ABSTRACT

Lipid Studies in Breath-Hold Diving Mammals and Obese, Pre-diabetic Mice

Kathryn Young, Ph.D.

Mentor: Myeongwoo Lee, Ph.D.

Lipidology, or the scientific investigation of cellular lipids in biological systems, as well as their pathways and networks, is becoming an increasingly integral component in the investigation of metabolic disease and, outside the healthcare fields, in the elucidation of adaptations to specific environmental stressors. Sphingolipid and whole phospholipid profiles were examined in several species of marine mammals (and in two tissue types in one marine mammal species), as well as in the skeletal muscle of obese, pre-diabetic mice. The fatty acid composition of the cells (the sum total of intracellular lipid and the fatty acids of membrane phospholipids) was also examined in obese, pre-diabetic skeletal muscle of the mouse.

Marine mammal species were compared based on diving ability. Shallower divers (harbor seals, *Phoca vitulina*) demonstrated significantly higher C16 ceramide, 16:0 sphingomyelin, and dipalmitoylphosphatidylcholine (DPPC) levels compared to deeper divers (Weddell seals, *Leptonychotes weddellii*). Additionally, in comparing ringed seal (*Pusa hispida*) skeletal muscle to liver tissue, liver tissue showed significantly higher levels of C16:0 sphingomyelin, dipalmitoylphosphatidlycholine, and C16

ceramide. Pre-diabetic mice were found to have no significant changes in sphingolipids or whole phospholipids after high-fat diet (HFD) feeding compared to controls, but had statistically significant elevations of PUFAs (particularly omega-6s), and statistically significant declines in MUFAs.

These results suggest that harbor seals are under greater stress than Weddell seals, based on upregulated levels of C16 ceramide and 16:0 sphingomyelin, and this increased stress likely results from an increased time at depth. Likewise, the liver of ringed seals shows significant signs of physiological stress, but here, the difference may be one of varying blood flow between the liver and skeletal muscle during diving. DPPC levels were higher in ringed seal liver, as well as harbor seal skeletal muscle, suggesting these tissues are more susceptible to hydrostatic pressure-related complications. Finally, the HFD-fed mice demonstrated a trend toward pro-inflammatory fatty acid pools with an increase in PUFAs, and specifically N6s. The results reported here contribute to our knowledge of the importance of biological membranes and the composition of fatty acid pools within cells to healthy physiological functioning.

Lipid Studies in Breath-Hold Diving Mammals and Obese, Pre-diabetic Mice

by

Kathryn Young, B.S., M.S.

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Approved by the Dissertation Committee

_____

Myeongwoo Lee, Ph.D., Chairperson

_____

Ann E. Rushing, Ph.D.

_____

Bessie W. Kebaara, Ph.D.

_____

Cheolho Sim, Ph.D.

_____

Dennis A. Johnston, Ph.D.

_____

Thomas J. Hawke, Ph.D.

Accepted by the Graduate School

December 2013

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J. Larry Lyon, Ph.D., Dean

Page bearing signatures is kept on file in the Graduate School.

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HIF-1α	hypoxia-inducible factor-1alpha
ATP	adenosine triphosphate
ADL	aerobic dive limit
PUFAs	polyunsaturated fatty acids
MUFAs	monounsaturated fatty acids
SFAs	saturated fatty acids
ROS	reactive oxygen species
S1P	sphingosine-1-phsophate
HFD	high-fat diet
T2DM	type 2 diabetes mellitus
GLUT4	glucose transporter 4
IRS	insulin-receptor substrate
PI(3)K	phosphatidylinositol-3-OH kinase
MAP	mitogen-activated protein
FFA	free fatty acid
LC-ACoA	long chain fatty acyl-CoA
DAG	diacylglycerol
TAG	triacylglycerol
PKB	protein kinase B
PP2A	protein phosphatase 2A
TNF-α	tumor necrosis factor α
SPT	serine palmitoyltransferase
IGT	impaired glucose tolerance
PGE_2	prostaglandin E2
COX-2	cyclooxygenase-2
N3	omega-3 fatty acids
N6	omega-6 fatty acids
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FasL	Fas ligand
LASS	longevity assurance
SPLs	sphingolipids
PLs	phospholipids
LC-HRMS	liquid chromatography-high resolution mass spectrometry
PE	phosphatidylethanolamine
PC	phosphocholine
PG	phosphatidylglycerol
SM	sphingomyelin
PS	phosphatidylserine
$CHCl_3$	chloroform
MeOH	methanol

PTFE	polytetrafluoroethylene
GC	gas chromatography
LTQ	linear trap quadropole
ESI	electrospray ionization
FWHM	full width at half maximum
a.u.	arbitrary units
HCOOH	formic acid
CCV	continuous calibration verification
MS/MSD	matrix spike/matrix spike duplicate
DPPC	dipalmitoylphosphatidylcholine
HOAD	β-hydroxyacyl-CoA
CS	citrate synthase
LDH	lactate dehydrogenase
IMCL	intramyocellular lipid
IPGTT	intraperitoneal glucose tolerance test
GC-FID	gas chromatography-flame ionization detector
DCM	dichloromethane
FAME	fatty acid methyl ester
IMTG	intramuscular triglyceride
PCR	polymerase chain reaction
TLC	thin layer chromatography
NFS	Northern fur seal
SSL	Steller sea lion
GC-MS	gas chromatography-mass spectrometry
ANOVA	analysis of variance
HPLC	high-performance liquid chromatography
NMR	nuclear magnetic resonance
EI	electron ionization
FA	fatty acid
NIST	National Institute of Standards and Technology
SI	similarity index
HSD	honestly significant difference

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CHAPTER ONE

Introduction

Marine Mammal Studies Rationale

Marine mammals represent a diverse assemblage of species which depend on the ocean for most or all of their life histories. As a group, marine mammals have accumulated a suite of adaptations which allow them to cope with the unique stressors of living in the ocean. These adaptations include increased insulation, increased blood volume, hematocrit, and red blood cell mass, countercurrent heat exchange systems, elevated levels of antioxidants, and sensory adaptations allowing for communication through high frequency sounds (Zenteno-Savin et al. 2002; Berta et al. 2006). Likely the most well-known adaptation of marine mammals, although conserved across all mammalians, is the dive response, characterized by selective peripheral vasoconstriction, bradycardia, and blood shift to the thoracic cavities (Elsner 1969). These adaptations, allowing for extended dive bouts and foraging while breath-holding, make marine mammals unique in that they can routinely push the limits of their physiological capacity.

While these adaptations offer extended dive times, marine mammals are still subject to limited oxygen stores, and lengthy or repetitive dive bouts often result in tissue-level hypoxia (Johnson et al. 2004; Johnson et al. 2005; Vazquez-Medina et al. 2011). Due to selective shunting of blood to, in particular, the central nervous system, many marine mammal organs, including the skeletal muscle, become hypoxic during diving due to reduced oxygen stores (Elsner 1999). In addition, though hypoxia-inducible factor-1alpha (HIF-1α) levels have not been measured for these tissues, blood

flow is severely restricted to the liver, kidneys, and gastrointestinal tract (Davis and Kanatous 1999). Hypoxia presents a potentially lethal situation for most mammals, as does the related reperfusion of tissues which typically follows these ischemic events. Diving mammals, however, have shown remarkable resistance to this physiological condition. In fact, they show very little evidence of pathological damage (e.g., lipid peroxidation or protein carbonyls) from dive-associated ischemia-reperfusion and resulting oxidative stress (Elsner et al. 1998; Wilhem Filho et al. 2002; Zenteno-Savin et al. 2002; Vazquez-Medina et al. 2007). For example, White et al. (1990) subjected harbor seal (*Phoca vitulina*) and common pig (*Sus scrofa*) to acute hypoxia until such point as cessation of cardiac output occurred. The harbor seal heart recovered rapidly, but the pig heart was not able to be revived. Clearly, marine mammals have a unique ability to protect themselves from oxidative damage.

Johnson et al. (2004) first identified HIF-1α in ringed seal (*Phoca hispida*) tissue, and then compared its expression across various tissue types in the seal, alongside levels of oxidized proteins in these same tissues. Within the ringed seal, the only marine mammal to have been thoroughly investigated in terms of HIF-1α expression to date, HIF-1α levels were significantly higher in the lung and skeletal muscle, and lower in the heart muscle and liver (Johnson et al. 2004). Upregulated levels of HIF-1α indicate hypoxia, as this protein is preferentially expressed under hypoxic conditions as a protective mechanism against oxidative stress (Semenza 2001; Bilton and Booker 2003), but degraded by proteases under normoxia (Maxwell et al. 1999; Pereira et al. 2003). Additionally, levels of oxidized protein levels were lowest in the lung and heart tissue, and higher in skeletal muscle. These results likely indicate an attempt by the organism to

ward off injury due to ischemia-reperfusion of these tissues associated with repetitive diving bouts, and indicate some level of protective success in that lung tissue revealed upregulated HIF-1α but low levels of oxidized proteins (Johnson et al. 2004). In addition to coping with hypoxia, marine mammals undergo carbon dioxide and lactate accumulation in the blood and muscle during diving, inducing acidosis over time (Berta et al. 2006). Deep-diving species such as sperm whales (*Physeter catodon*) and southern elephant seals (*Mirounga leonina*) can dive to over 1000 m and remain submerged for over 2 hours, while shallower divers, such as hooded seals (*Cystophora cristata*) undergo oxygen deprivation for bouts of 5 to 25 minutes (Hindell et al. 1991; Kooyman and Kooyman 1995; Folkow and Blix 1999).

Deep-diving species, especially, have developed physiological adaptations in their skeletal muscle, among other tissues, which allow them to sustain an aerobic, lipid-based metabolism under these hypoxic conditions (Kanatous et al. 1999). The cellular mechanisms utilized by marine mammals to produce adenosine triphosphate (ATP) from macromolecules are no different from those used by terrestrial mammals; however, since marine mammal diets are typically composed of solely lipids and protein (while terrestrial mammals often incorporate carbohydrates), these animals have evolved the capacity to exercise aerobically based only on lipids as a fuel source (Trumble et al. 2003). Due to the extensive dives of certain species, marine mammals must ration their stored oxygen during dives while dealing with very high concentrations of lactate produced during anaerobic glycolysis. Terrestrial mammals would find similar levels of lactate production and consequent acidosis physiologically disruptive or even lethal (Berta et al. 2006). In order to counter the decreasing pH resulting from anaerobic

metabolism and lactate production, marine mammals have developed enhanced non-carbonate plasma buffering capacities in order to hold tissue pH levels relatively constant (Boutilier et al. 1993). These metabolic adaptations during diving increase an animal's aerobic dive limit (ADL), which is the longest dive that an animal can make while relying primarily on oxygen stores in the blood (hemoglobin) and muscle (myoglobin) to sustain aerobic metabolism (Kooyman et al. 1983). Previous studies have also indicated additional adaptations to energy and oxygen utilization in skeletal muscle, which include: 1) an increased aerobic capacity (depending upon the exercise intensity required), 2) a reliance on fatty acid catabolism for aerobic ATP production, 3) enhanced oxygen storage and diffusion capacity, including higher hematocrit levels, and 4) a reduced dependency on blood-borne oxygen and metabolites compared to terrestrial mammals (e.g., decreased capillary density) (Davis et al. 1991; Davis and Kanatous 1999; Kanatous et al. 1999; Wenger et al. 2000; Polasek and Davis 2001). Additionally, the large body mass of most marine mammals is considered an adaptation because increased body size is correlated with decreased metabolic rate, another mechanism to conserve oxygen (Ramirez et al. 2007).

Metabolic rate, despite the multivariate complexities in its determination, is rather predictable among endotherms based entirely on relative body mass (Hulbert 2007). Recent research suggests that the fatty acid composition of tissue phospholipids varies systematically among species, and that this composition largely determines the metabolic rate characteristic of each species (Hulbert et al. 2007). In general, Hulbert (2007) found that as species size increases, there is a decrease in mass-specific metabolic rate and a decrease in membrane polyunsaturation. Membrane-associated processes are significant

energy sinks, and experiments (e.g., Turner et al. 2003) have shown that polyunsaturated membranes are associated with fast membrane-associated processes. This hypothesis has been coined the 'membrane pacemaker theory of metabolism', and appears to hold true for many endotherms (Hulbert 2007). Marine mammals were not among the species examined during the development of this hypothesis, but they may represent an exception to the rule. Based on previous energetic studies involving marine mammals (Singer et al. 1993; Berta et al. 2006), it appears most species have higher metabolic rates than would be predicted based upon the Kleiber curve. Pinnipeds appear to have metabolic rates 1.5 to 3 times greater than those of similarly-sized terrestrial mammals (Kooyman 1981), while cetaceans also fall significantly above the expected Kleiber metabolic rate (Irving et al. 1941; Pierce 1970; Hampton et al. 1971; Ridgway and Patton 1971; Kasting et al. 1989). Therefore, based on the hypothesis mentioned above, marine mammal membranes should be largely characterized by polyunsaturated fatty acids (PUFAs). Indeed, recent research suggests at least some agreement with this hypothesis (e.g., Weddell seal pups). PUFAs in these organisms are increased in skeletal muscle membranes in the younger animals, later converting to larger proportions of monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs) (Trumble and Kanatous 2012). This adaptation may allow pups to keep warm in these extreme polar climates by helping to maintain a high metabolism, while the reduction in PUFAs as Weddell seals age may help to offset rates of reactive oxygen species (ROS) production during sustained lipid metabolism and reperfusion of peripheral skeletal muscle after diving bouts (Trumble and Kanatous 2012).

Marine mammals, in general, consume a diet high in PUFAs (Budge et al. 2006), so these fats could easily be incorporated directly into cell membranes (Trumble et al. 2010). Additionally, studies have shown that significant hydrostatic pressure, such as that affecting marine mammals as they dive, can induce changes in the lipid composition of cellular membranes (Chen et al. 1994; Barshtein et al. 1997). Polyunsaturated fatty acids in the cell membranes increases membrane fluidity making the composition advantageous in animals subjected to high pressures during deep dives (Hazel and Williams 1990). In other words, membranes composed largely of PUFAs would remain more fluid at depth than would those composed of mostly SFAs. However, should marine mammal membranes be chiefly comprised of PUFAs, these membranes would then be subject to high levels of oxidative stress, specifically lipid peroxidation (Tsalouhidou et al. 2006). Exposure to hypoxia results in an increase in intracellular ROS, which in turn becomes crucial for the maintenance of oxygen homeostasis, such that ROS are abundantly formed and can serve as signaling molecules that help to maintain homeostasis. Interestingly, restoration of blood flow following a prolonged dive bout (i.e., reperfusion) has also been linked to the generation of ROS, which in turn can lead to tissue damage through induction of apoptosis (Gottlieb et al. 1994). It has been reported that the increased ROS production resulting from hypoxia may damage the PUFAs in cellular membranous structures causing a reduction in fluidity, permeability, and excitability which ultimately may alter the function of membrane-bound enzymes (Chan et al. 1985; Buettner 1993). For example, in rodent cells with decreased membrane fluidity, the membrane fails to maintain tonic gradients often resulting in neurological pathologies frequently associated with high altitude sickness (Rodnekov et

al. 2005). Prolonged periods of oxidative stress and lipid peroxidation are known to result in the degradation of membrane integrity, and eventually, cell death. This is a tradeoff marine mammals must continually confront which is not usually present in terrestrial species.

Another suite of characteristics marine mammals are likely to utilize to adapt to a diving lifestyle, beyond what might be implied by Hulbert (2007), are unique, complex lipid compositions in membranes and cell signaling pathways. While marine mammal membrane lipid compositions are clearly influenced by diet (Budge et al. 2006), studies in rats have shown that chronic, intermittent hypoxia (common in marine mammals) can alter the fatty acid profile of both nervous and myocardial tissue (Mourek et al. 1992; Jezkova et al. 2002). Lipids are an important component of not only fuel use and membrane compositions, but they also serve as critical cell signaling initiators or as secondary messengers in signal transduction pathways (Pfeilschifter and Huwiler 2000; Hannun and Obeid 2002; Futerman and Hannun 2004; Hannun and Obeid 2008). Cell signaling pathways are increasingly found to involve a class of lipids known as sphingolipids. These molecules are comprised of a backbone sphingoid base, and depending upon the species of sphingolipid, another hydrocarbon chain and a headgroup, the latter of which largely defines the chemistry of the molecule (Zheng et al. 2006). Sphingolipids act as both structural components of cell membranes and also as signaling molecules capable of inducing physiological processes as diverse as cell apoptosis, differentiation, proliferation, and cell cycle arrest (Maceyka et al. 2002; Spiegel and Milstien 2003; Ogretmen and Hannun 2004; Bruni and Donati 2008; Hannun and Obeid

2008; Haynes et al. 2009). Most recent research into sphingolipids has focused on a single class, the ceramides.

Ceramides, a group of bioactive sphingolipids, are now widely known to indicate and regulate biological responses to stressors, and can initiate signaling cascades involving both protein kinases and protein phosphatases which result in cell senescence and apoptosis (Hannun and Obeid 2002; Ruvolo 2003; Ogretmen and Hannun 2004). Ceramide accumulation is spurred by cytokines, growth factors, and other environmental stress stimuli which then employ the sphingomyelin-signaling pathway, resulting in the generation of ceramides (Pfeilschifter and Huwiler 2000). One example of an environmental stressor highly relevant to diving marine mammals is the production of ROS. ROS signaling is largely thought to operate through sphingolipid metabolites (Maceyka et al. 2007), and so the levels of ceramides present in the cellular membranes of marine mammals may indicate the levels to which these mammals are susceptible to the extreme stressors present in their environments. Another sphingolipid class, sphingosine-1-phosphate (S1P), has opposing effects to those of ceramides, and often promotes cell proliferation and survival (Maceyka et al. 2007). The ratio, then, of S1P to ceramides in the cell membranes may actually better illustrate the current stress level of the organism under question. Marine mammals may be characterized by unique sphingolipid membrane signatures based on their individual environmental stressors and are likely to employ distinctive cell signaling pathways, inferred by these sphingolipid compositions, compared to terrestrial mammals.

Arguably, the most pressing environmental stressor to which marine mammals must adapt is chronic, intermittent hypoxia during dives. Interestingly, hypoxia may

induce the production of a variety of lipids, including sphingolipids, as second messengers in adaptive cell signaling pathways (Huwiler and Pfeilschifter 2006). Sphingolipid concentrations and compositions in cell membranes may also be influenced by hypoxic conditions. Recent research in mammalian smooth muscle has shown that hypoxia can induce an increase in cellular S1P and a decrease in ceramide due to hypoxia-inhibited sphingomyelinase activity (Yun and Kester 2002). Other studies have shown increases in ceramide levels as a result of hypoxia in renal tubular epithelial cells of rats (Basnakian et al. 2005) and in cardiomyocytes (Bielawska et al. 1997). Chronic, intermittent hypoxia is likely to induce similar changes in marine mammal sphingolipid profiles, and the direction and composition of these changes may shed light on the ways in which these organisms are coping with this environmental stressor. Additionally, lipid profiles are likely to be organ-specific, as each organ system in a marine mammal may be influenced by different variables of the extreme environment in which these animals live (e.g., high pressures, increased levels of ROS, cold temperatures, chronic, intermittent hypoxia, differential vasoconstriction, etc.).

Marine mammals thus provide an ideal model for studies involving hypoxic stress and lipid signaling. These mammals have adapted to, and thrive in, extreme environments and conditions which would prove pathological for terrestrial mammals. For example, chronic, intermittent hypoxia in humans is correlated, among other conditions, with obstructive sleep apnea and cardiovascular disease (Semenza 2009). The mechanisms through which marine mammals avoid the oxidative stress which plagues other mammalian species under similar circumstances are extremely interesting, and the results of these investigations may provide clues as to better treatments for human

and veterinary pathologies involving periods of hypoxia or significant lipid signaling. The fact that marine mammals do not exhibit an abnormally shortened life span is indicative that these organisms are well equipped to deal with the physiological stress of chronic, intermittent hypoxia (Johnson et al. 2005). A complete lipid profile of marine mammal membranes, including sphingolipids, those domains integral to cell signaling, has never before been characterized in this group of organisms. This dissertation, in part, utilized a shotgun approach to lipidomics of marine mammal membranes and intracellular lipid, and while the characterization is, as yet, not fully complete, the information discovered here presents interesting indications of some of the changes inherent in marine mammal tissue compared to that of typical terrestrial mammals.

Marine Mammal Studies Specific Aims/Hypotheses

Specific Aim I: To test the hypotheses that marine mammal sphingolipid and whole phospholipid profiles in skeletal muscle will correlate with diving ability.

H_{01}: The skeletal muscle whole phospholipid profiles will not differ between deep-diving Weddell seals (*Leptonychotes weddellii*) and shallow-diving harbor seals (*Phoca vitulina*).

H_{02}: The skeletal muscle sphingolipid profiles will not differ between deep-diving Weddell seals and shallow-diving harbor seals.

Specific Aim II: To test the hypotheses that ringed seal (*Pusa hispida*) sphingolipid and whole phospholipid profiles will differ between skeletal muscle and liver tissue.

H_{01}: Ringed seal skeletal muscle whole phospholipid and sphingolipid profiles will not differ from those of liver whole phospholipid and sphingolipid profiles in the same species.

H_{02}: Ringed seal skeletal muscle sphingolipid and whole phospholipid profiles will not differ from those of liver sphingolipid and whole phospholipid profiles in the same species.

High Fat Diet Mouse Study Rationale

High-fat diet (HFD) consumption is a major contributor to insulin resistance, a condition characterized by an inability of skeletal muscle to effectively store carbohydrate, an inability of liver and adipose tissue to, respectively, reduce glucose output and store fat, and most notably, the likely development of impaired glucose tolerance (Kewalramani et al. 2010). In other words, insulin resistance represents an impaired metabolic response to the release of insulin. This condition is a significant risk factor for, among other diseases, obesity, polycystic ovarian disease, type 2 diabetes mellitus (T2DM), and cardiovascular disease (Straczkowski and Kowalska 2008). In healthy individuals, insulin, secreted by the pancreas, stimulates the uptake of glucose by skeletal muscle through a signal transduction cascade, resulting in the mobilization of the protein glucose transporter 4 (GLUT4) from intracellular spaces to the surface of skeletal muscle (Saltiel and Kahn 2001), allowing for the movement and storage of glucose in the cell. The signaling cascade involving insulin is a process far from simple. In brief, the receptor for the insulin molecule is a tyrosine kinase. This kinase undergoes autophosphorylation, subsequently catalyzing the phosphorylation of intracellular proteins, the best known of which are insulin-receptor substrate (IRS) proteins, such as

IRS-1 (White 2001). Once phosphorylated, these proteins then activate additional downstream targets, such as phosphatidylinositol-3-OH kinase (PI(3)K) and the mitogen-activated protein (MAP) kinase cascade (Saltiel and Kahn 2001). These and other downstream targets of insulin organize to activate enzymes, regulate vesicle traffic (such as GLUT4 movement), and coordinate gene expression, the end result of which is the appropriate regulation of glucose, lipid, and protein metabolism.

With such a complicated system, any disruption or deficiency in these pathways can create severe pathophysiological consequences. Numerous studies (e.g., Schmitz-Peiffer et al. 1999; Turner et al. 2007; Hancock et al. 2008; Holland et al. 2011) have identified inconsistencies or disruptions in the insulin signaling pathway thought to contribute to insulin resistance, but the causes of insulin resistance are extremely complex, likely multifactorial, and still under investigation. However, decreased uptake of glucose by skeletal muscle is known to be the critical step in the development of insulin resistance (Vaag et al. 1992). Some studies have implicated intramyocellular lipid accumulation as a condition resulting in the impairment of insulin action (e.g., Pan et al. 1997; Jacob et al. 1999; Perseghin et al. 1999; Itani et al. 2002; Petersen et al. 2004), while others imply the blocking of insulin action is caused by a disruption in the sphingomyelin signaling pathway (e.g., Summers and Nelson 2005; Summers 2006; Holland and Summers 2008), in which the second messenger is ceramide (Kolesnick 2002).

In free fatty acid (FFA)-induced insulin resistance, caused by HFD-feeding, excess adipose tissue, or a combination, the initial step in the development of the disease appears to be an impairment of glucose transport (Dresner et al. 1999). FFA decrease

insulin-stimulated uptake of glucose through the inhibition of insulin signaling (Straczkowski and Kowalska 2008). When FFA enter skeletal muscle cells, they form long-chain fatty acyl-CoA (LC-ACoA), which are utilized in the synthesis of sphingolipids (e.g., ceramide) or glycerolipids, such as diacylglycerol (DAG) or triacylglycerol (TAG). Both DAG and ceramide are able to inhibit insulin action within muscle cells, the end result of which is a decreased insulin-stimulated movement of GLUT4 to the plasma membrane, and therefore, decreased transport of glucose into muscle cells (Straczkowski and Kowalska 2008). In this way, FFA modify the intracellular lipid pool, resulting in the inhibition of insulin signaling (Shulman 2000; Unger 2003).

Elevated TAG content in muscle cells is actually thought to be protective against lipotoxicity and does not seem to exert disadvantageous metabolic constraints (Unger 2003; Liu et al. 2007; Schenk and Horowitz 2007), despite its negative correlation to whole-body insulin sensitivity (Pan et al. 1997; Perseghin et al. 1999). DAG, however, does not provide the same protective effect to skeletal muscle cells and is likely an important lipid correlate to insulin resistance (Straczkowski and Kowalska 2008). Ceramide, another key player in the blocking of insulin action, seems to exert its effects by decreasing insulin stimulation of protein kinase B (PKB/Akt) (Schmitz-Pfeiffer et al. 1999), both through stimulating the dephosphorylation of protein phosphatase 2A (PP2A) by Akt or through the translocation of Akt to the plasma membrane (Stratford et al. 2004). The consequence of this insulin-blocking action is a significant decrease in the transportation of GLUT4 to the skeletal muscle plasma membrane (Summers et al. 1998; JeBailey et al. 2007). Saturated fatty acids are also though to desensitize skeletal muscle

to the actions of insulin, and ceramide, here too, may play a key role (Straczkowski and Kowalska 2008). In addition, ceramide interacts with tumor necrosis factor α (TNF-α) and glucocorticoids to induce insulin resistance, the former through the activation of sphinomyelinases (Kanety et al. 1996; Peraldi et al. 1996), and the latter through the stimulation of sphingomyelinases and serine palmitoyltransferase (SPT) (Lepine et al. 2002; Linn et al. 2002) on a glucocorticoid-induced, sphingomyelin-enriched membrane (Murray et al. 1979).

Skeletal muscle is an important repository for insulin-stimulated glucose uptake, accounting for 70-80% of whole body glucose uptake (DeFronzo et al. 1981). A decrease in insulin-stimulated glucose uptake by skeletal muscle is one of the clear mechanisms responsible for insulin resistance development (Vaag et al. 1992; Straczkowski et al. 2004), and makes skeletal muscle an obvious tissue of investigational interest in the development of this disease. Type 2 diabetes involves a gradual progression from normal glucose tolerance to impaired glucose tolerance, with a subsequent decline in the function of pancreatic beta cells (Toye et al. 2005). With the decline of beta cell function comes a reduction in insulin secretion, resulting in overt hyperglycemia (Srinivasan and Ramarao 2007). While insulin resistance greatly enhances the risk of developing diabetes through increased demand on already stressed beta cells, the condition by itself does not directly result in diabetes (Toye et al. 2005). Elevated plasma FFA (either from HFD feeding, excessive adipose tissue, or both) combined with a relatively sedentary lifestyle (i.e., reduced lipid oxidation in skeletal muscle), often results in a substantial increase in the deposition of excessive intramyocellular lipid (Corcoran et al. 2007), worsening insulin resistance and glycemic control. Left unchecked, these conditions lead directly to a suite

of metabolic diseases not limited to obesity, pre-diabetes, and eventually, full-blown T2DM. Additionally, the increased availability and delivery of free fatty acids to peripheral skeletal muscle and liver is the main association between insulin resistance and obesity (Shulman 2000).

These metabolic derangements are leading to a serious health crisis in North America. For example, in the United States alone, an estimated 79 million people are pre-diabetic (characterized by impaired glucose tolerance, not quite to the level of T2DM). Even more astounding is that, of these 79 million, over 2 million are under the age of 20, an alarming trend in an age group previously unaffected by these metabolic disorders (ADA 2011). As skeletal muscle is critical in the roles of insulin-stimulated glucose disposal and energy expenditure, elucidating changes that occur to this tissue upon HFD feeding is paramount to understanding the causes and mechanisms behind insulin resistance and the suite of metabolic conditions associated with insulin resistance.

Generally, the consumption of a HFD by sedentary organisms leads to excess lipid accumulation in the muscle, which often subsequently leads to insulin resistance, obesity, pre-diabetes, and eventually, frank T2DM. Although many changes are likely to occur in the lipid profile of an organism under conditions of a HFD, ceramide in particular is thought to be at least partially responsible for the instigation of insulin resistance in skeletal muscle (Schmitz-Peiffer et al. 1999; Schubert et al. 2000; Straczkowski et al. 2007). Increases in ceramide in the lipid profile, then, are often seen associated with these metabolic conditions. Despite the clear importance of ceramide to insulin resistance however, ceramides are not the only lipids thought to be influential in insulin resistance (Straczkowski and Kowalska 2008). Indeed, for example, several other

studies have shown insulin action is improved with a greater percentage of polyunsaturated fatty acids in the membrane phospholipid profile (Vessby et al. 1994; Pan et al. 1995; Haugaard et al. 2006). This is possible and likely because dietary trends and interventions are known to have the capability to change the phospholipid composition of the membranes of cells throughout the body (Popp-Snijders et al. 1986).

While a few fatty acid groups (polyunsaturated fatty acids; Vessby et al. 1994; Pan et al. 1995; Haugaard et al. 2006) and sphingolipids (ceramides; Schmitz-Peiffer et al. 1999; Schubert et al. 2000; Straczkowski et al. 2007) have been investigated in correlation to HFD and insulin resistance, a broader study looking at species of whole phospholipids, sphingolipids, and the fatty acid composition in skeletal muscle of animals subjected to these conditions has not been undertaken. Elucidating which fatty acids, whole phospholipids, and sphingolipids change in response to HFD and insulin resistance may provide treatment ideas, as many of the phospholipids, in particular, are heavily linked to dietary intake, and therefore their composition within the cellular membrane may be induced or changed through dietary intervention (Popp-Snijders et al. 1986), a seemingly simple option for treatment. Additionally, sphingolipids are inherently tied to cell signaling pathways, and thus their relative changes in the context of a HFD and insulin resistance may provide valuable insight into the complex suite of metabolic diseases associated with insulin resistance.

Because human trials are inherently complicated, model organisms have been sought out and utilized for the study of diet-induced insulin resistance and T2DM. The high-fat diet-fed mouse, specifically the C57BL/6J mouse, has become a model system for insulin resistance (impaired glucose tolerance) and T2DM (Surwit et al. 1988;

Winzell and Ahren 2004; Toye et al. 2005; Srinivasan and Ramarao 2007). Until recently, most rodent models for T2DM were based on single genetic defects or traumatic chemical destruction of β-cells (e.g., Rerup 1970; Rossini et al. 1977; Pelleymounter et al. 1995; Ahren et al. 1996; Chen et al. 1996; Crouse et al. 1998). For example, the obese mouse (C57BL/6H *ob/ob*) is obese due to a single genetic mutation and has innate endocrine pathology which is not typically present in human development of T2DM (Coleman 1978). Two species of desert rodents have previously been used as model organisms for diet-induced obesity, but these organisms have no known diabetes-resistant strains for utilization as controls (Cameron et al. 1972; Schmidt-Nielsen et al. 1964). These characteristics made these former models unrealistic as a template for the true human disease. C57BL/6J mice fed a high-fat diet were first introduced in 1988 by Surwit et al., and have proven to be a robust model compared to other rodent strains (e.g., West et al. 1992; Ahren et al. 1998). These mice, when fed a HFD, gain weight, maintain a level of hyperglycemia, and develop worsening hyperinsulinemia, a key attribute of insulin resistance. After only one week on the HFD, the C57BL/6J mice show impaired glucose tolerance. The model mouse rapidly demonstrates both impaired glucose tolerance (IGT) and islet dysfunction, two important characteristics of insulin resistance, and most importantly, presents a realistic analogue for the development of T2DM in humans. The C57BL/6J mouse has been inbred to contain a genetic factor which predisposes the animal to T2DM after becoming obese (Surwit et al. 1988), and additionally demonstrates peripheral leptin resistance (Srinivasan and Ramarao 2007). Lean C57BL/6J mice remain euglycemic on a baseline diet, and thus serve as an appropriate control for interventional studies, although there is some evidence (e.g., Kaku

et al. 1988; Kayo et al. 2000) that a complex, subclinical abnormality in insulin secretion is present in the lean C57BL/6J mice. Also of interest, male mice of the C57BL/6J strain are substantially less glucose-tolerant than females, likely due to hormonal differences (Leiter et al. 1998; Hirayama et al. 1999; Kim et al. 2001). Thus, the C57BL/6J mouse seems to be an ideal model organism for the study of insulin resistance and T2DM.

High Fat Diet Mouse Study Specific Aim/Hypothesis

Specific Aim III: To test the hypotheses that standard-diet-fed mouse fatty acid, whole phospholipid, and sphingolipid profiles in the skeletal muscle of hamstrings will differ from those of mice fed a HFD.

H_{01}: The skeletal muscle fatty acid profile will not differ between standard-diet-fed mice and mice fed a HFD.

H_{02}: The skeletal muscle sphingolipid profile will not differ between standard-diet-fed mice and mice fed a HFD.

H_{03}: The skeletal muscle whole phospholipid profile will not differ between standard-diet-fed mice and mice fed a HFD.

Rationale for the Measurement and Comparison of Lipids of Interest

Saturated Fatty Acids

Saturated fatty acids (SFAs) are commonly consumed as part of a standard Western diet. The consumption of SFAs has been correlated to the development of insulin resistance in humans (Corcoran et al. 2007). Because these fats are less readily oxidized, they tend to accumulate in skeletal muscle as harmful bioactive lipids (e.g., ceramides and DAG) (Lee et al. 2006). In particular, the saturated fats palmitate and

stearate have been shown to block the protein complex Akt/PKB, normally activated by insulin (Chavez et al. 2003). In marine mammals, saturated fats are often found in adult cell membranes due to their lower likelihood of damage from reactive oxygen species (ROS) (Trumble and Kanatous 2012), and thus play a critical role in protecting the animal from the effects of hypoxia. In the immature rat brain, hypoxia resulted in a decrease in SFAs, as these fats are not able to take on additional hydrogen molecules in low pH conditions (Mourek et al. 1992). The role of SFAs in hypoxia tolerance in marine mammals and insulin resistance in rodent models of T2DM are of primary interest.

Monounsaturated Fatty Acids

Monounsaturated fatty acids (MUFAs) are a class of fats containing only a single double bond. These fatty acids are commonly found in olive oil, mostly as the compound oleic acid, and are associated with enhanced insulin sensitivity (Marshall et al. 1997; Soriguer et al. 2004), a critical factor in the determination of T2DM development. In immature rat brains, the proportion of MUFAs was significantly decreased under high pH conditions (Mourek et al. 1992). In marine mammal membranes, MUFAs are increased in adults due to their protective effects against ROS damage compared to polyunsaturated fatty acids (Trumble and Kanatous 2012). Finally, in chicken skeletal muscle, a diet supplemented by olive oil reduced ROS production induced by heat stress (Mujahid et al. 2009). The protective effects of MUFAs appear to be far-reaching and are of interest in the conditions of ischemia/reperfusion and insulin resistance.

Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFAs) include many subclasses of fats, such as omega-3s and omega-6s. PUFAs are important in insulin resistance, as studies have shown muscle membranes with higher percentages of PUFAs demonstrate greater insulin action (Pan et al. 1995). PUFAs are also preferentially oxidized over saturated fats, and are therefore less likely to cause an accumulation of intramuscular triglycerides (Corcoran et al. 2007). In marine mammals, PUFAs are used for fuel in order to conserve precious oxygen. Additionally, in Weddell seal pups, PUFAs are common in cell membranes until the animals age and start to dive regularly, when they are then influenced by the effects of hypoxia (Trumble and Kanatous 2012). Finally, PUFAs play prominent roles in lipid signaling in hypoxia, where hypoxia is known to increase levels of inflammatory eicosanoids (e.g., prostaglandin E2 (PGE_2) and the eicosanoid precursor, arachidonic acid) (Jelkmann et al. 1985) and influence levels of additional prostaglandins via cyclooxygenase-2 (COX-2) expression (Chida and Voelkel 1996; Huwiler and Pfeilschifter 2006).

Omega-3 Fatty Acids

Omega-3 fatty acids (N3), a subclass of PUFAs including the well-known docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are largely thought to be healthful due to their cardiovascular benefits and protection against high-fat diet-induced insulin resistance (Storlien et al. 1987; Ikemoto et al. 1996). A major source of omega-3s is fish oil, which decreases whole-body abundance of lipid metabolites by accentuating the catabolism of fatty acids in tissues sensitive to insulin (e.g., skeletal muscle) (Neschen et al. 2007). Long-chain omega-3s have been shown to decrease fasting insulin levels in

rodents (Haugaard et al. 2006). Omega-3s have also shown protective effects against retinal angiogenesis under the hypoxic conditions of the mouse eye (Connor et al. 2007) and additionally, are increased under hypoxia in immature rat brain, which is thought to be protective against high hydrogen levels (i.e., low pH) in the developing brain (Mourek et al. 1992). Finally, omega-3s embedded as phospholipids protect the rat heart from ischemia/reperfusion injury (Jezkova et al. 2002), a condition which marine mammals must defy continually. This class of fatty acids is of interest to many investigations.

Omega-6 Fatty Acids

Omega-6 fatty acids (N6), a subclass of PUFAs including the well-known arachidonic acid, are a necessary, but at times, demonized group of fatty acids, largely because the Western diet contains an overabundance of the group (Simopoulos 2002). The effects of omega-6s on insulin sensitivity seem to range between those of omega-3s and saturated fats (Storlien et al. 1991). Omega-6 fatty acids have been shown to blunt signaling of IRS-1 and reduce GLUT4 levels (Taouis et al. 2002), but other studies have shown arachidonic acid to be effective in preventing diabetes in male Wistar rats (Suresh and Das 2003). In immature nervous tissue of the rat, low oxygen levels did not affect omega-6 fatty acid levels (Mourek et al. 1992). Additionally, adaptation to hypoxia in rat heart tissue resulted in a decrease in omega-6 fatty acids in phospholipids (Jezkova et al. 2002). The degree to which this subclass of fatty acids is influenced by hypoxia or affects insulin sensitivity is of interest in these investigations.

C16:0 Ceramide

C16:0 ceramide (C16 ceramide) is one of the most common ceramide species in non-neuronal mammalian tissue (Pewzner-Jung et al. 2006; Seumois et al. 2007), and has been proposed to be the chief species elevated during apoptotic events induced by a multitude of stimuli (e.g., Thomas et al. 1999; Kroesen et al. 2001; Eto et al. 2003; Kroesen et al. 2003; Osawa et al. 2005; Eto et al. 2006). Ceramides, membrane lipids, play a key role in the eukaryotic stress response (Sawai and Hannun 1999; Hannun and Luberto 2000; Pfeilschifter and Huwiler 2000). These molecules, as bioactive intermediates, mediate several anti-proliferative cellular responses, such as cell senescence, apoptosis, and cell cycle arrest, through their accumulation intracellularly (Hannun 1996; Hannun and Luberto 2000; Ogretmen and Hannun 2004). This cellular accumulation of ceramides is set on course by stressors, the most well-known of which include Fas ligand (FasL), oxidative stress, TNF-α, ionizing radiation, hypoxia, anticancer therapies, injury, infections, and ultraviolet light (Hannun and Luberto 2000; Seumois et al. 2007; Bikman and Summers 2011).

Ceramides are formed within the cell through three pathways: *de novo* synthesis, the salvage pathway, and through the hydrolysis of the sphingolipid sphingomyelin. *De novo* synthesis is, by far, the best characterized pathway. In *de novo* synthesis, SPT and ceramide synthase coordinate to generate ceramides (Seumois et al. 2007), which takes place in the endoplasmic reticulum (Novgorodov and Gudz 2009). SPT catalyzes serine and palmitoyl-CoA condensation to form 3-ketosphinganine, which is subsequently reduced to sphinganine, and eventually N-acylated by ceramide synthase to form dihydroceramide. To produce the final product, ceramide, a double bond is introduced by

dihydroceramide desaturase (Seumois et al. 2007). De novo synthesis is the primary source of C16 ceramide (Grosch et al. 2012). Many of the enzymes required for this process are induced to action by inflammation common in obesity (Novgorodov and Gudz 2009). Ceramide levels intracellularly are tightly regulated, and the rapid conversion of ceramides into less harmful sphingolipids controls their participation in the apoptotic signaling pathways (Novgorodov and Gudz 2009). Further, pharmacological interventions to inhibit the production of these necessary enzymes in *de novo* synthesis appears to result in improved glucose tolerance and potentially reverse insulin resistance, including that caused by high-fat feeding (Holland et al. 2007).

Sphingomyelin hydrolysis is a second pathway through which ceramide is produced. In this pathway, the phosphocholine head group of sphingomyelin is hydrolyzed by spingomyelinase enzymes, which vary based on cellular location and pH requirements (Claus et al. 2009; Boulgaropoulos et al. 2010). Sphingomyelinases are known to be upregulated by TNF-α, a cytokine correlated with insulin resistance (Bikman and Summers 2011). Finally, the salvage pathway can be utilized to produce ceramides, which is simply the recycling of more complex sphingolipids into ceramides (Bikman and Summers 2011). Higher-order sphingolipids, such as sphingomyelin and glycosphingolipids, are degraded by enzymes within acidic compartments to form ceramide (Riboni et al. 1994; Kitatani et al. 2008; Kitatani et al. 2009). Ceramide is then degraded further into sphingosine and FFAs, which then enter the cytosol and are reconverted to ceramide by ceramidase (Riboni et al. 1998). In terms of pure quantity, the salvage pathway may be the most important of the three production mechanisms, as it

can contribute over half of the sphingolipid pool within a cell (Bikman and Summers 2011).

Ceramides most commonly contain acyl chain lengths of between 16-24 carbon atoms (Seumois et al. 2007), and their production is regulated by a family of mammalian ceramide synthase genes known as LASS (longevity assurance) genes (Pewzner-Jung et al. 2006). Each Lass gene, of which there are six paralogs in humans and mice, encode multi-transmembrane proteins in the endoplasmic reticulum, and are responsible for the production of ceramides of different chain lengths. LASS5 is largely responsible for the production of C16 ceramide (Pewzner-Jung et al. 2006). The chain length of ceramides is critical to physiological function, and is likely the reason mammals have multiple ceramide synthase genes encoding different isoforms. The pathway through which ceramide is created is also important to physiological function. C16 ceramide, derived from the sphingomyelin pathway, increases in primary hepatocytes during TNF-α-induced apoptosis (Osawa et al. 2005), while de novo synthesis of C16 ceramide is elevated in prostate cancer cells (Eto et al. 2003) and in Ramos cells during β-cell receptor engagement (Kroesen et al. 2001). The temporal pattern of ceramide accumulation also appears to be chain-length-specific, as C16 ceramide elevates after only 6 hours in Ramos cells, while C24 ceramide is not elevated until the 24 hour mark (Kroesen et al. 2003). Interestingly, C16 ceramide has been shown to be upregulated following hypoxia/reoxygenation events (Jin et al. 2008).

Ceramide accumulation in skeletal muscle has been linked to insulin resistance in a number of studies (e.g., Turinsky et al. 1990; Schmitz-Peiffer et al. 1999; Hajduch et al. 2001; Adams et al. 2004; Zendzian-Piotrowska et al. 2006; Straczkowski et al. 2007).

While many investigations to date have simply quantified total levels of ceramides, others have measured individual ceramide species, linked ceramide levels to specific muscle types, or both. Turinsky et al. (1990) measured total ceramide levels in obese, insulin-resistant Zucker rats and found significantly higher levels of ceramides in the obese mice compared to lean controls. Additionally, in lean rats, the *plantaris* muscle (primarily fast-twitch) demonstrated much higher levels of ceramides than the primarily slow-twich *soleus* muscle. The obese mice showed proportionately higher levels of ceramides in both muscle types. Turinsky et al. (1990) also experimented with the same rat skeletal muscle made insulin-resistant via nerve interruption, and the same trends were found (higher levels of ceramide in *plantaris* compared to *soleus* muscle, and higher levels of ceramide, proportionately, in both muscle after denervation). Adams et al. (2004) quantified several ceramide species in the *vastus lateralis* muscle of obese, insulin-resistant humans. The *vastus lateralis* muscle is characterized by a mixture of slow oxidative, fast oxidative glycolytic, and fast glycolytic fibers (Oberbach et al. 2006). Adams et al. (2004) found proportionate, yet abundant, levels of C24, C24:1, and C18 ceramides in the muscle. Straczkowski et al. (2007) also biopsied the *vastus lateralis* muscle, and found elevated levels of total ceramides in overweight or obese men, and also in lean offspring of diabetic patients. Obese participants with impaired glucose tolerance showed higher levels of ceramides compared to obese men with normal glucose tolerance. Finally, Straczkowski et al. (2007) found a strong negative correlation between total ceramide levels and insulin sensitivity. Zendzian-Piotrowska et al. (2006) found that a high-fat diet increased the levels of total ceramide in the *soleus* and red (oxidative) portion of rat *gastrocnemius*, but not in the white (glycolytic) *gastrocnemius*.

Control rats in the Zendzian-Piotrowska et al. (2006) study showed higher levels of ceramide in the *soleus* muscle compared to either portion of the *gastrocnemius*. Clearly the levels of ceramide in insulin-resistant, HFD mice are of interest.

Ceramides, due to their involvement in the stress response, are known to play a role in hypoxic tissues, acting directly or indirectly to cause mitochondrial malfunction within these tissues (Novgorodov and Gudz 2009). In a neonatal mouse model, C16:0 ceramide decreased in the hypoxic tissue, while its precursor C16:0-dihydroceramide increased in the same tissue. Thus, C16:0-dihydroceramide may provide an adaptive response to hypoxia in mouse cardiac tissue (Noureddine et al. 2008). Most mammalian heart models (rat, mouse, and rabbit) show an increase in ceramides after ischemic episodes (e.g., Bielawska et al. 1997; Argaud et al. 2004). Specifically, the rabbit model indicates ceramide increases, and therefore the lifespan of individual ceramide molecules, is likely not very long, on the order of 10 minutes (Argaud et al. 2004). Finally, while C16:0 is the most abundant ceramide in mammalian heart tissue, its proportionate increase due to ischemia is less than other species (e.g., C18:2 ceramide) (Beresewicz et al. 2002). In liver, ceramides also increased transiently within the tissue in response to ischemia and subsequent reperfusion (Bradham et al. 1997; Llacuna et al. 2006). Studying ceramide levels under conditions of hypoxia in animals likely well-adapted to such physiological strain (i.e., marine mammals) could provide some interesting comparisons.

Sphingosine, C18:0 diacylglycerol, C16:0 sphingomyelin, C14:0 phosphoethanolamine, C16:0 phosphatidylglycerol, C14:0 phosphatidylserine, C16 Lyso

phosphatidylcholine, C16:0 phosphatidylcholine dipalmitoylphosphatidylcholine, and sphingosine-1-phosphate (S1P) are additional lipids of interest.

Dissertation Theme

Biological membranes separate the world of the cell from the extracellular environment, as well as organelles from surrounding intracellular spaces. These biological boundaries, however, are not static, but rather highly dyanamic and interactive (Prinetti and Sonnino 2010). Membranes are composed principally of phospholipids, in a bilayer, with relatively fewer embedded proteins, and as such, the physical properties of membranes are determined to a large extent by their phospholipid composition. At typical physiological temperature (37º), fatty acid chains are in a fluid state, and thus the model of Singer and Nicolson (1972) was born. The Fluid Mosaic Model has been a staple model of membrane investigations since it was first proposed in 1972 (Singer and Nicolson 1972). This model posits that the lipid bilayer of biological membranes is two dimensional in its assembly of lipids and proteins. While there is structure to this membrane, it is very much a fluid and changing environment, and can change if environmental conditions dictate. While the Fluid Mosaic Model is adequate in explaining many biological membranes or portions of biological membranes at one point in time, it is not an extensive nor accurate characterization of most biological membranes (Catala 2012).

Studies in the early 1980's suggested that membrane lipids might have the ability to aggregate laterally to some extent, creating a second level of order within the membrane (Prinetti and Sonnino 2010). The lipid raft hypothesis, in 1988, acknowledged that multiple phases in the membrane lipid environment could create intramembrane

domains, largely comprised of cholesterol, proteins, and sphingolipids distinct from surrounding unsaturated phospholipids (Simons and van Meer 1988; Catala 2012). These rafts are critical to proper physiological functioning, as they are a major site for the induction of cell signaling, among other roles (Simons and Toomre 2000; Helms and Zurzolo 2004). The fluidity of a membrane, conserved through appropriate levels of polyunsaturated fatty acids in phospholipids, is at the heart of its function. Even small changes to this physical characteristic can cause pathological physiological processes (e.g., the loss of functioning of protein surface receptors in insulin signaling) (Catala 2012), while augmented fluidity appears to increase the speed with which protein-protein interactions can occur within the membrane bilayer (Niu et al. 2004). The plasticity in fluidity is maintained through modification of membrane lipids, either via *de novo* synthesis, selective distribution, or remodeling of localized portions of the membrane (Catala 2012).

In particular the peroxidation of lipids of biological membranes can cause permeability and fluidity changes (Nigam and Schewe 2000), and specific oxidized phospholipids can act as cell signaling molecules (Cole et al. 2003; Walton et al. 2003; Tyurina et al. 2004; Greenberg et al. 2006; Maskrey et al. 2007). As membrane lipids continue to oxidize, if the molecules are not remodeled, they accumulate numerous polar moieties on the fatty acid chains, which extend into the aqueous milieu akin to whiskers (Catala 2012). This new model of biological membranes has been named the Lipid Whisker Model, and takes into account the physical and functional changes correlated with membrane lipid oxidation (Greenberg et al. 2008). Changes to the structure and function of membrane lipids, as well as the overall lipid composition of membranes, is a

central theme in this dissertation, both in the context of diving marine mammals and insulin-resistant mice.

Cell signaling is the secondary theme of this dissertation, and has only relatively recently included a central role for lipids. The groundbreaking discovery of Berridge (1984) opened the door for studies of lipid signaling by unveiling the signal transduction pathway of phosphoinositide. Since 1984, additional bioactive lipids have been identified to play a role in signaling, including sphingosine-1-phosphate (S1P) and ceramide, both important classes of sphingolipids (Serhan et al. 1996). Diacylglycerol (DAG) is another lipid messenger directly involved in cell signaling, and given its known accumulation in insulin-resistant tissues, may play a role in the development of type 2 diabetes mellitus (T2DM) (e.g., Turinsky et al. 1990). Cell signaling, and lipid signaling in particular, is critical to proper physiological functioning of cells and tissues, and both hypoxia-adapted marine mammals and insulin-resistant mice are likely to demonstrate unique patterns or uses of these bioactive lipid messengers.

While additional lipid classes were examined (e.g., fatty acids of the intramyocellular lipid pool), this dissertation primarily investigates components of the biological membrane of both marine mammal species and obese, insulin resistant mice, tying findings to their respective environmental constraints and health. The overarching premise of this dissertation, then, is found in the importance of biological membranes and lipids, in both structural and signaling capacities, to the proper functioning of mammalian cells of all species, both terrestrial and aquatic.

CHAPTER TWO

Phospholipid and Sphingolipid Profiles in Harbor Seals (*Phoca vitulina*) and Weddell seals (*Leptonychotes weddellii*): A First Descriptive Comparison of Complex Lipids in marine mammals

Introduction

Sphingolipids (SPLs), a class of complex lipid species, are known primarily for their role in cell signaling in terrestrial mammals (Dickson 1998). By acting as both structural components of cell membranes and also as signaling molecules, these lipids are capable of inducing physiological processes as diverse as cell apoptosis, differentiation, proliferation, and cell cycle arrest (Maceyka et al. 2002; Spiegel and Milstien 2003; Ogretmen and Hannun 2004; Bruni and Donati 2008; Hannun and Obeid 2008; Haynes et al. 2009). Commonly studied SPL species, such as ceramide and sphingosine-1-phosphate (S1P), have been implicated in the causation and inhibition of several terrestrial mammalian disease processes, respectively. The results of these cell signaling processes often occur via the production of bioactive metabolites, utilized as secondary messengers in signaling pathways. Additionally, SPLs influence cell membrane structure, interact with the extracellular matrix and adjacent cells, and contribute to specialized structures key to vesicular traffic within and between cells (Zheng et al. 2006). Cell signaling, and the accompanied adaptational processes, are often complemented by chronic and sometimes rapid remodeling of membrane lipids, through the utilization of activated lipases (Huwiler and Pfeilschifter 2006). The largest class of lipids involved in membranes is phospholipids (PLs), which define the permeability layer of cells and their organelles (Dowhan 1997), play an active role in influencing the

properties of proteins involved in cell signaling, and can serve as intracellular or extracellular signaling components (Alberts et al. 2002).

Aquatic mammals are under great physiological pressure to conserve oxygen and cope with bouts of tissue-level hypoxia and oxidative stress (Johnson et al. 2004; Johnson et al. 2005). In order to sustain this aerobic metabolism, diving mammals undergo a dive response, characterized by apnea, bradycardia, and peripheral vasoconstriction, allowing the animal to survive on a finite supply of internally stored oxygen (Irving 1939). Thus, when marine mammals are actively foraging, they are breath-hold diving, their actively contracting muscles receive very little to no blood flow, and they are forced to rely solely upon onboard stores of oxygen to sustain an aerobic metabolism fueled by lipids (Fahlman et al. 2008; Williams et al. 2011; Shiomi et al. 2012). Not only must these organisms maintain an aerobic metabolism under dwindling oxygen supplies, marine mammals must battle increasing hydrostatic pressure during their dives, pressure which would likely prove fatal in terrestrial mammals. For every 10 m increase in depth, these mammals experience an additional atmosphere of hydrostatic pressure. In terrestrial mammals, these extreme pressure increases could cause convulsions, nitrogen narcosis, and internal bleeding (Zapol 1987). Species such as harbor (*Phoca vitulina*) and Weddell (*Leptonychotes weddellii*) seals combat these pressure changes through collapsible lungs, strong exhalations pre-dive, and potentially, cortisol increases to prevent nervous system excitability (Zapol 1987). The length and frequency of marine mammal diving bouts, then, creates unique physiological stressors for these organisms, based largely upon their respective diving behaviors and prevailing environmental conditions.

Both harbor and Weddell seals experience intermittent hypoxia in marginal skeletal muscle due to the peripheral vasoconstriction commonly employed during dives (Hochachka and Mottishaw 1998). Hypoxic stress is thought to be modulated in terrestrial mammals through cell signaling, the transduction of molecular cues between and among cells for the purpose of transmitting physiological information. In a variety of cell lines, hypoxia has been shown to increase the lipids PGE_2 (rat mesangial cells; Petry et al. 2005), arachidonic acid (rat neuroblastoma cells; Petroni et al. 2002), diacylglycerol (HeLa and T-Cells; Aragones et al. 2001 and Temes et al. 2004, respectively), as well as ceramides (e.g., Basnakian et al. 2005). In marine mammals, lipids play a central regulatory role in cell signaling for a variety of adaptational purposes. For example, myoglobin levels are thought to be regulated in diving mammals primarily through compositional and quantitative changes of lipids (de Miranda Jr. et al. 2012), with secondary regulation potentially spurred by calcium signaling and levels of physical activity (Kanatous et al. 2008b, 2009; Ptitsyn et al. 2010; Trumble and Kanatous 2012). As myoglobin has been shown to increase during the weaning period of phocids before they begin diving (Burns et al. 2005; Burns et al. 2007; Clark et al. 2007), and the metabolism of lipid classes appears to change ontogenetically in marine mammals (Trumble et al. 2010), it seems likely the regulation of myoglobin would be dependent on its interplay with specific fatty acids or SPLs (Trumble and Kanatous 2012). In addition to changes induced by hypoxia, many aquatic organisms adapt to hydrostatic pressure (and the accompanying cold environmental conditions at depth) by changes in cell membrane composition and fluidity. For instance, deep sea organisms are known to reduce their membrane cholesterol concentrations in order to enhance fluidity at depth, in

cold waters (Hazel and Williams 1990). Additionally, terrestrial mammals trend toward a higher ratio of long-chain fatty acids in their pulmonary surfactant cell membranes, whereas aquatic mammals generally utilize PLs with shorter-chain fatty acid tails, which increases membrane fluidity (Foot et al. 2006). Though changes in lipid profiles have not been examined in light of hypoxic status nor pressure/temperature differences, studies in terrestrial species, and evidence of lipid signaling in marine mammals for other adaptational purposes, lends credence to the idea that these pathways are utilized in marine mammals as part of an active, diving lifestyle.

Because complex lipids have such a critical function in many terrestrial mammals, we sought to compare levels of various SPLs and PLs in two species of marine mammals, the harbor seal and the Weddell seal. Our hypothesis is that the PL and SPL compositions will differ between the shallower-diving, more temperate-adapted harbor seal and the deep-diving, polar-inhabiting Weddell seal. The mechanisms through which marine mammals avoid the oxidative stress which plagues terrestrial mammalian species under similar circumstances (e.g., chronic obstructive sleep apnea) are extremely interesting, and the results of these comparisons may provide clues as to better treatments for human and veterinary pathologies involving periods of hypoxia or significant lipid signaling. Additionally, SPLs and whole PLs have never been described and quantified in any marine mammal species before, so this information represents a unique look at lipids in an area of complete novelty.

Materials and Methods

Animals and Experimental Procedures

Weddell seal skeletal muscle biopsies (*longissimus dorsi*; n = 5 adult males) were subsampled from a larger project in McMurdo Sound during October through December 2005 and 2006 (Kanatous et al. 2008a; Trumble et al. 2010). Adults, showing no visible indication of recent diving (resting/dry pelage), were immobilized while on the ice with an intravenous injection of telezol (dorsal sinus, 0.1 mg/kg, Wheatley et al. 2006) and subsequently weighed to the nearest 0.5 kg using an electronic scale (San Diego Scale Inc., San Diego, CA, USA) mounted on a cross-beam sling. To access the *longissimus dorsi* (Kanatous et al. 2002) muscle, a 2 cm^2 section was shaved and cleaned using alcohol and a betadine solution before a 1 cm incision was made using a sterile scalpel blade (no. 10). Biopsies were collected (30-50 mg taken under local anesthetic; 1 ml, Lydocaine®) using a 6mm biopsy cannula (Depuy, Warsaw, IN, USA) and immediately stored in liquid nitrogen. Adults were detained for less than 60 minutes and were released when the animal had regained full locomotion.

Harbor seal *longissimus dorsi* muscle biopsies (n = 5 adult males) were collected from research conducted by the Alaska Department of Fish and Game under a permit issued to SJT. The animals were netted and subsequently allowed to relax for several hours before sample collection. Biopsies were collected as stated above and immediately placed in a liquid nitrogen dry shipper (Taylor Wharton Cryogenics, Theodore, AL, USA). Samples were shipped and stored at -80°C until laboratory analysis commenced.

Sphingolipid and Phospholipid Analysis

Sphingolipid and whole PL analysis (identification and quantification) was accomplished via liquid chromatography-high resolution mass spectrometry (LC-HRMS). Briefly, SPL and PL internal standards for LC-HRMS included (Avanti Polar Lipids, Alabaster, AL; 14:0 D54 phosphatidylethanolamine (PE) (1.5 ng/nL), 16:0 D31 lyso phosphocholine (PC) (1 ng/nL), 16:0 D62 phosphatidylglycerol (PG) (1.75 ng/nL), 16:0 D31 ceramide (1.25 ng/nL), 16:0 PC D04 (1.25 ng/nL), sphingosine D7 (1 ng/nL), 1,3-18:0 diacylglycerol (DAG)-d5 (1.25 ng/nL), 16:0 D31 sphingomyelin (SM) (1 ng/nL), and 14:0 D54 phosphatidylserine (PS) (1.25 ng/nL)).

Biopsies (25 mg) were ground with diatomaceous earth (EMD Chemicals, Rockland, MA) using mortar and pestle to absorb any remaining water and homogenize the sample. Each sample was placed in a thimble and run through a Foss SoxTec 2043 (Eden Prairie, MN) using a solvent mixture of 65/35 chloroform/methanol ($CHCl_3$/MeOH), with a protocol as follows: samples were boiled for 25 minutes at 160°C, and rinsed for 30 min at 100°C. Lipid extracts were subsequently dried under a stream of nitrogen. Each sample was then reconstituted in 4 ml MeOH and placed in a 4 mL glass vial with polytetrafluoroethylene (PTFE) cap (Sigma-Aldrich, St. Louis, MO). From the 4 mL of each sample, 475 µL was removed from the glass vial and placed in a 1.5 mL plastic micro-centrifuge tube (VWR, West Chester, PA), and all samples were ultracentrifuged for 10 min @10,000*g*. Each sample was then placed in a 2 mL amber gas chromatography (GC) vial (Sigma-Aldrich, St. Louis, MO) and spiked with 25 µL of the above-mentioned internal standard mixture at a concentration of 1 ppm/compound.

Extracts were then analyzed on an Accela liquid chromatograph coupled to a linear trap quadropole (LTQ) Orbitrap Discovery mass spectrometer (Thermo Electron, Bremen, Germany) using both positive and negative electrospray ionization (+ESI/-ESI). Final extracts were injected (2 µL) into the LC system consisting of a 15 cm x 2.1 mm (5 µm, 80 Å) Extended-C18 column (Agilent Technologies, Palo Alto, CA). A binary mobile phase gradient containing 0.1% (v/v) formic acid (HCOOH) in water (A) and 0.1% (v/v) formic acid in MeOH (B) was applied as follows: 50% A for 3 min, to 98% B in 9 min, held for 3 min, back to 50% A in 1 min, and equilibrated for 2 min at 50% A. Additional chromatographic parameters were as follows: column temperature, 50°C; flow rate, 500 µL/min. Full-scan accurate mass spectra (m/z range: 200-1000) of eluting compounds were obtained at high resolution (30,000 full width at half maximum (FWHM)) on the Orbitrap mass analyzer using internal calibration (accuracy of measurements < 2 ppm) and processed using XCalibur v.2.0.7 software. Electrospray source conditions were: sheath and auxiliary gas flow 55 and 10 arbitrary units (a.u.), respectively; heated capillary temperature 300°C; electrospray voltage 0.5 kV for both +ESI and –ESI; capillary voltage 44 V for +ESI and 35 V for –ESI; tube lens voltage 85 V for +ESI and 88 V for –ESI.

Lipid concentrations were determined using an isotope dilution approach. Lipid stock solutions containing 100 µg/mL or 200 µg/mL were serially diluted into MeOH with 0.1% HCOOH to prepare 10 calibration points ranging from 2.5 ng/mL to 5 µg/mL. Constant amounts of lipid isotopes (molecular formula and amounts) were added to both calibrators and samples as internal standards using a stock solution concentration prepared in methanol. Calibration curves were prepared by plotting the ratio of observed

peak areas for the analyte and internal standard, respectively, versus analyte concentration. Calibration data were subjected to a linear regression that was forced through the origin, resulting in regression coefficients (r^2) exceeding 0.99 for all targeted lipids. During LC-HRMS analyses, calibration was monitored through the use of blanks and continuous calibration verification (CCV) standards meeting an acceptability criterion of ±15%. Two identical matrix spikes (i.e. one matrix spike/matrix spike duplicate pair, MS/MSD) were prepared by adding a known amount of lipids to analyzed samples, increasing total concentration by approximately a factor of two. These matrix spikes were run every 12 samples. Recovery percentage of spiked amounts (criteria 85-115%) as well as MS/MSD relative percentage difference (criteria 15%) were met for quality-assurance purposes.

S1P extraction utilized a butanolic lipid extraction method, modified from Baker et al. (2001). Briefly, 75 µl of skeletal muscle homogenate was mixed with a phosphate buffer (containing 30 mM citric acid and 40 mM Na2HPO4). After vortexing, 1 ml 1-butanol and 500 µl water-saturated 1-butanol was added to the sample. After mixing for 15 seconds, the sample was ultracentrifuged @10,000*g* for 10 min. The upper butanol phase containing the S1P was isolated, evaporated to dryness under nitrogen, and redissolved in 4 ml MeOH for analysis by LC-HRMS (as above).

Statistical Analyses

Comparison of results between treatment groups (independent variable, species; dependent variable, lipid concentration) was determined by unpaired Student's T-test. Analyses were conducted by using SAS® (Version 9.4). P values ≤ 0.05 were considered significant. All data presented are mean ± SE.

Results

Changes in Skeletal Muscle Sphingolipid Profiles

C16 ceramide was significantly increased in harbor seals compared to Weddell seals (harbor: $n = 5$, $\bar{x} = 6.04 \pm 0.99$ µg/g); Weddell: $n = 5$, $\bar{x} = 1.42 \pm 0.22$ µg/g); $p = 0.0019$; Figure 1). Harbor seals also had a significant increase in 16:0 sphingomyelin

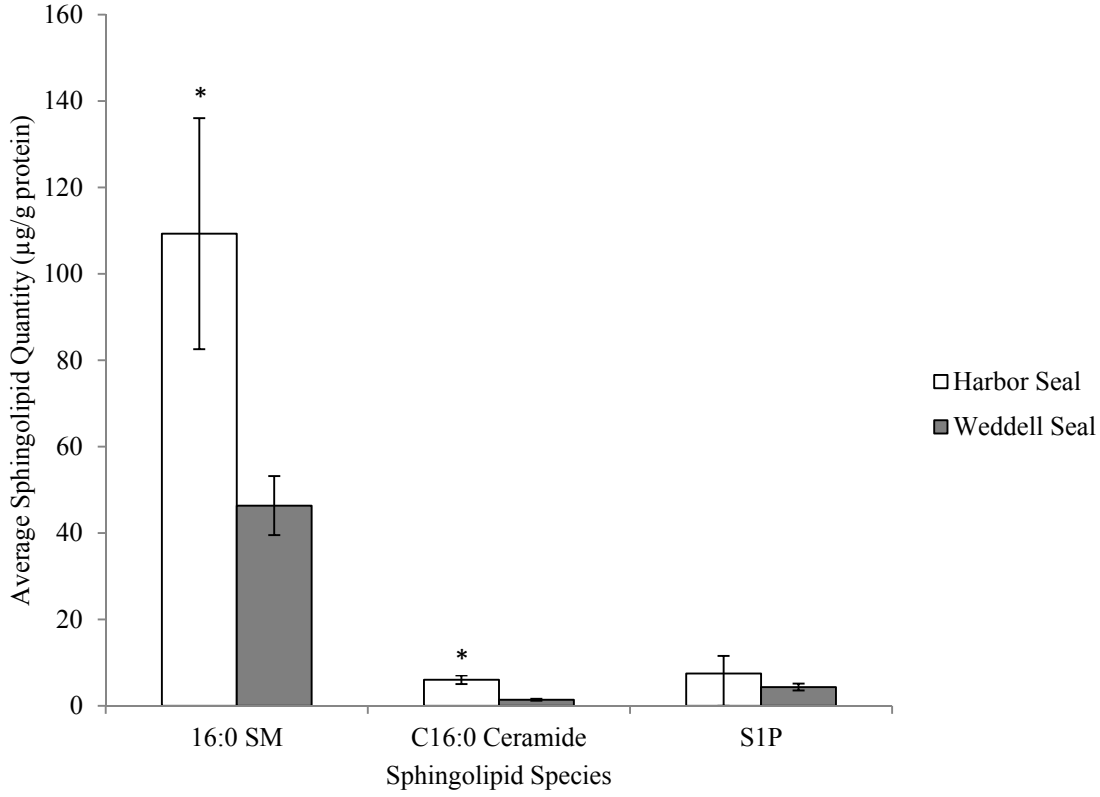

Figure 1. Average sphingolipid quantities in harbor seals ($n = 5$) vs. Weddell seals ($n = 5$). Values are means ± SE, * indicates significant environmental effect.

46.35 ± 6.83 µg/g; $p = 0.05$; Figure 1). Though a total of 5 SPL species were tested, 3 species (those mentioned here, and S1P) were found at measurable concentrations.

Changes in Skeletal Muscle Phospholipid Profiles

Only one PL, 16:0 dipalmitoylphosphatidylcholine (16:0 PC DPPC, or DPPC), was significantly increased in Weddell seals when compared to harbor seals (harbor: $n = 5$, $\bar{x} = 31.34 \pm 7.55$ µg/g; Weddell: $n = 5$, $\bar{x} = 11.09 \pm 1.08$ µg/g; $p = 0.03$; Figure 2). C16 lyso phosphatidylcholine, 14:0 phosphoethanolamine, and 16:0 phosphoglycerol did not

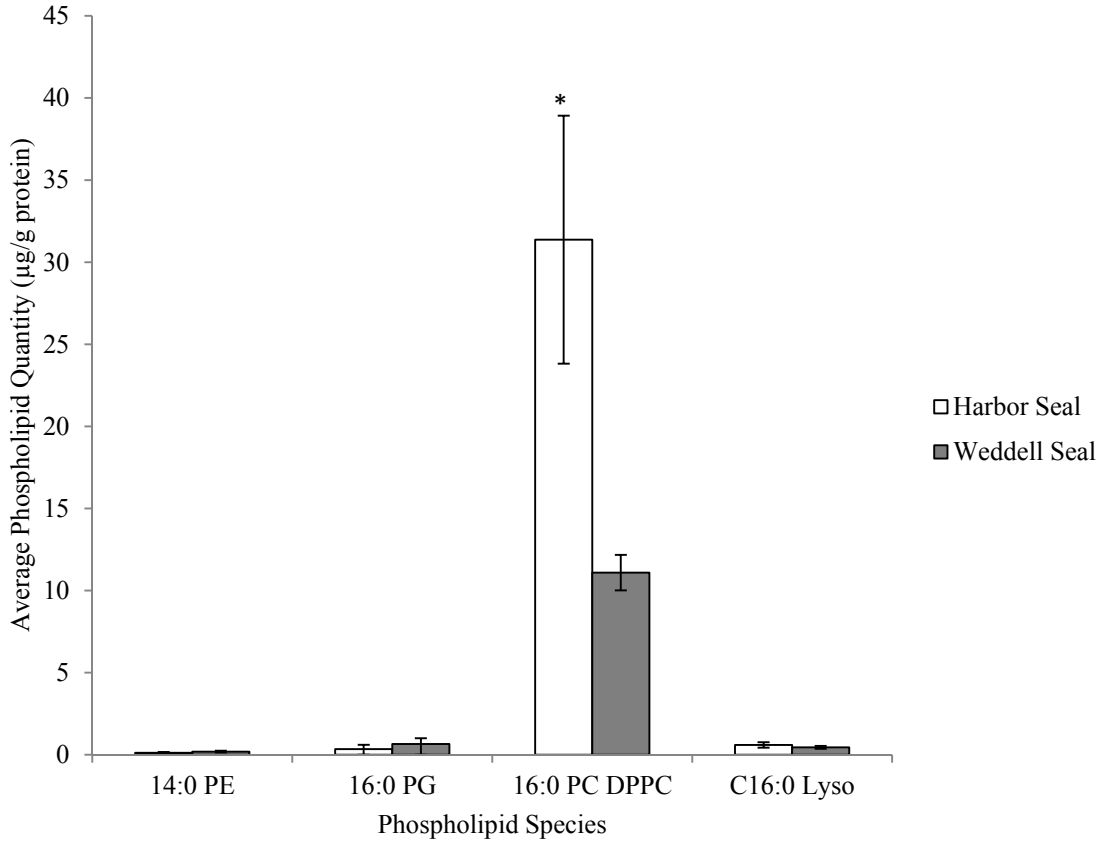

Figure 2. Average phospholipid quantities in harbor seals ($n = 5$) vs. Weddell seals ($n = 5$). Values are means ± SE, * indicates significant environmental effect.

differ significantly between species. Five PL species were analyzed with 4 of the 5 species (those mentioned here) found at measurable concentrations.

Correlations Between Animal Masses and Complex Lipids

A significant positive linear correlation was found between harbor seal body mass and C16 ceramide levels ($R^2 = 0.73$; Figure 3). This correlation was not found in Weddell seals. Additionally, a negative linear correlation was found between harbor seal body mass and 16:0 PC DPPC ($R^2 = 0.93$; Figure 4). Finally, a significant positive linear correlation was found between 16:0 PC DPPC and C16 ceramide in Weddell seals ($R^2 = 0.77$; Figure 5).

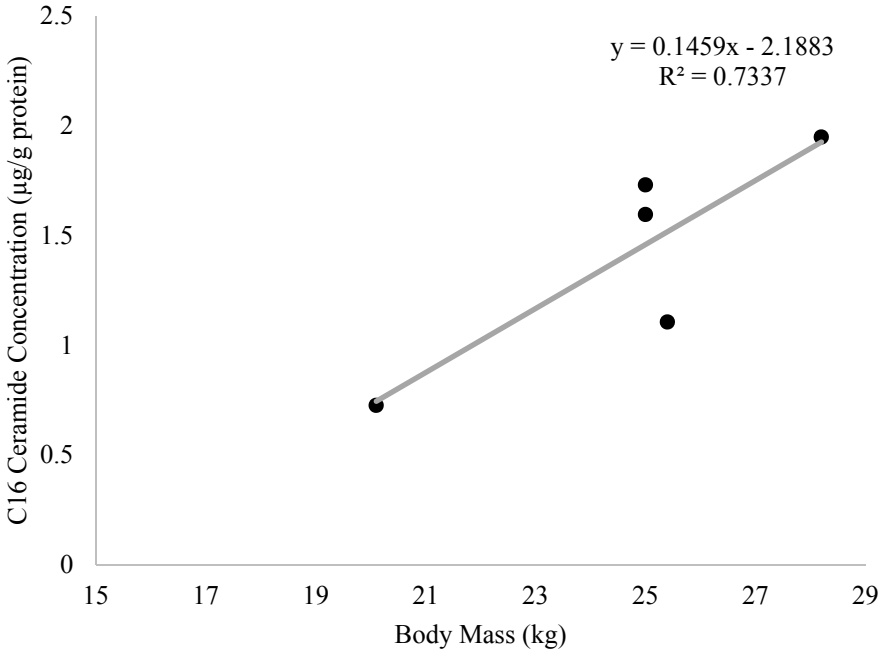

Figure 3. Individual harbor seal body mass (n = 5) vs. paired C16 ceramide levels.

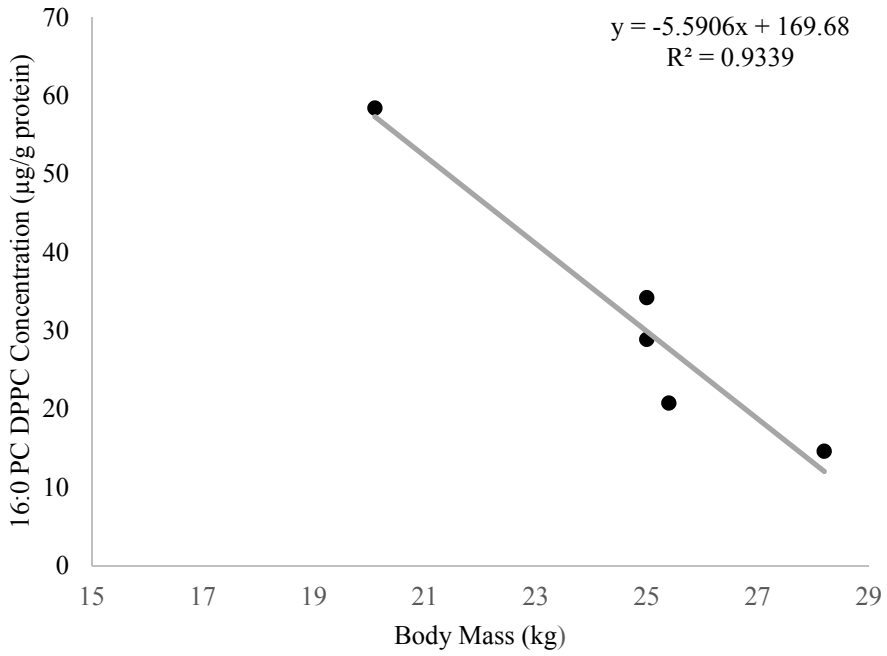

Figure 4. Individual harbor seal body mass (n = 5) vs. paired 16:0 PC DPPC levels.

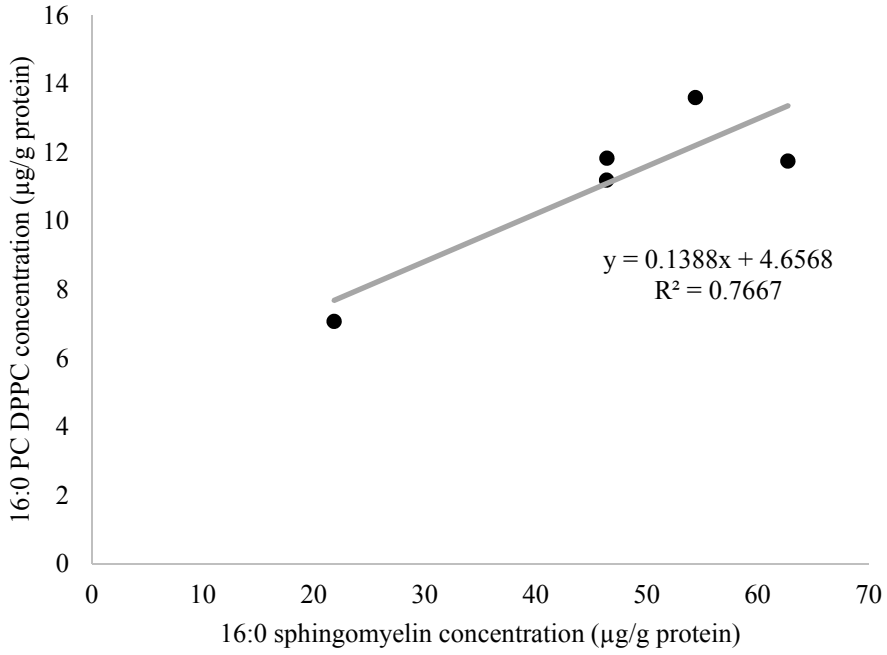

Figure 5. Individual Weddell seal (n = 5) 16:0 sphingomyelin concentrations vs. paired 16:0 PC DPPC concentration.

Discussion

Results presented here show harbor seals have significantly higher levels of the SPLs C16 ceramide and 16:0 sphingomyelin present in skeletal muscle cell membranes compared to Weddell seals (Figure 1), as well as significantly higher levels of the PL 16:0 dipalmitoylphosphatidylcholine (16:0 PC DPPC) (Figure 2). A positive linear correlation was discovered between harbor seal body mass and C16 ceramide levels (Figure 3). Additionally, a negative linear correlation was found in harbor seals between body mass and 16:0 PC DPPC (Figure 4). Finally, a significant positive linear correlation was reported between 16:0 sphingomyelin and 16:0 PC DPPC in the skeletal muscles of Weddell seals (Figure 5).

Cell signaling pathways are increasingly found to involve a class of lipids known as SPLs. Sphingolipids act in primarily two fashions: as a structural component in cell membranes, and also as signaling molecules in signal transduction pathways, capable of inducing widely diverse and critical physiological processes largely responsible for the outcome and growth of the cell (Maceyka et al. 2002; Spiegel and Milstien 2003; Ogretmen and Hannun 2004; Bruni and Donati 2008; Hannun and Obeid 2008; Haynes et al. 2009). Most recent research into SPLs has focused on a single class, the ceramides.

Ceramides are widely known indicators and regulators of biological responses to stressors, and can initiate signaling cascades involving protein kinases and protein phosphatases, both of which may result in cell senescence and apoptosis (Hannun and Obeid 2002; Ruvolo 2003). Ceramide accumulation in the cell is spurred by cytokines, growth factors, and other environmental stress stimuli which then employ the sphingomyelin-signaling pathway, resulting in the generation of ceramides (Pfeilschifter

and Huwiler 2000). Interestingly, hypoxia signaling pathways may employ a variety of lipids, including SPLs, as second messengers in adaptive cell signaling pathways (Huwiler and Pfeilschifter 2006). Marine mammals, with, as yet uncharacterized SPL profiles, may be associated with unique SPL membrane signatures based on their individual environmental stressors and life histories, and are likely to employ distinctive cell signaling pathways compared to terrestrial mammals. Sphingolipid concentrations and compositions in marine mammal cell membranes may also differ in ways which reflect adaptations to hypoxic conditions. Recent research in rat smooth muscle has shown that hypoxic conditions can induce an increase in cellular S1P and a decrease in ceramide due to hypoxia-inhibited sphingomyelinase activity (Yun and Kester 2002). Other studies have shown increases in ceramide levels as a result of hypoxia exposure in renal tubular epithelial cells of rats (Basnakian et al. 2005) and in rat neonatal cardiomyocytes (Bielawska et al. 1997). Chronic, intermittent hypoxia, as is found in diving seals, is likely to induce similar changes in marine mammal SPL profiles, and the direction and composition of these changes may shed light on the ways in which these organisms are adapting to this environmental stressor. While the causes of the differences reported here between harbor and Weddell seal SPLs are unclear, several questions arise: is hypoxia tolerance dictated by membrane SPL and PL composition, or does hypoxia, as a stressor, induce a downstream change in the membrane composition dependent, at least in part, upon the degree of hypoxia experienced by these mammals?

C16 ceramide was found in significantly higher levels in the relatively shallower-diving harbor seal compared to the Weddell seal. Additionally, 16:0 sphingomyelin, a SPL derived from ceramide (Zheng et al. 2006) was found in significantly elevated levels

in harbor seals compared to Weddell seals. Ceramides are thought to correlate with physiological stressors, and may indicate (1) that the deeper dives of Weddell seals do not elicit much physiological stress or (2) hypoxic stress caused by diving is more defined, in terms of physiological stress, by the frequency and total time diving each day more than the average depth and duration of each individual dive. In other words, Weddell seals may be experiencing less physiological stress from their diving patterns compared to harbor seals, or alternatively, harbor seal diving patterns are a more significant stressor to phocids. Because harbor seals have a higher mass-specific metabolic rate compared to Weddell seals (see Davis et al. 1985 and Castellini et al. 1992), these phocids may be required to forage more frequently, forcing the animals into a pattern of high-frequency breath-hold diving to which they may be less well-adapted. Additional, as yet unexplored, differences in ecological parameters (e.g., abundance of food, competition levels, and predation rates) may also help to explain these differences.

While diving patterns may play a role in these SPL differences between marine mammal species, body weight and prevailing environmental differences may also underlie the reported differences. Weddell seals are considerably larger than harbor seals (adult Weddell seals here averaged 375 kg, compared to 25 kg harbor seals), and inhabit a colder and more extreme environment, including water temperatures that fall below -1.9ºC at times (Zapol 1987). Both these characteristics may help to explain the SPL differences described in this study. C16 ceramide was shown to increase with increasing body weight in harbor seals. This may indicate that the smaller harbor seals, with less skeletal muscle undergoing oxygen deprivation, are better adapted to the stresses of diving and hypoxia than larger organisms of the same species. The warmer climate

inhabited by harbor seals may provide an explanation as to their comparatively higher levels of ceramide, as heat stress has been correlated to severely increased ceramide in the model organism, yeast (*Saccharomyces cerevisiae*) (e.g., Jenkins et al. 1997; Jenkins et al. 2002), as well as several mammalian cell lines (NIH WT-3T3, Chang et al. 1995; HL-60, Kondo et al. 2000; Molt-4, Jenkins 2003). Additionally, the higher levels of C16 ceramide and 16:0 sphingomyelin in harbor seals may be related to their greater environmental variability, resulting in a greater expression of these lipids as an adaptation to temperature effects, for example. Phenotypic plasticity, as suggested here, has been demonstrated in killer whale (*Orcinus orca*) fin shape and pigmentation patterns (Foote et al. 2009) and several Antarctic species, including Antarctic fur seals (*Arctocephalus gazella*) in the context of climate change (Forcada et al. 2008). This greater plasticity may prove beneficial as the global climate, especially at the poles, continues to change rapidly. As the importance of SPLs to diving adaptations becomes more defined, the exact contribution of each of these factors to differences found in marine mammal sphingolipid profiles will become clearer.

Whole PL changes in skeletal muscle (as opposed to the fatty acid changes seen within PLs) have very rarely been studied in relation to hypoxic stress, and never in relation to marine mammal diving adaptations. The PL 16:0 PC DPPC, found in significantly higher levels in harbor seals than Weddell seals, is one of the four most common PLs found in eukaryotic cell membranes (Alberts et al. 2002). In mammals, this PL is most commonly found associated with lung tissue, specifically in the protective pulmonary surfactant (Veldhuizen et al. 2000; Tonks et al. 2001). Within lung tissue, DPPC is known to have an anti-inflammatory effect, as well as reducing surface tension

within the pulmonary surfactant. When compressed, DPPC can pack into a gel-like phase that is easily able to resist very high surface pressures (Veldhuizen et al. 2000). Additionally, Foot et al. (2006) found that DPPC is found in higher concentrations in marine mammal lung surfactant compared to terrestrial species, implying that diving mammals require a surfactant with anti-adhesive properties, greater fluidity, and the ability to endure repeated total collapse and reinflation of the lung due to great increases in hydrostatic pressure. In diving marine mammals, the ability for cell membranes to counteract high pressure environments (e.g., during deep dives) would seem to be highly advantageous. The significantly higher levels of DPPC were hypothesized to be found in the deeper-diving Weddell seals, as these organisms must deal with higher average pressures than harbor seals. However, once again, the estimated frequency of the hypoxic bouts (i.e., dives) and total time at depth may be the driving force behind these PL adaptations, rather than the pure depth of average dives. In other words, with each dive, nearly all of which exceed the depth required to induce lung collapse in either species (estimated at 70m; Kooyman and Ponganis 1998), representing an increase in pressure to the organism, the absolute number and total duration of these higher-pressure events may play a more important role in adaptation than the maximum depth of these dives over time, resulting in higher levels of the PL DPPC in harbor seals.

In conclusion, it would appear that changes or differences in cellular SPLs and whole PLs in marine mammals may be a result of the frequency of hypoxic bouts and an interaction with body size. Additionally, environmental conditions may also contribute to the changes described here. Because of a changing (largely warming) global climate (Vaughan et al. 2003; Meredith and King 2005; Stroeve et al. 2007), more research needs

to be undertaken to understand the impact of climate and phenotypic plasticity in these mammalian divers. These findings should further elucidate the trends seen in this study, and provide further clues to hypoxia tolerance in marine mammals. Finally, differences between harbor and Weddell seal complex lipids discovered here provide potentially important implications for human afflictions such as chronic obstructive sleep apnea, and tumorgenesis, the former of which may show a very different SPL and PL profile than a growing tumor, based solely on the type of hypoxia involved.

CHAPTER THREE

Phospholipid and Sphingolipid Profile Comparisons in Ringed Seal (*Pusa hispida*) Skeletal Muscle and Liver Tissue

Introduction

Sphingolipids (SPLs) are a class of complex lipid species known primarily for their role in cell signaling in terrestrial mammals (Dickson 1998). These lipids are capable of inducing physiological processes as diverse as cell apoptosis, differentiation, proliferation, and cell cycle arrest by acting as both structural components of cell membranes and also as signaling molecules (Maceyka et al. 2002; Spiegel and Milstien 2003; Ogretmen and Hannun 2004; Bruni and Donati 2008; Hannun and Obeid 2008; Haynes et al. 2009). Ceramide and sphingosine-1-phosphate (S1P), commonly studied SPL species, have been implicated in many processes involved with the pathology and prevention of disease in terrestrial mammals, respectively. The production of bioactive metabolites, utilized as secondary messengers in signaling pathways, are often the result of these signal transduction processes. In addition, SPLs interact with the extracellular matrix and adjacent cells, influence cell membrane structure, and contribute to specialized structures key to vesicular traffic between and within cells (Zheng et al. 2006). Chronic and often rapid remodeling of membrane lipids, through the utilization of activated lipases, often accompanies cell signaling and its associated adaptational processes (Huwiler and Pfeilschifter 2006). Phospholipids (PLs) are the largest class of lipids involved in membranes. These lipids play an active role in influencing the properties of proteins involved in cell signaling, line cells and their organelles with a

layer of semi-permeability (Dowhan 1997), and can serve as intracellular or extracellular signaling components (Alberts et al. 2002).

As diving mammals, ringed seals (*Pusa hispida*) must tolerate a somewhat unique physiological burden of conserving oxygen and coping with bouts of oxidative stress and tissue-level hypoxia (Johnson et al. 2004; Johnson et al. 2005). In order to sustain an aerobic metabolism, aquatic mammals undergo a conserved dive response, characterized by peripheral vasoconstriction, apnea, and bradycardia (Irving 1939). This suite of traits allows the animal to survive on a finite and dwindling supply of internally stored oxygen, even while swimming and foraging. Therefore, when ringed seals are actively diving, they rely upon solely onboard stores of oxygen to sustain their lipid-based aerobic metabolism, and their actively contracting muscles receive very little to no blood flow (Fahlman et al. 2008; Williams et al. 2011; Shiomi et al. 2012). In addition to these physiological constraints, diving mammals must consistently confront increasing hydrostatic pressure during their dives, at levels which would likely prove fatal in terrestrial mammals. Aquatic mammals experience a further atmosphere of hydrostatic pressure for every 10 m increase in depth. In terrestrial mammals, these extreme pressure increases could cause nitrogen narcosis, convulsions, internal bleeding, and even death (Zapol 1987). Species such as the ringed seal combat these pressure changes through strong exhalations pre-dive, collapsible lungs, and potentially, cortisol increases to prevent nervous system excitability and thus, convulsions (Zapol 1987).

Hypoxic stress is thought to be tempered in terrestrial mammals, in large part, through the transduction of molecular cues between and among cells for the purpose of transmitting physiological information, or cell signaling. Often, these signal transduction

molecules are in the form of lipids. Lipids play a central regulatory role in cell signaling in marine mammals for a variety of adaptational purposes. For example, in diving mammals, myoglobin levels are thought to be regulated primarily through qualitative and quantitative changes of lipids (de Miranda Jr. et al. 2012), with calcium signaling and levels of physical activity serving as sources of secondary regulation (Kanatous et al. 2008b, 2009; Ptitsyn et al. 2010; Trumble and Kanatous 2012). As myoglobin levels have been shown to increase during the weaning period of phocid pups (Burns et al. 2005; Burns et al. 2007; Clark et al. 2007), and the metabolism of lipid classes in marine mammals appears to change ontogenetically (Trumble et al. 2010), it seems likely the regulation of myoglobin would be dependent on interaction with governing molecules such as fatty acids or SPLs (Trumble and Kanatous 2012). Thus, we hypothesize that, in addition to elevated levels of antioxidants within diving mammal tissues, a second adaptation in coping with hypoxic stress is through differential cell signaling pathways and resulting compositional changes to the complex lipids within marine mammal membranes. Because marine mammal organs receive variable blood supply during dives, liver and skeletal muscle tissues are likely to react and adapt differently to the stresses of a diving lifestyle (Davis and Kanatous 1999), and these adaptations may be reflected in specific lipids known to be correlated to environmental and physiological stressors (e.g., ceramides; Sawai and Hannun 1999).

Many aquatic organisms, in addition to the challenges created by hypoxia, must cope with dynamic hydrostatic pressure changes (and the accompanying cold environmental conditions at depth). These organisms often do so by varying cell membrane composition and fluidity. As an example, deep sea organisms enhance

membrane fluidity at cold, high-pressure depths by reducing their membrane cholesterol concentrations (Hazel and Williams 1990). In liver tissue of marine teleosts, studies have shown mitochondrial membrane phospholipids to contain a significantly higher percentage of unsaturated fatty acids to compensate for high pressures and low temperatures (e.g., Cossins and Macdonald 1986). Changes in lipid profiles have not been studied in aquatic mammals consistently challenged by hypoxia and rapid changes in pressures and temperatures. However, studies in terrestrial species, and evidence of lipid signaling in marine mammals for other adaptational purposes, supports the idea that these unique cell signaling pathways may be utilized in diving mammals as part of an active, diving lifestyle, and that the resulting lipid profile differences likely vary between organs due to differential blood flow patterns.

Because complex lipids have such a critical function in many terrestrial mammals, we sought to compare levels of various SPLs and PLs in two organs in the ringed seal, the liver and skeletal muscle. Our hypothesis is that the PL and SPL compositions will differ between the liver, an organ receiving significantly shunted convective oxygen transport and blood flow (Davis and Kanatous 1999) and the skeletal muscle, tissue which becomes hypoxic at the onset of diving, yet must continue to function at activity levels above those at rest due to increased exercise during diving (Davis 2013). The ways in which marine mammals limit oxidative stress, the same stress which causes pathologies such as obstructive sleep apnea in terrestrial mammals, are fascinating. The results of these comparisons between organs may provide critical information towards the treatment of human and veterinary pathologies involving periods of hypoxia, rapid pressure and temperature changes, or significant lipid signaling. Additionally, SPLs and

whole PLs have never been compared in marine mammal organs before, so this information represents a unique and novel glance at complex lipids in a species living at physiological extremes.

Materials and Methods

Animals and Experimental Procedures

Ringed seal skeletal muscle (*longissimus dorsi;* n = 5 adults) and liver samples (n = 5 adults) were sampled during subsistence hunts in Sisualik, AK during two different sampling periods; October 2008 and May 2009 (Kathy Frost, Alaska Department of Fish and Game). Samples were collected immediately upon harvest, placed in 2.0 ml cryovials (Sigma-Aldrich, St. Louis, MO), and shipped overnight on dry ice. All samples were subsequently stored at -80°C until laboratory analysis.

Sphingolipid and whole PL analysis, both quantification and identification, was accomplished through liquid chromatography-high resolution mass spectrometry (LC-HRMS). An internal standard mixture was prepared for LC-HRMS analysis utilizing the following compounds (Avanti Polar Lipids, Alabaster, AL): 14:0 D54 phosphatidylserine (PS) (1.25 ng/nL), 16:0 D31 sphingomyelin (SM) (1 ng/nL), 1,3-18:0 diacylglycerol (DG)-d5 (1.25 ng/nL), 16:0 PC D04 (1.25 ng/nL), sphingosine D7 (1 ng/nL), 16:0 D31 ceramide (1.25 ng/nL), 16:0 D62 phosphatidylglycerol (PG) (1.75 ng/nL), 16:0 D31 lyso phosphocholine (PC) (1 ng/nL), and 14:0 D54 phosphatidylethanolamine (PE) (1.5 ng/nL).

Skeletal muscle and liver tissue biopsies, frozen at -80°C, in sizes averaging 25 mg, were ground with diatomaceous earth (EMD Chemicals, Rockland, MA) with mortar and pestle to dehydrate and homogenize the sample. Each sample was subsequently

placed in a woven thimble and run through a Foss SoxTec 2043 (Eden Prairie, MN) to extract lipids, using a solvent mixture of 65/35 chloroform/methanol ($CHCl_3$/MeOH). The samples were boiled at 160°C for 25 minutes, and then rinsed at 100°C for 30 min. To prevent oxidation, lipid extracts were then dried under a stream of nitrogen. Each sample was subsequently diluted in 4 ml MeOH and placed in a 4 ml glass vial with a polytetrafluoroethylene (PTFE) cap (Sigma-Aldrich, St. Louis, MO). 475 µl of the total 4 ml of each sample was removed from the glass vial and placed in a 1.5 ml plastic micro-centrifuge tube (VWR, West Chester, PA), and all samples were ultracentrifuged @10,000*g* for 10 min. Each sample, placed in a 2 ml amber gas chromatography (GC) vial (Sigma-Aldrich, St. Louis, MO), was spiked with 25 µl of the above-mentioned internal standard mix at a concentration of roughly 1 ppm/compound.

An Accela liquid chromatograph coupled to a linear trap quadropole (LTQ) Orbitrap Discovery mass spectrometer (Thermo Electron, Bremen, Germany) using both positive and negative electrospray ionization (+ESI/-ESI) was used to analyze extracts. Final extracts were injected (2 µL) into the LC system utilizing a 15 cm x2.1 mm (5 µm, 80 Å) Extended-C18 column (Agilent Technologies, Palo Alto, CA). A gradient of binary mobile phases containing 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in MeOH (B) was applied as follows: 3 min at 50% A, to 98% B in 9 min, held steady for 3 min, back to 50% A in 1 min, and held again for 2 min at 50% A. Additional chromatographic parameters included the following: flow rate, 500 µl/min; column temperature, 50°C. High resolution (30,000 full width at half maximum (FWHM)) full-scan accurate mass spectra (m/z range: 200-1000) of eluting compounds were obtained on the Orbitrap mass analyzer using internal calibration (accuracy of

measurements < 2 ppm) and subsequently processed using XCalibur v.2.0.7 software. Electrospray source conditions were: heated capillary temperature 300°C; sheath and auxiliary gas flow 55 and 10 arbitrary units (a.u.), respectively; electrospray voltage 0.5 kV for both +ESI and –ESI; tube lens voltage 85 V for +ESI and 88 V for –ESI; capillary voltage 44 V for +ESI and 35 V for –ESI.

An isotope dilution approach was used to determine lipid concentrations. Ten calibration points ranging from 2.5 ng/ml to 5 μg/ml were created using lipid stock solutions containing 100 μg/ml or 200 μg/ml serially diluted into MeOH with 0.1% formic acid (HCOOH). Constant amounts, defined both by amounts and molecular formula, of lipid isotopes were added to both calibrators and samples as internal standards using a stock solution concentration prepared in methanol. Calibration curves were prepared by plotting the analyte concentration versus the ratio of observed peak areas for both the analyte and internal standard, respectively. Regression coefficients (r^2) exceeding 0.99 for all targeted lipids were accomplished for all calibration data subjected to a linear regression forced through the origin. Calibration was monitored during LC-HRMS analyses through the use of blanks and continuous calibration verification (CCV) standards meeting an acceptability criterion of ±15%. Two identical matrix spikes (i.e. one matrix spike/matrix spike duplicate pair, MS/MSD), increasing total concentration by approximately a factor of two, were prepared by adding a known amount of lipids to analyzed samples, and were run every 12 samples. For quality-assurance purposes, recovery percentages of spiked amounts (criteria 85-115%) as well as MS/MSD relative percentage difference (criteria 15%) were achieved.

The extraction of S1P used a butanolic lipid approach, modified from Baker et al. (2001). A phosphate buffer (containing 30 mM citric acid and 40 mM Na2HPO4) was mixed with 75 µl of skeletal muscle or liver homogenate. After vortexing, the sample was mixed with 1 ml 1-butanol and 500 µl water-saturated 1-butanol. After mixing for 15 seconds, the sample was centrifuged for 10 min @10,000*g*. The upper butanol phase containing the S1P was extracted, evaporated under nitrogen to dryness, and resuspended in 4 ml MeOH for analysis by LC-HRMS (as above). The remaining steps of preparation/analysis for LC-HRMS are identical to those already described.

Treatment group comparison results (independent variable, organ; dependent variable, lipid concentration) was determined by unpaired Student's T-test. Linear regressions were conducted using body mass (independent variable) against C16 ceramide concentration in the skeletal muscle (dependent variable), skeletal muscle C16 ceramide concentration (independent variable) against DPPC concentration in skeletal muscle (dependent variable), and skeletal muscle C16 ceramide concentration (independent variable) against 16:0 SM skeletal muscle concentration (dependent variable). Analyses were conducted by using SAS® (Version 9.4) and PHStat (Version 2.8), respectively. P values ≤ 0.05 were considered significant. All data presented are mean ± SE.

Results

Rationale for Pooling of Samples

Because marine mammal samples are notoriously difficult to acquire, ringed seal liver and skeletal muscle samples were not able to be paired, and thus were pooled for

analysis. Dietary influence on sphingolipid metabolism in organs beyond the gastrointestinal system is minimal (Merrill Jr. et al. 1995). When sphingolipids are broken down in the intestine, they are hydrolyzed to ceramides and sphingosines (Vesper et al. 1999). Once taken up by intestinal cells, most of these sphingolipid products are broken down to individual constituent fatty acids, which are then burned for fuel or stored, or are reformed into more complex sphingolipids which generally remain within the intestine (Nilsson 1968; Schmelz et al. 1994). Samples were also pooled due to their similarity. No individual sample was an outlier in any sphingolipid or phospholipid analysis, nor was any sample an outlier in the analysis of fatty acids (unpublished data). Additionally, studies have shown that dietary lipid intake does not necessarily influence membrane lipid composition (e.g., Ayre and Hulbert 1996).

Changes in Skeletal Muscle and Liver Sphingolipid Profiles

C16 ceramide was significantly increased in ringed seal liver compared to skeletal muscle (liver: $n = 5$, $\bar{x} = 15.114 \pm 3.75$ µg/g; skeletal muscle: $n = 5$, $\bar{x} = 2.972 \pm 0.44$ µg/g; $p = 0.0123$; Figure 6). Ringed seal liver also had a significant increase in 16:0 sphingomyelin (SM) when compared to skeletal muscle (liver: $n = 5$, $\bar{x} = 175.46 \pm 33.24$ µg/g; skeletal muscle: $n = 5$, $\bar{x} = 72.64 \pm 9.52$ µg/g; $p = 0.0178$; Figure 6). Though a total of 5 SPL species were tested, 3 species (those mentioned here as well as S1P) were found at measurable concentrations.

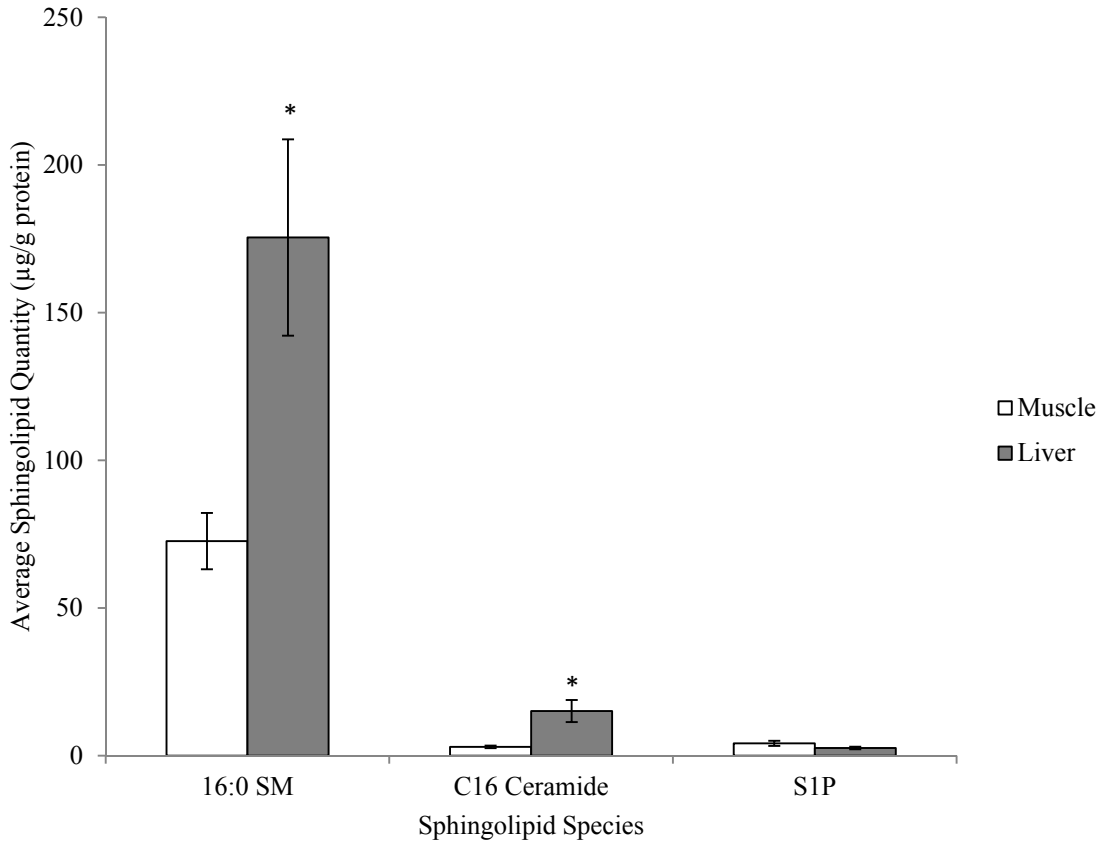

Figure 6. Average sphingolipid quantity in ringed seal skeletal muscle and liver. Values are means ± SE, * indicates significant environmental effect.

Changes in Skeletal Muscle Phospholipid Profiles

Only one PL, 16:0 dipalmitoylphosphatidylcholine (16:0 PC DPPC, or DPPC), was significantly increased in ringed seal liver when compared to skeletal muscle (liver: $n = 5$, $\bar{x} = 55.802 \pm 12.46$ µg/g; skeletal muscle: $n = 5$, $\bar{x} = 17.984 \pm 4.59$ µg/g; $p = 0.0215$; Figure 7). C16 lyso phosphatidylcholine, 14:0 phosphoethanolamine, and 16:0 phosphoglycerol did not differ significantly between ringed seal liver and skeletal muscle. Five PL species were examined, with 4 of the 5 species were found at measurable concentrations.

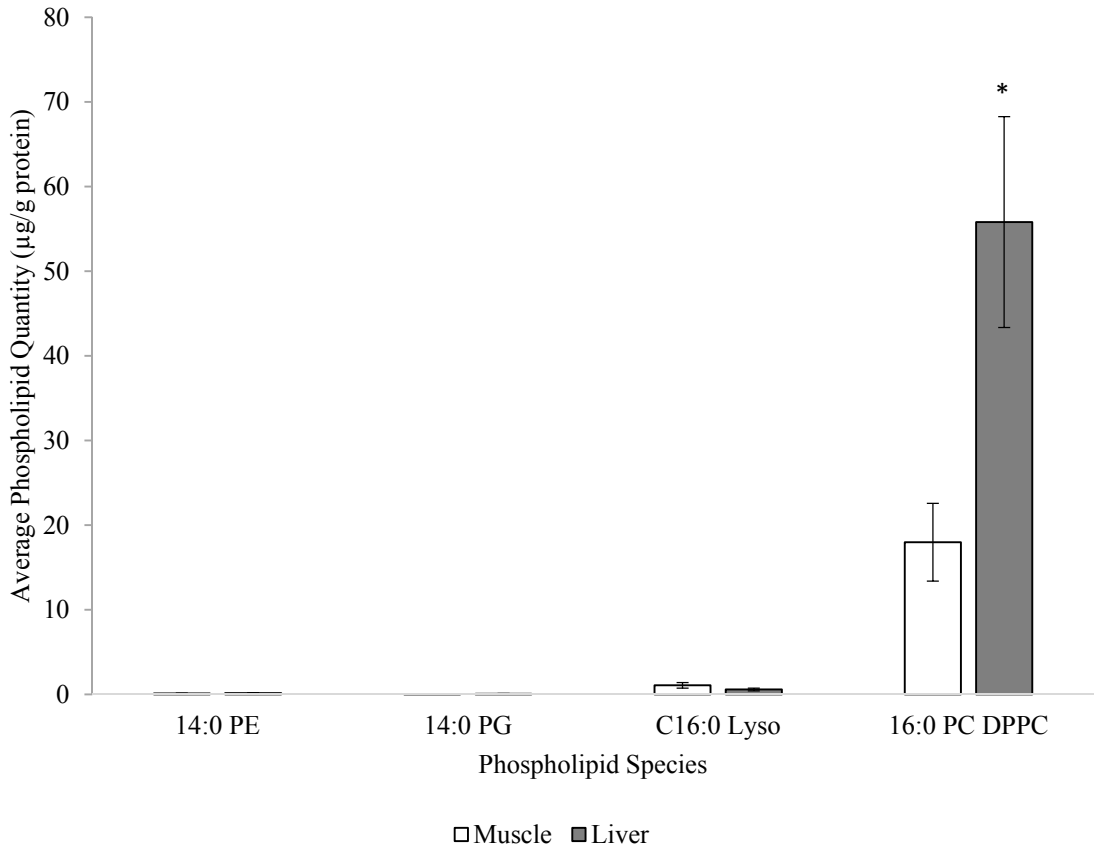

Figure 7. Average phospholipid quantity in ringed seal skeletal muscle and liver. Values are means ± SE, * indicates significant environmental effect.

Correlations Between Animal Masses and Complex Lipids

A significant positive linear correlation was found between ringed seal body mass and C16 ceramide levels in skeletal muscle ($R^2 = 0.73$; Figure 8). This correlation was not evident in ringed seal liver analyses. Additionally, a positive linear correlation was found between ringed seal C16 ceramide concentration and 16:0 PC DPPC concentration in skeletal muscle ($R^2 = 0.68$; Figure 9). Finally, a significant positive linear correlation was found between C16 ceramide concentration and 16:0 SM levels in ringed seal skeletal muscle ($R^2 = 0.61$; Figure 10).

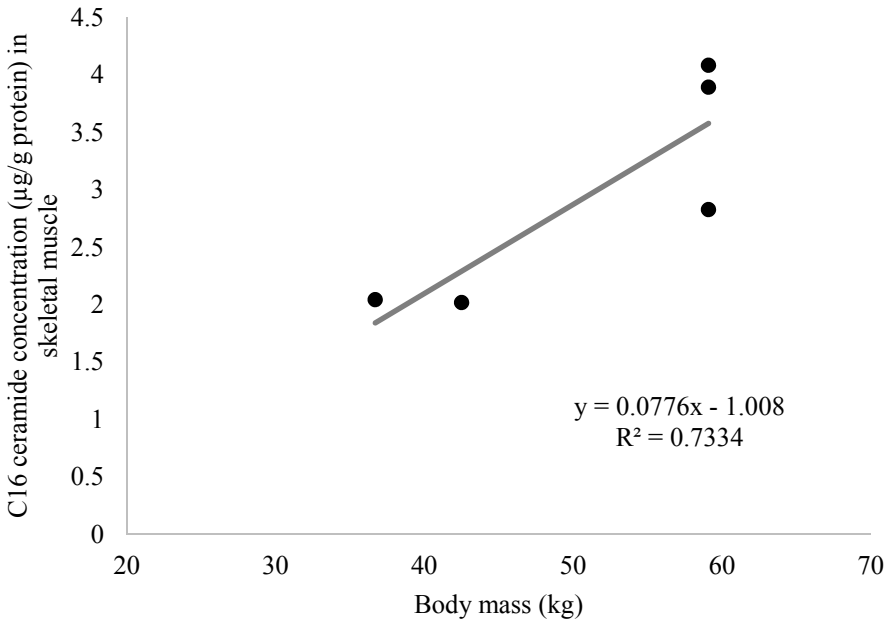

Figure 8. Individual ringed seal body mass (n = 5) vs. paired C16 ceramide skeletal muscle levels.

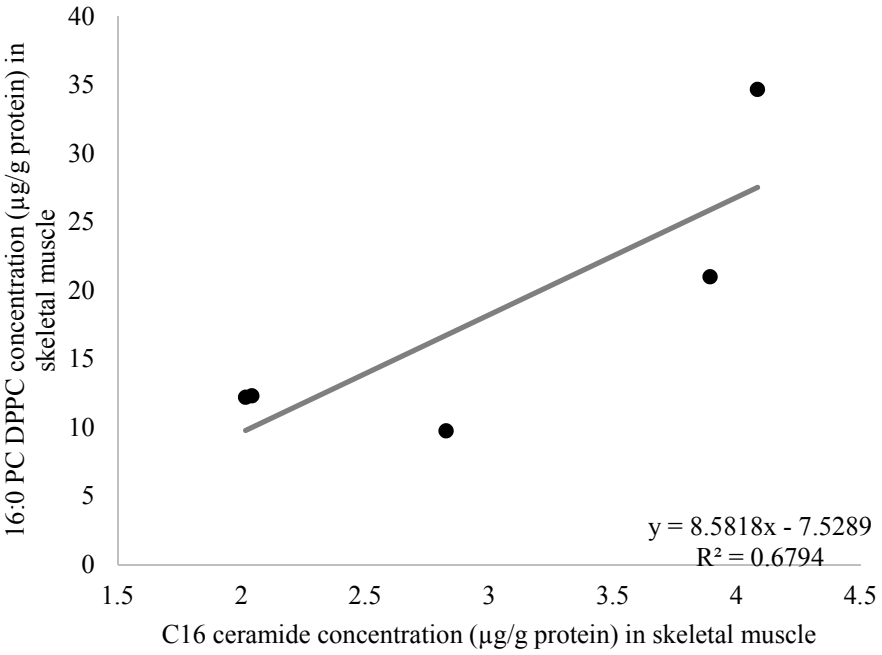

Figure 9. Individual ringed seal (n = 5) C16 ceramide concentration vs. paired 16:0 PC DPPC concentration in skeletal muscle.

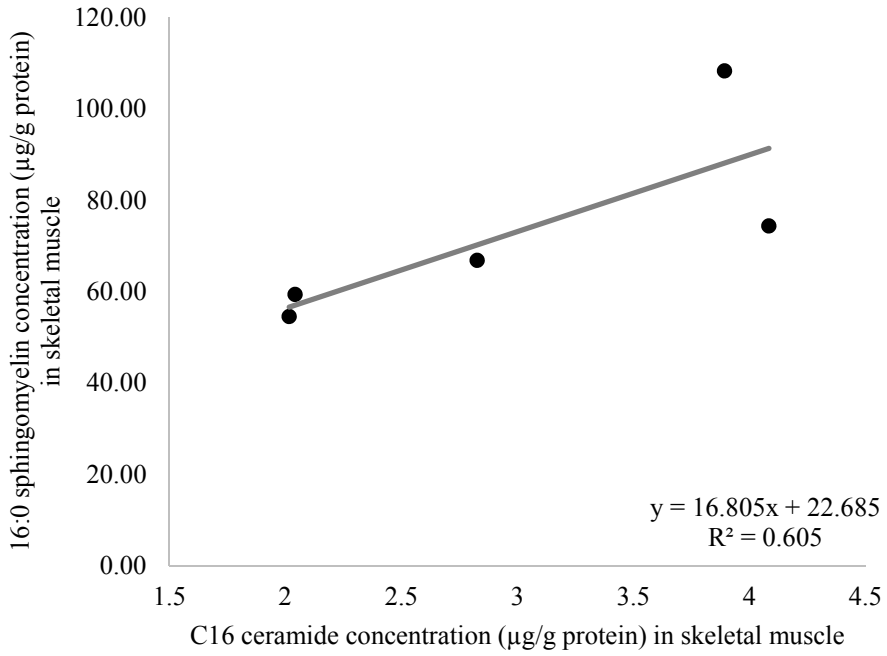

Figure 10. Individual ringed seal (n = 5) C16 ceramide concentration vs. paired 16:0 sphingomyelin concentration in skeletal muscle.

Discussion

Results presented here indicate ringed seal liver has significantly higher levels of the SPLs C16 ceramide and 16:0 sphingomyelin compared to skeletal muscle (Figure 6), as well as significantly higher levels of the PL 16:0 dipalmitoylphosphatidylcholine (16:0 PC DPPC) (Figure 7). A positive linear correlation was discovered between ringed seal body mass and C16 ceramide levels in skeletal muscle (Figure 8). Additionally, a positive linear correlation was found between C16 ceramide and 16:0 PC DPPC concentrations in ringed seal skeletal muscle (Figure 9). Finally, a significant positive correlation was reported between C16 ceramide and 16:0 SM levels in ringed seal skeletal muscle (Figure 10).

Aquatic mammals such as the ringed seal employ selective peripheral vasoconstriction during dives, resulting in intermittent hypoxia in marginal skeletal

muscle, the liver, and most bodily organs (Hochachka and Mottishaw 1998; Davis and Kanatous 1999). Very few studies in marine mammals have been accomplished tracking blood flow to splanchnic organs due to the inherent complexity of these experiments (i.e., Davis et al. 1983; Fuson et al. 2003). Mammalian blood flow, under resting conditions, typically perfuses splanchnic organs and the kidneys with roughly 61% of its total volume (Sapirstein 1958). Because most marine mammals are believed to dive within their aerobic dive limit (ADL, the longest duration dive an animal can make while maintaining an aerobic metabolism; Kooyman et al. 1980), the visceral organs receive adequate oxygen, despite selective vasoconstriction, to maintain an aerobic metabolism (Davis and Kanatous 1999). Thus, during most dives, the liver remains fully functional despite limited blood flow (Davis 1983). This is critical to physiological homeostasis in the aquatic mammal, as these organisms must feed and assimilate this food, while submerged, in addition to processing accumulated lactate upon surfacing (Davis 2013).

In a study on harbor seals, the only such study to report metabolic enzymatic adaptations in diving mammal splanchnic organs, the liver portrayed significantly higher mitochondrial density, elevated β-hydroxyacyl-CoA (HOAD) levels, an indication of lipid-based, aerobic potential, and elevated citrate synthase (CS) levels, a measure of aerobic capacity, compared to terrestrial mammals (Fuson et al. 2003). In addition, lactate dehydrogenase (LDH), an indicator of anaerobic capacity, was significantly elevated in liver tissue, which implies these organisms can tolerate some level of anaerobic metabolism if required (Fuson et al. 2003). Because the liver is dependent on blood flow and typically processes more blood than required to maintain homeostasis, blood flow to this organ can be reduced to approximately 20% of normal without

negative consequences to physiological functioning (Nishizaki et al. 2001; Levey and Coresh 2012). This ability of the liver to function at extremely low blood flow enables the organism to salvage much of the rest of the oxygen stores within the limited circulating blood for other tissues, particularly nervous tissue and active swimming muscles.

Blood flow is not only shunted to the splanchnic organs of diving mammals, but also to the skeletal muscle, even during exercise when the tissue is forced into an increased (from rest) metabolism. A normal, terrestrial response to exercise includes increased perfusion of skeletal muscle, but due to the dive response of marine mammals, bradycardia and peripheral vasoconstriction occur, limiting blood flow to the muscle. The degree to which the organism is exercising, in part, determines the amount of blood flow to skeletal muscle (e.g., increased exercise results in a lessened bradycardia and vasoconstriction compared to a lower-intensity dive). Interestingly, at some level, hypoxia in skeletal muscle is required in order to induce the dissociation of oxygen from myoglobin in the muscle (Guyton et al. 1995). The skeletal muscle of diving mammals, similar to the liver, has increased mitochondrial volume density compared to comparable muscles in terrestrial species (Kanatous et al. 1999). This same tissue also has increased levels of the enzymes CS, HOAD, and LDH (Kanatous et al. 1999; Polasek et al. 2006).

A comparison of liver to skeletal muscle tissue in a diving mammal will likely provide insight into adaptations to hypoxia. In rats, isolated hepatocytes have been shown to produce a large quantity of reactive oxygen species (ROS) after reoxygenation following anoxic conditions (Caraceni et al. 1994a; Caraceni et al. 1994b). Most of the ROS produced in these hepatocytes were derived from the mitochondria (Caraceni et al.

1995). As the liver in diving mammals is significantly saturated with mitochondrial volume compared to terrestrial mammals, and generally receives less blood flow compared to skeletal muscle during dives, this tissue would seem to be at a higher risk of oxidative damage. Chronic, intermittent hypoxia (and associated reperfusion) has been shown to increase the production of ROS in many tissues (e.g., Elsner et al. 1998). Because marine mammal tissues generally show no negative effects from this increased ROS production, adaptations to chronic, intermittent hypoxia are likely at play. One of these adaptations appears to be an increase in the antioxidant status of tissues within diving mammals compared to terrestrial mammals (e.g., Zenteno-Savin et al. 2002; Vazquez-Medina et al. 2012).

Ringed seals have not been characterized with respect to sphingolipid profiles, yet they may employ distinctive cell signaling pathways compared to terrestrial mammals based on their individual environmental stressors, and therefore may demonstrate a unique SPL membrane signature. Cell signaling pathways utilizing SPLs have recently been correlated with responses to hypoxia in terrestrial species (Huwiler and Pfeilschifter 2006). Ceramides are often considered the lipid mediators of the eukaryotic stress response (e.g., Jenkins et al. 1997; Jenkins et al. 2002). Recent research in rodent smooth muscle has shown that hypoxia can induce a decrease in ceramide and an increase in cellular S1P due to sphingomyelinase activity inhibited by low oxygen conditions (Yun and Kester 2002). Other studies in rats have shown increases in ceramide levels as a result of hypoxia in cardiomyocytes (Bielawska et al. 1997) and in renal tubular epithelial cells (Basnakian et al. 2005). Hypoxia has been demonstrated to increase lipid concentrations in a variety of other mammalian cell lines. For example, low oxygen

conditions caused an increase in the lipid PGE_2 in rat mesangial cells (Petry et al. 2005), diacylglycerol in HeLa and T-Cells (Aragones et al. 2001 and Temes et al. 2004, respectively), arachidonic acid in rat neuroblastoma cells (Petroni et al. 2002), in addition to an increase in ceramides in many cell lines (e.g., Basnakian et al. 2005).

C16 ceramide was found in significantly higher levels in liver tissue of ringed seals compared to skeletal muscle. Additionally, 16:0 sphingomyelin, a SPL derived from ceramide (Zheng et al. 2006), and therefore, a natural correlate to ceramide, was significantly elevated in ringed seal liver compared to skeletal muscle. In the skeletal muscle of ringed seals, a positive correlation was reported between C16 ceramide and 16:0 SM (Figure 10), likely indicating that the production of ceramides is via de novo synthesis rather than from a pool of SM via the salvage pathway (Grosch et al. 2012), which would otherwise lead to an expected negative correlation between C16 ceramide and 16:0 SM. Because ceramides are thought to correlate to physiological stressors, this may indicate that breath-hold diving in ringed seals creates a more prominent stressor to the liver due to the organ's essential role in survival and metabolic maintenance during diving. During longer dives, the liver potentially undergoes anaerobic metabolism (e.g., Valtin 1973; Adams and Cain 1983; Nelson et al. 1988), which is in stark contrast to the skeletal muscle, which is able to largely maintain an aerobic metabolism due to the large amounts of stored myoglobin (Davis and Kanatous 1999). In other words, ringed seal skeletal muscle may be so well-adapted to routine diving that the dives themselves do not cause an extreme amount of physiological stress to the tissue, as opposed to the liver, which is absolutely critical to the health of the seal (e.g., detoxification, bile production, etc.) and must remain fully metabolically active during dives. The liver, therefore, shows

a stress response in the form of increased C16 ceramide and 16:0 SM in the ringed seal. This result may not be surprising, as previous studies in ringed seals (e.g., Johnson et al. 2004) have shown reduced levels of HIF-1α in liver tissue compared to skeletal muscle. HIF-1α is thought to play a protective role against protein oxidation in mammalian tissue under low oxygen conditions (e.g., Bilton and Booker 2003), so the lower levels of this protein in liver tissue reported by Johnson et al. (2004) implies that the liver may be at increased risk of oxidative stress due to diving. This may explain why indications of stress (i.e., ceramides) were upregulated in liver tissue more so than in skeletal muscle. Though hepatic function appears to remain intact during dives despite severely reduced blood flow (Davis et al. 1983), the organ may still be exhibiting a response to oxidative stress. While this stress response may be a result of reduced blood flow, it may also be explained by the role of the liver repeatedly clearing accumulated lactate upon the organism surfacing. Elevated LDH levels in the liver may signify an enhanced ability by the liver of diving mammals to process this lactate compared to terrestrial mammals. However, extended dives yielding large amounts of lactate may stress the liver and result in an accumulation of ceramides greater than that found in the skeletal muscle, despite higher levels of many antioxidant enzyme systems in ringed seal liver compared to skeletal muscle (Vazquez-Medina et al. 2006).

Several other environmental factors may result in increased liver ceramide levels in the ringed seal. Ringed seals are among the most common seal species in the Arctic (Smith 1987). Because these seals live in the Polar Regions, they must continually cope with some of the most extreme environmental conditions on earth. However, a continually warming Arctic climate (e.g., Meier et al. 2004; Ferguson et al. 2005) puts

this species at risk (and likely under significant stress, including thermal) due to their absolute dependence on plentiful sea ice for breeding, pupping, nursing, and protection from predation (Reeves et al. 2002). In the model organism, yeast (*Saccharomyces cerevisiae*), heat stress has been correlated to strikingly increased ceramide levels (e.g., Jenkins et al. 1997; Jenkins et al. 2002). This same trend has been demonstrated in several cell lines in mammals, including HL-60 (Kondo et al. 2000), NIH WT-3T3 (Chang et al. 1995), and Molt-4 (Jenkins 2003). In addition, because harbor seals inhabit a circumpolar distribution, they may display increased phenotypic plasticity, resulting in a greater expression of C16 ceramide and 16:0 SM as an adaptation to thermal stress. Faced with climate change, phenotypic plasticity has been reported in Antarctic fur seals (*Arctocephalus gazella*) (Forcada et al. 2008). Additionally, killer whale (*Orcinus orca*) pigmentation patterns and fin shape have been suggested to differ due to phenotypic plasticity (Foote et al. 2009). The ability to adapt to a changing climate may prove invaluable to the ringed seal as it faces a continual decline in habitat. While hypoxic stress is likely the main mechanism at work in increasing ceramide levels in the liver, the size of the seal may also be a factor in the amount of stress sensed by the organism. As ringed seal body mass increased, the level of ceramides in skeletal muscle also increased (Figure 8). While skeletal muscle still showed lower levels of ceramides compared to liver tissue, the larger amounts of this SPL in the heavier ringed seals may indicate that the larger organisms, with a larger supply of only intermittently perfused skeletal muscle, may be less well-adapted to the stresses of diving.

In relation to hypoxia, whole phospholipid changes (as opposed to the fatty acid changes seen within phospholipids) have very rarely been studied, and never measured in

marine mammal organ tissue. The PL 16:0 PC (DPPC) was found in significantly higher levels in ringed seal liver compared to skeletal muscle. Dipalmioylphosphatidylcholine is one of the four most common phospholipids found in eukaryotic cell membranes (Alberts et al. 2002). This phospholipid is most commonly found associated with lung tissue in mammals, specifically in the protective pulmonary surfactant (Veldhuizen et al. 2000; Tonks et al. 2001). DPPC is known to have an anti-inflammatory effect within the lung tissue, as well as reducing surface tension within the pulmonary surfactant. This lipid, when compressed, can pack into a gel-like phase that is easily able to resist very high surface pressures (Veldhuizen et al. 2000). In addition, DPPC has been reported in higher concentrations in marine mammal lung surfactant compared to that of terrestrial species (Foot et al. 2006), which implies that the great increases in hydrostatic pressure endured by marine mammals requires a surfactant with greater fluidity, strong anti-adhesive properties, and the ability to tolerate continual collapse and reinflation of the lungs (Foot et al. 2006). The ability for cell membranes to counteract high pressure environments (e.g., during deep dives) would seem to be highly advantageous for marine mammals, The significantly higher levels of DPPC were hypothesized to be found in the ringed seal liver, as this organ must remain fully functional and metabolically active during breath-hold diving without the aid of myoglobin. Indeed, DPPC was significantly elevated in ringed seal liver compared to skeletal muscle. This likely relates to the chronic ischemia/reperfusion seen in skeletal muscle associated with breath-hold diving, where blood flow is only intermittently perfused to this tissue to spare oxygen for the absolutely essential organs (e.g., the heart, adrenals, brain, and lungs) (e.g., Kerem and Elsner 1973; Zapol et al. 1979; Hill et al. 1987). The average dive depth of ringed seals

(Reeves et al. 2002) exceeds that required to induce lung collapse (estimated at 70 m; Kooyman and Ponganis 1998). As DPPC is usually associated with conditions commonly influenced by pressure changes, a functioning liver would benefit more from the pressure-protective effects of DPPC than would peripheral skeletal muscle, which would be susceptible to some form of compression during diving due to the intermittent retraction of blood flow. Even in skeletal muscle however (Figure 9), evidence points to the higher stress loads requiring a greater protection from dynamic changes in hydrostatic pressure.

In conclusion, blood flow patterns in relation to hypoxia and body size may result in the difference reported between ringed seal liver and skeletal muscle tissue. In addition, a warming Arctic climate, and subsequent reduction in sea ice habitat, may also contribute to the changes shown here. Due to the immediacy of the global warming challenge, more research needs to be carried out to understand the role phenotypic plasticity may play in the adaptation, or lack thereof, or ringed seals to climate change. Future studies will further delineate the changes described here and provide additional clues to organ-specific hypoxia tolerance in marine mammals. Finally, these findings between liver and skeletal muscle tissue have potentially important implications for human afflictions such as congestive heart failure (a disease characterized by swelling and pressure changes), and afflictions dealing with hypoxia or low blood flow to critical organs (e.g., liver), such as cirrhosis. The mechanisms behind differences in organ responses to these environmental or pathological conditions may prove insightful to the medical field.

CHAPTER FOUR

High Fat Diet Drastically Alters Mouse Skeletal Muscle Fatty Acid Composition But Does Not Largely Influence Sphingolipid or Whole Phospholipid Compositions

Introduction

Insulin resistance and pre-diabetes are rapidly becoming epidemics in developed countries. Insulin resistance is a key feature of type 2 diabetes mellitus (T2DM), and a necessary precursor to pre-diabetes, a condition of chronic, mildly elevated blood glucose levels (Olefsky et al. 1973). North America is quickly becoming the epicenter for a serious health crisis involving these metabolic conditions. In the United States alone, for example, the American Diabetes Association reports an estimated 79 million people are pre-diabetic. However, even more alarming is the statistic that of these 79 million, over 2 million pre-diabetics in this country are under the age of 20, an age range once presumed to be metabolically healthy (ADA 2011).

Skeletal muscle, as a major glucose sink for the body, plays a chief role in insulin resistance, as it comprises 30-40% of body mass, on average, and accounts for roughly 80% of the insulin-stimulated uptake of glucose (DeFronzo et al. 1981; DeFronzo et al. 1985). High fat diet (HFD) consumption leads directly to the accumulation of excessive skeletal muscle lipid (through decreased insulin-stimulated glucose uptake), which in turn can lead to a suite of metabolic diseases not limited to obesity, insulin resistance, pre-diabetes, and eventually T2DM. Clarifying changes that occur to this skeletal muscle, a tissue so critical to energy expenditure and insulin-stimulated glucose disposal, is a top

priority in understanding the causes and mechanisms behind insulin resistance and the suite of metabolic conditions associated with insulin resistance.

Several studies linking changes in fatty acid composition to insulin resistance have been conducted, the majority of which demonstrate an increased proportion of saturated fatty acids (SFA) in skeletal muscle phospholipids (de Wilde et al. 2008; Haugaard et al. 2006; Hulbert et al. 2005; Pan et al. 1995; Vessby et al. 1994). Additional studies involving polyunsaturated fatty acids (PUFAs) (Haugaard et al. 2006; Pan et al. 1995; Vessby et al. 1994) and sphingolipids (ceramides; (Schmitz-Peiffer et al. 1999; Schubert et al. 2000; Straczkowski et al. 2007); diacylglycerol (DAGs); (e.g., Itani et al. 2002, Moro et al. 2009; Straczkowski et al. 2007)) have shown clear positive correlations with insulin resistance. Further, changes to the intramyocellular lipid (IMCL) content within skeletal muscle has provided information on insulin signaling; specifically, higher SFA content within the IMCL pool has been linked to a decrease in the insulin signaling components PKB/Akt (Storz et al.1999). However, a broader study assessing concurrent changes in whole phospholipids and sphingolipids with changes in fatty acids has not been undertaken. Elucidating which fatty acids, whole phospholipids, and sphingolipids change in response to HFD and insulin resistance may provide pathological insights, as many of the phospholipids, in particular, are heavily linked to dietary intake, and as such, may be manipulated through dietary intervention (Popp-Snijders et al. 1986). As well, sphingolipids, as key players in signal transduction pathways, may provide valuable insight into the complex suite of metabolic diseases associated with insulin resistance in the context of a HFD. Finally, many of the IMCL species are altered intracellularly to become bioactive lipids such as ceramide or DAG,

both of which are known to be correlated to some degree with insulin resistance (e.g., Kelley et al. 2002). This has important implications when the large quantity of the IMCL pool within skeletal muscle, especially insulin-resistant skeletal muscle, is considered.

Therefore, the aim of the present study was to test the hypotheses that ceramides, monounsaturated fatty acids (MUFAs), and SFAs will increase in pre-diabetic mice, as a result of HFD feeding and resultant insulin resistance. To study the effects of HFD feeding on C57BL/6J mice, the mice were fed a nearly 61% fat-based diet for 8 weeks. We then analyzed the composition of the IMCL/phospholipid fatty acid pool using gas chromatography, and characterized the composition of the sphingolipid and whole phospholipid profiles in these cell membranes using LC-HRMS.

Materials and Methods

Animals and Experimental Procedures

All experimental protocols were approved by the York University Animal Care Committee in accordance with Canadian Council for Animal Care Guidelines. Male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in temperature and humidity-controlled facility with a 12/12 h light/dark cycle and had *ad libitum* access to water and food. After an initial acclimatization, animals (10 weeks of age, n = 6 per group) were randomly assigned to either a high fat diet [HFD; TestDiet, cat#58126: energy (kcal/g) from protein (18.3%), fat (60.9%), carbohydrate (20.1%)] or standard mouse chow [LabDiet 5015 Mouse Diet: energy (kcal/g) from protein (20%), fat (25%), carbohydrate (55%)].

Following 8 weeks on the diet, mice were fasted overnight (16 hrs) and anaesthetized, with the hamstring muscle harvested. At the time of harvest, all HFD mice displayed elevated fasting insulin, elevated insulin at the 45-min mark of an intraperitoneal glucose tolerance test (IPGTT) performed after an overnight fast, and were determined to be obese with insulin resistance (i.e., pre-diabetic). All samples were frozen and stored at -80°C until analyzed. These mice were also used in an additional study (Shortreed et al. 2009).

Lipid Extraction and Fatty Acid Analysis

The fatty acid composition of the combined IMCL/phospholipid pool was analyzed through gas chromatography. Lipid extraction procedures included dehydrating each sample via diatomaceous earth (approximately 5g, J.T. Baker, Phillipsburg, NJ) before running through a lipid extractor (Foss Soxtec 2043, Eden Prairie, MN) using a 2:1 mixture of chloroform:methanol (Sigma-Aldrich, St. Louis, MO) as the solvent. Laboratory conditions were maintained across all extractions. Excess solvent was evaporated under a nitrogen stream (1.5 L/min @37°C) to prevent lipid oxidation. The resulting lipid extract bound for analysis by gas chromatography-flame ionization detector (GC-FID) was then derivatized using a method similar to Budge et al. (2006). Samples were heated at 100°C for 1 h after being mixed with 1.5 ml dichloromethane (DCM) and 3 ml Hilditch reagent (Sigma Aldrich, St. Louis, MO). The lipids were then extracted by repeated centrifugation separation (100 x *g* for 2-5 min) with DCM (3 ml for first centrifugation, 1 ml for latter two) and deionized water (1 ml for each centrifugation). The lipid layer (bottom) was then dehydrated with sodium sulfate (approximately 0.5 g) and heated in a water bath to evaporate any excess solvent. Each

transesterified sample was then analyzed for fatty acid methyl ester (FAME) on a GC-FID (Varian 430-GC and Varian CP-8400 AutoSampler) using a CP-Select column (CP7419, Varian) 100 m x 0.25 mm ID x 0.25 µm. GC-FID protocols follow Budge *et al.* (2006) with the following modifications: the injector temperature was 250°C with a 1 µl injector split ratio 100:1; the column length was 100 m. Column flow was 1.0 ml min^{-1} programmed at 210°C for 9.0 min and ramped for 7.7 min at 15°C min^{-1} to 260°C. Detector temperature was set at 300°C with an air flow of 300 ml min^{-1} and a hydrogen flow of 30 ml min^{-1}. The internal standard was the FAME C19:0 (Fluka 72332). A set of 37 standard historical marine FAME (Supelco®37 component FAME mix, Supelco®, Bellefonte, PA; additional FAME species, Sigma-Aldrich, St. Louis, MO) were used in the GC-FID analysis, primarily for their physiological similarity to fatty acid profiles commonly found in mammals, and were of the highest purity available. For each level of calibration and standard curve generated, all FAME target analytes were present at equal concentrations with ranges between 0.25 µg/µl and 10 µg/µl for each compound.

Sphingolipid and Phospholipid Analysis

Liquid chromatography-high resolution mass spectrometry (LC-HRMS) was utilized for the identification and quantification of sphingolipids and whole phospholipids. An internal standard mixture (Avanti Polar Lipids, Alabaster, AL) was created for LC-HRMS analysis: 14:0 D54 phosphatidylethanolamine (PE) (1.5 ng/nl), 16:0 PC D04 (1.25 ng/nl), 16:0 D31 lyso phosphocholine (PC) (1 ng/nl), 16:0 D31 Ceramide (1.25 ng/nl), 16:0 D62 phosphatidylglycerol (PG) (1.75 ng/nl), sphingosine D7 (1 ng/nl), 1,3-18:0 diacylglycerol (DG)-d5 (1.25 ng/nl), 14:0 D54 phosphatidylserine (PS) (1.25 ng/nl), and 16:0 D31 sphingomyelin (SM) (1 ng/nl).

Mouse skeletal muscle tissue biopsies, frozen at -80°C, in sizes ranging from 20 to 63 mg, were each ground with diatomaceous earth (EMD Chemicals, Rockland, MA) with mortar and pestle to dehydrate and homogenize the sample. Each sample was placed in a thimble and run through a lipid extraction device, Foss SoxTec 2043 (Eden Prairie, MN), using a solvent mixture of 65/35 chloroform/methanol ($CHCl_3$/MeOH). Samples, at 160°C, were boiled for 25 minutes, and then rinsed for 30 min at 100°C. Lipid extracts were then dried under a stream of nitrogen to prevent oxidation. Each sample was then resuspended in 4 ml MeOH and placed in a 4 ml glass vial with polytetrafluoroethylene (PTFE) cap (Sigma-Aldrich, St. Louis, MO). From the 4 ml of each sample, 475 µl was removed from the glass vial and placed in a 1.5 ml plastic micro-centrifuge tube (VWR, West Chester, PA), and all samples were ultracentrifuged @10,000*g* for 10 min. Each sample was then placed in a 2 ml amber gas chromatography (GC) vial (Sigma-Aldrich, St. Louis, MO) and spiked with 25 µl of the above-mentioned internal standard mix, roughly at a concentration of 1 ppm/compound.

An Accela liquid chromatograph was used to analyze extracts, and was coupled to an linear trap quadropole (LTQ) Orbitrap Discovery mass spectrometer (Thermo Electron, Bremen, Germany) using both positive and negative electrospray ionization (+ESI/-ESI). 2 µl of final extracts were injected into the LC system consisting of a 15 cm x 2.1 mm (5 µm, 80 Å) Extended-C18 column (Agilent Technologies, Palo Alto, CA). A binary mobile phase gradient containing 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in MeOH (B) was applied as follows: 3 min at 50% A, to 98% B over 9 min, held steady for 3 min, back to 50% A over 1 min, and held again for 2 min at 50% A. Additional chromatographic parameters were as follows: column temperature, 50°C

and flow rate, 500 µl/min. Full-scan accurate mass spectra (m/z range: 200-1000) were obtained at high resolution (30,000 full width at half maximum FWHM) for eluting compounds on the Orbitrap mass analyzer using internal calibration (accuracy of measurements < 2 ppm) and processed using XCalibur v.2.0.7 software. Electrospray source conditions were: tube lens voltage 85 V for +ESI and 88 V for –ESI; sheath and auxiliary gas flow 55 and 10 arbitrary units (a.u.), respectively; electrospray voltage 0.5 kV for both +ESI and –ESI; heated capillary temperature 300°C; capillary voltage 44 V for +ESI and 35 V for –ESI.

To determine lipid concentrations, an isotope dilution approach was used. To prepare 10 calibration points between 2.5 ng/ml to 5 µg/ml, lipid stock solutions containing 100 µg/ml or 200 µg/ml were serially diluted into MeOH with 0.1% HCOOH. Constant amounts of lipid isotopes (molecular formula and amounts) were added to both samples and calibrators as internal standards using a stock solution concentration prepared in methanol. Calibration curves were prepared by plotting the ratio of observed peak areas for both the internal standard and analyte, respectively, versus analyte concentration. Calibration data were subjected to a linear regression that was forced through the origin, presenting regression coefficients (r^2) exceeding 0.99 for all targeted lipids. During LC-HRMS analyses, calibration was monitored through the use of continuous calibration verification (CCV) standards and blanks meeting an acceptability criterion of ±15%. Two identical matrix spikes (i.e. one matrix spike/matrix spike duplicate pair, MS/MSD) were prepared by adding a known amount of lipids to analyzed samples, thereby increasing total concentration by approximately a factor of two, and were run every 12 samples. Recovery percentage of spiked amounts (criteria 85-115%)

as well as MS/MSD relative percentage difference (criteria 15%) were met to ensure quality data.

Statistical Analyses

Statistical analysis was performed using ANOVA, analysis of variance. All statistics were run using the program SAS®. P values less than 0.05 were considered significant. All data presented here are mean ± standard error of the mean.

Results

Fatty Acid Composition of Skeletal Muscle Combined IMCL/Phospholipid Pool

Skeletal muscle fatty acids of HFD-fed mice contained significantly more PUFAs than fatty acids of control mice ($p < 0.05$; Figure 11). Concentrations of MUFAs were significantly higher in control mice than in HFD-fed mice ($p < 0.05$; Figure 11). Additionally, quantities of SFAs were not statistically different between the control and HFD-fed mice (Figure 11).

Whereas the amount of N3 PUFA was significantly lower in the skeletal muscle fatty acid pool of HFD-fed mice versus the control mice (Figure 12), the amount of N6 PUFA was significantly higher in HFD-fed mice compared to control mice ($p < 0.05$).

Significantly lower amounts of MUFA were found in the skeletal muscle lipid pool of HFD-fed mice versus control mice. This reduction in MUFA of HFD-fed mice was paralleled by a HFD-induced increase in PUFA, with no statistical change between mouse groups for SFA. Furthermore, significantly lower N3 levels were recovered in HFD-fed mice, with a significantly greater amount of N6 recovered in the HFD-fed mice compared to controls.

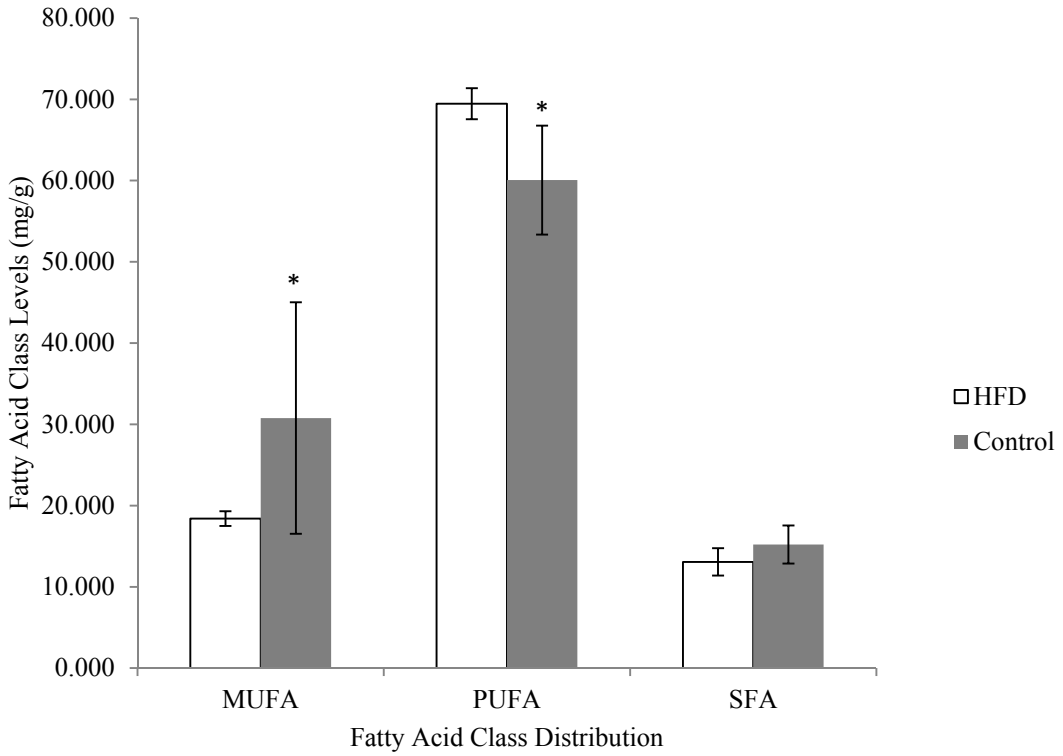

Figure 11. HFD-feeding for 8 weeks drastically changes fatty acid class (MUFA, PUFA, and SFA) distributions in mouse hamstring skeletal muscle. Values are means ± SE (n=6), * indicates significant diet effect.

Skeletal muscle fatty acids of HFD-fed mice contained significantly more C18:2n-6(t) than control mice, while control mice demonstrated significantly more C14:1, C18:1n-11(c), C22:1n-9, C15:0, C24:0, and C22:6n-3 than HFD-fed mice (Table 1).

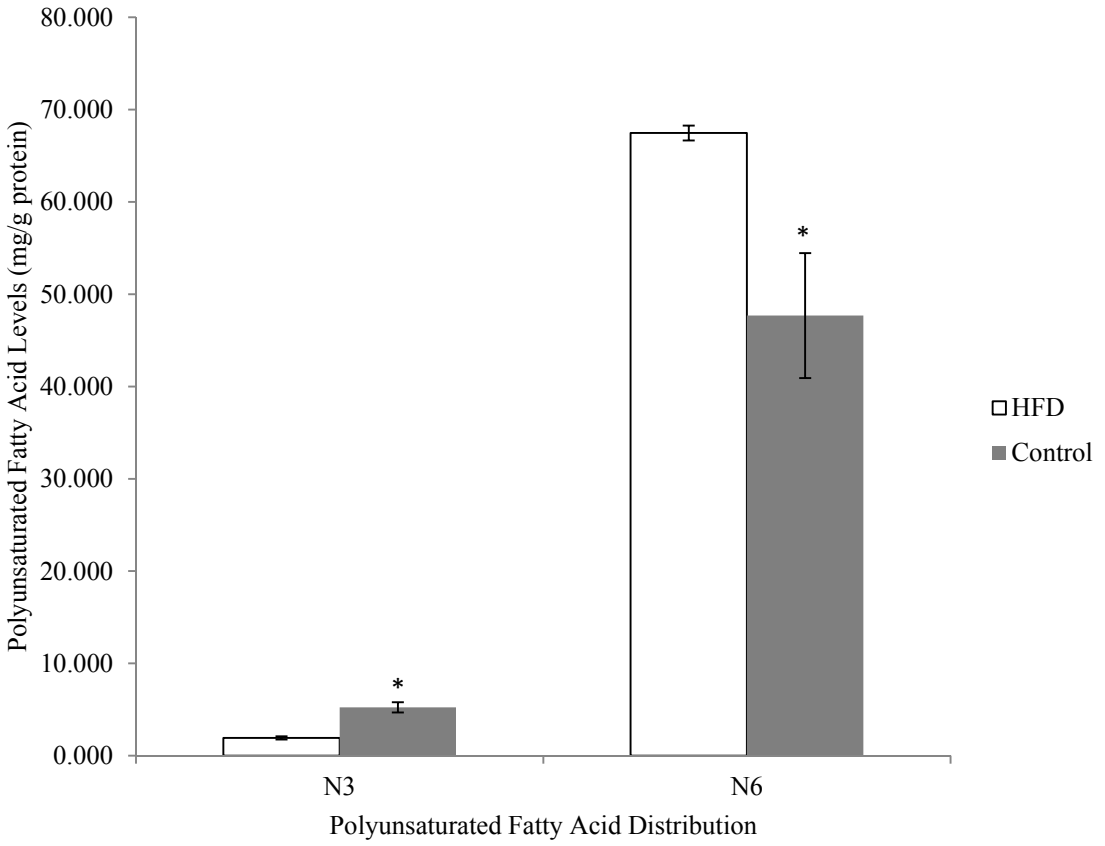

Figure 12. HFD-feeding for 8 weeks drastically changes the polyunsaturated N3 and N6 levels. Values are means ± SE (n=6), * indicates significant diet effect.

Table 1. Specific changes in fatty acid species (µg/g protein) in mouse hamstring muscle after 8 weeks of HFD feeding. All changes shown are statistically significant ($p < 0.05$).

Treatment	C14:1	C18:1n-11(c)	C22:1n-9	C15:0	C24:0	C18:2n-6(t)	C22:6n-3
Control	0.96 ± 0.56	0.30 ± 0.07	1.54 ± 0.59	0.52 ± 0.08	1.74 ± 1.03	28.49 ± 10.68	3.90 ± 1.53
HFD	0.35 ± 0.10	0.21 ± 0.06	0.74 ± 0.23	0.37 ±0.13	0.4 ± 0.18	48.37 ± 2.9	1.33 ± 0.45

Changes in Skeletal Muscle Sphingolipid and Whole Phospholipid Profiles

Changes were reported in sphingolipid and whole phospholipid composition profiles; however, none were statistically significant (Table 2). Though a total of 10 sphingolipid and whole phospholipid species were tested, only 3 species were found in

measurable amounts using LC-HRMS. These three species were 16:0 sphingomyelin (16:0 SM), 16:0 phosphocholine (DPPC), and C16 Ceramide ($p > 0.05$). Both ceramide and SM levels were elevated in HFD-fed mice ($p > 0.05$), while DPPC was found to be elevated in control mice ($p > 0.05$).

Table 2. Changes in measureable sphingolipid and whole phospholipid species (μg/g protein) in hamstring skeletal muscle after 8 weeks of HFD-feeding. No changes shown are statistically significant ($p > 0.05$).

Treatment	16:0 SM	DPPC	C16:0 Ceramide
Control	11.55 ± 5.98	39.68 ± 19.92	0.67 ± 0.31
HFD	12.69 ± 8.50	31.26 ± 19.17	0.77 ± 0.35

Discussion

Type 2 diabetes mellitus and lipid-induced insulin resistance have been associated with changes in skeletal muscle cell membrane phospholipid fatty acid, IMCL, and sphingolipid profiles. In the current study, we examined the hypothesis that 8 weeks of HFD consumption, resulting in insulin resistance, would lead to an increase in ceramide, MUFAs, and SFAs in mouse skeletal muscle tissue. The HFD utilized in this study contained 14% MUFAs, 13.68% SFAs, 0.39% N3, and 4.76% N6 (5.10 kcal/g), while the control chow contained 3.87% MUFAs, 3.71% SFAs, 0.21% N3, and 2.07% N6 (4.68 kcal/g). We found the skeletal muscle fatty acid pool of HFD-fed mice contained relatively less MUFA than that of control mice. This is unexpected given the relatively higher levels of MUFAs in the HFD, as well as higher calorie intake (assuming the mice

consumed equal portions of chow). Additionally, relative PUFA levels were higher in HFD-fed mice, whereas SFA levels were similar between HFD-fed and control mice. Also surprising was the lack of a difference in SFA levels in the HFD-fed and control mice, given the much greater percentage of SFAs in the HFD chow compared to the control chow. Omega-3 fatty acids are correlated to greater insulin sensitivity (Kelley et al. 2002), so the significantly higher levels found in the control mice, or the significantly lower levels found in the insulin-resistant HFD-fed mice, are logical.

Prior studies using dietary intervention in rats showed that diets high in SFA and MUFAs led to higher levels of muscle triglycerides as well as insulin resistance, while diets enriched with N3 PUFAs had an opposing effect (Kelley et al. 2002). The decreased levels of N3 PUFAs in the fatty acid profile of the HFD-fed mice in this study corroborate past findings as the mice were fed a diet known to lead to increased IMCL and insulin resistance. That the fatty acid pool was largely composed of PUFAs, and not the less readily oxidized SFAs, implies that some of the excess dietary SFAs may have been converted to bioactive lipid metabolites (e.g., ceramides and DAG), while the PUFAs from the diet were likely channeled into intramuscular triglyceride (IMTG) or free fatty acids within the body (Lee et al. 2006). Alternatively, since the HFD-fed mice gained significant weight on the diet (to the level of obesity), the excess SFAs may have been stored in their large adipose depots. Saturated fatty acids within skeletal muscle, in particular palmitate, have been shown to inhibit insulin signaling molecules PKB/Akt, and therefore, insulin-stimulated glucose uptake. This is largely accomplished through the conversion of SFAs into ceramides, a sphingolipid derivative of palmitate (Kelley et al. 2002).

Cholesterol levels in the two chows differed significantly (301 ppm in the HFD versus only 28 ppm in the standard chow). Cholesterol is an essential component of any cell membrane, and can comprise up to 50% of the cell boundary (Dahl and Dahl 1988; Yeagle 1993). Excess cholesterol in a membrane can induce membrane stiffness, or reduce fluidity, by reducing the movement of phospholipids within the membrane (Ohvo-Rekila et al. 2002). While historical governmental dietary recommendations have supported the idea of a low cholesterol diet for cardiovascular (and general) health, recent studies have shown no significant correlation between high cholesterol dietary intake and poor health outcomes (e.g., Djousse and Gaziano 2009; Lecerf and de Lorgeril 2011). This is likely because the levels of cholesterol in membranes, both through biosynthesis and uptake, are highly regulated by feed-back mechanisms (Goldstein and Brown 1990; Brown and Goldstein 1997; Brown and Goldstein 1998; Brown and Goldstein 1999). Thus, the high cholesterol content of the HFD here is unlikely to influence membrane fluidity to a large degree.

While earlier studies linked large pools of IMCL with insulin resistance in skeletal muscle, more recent research (e.g., Corcoran et al. 2007) has shown the issue is a lack of turnover of the fatty acids comprising IMCL, rather than the quantity of the fatty acids stored that is correlated with insulin resistance. In other words, in insulin resistant skeletal muscle, there is a large oversupply of IMCL, but diminished oxidation, and therefore, an imbalance between fatty acid availability, uptake, and use (Corcoran et al. 2007). In healthy skeletal muscle tissue, the muscle retains the ability to utilize both glucose and fatty acids as sources of fuel, and further, the ability to transition between the

two. In insulin resistant skeletal muscle, this ability to transition is largely lost and is one of the defining characteristics of the metabolic disease (Kelley et al. 2002).

An additional change typically occurring in insulin resistant skeletal muscle is a transformation of the phospholipid composition of the cell membrane. The fatty acid composition of any cell membrane, including that of skeletal muscle, is important to that membrane's fluidity, permeability, and relative functionality (Pan et al. 1994; Haag and Dippenaar 2005). The more saturated a membrane, the less fluid that membrane is; alternatively, the more saturated a membrane, the less permeable that membrane is to extracellular molecules. Saturated fatty acids can pack more closely due to their lack of double bonds, and in this way, decrease membrane fluidity and leakiness to both protons and ions. A resulting effect of this saturation and decrease in leakiness is a reduction in metabolic rate, since leakiness across membranes is related to proton and ion pumping, a significant energy expenditure of the cell. As expected, the reverse is also true, with an increase in PUFA species in cell membranes increasing the leakiness of cell membranes, and thus the cell's energy expenditure, and metabolic rate (Kelley et al. 2002).

Another change resulting from saturation of phospholipid membranes is the membrane's reception to the activity of insulin. Studies have consistently shown a more saturated phospholipid profile in insulin resistant skeletal muscle (Borkman et al. 1993; Vessby et al. 1994; Pan et al. 1995). Additionally, a reduction in insulin sensitivity has been associated with higher levels of N6 PUFAs in phospholipids and lower levels of N3 PUFAs (Borkman et al. 1993; Vessby et al. 1994; Pan et al. 1995; Haugaard et al. 2006). The binding of insulin to receptors on skeletal muscle membranes initiates the glycogen synthesis in muscle, the process most impaired in insulin resistant tissue. The rate of this

ability to synthesize glycogen allows for the variance in insulin sensitivity among subjects and body tissues. Polyunsaturated fatty acid-rich membranes generally have more insulin receptors, and therefore better insulin activity, with the reverse also holding true (Borkman et al. 1993). Omega-3 fatty acids have a protective effect on insulin sensitivity, even under a generally SFA diet (Storlien et al. 1991). Here, we found that, of the whole skeletal muscle membrane phospholipids of measurable levels, those of HFD-fed mice differed little from those of control mice. This is not unusual, as changes in dietary fatty acid profiles don't always induce changes in phospholipid composition in skeletal muscle (Ayre and Hulbert 1996).

The sphingolipid composition of cell membranes has also been examined in relation to insulin resistance, particularly with regard to ceramides. Ceramides have been thought to contribute to the induction of insulin resistance in skeletal muscle, largely through inhibiting insulin action by altering various kinases, transcription factors, and phosphatases (Schmitz-Peiffer et al. 1999; Schubert et al. 2000; Straczkowski et al. 2007). While ceramides were elevated in the HFD-fed mice, they were not statistically so. The lack of a significant increase in ceramide levels could be the result of a multitude of factors. As the hamstring is a predominately glycolytic muscle, ceramides in this particular muscle may not accumulate as they would in more oxidative muscles (e.g., Zendzian-Piotrowska et al. 2006). Lee et al. (2006) also found no elevation of ceramide levels in rats fed either a high SFA or PUFA diet after 8 weeks, thus our findings have precedence. Ceramides are often produced in the cell by the breakdown of sphingomyelins and while we found no differences between treatments, we did show elevated trends which have been positively correlated with obesity (Zeghari et al. 2000a;

Zeghari et al. 2000b). This positive correlation may be due to the link between ceramide accumulation within tissues and metabolic alterations, which can include catabolism through the downregulation of nutrient transport proteins (Bikman and Summers 2011). In numerous rodents studies (e.g., Summers 2006; Polya and Parsons 1973; Bismuth et al. 2008), inhibiting ceramide production can either delay or completely prevent the onset of metabolic diseases (e.g., T2DM) in the obese. Ceramides are now known to increase in response to nearly all stress stimuli, including inflammation (common in obesity), hypoxia, and oxidative stress (Bikman and Summers 2011).

Diacylglycerols (DAGs) have historically been implicated in the pathogenesis of insulin resistance, yet newer studies (Bruce et al. 2006; Vistisen et al. 2008; Amati 2012) have cast doubt upon this theory. While DAGs were evaluated in the current study, their levels were not measureable. This is likely a methodological issue with LC-HRMS, as we used a high-efficiency, shotgun approach to measuring ten sphingolipid/phospholipid species simultaneously. Future studies could utilize less efficient, but more precise mass spectrometry methods to look at individual sphingolipids within the skeletal muscle cells.

While not many conclusions can be drawn from the sphingolipid and whole phospholipid comparisons between the HFD-fed and control mice, much can be said regarding the changes in fatty acid composition of the IMCL/phospholipid pool in the skeletal muscle tissue. N6 PUFAs are known to be inflammatory in nature, while N3 PUFAs are much the reverse (anti-inflammatory). Additionally, N3 PUFAs are thought to be critical to normal brain development and function in juveniles. Given the increasing numbers of juveniles in North America with insulin resistance, the decrease in N3 and resultant increase in N6 PUFAS is worrisome. Beyond insulin resistance, a

decrease in N3 PUFA levels after HFD feeding could have implications in protection from cancer, as N3s are known to direct apoptosis in tumor cells (Schley et al. 2007; Kang et al. 2010; Fukui et al. 2013).

In conclusion, our results show that HFD-feeding increases the PUFA levels, particularly N6 PUFAs, and decreases the MUFA levels and N3 PUFAs in insulin-resistant mice IMCL/phospholipid fatty acid pools in skeletal muscle. Additionally, no significant changes in sphingolipid or whole phospholipid compositions were detected. These findings support, though not strongly, that diets high in saturated fats alter the cellular fatty acid composition and moderately increase ceramides and sphingomyelin.

CHAPTER FIVE

Conclusions and Future Directions

Hypoxia and Marine Mammals

Results reported here suggest that marine mammals utilize a variety of methods to combat tissue-level hypoxia, including, but not limited to, elevated enzyme levels (Fuson et al. 2003), specific antioxidant systems (Zenteno-Savin et al. 2002; Vazquez-Medina et al. 2012), and cell signaling (de Miranda et al. 2012). Levels of C16 ceramide, a lipid correlated to environmental and physiological stressors, as well as the C16 ceramide correlate 16:0 sphingomyelin, were significantly higher in harbor seal skeletal muscle compared to that of Weddell seal, and significantly higher in ringed seal liver compared to skeletal muscle. These data point to diving patterns dictating stress levels, where harbor seals spend more time at depth, and therefore a greater portion of their day coping with hypoxia, compared to Weddell seals. Body mass provides an additional source of potential stress, as relatively greater levels of C16 ceramide were recovered from the larger harbor seals. A larger body size implies a larger skeletal muscle mass, tissue which becomes hypoxic during breath-hold diving. In the ringed seal, liver tissue receives only 20% of its normal blood flow during longer-duration dives (Davis et al. 1983). Because diving mammal liver tissue is saturated with mitochondria compared to terrestrial mammals (Fuson et al. 2003), the risk of damage from mitochondrial-derived ROS production is extremely high. These high levels of oxidative stress in the tissue, in addition to the fact that the liver must remain fully functional during dives (Davis 2013), results in an increased level of C16 ceramide, a known indicator of stress levels.

Further, in these same tissues (harbor seal skeletal muscle and ringed seal liver), the phospholipid DPPC was significantly elevated. DPPC is typically found in the membranes of cells where pressure changes are common (e.g., mammalian lung surfactant; Veldhuizen et al. 2000; Tonks et al. 2011). When this lipid is compressed, it provides protection from high surface pressures and helps to maintain fluidity within the membrane (Veldhuizen et al. 2000). In harbor seals, diving pattern likely dictate the elevated levels of this phospholipid, as they spend more total time at depth (and therefore, at higher hydrostatic pressures) compared to Weddell seals. In ringed seal liver, the critical importance of the organ to physiological homeostasis necessitates the organ be better protected from dynamic pressure changes than the skeletal muscle.

While the data presented here point clearly to some of the many adaptations of marine mammals to hypoxia, future studies could solidify these results even further. Johnson et al. (2004) have shown seal lung DNA PCR products to be extremely similar to those of mice, resulting in the ability to measure HIF-1α levels in seal tissue (specifically ringed seal). At date of publication, no additional marine mammal species have been thoroughly investigated in relation to HIF-1α levels (though Vazquez-Medina et al. (2011) have reported upregulated levels of HIF-1α in the blood of northern elephant seal (*Mirounga angustirostris*) pups correlated to apnea). The work of Johnson et al. (2004) has demonstrated that seal tissues do become hypoxic, as evidenced by significantly upregulated HIF-1α levels. HIF-1α levels of the organisms studied here were not measured due to financial constraints, but the demonstration of these proteins and hypoxic status in a closely related species indicates the hypoxia pathway is likely conserved across all species of marine and terrestrial mammals.

Future studies correlating hypoxia in marine mammal tissues to diving patterns and blood flow changes should utilize larger sample sizes, if available. Due to permitting issues and the endangered status of many marine mammal species, acquiring tissue samples is notoriously difficult. For this reason, the sample sizes in this dissertation are low (but not uncommonly low for these types of studies), and samples were collected from varying sites and individuals. While the author was not present for tissue collection, each sample was visually inspected before analysis, and those that did not meet the author's standards (e.g., muscle tissue appearing brown/spoiled in color) were not used in these studies. Subsequent studies should attempt to include hands-on sampling, and a streamlined process involving the same personnel from study design/data collection through to data analysis and discusssion.

Additionally, as tissue collection occurred in remote areas, measurement of the major stress-induced lipid, C16 ceramide, may have been unavoidably biased. Ceramide levels in response to known sources of environmental stressors (ionizing radiation and Fas ligand) were measured by Thomas et al. (1999) and shown to elevate in Jurkat (T-cell leukemia) cells after only two hours. Depending upon the stressor, ceramide levels upon stressor initiation are only modest, but increase significantly after several hours. During the early time periods (30 min to 2 hours), no significant increases in C16 ceramides were noticed, but ceramides were increased 6-fold after the 2-hour time point (Thomas et al. 1999). Once the offending stressor has ceased, ceramide levels tend to return to normal rapidly, on the order of 30 minutes or less (Dressler et al. 1992; Cifone et al. 1994; Haimovitz-Friedman et al. 1994). While the author is not aware of longitudinal ceramide level studies with oxidative stress, since ceramides regulate the general eukaryotic stress

response (Hannun and Luberto 2000), some conservation of time frames is likely. As the author was not present during sample collection, the length of time between diving bouts and biopsy collection for each organism is not known, and is likely to be variable, both between species and among organisms. This factor may have influenced results, but given the difficult nature of marine mammal sample collection, would be difficult to avoid. A study in the future where the variability in sample collection is more strictly controlled is clearly warranted.

One future direction or area of interest involving hypoxia is in its relation to the hormone leptin. Leptin is largely known for its role in the regulation of an organism's fat mass, but recent studies have indicated that hypoxia increases the expression of leptin in adipocytes (Grosfeld et al. 2002). Leptin can stimulate angiogenesis (Sierra-Honigmann et al. 1998), and in humans subjected to high altitude hypoxia, leptin levels were significantly upregulated (Tschop et al. 1998). While leptin levels have been measured in marine mammals in relation to fat mass (e.g., Ortiz et al. 2001), they have not been evaluated in correlation to the hypoxic status of a diving mammal. If leptin is involved in angiogenesis (or angiostatic activity, as has been cited by at least one group, Cohen et al. 2001), the mechanisms through which marine mammals have adapted to diving and chronic, intermittent hypoxia may become more clear.

HFD-Fed, Insulin-Resistant Mice

This dissertation reports HFD-fed, insulin-resistant mice show significantly higher levels of PUFAs, significantly lower levels of MUFAs, and no difference in SFAs compared to control mice. Additionally, HFD-fed mice demonstrated significantly higher levels of the inflammatory N6 PUFAs, and significantly lower levels of the anti-

inflammatory N3 PUFAs compared to control mice. Finally, no significant changes were found between HFD-fed mice and their controls. These changes in fatty acid levels seen in HFD-fed, insulin-resistant mice, when taken in context with the dietary makeup of the HFD, suggest the SFAs were likely stored in adipose tissue (not the sampled skeletal muscle). The higher concentrations of N6 and lower concentrations of N3 match the phenotype of insulin sensitivity (e.g., Kelley et al. 2002), and were therefore expected. The lack of an increase in C16 ceramide, while somewhat surprising given ceramide is a known inhibitor of insulin signaling (e.g., Schmitz-Peiffer et al. 1999; Schubert et al. 2000; Straczkowski et al. 2007), may relate to the fiber type distribution of the hamstring muscle sampled, a largely glycolytic muscle, which in other studies (e.g., Zendzian-Piotrowska et al. 2006), has shown a lack of accumulation of ceramide despite insulin resistance.

Future studies on HFD-fed, insulin-resistant mice should heavily consider the exact makeup of the HFD utilized in the study. The diet used for HFD-feeding in this insulin-resistant mouse study, consisting of nearly 60% energy from fat, would be considered extreme from the perspective of a human diet. A typical Western diet averages a macronutrient composition of approximately 51.8% carbohydrate, 15.4% protein, and only 32.8% fat (Cordain et al. 2005). The type of diet utilized in this study rapidly induces obesity in the animals, which in the C57BL/6 mouse, is a precursor to T2DM (e.g., Surwit et al. 1988; Ghibaudi et al. 2002; Johnston et al. 2007). Rapid onset of obesity in the model organism allows for the testing of compounds of interest in a more time-efficient manner. The HFD here accomplishes this without demonstrated hyperphagia in the animals (West et al. 1992). In other words, obesity is induced, in

many cases, with a similar caloric content compared to the control chow. The diet utilized in the HFD study here did have a higher caloric value (5.10 versus 4.68 kcal/g), but since the mice were fed *ad libitum*, this does not necessarily indicate the obese mice ingested more total calories than the controls.

Measurement and Comparison of Lipids of Interest

While method development for LC-HRMS was extremely successful (measuring 10 sphingolipids/whole phospholipids simultaneously), future studies may even improve upon the lipid species tested and the methods used. C16 ceramide is most commonly found in lung and brain tissue (Xu et al. 2005; Becker et al. 2008), and the ceramide synthase gene (LASS5) encoding the production of C16 ceramide was the first to be purified (Lahiri and Futerman 2005). Ceramide synthase-4 is among the least-studied of the LASS genes, but has been found in liver tissue (Laviad et al. 2008). Ceramide synthase-2 has been found in high levels in the liver and moderate levels in skeletal muscle (Cai et al. 2003), while ceramide synthase-1 has been found in low levels in skeletal muscle (Mizutani et al. 2005). While the author would like to investigate additional ceramide levels in the future (specifically those known to be expressed in higher levels in skeletal muscle), C16 ceramide, given its known upregulation in hypoxic conditions (Jin et al. 2008), was an appropriate species to measure. The fact that only one species of ceramide (C16) was measured could certainly confound the results in both marine mammal and mouse tissue, but without the ability to measure other ceramide species (due to a lack of standard availability for other ceramide species), the direction of potential changes to the analysis cannot be known. The comparison of levels of all ceramide species in these tissues is an important future direction.

Future studies examining fatty acid composition could also utilize more effective separation methods. Fatty acid analysis in both marine mammal and pre-diabetic mouse tissues was complicated by the fact that thin layer chromatography (TLC) was not a methodological option, and so the fatty acids from intracellular lipids were not able to be separated from fatty acids of membrane phospholipids. This made discussion of membrane phospholipids forcibly limited to the whole phospholipids measured using LC-HRMS. The separation of fatty acids recovered from intracellular lipid pools from those of membranes is an important future direction to explore.

Dissertation Theme Conclusions

Hypoxia and ischemia-reperfusion are serious stressors to all mammalian species. The fact that marine mammals tolerate these physiological conditions with such aptitude makes them an invaluable model system for hypoxia tolerance. Hypoxia plays a central role in the pathophysiology of many common human conditions, including pulmonary hypertension, cancer, intrauterine growth retardation, and preeclampsia (Lal et al. 2001; Sowter et al. 2001; Buchler et al. 2003; Zhong et al. 1999), just to name a few. HIF-1α also participates in the new growth of myocardial tissue post-ischemia, and helps protect the brain from ischemia via a process known as late-phase conditioning (Sodhi et al. 2001). The interesting results discussed here on the interplay between lipid species and hypoxia in model organisms may help to unlock the mechanisms through which hypoxia influences various pathological conditions and the ways in which HIF-1α helps to protect against ischemic injury.

HFD-induced insulin resistance in C57BL/6J mice serves as a fairly accurate model for the development of the disease in humans, and unfortunately, is a likely

preview for the health of millions of people in developed nations in the near future (ADA 2011). The results reported here serve as evidence that diets high in saturated fats can be pathogenic in the right physiological context (namely, a predisposition for obesity), and that some inclusion of the anti-inflammatory N3 fatty acids to the diet is not enough to prevent insulin resistance.

The study of lipids and signal transduction pathways links these studies of marine mammal adaptations to hypoxia to those of HFD-fed, insulin-resistant mice. Hypoxia and insulin resistance are both significant physiological stressors which utilize lipids as secondary messengers in cell signaling pathways, and result in changes to lipid profiles of various tissues. This dissertation contributes to that block of knowledge by deciphering important changes to the lipid profiles in marine mammals and pre-diabetic mice, several of which are known secondary messengers in signal transduction pathways (e.g., diacylglycerol; Turinsky et al. 1990) or the result of cell signaling pathways (e.g., C16 ceramide via sphingomyelinase signaling pathway; Grosch et al. 2012). This dissertation has elucidated important changes to mammalian tissues from two very different perspectives: one of pathological disease (insulin-resistant mice), and one of adaptation to physiological extremes (diving mammals). These results have contributed to our understanding of the importance of biological membranes to the proper functioning of cells and tissues in a variety of organisms. Additionally, the signaling capabilities of various lipid species studied and the pathways they embark upon to provide physiological buffers to environmental conditions (marine mammals) or pathological situations (insulin resistance) is key to the understanding of signal transduction pathways across species and variables.

APPENDICES

APPENDIX A

Table A1. Marine mammal sphingolipid and phospholipid data

SSN	SM	PE	PG	DPPC	Cer	Lyso	S1P	Cer:S1P
B1	61.57	0.26	0.06	14.98	2.36	1.43	5.18	0.45
B2	40.17	0.16	0.04	8.46	1.80	1.65	4.75	0.38
B3	64.19	0.16	0.01	16.45	2.02	2.30	2.39	0.85
B4	53.40	0.15	0.02	11.12	2.19	1.33	3.53	0.62
B5	50.48	0.07	0.18	13.55	2.15	0.74	2.01	1.07
BH1	89.35	0.12	0.12	61.05	7.13	1.50	4.47	1.59
BH2	107.49	0.09	0.70	50.31	7.39	0.93	2.92	2.53
BH3	162.22	0.05	0.34	63.83	4.57	1.17	3.94	1.16
BH4	99.39	0.16	0.25	40.52	7.51	1.13	4.42	1.70
BH5	142.35	0.15	0.20	44.46	6.08	2.05	2.37	2.57
H1	60.01	0.26	1.38	20.75	6.13	0.85	23.40	0.26
H2	189.31	0.16	0.03	34.21	7.74	0.69	6.33	1.22
H3	156.83	0.16	0.21	58.42	8.63	1.03	3.78	2.28
H4	61.41	0.00	0.03	14.59	3.31	0.19	2.19	1.51
H5	79.00	0.04	0.07	28.88	4.37	0.23	1.73	2.53
NFS1	182.65	0.67	0.00	29.18	6.85	1.27	27.79	0.25
NFS2	90.07	0.45	0.18	17.10	4.14	0.87	11.44	0.36
NFS3	69.54	1.47	0.34	33.06	11.55	2.55	32.64	0.35
NFS4	68.45	0.60	0.20	22.23	7.20	1.72	17.59	0.41
NFS5	39.91	0.95	0.16	10.20	9.57	2.66	33.80	0.28
R1	108.26	0.24	0.14	20.99	3.89	1.83	7.23	0.54
R2	59.33	0.09	0.03	12.31	2.04	0.96	2.52	0.81
R3	66.80	0.11	0.03	9.75	2.83	0.40	4.89	0.58
R4	54.49	0.10	0.01	12.20	2.02	1.87	3.60	0.56
R5	74.34	0.09	0.03	34.67	4.08	0.32	2.66	1.54
RH1	152.15	0.13	0.08	36.18	7.19	0.64	4.72	1.52
RH2	90.95	0.14	0.34	47.11	3.80	0.75	3.19	1.19
RH3	51.74	0.06	0.05	24.24	4.52	0.99	1.62	2.79
RH4	102.32	0.10	0.16	61.82	3.39	0.84	1.74	1.95
RH5	92.55	0.15	0.07	48.47	3.61	0.59	2.92	1.24
RL1	46.53	0.11	0.01	7.91	2.00	0.37	3.88	0.51
RL2	191.35	0.12	0.05	56.93	13.98	1.20	3.05	4.59
RL3	227.39	0.17	0.13	76.69	17.37	0.51	2.68	6.48
RL4	224.18	0.17	0.06	64.36	17.19	0.28	1.89	9.12
RL5	187.85	0.20	0.20	73.12	25.03	0.57	1.41	17.81
S1	81.68	0.12	0.08	21.66	4.83	0.27	2.84	1.70
S2	83.43	0.07	0.02	13.44	2.94	0.22	2.04	1.44
SH1	192.31	0.27	0.12	72.73	19.30	0.45	1.77	10.92
SH2	80.47	0.18	0.08	48.65	9.16	0.55	4.44	2.06
SL1	328.17	0.27	0.06	87.75	17.99	0.34	3.54	5.08
SL2	86.23	0.24	0.40	54.98	8.25	0.97	0.00	0.00
W1	59.28	0.22	0.07	20.37	1.83	1.59	4.01	0.46
W2	39.45	0.15	0.02	8.69	1.55	1.36	4.27	0.36
W3	34.15	0.11	0.02	10.96	1.22	0.79	3.15	0.39
W4	39.53	0.11	0.02	11.24	1.43	1.03	2.50	0.57
W5	86.85	0.14	1.49	12.11	1.54	0.59	3.57	0.43
Wd1	46.39	0.12	1.33	11.20	1.10	0.37	4.00	0.28
Wd2	62.72	0.18	1.67	11.75	1.73	0.44	3.77	0.46
Wd3	21.83	0.06	0.04	7.07	0.73	0.18	1.81	0.40
Wd4	46.42	0.37	0.10	11.83	1.95	0.65	6.42	0.30
Wd5	54.38	0.24	0.17	13.60	1.60	0.62	5.77	0.28
MC1	14.07	0.02	0.00	17.06	1.44	0.25	4.26	0.34
MC2	16.36	0.03	0.00	18.97	1.21	0.19	4.35	0.28
P1	28.64	0.04	0.00	49.85	2.01	0.32	5.27	0.38
P2	30.90	0.00	0.00	34.30	3.67	0.18	5.80	0.63
P3	2.01	0.01	0.09	0.00	0.80	0.13	3.29	0.24
P4	17.23	0.01	0.00	35.10	1.61	0.17	4.71	0.34
MCH1	79.80	0.02	0.00	144.00	20.81	4.17	2.14	9.70
MCH2	52.07	0.02	0.00	130.93	9.69	0.40	1.65	5.88
PH1	44.75	0.02	0.00	67.62	8.58	0.65	1.33	6.46
PH2	54.19	0.02	0.00	94.71	13.67	0.44	3.10	4.41
PH3	68.64	0.02	0.00	110.33	15.99	0.98	1.36	11.74
PH4	49.35	0.01	0.00	100.53	9.01	0.77	1.71	5.28
MCL1	57.33	0.01	0.00	51.77	32.09	0.46	2.14	15.02
MCL2	97.62	0.00	0.00	90.90	56.32	2.38	1.40	40.19
PL1	66.94	0.02	0.00	126.04	81.65	2.20	1.74	46.87
PL2	58.11	0.00	0.00	93.04	62.04	2.45	1.50	41.35
PL3	124.10	0.01	0.00	136.63	59.31	5.51	2.02	29.38
PL4	76.79	0.03	0.00	82.05	32.49	0.97	2.34	13.89

APPENDIX B

Table B1. Marine mammal IMCL/phospholipid fatty acid data

Treatment	SSN	Tissue	MUFA	PUFA	SAT	N3	N6
B	1	M	53.934	140.592	56.661	46.056	67.872
B	2	M	33.852	86.583	50.778	29.295	39.711
B	3	M	33.904	92.352	44.928	32.24	43.68
B	4	M	38.779	110.903	62.491	40.261	43.966
B	5	M	37.03	106.49	57.04	35.88	52.44
B	1	H	0	0	0	0	0
B	2	H	0	0	0	0	0
B	3	H	0	0	0	0	0
B	4	H	0	0	0	0	0
B	5	H	0	0	0	0	0
H	1	M	0	0	0	0	0
H	2	M	41.724	114.192	66.856	39.772	57.34
H	3	M	0	0	0	0	0
H	4	M	23.94	76.19	43.13	27.17	34.96
H	5	M	27.3	83.538	44.044	27.118	43.862
NFS	1	M	0	0	0	0	0
NFS	2	M	0	0	0	0	0
NFS	3	M	0	0	0	0	0
NFS	4	M	0	0	0	0	0
NFS	5	M	0	0	0	0	0
R	1	M	23.6	66.6	52.6	30.8	21.6
R	2	M	19.572	111.84	47.532	33.785	58.017
R	3	M	13.26	74.63	39.44	23.12	36.55
R	4	M	19.8	87.285	48.51	25.905	44.715
R	5	M	18.762	56.994	33.748	18.29	32.096
R	1	H	30.788	73.1	41.968	27.348	35.432
R	2	H	35.34	91.77	52.25	26.79	52.25
R	3	H	37.769	92.543	40.812	30.43	47.793
R	4	H	40.392	104.112	61.344	34.128	52.92
R	5	H	66.708	183.992	105.512	57.988	98.972
R	1	L	30.4	76.57	40.28	29.83	34.01
R	2	L	38.194	87.688	56.952	33.9	41.358
R	3	L	26.578	80.122	43.068	25.608	37.83
R	4	L	36.36	87.264	42.622	30.502	41.612
R	5	L	32.832	84.48	39.552	26.88	45.12
S	1	M	41.87	109.71	72.08	33.125	59.36
S	2	M	0	0	0	0	0
S	1	H	51.152	129.548	59.77	42.256	66.442
S	2	H	35.805	90.09	47.817	34.188	52.206
S	1	L	25.239	82.698	54.595	22.196	46.54
S	2	L	31.104	79.104	31.296	27.648	35.328
W	1	M	37.411	107.635	55.594	34.903	51.205
W	2	M	29.58	72.828	42.432	15.3	45.696
W	3	M	38.61	97.305	53.43	30.81	49.725
W	4	M	17.955	73.359	36.936	18.297	40.014
W	5	M	31.416	80.976	34.608	30.072	38.976
Wd	1	M	38.247	85.644	42.09	28.914	44.286
Wd	2	M	0	0	0	0	0
Wd	3	M	0	0	0	0	0
Wd	4	M	0	0	0	0	0
Wd	5	M	0	0	0	0	0
MC	1	M	21.375	76.5	65.925	26.325	36
MC	2	M	36.099	80.411	44.312	23.684	43.166
P	1	M	45.65	110.275	66.825	38.5	55.275
P	2	M	36.4	105.84	68.32	46.2	43.4
P	3	M	40.689	106.92	61.182	44.253	47.52
P	4	M	25.365	88.92	64.125	36.195	41.61
MC	1	H	44.608	95.744	59.568	35.088	45.968
MC	2	H	45.375	119.25	85.125	37.875	55.5
P	1	H	16.171	40.82	32.028	12.56	21.666
P	2	H	27.832	89.744	57.368	36.636	42.6
P	3	H	53.138	144.418	81.174	42.706	69.112
P	4	H	43.47	104.22	53.73	42.12	57.51
MC	1	L	47.808	103.968	67.968	34.56	46.656
MC	2	L	52.954	121.858	63.162	36.047	54.23
P	1	L	47.173	111.926	66.511	37.504	51.568
P	2	L	27.777	76.633	42.158	24.231	39.006
P	3	L	34.02	86.31	48.09	30.03	42.63
P	4	L	46.76	125.72	58.24	39.2	66.92

APPENDIX C

Table C1. High-fat diet-fed (HFD) vs. control mouse fatty acid data

Muscle Sample	147	148	149	151	152	155	157	158	159	162	163	166	Avg HFD	Avg Con
Treatment	HFD	HFD	HFD	HFD	HFD	HFD	C	C	C	C	C	C		
MUFA	17.998	17.703	18.954	19.720	17.634	12.797	26.132	22.213	21.830	37.033	20.372	57.070	17.468	30.775
PUFA	69.975	69.899	68.223	66.280	71.152	71.243	59.250	65.126	64.283	48.765	62.902	23.771	69.462	54.016
SAT	12.027	12.398	12.823	14.000	11.214	15.960	14.617	12.661	13.886	14.202	16.726	19.159	13.070	15.209
N3	1.330	2.086	1.501	2.387	2.311	1.907	4.373	3.411	4.841	7.208	5.383	6.223	1.920	5.240
N6	68.645	67.813	66.722	63.828	68.841	68.967	48.621	61.715	59.443	41.557	57.236	17.547	67.469	47.686
C12:0	0.250	0.271	0.293	0.398	0.153	0.120	0.416	0.335	0.402	0.485	0.250	0.857	0.247	0.458
C13:0	0.19	0.183	0.254	0.344	0.107	0.162	0.375	0.245	0.307	0.429	2.087	0.735	0.206	0.696
C14:0	0.73	0.812	0.871	0.892	0.717	0.504	0.781	0.824	0.826	0.801	0.716	1.000	0.755	0.825
C14:1	0.37	0.388	0.356	0.495	0.247	0.230	0.687	0.624	0.540	0.767	2.042	1.102	0.347	0.960
C15:0	0.33	0.381	0.502	0.538	0.266	0.191	0.573	0.476	0.477	0.620	0.394	0.551	0.368	0.515
C15:1	0.25	0.278	0.363	0.473	0.156	0.379	0.479	0.360	0.402	0.530	0.144	0.531	0.316	0.408
C16:0	7.98	7.999	8.269	8.237	7.825	5.787	6.403	7.550	7.086	7.750	5.882	8.019	7.683	7.115
C16:1	5.82	6.243	6.729	5.806	7.394	4.457	11.234	9.822	7.489	16.289	8.246	31.320	6.075	14.067
C17:0	0.49	0.512	0.585	0.731	0.510	7.545	0.458	0.451	0.424	0.654	0.427	0.939	1.728	0.559
C17:1	0.47	0.490	0.509	0.559	0.446	0.418	0.479	0.502	0.498	0.451	0.361	0.612	0.482	0.484
C18:0	0.00	0	0.000	0.000	0.000	0.078	0.000	0.000	0.000	0.000	1.781	0.000	0.013	0.297
C18:1n-9(t)	9.47	8.189	9.013	9.710	7.367	5.933	10.349	8.290	9.808	14.834	6.448	20.159	8.280	11.648
C18:1n-9(c)	0.50	0.498	0.464	0.570	0.391	0.175	0.416	0.406	0.413	0.553	0.294	0.000	0.432	0.347
C18:2n-6(t)	48.97	47.878	46.667	44.484	49.080	53.161	27.121	39.547	34.403	25.313	34.989	9.549	48.373	28.487
C18:1n-11(c)	0.20	0.242	0.223	0.301	0.162	0.128	0.281	0.232	0.297	0.316	0.250	0.428	0.210	0.301
C18:2n-6(c)	0.10	0.161	0.178	0.204	0.098	0.428	0.000	0.161	0.254	0.000	0.105	0.000	0.196	0.087
C18:3n-6	18.73	18.787	18.776	17.817	18.989	14.696	19.198	20.919	23.345	14.755	19.478	6.060	17.967	17.293
C20:0	0.22	0.000	0.134	0.161	0.070	0.376	0.271	0.135	0.222	0.305	0.133	0.469	0.161	0.256
C18:3n-3	0.00	0.000	0.000	0.000	0.104	0.724	0.000	0.000	0.000	0.000	0.100	0.000	0.138	0.017
C20:1	0.34	0.381	0.439	0.613	0.324	0.528	0.656	0.521	0.763	0.406	0.477	0.449	0.438	0.545
C21:0	0.98	1.244	0.738	1.000	0.779	0.575	1.229	1.165	1.684	1.286	0.932	3.918	0.885	1.702
C20:2	0.00	0.000	0.000	0.000	0.000	0.246	6.257	0.000	0.000	0.000	0.000	0.000	0.041	1.043
C20:3n-6	0.66	0.695	0.757	0.925	0.565	0.556	1.499	0.779	0.985	0.959	0.583	1.163	0.693	0.995
C22:0	0.32	0.388	0.458	0.839	0.488	0.327	0.510	0.579	0.604	0.756	0.361	0.898	0.470	0.618
C20:3n-3	0.06	0.073	0.134	0.215	0.046	0.031	0.104	0.071	0.212	0.135	1.182	0.367	0.094	0.345
C22:1n-9	0.46	0.842	0.731	0.903	1.047	0.481	1.229	1.255	1.356	2.493	0.921	2.000	0.745	1.542
C23:0	0.15	0.198	0.204	0.194	0.104	0.073	0.364	0.354	0.254	0.237	1.121	0.265	0.153	0.433
C20:4n-6	0.18	0.293	0.343	0.398	0.110	0.125	0.802	0.309	0.455	0.530	2.081	0.775	0.241	0.825
C22:2	0.00	0.000	0.000	0.065	0.000	0.123	0.000	0.000	0.000	0.000	0.283	0.000	0.031	0.047
C24:0	0.39	0.410	0.515	0.667	0.195	0.222	3.238	0.547	1.599	0.880	2.642	1.510	0.400	1.736
C24:1	0.12	0.154	0.127	0.290	0.101	0.068	0.323	0.200	0.265	0.395	1.188	0.469	0.143	0.473
C20:5n-3	0.22	0.315	0.210	0.419	0.101	0.264	0.312	0.238	2.097	0.372	1.054	0.633	0.255	0.784
C22:5n-3	0.09	0.124	0.108	0.183	0.061	0.050	0.177	0.122	0.180	0.214	0.100	0.347	0.102	0.190
C22:6n-3	0.96	1.573	1.049	1.570	2.000	0.839	3.779	2.980	2.351	6.486	2.947	4.877	1.332	3.903

APPENDIX D

Table D1. Total marine mammal statiscal analysis

MC: Mouse Control; P: PUFA Mouse Control; Wd: Weddell; W: Walrus; B: Bearded; R: Ringed; S: Spotted; NF: Northern Fur; H: Harbor

Lipid	Analysis of Variance Values	Tukey Differences								
Sphingomyelin (SM)	F=3.43; p=0.0066	MC	P	Wd	W	B	R	S	NF	H
				A	A	A	A	A	A	A
		B	B	B	B	B	B	B	B	
PE	F=10.34; p<0.0001	P	MC	S	H	R	W	B	Wd	NF
										A
		B	B	B	B	B	B	B	B	
PG	F=1.10; p=0.3867	No Difference								
DPPC	F=2.12; p=0.06	No Difference								
Ceramide	F=11.36; p<0.0001	MC	Wd	W	P	B	R	S	H	NF
								A	A	A
							B	B	B	
		C	C	C	C	C	C	C		
Lyso	F=5.64; p=0.0002	P	MC	S	Wd	H	W	R	B	NF
							A	A	A	A
			B	B	B	B	B	B	B	
		C	C	C	C	C	C	C		
Sphingosine-1-Phosphate (S1P)	F=8.81; p<0.0001	S	W	B	R	MC	Wd	P	H	NF
										A
		B	B	B	B	B	B	B	B	
C6:0	F=0.97; p=0.48	No Difference								
C8:0	F=2.32; p=0.07	No Difference								
C10:0	F=0.97; p=0.48	No Difference								
C11:0	F=1.68; p=0.17	No Difference								
C12:0	F=1.41; p=0.26	No Difference								
C13:0	F=0.44; p=0.86	No Difference								
C14:0	F=2.21; p=0.08	No Difference								
C14:1	F=0.98; p=0.48	No Difference								
C15:0	F=1.07; p=0.42	No Difference								
C15:1	F=2.44; p=0.06	No Difference								
C16:0	F=0.79; p=0.61	No Difference								
C16:1	F=0.70; p=0.67	No Difference								
C17:0	F=0.51; p=0.82	No Difference								
C17:1	F=2.13; p=0.09	No Difference								
C18:0	F=0.65; p=0.71	No Difference								
C18:1n-9(t)	F=3.00; p=0.02	H	R	W	Wd	B	P	S	MC	
		A	A	A	A	A	A	A	A	
C18:1n-9(c)	F=0.23; p=0.97	No Difference								
C18:2n-6(t)	F=0.32; p=0.93	No Difference								
C18:2n-6(c)	F=0.77; p=0.62	No Difference								
C18:3n-6	F=2.19; p=0.08	No Difference								
C18:3n-3(c)	F=4.20; p=0.0066	Wd	S	H	W	MC	B	R	P	
			A			A	A	A	A	
		B	B	B	B	B	B	B		
C20:0	F=13.68; p<0.0001	R	Wd	W	B	P	MC	H	S	
									A	
		B	B	B	B	B	B	B		
C20:1	F=0.4132; p=0.88	No Difference								
C21:0	F=1.20; p=0.35	No Difference								
C20:2	F=1.39; p=0.27	No Difference								
C20:3n-6	F=2.38; p=0.07	No Difference								
C22:0	F=0.53; p=0.80	No Difference								
C20:3n-3	F=1.89; p=0.13	No Difference								
C22:1n-9	F=4.34; p=0.0056	R	MC	P	W	Wd	H	B	S	
			A	A	A	A	A	A	A	
		B	B	B	B	B			B	
C23:0	F=2.23; p=0.08	No Difference								
C22:2	F=0.94; p=0.5	No Difference								
C20:4n-6	F=1.49; p=0.23	No Difference								
C24:0	F=1.10; p=0.41	No Difference								
C20:5n-3	F=1.84; p=0.14	No Difference								
C22:6n-3	F=0.88; p=0.54	No Difference								

APPENDIX E

Comparing Gas Chromatographic Techniques Used in Fatty Acid Profiling of Northern Fur Seals (*Callorhin usursinus)* and Steller Sea Lions (*Eumetopias jubatus*) from Lovushki Island Complex, Russia

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Abstract

Northern fur seal (NFS, *Callorhinus ursinus*, n = 22) and Steller sea lion (SSL, *Eumetopias jubatus*, n = 12) blubber samples were collected from adults occupying the same rookery near the Lovushki Island complex, Russia. The objective of this study was to compare identified fatty acid methyl esters (FAMEs) using gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS) between species for each lipid class (saturated, SFA; monounsaturated, MUFA; polyunsaturated, PUFA). GC-FID identified an average of 26 FAMEs from each species against a set of 37 FAMEs. ANOVA detected differences between detectors and species, with GC-MS recovering greater numbers of total FAMEs but with fewer SFA detected. Interestingly, the GC-MS recovered greater numbers of FAMEs for NFS when compared to SSL. The use of both GC-FID and GC-MS, rather than solely one method, seems appropriate in order to avoid drawing spurious conclusions regarding potential resource partitioning in ecological studies.

Introduction

There are several approaches commonly used in which to identify fatty acids or their derivatives (fatty acid methyl esters, FAME), including gas chromatography-flame ionization detector (GC-FID), gas chromatography- mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) spectroscopy and silver ion thin-layer chromatography (TLC, Skoog et al. 1998). These methods have been used to identify the common fatty acids of animal (Budge et al. 2006) and plant (Glew et al. 1997) origin, with chains typically numbering from 12 to 24 carbon atoms, including zero to six double bonds, as well as specialized non-methylene-interrupted double bonds (Budge et al. 2007). Of these techniques, gas chromatography (GC) along with any one of a number of detectors (Harris. 2003), offers a simple, rapid and relatively inexpensive method for the identification or quantification of FAME in lipid research. While somewhat dependent on the detector, GC has a straightforward derivatization procedure (e.g., Budge et al. 2006), uses readily available reagents and has simple preparatory requirements. Another important asset of GC is that it is not typically necessary to isolate lipid components in pure form, as may be required by other methods (e.g., NMR spectroscopy). Currently, a considerable number of lipid researchers use GC-FID for FAME analysis in topics ranging from agriculture to biomedicine to ecology. However, there has been a dramatic increase in fatty acid analysis and interpretation in animals inhabiting the marine environment, specifically marine mammals. Topics of current research interest include, for example, climate change (Cooper et al. 2009), predator-prey dynamics (Iverson et al. 1997), and age-related lipid changes (Trumble et al. 2010).

As beneficial as GC is, as with any method, there are limits associated with its use; GC-MS spectra may not always contain ions indicative of structural features (e.g., the positions of double bonds in the aliphatic chain cannot always be definitively determined) or for GC-FID, there can be a failure to differentiate between *cis* and *trans* isomers, causing misidentification of FAME.

Identification using GC-FID may also be hampered by contaminants or coeluting compounds. Another limitation of using GC-FID is obtaining adequate standards, as standards are not available for many of the fatty acids found in mammalian tissue, especially for the more complicated polyunsaturated fatty acids. Therefore, there are instances when FAME analysis is best served by a combination of GC-MS and GC-FID, either for confirmatory purposes (to ensure the correct identification of a peak) or as an exploratory guide for further work (e.g., Best et al. 2003; Newland et al. 2009).

Materials and Methods

Our investigation compares FAME using commonly utilized GC detectors in the blubber tissue of sympatric pinniped species inhabiting remote Eastern Asia. As part of a larger study on resource partitioning, these samples were collected from the Lovushki Island complex, Russia (Figure E1), from sexually dimorphic and piscivorous adult Northern fur seals (NFS, *Callorhinus ursinus*) and Steller sea lions (SSL, *Eumetopias jubatus*). Approximately half of the total SSL breeding population in Russian waters occurs on these rookeries (Burkanov and Loughlin. 2005) and after declining in numbers for 20 years, the population has remained relatively stable from 1995 through 2005 (Burkanov and Loughlin. 2005). In contrast, a rapid increase in NFS population numbers

on Lovushki Island began in the 1950's resulting in the current population being near their historic high (Burkanov et al. 2007).

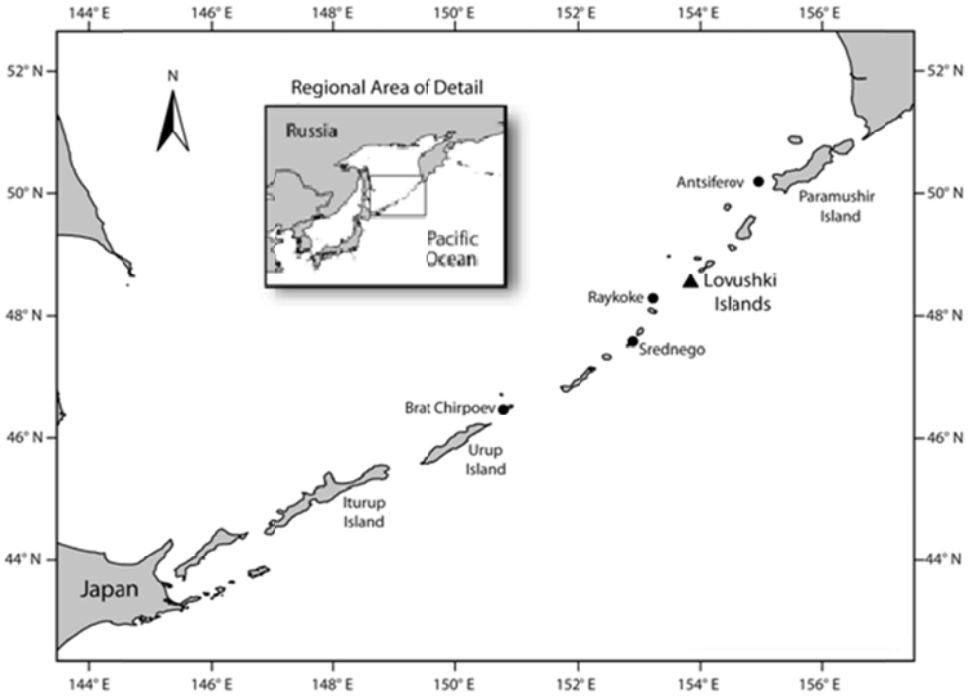

Figure E1. Lovushki Island Complex of the Kuril Island Chain, Russia (modified from Waite et al. 2012).

The goal of this study is to establish and compare FAME analysis from blubber samples from NFS and SSL using both GC-FID and GC-MS techniques, and to determine which particular technique, if not a combination, provides the best method for characterizing blubber in these sympatrically breeding marine mammals. A secondary objective is to establish similarity indices of lipid profiles using each technique. Given the common use of GC techniques to describe FAME, we feel comparing results from samples taken from a field study will provide information on the limits of each technique and thus allow researchers to make more informed decisions regarding methodology during ecological studies.

Blubber samples from juvenile male and non-lactating female Northern fur seal (NFS, n=22) and Steller sea lions (SSL, n=12) were collected from the same rookery near the Lovushki Island complex (Figure E1, Kuril Island chain) in Russia (48º 32.617' N, 153º 40.417' E) during the breeding season of 2008. To access the blubber sample from the ventral hip area, a 2 cm^2 area was shaved and then cleaned using a solution of 70% ethyl alcohol and betadine before each incision.

Biopsies were collected (30-50 mg taken under general anesthetic; 1 ml, Isoflurane) using a 4 mm biopsy cannula (Depuy, Warsaw, IN, USA). All blubber samples were frozen and stored inliquid nitrogen until later analysis. All samples were weighed after thawing to the nearest 0.001g (wwt).

Lipid extraction procedures included drying each sample with sodium sulfate (approximately 50 g, J.T. Baker, Phillipsburg, NJ) before running through an Accelerated Solvent Extractor (Dionex ASE 350, Sunnyvale, CA), using up to 100 ml dichloromethane (VWR, West Chester, PA) as the solvent. Similar laboratory conditions were maintained for all extractions. Excess solvent was evaporated under a steady stream of nitrogen (1.5 L/min @ 37C). The resulting lipid extract was then transesterified using a method similar to Budge et al. (2006). Briefly, the samples were added to 1.5 ml dichloromethane (DCM) and 3 ml Hilditch reagent (Sigma Aldrich, St. Louis, MO), and then heated at 100°C for 1h. The lipids were then separated out by extraction by repeated centrifugation (100 X*g* for 2-5 min) with DCM (3 ml for first centrifugation, 1 ml for latter two) and deionized water (1 ml for each centrifugation). The lipid layer (bottom) was then dried with sodium sulfate (approximately 0.5 g), and heated in a water bath to evaporate solvent. Each transesterified sample was divided into two equal aliquots and

analyzed for FAME on a GC-FID (Varian 430-GC and Varian CP-8400 AutoSampler) and subsequently on an Electron Ionization GC-MS (Varian GC 3900/MS Saturn 2100T) using, in both cases, the same CP-Select column (CP7419, Varian) 100 m x 0.25 mm ID x 0.25 um. GC-FID protocols followed Budge et al (2006) with the following modifications: the column length was 100m; the injector temperature was 250^{o}C with a 1μl injector split ratio 100:1. Column flow was 1.0 ml min^{-1} programmed at 210^{o}C for 9.0 minutes and ramped at 15^{o}C min^{-1} to 260^{o}C for 7.7 minutes. Detector temperature was set at 300^{o}C with a hydrogen flow of 30 ml min^{-1} and airflow of 300 ml min^{-1}. The internal standard was C19:0 (Fluka 72332).

A set of 37 standard historical marine FAME (Supelco® 37 component FAME mix) were used in the GC-FID analysis (Table E1). The FAME were obtained from Sigma-Aldrich (St. Louis, MO) and Supelco® (Bellefonte, PA), chosen primarily for their physiological relevance to fatty acids commonly found in marine organisms, and were of the highest purity available. Blanks were run between each true sample. For each level of calibration, all FAME target analytes were present at equal concentrations with concentrations between 0.25 μg/μL and 10 μg/μL for each standard curve generated.

For GC-MS analysis, the same standards (including the C19:0 internal standard) were analyzed on an Electron Ionization (EI) mass spectrometer with the same flow rates and temperature programs as above. These standards were used to determine FA elution order, and to confirm that the same standards utilized with the GC-FID are also properly identified on the GC-MS. The NIST (National Institute of Standards and Technology) library was also utilized with GC-MS to confirm peaks matched with standards (utilizing both retention times and structural details), and additionally, to identify novel peaks in

our samples using highest probability methods with retention times, structural details, and isotopic patterns (Figure E2).

Table E1. FAME standards with elution order

Elution Order	FAME Formula	Systematic Name	Common Name
1	C4:0	Butanoic acid, methyl ester	Butyric acid methyl ester
2	C6:0	Hexanoic acid, methyl ester	Caproic acid methyl ester
3	C8:0	Octanoic acid, methyl ester	Caprylic acid methyl ester
4	C10:0	Decanoic acid, methyl ester	Capric acid methyl ester
5	C11:0	Undecanoic acid, methyl ester	Undecylic acid methyl ester
6	C12:0	Dodecanoic acid, methyl ester	Lauric acid methyl ester
7	C13:0	Tridecanoic acid, methyl ester	Tridecylic acid methyl ester
8	C14:0	Tetradecanoic acid, methyl ester	Methyl myristate
9	C14:1	9-Tetradecenoic acid, methyl ester	Myristoleic acid methyl ester
10	C15:0	Pentadecanoic acid, methyl ester	Pentadecylic acid methyl ester
11	C15:1	*cis*-10-Pentadecenoic acid, methyl ester	None
12	C16:0	Hexadecanoic acid, methyl ester	Methyl palmitate
13	C16:1	9-Hexadecenoic acid, methyl ester	Palmitoleic acid methyl ester
14	C17:0	Heptadecanoic acid, methyl ester	Methyl margarate
15	C17:1	*cis*-10-Heptadecenoic acid, methyl ester	Heptadecenoic acid methyl ester
16	C18:0	Octadecanoic acid, methyl ester	Methyl stearate
17	C18:1n-9(t)	*trans*-9-Octadecenoic acid, methyl ester	*trans*-Methyl oleate
18	C18:1n-9(c)	*cis*-9-Octadecenoic acid, methyl ester	*cis*-Methyl oleate
19	C18:2n-6(t)	*trans*,*trans*-9,12-Octadecadienoic acid, methyl ester	*trans*-Methyl linoleate
20	C18:2n-6(c)	*cis*,cis-9,12-Octadecadienoic acid, methyl ester	*cis*-Methyl linoleate
21	C18:3n-6	6,9,12-Octadecatrienoic acid, methyl ester	GLA, methyl ester
22	C20:0	Eicosanoic acid, methyl ester	Methyl arachidate
23	C18:3n-3	9,12,15-Octadecatrienoic acid, methyl ester	ALA, methyl ester
24	C20:1	*cis*-11-Eicosenoic acid, methyl ester	Gondoic acid methyl ester
25	C21:0	Heneicosanoic acid, methyl ester	None
26	C20:2	*cis*-11,14-Eicosadienoic acid, methyl ester	Eicosadienoic acid methyl ester
27	C20:3n-6	*cis*-8,11,14-Eicosatrienoic acid, methyl ester	DGLA, methyl ester
28	C22:0	Docosanoic acid, methyl ester	Methyl behenate
29	C20:3n-3	11,14,17-Eicosatrienoic acid, methyl ester	ETE, methyl ester
30	C22:1n-9	13-Docosenoic acid, methyl ester	Methyl erucate
31	C23:0	Tricosanoic acid, methyl ester	Tricosylic acid methyl ester
32	C20:4n-6	5,8,11,14-Eicosatetraenoic acid, methyl ester	Methyl arachadonate
33	C22:2	*cis*-13,16-Docosadienoic acid, methyl ester	Docosadienoic acid methyl ester
34	C24:0	Tetracosanoic acid, methyl ester	Lignoceric acid methyl ester
35	C24:1	Nervonic acid methyl ester	Nervonic acid methyl ester
36	C20:5n-3	5,8,11,14,17-Eicosapentaenoic acid, methyl ester	EPA, methyl ester
37	C22:6n-3	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester	DHA, methyl ester
N/A (IS)	C19:0	Nonadecanoic acid, methyl ester	None

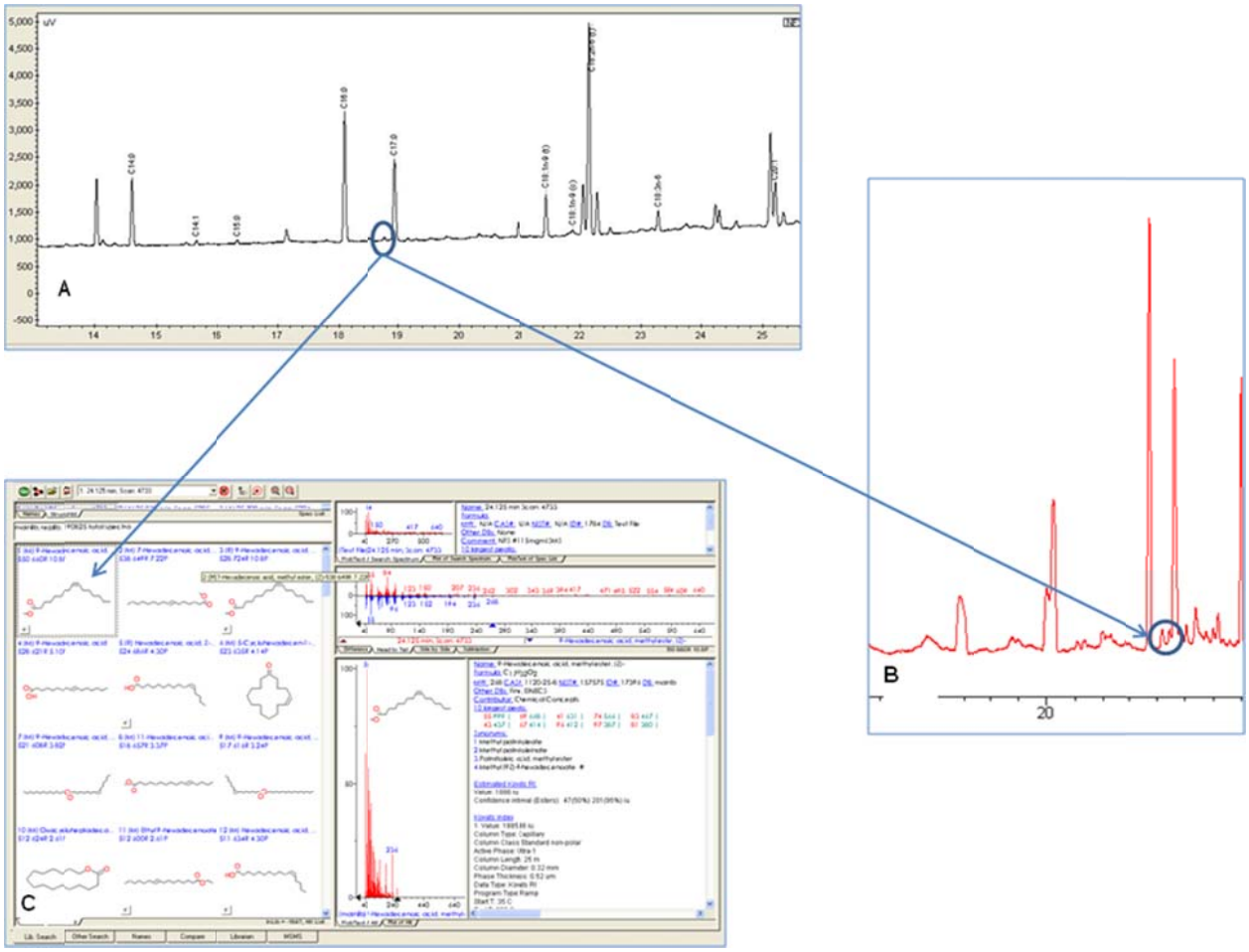

Figure E2. Comparison of same sample run through GC-FID and GC-MS; A. Magnified portion of GC-FID spectrum for a Northern fur seal sample, showing unidentified peak of interest (circled), B. Magnified portion of GC-MS spectrum of same sample, showing unidentified peak of interest (circled), now identified in (C), C. NIST library finding for the unidentified peak of interest using GC-MS

A Sørensen similarity index (SI) was used to determine differences in FAME detected between blubber samples, using GC-FID and GC-MS. Each fatty acid recovered and identified from the blubber samples was applied to the following formula: QS = 2C/A+B, where *A* and *B* are the number of fatty acid species identified for NFS (A) and SSL (B), respectively, and *C* is the number of fatty acids identified shared by the two marine mammals. SI calculations were performed for both GC-FID and the combination of GC-FID and GC-MS to establish differences between the protocols.

The commercially available standard mixture of 37 FAME (Supelco®) was used as a control in the determination of response factor for each GC detector and analyzed in triplicate before and after each series of samples run by both GC–FID and GC–MS. To test differences in the number of fatty acids between species (SSL, NFS) with detector (MS, FID) and lipid class (SFA, MUFA, PUFA), a fixed-factor ANOVA was performed (SPSS v.17). ATukey's HSD test was used to determine among group differences. Statistical significance was $P < 0.05$. Both detectors (GC-MS and GC-FID) used to identify fatty acids from blubber samples of SSLs and NFSs completely and correctly identified and recovered all FAMEs from the Supelco® 37 mixture during all controlled tests ($P > 0.05$). Therefore, we judge any differences in recovered FAMEs between the genuine field samples of NFS and SSL are, therefore, due solely to differences in the blubber composition of the sample, and not the methodology, as both detectors clearly identified all standards.

Results

GC-FID methods identified 30 FAME from NFS samples and 31 FAME from SSL samples, against a standard set of 37 FAME (Table E2, $P > 0.05$).

This equaled a 98% similarity on recovered FAMEs between both species inhabiting the same rookery. GC-MS methods identified a total of 43 FAME for NFS, including 4 not identified via GC-FID (C18:1n-13(t), C18:1n-12(t), C20:2n-6(t), C22:4n-7(t)) whereas a total of 27 FAME were identified by GC-MS for SSL ($P < 0.01$, Figure E3), including 4 not identified via GC-FID methods (C18:1n-8(t), C21:1n-9T), C16:2n-4(t), C18:3n-3(cis)). An additional 5 FAME (C18:1n-7(t), C18:3n-3, C21:4n-6(cis), C20:4n-3, C17:1(branched)) were identified by GC-MS which were common to both

species. Furthermore, 7 FAME found in NFS (C16:1n-7(cis), C23:1n-9(cis), C24:1n-9(t), C20:3n-3(t), C20:5n-3(t), C22:5n-3(cis), C22:6n-3(t)) and 2 found in SSL (C22:1n-9(t), C20:2n-6) showed superior structural characterization using mass spectrometry (Figure E3). Further structural characterization of 5 FAME common to both species (C16:1n-9(t), C20:1n-9(t), C22:1n-9(cis), C20:5n-3(cis), C22:6n-3(cis)) was achieved using GC-MS. The SI calculation performed on GC-MS data determined a 51% similarity between species based on FAME composition.

While a significantly greater number of saturated FAMEs were recovered using GC-FID for both species ($P < 0.05$), the GC-MS detector recovered a significantly greater number of unsaturated FAMEs for the NFS ($P < 0.05$). No difference was found in the number of unsaturated FAMEs recovered for SSL using both detectors (Figure E3, $P > 0.05$). For the GC-MS, we observed significantly greater FAMES recovered for the NFS when compared to the SSL (Figure E3, $P < 0.05$ for all lipid classes). When combining FAME profiles from both GC-FID and GC-MS, additional structural information and newly-identified fatty acids were found in both NFS and SSL samples (Table E2, Figure E3). Together, the methods of GC-FID and GC-MS identified a total of 61 individual fatty acids when compiling both NFS and SSL samples. A total of 31 FA were identified by GC-FID, and 51 by GC-MS, representing 21 FA's through GC-MS which were either better characterized as to bond structure or newly identified; a 64.5% increase in FA identification was achieved using GC-MS. Combining methods resulted in an 81% agreement in fatty acids identified between SSL and NFS inhabiting the same rookery. GC-MS profiles indicate a greater number of MUFAs and PUFAs (listed above) compared to saturated FAs for both species (Table E2, Figure E3).

Table E2. Fatty acids by method, class, and species

Fatty Acids	GC-FID		GC-MS		GC-FID and GC-MS Combined	
	NFS	SSL	NFS	SSL	NFS	SSL
Saturated						
C12:0	X	X	X	X	X	X
C13:0	X	X			X	X
C14:0	X	X	X	X	X	X
C15:0	X	X	X		X	X
C16:0	X	X	X		X	X
C17:0	X	X	X	X	X	X
C18:0	X	X	X	X	X	X
C20:0	X	X	X	X	X	X
C21:0	X	X	X		X	X
C22:0	X	X	X		X	X
C23:0	X	X	X		X	X
C24:0	X	X	X		X	X
	X	X	X		X	X
Monounsaturated						
C14:1	X	X			X	X
C15:1	X	X	X	X	X	X
C16:1	X	X			X	
C16:1n-7(c)			X		X	X
C16:1n-9(t)			X	X	X	X
C17:1	X	X			X	X
C18:1n-7(t)			X	X	X	X
C18:1n-8(t)				X		X
C18:1n-9(t)	X	X			X	X
C18:1n-9(c)	X	X	X	X	X	X
C18:1n-12(t)			X		X	
C18:1n-13(t)				X	X	
C19:1n-9(t)				X	X	
C20:1	X	X	X		X	X
C20:1n-9(t)			X	X	X	X
C21:1n-9(t)				X		X
C22:1n-9	X	X	X		X	X
C22:1n-9(c)			X	X	X	X
C22:1n-9(t)				X		X
C23:1n-9(c)			X		X	
C24:1	X	X	X	X	X	X
C24:1n-9(t)			X		X	
Polyunsaturated						
C16:2n-4(t)				X		X
C18:2n-6(t)	X	X	X	X	X	X
C18:2n-6(c)	X	X	X	X	X	X
C18:3n-3			X	X	X	X
C18:3n-3(c)			X	X	X	X
C18:3n-6	X	X	X		X	X
C20:2	X	X	X		X	X
C20:2n-6				X		X
C20:2n-6(t)			X		X	
C20:3n-3(t)			X	X	X	X
C20:4n-3			X		X	
C20:3n-3	X	X			X	X
C20:3n-6		X			X	X
C20:4n-6	X	X			X	X
C21:4n-6(c)			X	X	X	X
C22:2	X	X			X	X
C20:5n-3	X	X			X	X
C20:5n-3(c)			X	X	X	X
C20:5n-3(t)			X		X	
C21:5n-3(t)			X		X	X
C22:4n-7(t)			X		X	
C22:5n-3(c)			X		X	
C22:6n-3	X	X		X	X	
C22:6n-3(c)			X	X	X	X
C22:6n-3(t)			X		X	X
TOTAL	29	30	43	27	53	46

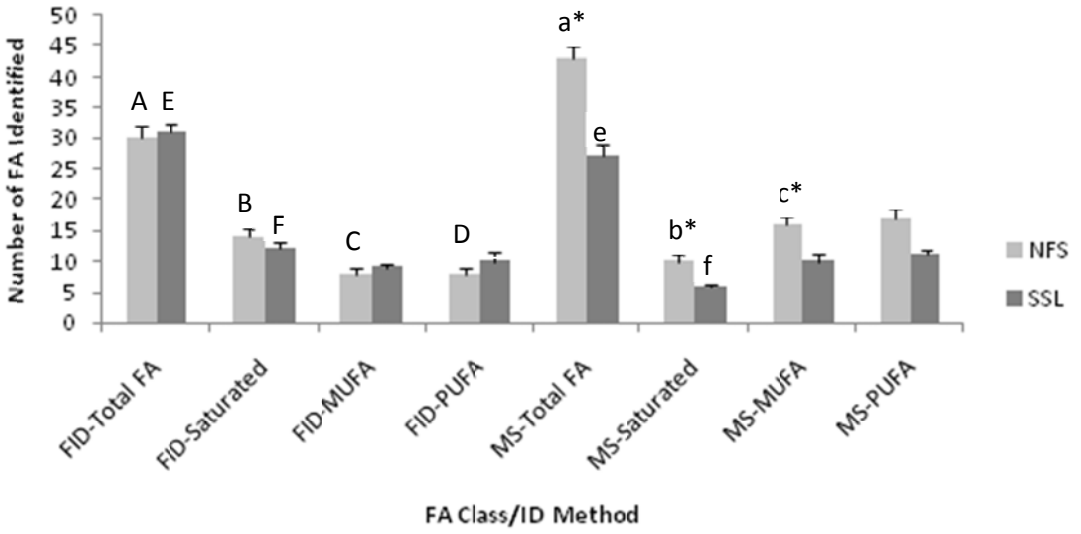

Figure E3. Comparison of FAME identification methods by lipid class, total FAMEs recovered and between species (NFS, SSL); Large and small case letters indicate statistical significance ($P < 0.05$) whereas * indicates significance between species. Error bars represent $\pm$SE.

In our study, a greater number of saturated fatty acids in both species were found using GC-FID, while the identification of unsaturates, both MUFAs and PUFAs, was best resolved via GC-MS (Figure E3).

Therefore, we identified a greater number of fatty acids in SSL blubber using GC-FID when compared to the GC-MS, whereas GC-MS identified a greater number of FAMEs from NFS blubber samples. GC-FID analysis revealed a high degree of similarity (98%) of fatty acids between species whereas GC-MS revealed a relatively lower degree of fatty acid signature similarity (51%). When combining both methods the total number of fatty acids identified increased over using each method individually (Table E2).

Discussion

Increased numbers of MUFAs and PUFAs were identified using GC-MS compared to GC-FID, implicating the GC-FID as an inferior method for identifying unsaturated fatty acids in these blubber samples. Typically, the GC-MS is regarded as an improvement in resolution when compared to GC-FID; the method provides greater selectivity and sensitivity over GC-FID, the ability to confirm compounds based on both retention time and additional spectral data, and an enhanced ability to separate coeluting peaks based on unique ions (Dodds et al. 2005). It has been reported that using the GC-MS as a detector leads to much greater distinguishing ability between lipids, and even among similar FAME groups (e.g., C18:Xn-x). In particular, characteristic fragmentation patterns exist for FA, with prominent fragments of m/z=43 in saturates, and m/z=41 in unsaturates (Wetzel and Reynolds. 2004). However, our data indicated a decreased capability for GC-MS to identify saturated fatty acids compared to GC-FID in free-ranging marine mammal species. This result is likely due to error associated with instrument overloading. The ion trap of the MS is more sensitive to sample overload, whereas this issue is not common in GC-FID (Hilkert et al. 1999). These findings may present serious implications for studies both in the past and the future utilizing one detection method for identifying and quantifying fatty acids (see below). GC-MS identification of FAMEs can be accomplished by retention time matching, and for confirmation, by referencing the NIST library for structural identification. In this study, we found GC-MS had a greater ability to resolve unsaturated fatty acids (Figure E3).

We also observed a failure by GC-FID to identify many MUFAs and PUFAs, which may lead to incomplete or incorrect conclusions pertaining to specific research

involving resource use and dietary habits of marine mammal species. For example, many resource partitioning and dietary studies (Best et al. 2003; Cooper et al. 2009; Newland et al. 2009) use primarily GC-FID detection to identify FAMEs, with subsequent use of GC-MS to verify FID peaks. Using GC-FID as the sole detector limits the investigator in that several fatty acids may elute with the same retention time, and the possibility of similar non-FA lipids eluting also exists. Therefore, any results from GC-FID where peaks are tightly clustered are likely to be associated with significant error in identification (Budge et al. 2008). Results from this study show that specific FAMEs or FAs recovered from blubber and subsequently used in analysis to determine dietary habits may be best served using both GC detection methods. In other words, combining the methods of both GC-FID and GC-MS would seem to lend greater accuracy to FA blubber composition studies, and more broadly, to applications in resource partitioning and competition studies. Accurate determination of the fatty acid profile in marine mammals with the use of both GC-FID and GC-MS will add validity to ecological and physiological interpretations made with these data. Although this study was only comparing methods for identifying fatty acids, calibration curves for both detectors could be created, allowing for a complete and quantitative analysis of all fatty acids in the tissue (usually reported in relative percentage).

Surprisingly, we found that the GC-MS consistently recovered greater numbers of FAMEs for NFS when compared to SSL (Figure E3). Fatty acids are known to stratify in the blubber layers of marine mammals (Käkelä and Hyvärinen. 1996; Best et al. 2003; Thiemann et al. 2004) and while these stratification patterns can be species-specific, generally, polyunsaturated fatty acids are found closer to the muscle layers (i.e., deeper),

while more saturated fatty acids (particularly from *de novo* synthesis) are found in the outermost layers (Cooper. 2004). Our species difference identified by GC-MS, then, does not seem to implicate a sampling error based on blubber depth sampled, as the number of fatty acids identified in NFS by GC-MS was higher across all FA classes. Therefore, we deem this species difference to be a factor of detector differences (discussed above) and diet (see next paragraph).

Given the importance that competition has on community structure and the patchy nature of resources, many species populating similar habitats demonstrate some degree of resource partitioning as a means to reduce inter- and intra-specific competition (Bolnick et al. 2003; Newland et al. 2009).

Traditionally, pinniped diet or dietary overlap between or among species has been determined from the identification of prey hard parts collected from stomachs, colons, spewings (regurgitations) or scat (fecal) samples (e.g., Lucas 1899; Antonelis and Perez 1984; Sinclair et al. 1994; Yonezaki et al. 2003; Gundmundson et al. 2006, Zeppelin and Ream 2006; Yonezaki et al. 2008). Our results indicated that there may be a significant level of resource partitioning between NFS and SSL, but these findings are, importantly, inherent on the use of both GC-FID and GC-MS as a means to detect FAMEs in the blubber. While food habit data on our two species is sparse in Russian waters, studies conducted in the 1950's and 1960's revealed some dietary overlap, with walleye pollock (*Theragra chalcogramma*) being a primary prey item (Belkin 1966). Additionally, while some overlap was detected, Kuzin et al. (1977) used stomach content analysis to show a clear partitioning of foraging resources between these pinniped species. Their data indicated that the NFS diet was composed of 60.1% pollock, 21.7% salmon

(*Oncorhynchus* sp.), 14.1% anchovy (*Engraulis* sp.), and 4.1% squid, while the diet of SSL consisted of 37.5% squid, 35.6% rockfish (*Sebastes* sp.), and 26.8% pollock. Our findings are, therefore, substantiated by previous dietary studies. The fatty acid profiles of these prey species, in large part, explain the species differences we see in GC-MS detection. NFS fatty acid detection by GC-MS, while higher across all FA classes, shows particularly large recoveries for MUFAs and PUFAs. NFS prey are composed largely of high-PUFA and MUFA fish (pollock, salmon, squid), with only a small percentage of diet determined by anchovy (with a composition more saturated).

SSL diet, on the other hand, is composed in larger proportions of species comparatively lower in unsaturated fatty acids (rockfish), and a smaller proportion of species higher in PUFAs (squid and Pollock) (Iverson et al. 2002; Kaya and Turan 2010). Therefore, the difference we see in GC-MS detection between NFS and SSL can be explained by the relative proportion of their diets composed of unsaturated fatty acids as composed to SFA. Had we used solely GC-FID to investigate potential resource partitioning between these animals, our results would be drastically different (98% SI for GC-FID versus 81% SI in combined methods), failing to identify many of the unsaturated fatty acids present in the samples. Additionally, had we used solely GC-MS, our analysis would have failed to identify many of the common saturated fatty acids that were clearly present in the samples. Thus, using a combination of both GC-FID and GC-MS provided, we believe, a more complete analysis of fatty acids.

FA signature analysis is considered to be a robust method in determining dietary preferences (Iverson et al. 2004). However, a few caveats in GC work do exist, limiting the scope of our results. Although the differences seen between NFS and SSL can likely

be attributed to differences in prey composition, this assumes ideal, representative samples from both species have been obtained. In other words, some differences in blubber FA composition between the species may simply stand as an artifact of sampling technique. However, because of the substantially different FA composition between the two species, a reasonable assumption can be made that SSL and NFS in our study location are largely feeding on different prey. As SSL and NFS are both otariids with a similar life histories, the FA differences we have identified in depot lipids are almost certainly representative of a recent diet, rather than any difference resulting from the way in which prey are digested (Falk-Petersen et al. 2009). Despite these caveats, gas chromatographic fatty acid analysis remains one of the most rigorous methods to study dietary habits of marine organisms in remote locations, and combining detectors in marine mammal studies provides a more complete and reliable fatty acid profile.

GC-FID/MS analysis of marine mammal blubber revealed many individual methyl esters ranging in total carbon number from C12 to C24. However, FAME detection using FID provided greater resolution for saturates, whereas GC-MS demonstrated higher sensitivity for unsaturates. This information could prove highly beneficial with broad implications in studies involving dietary overlap and FA stratification in blubber.

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