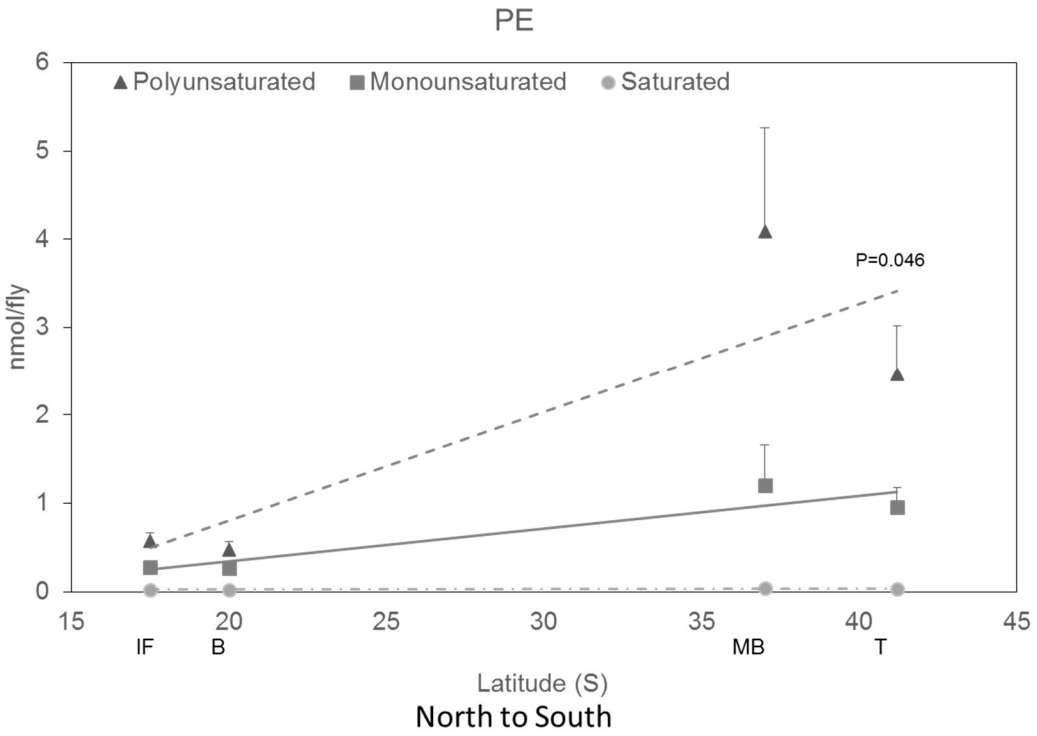

Figure 2.7A. Latitudinal trends of relative abundance for polyunsaturated (dashed line with p=0.048), monounsaturated (solid line), and saturated (dashed and dotted line) PE in male *D. melanogaster*, calculated based on nmol per fly, error bars represent the SE (n = 4).

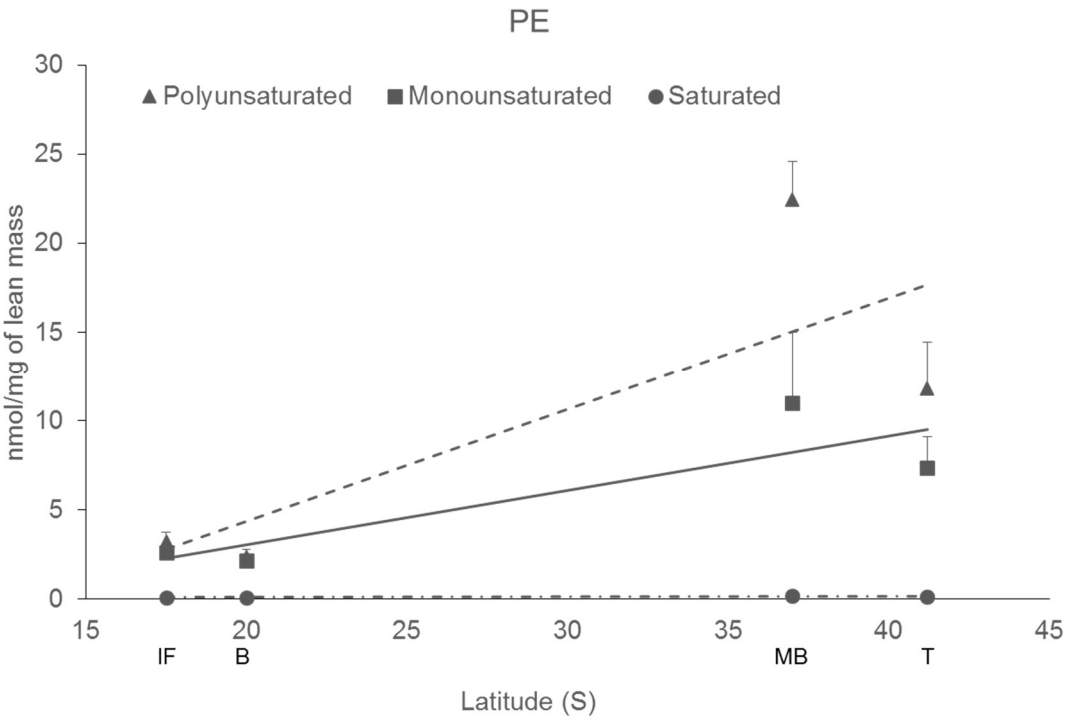

Figure 2.7B. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) PE in male *D. melanogaster*, calculated based on nmol per mg of lean mass, error bars represent the SE (n = 4).

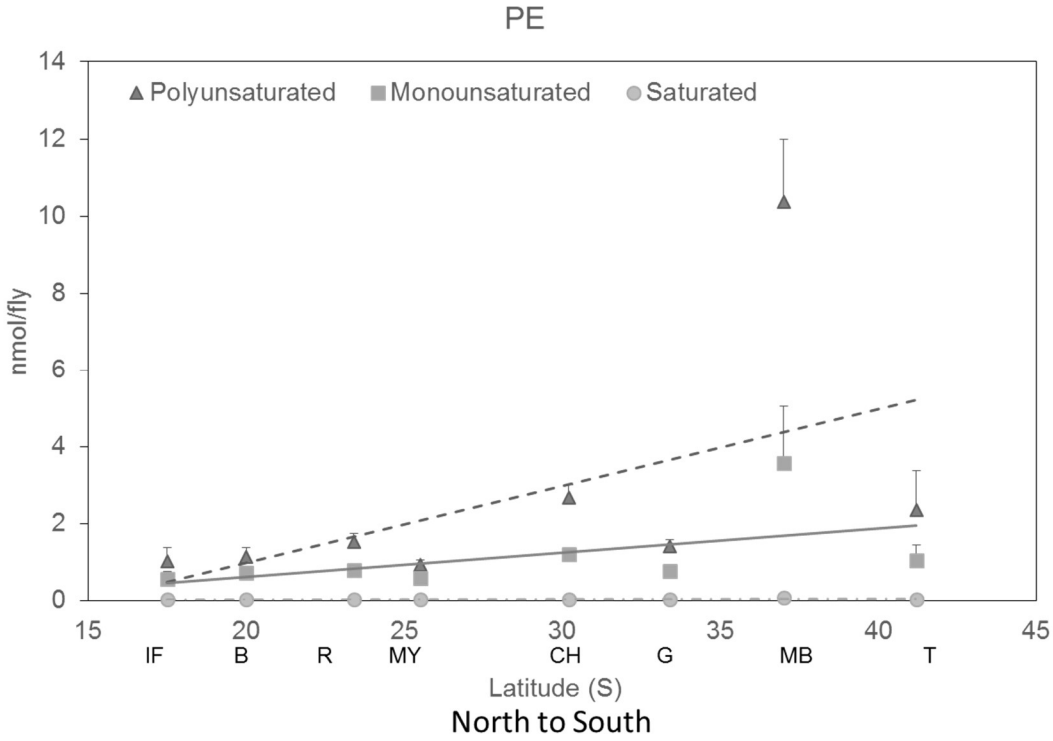

Figure 2.7C. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) PE in female *D. melanogaster*, calculated based on nmol per fly, error bars represent the SE (n = 4).

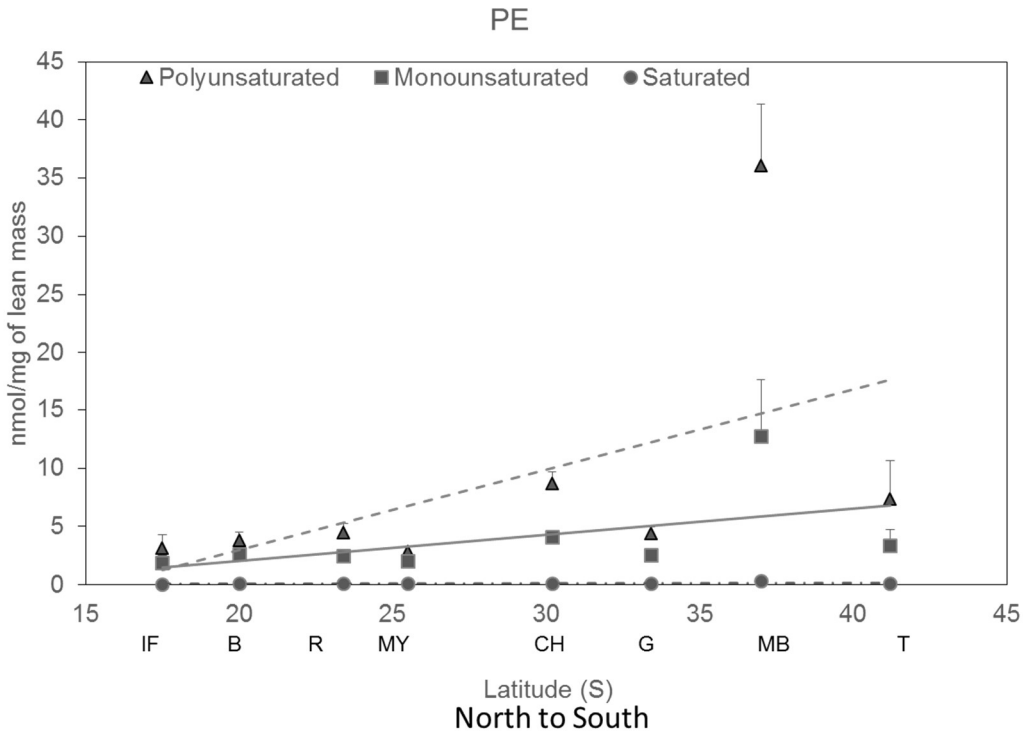

Figure 2.7D. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) PE in female *D. melanogaster*, calculated based on nmol per mg of lean mass, error bars represent the SE (n = 4).

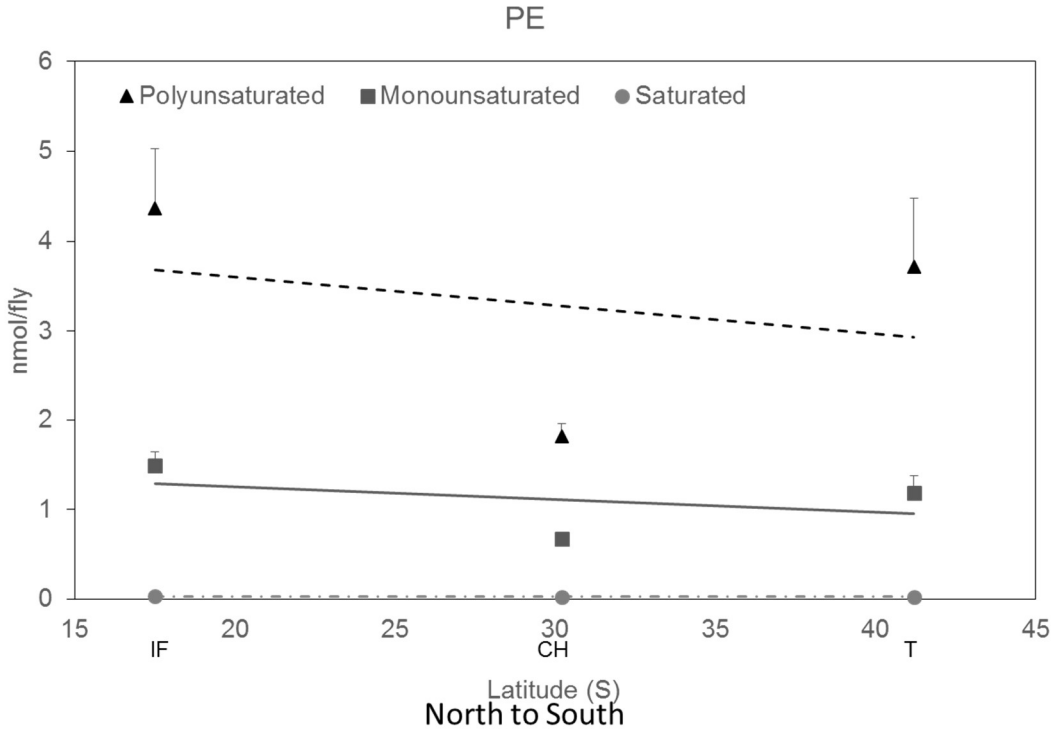

Figure 2.7E. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) PE in male *D. simulans*, calculated based on nmol per fly, error bars represent the SE (n = 4).

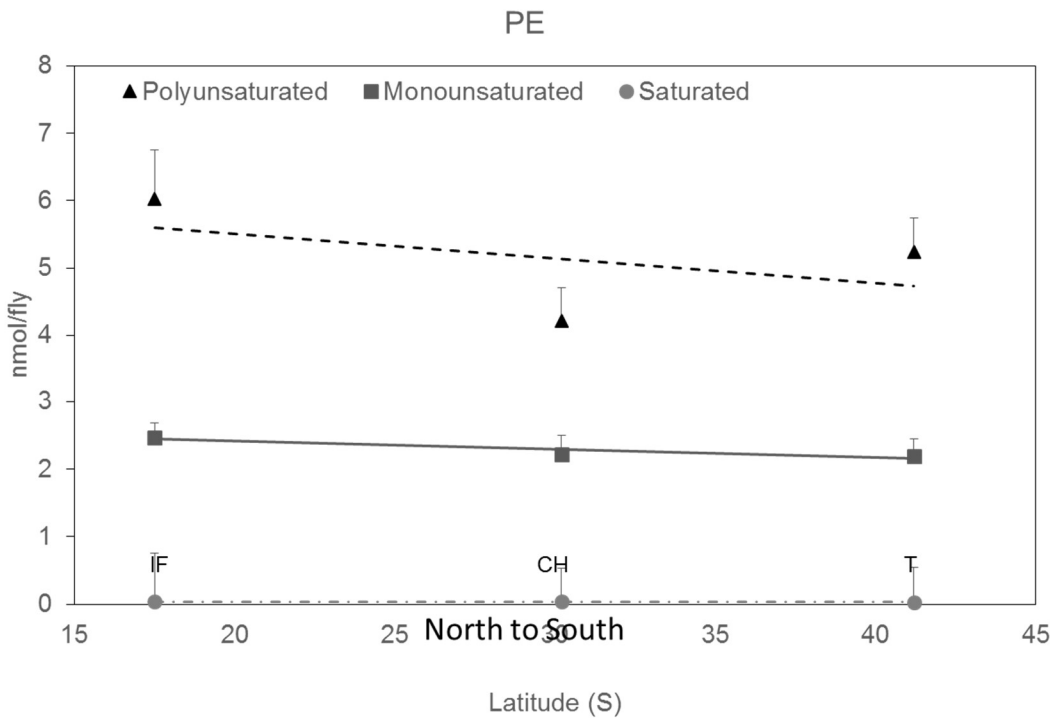

Figure 2.7F. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) PE in female *D. simulans*, calculated based on nmol per fly, error bars represent the SE (n = 4).

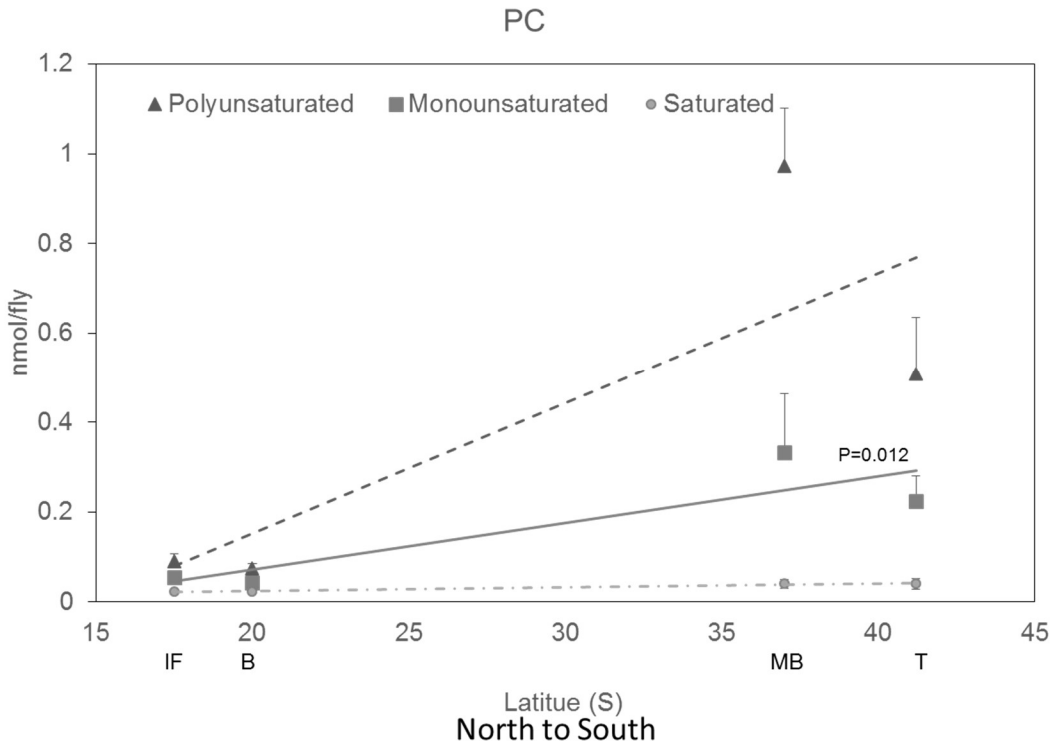

Figure 2.8A. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line with p=0.012), and saturated (dashed and dotted line) PC in male *D. melanogaster*, calculated based on nmol per fly, error bars represent the SE (n = 4).

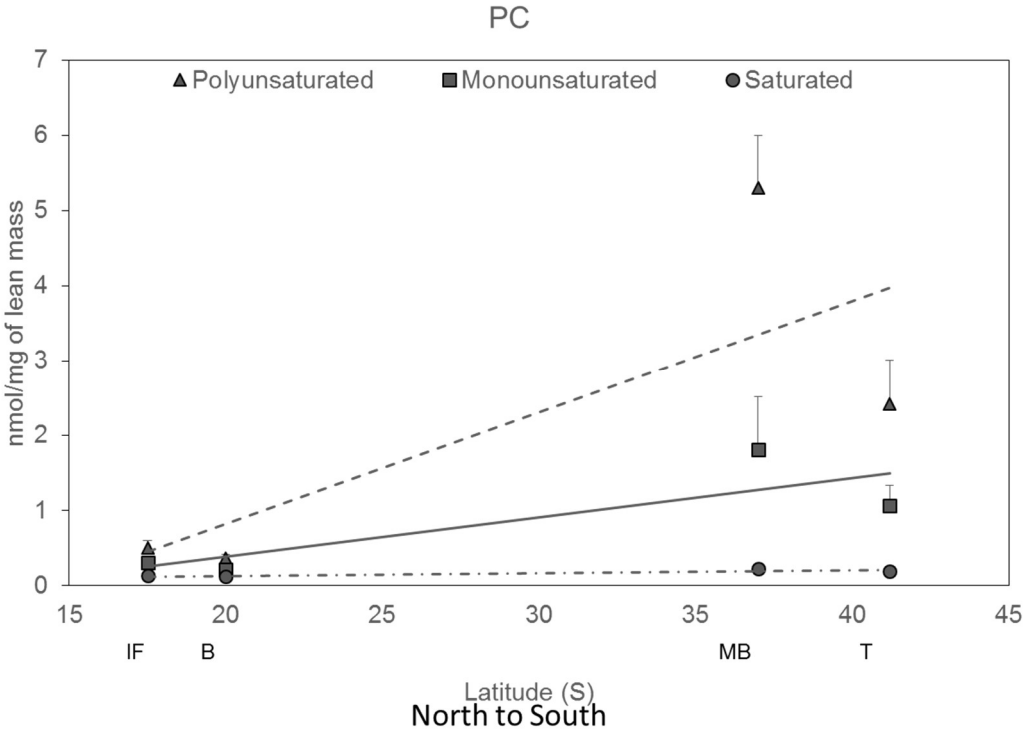

Figure 2.8B. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) PC in male *D. melanogaster*, calculated based on nmol per mg of lean mass, error bars represent the SE (n = 4).

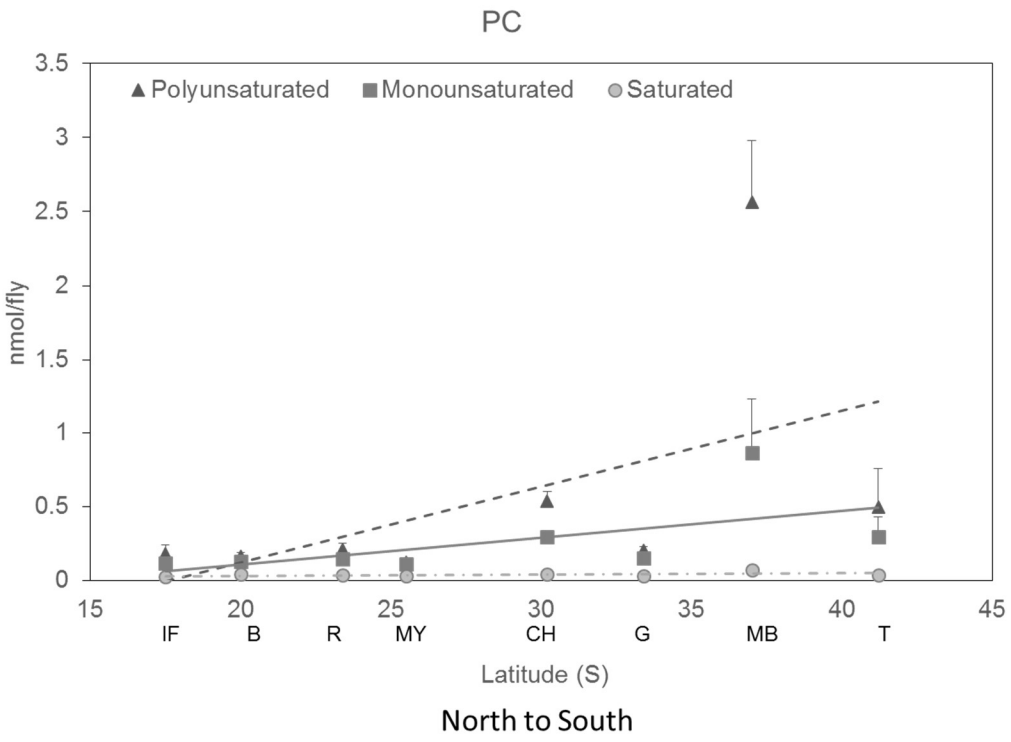

Figure 2.8C. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) PC in female *D. melanogaster*, calculated based on nmol per fly, error bars represent the SE (n = 4).

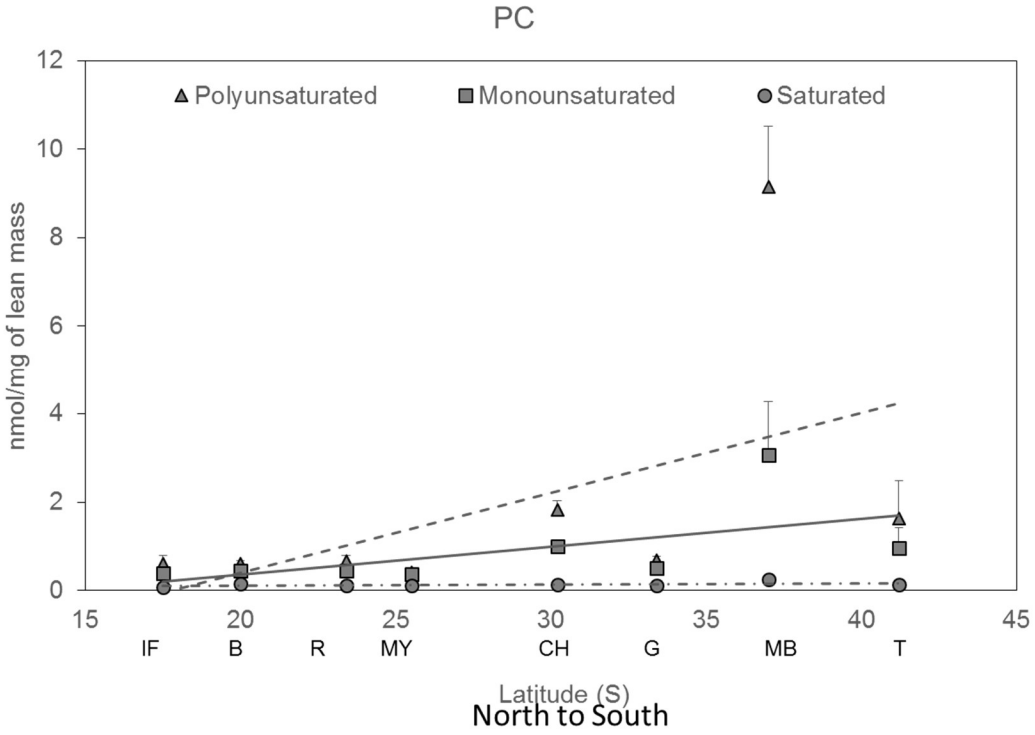

Figure 2.8D. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) PC in female *D. melanogaster*, calculated based on nmol per mg of lean mass, error bars represent the SE (n = 4).

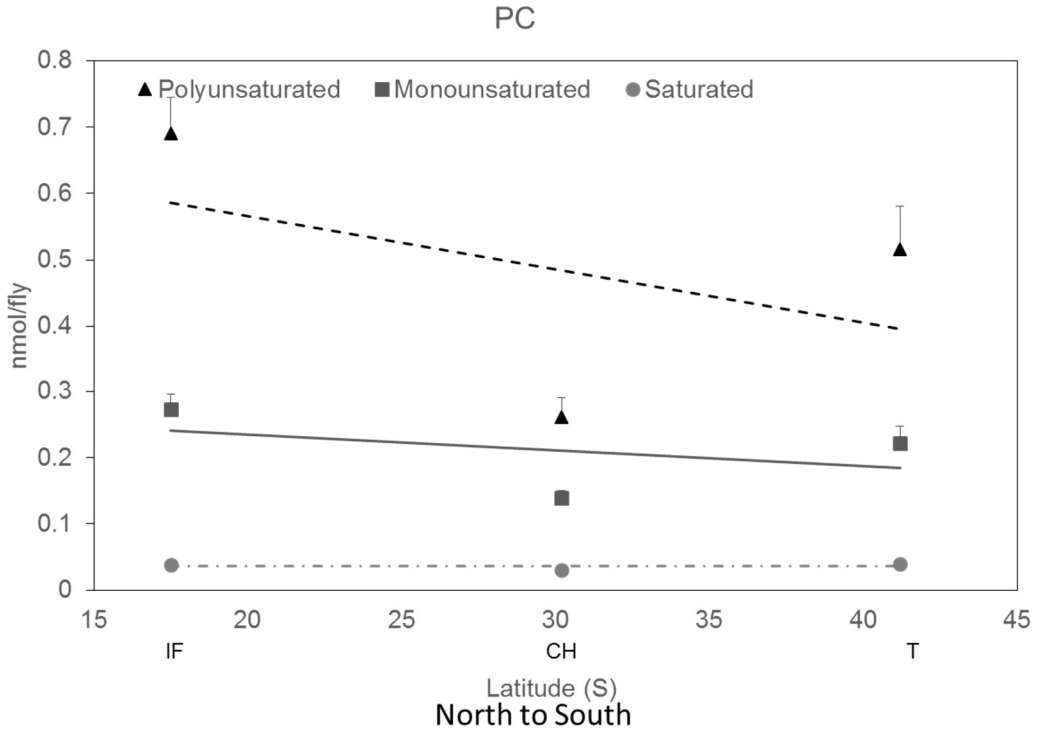

Figure 2.8E. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) PC in male *D. simulans*, calculated based on nmol per fly, error bars represent the SE (n = 4).

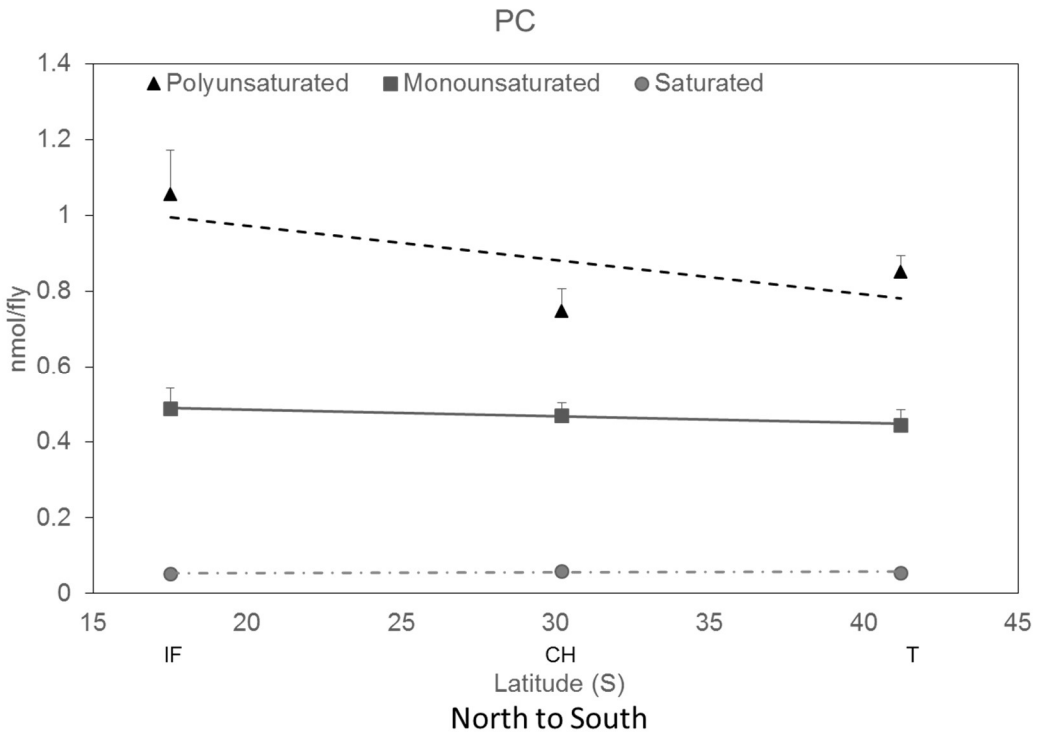

Figure 2.8F. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) PC in female *D. simulans*, calculated based on nmol per fly, error bars represent the SE (n = 4).

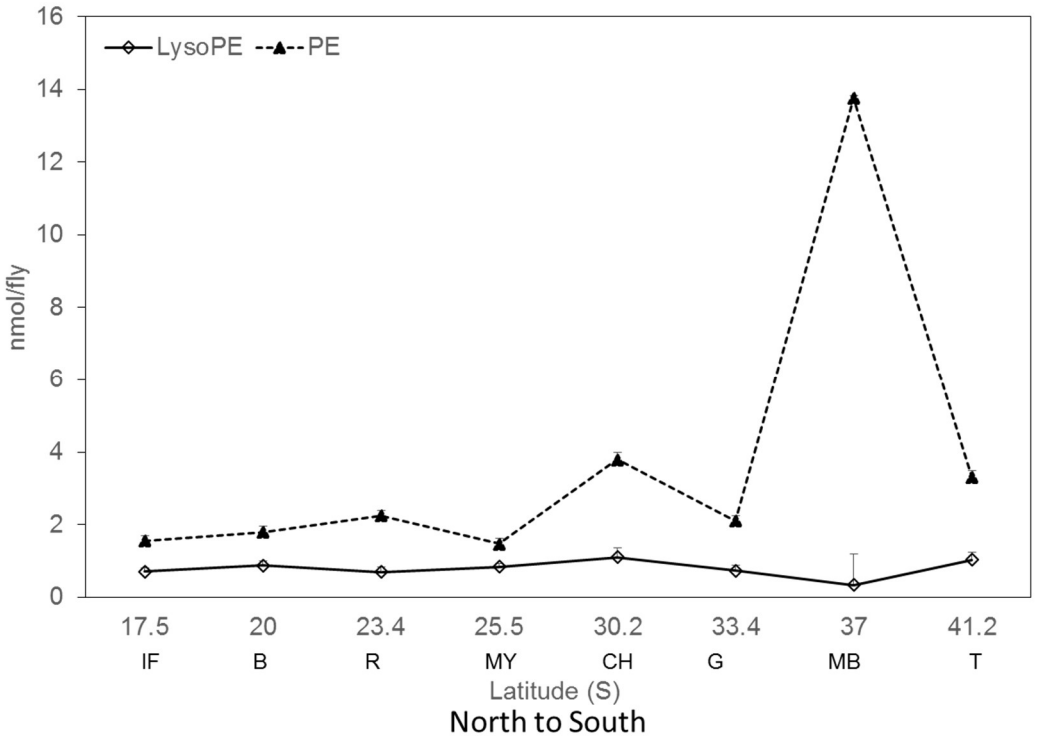

Figure 2.9A. Latitudinal trends in relative abundance of lysoPE and PE in *D. melanogaster* females normalized to nmol per fly, error bars represent the SE (n = 4).

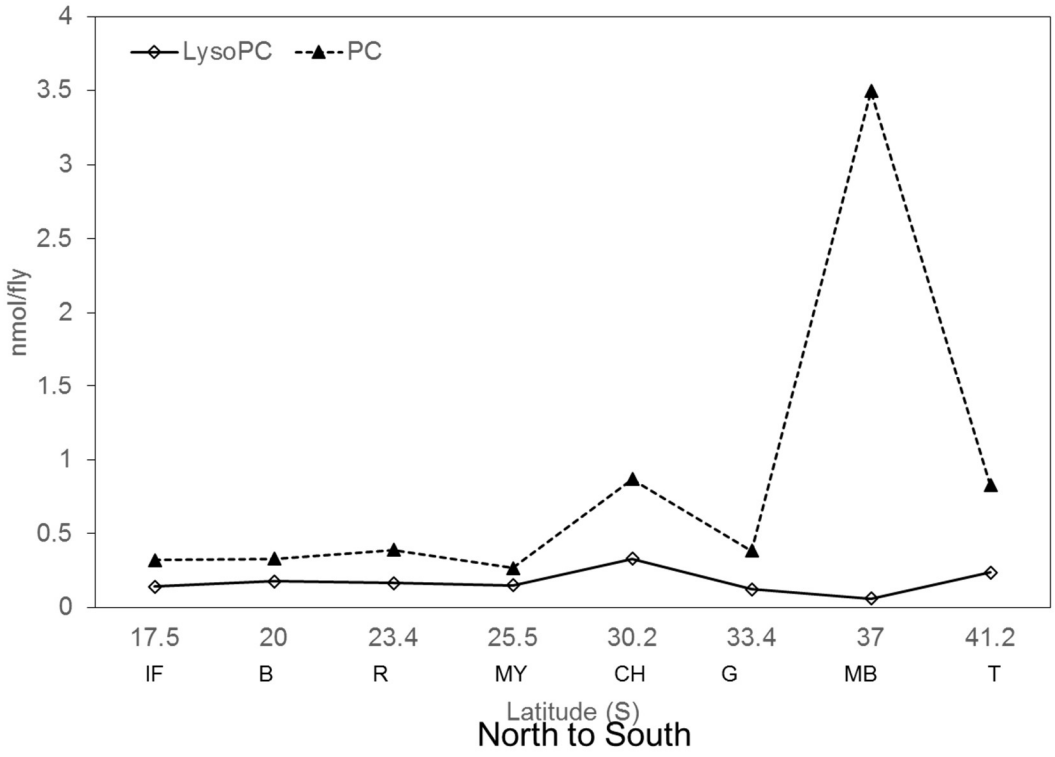

Figure 2.9B. Latitudinal trends in relative abundance of lysoPC and PC in *D. melanogaster* females normalized to nmol per fly, error bars represent the SE (n = 4).

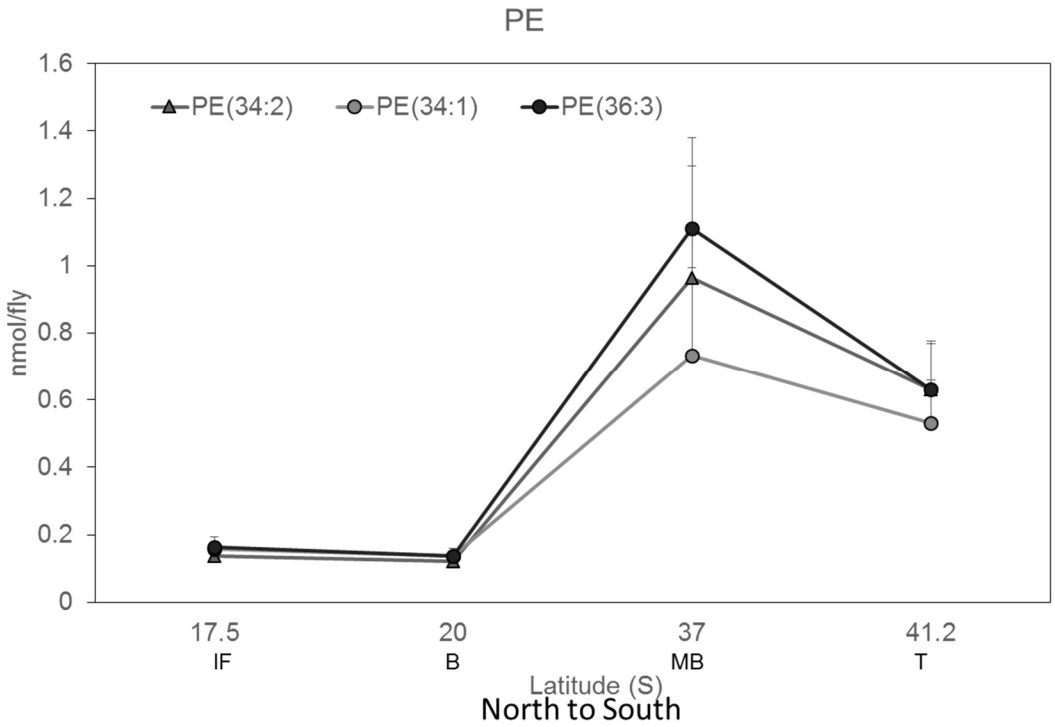

Figure 2.10A. Latitudinal trends of relative abundance for the three predominant PE species in *D. melanogaster* males in nmol per fly, error bars represent the SE (n = 4).

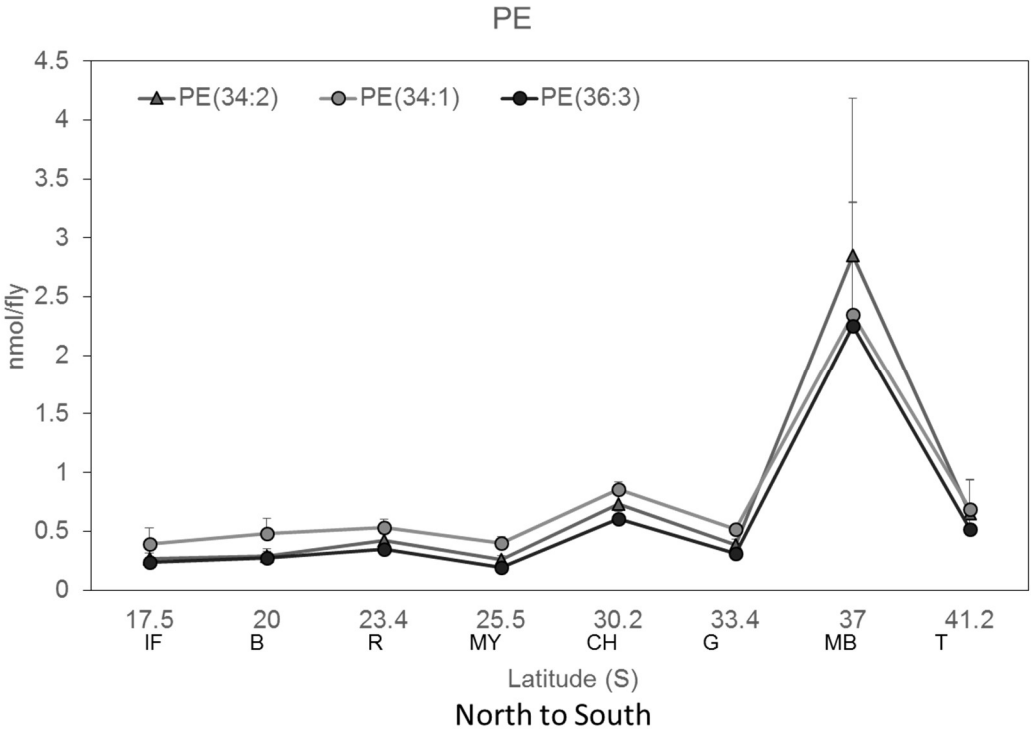

Figure 2.10B. Latitudinal trends of relative abundance for the three predominant PE species in *D. melanogaster* females in nmol per fly, error bars represent the SE (n = 4).

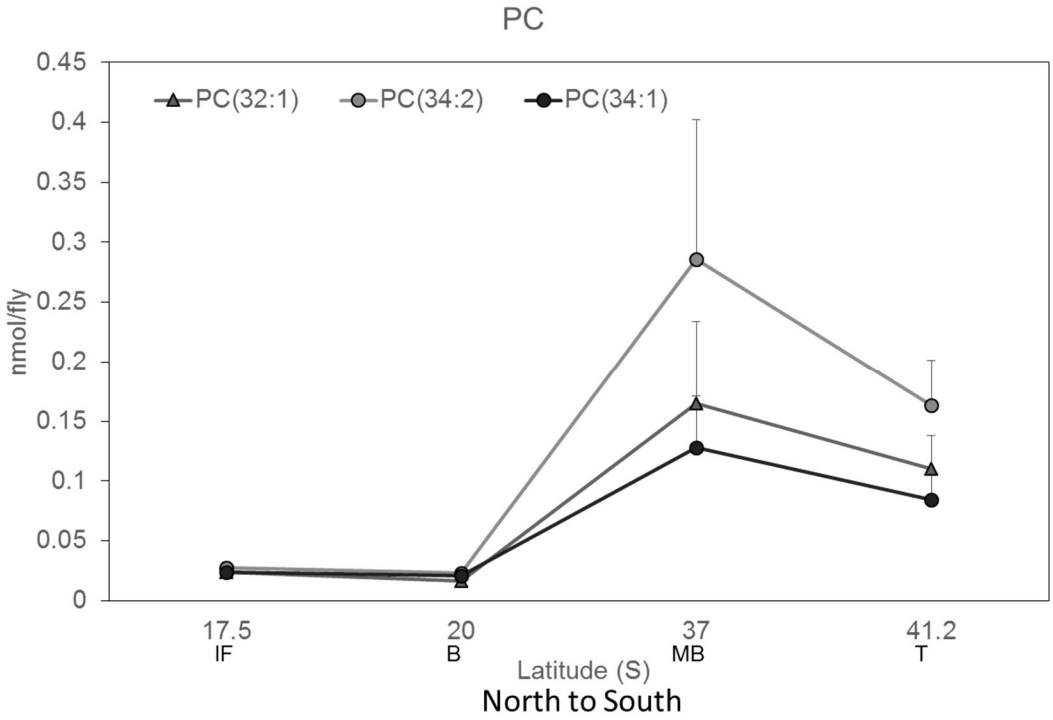

Figure 2.10C. Latitudinal trends of relative abundance for the three predominant PC species in *D. melanogaster* males in nmol per fly, error bars represent the SE (n = 4).

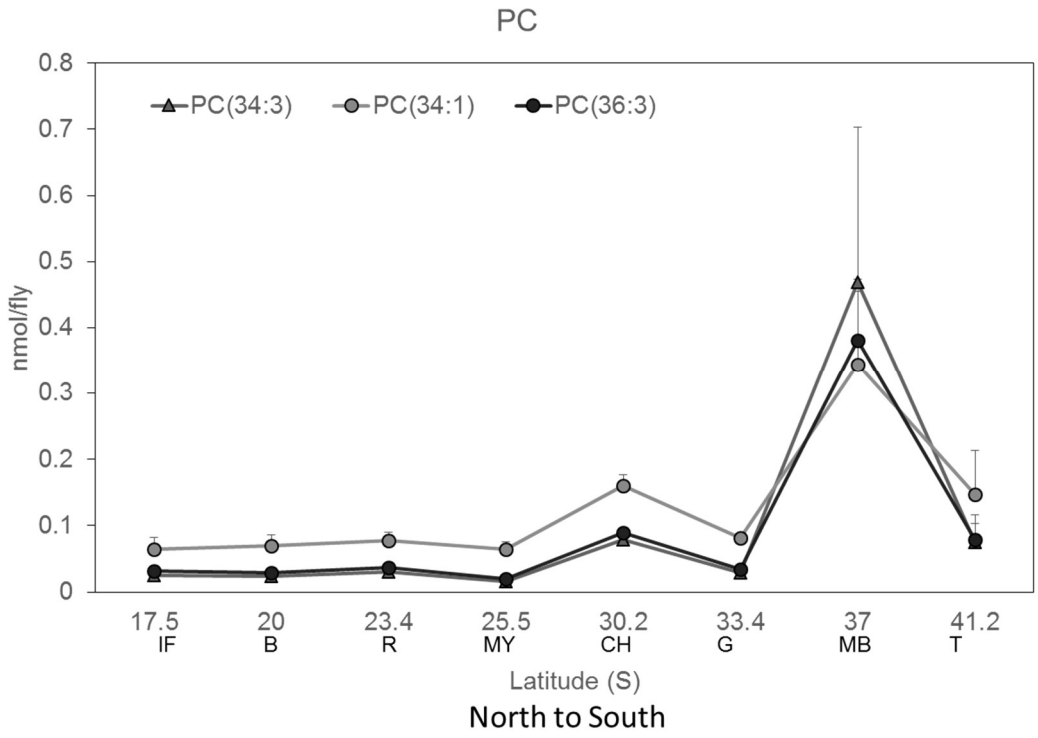

Figure 2.10D. Latitudinal trends of relative abundance for the three predominant PC species in *D. melanogaster* females in nmol per fly, error bars represent the SE (n = 4).

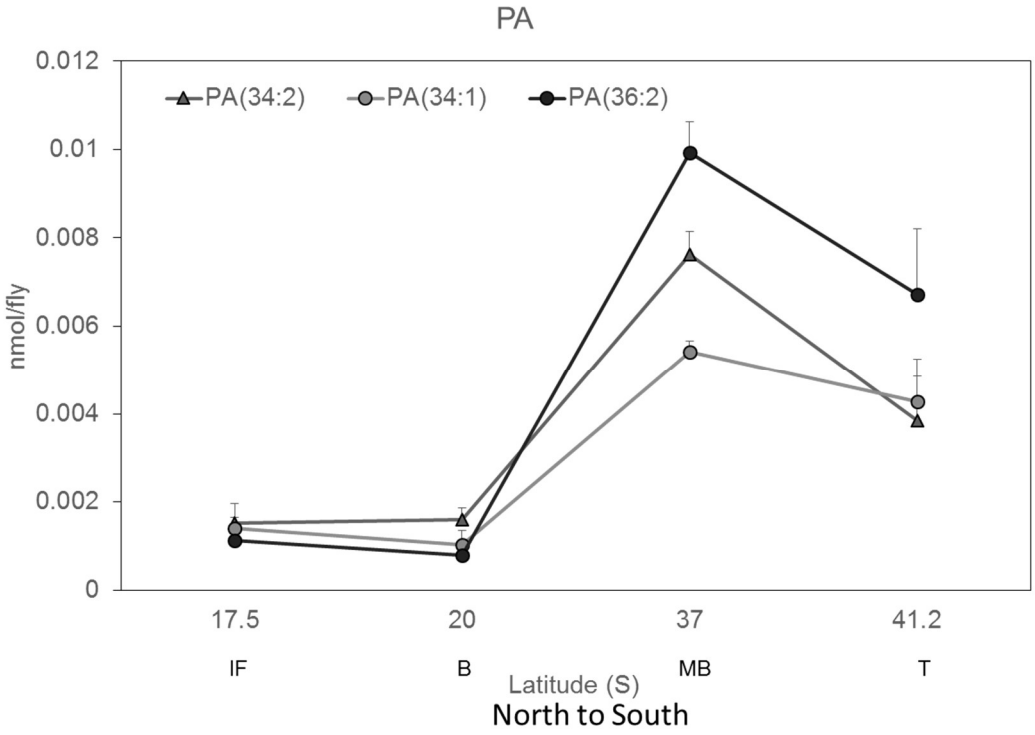

Figure 2.10E. Latitudinal trends of relative abundance for the three predominant PA species in *D. melanogaster* males in nmol per fly, error bars represent the SE (n = 4).

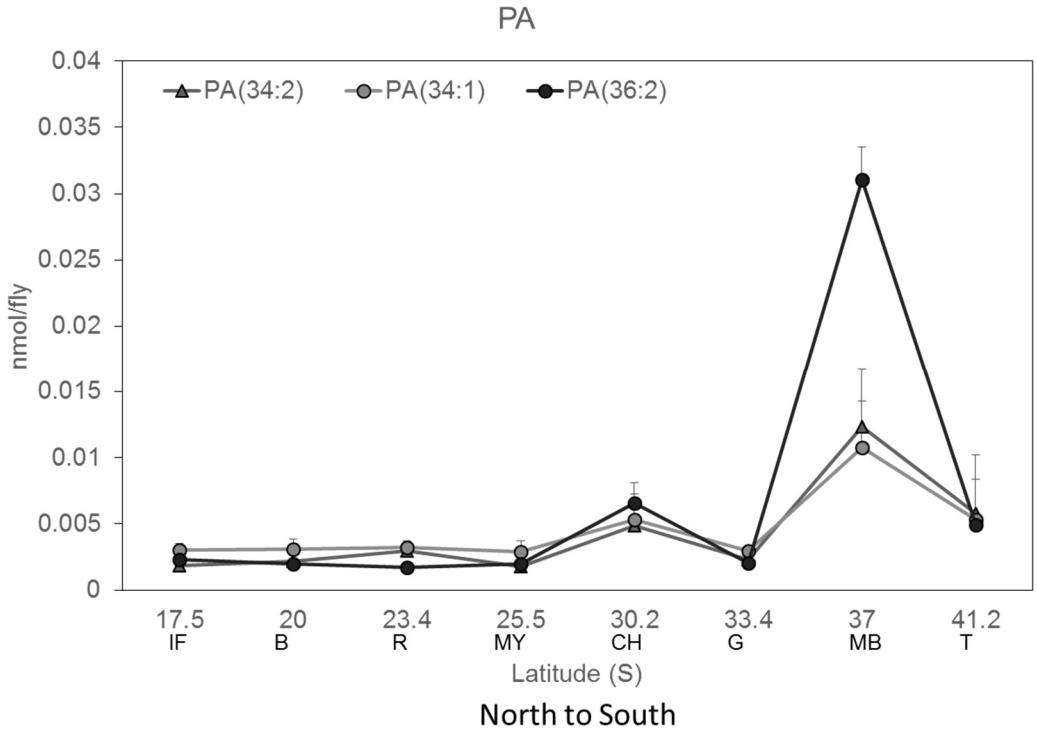

Figure 2.10F. Latitudinal trends of relative abundance for the three predominant PA species in *D. melanogaster* females in nmol per fly, error bars represent the SE (n = 4).

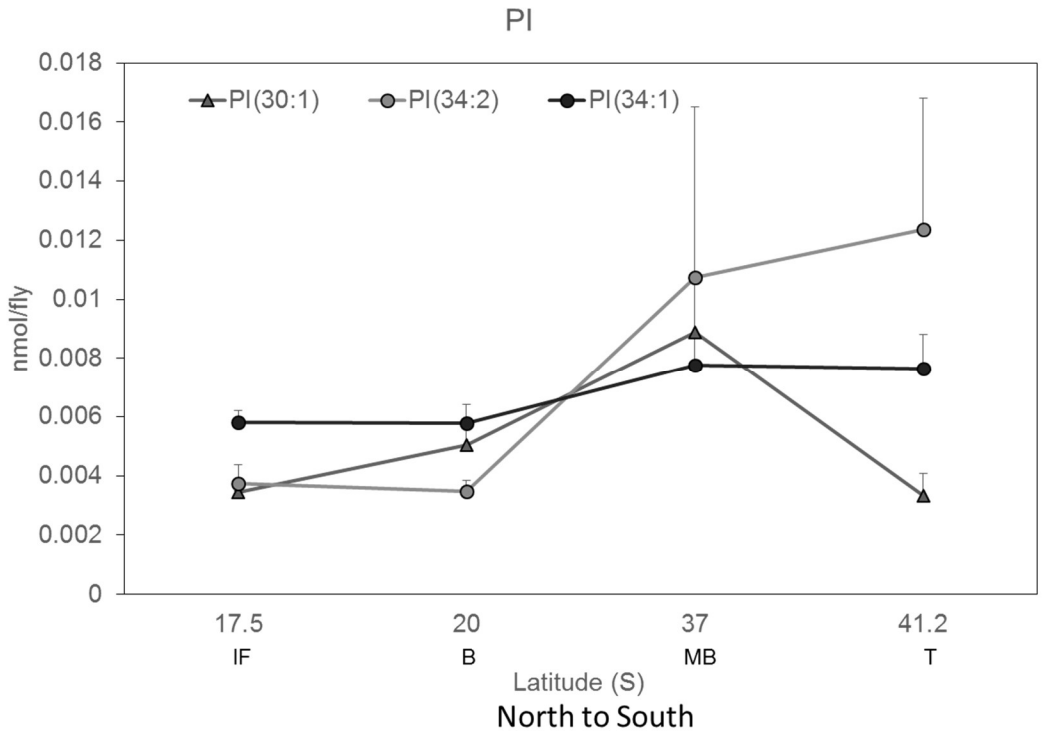

Figure 2.10G. Latitudinal trends of relative abundance for the three predominant PI species in *D. melanogaster* males in nmol per fly, error bars represent the SE (n = 4).

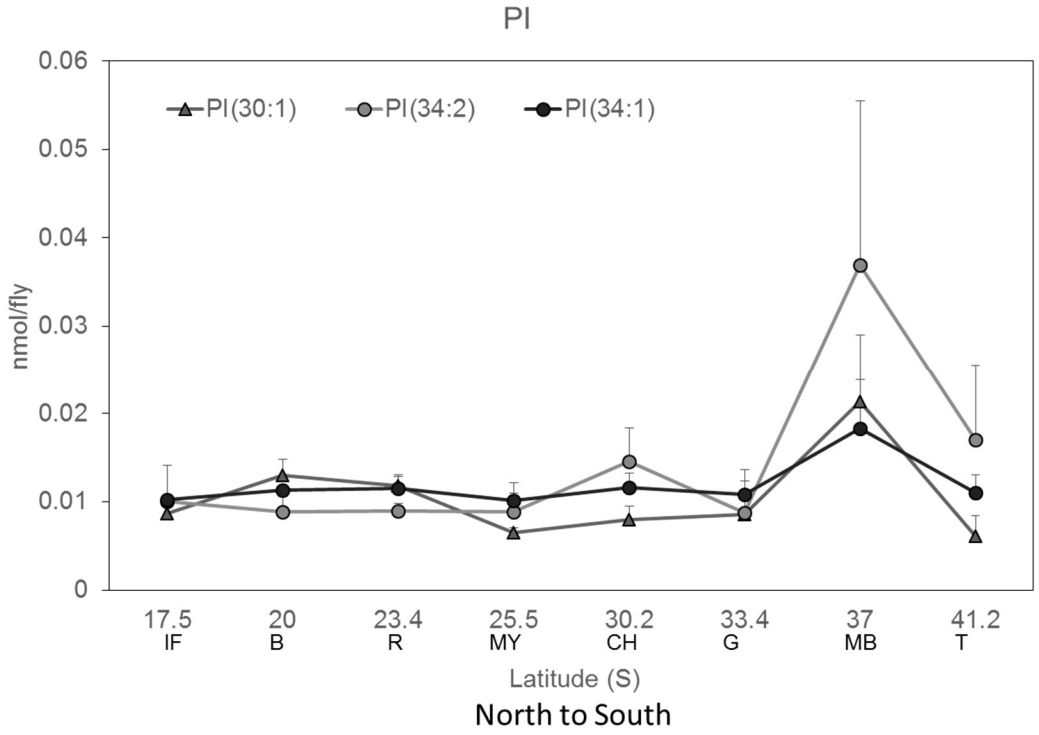

Figure 2.10H. Latitudinal trends of relative abundance for the three predominant PI species in *D. melanogaster* females in nmol per fly, error bars represent the SE (n = 4).

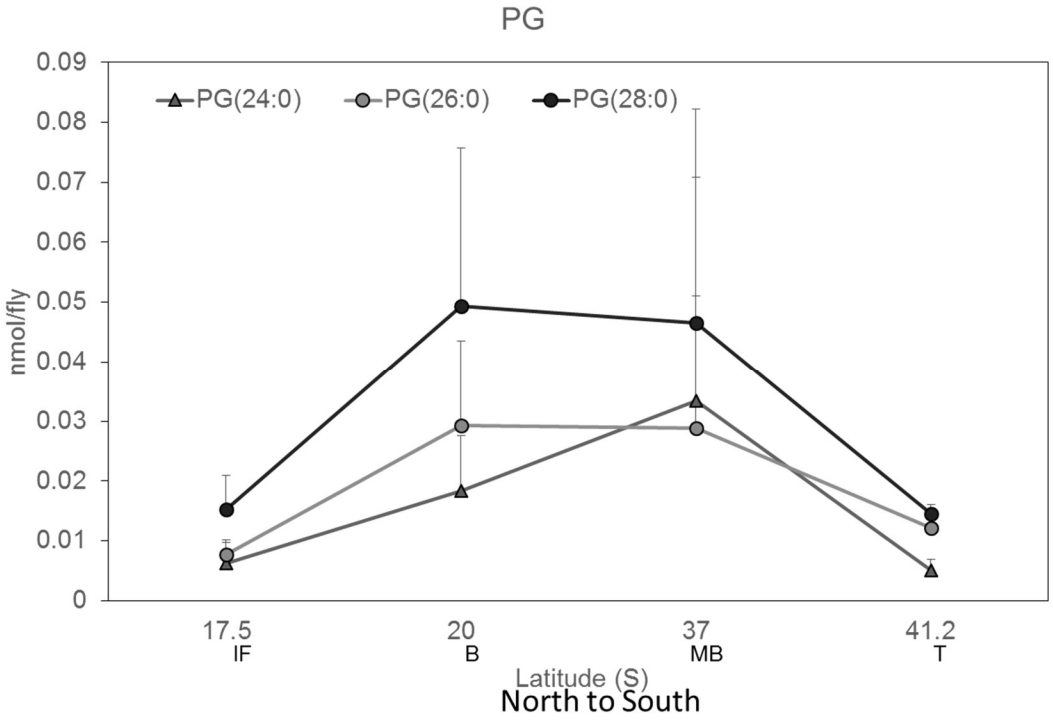

Figure 2.10I. Latitudinal trends of relative abundance for the three predominant PG species in *D. melanogaster* males in nmol per fly, error bars represent the SE (n = 4).

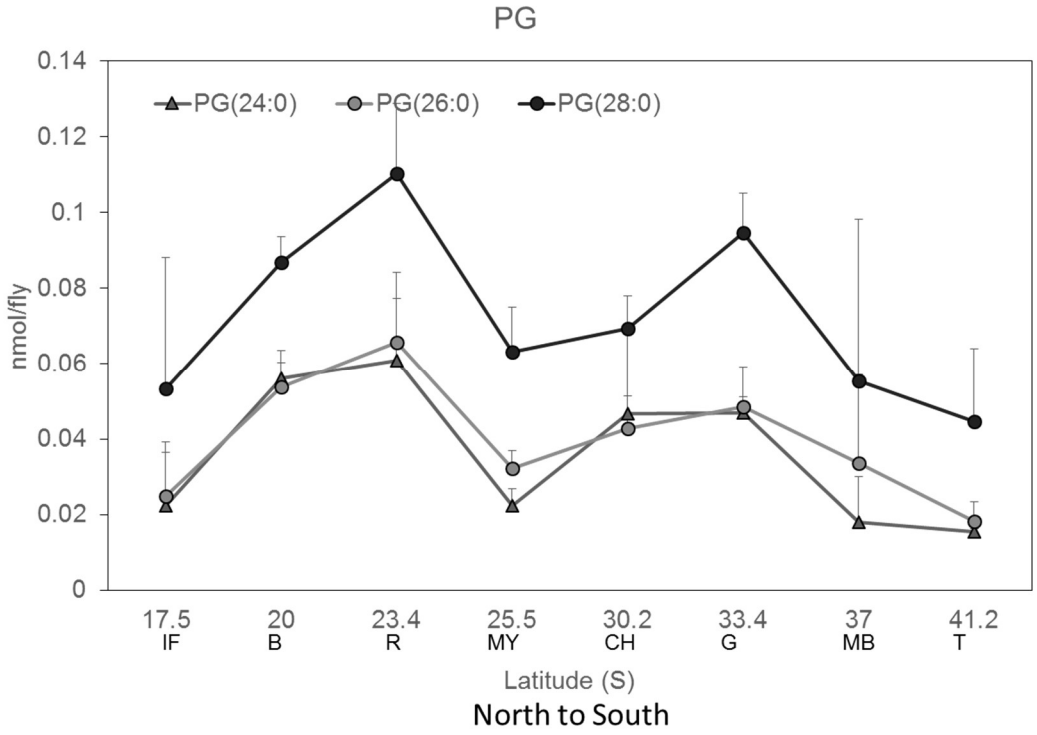

Figure 2.10J. Latitudinal trends of relative abundance for the three predominant PG species in *D. melanogaster* females in nmol per fly, error bars represent the SE (n = 4).

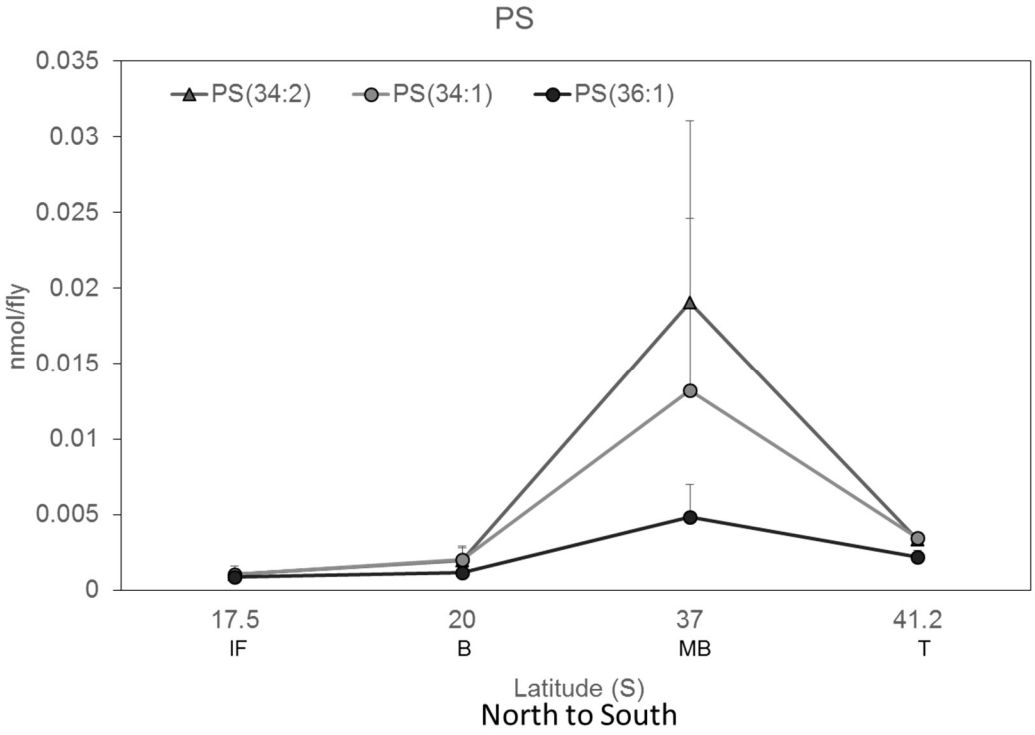

Figure 2.10K. Latitudinal trends of relative abundance for the three predominant PS species in *D. melanogaster* males in nmol per fly, error bars represent the SE (n = 4).

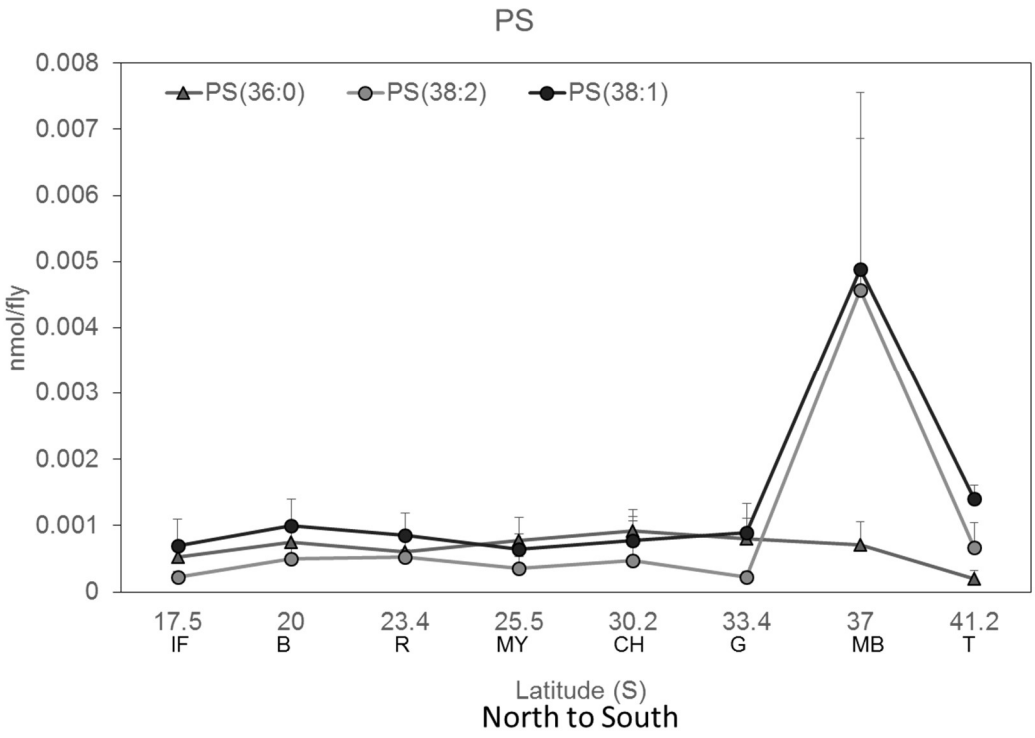

Figure 2.10L. Latitudinal trends of relative abundance for the three predominant PS species in *D. melanogaster* females in nmol per fly, error bars represent the SE (n = 4).

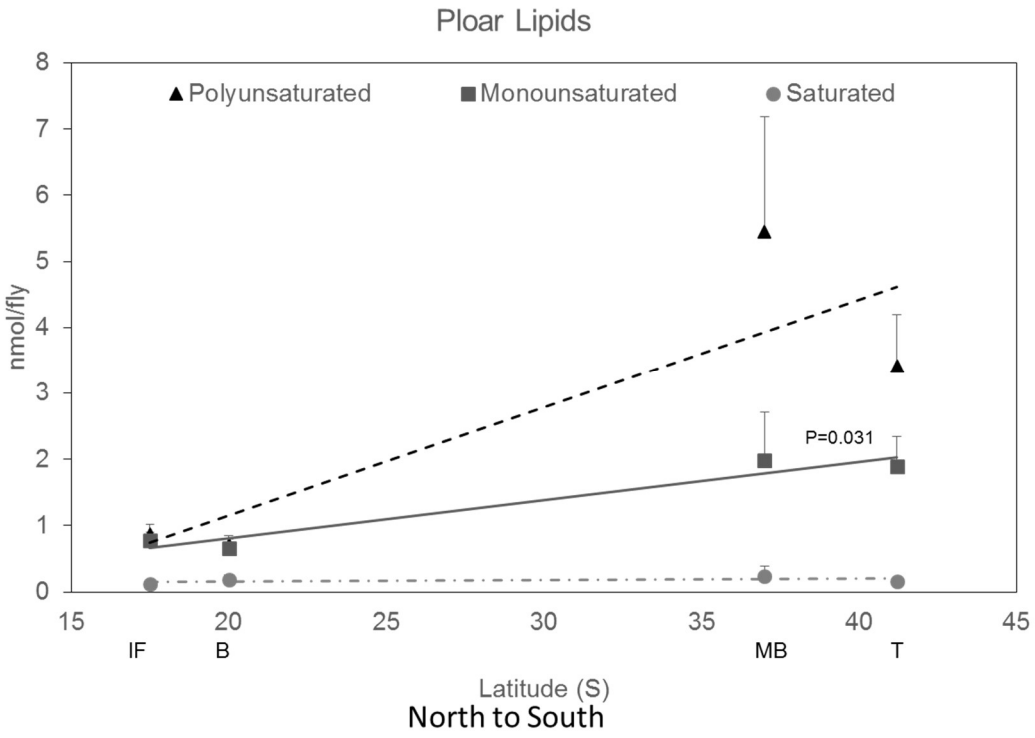

Figure 2.11A. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line with p=0.031), and saturated (dashed and dotted line) polar lipids in male *D. melanogaster*, calculated based on nmol per mg of lean mass, error bars represent the SE (n = 4).

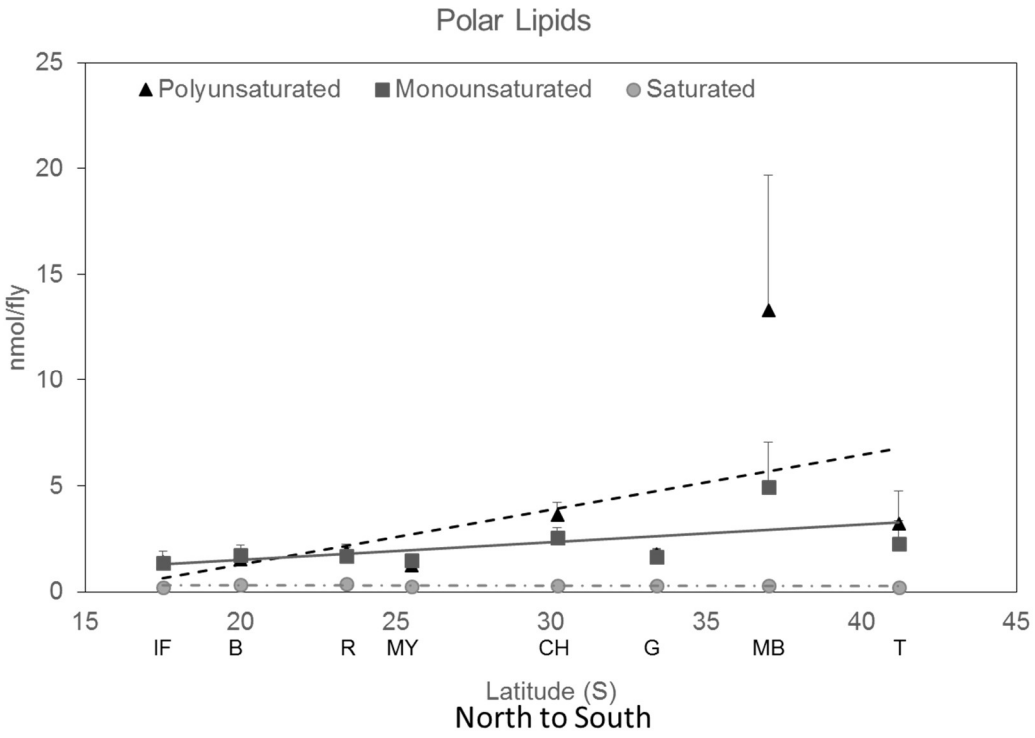

Figure 2.11B. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) polar lipids in female *D. melanogaster*, calculated based on nmol per mg of lean mass, error bars represent the SE (n = 4).

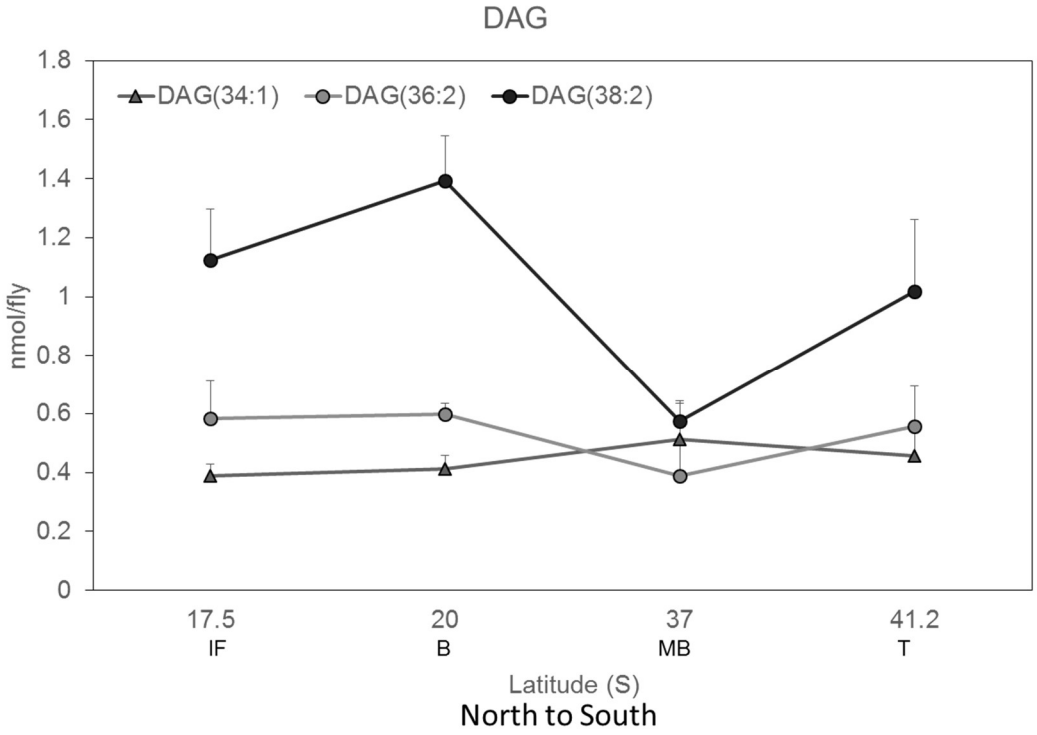

Figure 2.12A. Latitudinal trends of relative abundance for the three predominant DAG species in *D. melanogaster* males in nmol per fly, error bars represent the SE (n = 4).

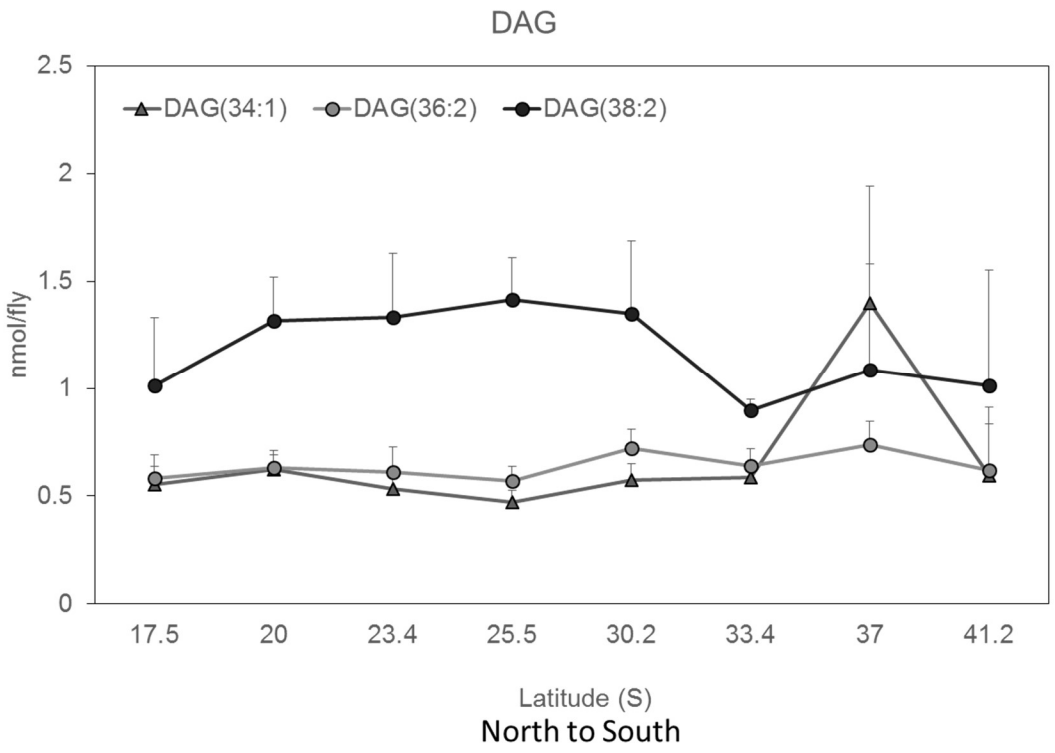

Figure 2.12B. Latitudinal trends of relative abundance for the three predominant DAG species in *D. melanogaster* females in nmol per fly, error bars represent the SE (n = 4).

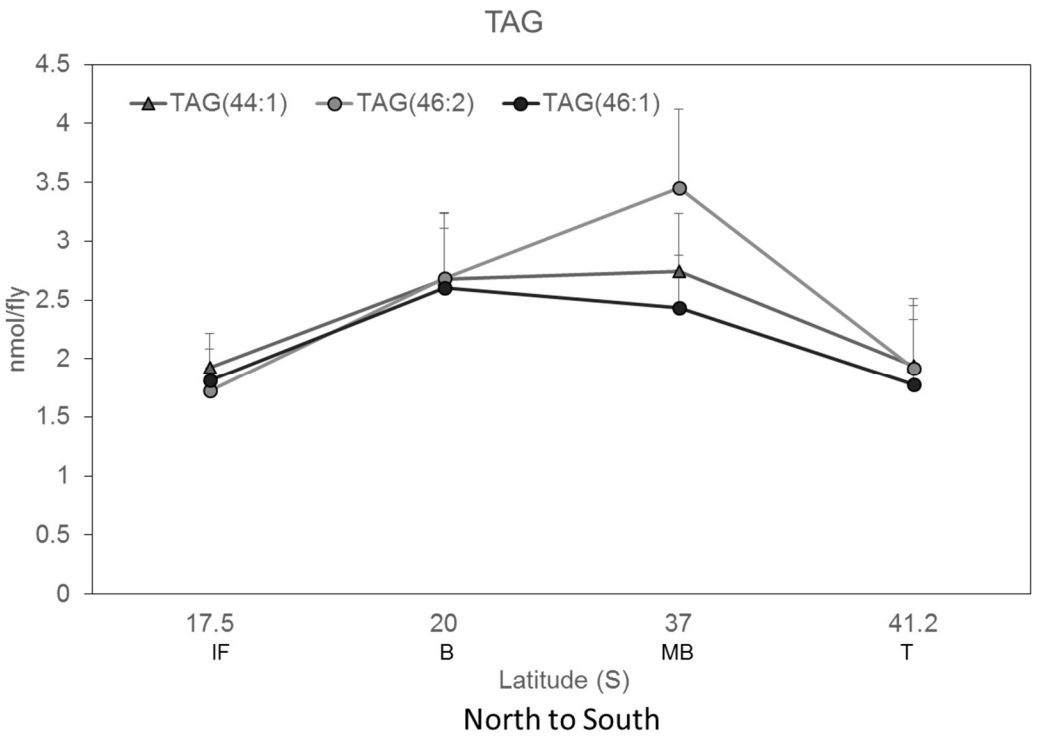

Figure 2.12C. Latitudinal trends of relative abundance for the three predominant TAG species in *D. melanogaster* males in nmol per fly, error bars represent the SE (n = 4).

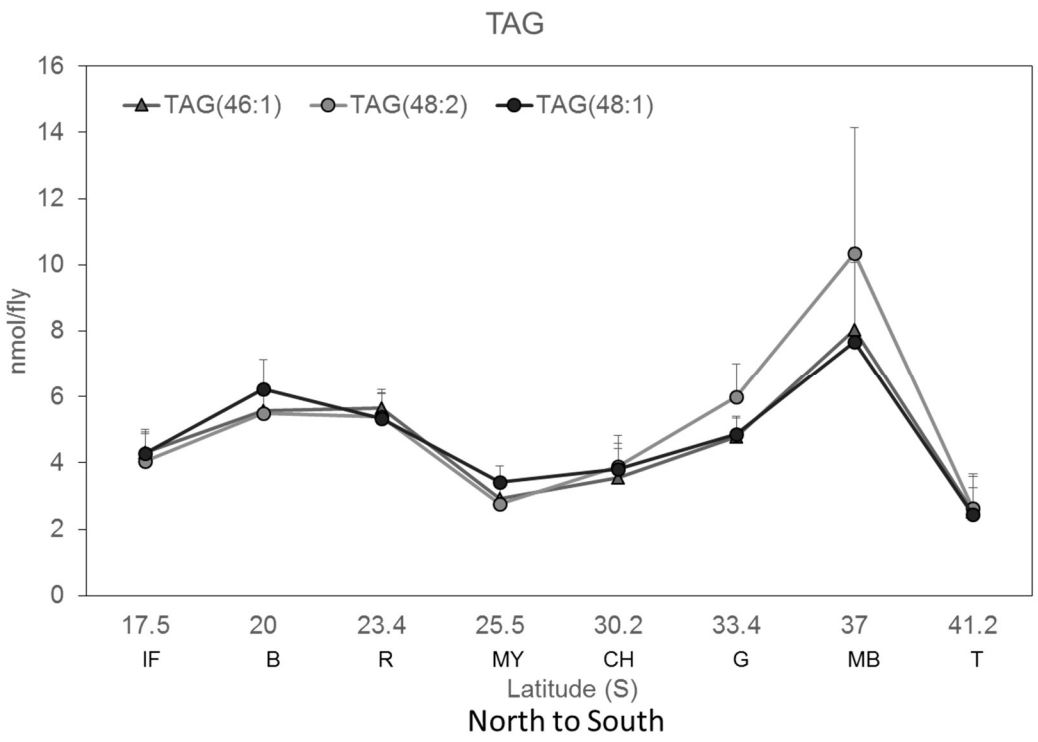

Figure 2.12D. Latitudinal trends of relative abundance for the three predominant TAG species in *D. melanogaster* females in nmol per fly, error bars represent the SE (n = 4).

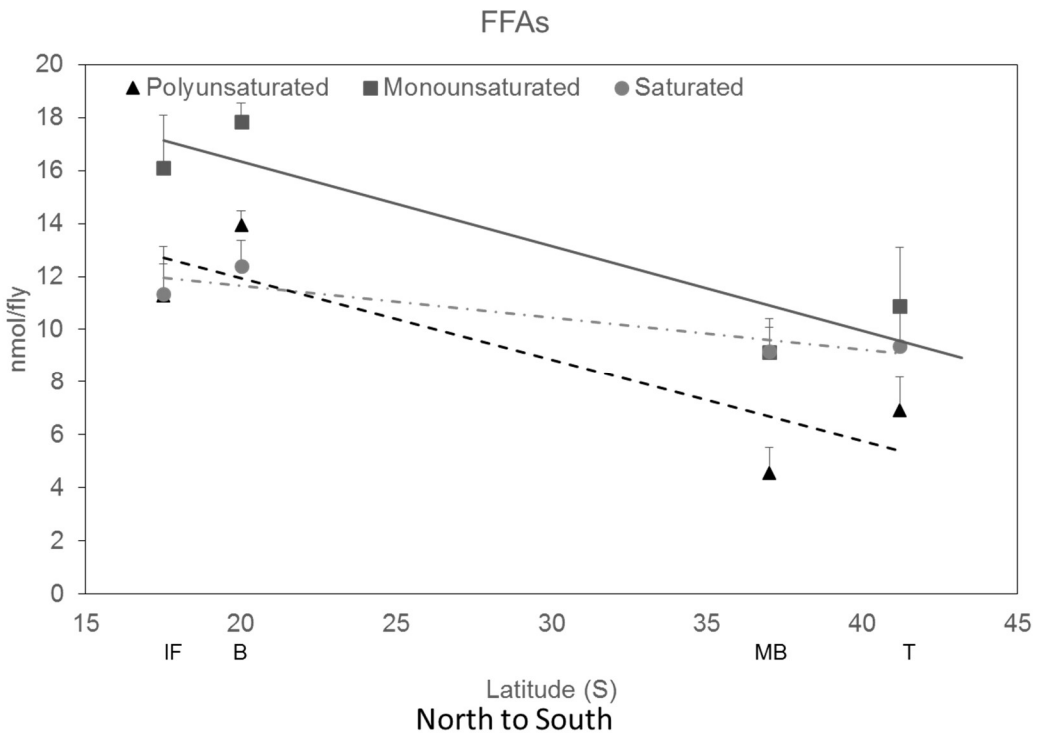

Figure 2.13A. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) free fatty acids in male *D. melanogaster*, calculated based on nmol per mg of lean mass, error bars represent the SE (n = 4).

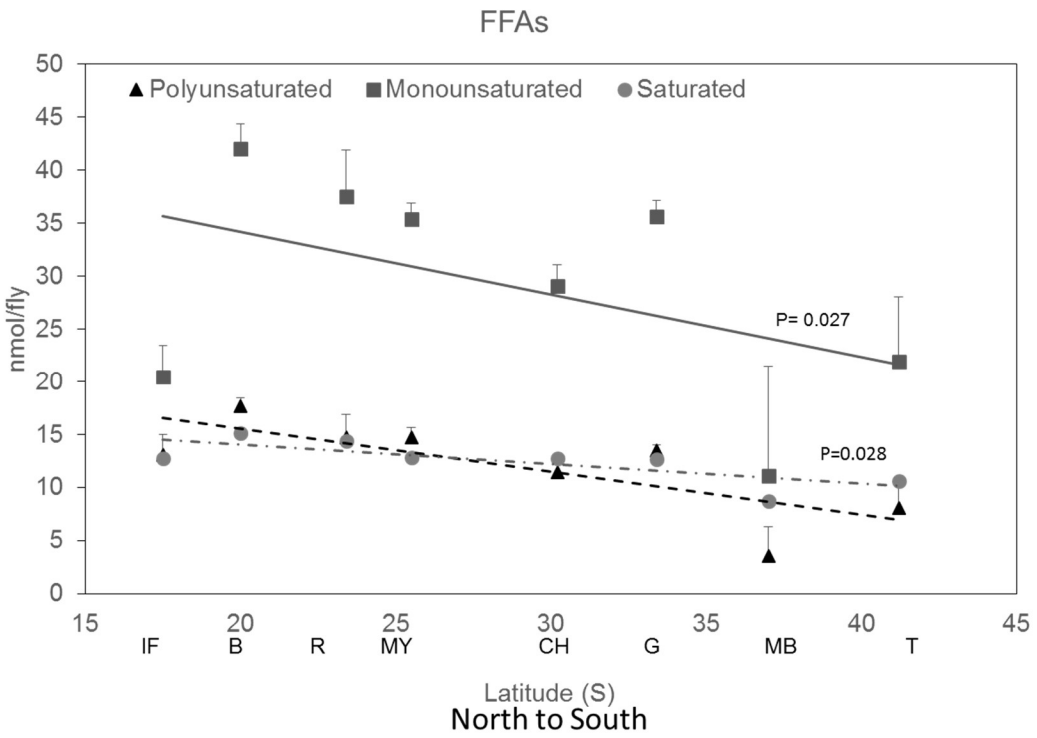

Figure 2.13B. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) free fatty acids in female *D. melanogaster*, calculated based on nmol per mg of lean mass, error bars represent the SE (n = 4).

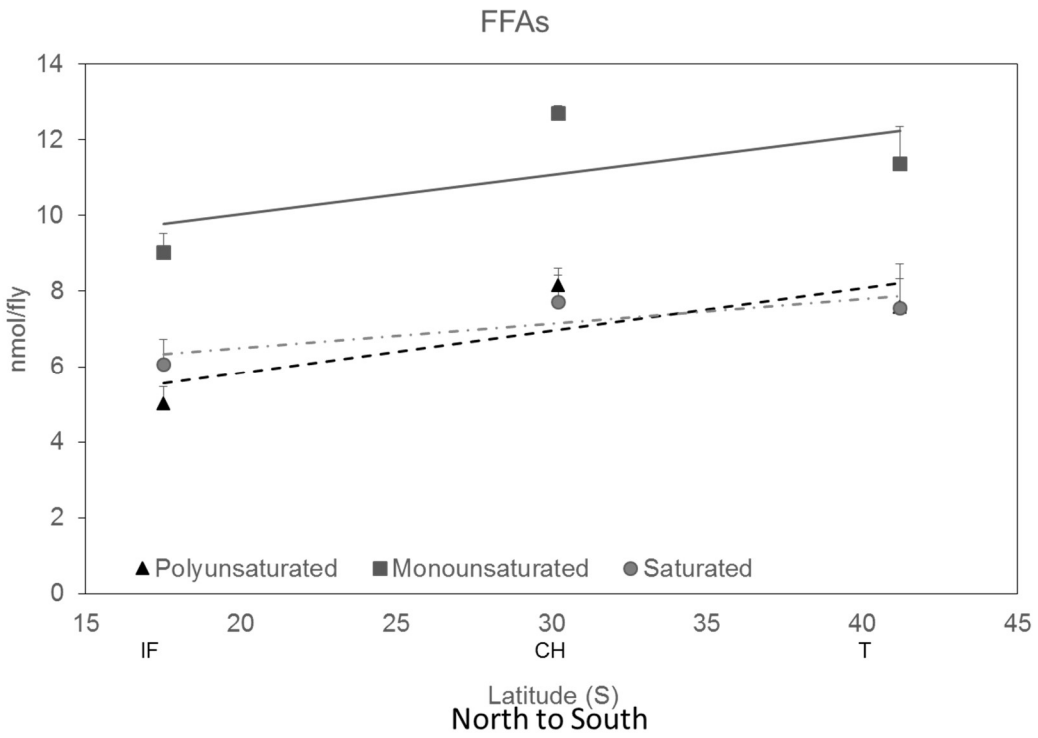

Figure 2.13C. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) free fatty acids in male *D. simulans*, calculated based on nmol per mg of lean mass, error bars represent the SE (n = 4).

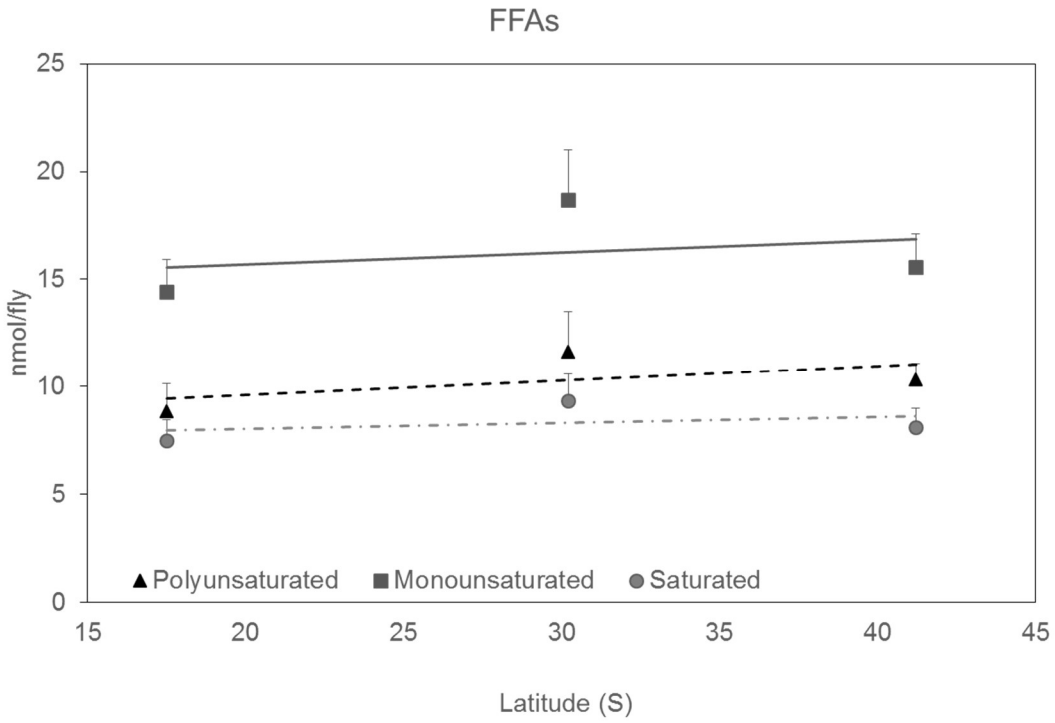

Figure 2.13D. Latitudinal trends of relative abundance for polyunsaturated (dashed line), monounsaturated (solid line), and saturated (dashed and dotted line) free fatty acids in female *D. simulans*, calculated based on nmol per mg of lean mass, error bars represent the SE (n = 4).

CHAPTER 3

LIPIDOME RESPONSE TO LABORATORY SELECTION TO DESICCATION SURVIVAL, TOLERANCE TO ELEVATED HEAT KNOCKDOWN AND CHILL-COMA RECOVERY

3.1 Introduction

Laboratory selection experiments using *Drosophila*, and other organisms, are well-established approaches in basic and applied research. The method is to select a subset of individuals in a population (line) for breeding each generation or allow a subset of individuals to naturally prevail in competition for representation in the next generation for evolution to occur in the laboratory. Over generations, the selected lines will diverge from control lines based on progressive amplification of genetic differentiation. The direct and indirect (correlated) responses to selection can be investigated. Motivations for studies of the correlated responses including information about the life-history and stress resistance traits as well as underlying biochemical, physiological etc. basis of divergence.

Often ether-extracted lipids, or sometimes specifically triglycerides, have been investigated as a correlated response to stress selection. An example of laboratory selection experiment on *D. melanogaster* in which stress responses were investigated was reported in Rose (1984) and Service et al. (1985). In this case, lines selected for longevity and late reproduction showed correlated responses of increased desiccation resistance, as well as increased glycogen and lipid abundance. Moreover, selection for reduced longevity was associated with decreased lipid abundance (Zwann 1995). An

consideration is how often responses to laboratory life-history or stress selection are associated with an increase in triglyceride abundance in the selected lines.

Although we did not conduct selection for starvation resistance in this study, studies of starvation resistance of *D. melanogaster* have been conducted numerous times and the results of such studies are relevant to the present research. For example, an early selection experiment for starvation resistance observed that starvation selected lines had high levels of lipid and total energy stores which would include glycogen (Djawdan et al. 1998). In another study, selection on female starvation survival resulted in an increase in lipids and multiple stress resistance (Harshman et al. 1999, Harshman and Schmid 1998). In Harshman et al. (1999) the starvation selected lines exhibited a correlated increase in ethanol exposure survival suggesting the cell membranes had responded to selection allowing for resistance to ethanol (Logan-Garbisch. 2015, Montooth et al. 2006, Scheitz et al. 2013). In an experiment selecting for resistance to different stresses, including starvation resistance, lipid abundance increased in the starvation resistant lines (Bubliy and Loeschcke 2005). In Bubliy and Loeschcke (2005), lines were selected for cold or heat survival and tolerance to knockdown at elevated temperature. All selected lines exhibited increased starvation resistance as a correlated response to selection. Thus, it is possible that all these selected lines had increased triglyceride stores that allowed the adults to live longer under starvation condition. A long-term selection experiment for starvation resistance finds a two-fold increase in triglyceride stores in adult flies at two ages and cardiac dysfunction states similar to that observed for obesity in humans (Hardy et al. 2015). There are a number of potential generalities that arise from such studies, including the possibility that multiple stress resistance is a typical outcome for selection

on one stress trait and the possibility that increased lipid stores typically underlies the response to selection for stress resistance.

Selection for desiccation resistance was part of the present study. In an early study, desiccation-selected and control lines did not differ in the amount of lipid extractable by ether (Hoffmann and Parsons 1989). In a parallel selection experiment independently for starvation survival (described in the paragraph above) and desiccation resistance, there was no correlation with lipid or total energy stores and desiccation resistance (Djawdan et al. 1998). There was a significant correlation between carbohydrate stores and desiccation resistance in females, but not males. In Chippindale et al. (1998) a comparison between desiccation-selected and control lines was conducted and the control lines accumulated more lipids perhaps due to periodic starvation in the many generations of selection. In these lines, there was no relative increase in adult surface lipids, which would include cuticular hydrocarbons, in the desiccation-selected lines, but the hydrocarbons from the desiccation-resistant flies were longer and melted at a higher temperature (Gibbs et al. 1997). Another set of lines selected for adult desiccation resistance found evidence for increased whole-body lipid storage (Telonis-Scott et al. 2006). Overall, the evidence for an increase in lipid storage in desiccation-selected *D. melanogaster* lines is mixed.

The present study includes lines selected for relatively rapid chill-coma recovery. Gerken et al. (2016) selected for chill-coma recovery and investigated a range of other environmental stresses as potential correlated responses to selection in *D. melanogaster*. They found that there was no correlation between chill-coma recovery and survival after acute or chronic cold, heat tolerance or starvation survival. No evidence for multiple

stress resistance associated with chill-coma recovery, nor any indirect evidence for storage lipid accumulation (increased starvation survival) was observed. Hoffmann et al. (2005) and Anderson et al. (2005) tested lines selected for starvation resistance in terms of chill-coma recovery and tested lines selected for chill-coma recovery in terms of starvation resistance. Selection for starvation resistance reduced chill-coma recovery. Reciprocally, selection for chill-coma recovery reduced starvation survival implying reduced triglyceride levels in the chill-coma lines. The evidence for a negative trade-off between chill-coma survival and starvation resistance was found to be robust.

A ramping method for elevated temperature selection was used in the present study. Selection for survival under high temperature or slower knockdown time under elevated temperature has been previously investigated. The elevated temperature selection experiments have investigated heat shock protein gene expression (Folk et al. 2006, Pappas et al. 2007). Lipid abundance is not commonly investigated in *D. melanogaster* elevated heat selection experiments. However, (Malmendal et al. 2013) found that fatty acid abundance was reduced in lines selected by continuous exposure to 30°C. Although not a selection experiment, a study based on laboratory strains and a wild-caught population provides insight into energy reserves and thermal stress (Klepsatel et al. 2016). The elevated temperature can cause a decline in body fat, which is at least partially caused by apoptosis of fat body cells. Thus, there can be an irreversible decline in fat body lipid reserve storage capacity. A compelling argument (Klepsatel et al. 2016) is that a functional heat shock response is essential to protect flies from temperature-mediated fat body reduction in energy storage function.

In this study, we investigated the lipidomic correlated responses to selection for high temperature knockdown tolerance, desiccation survival, and chill coma recovery. The flies used for the selection experiment were derived from Melbourne, which is a location on the eastern coast of Australian latitudinal cline. In this study, insight into the lipid basis of stress trait evolution has been investigated in terms of lipid compositional changes.

3.2 Methods

Stress selection

All fly culture and stress selection experiment procedures described below are obtained from materials and methods described in Hangartner and Hoffmann 2016 (unpublished manuscript).

Flies for lipid extraction and fly culture conditions

The sample flies for lipid extraction of the stress selected lines were virgin females reared at 19°C as the selection was conducted on virgin flies at the same temperature. Flies were maintained in 12 : 12 hours light : dark cycles in 250ml bottles at 19°C. These flies were cultured on a standard *Drosophila* diet consisting of 7.3% yellow cornmeal, 3.5% dried yeast, 2% soy flour, 7.5% dextrose, 0.6% agar, 1.4% acid mix and 1.65% 4-methyl 4-hydroxybenzoate. The experimental flies were reared under controlled density-limited conditions by removing adult flies from bottles after 48 hours of oviposition.

There were five replicated lines for each stress selection condition and each of the two control lines. All selected and control lines (25 lines) were founded from *D. melanogaster* collected near Melbourne in May 2012. Offspring of 60 field-collected

females were pooled and mass-bred for two generations in the laboratory prior to desiccation and elevated heat tolerance selection, and six generations for chill-coma recovery selection and control lines. For mass breeding prior to selection, 5000 virgin flies of each sex were separated by sex under light CO_2 anesthesia and held at separate vials per sex at a density of 25 flies per vial.

Desiccations and elevated heat selection

Desiccation selection experiments were done separately for both sexes as virgins at 4-8 days post eclosion. Flies were desiccated in groups of 25 in glass vials topped with gauze in sealed glass tanks containing silica desiccant (Relative humidity < 10%) at 25°C until the approximate time to 90% mortality (LT90). The survivors were randomly allocated into five replicate lines comprised of 100-110 flies of each sex (200-210 flies total per replicate). For each of the following selected generations, 1000 females and 1000 males per line were stressed and the 10% most desiccant resistant adults (100-110 each sex, 200-210 in total) were kept as parents for the next generation.

Heat selection experiments were done separately for both sexes as virgins at 4-8 days post eclosion. Glass bottles (100ml) containing 100 flies were immersed in a circulating water bath at 39°C using a Ratek SP599 thermoregulator with an REXP24 controller (Ratek, Boronia, Vic, Australia). When 90% of the flies were knocked-down (did not move anymore when flashed with a torch), bottles were removed from the tank and the flies that were able to stand were randomly allocated into five replicate lines with 90-110 flies of each sex (190-210 in total) per replicate. For each of the following selected generations, 1000 females and 1000 males per line were stressed and the 10% most desiccant resistant adults (100-110 each sex, 200-210 in total) were kept as parents for

the next generation. Virgin flies were selected for every generation for seven generations, starting generation eight and thereafter, flies were selected every second generation and non-virgin flies were used. The controls were established in the same manner as the selected lines but not exposed to any treatment, and an equivalent number of flies were randomly allocated into six replicate lines at the end of the desiccation stress. Mortality was very low in the control and heat lines and all control and selected lines produced a similar number of offspring.

Chill-coma recovery selection

Chill-coma recovery selection lines were set up by placing groups of 100 virgin females or males in 250ml glass bottles. Flies were selected in the parental generation, then F1s were unselected, and F2s became the next selected parental population. For each selected generation 1000 flies (500 males and 500 females) were placed on ice for 4 hours and then returned room temperature for recovery. For each selected line the top 10% flies that recovered fastest from chill coma were kept as parents of the next generation, 100 males, and 100 females being placed in each of five bottles. Oviposition 3–5 days after selection was used for the next generation, such that selected females were likely to have re-mated with selected males. Every other generation was not selected because carry-over effects inhibit the selection response for cold stress (Watson and Hoffmann 1996). Instead for each of five bottles for each selected line, 25 males and 25 females were used as the parents of the next generation.

Cold control lines were not exposed to any treatment, but equivalent numbers of flies were randomly allocated into five replicate lines of 100 flies of each sex (200 in total) to

establish each replica lines. These control lines were maintained at equivalent population size as cold-selected lines.

Fly collection for lipid analysis

Virgin females were collected from density controlled vials (75 eggs per vials) and 20-50 virgin females were collected and under light CO_2 anesthesia. Flies were frozen 4 days post collection. Females were knocked down using CO_2, put on ice for about 10 minutes and then frozen at -80°C. Heat (H1-H5) and desiccation (D1-D5) selected lines and control lines (Control 1-5) were collected after 2 generations of relaxation after selection generation 19. Chill-coma recovery (Cold 1-5) selected lines and cold control (Cold Control 1-5) lines were collected after 2 generations of relaxation following selection generation 16.

Lipid extraction and analysis

Flies collected were transferred to University of Nebraska – Lincoln and were subject to a standard lipid extraction protocol (Bligh and Dyer. 1959). Each extraction was from 5 virgin female flies. Each tube with an aqueous (HPLC grade water) chloroform (BHT added to prevent lipid oxidation) – methanol solution and 5 flies, was inverted gently five times and left at room temperature (approximately 24°C) for 1 hour. The tubes were then held overnight at -20° C. The extraction tubes were removed from -20°C and placed at RT for one hour. An internal standard (15:0 TAG) was added to each vial based on the weight of 5 comparable flies and the approximate expected triglyceride content of the flies estimated; the internal standard was present at approximately 20-30% of the total triglyceride. Equal parts of chloroform and H_2O were added to the extraction solution. The samples were back-extracted three times and washed with a KCL solution. Once

extractions were made completed, the samples were dried under a gentle stream of nitrogen in 2 ml glass vials with Teflon caps. The dried samples were sent to Kansas State University for lipid analysis (described below).

The analysis of the lipid extraction was performed at the Kansas Lipidomics Research Center (KLRC). Drosophila samples with standards were introduced into a tandem mass spectrometer (Applied Biosystems API 4000) by continuous infusion insolvent into the electrospray ionization source. The ion fragments of the lipids were separated in an electric field and sequentially scanned to identify lipids by class with peaks within individual lipid classes corresponding to different lipid species. Quantification of each lipid species occurred by comparison to internal standards. The KLRC estimated the ratios of TAGs to polar lipids, DAGs to polar lipids and free fatty acids to polar lipids. For example, the normalized signal for total TAGs (nmol) was divided by the total nmol for polar lipids. This parameter was not an exact measurement of concentration, as the estimate of TAGs was not precise due to an inability to determine each of the three fatty acids present on each triglyceride molecule. The estimated ratios were not used for statistical analyses.

Statistical analysis

Statistical analyses were conducted using generalized linear mixed models (GLIMMX) in SAS 9.4. Multiple paired t-tests using GraphPad Prism V6.0 were to compare the concentration of free fatty acids between control lines and heat, desiccation, or chill-coma recovery selected lines. Two-way ANOVA was conducted using GraphPad Prism V6.0 to compare data expressed as per fly versus lean mass per fly.

3.3 Results

Relative abundance of lipids in classes: selected versus control lines

In this work, artificial laboratory selection conditions included desiccation, elevated heat tolerance, and chill-coma recovery was conducted. As shown in Tables 3.1A-D, abundance for different lipid classes of each set of selected lines (desiccation, elevated heat and chill-coma recovery) is compared to the appropriate control lines. The following description is based on statistically significant results with data expressed per fly.

Under desiccation selection, a significant decrease in PA and increases in lysoPE, lysoPC, and free fatty acids were shown (Table 3.1A). Heat selections indicated significance increases in total lipids, total neutral lipids, and DAG (Table 3.1B). Comparisons between desiccation and heat selected populations showed significant decreases of lysoPC and lysoPE in desiccation-selected lines (Table 3.1C). Importantly, for chill-coma recovery selected lines only statistically significant increases of PA and PI were shown (Table 3.1D).

Comparison of data expressed per fly versus lean mass per fly

Normalizations between calculated lipid abundances per single fly or per lean mass per fly were significantly different based on two-way ANOVA (p-value<0.001). Relative abundance for lipid classes per fly were shown in Table 3.1A-D, and abundances per lean mass per fly were shown in table 3.2A-D. Reporting statistically significance using the lean mass per fly data, significant decreases were observed in total polar lipids, PE, PC, PA, PI, and PS; and a significant increase in lysoPC when compared control to desiccation selected flies (Table 3.2A). For heat selected populations, significant

increases were observed in total lipids, total neutral lipids, and DAG (Table 3.2B). Additionally, when desiccation and heat selected lines are compared, significant increases in total lipids, total polar lipids, total neutral lipids, TAG, DAG, PC, PE, PI, and PS are present (Table 3.2C). Notably, in the comparison between cold control and chill-coma recovery selected flies, the only significant difference was an increase in PA (Table 3.2D).

Membrane lipid composition for stress-selected versus control populations

There was little evidence for changes in PE/PC in the selected versus control lines. A non-statistically significant increase of PE/PC ratio were observed for desiccation and chill-coma recovery selected populations (Fig 3.1 A and B). Abundances of both monounsaturated and polyunsaturated PE and PC exhibited no noticeable changes (Fig. 3.2A and Fig. 3.3A) when calculated per fly, but are marginally elevated in heat selected lines and slight demotion were shown in desiccation selected lines when normalized to per lean mass (Fig. 3.2B, and Fig. 3.3B). However, in the comparison of chill-coma selected and control lines on the basis of different degrees of saturation of PE and PC, significant decreases in both monounsaturated and polyunsaturated were observed in the selected lines whether normalized to per fly of per lean mass (Fig. 3.2C and D, Fig. 3.3C and D).

Trends of predominant lipid species in each lipid class

In the selection experiment, predominant species of lipids in different lipid classes sometimes changes in a similar manner in the same selected lines, but other times did not respond in concert. Generally, the following descriptions are based on results that are not

statistically significant, but are thought to be informative in terms of representing the variation in the data.

When examining phospholipids in *D. melanogaster* for the different selected and control populations, the dominated PE species are 34:2, 36:4 and 36:3 (where the first number indicates the number of carbon and the second number indicates the number of double bonds). No noticeable changes were shown when calculated per fly (Fig. 3.4A), but slight decreases of 0.3 nmol per lean mass per fly (Fig 3.4B) for these PE species were observed in desiccation selected population. No noticeable changes per fly were seen in heat selected line but slight elevations of 0.1 nmol per lean mass per fly were observed (Fig. 3.4A and B). For chill-coma recovery selected lines, there is a general decrease of 0.03 nmol per fly or 0.1nmol per lean mass per fly when compared to control lines (Fig. 3.4C and D). PC is another essential component of membrane structure, the most dominant species of PC are 32:2, 34:3, and 34:2 across all selected and control populations. A trend similar to PE species was shown in desiccation selected lines which slight decreases of 0.01 nmol per fly (Fig 3.5A) or 0.1 nmol per lean mass were shown (Fig. 3.5B) and no change was observed in either per fly or per lean mass for heat selected populations (Fig. 3.4A and B). There is a small decrease of 0.01 nmol per fly or 0.1nmol per lean mass per fly for chill-coma recovery selected lines when compared to control lines (Fig. 3.4C and D).

PA exhibits similar trends to PE or PC, where there was a decrease in abundance of the predominant species for desiccation selected populations, and no changes in abundance in heat selected populations when compared to control (Fig. 3.5A and B). PI showed a general decrease in desiccation selected and no change in heat selected for 34:2 and 36:3

for both per fly and per lean mass (Fig. 3.6A and B). However, PI (32:1) showed no change in desiccation selected and a decrease in heat selected when normalized to per fly (Fig. 3.6A). But when normalized to lean mass, the trend was similar to the other two species (Fig. 3.6B). Slight decreases in predominant species of PA and PI were shown in chill-coma selected lines in comparison to control lines (Fig. 3.5C and D, Fig. 3.6C and D).

PS species showed a similar trend to PE when normalized to per fly (Fig. 3.7A) but a different trend when normalized to lean mass, there are decreases in 36:3 and 36:2 but an elevation in desiccation lines for 34:2, and general increases for 34:2, 36:3, and 36:2 for heat selected lines (Fig. 3.7B). There is also a slight elevation for PS (36:2) and decreases of 34:2 and 36:3 for chill-coma recovery selected lines when compared to control lines (Fig 3.7C and D).

PG considered in the desiccation and heat selected lines, compared to control populations, showed slight increases in desiccation selected when normalized to per fly but slight decreases when normalized to per lean mass and a slight increase in heat selected for 32:1, 34:2, and 36:3 (Fig. 3.8A and B). Additionally, a general small increase for predominant species of PG is shown when normalized to per fly (Fig. 3.8C), and slight elevation in 34:2 and not much changed for 32:1 and 36:3 were observed when comparing chill-coma recovery selected lines to control lines normalized to per lean mass per fly (Fig. 3.8D).

Polyunsaturated polar lipids per fly did not exhibit a notable change in selected lines (Fig. 3.9 A), but when calculated to lean mass per fly indicated a slight decrease in desiccation selected populations while a slight increase was shown in heat selected populations (Fig.

3.9B). The opposite trend for polyunsaturated polar lipids was seen chill-coma recovery selected lines, where there is a decrease of polyunsaturated polar lipids in chill-coma recovery selected lines for both normalized to per fly or lean mass per fly when compared to control lines (Fig. 3.9C and D). Saturated and monounsaturated polar lipids maintained similar concentration across selected and control populations (Fig. 3.9A-D).

Neutral lipids such as DAG and TAG were also considered. DAG showed a general increase in desiccation selected lines and decrease in heat selected lines when calculated to per fly (Fig. 3.10A). A general decrease of predominant DAG species was shown for desiccation selected lines and increase in heat selected populations when calculated per lean mass (Fig 3.10B). General decreases for predominant DAG species were observed when compared chill-coma recovery selected lines to control lines for both normalized to per fly or lean mass per fly (Fig. 3.10C and D). Whether normalized to per fly or lean mass, small general decreases were observed for predominant TAG species in desiccation and chill-coma recovery selected lines compared to respective control lines (Fig. 3.11A-D), and there is an increase for predominant TAG species in heat selected lines (Fig. 3.11A and B).

Saturation level and chain length of lipids in selected and control populations

All of the differences between selected versus control populations described in this section are statistically significant. For desiccation selected lines, significant decreases of chain length can be observed in total lipids, total polar lipids, and total neutral lipids (Table 3.3A). Heat selected lines showed significant decreases in chain length in total lipids, total neutral lipids, TAG, and DAG (Table 3.3B). Comparing desiccation selected with heat selected groups, it is shown that significant decreases of chain length can be

seen in TAG, DAG, and PG, and significant increases in chain length in total neutral lipids, and PS (Table 3.3C). Additionally, a significant decrease of chain length was shown in DAG and increase in free fatty acids when compared cold control to chill-coma recovery selected lines (Table 3.3D).

Degrees of saturation showed a significant increase in desiccation selected free fatty acids when compared to control (Table 3.4A), while no significant changes were shown in heat selected lines (Table 3.4B). When comparing desiccation selected to heat selected lines, significant decreases of unsaturation were observed in total neutral lipids, free fatty acids, TAG, and DAG, while a significant increase was only exhibited in lysoPC (Table 3.4C). For cold control compared to chill-coma recovery selected, there were no significant changes for degrees of unsaturation (Table 3.4D).

3.4 Discussion

The selection experiments for virgin female and male knock-down tolerance to incrementally increased heat, quicker chill-coma recovery time and survival under desiccation resistance have been analyzed in terms of virgin female responses to selection. Flies extracted for lipidome analysis were reared at 19°C which is the rearing temperature used for the selection experiment. These are the mating status and temperatures used for selection. The temperature corresponds to winter conditions for D. melanogaster populations from eastern Australia (Hoffmann et al 2005).

PE and PC phospholipid ratio and structure (saturation level, chain length) is considered as a possible homeoviscous adaptation response to selection. Compared to the appropriate set of control lines there was no statistically significant response to any form of selection

in terms of change in the ratio of PE/PC (Fig. 3.1A and B). Thus, there was no support for homeoviscous adaptation in this important measure of membrane fluidity as a genetic response to stress selection that is manifest in this study. Cooper et al. (2014) did document an effect on PE/PC of laboratory selection based on alternating rearing temperature (16°C and 26°C) across generations. In our study, there were no statistically significant changes in PE or PC as a response to selection for any of the stress conditions. There was a statistically significant decrease in mono- and polyunsaturated PE and PC in the chill-coma recovery lines (Fig. 3.2C and 3.3C). This change in saturation is unexpected as a response to low-temperature selection because it would decrease membrane fluidity.

TAG changes are a general consideration in terms of responses to laboratory stress selection (Introduction). There was a statistically significant decrease in TAG in the desiccation-selected lines in the present study. Gibbs et al. (1997) and Chippendale et al. (1998) found decreased lipid stores in the one set of desiccation-selected lines. In another selection experiment for starvation survival (Telonis et al. 2006), there was an increase in ether-extractable lipid in desiccation-selected lines. It is not clear that total lipids are equivalent to TAG stores. A potentially relevant study is selection for different stress survival traits using *D. melanogaster* (Bubliy and Loeschcke 2005). In this case, selection for desiccation resistance, severe cold, and two elevated temperature stress resulted in increased starvation survival as a correlated response to selection. A generality gleaned from many studies is that starvation survival is often correlated with increased TAG storage. In the context of the literature on laboratory selection for increased desiccation

survival, our observation of decreased TAG matches the results in some studies, but not others.

A strongly statistically significant response to selection was an increase in DAG in elevated heat selected lines (Fig. 3.10A, Table 3B). Our interpretation of this observation is motivated in terms of Klepsatel et al. (2016) in which elevation of thermal stress can damage lipid storage capacity in *D. melanogaster*. The basis of this impact of elevated temperature is prospectively based on apoptosis of fat body cells. The damage by high temperature can be irreversible. As shown by heat shock gene knockdowns, a functional heat shock system can reduce high temperature damage to fat body cell. DAG is a precursor for the synthesis of relatively abundant TAGs in Drosophila adult bodies. When the storage capacity of TAG is diminished then a buildup in the precursor DAG is a possible outcome. The genetically selected lines in the present study may have evolved to parallel the phenotypic response to elevated thermal stress by somehow limiting TAG storage and promoting DAG buildup. It is interesting to note that there is a trend for the polar lipid class PS to increase in the elevated heat selected lines in our study (Table 3.2A-D) as PS signaling is known to mediate apoptosis.

Lyso-precursor forms of the major membrane phospholipids tended to increase in abundance in response to selection. A statistically significant increase in lysoPC and lysoPE was observed in the desiccation selected lines. Both lipid precursors appeared to increase in the chill-coma recovery and elevated heat knockdown lines, but these trends were not statistically significant. This increase in lyso-precursor forms was not correlated with any statistically significant changes in PC or PE as a result of selection.

One of the statistically significant changes in the free fatty acids in the present study was shown in chain length increase in the chill-coma selected lines (Table 3.3D). Free fatty acids are converted into a broad range of lipids including neutral lipids (storage), phospholipids (membranes and signaling), cuticular hydrocarbons etc. Longer chain phospholipids contribute to membrane rigidity which would not be expected to be characteristic of phospholipids selected for recovery after lengthy exposure to cold producing a coma state. It is not clear why chain length increased in free fatty acids selected for chill coma recovery.

The other significant change of free fatty acids in the selection experiment was that the abundance of free fatty acids increased in desiccation-selected lines (Table 3.3A). Free fatty acids are important for the production of cuticular hydrocarbons which can play an important role in controlling water loss through the cuticle. There was no increase in surface hydrocarbons in an earlier selection experiment for desiccation resistance (Gibbs et al. 1997). Gibbs et al. (1997) found that respiratory water loss was reduced in long-term desiccation selected lines. In Gibbs et al. (1997), there was no difference in surface lipids in the selected versus control lines, but cuticular hydrocarbons were longer and had a higher melting temperature. In future studies, it will be of interest to investigate free fatty acids in relationship to cuticular hydrocarbons in the context of desiccation resistance as it is selected in the laboratory and evolves in natural populations.

Selection for desiccation survival produced the greatest change in lipid profile characteristics. This includes significant increases in free fatty acids, lysoPE, and lysoPC, as well as a significant decrease in PA (Table 3.2A). This may be due to desiccation selection studies have a stronger selective pressure as experimental conditions selected

for survival. High mortality selection can contribute to a stronger adaptive response, as seen in (Stearns et al. 2000) when placing *D. melanogaster* under high and low mortality conditions, the population with high mortality had a stronger response for early maturation and shifting of the peak in reproduction age. Thus, it can be concluded that the high mortality rate in desiccation selection caused a stronger adaptive response than the low mortality heat tolerance and chill-coma recovery selection.

Selection for chill-coma survival produced the weakest response to selection. The only consistent statistically significant response was an increase in PA, but there was an indication of an increase in PI when data was expressed per fly (Table 3.1D). Selection experiments for cold and desiccation tolerance found that there were little correlations between the two (Sinclair et al. 2007). Another study that examined gene expression levels for several stress associated genes including *frost*, smp-30 and genes code for heat shock proteins showed that chill-coma recovery is quite different from desiccation stress (Sinclair et al. 2007). The selection response for chill-coma recovery was not correlated with other measures of stress resistance such as starvation or heat tolerance (Gerken et al. 2016). In general, these results indicate that chill-coma recovery is not positively correlated with other measures of stress resistance or tolerance. Another consideration is the mechanism underlying induction of chill-coma in insects. A review of the mechanisms that contribute to the chill-coma state (MacMillan and Sinclair 2011) is informative and can be compared to our selection results. Two general classes of mechanisms are based on nerve transmission parameters, specifically decreased resting membrane potential and disrupted action potential. However, another class of mechanisms is a loss of muscle and nerve excitability mediated by decreased membrane

fluidity and interference with synaptic exocytosis (MacMillan and Sinclair 2011). Overall, mechanisms of chill-coma induction need not be based on membrane fluidity. Our investigation of chill-coma recovery is based on a somewhat different trait than chill coma per se, but the mechanisms underlying chill coma could be relevant to our study. Our observation of a weak lipid profile selection response for chill-coma recovery selection implies that membrane fluidity is not strongly associated with the artificial selection mediated evolution of chill-coma recovery in the laboratory.

3.5 Literature cited

Anderson, A. R., A. A. Hoffmann, and S. W. McKechnie. 2005. 'Response to selection for rapid chill-coma recovery in Drosophila melanogaster: physiology and life-history traits', *Genet Res*, 85: 15-22.

Bligh, E. G., and W. J. Dyer. 1959. 'A rapid method of total lipid extraction and purification', *Can J Biochem Physiol*, 37: 911-7.

Bubliy, O. A., and V. Loeschcke. 2005. 'Correlated responses to selection for stress resistance and longevity in a laboratory population of Drosophila melanogaster', *J Evol Biol*, 18: 789-803.

Chippindale, A. K., A. G. Gibbs, M. Sheik, K. J. Yee, M. Djawdan, T. J. Bradley, and M. R. Rose. 1998. 'Resource acquisition and the evolution of stress resistance in Drosophila melanogaster', *Evolution*, 52: 1342-52.

Cooper, B. S., L. A. Hammad, N. P. Fisher, J. A. Karty, and K. L. Montooth. 2012. 'In a variable thermal environment selection favors greater plasticity of cell membranes in Drosophila melanogaster', *Evolution*, 66: 1976-84.

Djawdan, M., A. K. Chippindale, M. R. Rose, and T. J. Bradley. 1998. 'Metabolic reserves and evolved stress resistance in Drosophila melanogaster', *Physiol Zool*, 71: 584-94.

Folk, D. G., P. Zwollo, D. M. Rand, and G. W. Gilchrist. 2006. 'Selection on knockdown performance in Drosophila melanogaster impacts thermotolerance and heat-shock response differently in females and males', *J Exp Biol*, 209: 3964-73.

Gasser, M., M. Kaiser, D. Berrigan, and S. C. Stearns. 2000. 'Life-history correlates of evolution under high and low adult mortality', *Evolution*, 54: 1260-72.

Gerken, A. R., T. F. Mackay, and T. J. Morgan. 2016. 'Artificial selection on chill-coma recovery time in Drosophila melanogaster: Direct and correlated responses to selection', *J Therm Biol*, 59: 77-85.

Gibbs, A. G., A. K. Chippindale, and M. R. Rose. 1997. 'Physiological mechanisms of evolved desiccation resistance in Drosophila melanogaster', *J Exp Biol*, 200: 1821-32.

Hardy, C. M., R. T. Birse, M. J. Wolf, L. Yu, R. Bodmer, and A. G. Gibbs. 2015. 'Obesity-associated cardiac dysfunction in starvation-selected Drosophila melanogaster', *Am J Physiol Regul Integr Comp Physiol*, 309: R658-67.

Harshman, L. G., A. A. Hoffmann, and A. G. Clark. 1999. 'Selection for starvation resistance in Drosophila melanogaster: physiological correlates, enzyme activities and multiple stress responses', *Journal of Evolutionary Biology*, 12: 370-79.

Harshman, L. G., K. M. Moore, M. A. Sty, and M. M. Magwire. 1999. 'Stress resistance and longevity in selected lines of Drosophila melanogaster', *Neurobiology of Aging*, 20: 521-29.

Harshman, L. G., and J. L. Schmid. 1998. 'Evolution of starvation resistance in Drosophila melanogaster: Aspects of metabolism and counter-impact selection', *Evolution*, 52: 1679-85.

Hoffmann, A. A., R. Hallas, A. R. Anderson, and M. Telonis-Scott. 2005. 'Evidence for a robust sex-specific trade-off between cold resistance and starvation resistance in Drosophila melanogaster', *J Evol Biol*, 18: 804-10.

Hoffmann, A. A., and P. A. Parsons. 1989. 'Selection for increased desiccation resistance in Drosophila melanogaster: additive genetic control and correlated responses for other stresses', *Genetics*, 122: 837-45.

Klepsatel, P., M. Galikova, Y. Xu, and R. P. Kuhnlein. 2016. 'Thermal stress depletes energy reserves in Drosophila', *Sci Rep*, 6: 33667.

Logan-Garbisch T, Bortolazzo A, Luu P, Ford, A., Do, D., Khodabakhshi, P., and French, R. L. 2015. ' Developmental Ethanol Exposure Leads to Dysregulation of Lipid Metabolism and Oxidative Stress in Drosophila. ', *G3: Genes|Genomes|Genetics*, 5: 49-59.

Malmendal, A., J. G. Sorensen, J. Overgaard, M. Holmstrup, N. C. Nielsen, and V. Loeschcke. 2013. 'Metabolomic analysis of the selection response of Drosophila melanogaster to environmental stress: are there links to gene expression and phenotypic traits?', *Naturwissenschaften*, 100: 417-27.

Montooth, K. L., K. T. Siebenthall, and A. G. Clark. 2006. 'Membrane lipid physiology and toxin catabolism underlie ethanol and acetic acid tolerance in Drosophila melanogaster', *Journal of Experimental Biology*, 209: 3837-50.

Pappas, C., D. Hyde, K. Bowler, V. Loeschcke, and J. G. Sorensen. 2007. 'Post-eclosion decline in 'knock-down' thermal resistance and reduced effect of heat hardening in Drosophila melanogaster', *Comp Biochem Physiol A Mol Integr Physiol*, 146: 355-9.

Rion, S., and T. J. Kawecki. 2007. 'Evolutionary biology of starvation resistance: what we have learned from Drosophila', *J Evol Biol*, 20: 1655-64.

Rose, M.R. 1984. 'Laboratory evolution of postponed senescence in Drosophila melanogaster.', *Evolution*, 38 1004-10.

Scheitz, C. J. F., Y. Guo, A. M. Early, L. G. Harshman, and A. G. Clark. 2013. 'Heritability and Inter-Population Differences in Lipid Profiles of Drosophila melanogaster', *PLoS One*, 8.

Service, P. M., E. W. Hutchinson, M. D. Mackinley, and M. R. Rose. 1985. 'Resistance to Environmental-Stress in Drosophila-Melanogaster Selected for Postponed Senescence', *Physiological Zoology*, 58: 380-89.

Telonis-Scott, M., K. M. Guthridge, and A. A. Hoffmann. 2006. 'A new set of laboratory-selected Drosophila melanogaster lines for the analysis of desiccation resistance: response to selection, physiology and correlated responses', *J Exp Biol*, 209: 1837-47.

Watson, M. J. O., and A. A. Hoffmann. 1996. 'Acclimation, cross-generation effects, and the response to selection for increased cold resistance in Drosophila', *Evolution*, 50: 1182-92.

Zwann, B., R. Bijlsma and R.F. Hoekstra. 1995. '1995. Direct selection on life span in Drosophila melanogaster.', *Evolution*, 49: 649-59.

3.6 Tables

Table 3.1A. Relative abundance of different lipid classes per fly for control compared to desiccation selected lines. A positive slope indicates an increase of abundances and a negative slope indicates a decrease in abundance. Std error indicates standard errors with n=5.P-values less than 0.05 were notated with *, less than 0.01 with ** and less than 0.001 with ***.

Comparison	Lipid class	Slope	Std error	P-values
Control to Desiccation	Total lipids	7.7763	4.9297	0.1407
	Total polar lipids	0.5761	1.5001	0.7077
	Total neutral lipids	7.2001	3.8762	0.0879
	Free Fatty Acids	16.9253	5.8975	0.0141*
	TAG	-0.9114	1.9530	0.6491
	DAG	8.1115	2.3850	0.0053**
	PE	0.1097	0.9681	0.9117
	PC	-0.06940	0.2660	0.7986
	PA	-0.02690	0.008430	0.0078**
	PI	-0.1973	0.1598	0.2404
	PS	0.01215	0.06172	0.8472
	PG	0.04235	0.02616	0.1314
	lysoPE	0.3272	0.1111	0.0123*
	lysoPC	0.3784	0.08491	0.0008***

Table 3.1B. Relative abundance of different lipid classes per fly for control compared to heat selected lines. A positive slope indicates an increase of abundances and a negative slope indicates a decrease in abundance. Std error indicates standard errors with n=5. P-values less than 0.05 were notated with *, and less than 0.001 with ***.

Comparison	Lipid class	Slope	Std error	P-values
Control to Heat	Total lipids	12.6549	4.9297	0.0247*
	Total polar lipids	1.5603	1.5001	0.3188
	Total neutral lipids	11.0945	3.8762	0.0143*
	Free Fatty Acids	7.3525	5.8975	0.2363
	TAG	1.3873	1.9530	0.4911
	DAG	9.7072	2.3850	0.0016**
	PE	0.8574	0.9681	0.3932
	PC	0.3650	0.2660	0.1951
	PA	-0.01440	0.008430	0.1133
	PI	-0.02815	0.1598	0.8631
	PS	0.1150	0.06172	0.0869
	PG	0.02645	0.02616	0.3319
	lysoPE	0.1170	0.1111	0.3130
	lysoPC	0.1215	0.08491	0.1780

Table 3.1C. Relative abundance of different lipid classes per fly for desiccation selected lines compared to heat selected lines. A positive slope indicates an increase of abundances and a negative slope indicates a decrease in abundance. Std error indicates standard errors with n=5. P-values less than 0.05 were notated with *, and less than 0.01 with **.

Comparison	Lipid class	Slope	Std error	P-values
Desiccation to Heat	Total lipids	4.8786	4.9297	0.3419
	Total polar lipids	0.9842	1.5001	0.5242
	Total neutral lipids	3.8944	3.8762	0.3349
	Free Fatty Acids	-9.5727	5.8975	0.1305
	TAG	2.2987	1.9530	0.2620
	DAG	1.5957	2.3850	0.5161
	PE	0.7478	0.9681	0.4548
	PC	0.4344	0.2660	0.1284
	PA	0.01250	0.008430	0.1639
	PI	0.1692	0.1598	0.3104
	PS	0.1029	0.06172	0.1213
	PG	-0.01590	0.02616	0.5546
	lysoPE	-0.2101	0.1111	0.0831
	lysoPC	-0.2569	0.08491	0.0106*

Table 3.1D. Relative abundance of different lipid classes per fly for cold control compared to chill-com recovery selected lines. A positive slope indicates an increase of abundances and a negative slope indicates a decrease in abundance. Std error indicates standard errors with n=5.P-values less than 0.05 were notated with *.

Comparison	Lipid class	Slope	Std error	P-values
Chill-coma recovery to Cold Control	Total lipids	4.0722	2.8825	0.1954
	Total polar lipids	1.5129	0.8878	0.1268
	Total neutral lipids	2.5608	2.6670	0.3651
	Free Fatty Acids	5.8309	7.2127	0.4422
	TAG	1.3494	1.9482	0.5081
	DAG	1.2702	1.6833	0.4721
	PE	0.6369	0.6016	0.3207
	PC	0.2883	0.2014	0.1903
	PA	0.03883	0.01152	0.0098**
	PI	0.3623	0.1019	0.0075**
	PS	0.01175	0.04394	0.7959
	PG	-0.03121	0.01698	0.1033
	lysoPE	0.08340	0.07259	0.2838
	lysoPC	0.1210	0.06699	0.1086

Table 3.2A. Relative abundance of different lipid classes per mg of lean mass for control compared to desiccation selected lines. A positive slope indicates an increase of abundances and a negative slope indicates a decrease in abundance. Std error indicates standard errors with n=5. P-values less than 0.05 were notated with *, less than 0.01 with ** and less than 0.001 with ***.

Comparison	Lipid class	Slope	Std error	P-values
Control to Desiccation	Total lipids	-21.7820	17.5799	0.2390
	Total polar lipids	-13.3740	5.5281	0.0324*
	Total neutral lipids	-8.4085	12.2468	0.5054
	Free Fatty Acids	-11.9337	21.6299	0.5913
	TAG	-14.0802	5.8147	0.0322*
	DAG	5.6715	7.2539	0.4494
	PE	-8.7000	3.4258	0.0260*
	PC	-3.1333	1.1931	0.0221*
	PA	-0.1545	0.03429	0.0007***
	PI	-1.9237	0.5167	0.0029**
	PS	-0.5091	0.1945	0.0225*
	PG	-0.02987	0.09055	0.7472
	lysoPE	0.3580	0.3736	0.3568
	lysoPC	0.7183	0.2663	0.0194*

Table 3.2B. Relative abundance of different lipid classes per mg of lean mass for control compared to heat selected lines. A positive slope indicates an increase of abundances and

a negative slope indicates a decrease in abundance. Std error indicates standard errors with n=5. P-values less than 0.05 were notated with *, less than 0.01 with ** and less than 0.001 with ***.

Comparison	Lipid class	Slope	Std error	P-values
Control to Heat	Total lipids	43.7297	17.8097	0.0303*
	Total polar lipids	4.8007	5.6003	0.4081
	Total neutral lipids	37.7471	12.4069	0.0102*
	Free Fatty Acids	22.9527	21.7201	0.3114
	TAG	4.9204	5.8897	0.4198
	DAG	32.8237	7.3413	0.0008***
	PE	2.8377	3.4706	0.4295
	PC	1.1857	1.2087	0.3460
	PA	-0.05506	0.03474	0.1390
	PI	-0.2165	0.5234	0.6865
	PS	0.3152	0.1969	0.1353
	PG	0.05942	0.09170	0.5292
	lysoPE	0.3481	0.3755	0.3722
	lysoPC	0.3505	0.2688	0.2167

Table 3.2C. Relative abundance of different lipid classes per mg of lean mass for desiccation selected lines compared to heat selected lines. A positive slope indicates an increase of abundances and a negative slope indicates a decrease in abundance. Std error

indicates standard errors with n=5.P-values less than 0.05 were notated with *, less than 0.01 with ** and less than 0.001 with ***.

Comparison	Lipid class	Slope	Std error	P-values
Desiccation to Heat	Total lipids	65.5117	17.8097	0.0032**
	Total polar lipids	18.1747	5.6003	0.0070**
	Total neutral lipids	46.1557	12.4069	0.0029**
	Free Fatty Acids	34.8864	21.7201	0.1342
	TAG	19.0006	5.8897	0.0073**
	DAG	27.1522	7.3413	0.0030**
	PE	11.5377	3.4706	0.0061**
	PC	4.3190	1.2087	0.0038**
	PA	0.09940	0.03474	0.0143
	PI	1.7073	0.5234	0.0068**
	PS	0.8243	0.1969	0.0013**
	PG	0.08929	0.09170	0.3494
	lysoPE	-0.009910	0.3755	0.9794
	ysoPC	-0.3678	0.2688	0.1963

Table 3.2D. Relative abundance of different lipid classes per mg of lean mass for cold control compared to chill-coma recovery selected lines. A positive slope indicates an

increase of abundances and a negative slope indicates a decrease in abundance. Std error indicates standard errors with n=5. P-values less than 0.05 were notated with *, less than 0.01 with ** and less than 0.001 with ***.

Comparison	Lipid class	Slope	Std error	P-values
Chill-coma recovery to Cold Control	Total lipids	13.1346	14.9998	0.4067
	Total polar lipids	4.2041	5.4335	0.4613
	Total neutral lipids	8.9324	10.9614	0.4387
	Free Fatty Acids	16.3575	21.4931	0.4685
	TAG	4.8470	6.8668	0.5003
	DAG	4.2740	7.3511	0.5770
	PE	1.8369	3.4418	0.6081
	PC	0.7653	0.9121	0.4258
	PA	0.1073	0.03542	0.0163*
	PI	1.0939	0.5297	0.0728
	PS	-0.02301	0.1945	0.9087
	PG	-0.1076	0.07548	0.1917
	lysoPE	0.2046	0.2692	0.4692
	lysoPC	0.3313	0.3171	0.3267

Table 3.3A. Trends for chain length (number of carbons) in different lipid classes for control compared to desiccation selected lines. A positive slope indicates an increase of chain length and a negative slope indicates a decrease of chain length. Std error indicates

standard errors with n=5. P-values less than 0.05 were notated with *, less than 0.01 with ** and less than 0.001 with ***.

Comparison	Lipid class	Slope	Std error	P-values
Control to Desiccation	Total lipids	-0.8647	0.2686	0.0074**
	Total polar lipids	-0.5477	0.1475	0.0030**
	Total neutral lipids	-1.3089	0.3058	0.0011**
	Free Fatty Acids	-0.04975	0.01986	0.0277*
	TAG	0.05738	0.07769	0.4744
	DAG	0.04278	0.05335	0.4383
	PE	0.02173	0.03163	0.5051
	PC	-0.004045	0.03211	0.9018
	PA	-0.02830	0.1674	0.8686
	PI	0.03214	0.09105	0.7303
	PS	-0.1605	0.09041	0.1012
	PG	0.1997	0.2034	0.3456
	lysoPE	-0.01202	0.01912	0.5414
	lysoPC	-0.06382	0.03590	0.1007

Table 3.3B. Trends for chain length (number of carbons) in different lipids classes for control compared to heat selected lines. A positive slope indicates an increase of

abundances and vice versa. Std error indicates standard errors with n=5. P-values less than 0.05 were notated with *, less than 0.01 with ** and less than 0.001 with ***.

Comparison	Lipid class	Slope	Std error	P-values
Control to Heat	Total lipids	-0.6251	0.2686	0.0383*
	Total polar lipids	-0.1143	0.1475	0.4534
	Total neutral lipids	-1.2157	0.3058	0.0018**
	Free Fatty Acids	-0.02440	0.01986	0.2429
	TAG	-0.2149	0.07769	0.0171*
	DAG	-0.1361	0.05335	0.0254*
	PE	0.02032	0.03163	0.5325
	PC	0.04176	0.03211	0.2178
	PA	0.2527	0.1674	0.1571
	PI	-0.07385	0.09105	0.4331
	PS	0.04988	0.09041	0.5912
	PG	-0.3076	0.2034	0.1564
	lysoPE	0.00004	0.01912	0.9984
	lysoPC	-0.02233	0.03590	0.5456

Table 3.3C. Trends for chain length (number of carbons) in different lipid classes for desiccation selected lines compared to heat selected lines. A positive slope indicates an

increase of chain length and a negative slope indicates a decrease of chain length. Std error indicates standard errors with n=5. P-values less than 0.05 were notated with *, and less than 0.01 with **.

Comparison	Lipid class	Slope	Std error	P-values
Desiccation to Heat	Total lipids	0.2396	0.2686	0.3901
	Total polar lipids	0.4334	0.1475	0.0124*
	Total neutral lipids	0.09319	0.3058	0.7657
	Free Fatty Acids	0.02536	0.01986	0.2258
	TAG	-0.2723	0.07769	0.0043***
	DAG	-0.1789	0.05335	0.0057**
	PE	-0.001405	0.03163	0.9653
	PC	0.04580	0.03211	0.1792
	PA	0.2810	0.1674	0.1191
	PI	-0.1060	0.09105	0.2670
	PS	0.2104	0.09041	0.0383*
	PG	-0.5073	0.2034	0.0282*
	lysoPE	0.01206	0.01912	0.5401
	lysoPC	0.04149	0.03590	0.2702

Table 3.3D. Trends for chain length (number of carbons) in different lipid classes for cold control compared to chill-coma recovery selected lines. A positive slope indicates an

increase of chain length and a negative slope indicates a decrease of chain length. Std error indicates standard errors with n=5. P-values less than 0.05 were notated with *.

Comparison	Lipid class	Slope	Std error	P-values
Chill-coma recovery to Cold Control	Total lipids	-0.1329	0.3042	0.6738
	Total polar lipids	-0.05636	0.06784	0.4302
	Total neutral lipids	-0.1318	0.4233	0.7635
	Free Fatty Acids	0.04960	0.02012	0.0390*
	TAG	-0.2594	0.1956	0.2214
	DAG	-0.1957	0.07677	0.0342*
	PE	0.02197	0.02679	0.4359
	PC	-0.04229	0.04238	0.3476
	PA	-0.08333	0.1225	0.5154
	PI	0.06785	0.09676	0.5031
	PS	-0.07506	0.07550	0.3492
	PG	-0.04579	0.2128	0.8350
	lysoPE	-0.002790	0.01400	0.8470
	lysoPC	-0.009350	0.02236	0.6868

Table 3.4A. Trends for degrees of unsaturation (numbers of the double bond) in different lipid classes for control compared to desiccation selected lines. A positive slope indicates an increase of degrees of unsaturation and a negative slope indicates a decrease of

degrees of unsaturation. Std error indicates standard errors with n=5. P-values less than 0.05 were notated with *, less than 0.01 with ** and less than 0.001 with ***.

Comparison	Lipid class	Slope	Std error	P-values
Control to Desiccation	Total lipids	-0.02222	0.01663	0.2061
	Total polar lipids	-0.01858	0.02527	0.4761
	Total neutral lipids	0.00928	0.01117	0.4222
	Free Fatty Acids	0.07832	0.01823	0.0010***
	TAG	0.01909	0.01543	0.2396
	DAG	0.02412	0.01126	0.0534
	PE	0.02834	0.02479	0.2752
	PC	-0.005975	0.02121	0.7829
	PA	0.02607	0.04556	0.5777
	PI	0.05001	0.04277	0.2650
	PS	-0.02465	0.07014	0.7313
	PG	-0.01098	0.07162	0.8807
	lysoPE	-0.004514	0.01881	0.8144
	lysoPC	-0.01793	0.01390	0.2214

Table 3.4B. Trends for degrees of unsaturation (numbers of the double bond) in different lipid classes for control compared to heat selected lines. A positive slope indicates an

increase of the degree of unsaturation and a negative slope indicates a decrease of degrees of unsaturation. Std error indicates standard errors with n=5.

Comparison	Lipid class	Slope	Std error	P-values
Control to Heat	Total lipids	-0.03597	0.01663	0.0514
	Total polar lipids	0.01557	0.02527	0.5493
	Total neutral lipids	-0.02103	0.01117	0.0842
	Free Fatty Acids	0.00729	0.01823	0.6964
	TAG	-0.02399	0.01543	0.1459
	DAG	-0.003647	0.01126	0.7517
	PE	0.02499	0.02479	0.3332
	PC	0.01623	0.02121	0.4589
	PA	0.07393	0.04556	0.1306
	PI	0.01662	0.04277	0.7044
	PS	0.05565	0.07014	0.4429
	PG	-0.08184	0.07162	0.2755
	lysoPE	0.00545	0.01881	0.7770
	lysoPC	0.00779	0.01390	0.5856

Table 3.4C. Trends for degrees of unsaturation (numbers of the double bond) in different lipid classes for desiccation selected lines compared to heat selected lines. A positive

slope indicates an increase of degrees of unsaturation and a negative slope indicates a decrease of unsaturation. Std error indicates standard errors with n=5. P-values less than 0.05 were notated with *, and less than 0.01 with **.

Comparison	Lipid class	Slope	Std error	P-values
Desiccation to Heat	Total lipids	-0.01375	0.01663	0.4245
	Total polar lipids	0.03415	0.02527	0.2014
	Total neutral lipids	-0.03031	0.01117	0.0188*
	Free Fatty Acids	-0.07103	0.01823	0.0021**
	TAG	-0.04307	0.01543	0.0163*
	DAG	-0.02777	0.01126	0.0297*
	PE	-0.003347	0.02479	0.8948
	PC	0.02220	0.02121	0.3157
	PA	0.04785	0.04556	0.3142
	PI	-0.03339	0.04277	0.4501
	PS	0.08031	0.07014	0.2745
	PG	-0.07086	0.07162	0.3420
	lysoPE	0.00996	0.01881	0.6060
	lysoPC	0.02572	0.01390	0.0890

Table 3.4D. Trends for degrees of unsaturation (numbers of the double bond) in different lipid classes for cold control compared to chill-coma recovery selected lines. A positive

slope indicates an increase of degrees of unsaturation and a negative slope indicates a decrease of unsaturation. Std error indicates standard errors with n=5.

Comparison	Lipid class	Slope	Std error	P-values
Chill-coma recovery to Cold Control	Total lipids	-0.006395	0.02564	0.8093
	Total polar lipids	0.00005	0.02452	0.9983
	Total neutral lipids	-0.01690	0.01926	0.4057
	Free Fatty Acids	-0.003510	0.02707	0.9000
	TAG	-0.02004	0.02968	0.5187
	DAG	-0.01504	0.01596	0.3735
	PE	0.01446	0.02257	0.5396
	PC	-0.02010	0.02563	0.4554
	PA	0.06810	0.03241	0.0688
	PI	0.04433	0.04646	0.3680
	PS	-0.08514	0.06251	0.2103
	PG	-0.03050	0.06703	0.6612
	lysoPE	-0.002284	0.008532	0.7957
	lysoPC	0.00203	0.01074	0.8545

3.7 Figures

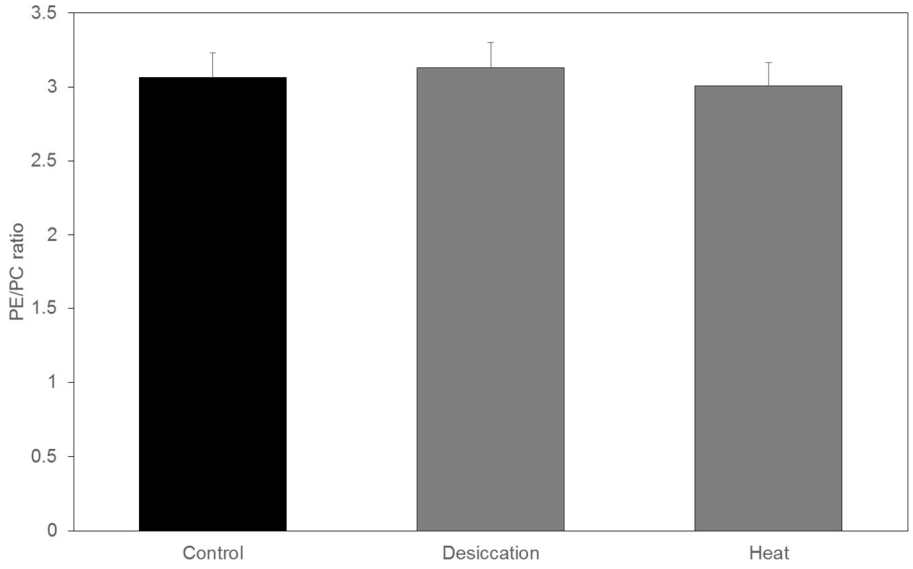

Fig. 3.1A. PE/PC ratio for control, desiccation and heat selected populations, error bars represent the SE (n = 5).

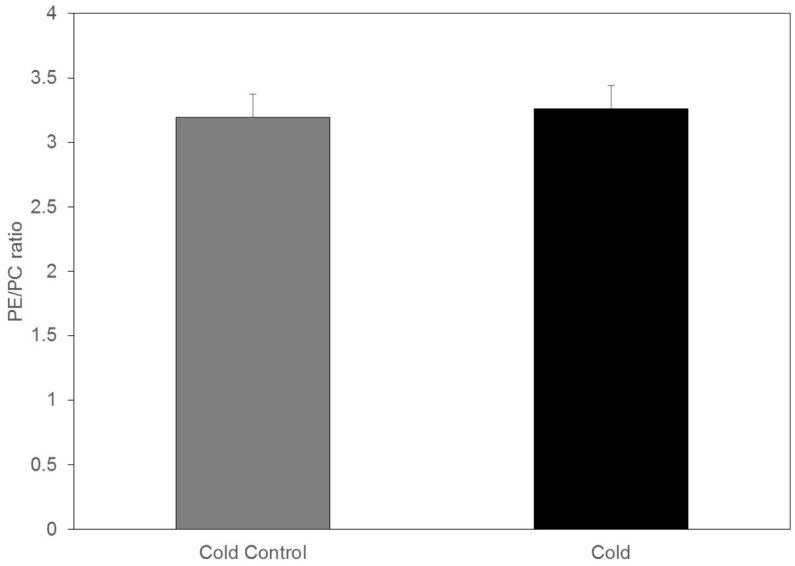

Fig. 3.1B. PE/PC ratio for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

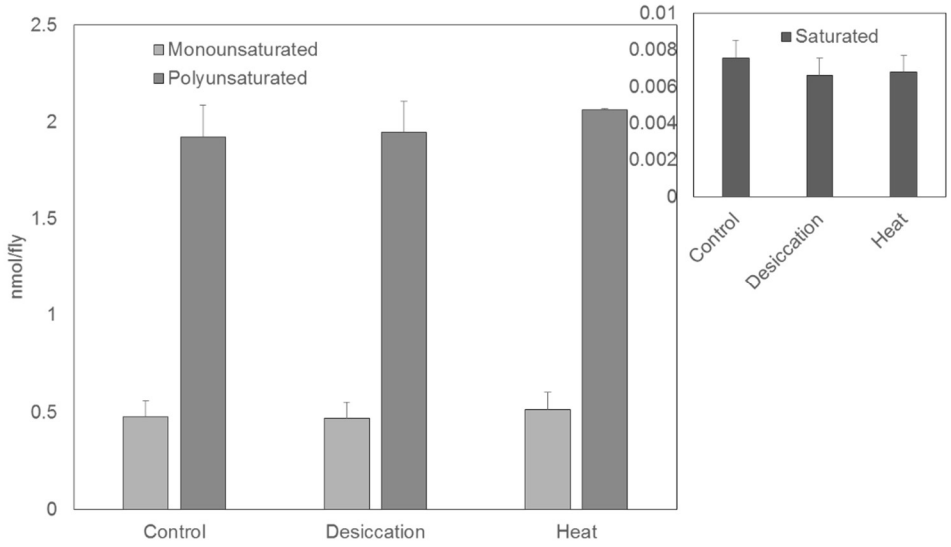

Fig. 3.2A. Concentration per fly of saturated, monounsaturated and polyunsaturated PE for control, desiccation and heat selections, error bars represent the SE (n = 5).

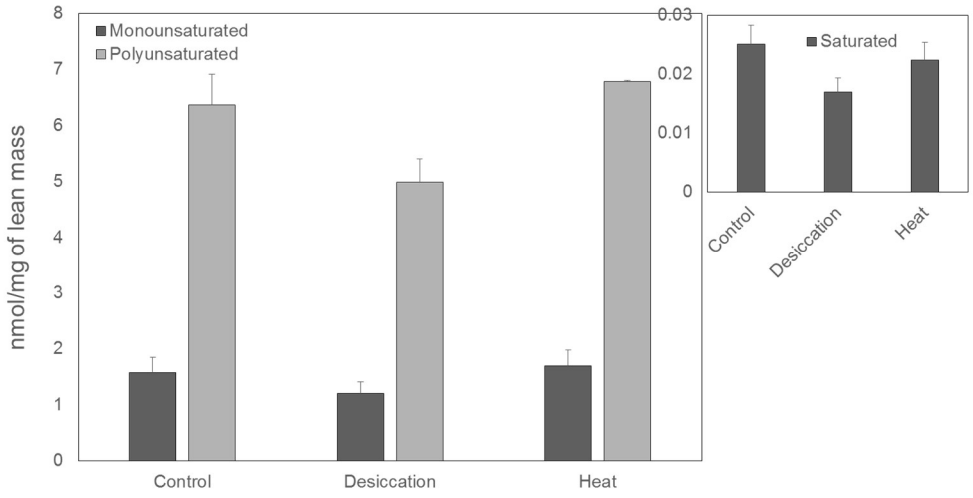

Fig. 3.2B. Concentration per lean mass of saturated, monounsaturated and polyunsaturated PE for control, desiccation and heat selections, error bars represent the SE (n = 5).

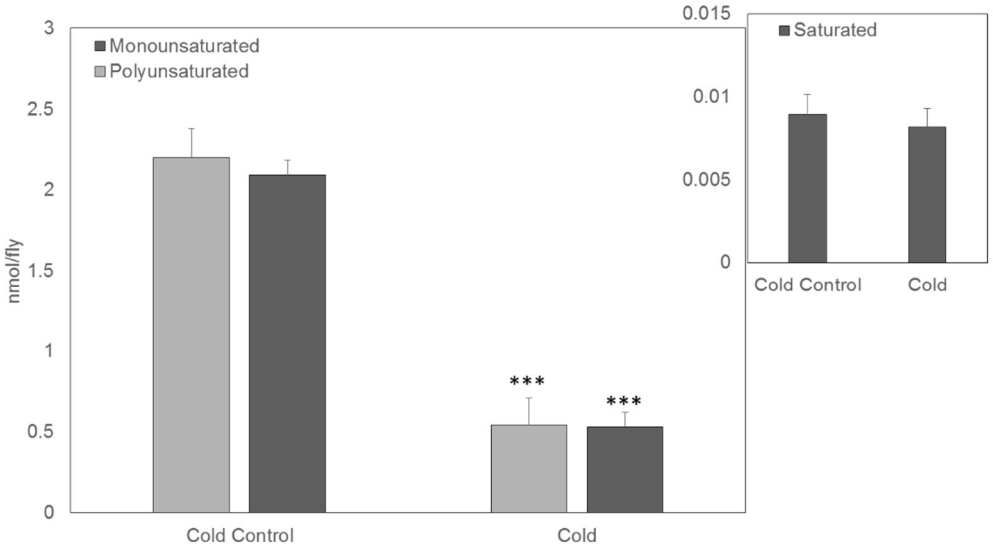

Fig. 3.2C. Concentration per fly of saturated, monounsaturated and polyunsaturated PE for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

*** indicates p-value<0.001.

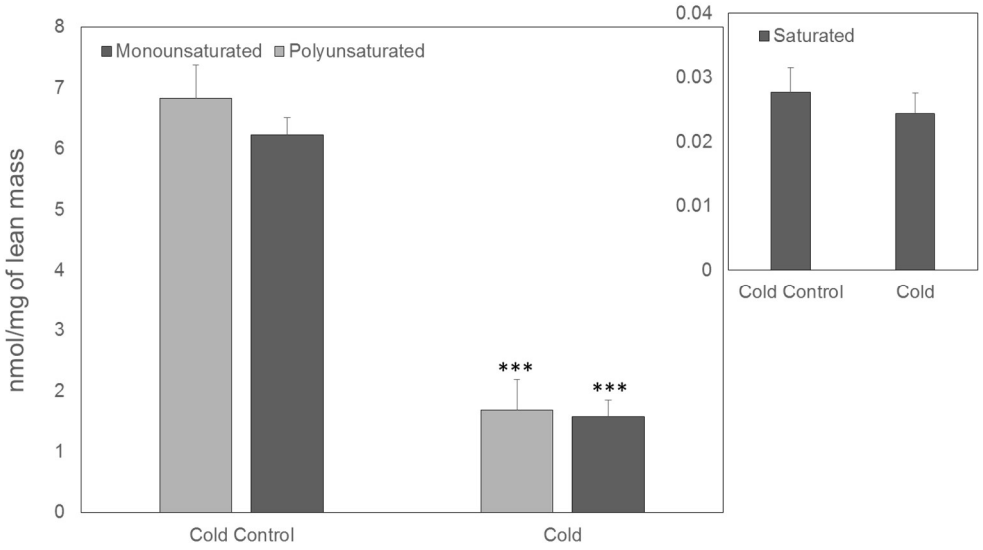

Fig. 3.2D. Concentration per lean mass of saturated, monounsaturated and polyunsaturated PE for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5). *** indicates p-value<0.001.

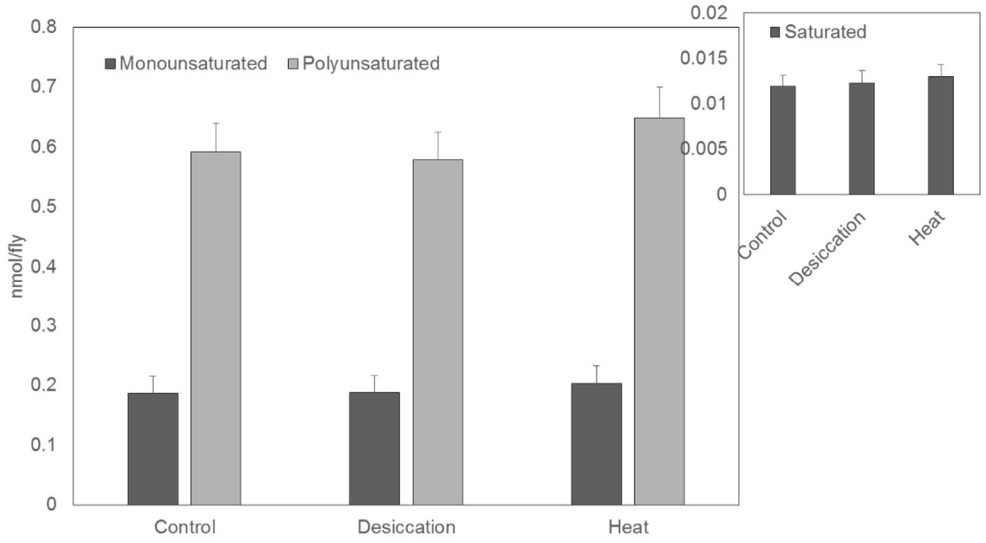

Fig. 3.3A. Concentration per fly of saturated, monounsaturated and polyunsaturated PC for control, desiccation and heat selections, error bars represent the SE (n = 5).

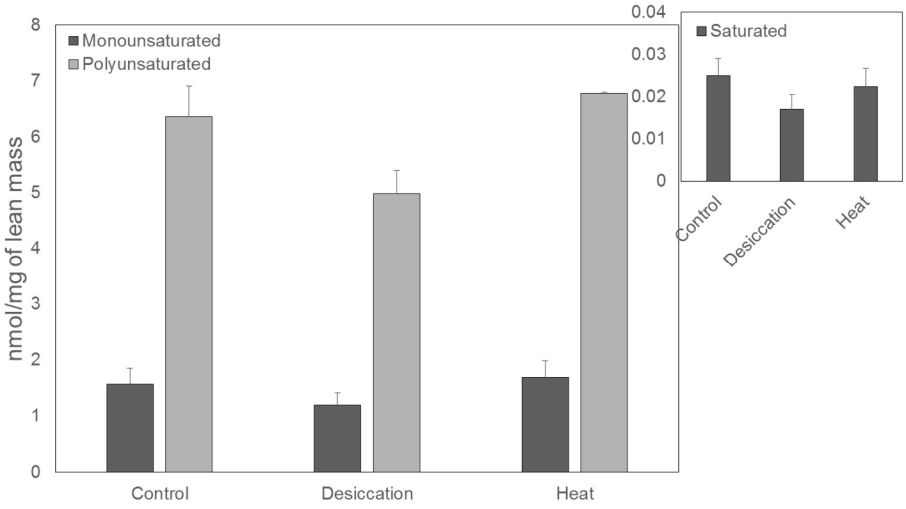

Fig. 3.3B. Concentration per lean mass of saturated, monounsaturated and polyunsaturated PC for control, desiccation and heat selections, error bars represent the SE (n = 5).

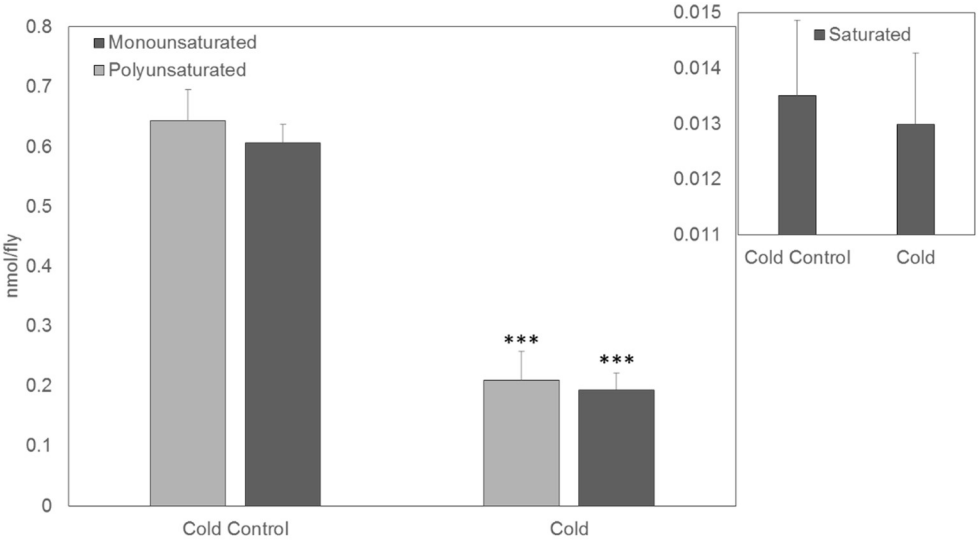

Fig. 3.3C. Concentration per fly of saturated, monounsaturated and polyunsaturated PC for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5). *** indicates p-value<0.001.

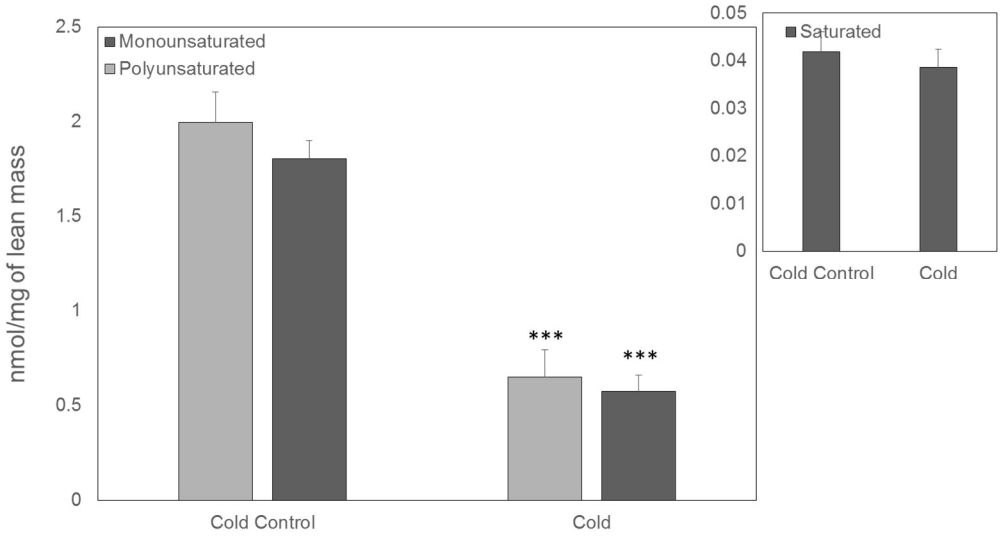

Fig. 3.3D. Concentration per fly of saturated, monounsaturated and polyunsaturated PC for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5). *** indicates p-value<0.001.

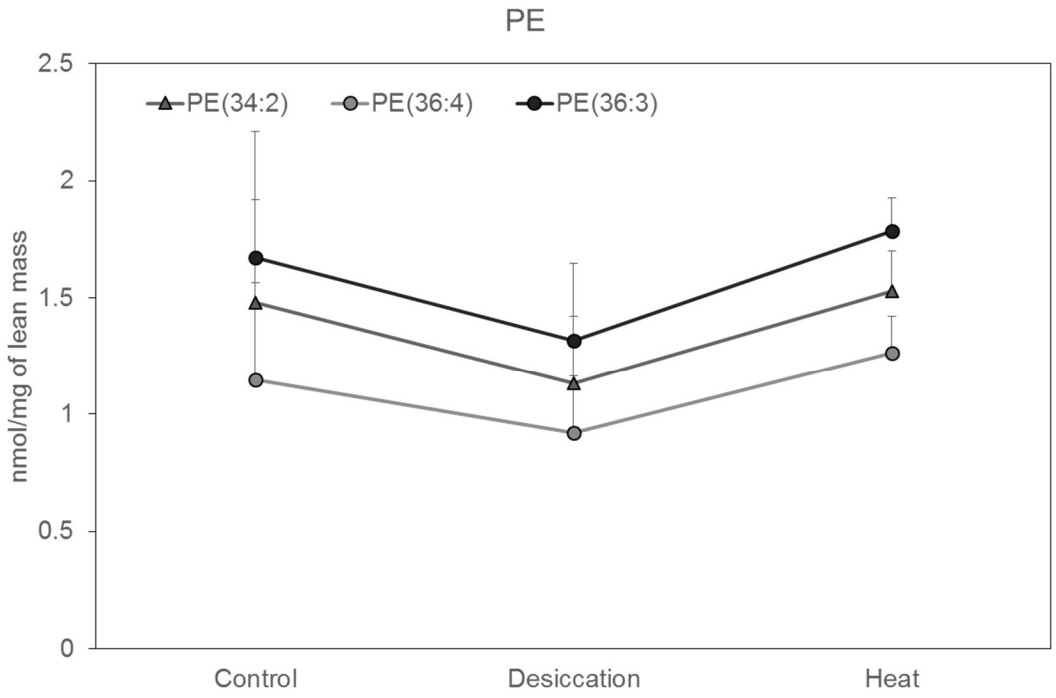

Fig. 3.4A. Trends of predominant PE species per fly for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

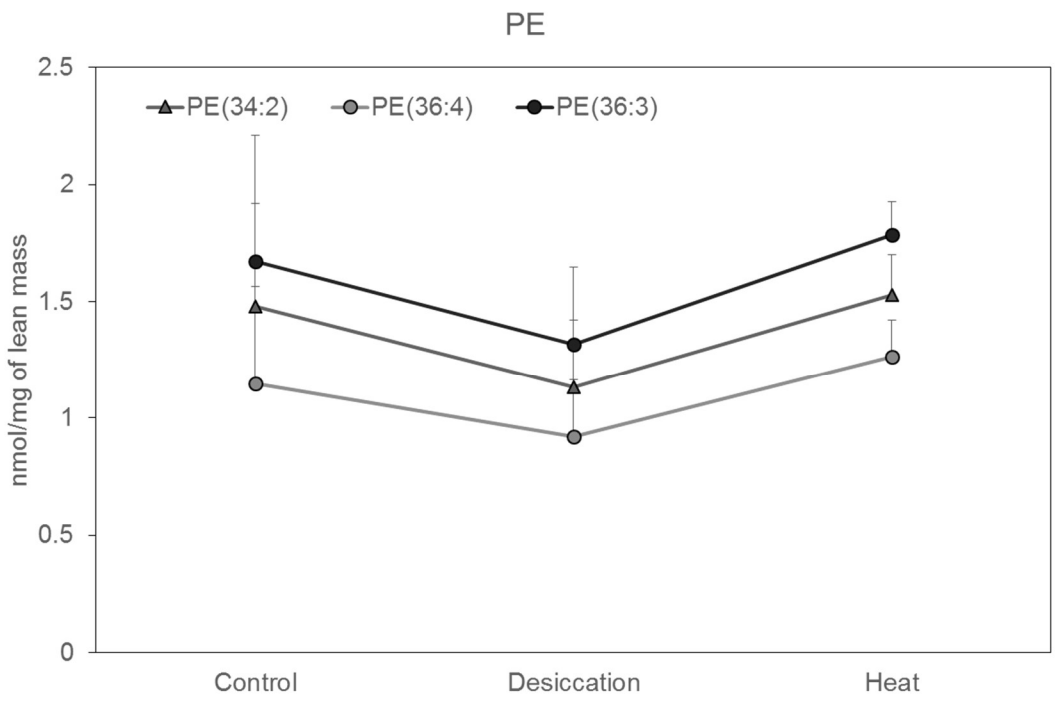

Fig. 3.4B. Trends of predominant PE species per mg of lean mass for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

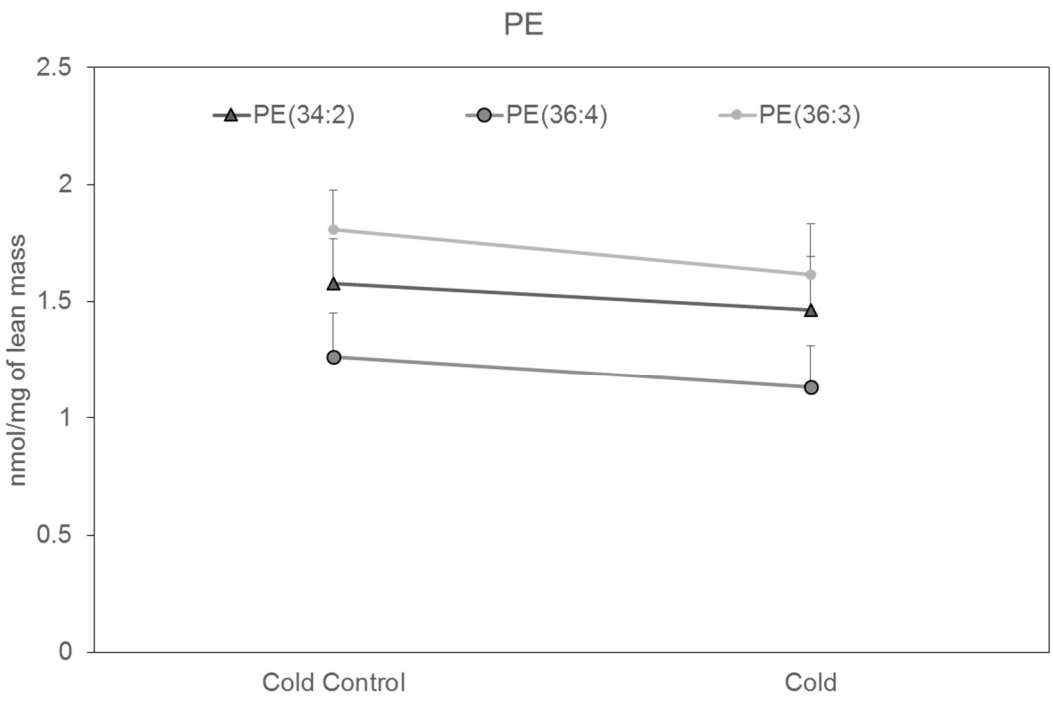

Fig. 3.4C. Trends of predominant PE species per fly for cold control and chill coma recovery (Cold) selected populations, error bars represent the SE (n = 5).

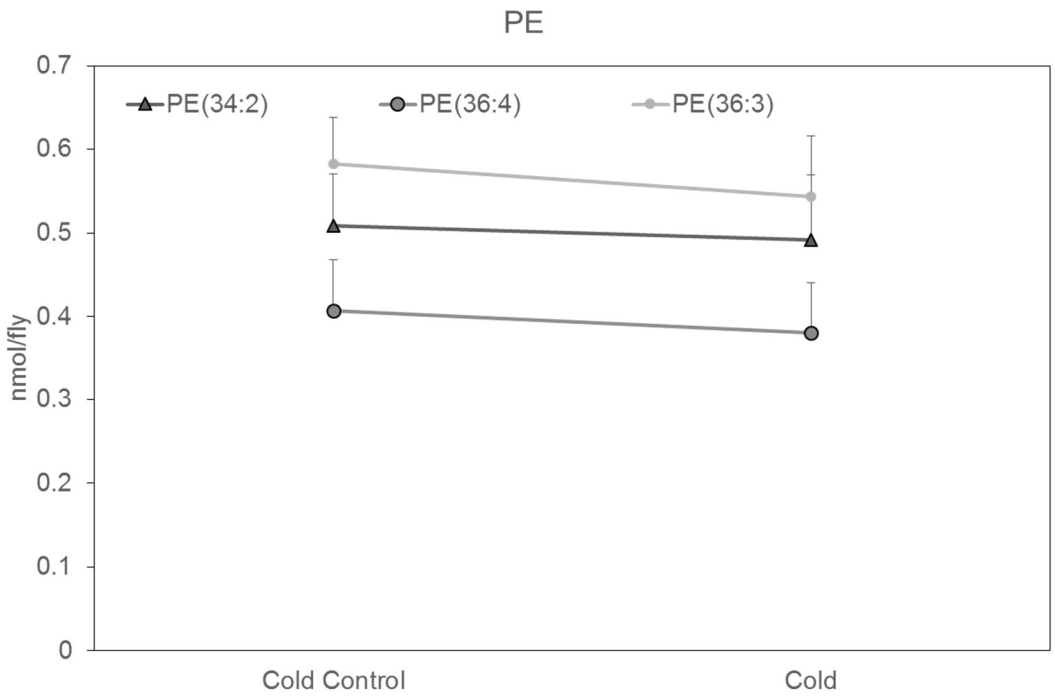

Fig. 3.4C. Trends of predominant PE species per fly for cold control and chill coma recovery (Cold) selected populations, error bars represent the SE (n = 5).

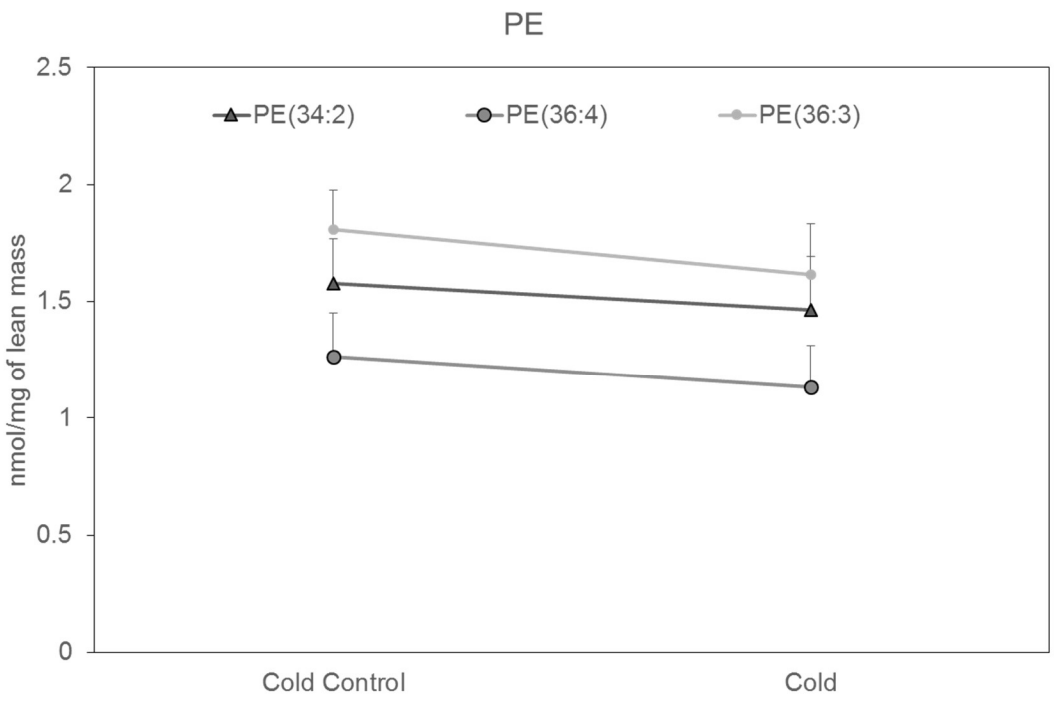

Fig. 3.4D. Trends of predominant PE species per mg of lean mass for cold control and chill coma recovery (Cold) selected populations, error bars represent the SE (n = 5).

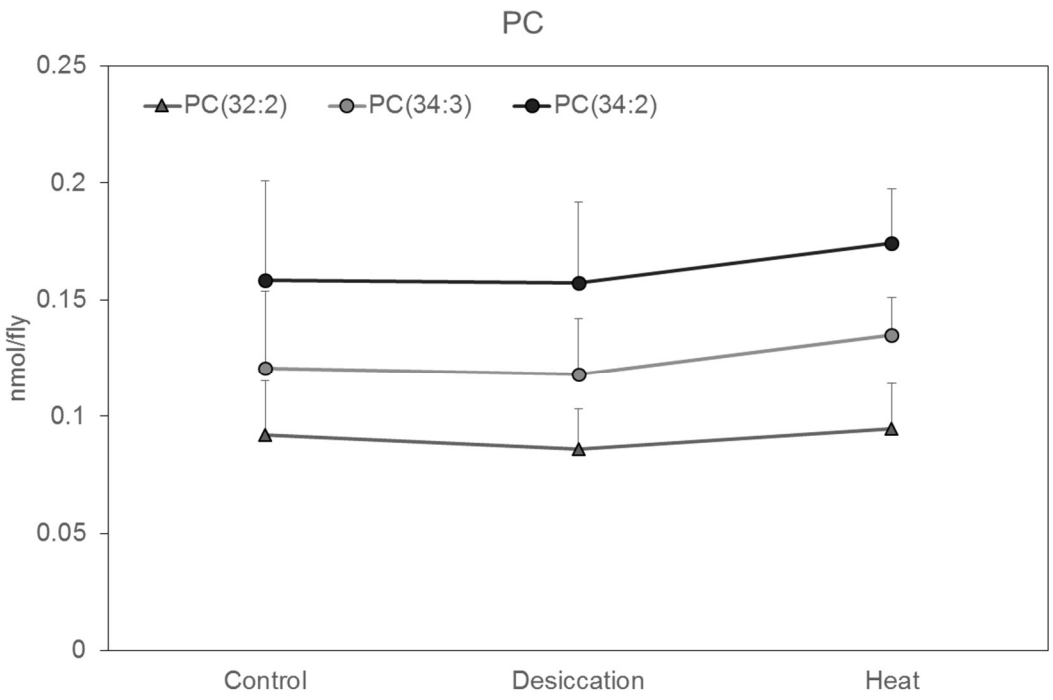

Fig. 3.5A. Trends of predominant PC species per fly for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

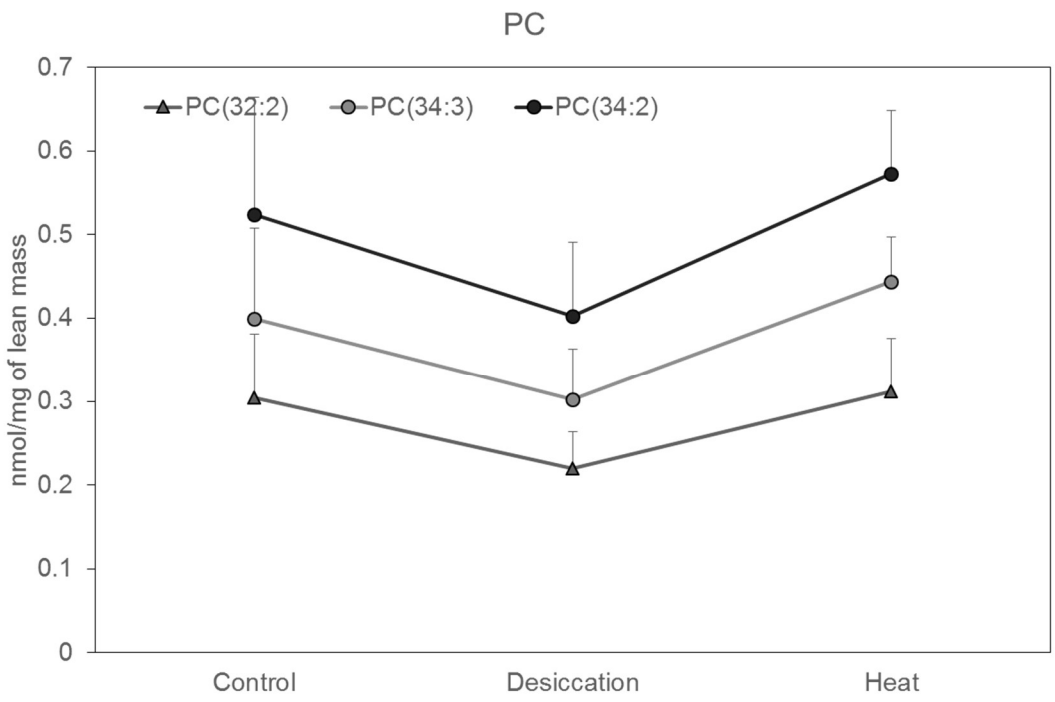

Fig. 3.5B. Trends of predominant PC species for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

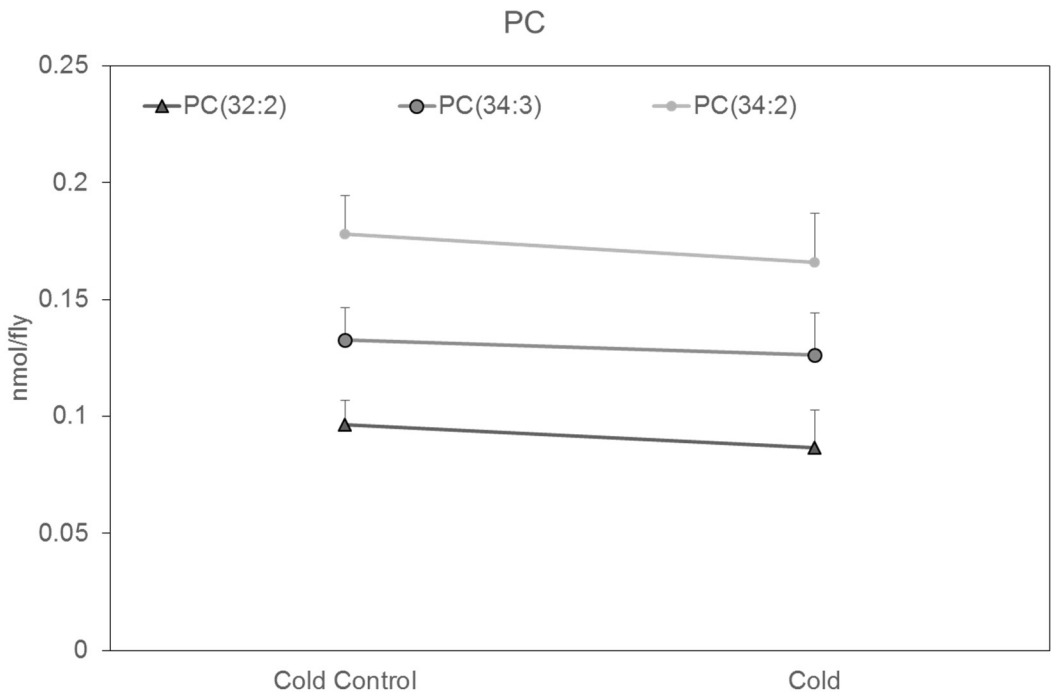

Fig. 3.5C. Trends for predominant PC species per fly for cold control chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

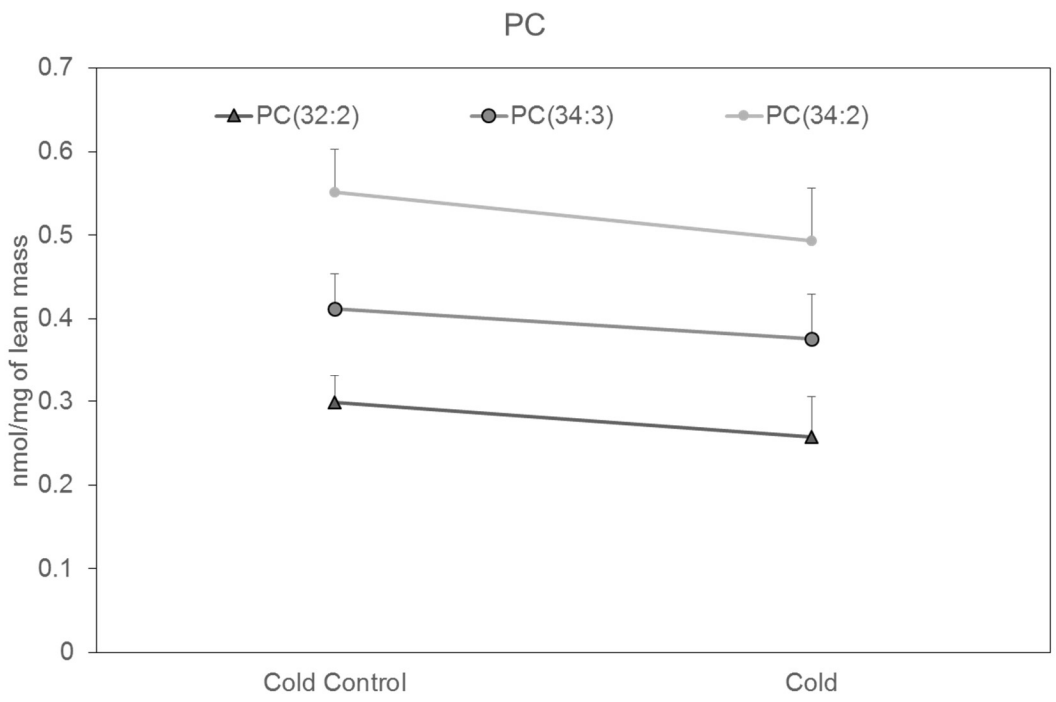

Fig. 3.5D. Trends for predominant PC species per fly for cold control chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

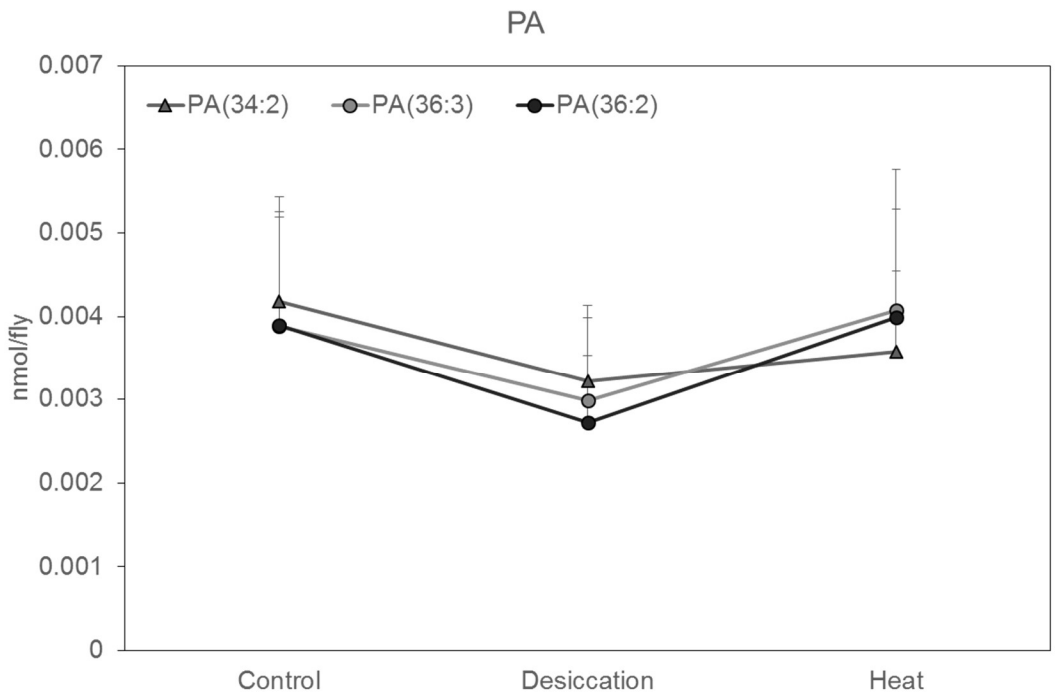

Fig. 3.6A. Trends of predominant PA species per fly for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

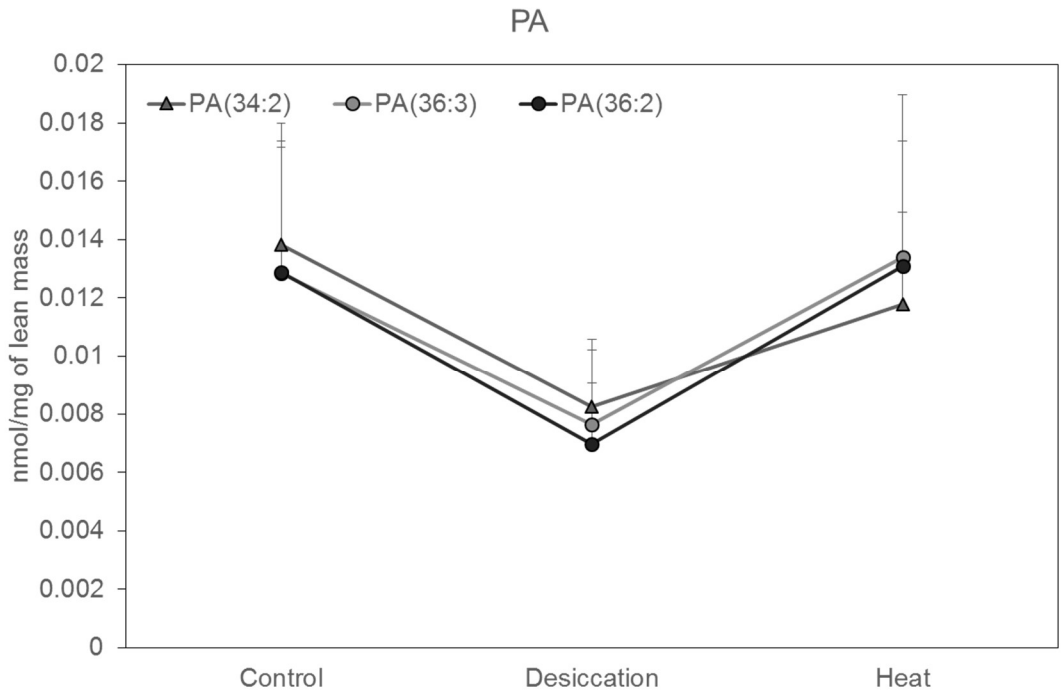

Fig. 3.6B. Trends of predominant PA species per lean mass for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

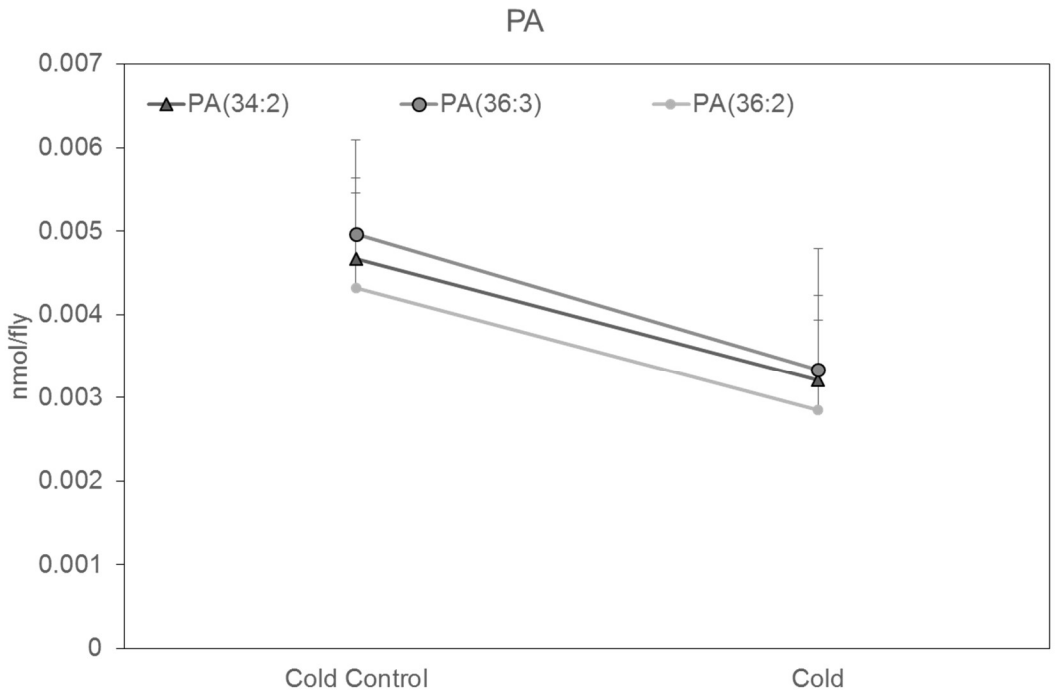

Fig. 3.6C. Trends for predominant PA species per fly for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

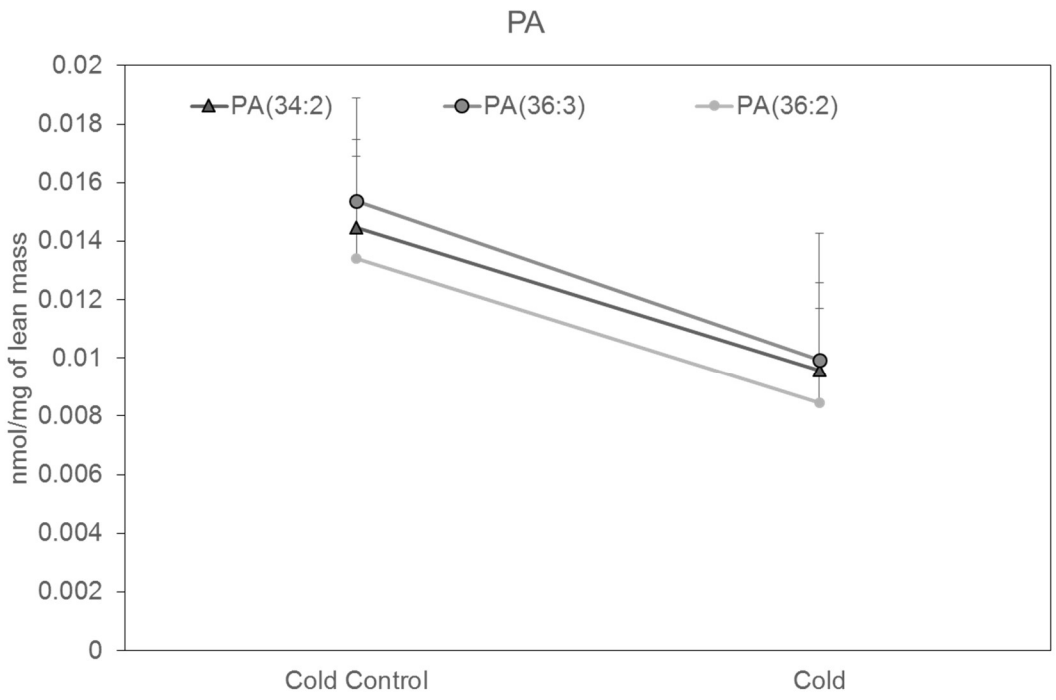

Fig. 3.6D. Trends for predominant PA species per lean mass for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

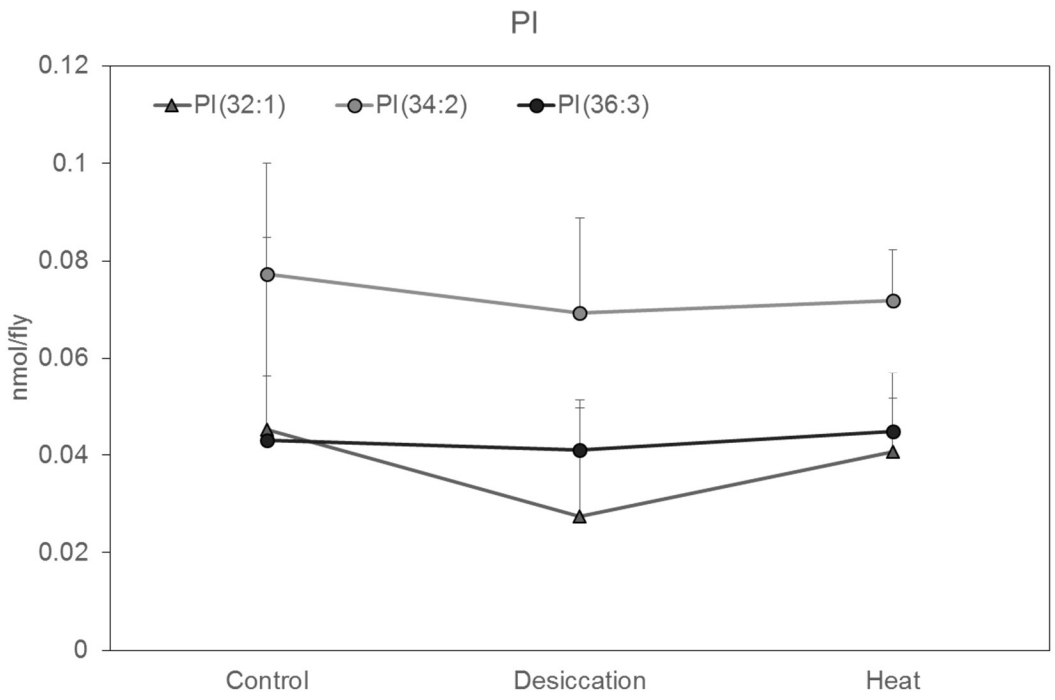

Fig. 3.7A. Trends of predominant PI species per fly for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

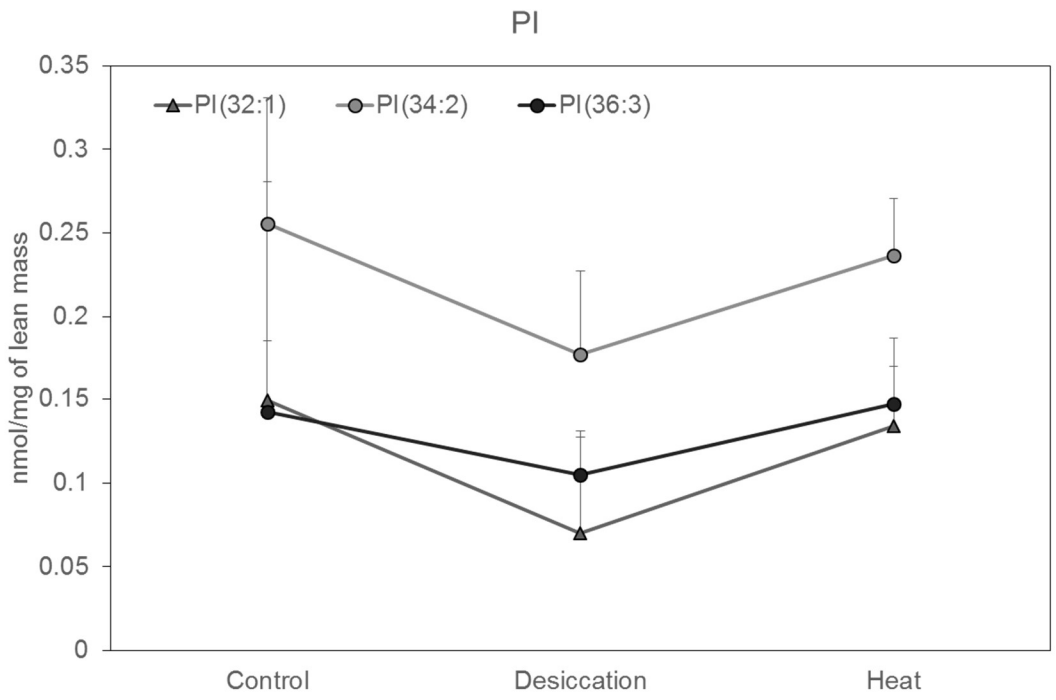

Fig. 3.7B. Trends of predominant PI species per lean mass for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

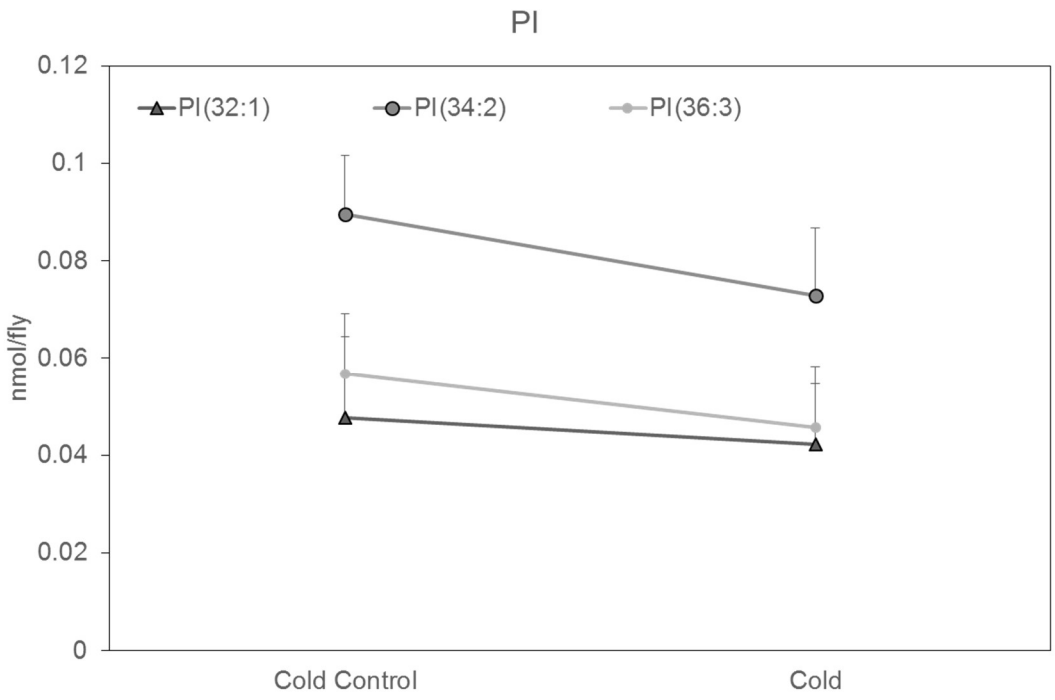

Fig. 3.7C. Trends for predominant PI species per fly for cold control and chill-coma selected (Cold) lines , error bars represent the SE (n = 5).

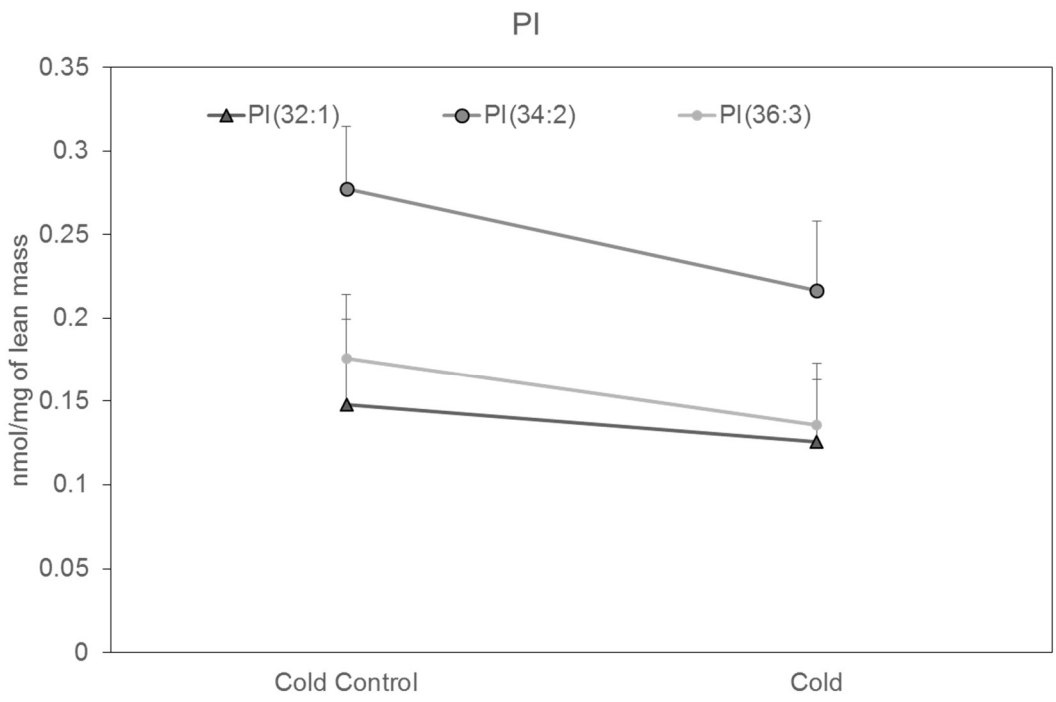

Fig. 3.7D. Trends for predominant PI species per mg of lean mass for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

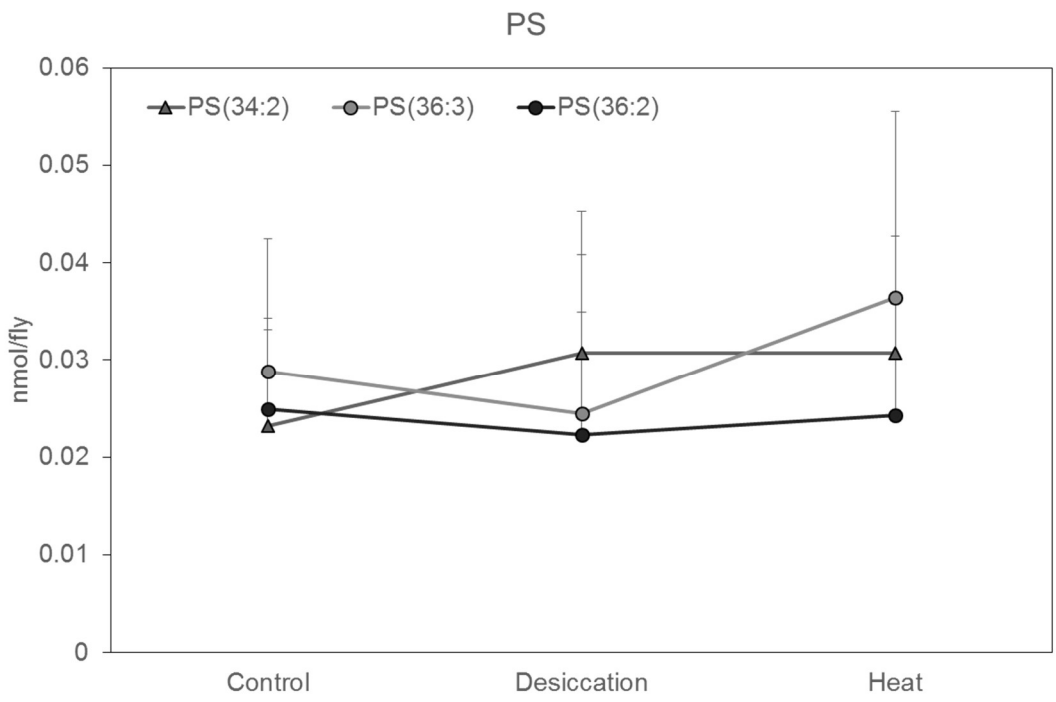

Fig. 3.8A. Trends of predominant PS species per fly for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

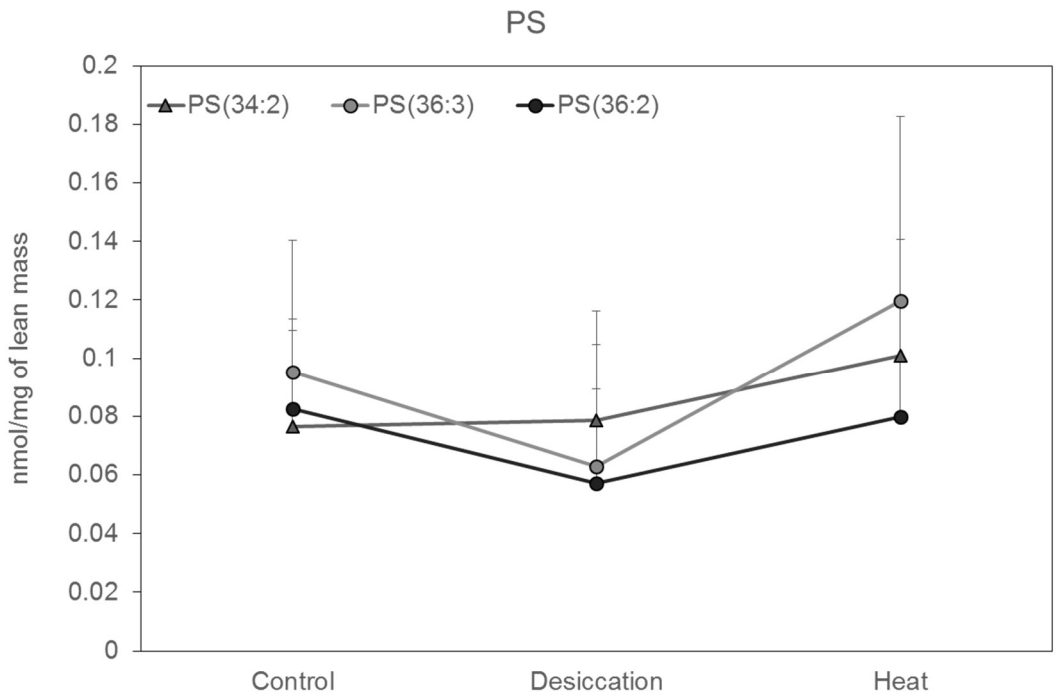

Fig. 3.8B. Trends of predominant PS species per mg of lean mass for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

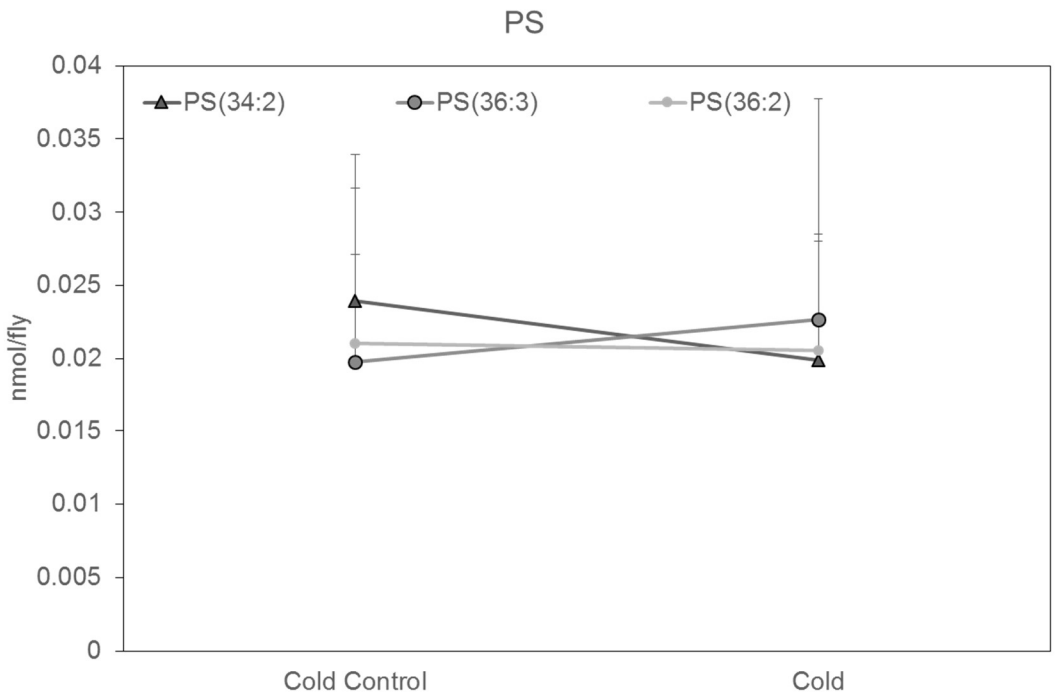

Fig. 3.8C. Trends for predominant PS species per fly for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

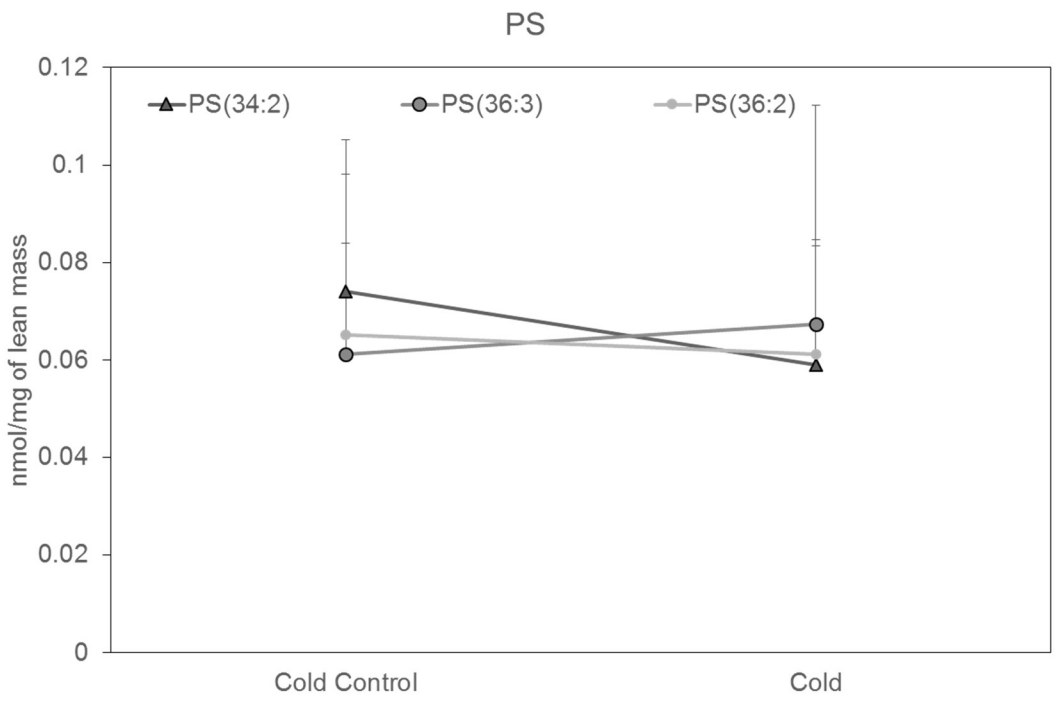

Fig. 3.8D. Trends for predominant PS species per mg of lean mass for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

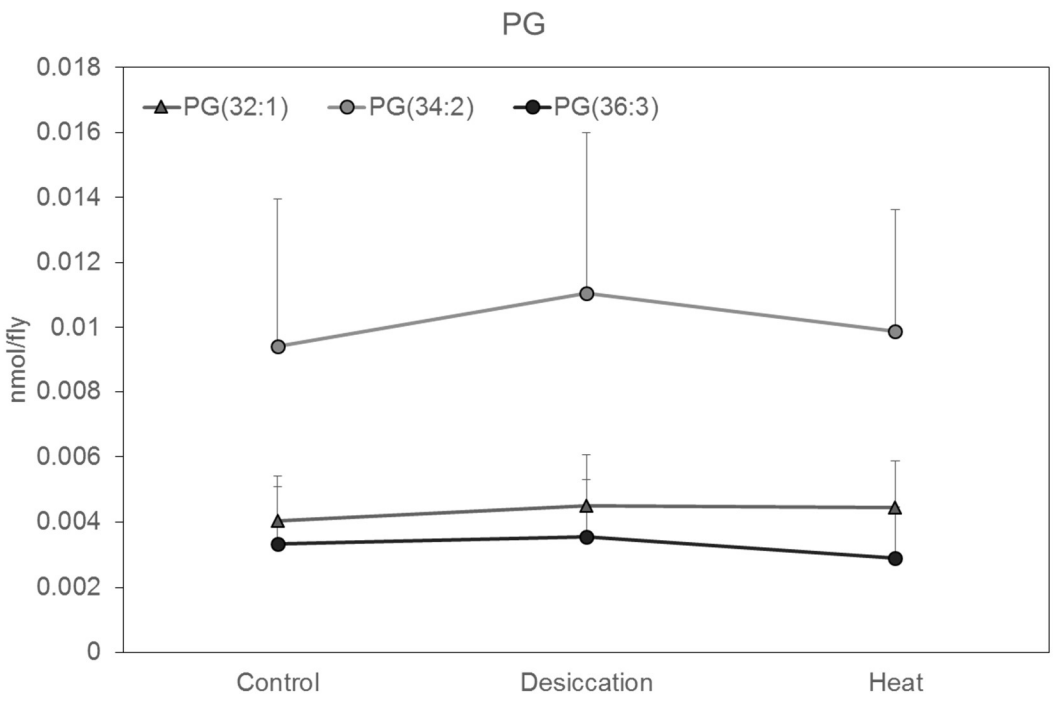

Fig. 3.9A. Trends of predominant PG species per fly for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

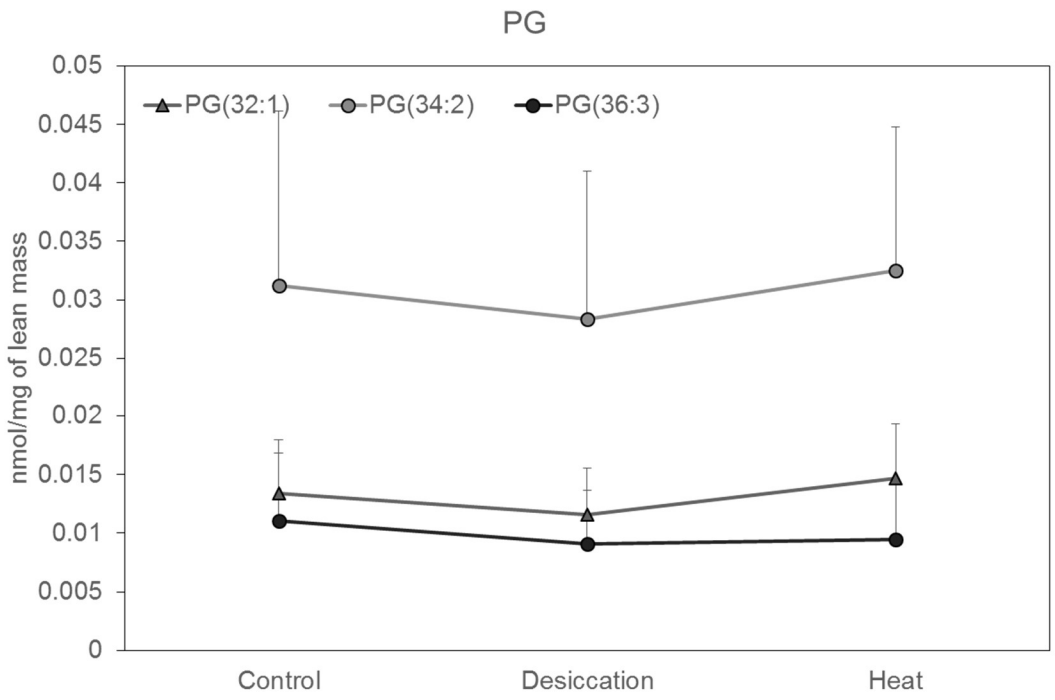

Fig. 3.9B. Trends of predominant PG species per mg of lean mass for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

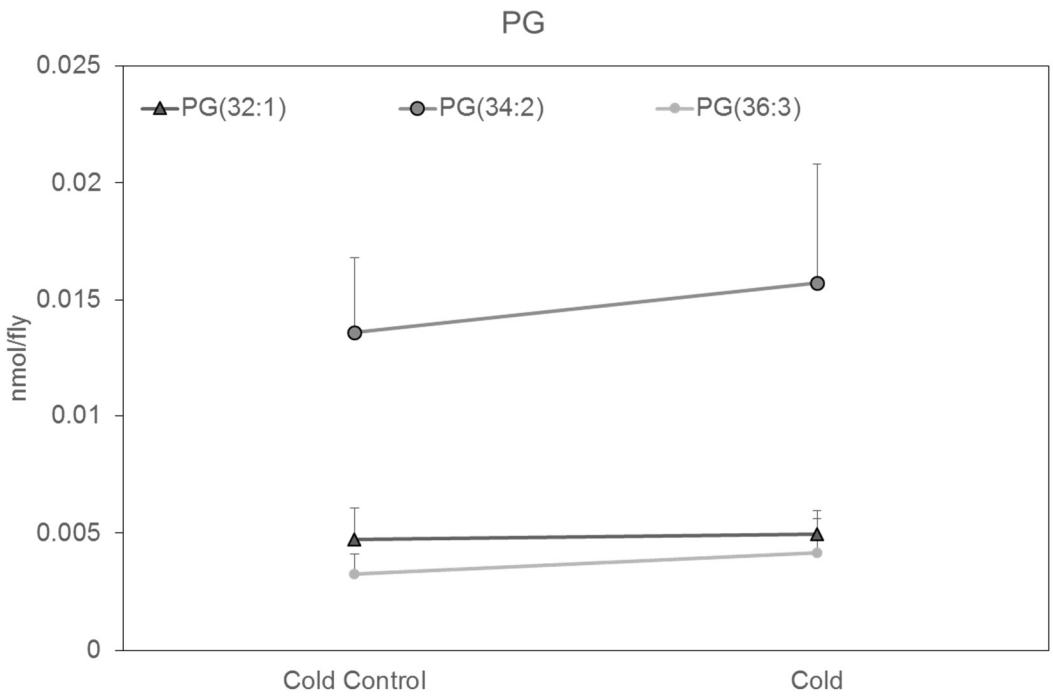

Fig. 3.9C. Trends for predominant PG species per fly for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

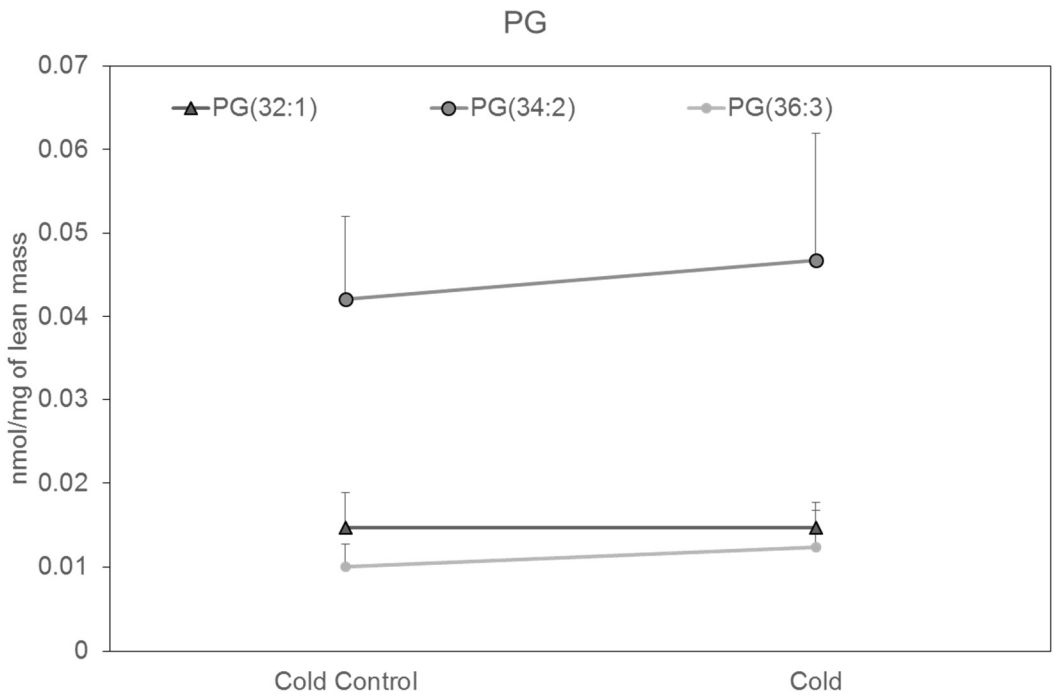

Fig. 3.9D. Trends for predominant PG species per mg of lean mass for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

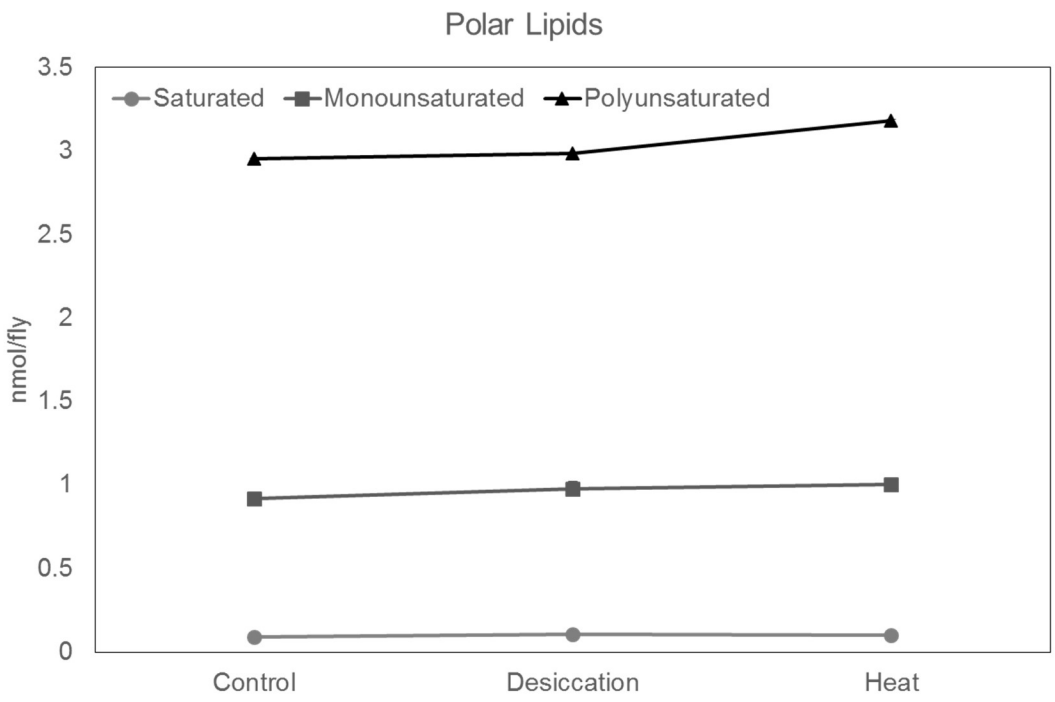

Fig. 3.10A. Trends of saturated, monounsaturated and polyunsaturated polar lipids per fly for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

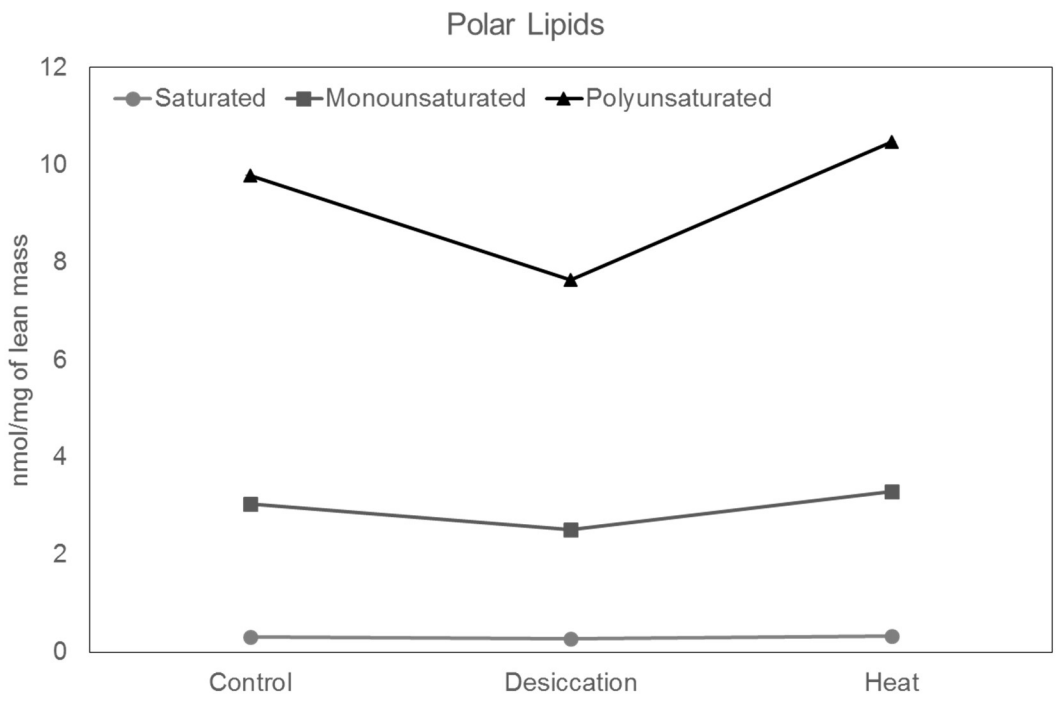

Fig. 3.10B. Trends of saturated, monounsaturated and polyunsaturated polar lipids per mg of lean mass for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

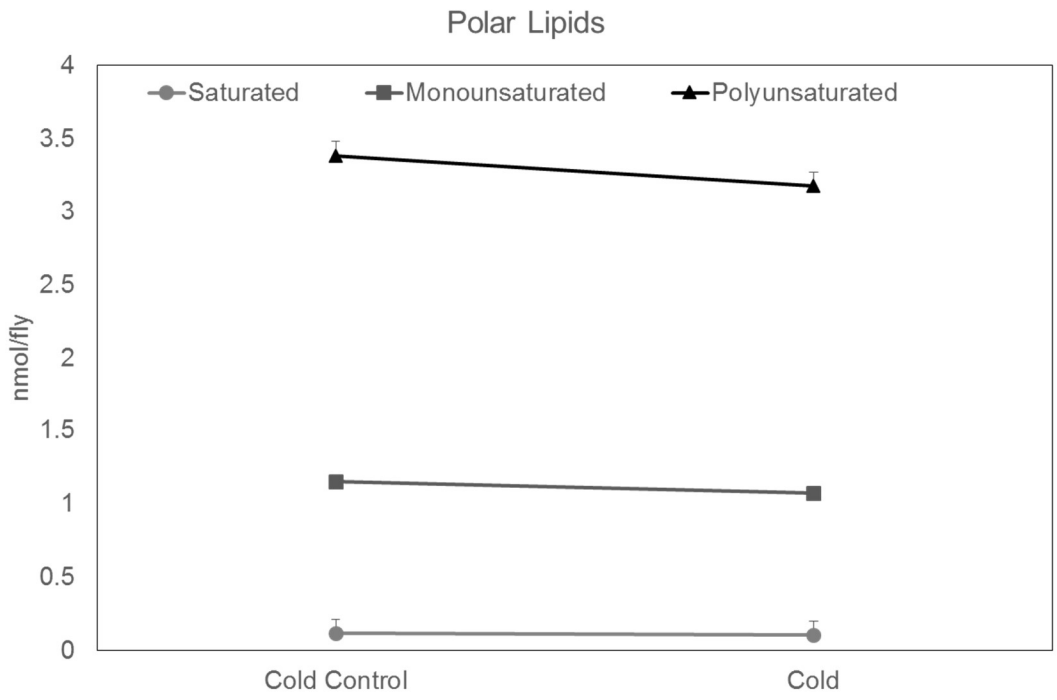

Fig. 3.10C. Trends for saturated, monounsaturated and polyunsaturated polar lipids per fly for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

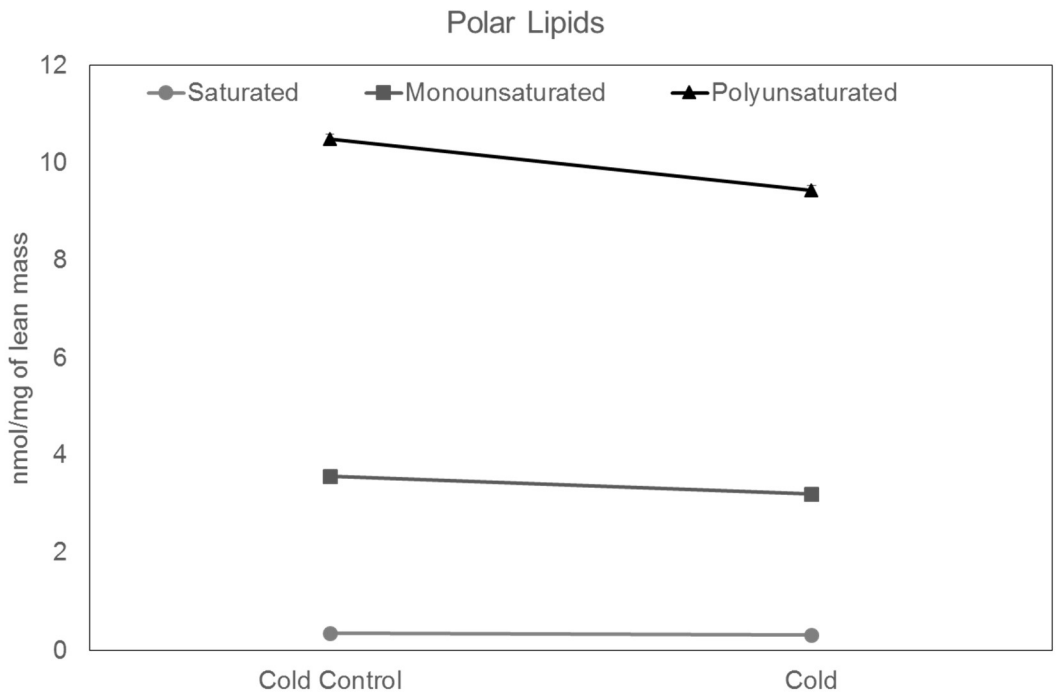

Fig. 3.10D. Trends for saturated, monounsaturated and polyunsaturated polar lipids per mg of lean mass for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

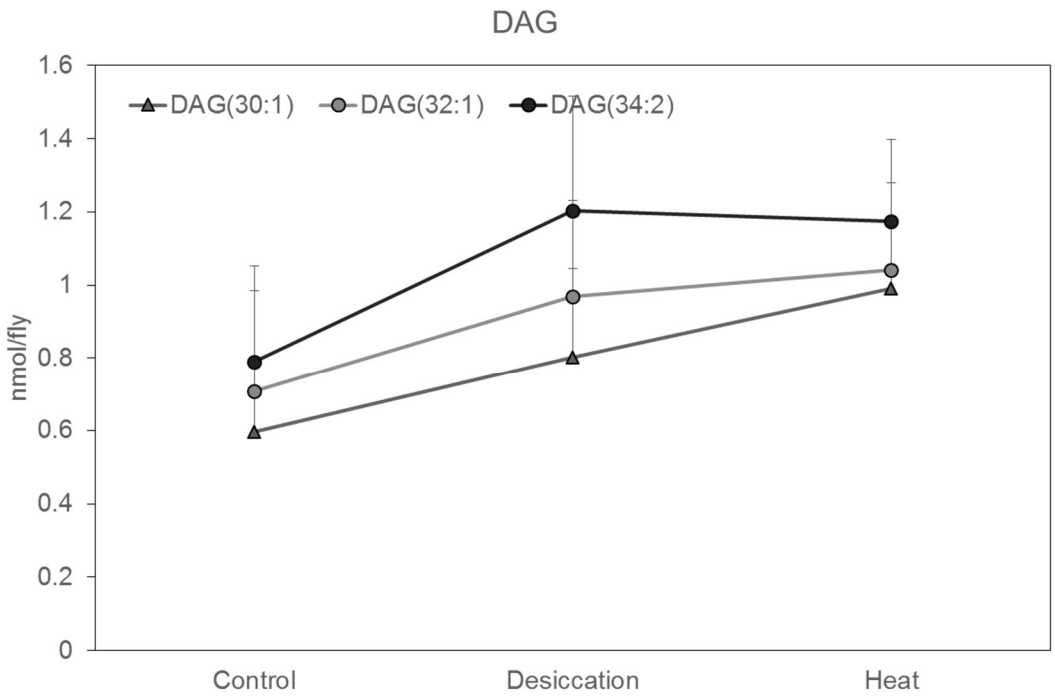

Fig. 3.11A. Trends of predominant DAG species per fly for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

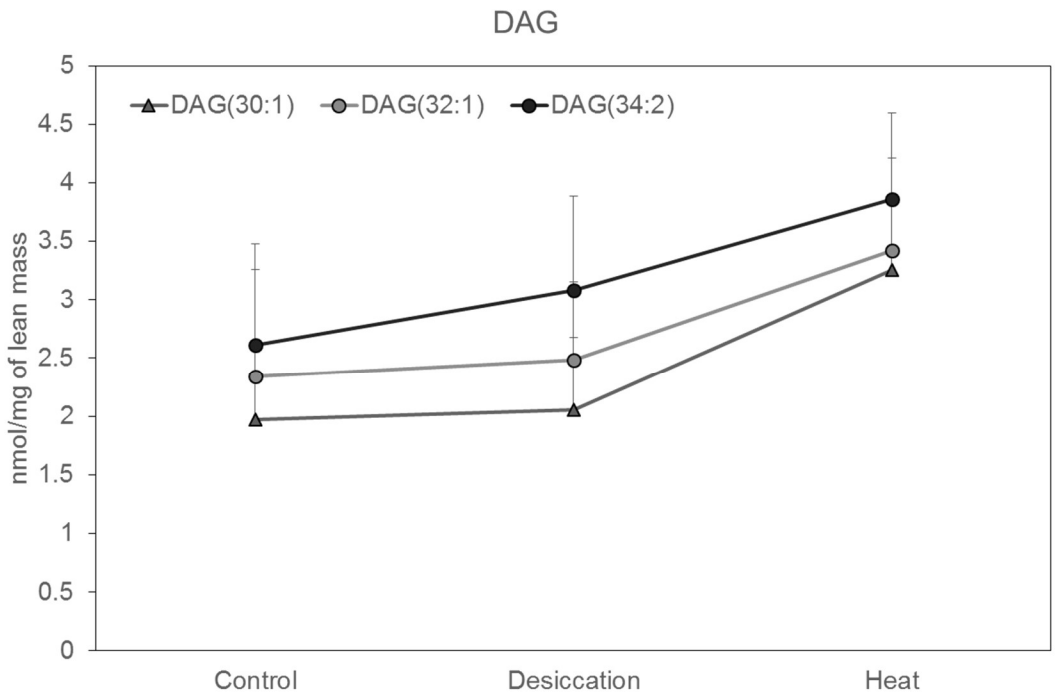

Fig. 3.11B. Trends of predominant DAG species per mg of lean mass for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

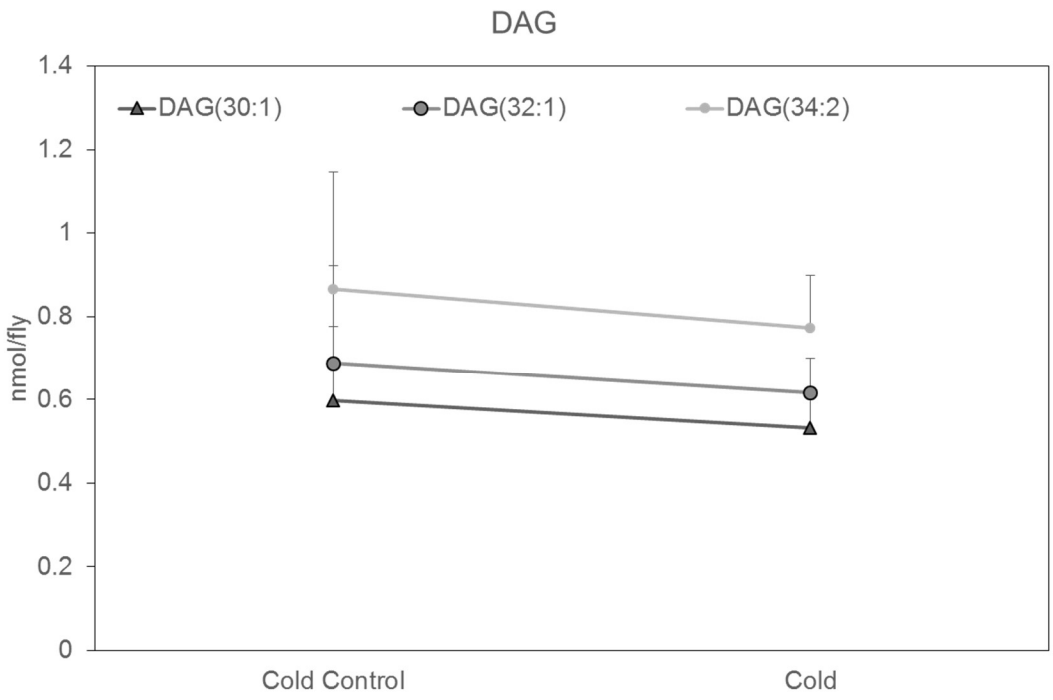

Fig. 3.11C. Trends for predominant DAG species per fly for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

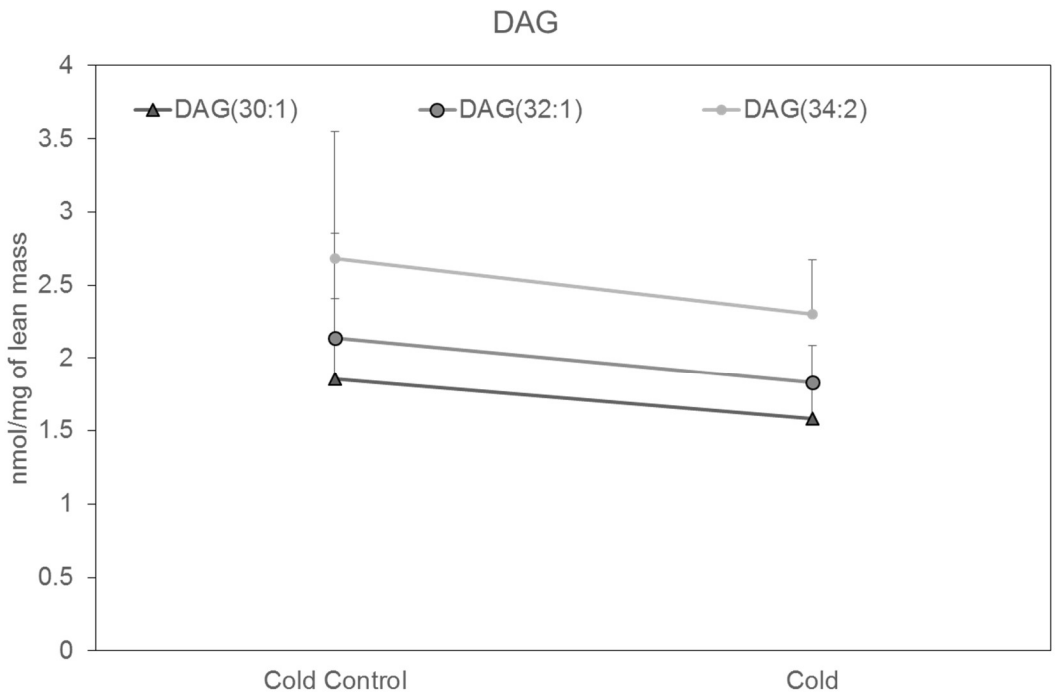

Fig. 3.11D. Trends for predominant DAG species per mg of lean mass for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

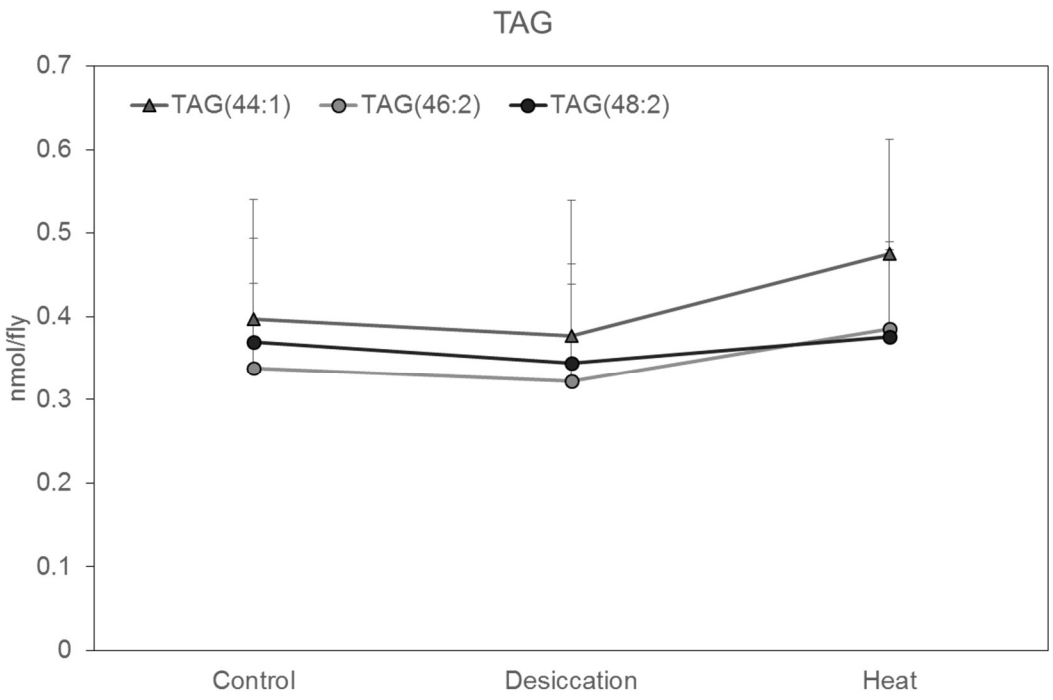

Fig. 3.12A. Trends of predominant TAG species per fly for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

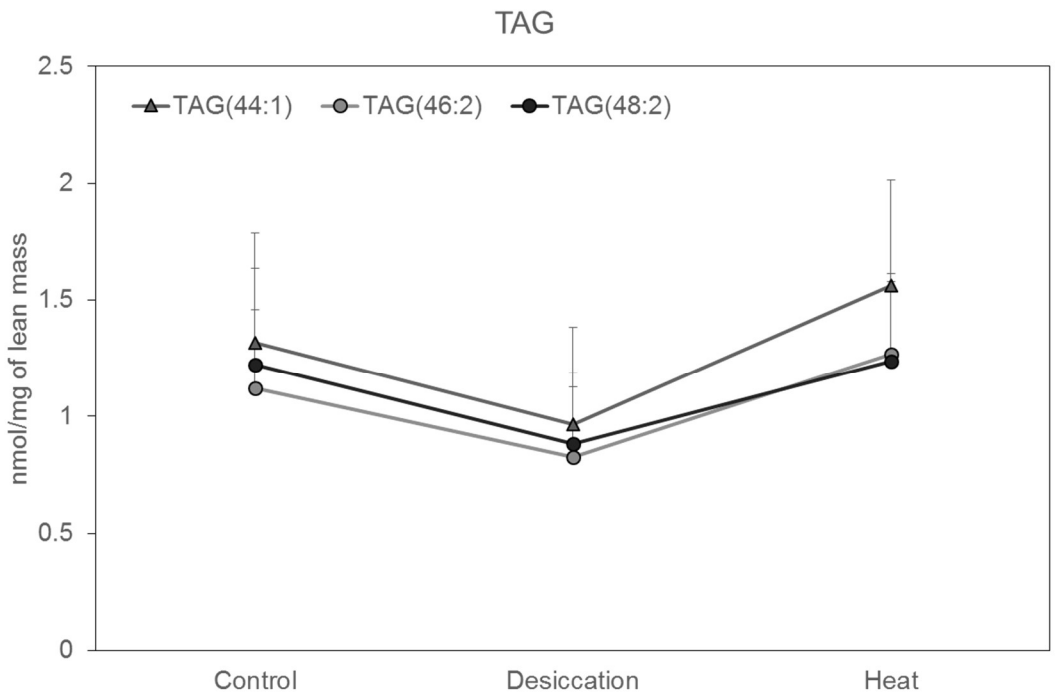

Fig. 3.12B. Trends of predominant TAG species per mg of lean mass for control, desiccation selected and heat selected populations, error bars represent the SE (n = 5).

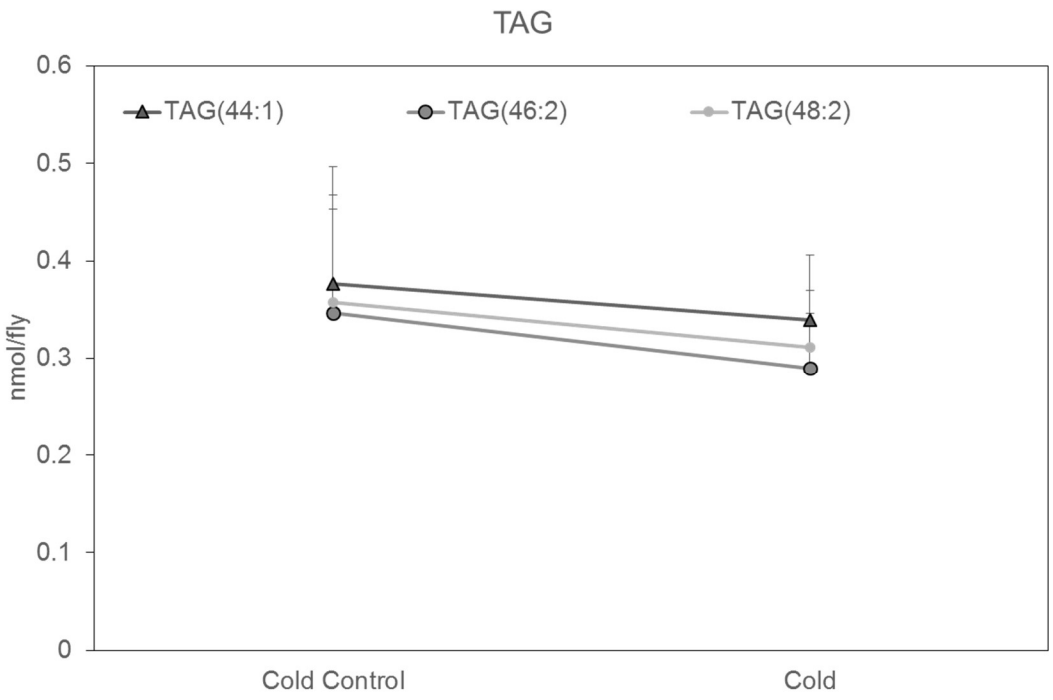

Fig. 3.12C. Trends for predominant TAG species per fly for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

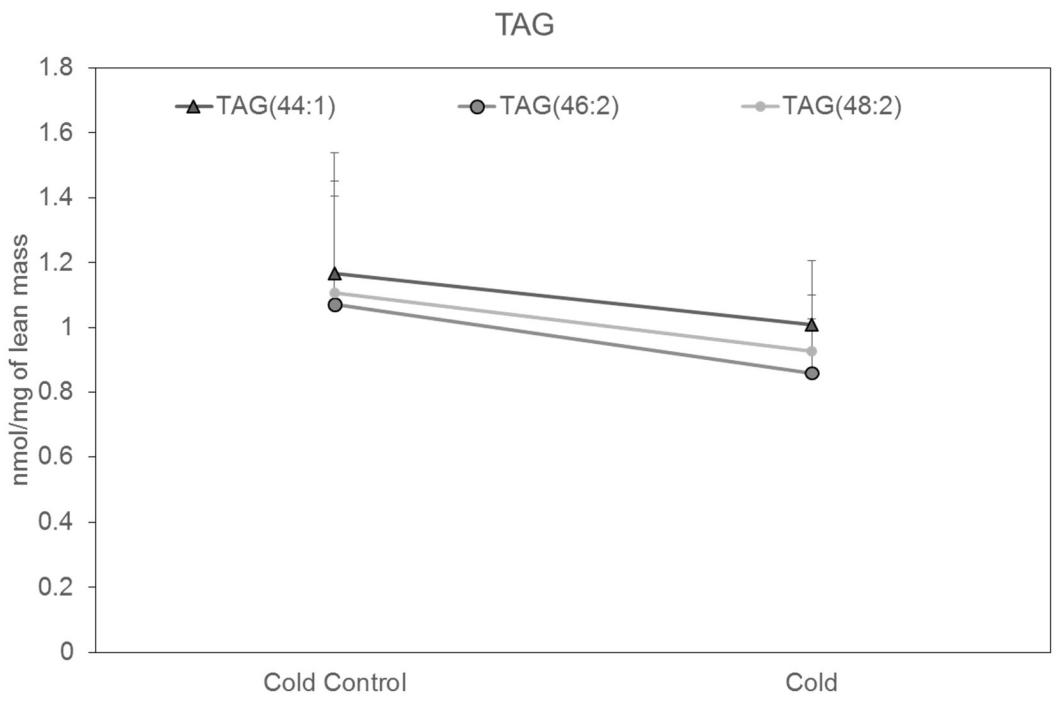

Fig. 3.12D. Trends for predominant TAG species per mg of lean mass for cold control and chill-coma selected (Cold) lines, error bars represent the SE (n = 5).

CHAPTER 4

KNOCKDOWNS OF KEY LIPID BIOSYNTHESIS GENES USING RNAi AND IMPACT ON STRESS TOLERANCE IN *DROSOPHILA MELANOGASTER*

4.1 Introduction

As ectothermic organisms, insects have evolved a series of distinct responses to external stress such high or low temperature, and scarcity of water. Survival of these types of stress often correlates with energy allocation requiring storage lipids from an energy reservoir such as the fat body. Membrane lipid composition is correlated with thermal stress tolerance (Cooper et al. 2011, Overgaard et al. 2006). Cuticular lipid is associated with desiccation stress tolerance (Gibbs et al. 1997).

In *D. melanogaster*, lipid abundance and structure is heritable (Scheitz et al. 2013). In this study based on 92 inbred lines derived from five geographic locations, unsaturated lipids showed greater heritability compared saturated lipids (Scheitz et al. 2013). Our goal in this study is to investigate lipid biosynthesis in relationship to environmental stress tolerance or survival.

It is common for organisms to encounter environmental hardship such as adverse temperatures. Heat stress results in a number of problems in animals. Increased body temperature has negative effects on proteins and other macromolecules, which can results in respiration rate increase, cell membranes can become damaged, and results in pH decrease (Neven 2000). The heat shock response system can provide somatic protection from heat stress. For example, small heat shock proteins (sHsp) interact with membrane lipids by affecting the hydrophobic core with a polar head group of membrane lipids to

prevent the formation of nonbilayer structure that would cause leakage or damage of membrane integrity during temperature stress (Tsvetkova et al. 2002).

Cold stress results in a chill coma in both mammals and insects, while occurring by distinct mechanisms (Macmillan and Sinclair 2011). Mammals enter chill coma once they reach a temperature that induces failure of circulatory and respiratory systems. In insects, chill coma is accompanied by disruptions of ion hemostasis in the neuromuscular system. One relevant mechanism is that cold temperature causes a decrease of membrane fluidity that results in the limiting of ion channel gating and ion pump activity, which disrupts resting potential. Upon the depletion of resting potential, an insect falls into a chill coma (Macmillan and Sinclair 2011).

Desiccation is another abiotic stress. Insects have evolved a variety of mechanisms which result in resistance to desiccation. *D. melanogaster*, for example, has evolved strategies to maintain water balance that includes decreasing metabolic rates and increasing water storage via glycogen storage (Djawdan et al. 1998, Gibbs et al. 1997). When compared across 20 species of *Drosophila,* it was shown that desert flies have a reduced rate of water loss, prolonging their survival under desiccation conditions (Gibbs and Matzkin 2001). Cuticular hydrocarbon chain length increases with desiccation resistance in different populations of *Drosophila* (Foley and Telonis-Scott 2011, Gibbs et al. 1997). There is evidence that cuticular lipids contribute to the retention of water in insects (Bazinet et al. 2010).

Lipid metabolism pathways in *Drosophila* are not fully understood, but it is assumed they conform to general lipid metabolic pathways that are defined in mammals due to the highly conserved lipid biosynthesis enzymes between *Drosophila* and mammals. Acetyl-

CoA is converted into saturated fatty acid via a multi-step fatty acid synthesis pathway. Then the saturated fatty acid such as a palmitic acid is converted into monounsaturated fatty acid via stearoyl-CoA desaturase. Saturated and unsaturated fatty acids can be combined with glycerol-3.phosphate to generate lysophosphatidate and phosphatidate with glycerol-3.phophate transferase (GPAT). Phosphatidate can be converted into diacylglycerol (DAG) with phosphatidic acid phosphatase (PAP). DAG is an important intermediate, as it can be converted into PC with phosphocholine cytidyltransferase (CT) (Jones et al. 1992) or other phosphatidylglycerol lipids such as PE or PC, as well as processed into storage lipid such as triacylglycerol (TAG) with diacylglycerol O-acyltransferase (DGAT) (Buszczak et al. 2002) as shown in (Fig. 4.1).

Based on conserved lipid biosynthesis pathways (Fig. 4.1), homologs of specific biosynthesis enzymes used in this study were identified in *D. melanogaster*. In *Drosophila*, there are two isoforms of stearoyl-Co A desaturases, desat1, and desat2. These desaturases provide essential roles in lipid metabolism as well as the maintenance and function of biological membranes. These stearoyl-CoA desaturases introduced the initial double bond in fatty acid synthase, which results in monounsaturated fatty acid that is essential for downstream lipid biosynthesis and synthesis of cuticular hydrocarbons (Dallerac et al. 2000, Fang et al. 2009, Matzkin and Markow 2009, Takahashi et al. 2001). These cuticular hydrocarbons provide the primary barrier against evaporative water loss (Bazinet et al. 2010, Chown et al. 2011). In addition, desaturation in membrane lipids may be a mechanism leading to a cold-hardening response in *D. melanogaster* (Overgaard et al. 2005).

GPAT in *Drosophila* was identified using molecular sequence prediction by comparison to other mammal GPAT genes. In *Drosophila*, multiple homologs of PAP were identified, as this particular enzyme is essential for embryo development, which can be due to its important role in phospholipid synthase. In *D. melanogaster*, humans, and several other higher eukaryotes, two isoforms of CT appear to be present (Lykidis et al. 1998), in *Drosophila*, they are CCT1 and CCT2 (Gupta and Schupbach 2003, Helmink and Friesen 2004). The specific role of each CCT isoform in cellular processes, especially the contribution of each isoform to cellular PC synthesis has been investigated (Tilley et al. 2008). DGAT was identified as an essential for oogenesis (Schupbach and Wieschaus 1991), as well as for storage lipid formation (Beller et al. 2008).

4.2 Methods

Fly culture, RNAi lines, and crosses

For maintaining RNAi fly stocks, the following culture conditions were used. Flies were maintained on a diet consisting of 10% yellow cornmeal, 8.3% torula yeast, 6% molasses, 0.7% agar, 0.6% propionic acid and 0.3% tegosept. All RNAi stocks and crosses were maintained at 25˚C unless otherwise noted.

RNAi knockdowns, presumably of specific genes, were generated by reciprocally crossing each of the following RNAi stock virgin flies: desat1 (CG5887), desat2 (CG5925), Cct1 (CG1049), GPAT (CG4625), DGAT (CG31991) or PAP (CG8804) as shown in (Fig. 3.1), with virgin driver tub-GAL4 flies. All RNAi stocks were obtained from the Vienna *Drosophila* RNAi Center (VDRC). Control flies were generated by crossing w1118 flies with driver tub-GAL4 flies. The w1118 stock (genetic background)

was used to generate the VDRC collection of RNAi lines. To collect virgin flies for crosses, 25 male and female flies were placed in glass cut bottles for an egg laying period of 48 hours. Eggs were collected and placed in groups of 100 in vials with fresh food, with thirty vials for each cross. Vials with black pupae were placed in a 17˚C incubator and checked every 12 hours for adult flies. The newly emerged virgin flies were separated into females and males. After the desired number of virgin flies were collected, females were monitored until day five post-eclosion to ensure they were virgins as indicated by an absence of larvae in the vials.

qPCR

The F_1 from crosses that generated knockdown (KD) flies, and control crosses, were collected as virgins at day 5 post-eclosion, flash frozen using liquid nitrogen. Ten flies were collected and combined for each sample used for qPCR. RNA was extracted from each sample of a total of 10 flies prepared as detailed above using a Qiagen RNeasy mini kit and reverse transcribed to cDNA using BioRad iScript™ cDNA synthesis kit for the production of a template for qPCR. Reactions were performed in a 96-well plate format using the Eppendorf Realplex² Mastercycler. Each qPCR reaction contained the following: 12.5µL of BioRad iQ SYBR Green Supermix, 500nM of each primer, cDNA, and filter purified H_2O up to a total volume of 25µL.

For all genes assayed, a standard curve was generated using serial dilutions of the PCR product amplicon using the primers specified for each gene. The PCR amplicons were subject to a cleanup step using a Qiagen PCR purification kit and the amplicon concentrations measured using a NanoDrop®. Total copy number/µL was calculated with the following equation: ([DNA]/molecular weight of fragment)) x (6.023×10^{23}). QPCR

amplification parameters were set at 95˚C (5 min), and the following for 40 cycles 95˚C (15 sec), 55˚C (15 sec) and 68˚C (15 sec). RT-PCR was performed using a 96-well plate. The expression of each gene was quantified and corrected for varying cDNA microgram inputs and was expressed as numbers of copies relative to those measured for Ribosomal protein 32 within the same RNA sample.

Rpl32	forward: 5'-AGCATACAGGCCCAAGATCGTGAA-3' reverse: 5'-TCTGTTGTCGATACCCTTGGGCTT-3'
Desat1	forward: 5'-CGTTGTCCCGCCGATTTGCTTAAT-3' reverse: 5'-TTGAGTGTAGGAGTGGCGAAAGCA-3'
Desat2	forward: 5'-CTTGGTTTGTGGCCACCATGTTCA-3' reverse: 5'-ACTTGTCGTATGGGCGATTTCCGA-3'
PAP	forward: 5'-CAGCGAGCAACAACAACATAC-3' reverse: 5'-GTACTTACCGCAGAGCAAGATTA-3'
DGAT	forward: 5'-GCAGACCGTAAGTAGGGAAATTA-3' reverse: 5'-GGTAAATCCAGAGCTCCAAGAA-3'
Cct1	forward: 5'-GTTCGAGTCGTGAGTCGTATATT-3' reverse: 5'-TCACACCGCCTGATCTTTG-3'
GPAT	forward: 5'-CTCAGCAAACAGCGAGTTTATG-3' reverse: 5'-GGAGGTAGCTGACTGAAAGAAA-3'

Stress Assays

The stress tolerance and survival assays described below were adapted from methods used in the Hoffmann laboratory at the University of Melbourne (Schiffer, Hangartner et al. 2013 and S. Hangartner pers. comm.)

Heat stress assays

Five 5 ml glass vials, each containing a single fly 4-6 day post-eclosion, was sealed and placed in a fish aquarium tank with the water temperature set to 38°C. Flies were then monitored until they dropped to the bottom of the vial and remained immobile. The time it took for them to fall and become immobile was recorded for each fly.

Chill-coma recovery

Flies age 4-6 days post-eclosion in groups of 50 were placed in a sealed 300 ml glass bottle and 4-6 bottles were placed in an ice water bath in a walk-in incubator set at 0°C. Each bottle remained in the ice water bath for 8 h. After removal from the ice water bath, flies were transferred to 25°C room temperature controlled room for recovery time scoring on a white sheet of paper. When a fly recovered (by standing up), the time of recovery was recorded and the fly removed by aspiration. Data were tabulated until all flies recovered with the exception of a few percent that were dead.

Desiccation stress

Flies in groups of 3 were placed in 15 ml assay glass vials topped with gauze in sealed 10-gallon glass tank with silica desiccant covering entire bottom surface for one-inch depth to maintain a low humidity. Each trial with 12 replicate assay vials was initiated with freshly- prepared silica desiccant. Flies were monitored every half an hour. The time to falling to the bottom of the vial and immobility was recorded for each fly until all flies in the tank were immobile (dead).

Statistical analysis

To determine statistical significance, multiple t-tests were performed using GraphPad Prism V6.0 as each of the RANi KD group was compared to control group. All p-values < 0.05 are denoted with *, p-values< 0.01 with ** and p-values <0.001 with ***.

4.3 Results

Assessment of specific gene mRNA abundance in the F1s of RNAi crosses

Gene expression in the controls and targeted RNAi knockdowns of specific genes was measured by quantitative real-time PCR (qPCR) to estimate the extent of mRNA expression. We tested the hypothesis of reduced gene expression corresponding to specific genes. The expression of targeted genes was significantly reduced in the RNAi flies (Fig. 4.2A and B). Desat1 KD expression level in males and females was reduced by 72% and 64% respectively. In Desat2 KD, the expression level for males was reduced by 77% and in females it was reduced by 75%. Cct1 KD expression level in males and females was reduced by 86% and 84%, respectively. Males and females of the GPAT KD exhibited reduced expression of 77% and 39%, respectively. In DGAT KD, expression level for males and females was reduced by 90% and 58% respectively. PAP mRNA was diminished in males and females with expression level reduced by 92% and 61%, respectively.

Knockdowns of lipid biosynthesis enzymes impact on heat tolerances.

In Desat1 KD males and females, as well as Desat2 KD females, there was a significant reduction in heat tolerance time compared to the control group (Fig. 3.3 A and B). In Desat1 KD males, there was a 4.5 minutes reduction of heat tolerance time compared to the control group (Fig 4.3A). In Desat1 KD and Desat2 KD females, heat tolerance time decreased 3.2 minutes and 3.5 minutes, respectively. Additional significant decreases in heat tolerance time were in GPAT KD and DGAT KD females, with a reduction of 4.5 and 3.5 minutes (Fig 4.3B).

For Cct1 KD, DGAT KD and PAP KD males, heat tolerance time was reduced approximately by 2 minutes (Fig. 4.3A). GPAT KD males showed a decreased heat tolerance time of approximately 3 minutes (Fig. 4.3A). In Cct1 KD females, heat tolerance time was decreased by 2.4 minutes (Fig. 4.3B). For PAP KD females, the decrease of tolerance time was approximately 30 seconds (Fig. 4.3B).

Chill-coma recovery after RNAi knockdown of lipid biosynthesis enzymes

All of the lipid biosynthesis gene RNAi knockdowns showed an increase in chill-coma recovery time (Fig. 4.4A and B). Control males and females started recovery soon after being placed at room temperature. The time for control flies to attain 50% recovery was 15 and 13 minutes, respectively. Desat2 KD males and females were impacted to greatest degree with a recovery time starting at about 12 minutes, and taking about 32 minutes for 50% of the flies to recover (Fig. 4.4 A and B). For Desat1 KD males and females, recovery started at about 11 minutes and took about 19 minutes for 50% of the flies to recover (Fig. 4.4 A and B). GPAT KD and Cct1 KD males started recovery at approximately 5 minutes and took about 21 minutes for half the flies to recover (Fig 4.4A). For Desat1 KD and PAP KD males, the flies started recovery at about 11 minutes and took about 22 minutes for 50% of the flies to recover (Fig. 4.3A). For GPAT KD females, recovery started at 6 minutes and took about 20 minutes for half of the flies to recover (Fig. 4.4B). Cct1 KD, DGAT KD, and PAP KD females exhibited a similar trend, with recovery started at 6 minutes and taking about 18 minutes for 50% of the flies to recover (Fig. 4.4B).

Desiccation survival after RNAi knockdown of lipid biosynthesis enzymes

In desiccation survival assays, flies were placed in conditions without food or water source in a tank filled with silica desiccant to reduce the humidity to a low level. Control males survived about 13 hours until half the flies were dead. GPAT KD males were the only group that appeared to have increased (not statistically significant) in survival time taking 13.5 hours until 50% mortality (Fig. 4.5A). In terms of 50% mortality, Desat1 KD males only lasted 8.2 hours and Desat2 KD males survived only 5.8 hours under desiccation (Fig. 4.5A). Cct1 KD and PAP KD males survived for 9.3 hours and 7.5 hours to 50% mortality, respectively (Fig. 4.5A).

Females survived longer under desiccation condition than control females that survived for an average of 26.7 hours until 50% mortality. All RNAi KD of specific lipid biosynthesis genes had reduced survival time (Fig. 4.5B). GPAT KD females survived the longest among the KD groups, with an average survival period of 22.8 hours. Desat2 KD and PAP KD females, on average, survived for about 20 hours (Fig. 4.5B). Desat1 KD and DGAT KD females survived an average of 17.9 and 18.6 hours respectively, and Cct1 KD females only lasted for an average of 12.6 hours under desiccation (Fig. 4.5B).

4.4 Discussion

The considerable difference between Desat1 and Desat2 knockdowns on chill-coma recovery emerged as a highly interesting feature of this study. Initially this discussion will address desaturase studies in *D. melanogaster*: a general perspective on desaturases, environmental studies in other laboratories addressing effects of individual desaturase genes often in the context of stress responses, life history impacts and lipid composition

of knockdowns of both desaturases investigated individually, knockdown effects of the each of the two desaturases on stress tolerance or survival reported in the Results of this chapter. A second noteworthy result is that every gene knockdown showed delayed chill-coma recovery in both males and females. Sexual dimorphism in stress trait vulnerability as a result of lipid biosynthesis gene knockdown was a third notable feature of this research. Desiccation was also highly impacted by lipid biosynthesis gene knockdowns. Females exhibited decreased desiccation survival associated with knockdown of all genes whereas males were impacted by four genes. An even greater instance of sexual dimorphism was exhibited by diminished heat tolerance after lipid biosynthesis gene knockdown. Four out of six genes reduced female heat tolerance, but only gene (Desat1) reduced heat tolerance in males.

General perspectives on Desat1 and Desat2 functions indicate that desaturases are important in mediating lipid structures, therefore, in organization of membrane lipids. Disruption of Desat1 and Desat2 mRNA negatively impacts fatty acid desaturation capability in *D. melanogaster*. A primary function of both desaturases isoform is to introduce the first double bond in the Δ9 position of fatty acids (Dallerac et al. 2000, Keays et al. 2011). These monounsaturated fatty acids are important precursors for the downstream synthesis of other lipids including cuticular, membrane, and storage lipids.

Studies on the biological impacts of desaturases generally considered only one of the two desaturase genes at a time which is important in the context of our studies that investigate both desaturases one at a time. Reduction of Desat1 in the fat body results in smaller pupae and adults with reduced body size, as well as reducing cell size of the fat cells by 26% (Parisi et al. 2013). Desat1 is expressed in tissues involved in the regulation of water

content such as malpighian tubule and rectal papillae which are important for water balances (Bousquet et al. 2012). In a study by Greenberg et al. (2003), differences in alleles of desaturase 2 were suggested to be associated with cold, desiccation and starvation tolerance. In this study, a site-directed gene replacement technique allowed for replacement of a geographically differentiated Desat2 allele from Zimbabwe into another population of *D. melanogaster* (Greenberg et al. 2003). Their results indicated that with the replacement of a Desat2 allele, while the genetic background remains the same, resulted in a significant reduction in cold tolerance, desiccation and starvation resistance (Greenberg et al. 2003). These data suggested that allelic differences in Desat2 are associated with cold and desiccation tolerances. However, this conclusion has been challenged. Coyne and Elwyn (2006) and Greenberg et al (2006), both repeated the study described in Greenberg et al (2003) and found no associations between allelic differences and cold tolerance or starvation and desiccation resistance (Coyne and Elwyn 2006a, 2006b, Greenberg et al. 2006).

In unpublished research (Ko, MS Thesis 2013), the effect of knocking down either Desat1 or Desat2 on life history traits and lipid composition was reported. Importantly, both genes were knocked down one at a time, which introduced a greater gene range that is normally employed in *Drosophila* studies of desaturase effects. Life-history traits such as fecundity and longevity were both negatively impacted by knockdowns of either desaturase genes. Female reproductive output was investigated by quantifying egg count and progeny number (Ko, MS Thesis, Fig. 5 and 6). Based on the thesis data, both Desat1 and Desat2 KD resulted in reduced longevity, with a spike in die-off at day 45 in both sexes (Ko, MS Thesis Fig. 7 and 8). Overall, only a few reductions were observed in

free fatty acid composition when Desat1 KD and Desat2 KD flies were reared on a standard diet. More extensive reductions in fatty acid composition were exhibited when flies were reared on a restricted diet with a minimal amount of fat. Larval survival was strongly impacted by Desat1 and Desat2 KD when reared on a minimal diet. A range of consequences was observed by disruptions of stearoyl-CoA desaturase gene expression, but knockdowns of the two isoforms of desaturases result in a similar pattern of negative life-history and biochemical consequences. Structurally, these two desaturase isoforms were highly conserved, with 94% match using CLUSTAL multiple sequence alignments (Fig. 4.6).

In the present work, there is a notable variation in chill-coma recovery between Desat1 and Desat2 knockdowns. This is the first documentation of the differential phenotypic impact of disruption of isoform gene expression. There is a strong increase in chill-coma recovery time in both sexes of Desat1 and Desat2 KD (Fig 4.4A and B). The effect of Desat2 is much greater than Desat1. The difference in recovery time suggests that there is variation in how these two enzyme isoforms function and the resulting effect on chill-coma recovery.

All knockdowns resulted in a delayed in chill-coma recovery time in both sexes. GPAT and PAP are important in synthesizing DAG, which is an essential intermediate for downstream conversion to triacylglycerol and phospholipids. If the pathway for synthesizing diacylglycerol is impacted, this can result in a reduction of lipids essential for fat storage or membrane structure. Although there are other ways for *Drosophila* to obtain diacylglycerol either by conversion of monoacylglycerol or by ingestion of TAG. The disruption to one of the pathway to synthesized DAG *de novo* may contribute to

impacted downstream production of membrane lipids such as PE. Cold temperature tends to increase PE to PC ratio and by increase PE composition level in cellular membranes (Overgaard et al. 2008). If there is a blockage in PE synthesis, membrane structures will be more rigid and can enter a dysfunction gel state induced by cold temperature. Another gene that would impact membrane structure is Cct1; as its knock down will cause a blockage in PC synthesis, which could result in leaky membranes. Intriguing questions arise from the observation that both sexes showed increased chill-coma recovery time after knockdowns of all lipid synthesis genes. There were no sexually dimorphic effects and all the tested lipid biosynthesis genes slowed chill-coma recovery.

There were sexual dimorphic differences in desiccation survival after knocking down lipid biosynthesis genes. All genes reduced female desiccation survival whereas four of six genes reduced male desiccation survival. For example, GPAT KD only significantly affected females, but both sexes were negatively impacted by PAP KD (Fig. 4.5A and B). Membrane lipids are only marginally associated with differential desiccation stress survival (Tomcala et al. 2006) and storage lipid is not affected by laboratory selection for desiccation survival in *D. melanogaster* or is slightly altered in selected lines (Introduction, Chapter 3).

The effect of knockdown on lipid biosynthesis genes was strongly dimorphic when tolerance to elevated heat was investigated in this study. For heat stress tolerance, the only male knockdown that showed a significant decrease in tolerance period was Desat1, where Desat1 and Desat2 both showed significant decreases of heat tolerance in females. This is another example of the difference between isoforms of desaturase genes, in this case, there was a sexual dimorphism in which in which Desat2 had no effect on males.

DGAT is a key enzyme for converting diacylglycerol to triacylglycerol, which is the major component in storage lipids. Disruption of TAG will negatively impact heat tolerances, as our data showed a slight reduction for heat tolerance time in DGAT KD males and a significant decrease for heat tolerance period for females (Fig. 4.3A and B). As *D. melanogasters* are ectotherms and their metabolic rate are highly impacted by environmental temperature, an increase metabolic rate can deplete energy reservoirs needed for heat stress tolerance. As has been shown that thermal stress can cause significant decreases in the TAG concentration in *D. melanogaster* (Klepsatel et al. 2016). This reduction is apparently a result of fat body cell apoptosis and the phenomenon may be relevant to the results observed in our investigations.

In general, the sexual dimorphism of the vulnerability of females to lipid biosynthesis gene knockdown is an interesting trend. Females have greater levels of lipids, perhaps driven by the requirements of egg production. It is possible that they are more vulnerable to somatic stress when lipid stores needed for both reproduction and stress tolerance are depleted.

4.5 Literature cited

Bazinet, A. L., K. E. Marshall, H. A. MacMillan, C. M. Williams, and B. J. Sinclair. 2010. 'Rapid changes in desiccation resistance in Drosophila melanogaster are facilitated by changes in cuticular permeability', *J Insect Physiol*, 56: 2006-12.

Beller, M., C. Sztalryd, N. Southall, M. Bell, H. Jackle, D. S. Auld, and B. Oliver. 2008. 'COPI complex is a regulator of lipid homeostasis', *PLoS Biol*, 6: e292.

Bousquet, F., T. Nojima, B. Houot, I. Chauvel, S. Chaudy, S. Dupas, D. Yamamoto, and J. F. Ferveur. 2012. 'Expression of a desaturase gene, desat1, in neural and nonneural tissues separately affects perception and emission of sex pheromones in Drosophila', *Proceedings of the National Academy of Sciences of the United States of America*, 109: 249-54.

Buszczak, M., X. H. Lu, W. A. Segraves, T. Y. Chang, and L. Cooley. 2002. 'Mutations in the midway gene disrupt a Drosophila acyl coenzyme A: Diacylglycerol acyltransferase', *Genetics*, 160: 1511-18.

Chippindale, A. K., A. G. Gibbs, M. Sheik, K. J. Yee, M. Djawdan, T. J. Bradley, and M. R. Rose. 1998. 'Resource acquisition and the evolution of stress resistance in Drosophila melanogaster', *Evolution*, 52: 1342-52.

Chown, S. L., J. G. Sorensen, and J. S. Terblanche. 2011. 'Water loss in insects: an environmental change perspective', *J Insect Physiol*, 57: 1070-84.

Cooper, B. S., L. A. Hammad, J. A. Karty, and K. L. Montooth. 2011. 'Evolution of membrane acclimation in experimentally evolved populations of Drosophila melanogaster', *Integrative and Comparative Biology*, 51: E177-E77.

Cooper, B. S., L. A. Hammad, and K. L. Montooth. 2014. 'Thermal adaptation of cellular membranes in natural populations of Drosophila melanogaster', *Funct Ecol*, 28: 886-94.

Coyne, J. A., and S. Elwyn. 2006a. 'Desaturase-2, environmental adaptation, and sexual isolation in Drosophila melanogaster', *Evolution*, 60: 626-27.

Coyne, J. A., and S. Elwyn. 2006b. 'Does the desaturase-2 locus in Drosophila melanogaster cause adaptation and sexual isolation?', *Evolution*, 60: 279-91.

Dallerac, R., C. Labeur, J. M. Jallon, D. C. Knippie, W. L. Roelofs, and C. Wicker-Thomas. 2000. 'A Delta 9 desaturase gene with a different substrate specificity is responsible for the cuticular diene hydrocarbon polymorphism in Drosophila melanogaster', *Proceedings of the National Academy of Sciences of the United States of America*, 97: 9449-54.

Djawdan, M., A. K. Chippindale, M. R. Rose, and T. J. Bradley. 1998. 'Metabolic reserves and evolved stress resistance in Drosophila melanogaster', *Physiol Zool*, 71: 584-94.

Fang, S., C. T. Ting, C. R. Lee, K. H. Chu, C. C. Wang, and S. C. Tsaur. 2009. 'Molecular evolution and functional diversification of fatty acid desaturases after recurrent gene duplication in Drosophila', *Mol Biol Evol*, 26: 1447-56.

Foley, B. R., and M. Telonis-Scott. 2011. 'Quantitative genetic analysis suggests causal association between cuticular hydrocarbon composition and desiccation survival in Drosophila melanogaster', *Heredity (Edinb)*, 106: 68-77.

Gibbs, A. G., A. K. Chippindale, and M. R. Rose. 1997. 'Physiological mechanisms of evolved desiccation resistance in Drosophila melanogaster', *J Exp Biol*, 200: 1821-32.

Gibbs, A. G., and L. M. Matzkin. 2001. 'Evolution of water balance in the genus Drosophila', *Journal of Experimental Biology*, 204: 2331-38.

Goto, S. G. 2000. 'Expression of Drosophila homologue of senescence marker protein-30 during cold acclimation', *J Insect Physiol*, 46: 1111-20.

Greenberg, A. J., J. R. Moran, J. A. Coyne, and C. I. Wu. 2003. 'Ecological adaptation during incipient speciation revealed by precise gene replacement', *Science*, 302: 1754-7.

Greenberg, A. J., J. R. Moran, and C. I. Wu. 2006. 'Proper control of genetic background with precise allele substitution: a comment on Coyne and Elwyn', *Evolution*, 60: 623-5; discussion 26-7.

Gupta, T., and T. Schupbach. 2003. 'Cct1, a phosphatidylcholine biosynthesis enzyme, is required for Drosophila oogenesis and ovarian morphogenesis', *Development*, 130: 6075-87.

Helmink, B. A., and J. A. Friesen. 2004. 'Characterization of a lipid activated CTP: phosphocholine cytidylyltransferase from Drosophila melanogaster', *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids*, 1683: 78-88.

Hoffmann, A. A., M. J. Blacket, S. W. McKechnie, L. Rako, M. Schiffer, R. V. Rane, R. T. Good, C. Robin, and S. F. Lee. 2012. 'A proline repeat polymorphism of the Frost gene of Drosophila melanogaster showing clinal variation but not associated with cold resistance', *Insect Mol Biol*, 21: 437-45.

Jones, H. E., J. L. Harwood, I. D. Bowen, and G. Griffiths. 1992. 'Lipid composition of subcellular membranes from larvae and prepupae of Drosophila melanogaster', *Lipids*, 27: 984-7.

Keays, M. C., D. Barker, C. Wicker-Thomas, and M. G. Ritchie. 2011. 'Signatures of selection and sex-specific expression variation of a novel duplicate during the evolution of the Drosophila desaturase gene family', *Mol Ecol*, 20: 3617-30.

Klepsatel, P., M. Galikova, Y. Xu, and R. P. Kuhnlein. 2016. 'Thermal stress depletes energy reserves in Drosophila', *Sci Rep*, 6: 33667.

Ko, L., Harshman, L.G., Black, P.N., Allen, J.W. Investigation of the life history and lipid metabolic changes of RNAi knockdown of the two major Δ^9 fatty acyl-CoA desaturases of *Drosophila melanogaster.*

Lykidis, A., K. G. Murti, and S. Jackowski. 1998. 'Cloning and characterization of a second human CTP:phosphocholine cytidylyltransferase', *J Biol Chem*, 273: 14022-9.

Macmillan, H. A., and B. J. Sinclair. 2011. 'Mechanisms underlying insect chill-coma', *J Insect Physiol*, 57: 12-20.

Marron, M. T., T. A. Markow, K. J. Kain, and A. G. Gibbs. 2003. 'Effects of starvation and desiccation on energy metabolism in desert and mesic Drosophila', *J Insect Physiol*, 49: 261-70.

Matzkin, L. M., and T. A. Markow. 2009. 'Transcriptional regulation of metabolism associated with the increased desiccation resistance of the cactophilic Drosophila mojavensis', *Genetics*, 182: 1279-88.

Neven, L. G. 2000. 'Physiological responses of insects to heat', *Postharvest Biology and Technology*, 21: 103-11.

Overgaard, J., J. G. Sorensen, S. O. Petersen, V. Loeschcke, and M. Holmstrup. 2005. 'Changes in membrane lipid composition following rapid cold hardening in Drosophila melanogaster', *J Insect Physiol*, 51: 1173-82.

Overgaard, J., J. G. Sorensen, S. O. Petersen, V. Loeschcke, and M. Holmstrup. 2006. 'Reorganization of membrane lipids during fast and slow cold hardening in Drosophila melanogaster', *Physiological Entomology*, 31: 328-35.

Overgaard, J., A. Tomcala, J. G. Sorensen, M. Holmstrup, P. H. Krogh, P. Simek, and V. Kostal. 2008. 'Effects of acclimation temperature on thermal tolerance and membrane phospholipid composition in the fruit fly Drosophila melanogaster', *J Insect Physiol*, 54: 619-29.

Parisi, F., S. Riccardo, S. Zola, C. Lora, D. Grifoni, L. M. Brown, and P. Bellosta. 2013. 'dMyc expression in the fat body affects DILP2 release and increases the expression of the fat desaturase Desat1 resulting in organismal growth', *Dev Biol*, 379: 64-75.

Parkash, R., P. Ranga, and D. D. Aggarwal. 2014. 'Developmental acclimation to low or high humidity conditions affect starvation and heat resistance of Drosophila melanogaster', *Comp Biochem Physiol A Mol Integr Physiol*, 175: 46-56.

Scheitz, C. J. F., Y. Guo, A. M. Early, L. G. Harshman, and A. G. Clark. 2013. 'Heritability and Inter-Population Differences in Lipid Profiles of Drosophila melanogaster', *PLoS One*, 8.

Schiffer, M., S. Hangartner, and A. A. Hoffmann (2013). "Assessing the relative importance of environmental effects, carry-over effects and species differences in

thermal stress resistance: a comparison of Drosophilids across field and laboratory generations." J Exp Biol **216**(Pt 20): 3790-3798.

Schmidt, P. S., L. Matzkin, M. Ippolito, and W. F. Eanes. 2005. 'Geographic variation in diapause incidence, life-history traits, and climatic adaptation in Drosophila melanogaster', *Evolution*, 59: 1721-32.

Schupbach, T., and E. Wieschaus. 1991. 'Female Sterile Mutations on the 2nd Chromosome of Drosophila-Melanogaster .2. Mutations Blocking Oogenesis or Altering Egg Morphology', *Genetics*, 129: 1119-36.

Takahashi, A., S. C. Tsaur, J. A. Coyne, and C. I. Wu. 2001. 'The nucleotide changes governing cuticular hydrocarbon variation and their evolution in Drosophila melanogaster', *Proc Natl Acad Sci U S A*, 98: 3920-5.

Telonis-Scott, M., C. M. Sgro, A. A. Hoffmann, and P. C. Griffin. 2016. 'Cross-Study Comparison Reveals Common Genomic, Network, and Functional Signatures of Desiccation Resistance in Drosophila melanogaster', *Mol Biol Evol*, 33: 1053-67.

Terblanche, J. S., A. A. Hoffmann, K. A. Mitchell, L. Rako, P. C. le Roux, and S. L. Chown. 2011. 'Ecologically relevant measures of tolerance to potentially lethal temperatures', *J Exp Biol*, 214: 3713-25.

Tilley, D. A., C. R. Evans, T. M. Larson, K. A. Edwards, and J. A. Friesen. 2008. 'Identification and Characterization of the Nuclear Isoform of Drosophila melanogaster CTP:Phosphocholine Cytidylyltransferase', *Biochemistry*, 47: 11838-46.

Tomcala, A., M. Tollarova, J. Overgaard, P. Simek, and V. Kostal. 2006. 'Seasonal acquisition of chill tolerance and restructuring of membrane glycerophospholipids

in an overwintering insect: triggering by low temperature, desiccation and diapause progression', *J Exp Biol*, 209: 4102-14.

Tsvetkova, N. M., I. Horvath, Z. Torok, W. F. Wolkers, Z. Balogi, N. Shigapova, L. M. Crowe, F. Tablin, E. Vierling, J. H. Crowe, and L. Vigh. 2002. 'Small heat-shock proteins regulate membrane lipid polymorphism', *Proc Natl Acad Sci U S A*, 99: 13504-9.

Wicker-Thomas, C., and J. Jallon. 2000. 'Role of Enhancer of zeste on the production of Drosophila melanogaster pheromonal hydrocarbons', *Naturwissenschaften*, 87: 76-9.

4.6 Figures

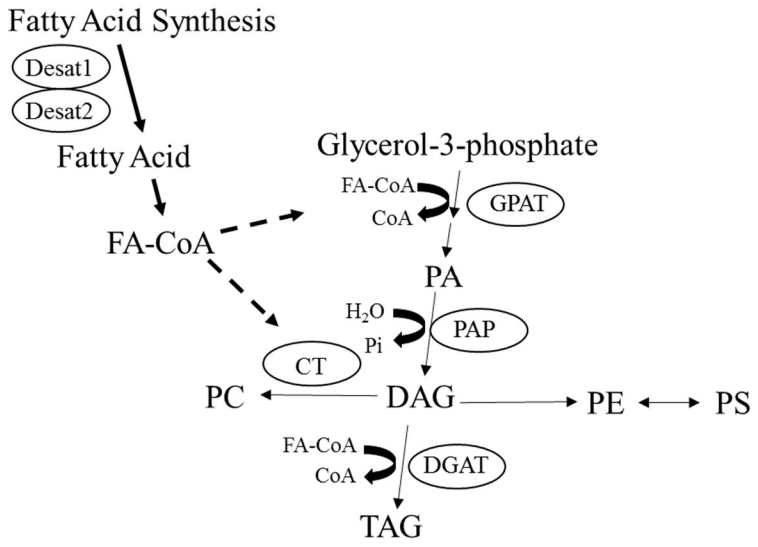

Figure 4.1. Schematic pathway for *D. melanogaster* lipid biosynthesis.

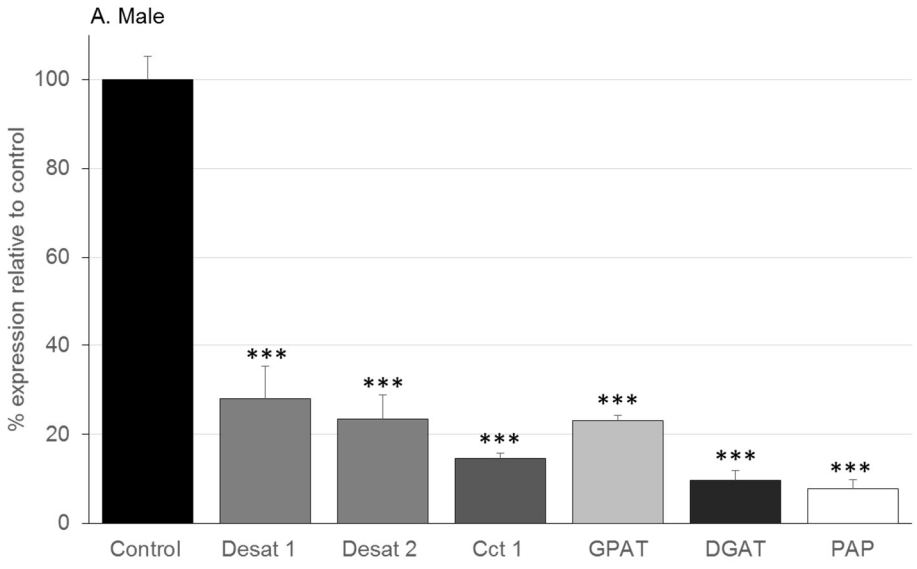

Figure 4.2A. Reduction of each targeted RNAi KD expression in the male of the knockout lines estimated using qPCR. Expression data were normalized against Rpl 32 and are presented as percentage expression relative to control; error bars represent the SE (n = 3); $p<0.001$ notated with ***.

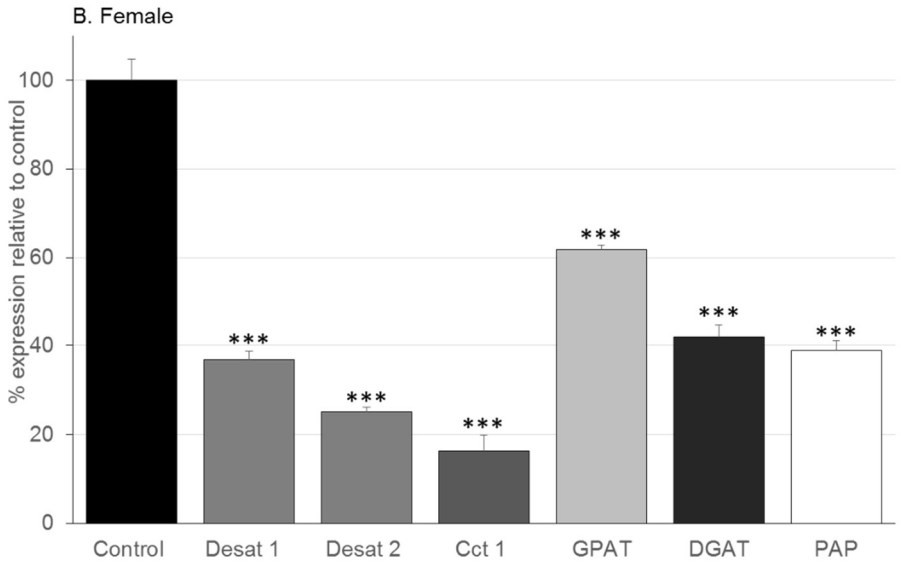

Figure 4.2B. Reduction of each targeted RNAi KD expression in the female of the knockout lines estimated using qPCR. Expression data were normalized against Rpl 32 and are presented as percentage expression relative to control; error bars represent the SE (n = 3); $p<0.001$ notated with ***.

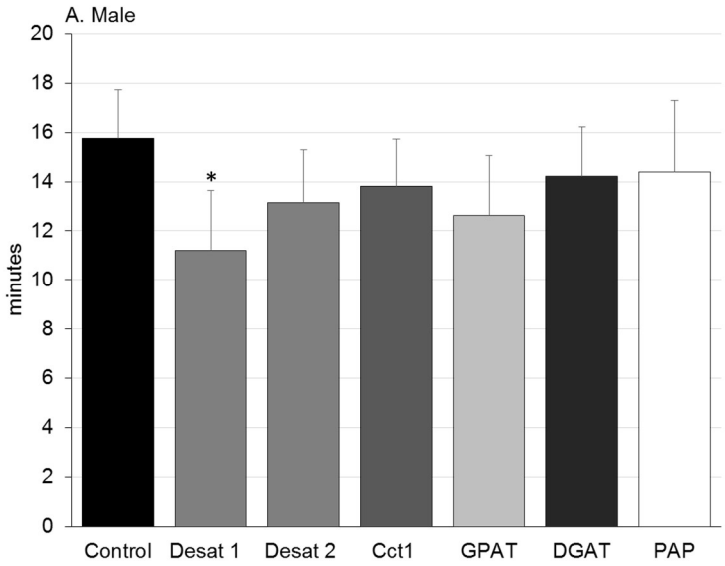

Figure 4.3A. Heat stress assay in males, error bars represent the SE (n = 3); $p<0.05$ notated with *.

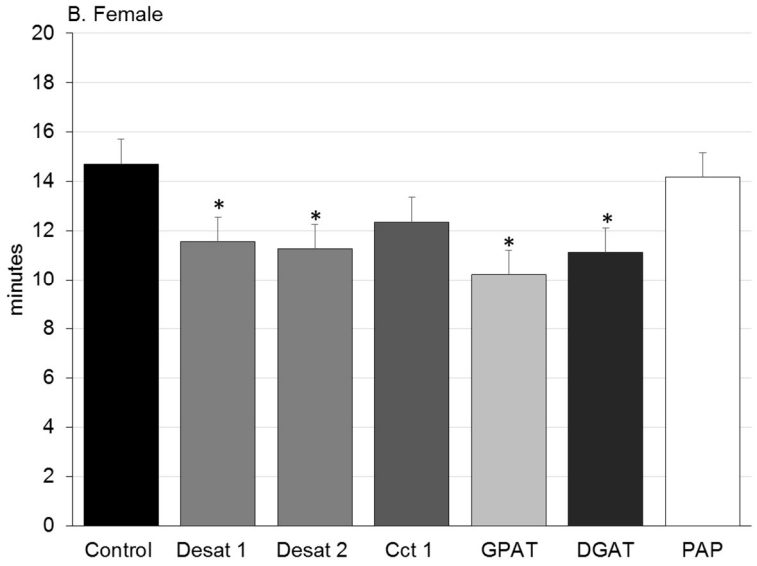

Figure 4.3B. Heat stress assay in males, error bars represent the SE (n = 3); $p<0.05$ notated with *

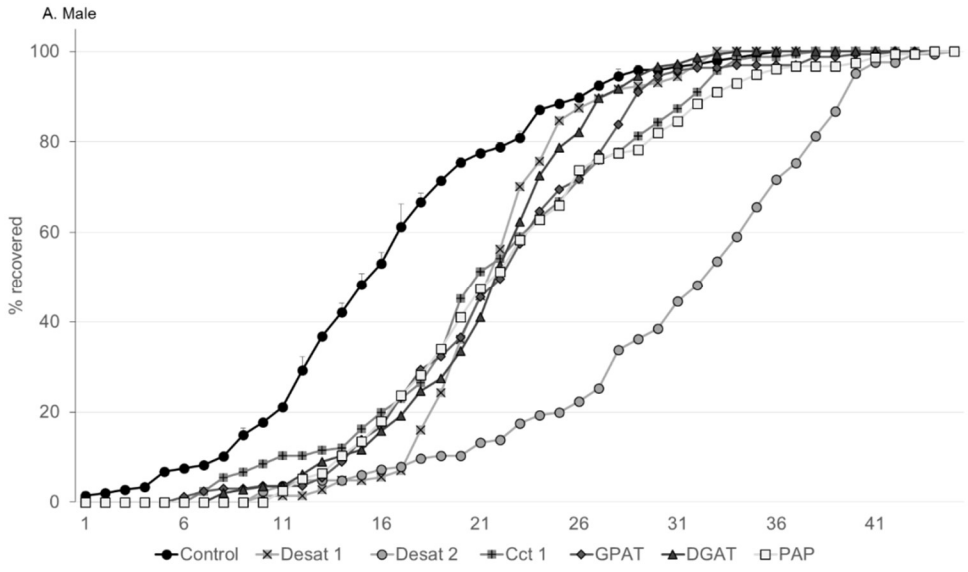

Figure 4.4A. Chill-coma recovery for males, error bars represent the SE (n = 3).

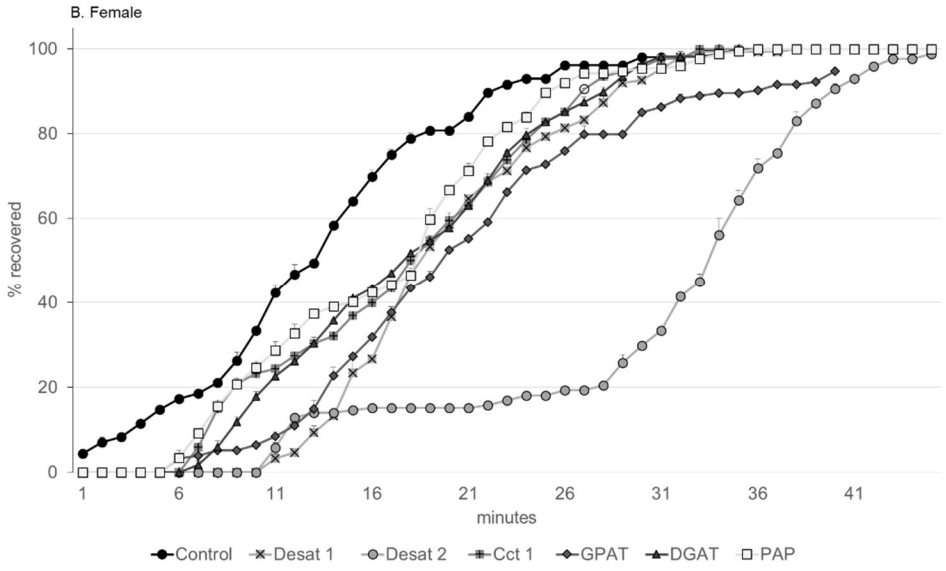

Figure 4.4B. Chill-coma recovery for females, error bars represent the SE (n = 3).

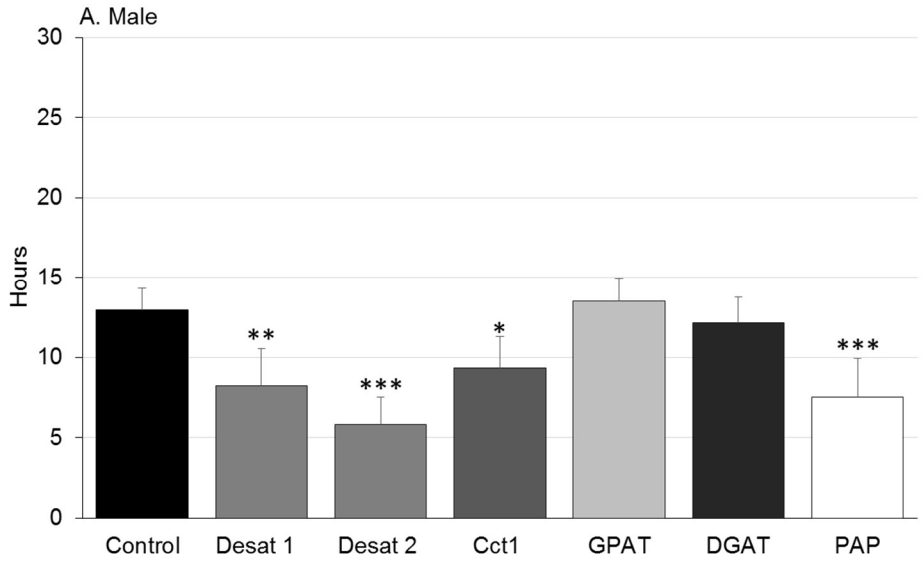

Figure 4.5A. Desiccation Stress assay for males, error bars represent the SE (n = 3); *p<0.05 notated with *, p<0.01 notated with **, p<0.001 notated with ***.

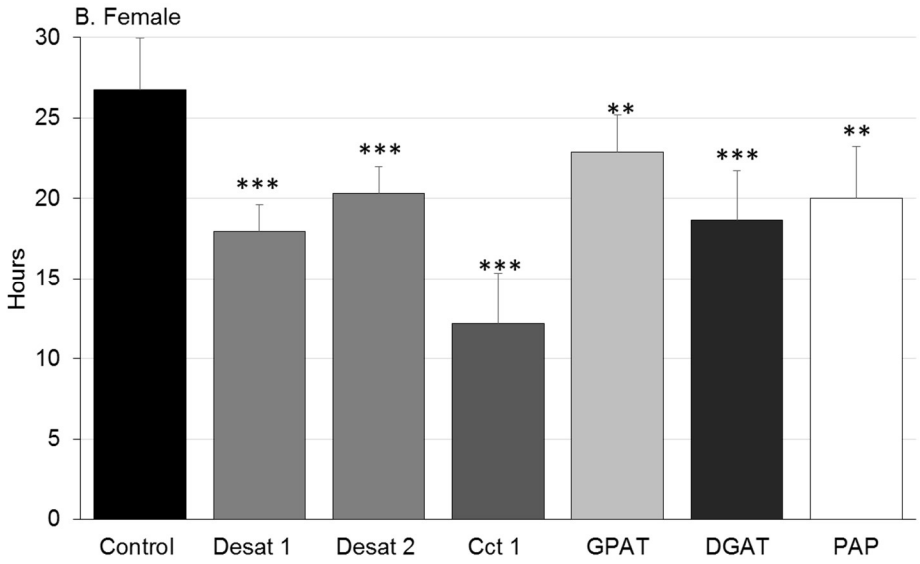

Figure 4.5B. Desiccation Stress assay for females, error bars represent the SE (n = 3); $p<0.01$ notated with **, $p<0.001$ notated with ***.

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Desat1;      MPPNAQAGAQSISDSLIAAASAAADAGQSPTKLQEDSTGVLFECDVETTDGGLVKDITVM
Desat2;      MAPYSRIYHQDKSSR--------------------ETGVLFEDDAQTVDGDLTTDRFQL
             *.* :.   *. *.                     .****** *.:*.**.*..*   :

Desat1;      KKAEKRRLKLVWRNIIAFGYLHLAALYGAYLMVTSAKWQTCILAYFLYVISGLGITAGAH
Desat2;      KRAEKRRLPLVWRNIILFALVHLAALYGLHSIFTRAKLATTLFAAGLYIIGMLGVTAGAH
             *.****** ******* *. :******* : :.* **  * ::*  **:*. **:*****

Desat1;      RLWAHRSYKAKWPLRVILVIFNTIAFQDAAYHWARDHRVHHKYSETDADPHNATRGFFFS
Desat2;      RLWAHRTYKAKWPLRLLLVIFNTIAFQDAVYHWARDHRVHHKYSETDADPHNATRGFFFS
             ******:********::************.******************************

Desat1;      HVGWLLCKKHPEVKAKGKGVDLSDLRADPILMFQKKYYMILMPIACFIIPTVVPMYAWGE
Desat2;      HVGWLLCKKHPDIKEKGRGLDLSDLRADPILMFQRKHYYILMPLACFVLPTVIPMVYWNE
             ***********::* **.*:**************.*:* ****:***::***:**  *.*

Desat1;      SFMNAWFVATMFRWCFILNVTWLVNSAAHKFGGRPYDKFINPSENISVAILAFGEGWHNY
Desat2;      TLASSWFVATMFRWCFQLNMTWLVNSAAHKFGNRPYDKTMNPTQNAFVSAFTFGEGWHNY
             :: .:*********** **:************.***** :**::*  *: ::********

Desat1;      HHVFPWDYKTAEFGKYSLNFTTAFIDFFAKIGWAYDLKTVSTDIIKKRVKRTGDGTHATW
Desat2;      HHAFPWDYKTAEWGCYSLNITTAFIDLFAKIGWAYDLKTVAPDVIQRRVLRTGDGSHELW
             **.*********:* ****:******:*************:.*:*:.** *****:*  *

Desat1;      GWGDVDQPKEEIEDAVITHKKSE
Desat2;      GWGDKDLTAEDARNVLLVDKSR-
             **** * . *:  :.::. *.
```

Figure 4.6. CLUSTAL multiple sequence alignment by MUSCLE (3.8)