ABSTRACT

Metabolic Lipid Regulation during Developmental Stress:
Fatty Acid Dynamics in Fasting Northern Elephant Seals

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Long chain fatty acids (LCFA) are important as fuel during exercise in humans, yet have pathological consequences during LCFA overabundance. The northern elephant seal (NES) (*Mirounga angustirostris*) is a temporally insulin resistant, deep-diving marine mammal reliant on fatty acid oxidation during seasonal fasting. Over the fast, NES females and weaned pups were found to oppositely utilize monounsaturated and saturated fatty acids, while polyunsaturated fatty acids generally increased in both age groups. Fatty acid transporter proteins (FATP) CD36, FATP1, FATP4 and binding protein 3 (FABP3) were detected in adult males, adult females, and pups. This thesis is the first documentation of these FATPs and FABPs in all NES age classes and expands current knowledge of lipid utilization in a mammal accustomed to a LCFA-rich diet.

Metabolic Lipid Regulation during Developmental Stress:
Fatty Acid Dynamics in Fasting Northern Elephant Seals

by

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A Thesis

Approved by the Department of Biology

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Robert Doyle, Ph.D., Chairperson

Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of

Master of Science

Approved by the Thesis Committee

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Accepted by the Graduate School

May 2014

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TABLE OF CONTENTS

LIST OF FIGURES

Figures

ABBREVIATIONS

βHBA	β-Hydroxybutyrate
FAT/CD36	Fatty Acid Translocase/Center of Differentiation 36
DHA	Docosahexaenoate
EF	Early Post-Wean Fast
EL	Early Lactation Fast
EPA	Eicosapentaenoate
FABP	Fatty Acid Binding Protein
FABPpm	Fatty Acid Binding Protein Plasma Membrane
FATP	Fatty Acid Transport Protein
IMTG	Intramuscular Triglyceride
IML	Intramuscular Lipid
LC-MS	Liquid Chromatography – Mass Spectrometry
LCFA	Long-Chain Fatty Acid
LF	Late Post-Wean Fast
LL	Late Lactation Fast
MUFA	Monounsaturated Fatty Acid
NEFA	Non-Esterified Fatty Acid
NES	Northern Elephant Seal
PPAR	Peroxisome Proliferator-Activated Receptor
PUFA	Polyunsaturated Fatty Acid
ROS	Reactive Oxygen Species

SFA	Saturated Fatty Acid
T2DM	Type 2 Diabetes Mellitus

ACKNOWLEDGEMENTS

The journey to completing this thesis has been quite the formative experience, both personally and professionally, and there are many people that played an integral role in this process. First, I would like to thank my advisor, Dr. Stephen Trumble for all of his support throughout these past years and allowing me to gain invaluable experience both in the laboratory and out in the field. He has provided opportunities unlike anything I have ever done before, and I will certainly use the lessons gleaned from them in the future. Secondly, I would like to thank my committee members: Dr. Cheolho Sim and Dr. Sascha Uskeno. Their input has helped shaped my thesis and also my perspectives on the future of the marine mammal field. I will always be grateful for their continuous patience, understanding, and thoughtful guidance. I would also like to thank Dr. Shane Kanatous; the incredible lessons I've learned from him are some of my favorite memories from the past two years. I am sincerely grateful for his patience, mentorship, and simply believing in me. I would also like to thank Dr. Daniel Crocker, whose guidance and leadership during sampling was invaluable, and I am certain that without it this thesis would not be possible. His humility and collaborative nature is worthy of great admiration and I believe science would make substantial progressive strides if more researchers shared his perspective. Dr. Bryan Gibbon also deserves a substantial thank you, as his help and advice in troubleshooting was critical to the success of my experiments.

A special thank you goes to my labmates, Colby Moore and Rebel Sanders. By having someone also doing Western blots in my lab, Colby made it infinitely more bearable to keep going during the many frustrating and head-scratching moments.

Rebel's willingness to help with anything, whether it was doing dishes or just being an ear to listen, was sincerely appreciated. I would also like to thank the now-Dr. Amber Schlater. I will always be grateful for her friendship, guidance in the field, and help in the lab. I would also like to thank Mo Jia; her wise advice and support in the lab was invaluable.

My friends and family are by far deserving of the most gratitude. While there are many people who supported me along the way, there are a few people in particular who deserve recognition. First, I would like to thank my best friend Sungshil Kim. Her friendship was undoubtedly heaven-sent, and her support in every aspect of my life is deserving of more thanks than I can include here. I couldn't imagine these past two years without her. Secondly, I am always grateful for the friendship and support of Jemilia Davis, Greg Steele, and Sarah Leger, who helped me maintain my grounding and perspective during the most difficult seasons. I would also like to thank Rebekah Childers, Nikki Georgacakis, Remi Davis, and countless other friends that supported me throughout this whole process. Whether it was helping with dishes, buying me late-night thesis writing snacks, or just simply providing an ear to listen, I will always be grateful to have been surrounded and supported by such wonderful people during two of my most challenging years.

Last but certainly not least, I would like to thank my family: Dad, Mom, Kim, and Aunt Jo. They have always supported all of my educational adventures, both in and outside of the continental US. Although half a country away, I am continuously blessed by their steadfast love and unwavering encouragement. I will never be able to fully express the extent of my gratitude for them during the past two years. Finally, I would

like to thank God for providing me with so many amazing opportunities for personal and professional growth, and being the bedrock of my life. All of the glory goes to Him for the success of my thesis and these past few years. While I never thought I would move to Texas, the Lord is continuously teaching me to find beauty in the process of being molded shaped by infinitely talented master.

I would like to thank Metabolon, Inc. for sample processing. We also thank the rangers and staff of Año Nuevo State Park for their support. This research was supported by the Baylor University Young Investigators Development Grant Program and the Baylor University Department of Biology. This work was collected under NMFS permit 87-1743 and approved by the Sonoma State Institute for Animal Care and Use Committee.

DEDICATION

To my sister Kimberly J. Robbins,

Always have the courage to take the road less traveled when chasing your dreams and may God always help you find the beauty and purpose in life's U-turns

CHAPTER ONE

Introduction and Overview

Long chain fatty acids (LCFAs) are well established to play an integral role in providing fuel to mammalian skeletal muscle; LCFA are the main fuel source for humans in moderate to low intensity exercise and their regulation has also been implicated in a wide range of metabolic diseases (Calder 1996; Hulbert et al. 2007; Long et al. 2011; Seifert et al. 2010; Trumble et al. 2010; Watt and Hoy 2012; Wood et al. 2009). Fatty acids play a critical role in maintaining the structure of cellular membranes, are valuable precursors to numerous hormones and neurotransmitters, and are necessary for cellular transport and signaling. Along with their importance as an intramuscular fuel source, there is increasing evidence that fatty acids are critical in regulating metabolism due to their prevalence in cell membranes. This is known as the *membrane-pacemaker theory* (Hulbert et al. 2007). This theory asserts the diverse mosaic of fatty acids within the cell membrane affects longevity based on their susceptibility to peroxidative damage. Cellular membranes are particularly vulnerable due to the solubility of reactive oxygen species (ROS) in lipid membrane bilayers. Specifically, membranes composed of polyunsaturated fatty acids (PUFA) have a greater degree of vulnerability to ROS than saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) (Hulbert et al. 2007).

While elevating antioxidant defenses can mitigate some of the negative affects of ROS, it may more advantageous for animals that inhabit stressful environments to compose their membranes with more ROS-resistant fatty acids than to upregulate the

production of antioxidants. This alteration of membrane composition has been observed in numerous species that inhabit challenging environments. While living in the extremely stressful environment of Antarctica, Weddell seals (*Leptonychotes weddellii*) have been observed to alter the composition of their intramuscular lipids with developmental state (Trumble et al. 2012; Wheatley et al. 2008). Similarly the longest living rodent, the naked mole rat (*Heterocephalus* glaber), was found to have lower levels of PUFAs within their tissue phospholipids in comparison to mice (*Mus* musculus) (Hulbert et al. 2007; Ramirez 2007). While there are numerous organisms that mitigate the negative effects of ROS by altering the composition of their cellular membranes, it is less lucid how this is mediated. Intake of specific fatty acids appear to play an important role for those organisms that are able to transiently alter their membrane composition, such as seasonally fasting organisms (Geiser and Kenagy 1987; Geiser 1991).

Marine mammals, particularly pinnipeds, display a unique suite of physiological adaptations that allow the maintenance of a lipid-based aerobic metabolism during diving. The northern elephant seal (*Mirounga angustirostris*) is the deepest diving pinniped and development of their diving abilities is crucial for successful foraging and predator avoidance. For general classification purposes, the northern elephant seal (NES) can be categorized into four distinct age classes: adult males, adult females, juveniles, and pups. Additional categories are assigned dependent upon various developmental stages, for instance during weaning, lactation, and molting (Le Boeuf and Laws 1994). Due to periods of prolonged fasting during seasonal haul-outs, all age classes are reliant on the successful foraging trips as adults and juveniles, and on successful nursing as pups for survival. NES experience an extended fast lasting 1-3 months, and often while

simultaneously under the energetic stress of lactation, breeding, and development (Champagne et al. 2012). The NES population selected for this study, located in the Año Nuevo State Reserve in Pescadero, California is a robust rookery that has been extensively studied for decades, making this population an ideal sample group.

While NES dive considerably deeper and for longer than Weddell seals (maximum recorded depth 1,764 m vs. 741 m, respectively), the Antarctic habitat of Weddell seals pose thermoregulatory challenges that necessitate the utilization of fatty acids for insulation and energy (Kanatous et al. 2002; Robinson et al. 2012). Earlier research has indicated the utilization of fatty acids in the similarly lipid rich diet of Weddell seals play a role in their diving development (Trumble et al. 2010). Specifically, the skeletal muscle of a Weddell seal pup begins at a highly aerobic, thermogenically favorable state, with polyunsaturated fatty acids (PUFAs) comprising the majority of FA in intramuscular membranes and storing saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) in the blubber; as pups develop into juveniles and adults, there is an increase in the metabolism of PUFAs, suggested to assist in oxygen conservation and diving development (Trumble et al. 2010). Also, there is a decrease in PUFAs in the intramuscular membranes with a corresponding increase in the prevalence of MUFAs and SFAs. Recently, the fatty acid blubber composition of NES post-wean fasting pups has been observed to be similar to Weddell seals, with a largest proportion of FAs within the blubber being MUFAs, followed by SFAs and PUFAs, respectively (Noren et al. 2013; Wheatley et al. 2008). Similar metabolic challenges, blubber composition, and lactation duration between Weddell seals and the NES make these species comparable. However, the seasonally fasting lifestyle of the NES and the

proximity of a robust rookery make the NES an ideal candidate for the study of fatty acid regulation (Eisert et al. 2013; Riedman and Ortiz 1979). Alterations in fatty acid metabolism have been observed across multiple age classes in the NES, including the significant increases of circulating non-esterified fatty acids (NEFA) with low β-hydroxybutyrate (βHBA) over the lactation fast in adult females, indicative of alternative form of ketone metabolism (Champagne et al. 2012; Houser et al. 2007). While the utilization of extraneous βHBA for *de novo* lipid synthesis by the mammary gland is a prospective pathway to circumvent ketoacidosis in lactating females, the lack of short chain and medium chain fatty acids in the milk suggests that circulating NEFA are responsible for the milk fat constituents (Eisert et al. 2013; Oftedal et al. 1988; Riedman and Ortiz 1979; Wheatley et al. 2008). There are many aspects to consider when studying fatty acid metabolism, but this study will primarily focus on the intracellular, intercellular, and circulating transport of fatty acids.

The study in the second chapter evaluates the potential utilization of specific fatty acids sampled from 10 NES mothers and 10 weaned pups, over the course of the lactation and post-wean fast. A mixture of eleven essential and non-essential SFA, MUFA, and PUFA were selected based on their prevalence in pacific hake (*Merluccius productus*) and the boreal clubhook squid (*Onychoteuthis borealijaponica*), two primary prey species of the NES (Condit and Le Boeuf 1984; Huynh and Kitts 2009; Takama et al. 1999). The importance of these fatty acids were corroborated by their presence the milk during lactation (Riedman and Ortiz 1979; Wheatley et al. 2008). Due to the limited nature of our data, we are unable to directly distinguish between circulating fatty acids destined for oxidation or incorporation into the pup blubber or adult female milk. However, our

current data will not only provide insight to the differential dynamics of fatty acids between mother and pup during these metabolically stressful seasons, but also possibly to other directly and indirectly related biochemical pathways, specifically with interactions involving catabolic protein, lipid, and glucose pathways. This study is the first to document circulating fatty acids in the NES during periods of physiological stress.

To begin investigating the dynamic role fatty acids play in the development of northern elephant seals, the preliminary detection of the fatty acid transporter proteins (FATPs) and fatty acid binding proteins (FABPs) is necessary. While there have been a few recent studies which have successfully detected Fatty Acid Translocase/Cluster of Differentiation 36 (FAT/CD36) in the adipose tissue of marine mammals, the critical role of FATPs and FABPs in skeletal muscle LCFA transport warrants further investigation to elucidate these proteins in the skeletal muscle of the NES (Castelli et al. 2014; Viscarra et al. 2012). The third chapter focuses on the immunodetection of fatty acid transporter proteins CD36, FATP1, and FATP4, and the fatty acid binding proteins FABP3 and FABP4 in NES adult males, adult females, and weaned pups. As the proteins involved in cytosolic and intercellular fatty acid transport, the detection of FABPs and FATPs is of considerable interest in an animal with high rates of lipolysis (Jeppesen et al. 2012; Stahl et al. 2001). Additionally, detection of these proteins is necessary to quantify their presence during the NES fast. The foundational results from this study will contribute to a greater understanding of the metabolic adaptations to the regulation of fatty acid utilization in a deep diving mammal, and can provide insight into the regulation of lipid-based insight into the regulation of lipid-based metabolic diseases in humans.

CHAPTER TWO

Assessing Lipid Mobilization Over The Lactation Period and Post-Wean Fast

Introduction

For adult female northern elephant seals, lactation is one of the most energetically expensive activities during reproduction . During lactation, females primarily rely on fatty acids derived from their subcutaneous blubber stores to supplement metabolic needs during a fast lasting several weeks (Wheatley et al. 2008). Unlike other lactating mammals, consistent incorporation of protein into mammary tissue during the fast requires the mobilization of amino acids from the mother's lean body protein (Champagne et al. 2012; Crocker et al. 2014). This is in contrast to other NES age classes, in which protein does not play a major role as an energy substrate (Adams and Costa 1993). Therefore, the energetic demands placed on adult females during simultaneous lactation and fasting are greater than during other life stages, underscoring the importance of energy conservation. One of the methods utilized by the NES and by other fasting mammals for energy conservation is a reduction in metabolic rate. A reduction in metabolic rate and the accompanying bradycardia has been observed in all age classes, both while diving and on the beach (Andrews et al. 1997). Hibernating mammals display a reduction in metabolic rate as well during hibernation. Studies evaluating the effect of essential fatty acid utilization during hibernation suggest specific fatty acids play a role in suppressing metabolism and extending hibernation duration. These studies indicated a diet rich in PUFA resulted in a lower body temperature and also increased the duration of unaroused hibernation. Conversely, diets rich in saturated fatty

acids resulted in a hibernation with higher body temperatures and more frequent arousals (Florant 1998). Ingestion of these metabolically beneficial fatty acids appears to be a priority for some hibernating species, who forage for PUFA-rich plants, and have been observed to extend their foraging range when these plants are scarce (Cunnane 1988; Florant 1998; Geiser et al. 1988). Further, mammalian hibernators were observed to be able to conserve LCFA PUFA on the middle position of triacylglyerols from white adipose tissue (Cunnane 1988). These studies suggest that the strategic conservation of specific fatty acid species may play an adaptational role in energy conservation and maintaining a low metabolic rate during periods of extending fasting.

From January to May, when the weaned pups are present on the beach, they experience a wide range of air temperatures ranging from 10 °C to 27 °C (McGinnis and Southworth 1971; Heath et al. 1977). Higher temperatures correspond with the end of the post-wean fast when weaned pups enter the water for their first foraging trip. Although the insulative properties of their thick blubber layer is thermogenically beneficial in cold ocean waters, blubber can exacerbate the hyperthermic challenges NES pups can face during the post-wean fast on the beach. While there is limited data on the thermoregulatory demands of adult females, post-weaned pups have been observed to have a higher thermal conductance in ambient air temperatures (20.9 °C) in comparison with cold water (3.8 °C) and warm water (14.5 °C) (Noren 2002). This illustrates their ability to regulate their temperature through the dynamic utilization of their countercurrent heat exchange, peripheral shunting and vasoconstriction, and the presence of thermal windows. While these are not unique to marine mammals, it does illustrate adaptational thermoregulatory capabilities are successfully utilized early in development.

While suckling, NES pups gain a substantial amount of weight, primarily due to the development of their blubber stores. However, blubber fatty acids are the primary energetic substrate for all age classes, therefore a delicate balance must be maintained: they must prevent hyperthermia and rely on fatty acid oxidation for fuel while retaining some their insulation for their upcoming foraging trip.

In addition to meeting their own energetic demands, females must also meet the needs of their nursing pup, which typically gain 100-200 kg over the four-week lactation period. Milk production is the most energetically expensive activity during the lactation fast, comprising 59% of their daily energy expenditure contrasted with dedicating 41% to their metabolism (Costa et al. 1986). While the lactation period is considerably shorter than other mammals (approximately 26 days), NES adult females produce some of the most fat-rich milk in the animal kingdom, which clarifies the need for a high energy expenditure, and could compensate for an abrupt weaning (McDonald and Crocker 2006).

Milk composition over the course of the fast is characterized by a substantial increase in unsaturated fatty acids, a concurrent decrease in water and stable protein content (Riedman and Ortiz, 1979). The decrease in water content underscores the need for water conservation in the female over the fast (Eisert et al. 2013). At its peak, the milk fat content can be well over 50% (Riedman and Ortiz 1979). Hooded seals, which have the shortest lactation period of any mammal, maintain a consistent percentage of protein, fat, and water content throughout lactation (Oftedal et al. 1988). Milk composition dynamics vary with species. While there has been debate surrounding the similarity of the maternal blubber, milk, and pup blubber fatty acid composition, these similarities vary

between species (Champagne et al. 2012; Grahl-Nielsen et al. 2000; Iverson et al. 1995). The NES and Weddell seals display a similar change in milk composition over the course of lactation, with an increasing amount of fat content and decrease in water content, in addition to a similar change in blubber fatty acid composition in pups (Eisert et al. 2013; Noren et al. 2013; Riedman and Ortiz 1979; Wheatley et al. 2008). The debate surrounding the source of maternal transfer is derived from the ambiguity in the current literature, despite our knowledge of pup chylomicra and blubber fatty acid composition. Until the blubber composition of NES adult females is elucidated or lactating females are supplemented with radiolabeled fatty acids, the primary source of these fatty acids, whether from the mammary glands, liberated from the adipose tissue, or a combination, will remain elusive and warrants further investigation (Riedman and Ortiz 1979).

The objective of this study was to determine the difference in lipid metabolic constituents during early and late fasting periods in weaned NES pups as well as early and late lactation periods in adult females, and hypothesize the dynamics of any detectable lipid-based biochemical pathways. This study will assist in investigating the final destination of fatty acids (i.e. oxidation, storage, maternal transfer) in a metabolically active mammal that relies on a lipid-based diet. NES lactating females and weaned pups are simultaneously experiencing similar yet distinctive physiological challenges during the lactation period. Specifically for the females, the primary metabolic challenges are due to concurrent energetic demands of lactation and fasting. Therefore, circulating fatty acids will be representative of the fatty acids present in the milk and the dynamic energetic needs of the female. For the weaned pups, the circulating fatty acids will be reflective of those ingested during nursing, as well as those stored and mobilized

to assist in facing thermoregulatory and developmental challenges. Although the circulating fatty acids will be variable throughout the fast, we hypothesize that there will be a consistent abundance of sentinel species such as linoleate, linolenate, and palmitate. Currently, the fatty acid composition of NES adult females is unknown, which complicates the maternal transfer debate in the NES. However, this study is the first to document circulating fatty acids in the NES and will provide valuable insight to our current knowledge of their lipid-based metabolism. Through measuring the difference in circulating fatty acids at the beginning and end of the fast for pups and lactating females, we are further able to understand the extent of utilization of specific fatty acid species and groups of fatty acids based on their degree of saturation.

Materials and Methods

Sample Site and Subjects

Samples for this study were collected from 10 adult lactating females and 10 weaned pups (4 female, 6 male) at the Año Nuevo state reserve in San Mateo County, CA (37.1° N, 122.3° W). Measurements for lactating females were taken during the early and late fasting period during the breeding season (5 and 22 days post-partum in January and March, respectively). Measurements were made on pups 16 (SD = 2) and 46 (SD = 1) days into the post-weaning fast. Adult females sampled in this study were unrelated to the pups (Champagne et al. 2013).

Sample Collection

Selected animals were immobilized with an induction dose of Telazol (1mg kg^{-1}) and given maintenance doses of diazepam and ketamine as needed (Fort Dodge Laboratories, Ft. Dodge, IA, USA). Administration was performed as previously described (Boaz et al. 2012). Fifteen minutes after the Telazol injection, blood samples were collected via the extradural vein using a 8.9 cm X 18g spinal needle. Metabolite assay blood samples were collected in chilled EDTA-containing vacutainers. Blood samples were placed on ice, transported to the lab, and centrifuged at 4 °C and stored at -80 °C until further analysis (Champagne et al. 2013).

Metabolite Analysis

Blood sample constituents were evaluated through metabolomics analysis performed by Metabolon Inc (Durham, NC, USA). Plasma extracts were analyzed through a dual-platform of gas and liquid chromatography, in addition to tandem mass spectrometry. The LC-MS utilized a Waters Acquity UPLC and a Thermo-Finnigan LTQ mass spectrometer (Champagne et al. 2013). The GC column was composed of 5% phenyl at 40° ramped to 300°, in conjunction with a Thermo-Finnigin Trace DSQ fast-scanning single quadrupole mass spectrometer. Identification of compounds was dependent upon the mass to charge ratio (m/z) and tandem mass spectral data by a comparison to a library containing ~1500 purified standards (Lawton et al. 2008). Collectively, 227 compounds were identified in the NES seal plasma samples through the Metabolon platform. Compound abundance was calculated as the area underneath its MS peak.

For this study, a total of 11 fatty acids were chosen based on their abundance in two of the primary prey species of the NES, pacific hake (*Merluccius productus*) and the boreal clubhook squid (*Onchoyeuthis borealijaponica*) (Condit and Le Boeuf 1984; Huynh and Kitts 2009; Takama et al. 1999). Specifically, three saturated fatty acids (SFAs; myristate, 14:0; palmitate, 16:0; and stearate, 18:0), three monounsaturated fatty acis (MUFAs; cis-vaccenate, 18:1n7; oleate, 18:1n9; palmitoleate, 16:1n7), and five polyunsaturated fatty acids (PUFA; arachidonate, 20:4n6; docosahexaenoate/DHA, 22:6n3; eicosapentaenoate/EPA, 20:5n3; linolenate, 18:3n3 or 18:3n6; and linoleate, 18:2n6) were selected. The abundance of these fatty acids during lactation was also taken into consideration during the selection process (Eisert et al. 2013; Grahl-Nielsen et al. 2000; Riedman and Ortiz 1979). These fatty acids represent the majority of marine-based fatty acids found in prey and adult female milk (milk: 93% of SFAs, 79% of MUFAs, and 62% of PUFAs; prey: 96% of SFAs, 76% of MUFAs, 91% of PUFAs) (Condit and Le Boeuf 1984; Huynh and Kitts 2009; Riedman and Ortiz 1979; Takama et al. 1999; Wheatley et al. 2008). In addition, the non-essential PUFA derivatives chosen are metabolically important for development (Calder et al. 1996). We also limited the additional compounds evaluated to those most related to lipid metabolism to provide context for the remainder of the study. Selection of compounds was performed prior to statistical analysis.

Statistical Analysis

Fatty acid abundance was evaluated by assessing the change in abundance in circulating stores across the duration of the fast (comparing the early lactation fast to late lactation fast, and comparing the early post-wean fast to the late post-wean fast). The

abundance was compared within each age class by transforming their relative abundance to an early to late fasting ratio for both groups. Paired t-tests were used to determine the significant difference between the early and late relative abundances within each group over the time course of the fast. In addition, significant differences between groups at the same period of the fast (early lactation vs. early post-wean fast and late lactation vs. late post-wean fast) were calculated using Welch's T-test assuming unequal variances. Analyses were performed using the software JMP version 10 (SAS institute, Cary NC) and Microsoft Excel (Office 2010). Statistical significance was determined at $p < 0.05$.

Results

Saturated Fatty Acids

For the SFAs evaluated, weaned pups displayed a significant increase in utilization of stearate (18:0) ($t = -4.02$, $p < 0.005$), while maintaining their utilization of both palmitate and myristate throughout the course of the fast (Figure 1). While females similarly maintained circulating palmitate throughout lactation, there was a significant decrease in utilization of myristate (14:0) ($t = 3.07$) and stearate ($t = 2.63$) (18:0) ($p < 0.05$).

Monounsaturated Fatty Acids

Oleate (18:1n9), a derivative of stearate (18:0), is the most prevalent MUFA found in NES prey. Consequently, it is also the MUFA with the highest abundance in milk during lactation and thus circulating during suckling (Puppione et al. 1996). Our data corroborates these earlier findings with a significant increase in utilization of oleate

for pups ($t = -6.73$, $p < 0.0001$) and a concurrent significant decrease for females ($t = 2.52$, $p < 0.05$), which may be due to maternal transfer or endogenous synthesis (Figure 2). Palmitoleate (16:1n7), a derivative of palmitate (16:0), is another considerably prevalent MUFA found circulating in plasma and in the milk of lactating adult females of numerous seal species (Eisert et al 2013; Riedman et al. 1979; Wheatley et al. 2008). Although the females displayed no difference in palmitoleate throughout lactation, pups significantly increased their utilization of palmitoleate ($t = -3.68$, $p < 0.05$) (Figure 2).

Polyunsaturated Fatty Acids

PUFAs were the most dynamic fatty acid class in between age groups during fasting periods. As expected, linoleate (18:2n6) ($t = 2.36$, $p < 0.05$) and linolenate (18:3n3) ($t = 2.19$, $p < 0.05$), two essential fatty acids, were both significantly increased in abundance in adult females over the course of lactation (Figure 3). Adult females also revealed a significant increase in abundance of DHA from early to late lactation ($t = 3.19$, $p < 0.05$). Overall, the observed trend for adult females was an increase of the presence of circulating PUFAs over the course of the lactating fast, as indicated by the lower early to late lactation sample ratios. For post-weaned fasting pups, there was a similar trend, with a significant increase in the abundance of circulating PUFAs over the course of the fast ($p < 0.05$), with the exception of linolenate, which was significantly decreased in abundance during the post-wean fast ($t = -6.02$, $p < 0.05$) (Figure 3).

Comparison Between Age Groups

For the comparison of the difference in the early to late fasting ratio between the adult females and the post-wean fasting pups, we utilized Welch's t-tests assuming unequal variances, and Wilcoxon rank tests when normality came into question. For the vast majority of fatty acid species evaluated, there was no significant difference between early to late fasting ratios between the adult females and pups (Figures 4, 5, 6). However, the one exception was oleate, which adult females had a significantly higher early to late fasting ratio than weaned pups (Figure 5C), which also corroborates our earlier observation of the significantly decreased utilization of oleate in adult females in contrast with weaned pups (Figure 2).

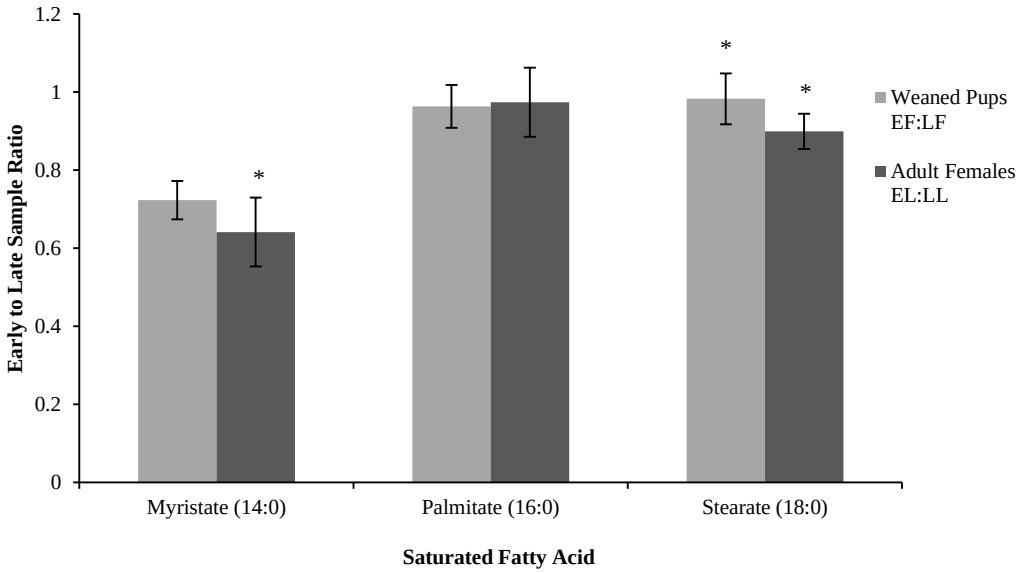

Figure 1. Comparison of saturated fatty acid utilization within age class (early lactation *vs.* late lactation, and early post-wean fast *vs*. late post-wean fast) at early and late fasting time points for the NES. EF = early post-wean fast, LF = late post-wean fast, EL = early lactation fast, LL = late lactation fast. Asterisks indicate a p-value < 0.05.

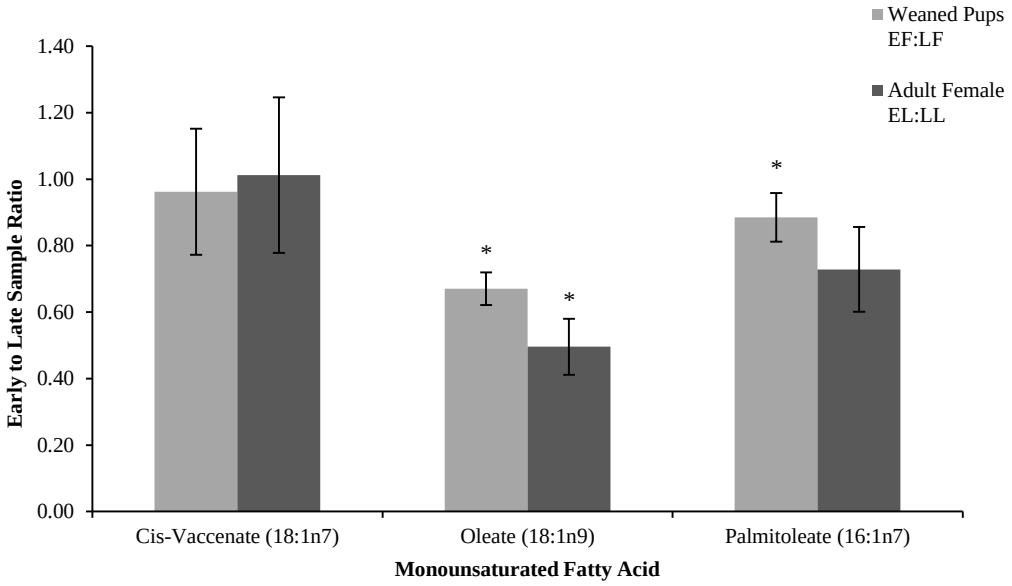

Figure 2. Comparison of monounsaturated fatty acid utilization within age class (early lactation *vs.* late lactation, and early post-wean fast *vs*. late post-wean fast) at early and late fasting time points for the NES. EF = early post-wean fast, LF = late post-wean fast, EL = early lactation fast, LL = late lactation fast. Asterisks indicate a p-value < 0.05.

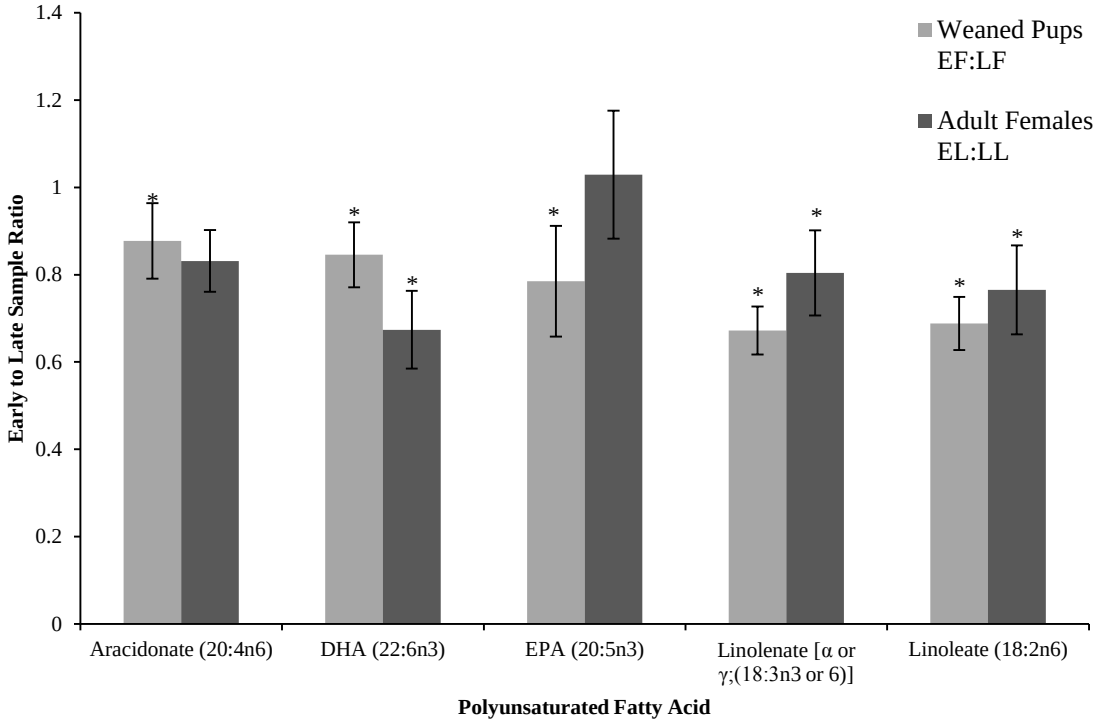

Figure 3. Comparison of polyunsaturated fatty acid utilization within age class (early lactation *vs.* late lactation, and early post-wean fast *vs*. late post-wean fast) at early and late fasting time points for the NES. EF = early post-wean fast, LF = late post-wean fast, EL = early lactation fast, LL = late lactation fast, DHA = docosahexaenoate, EPA = eicosapentaenoate. Asterisks indicate a p-value < 0.05.

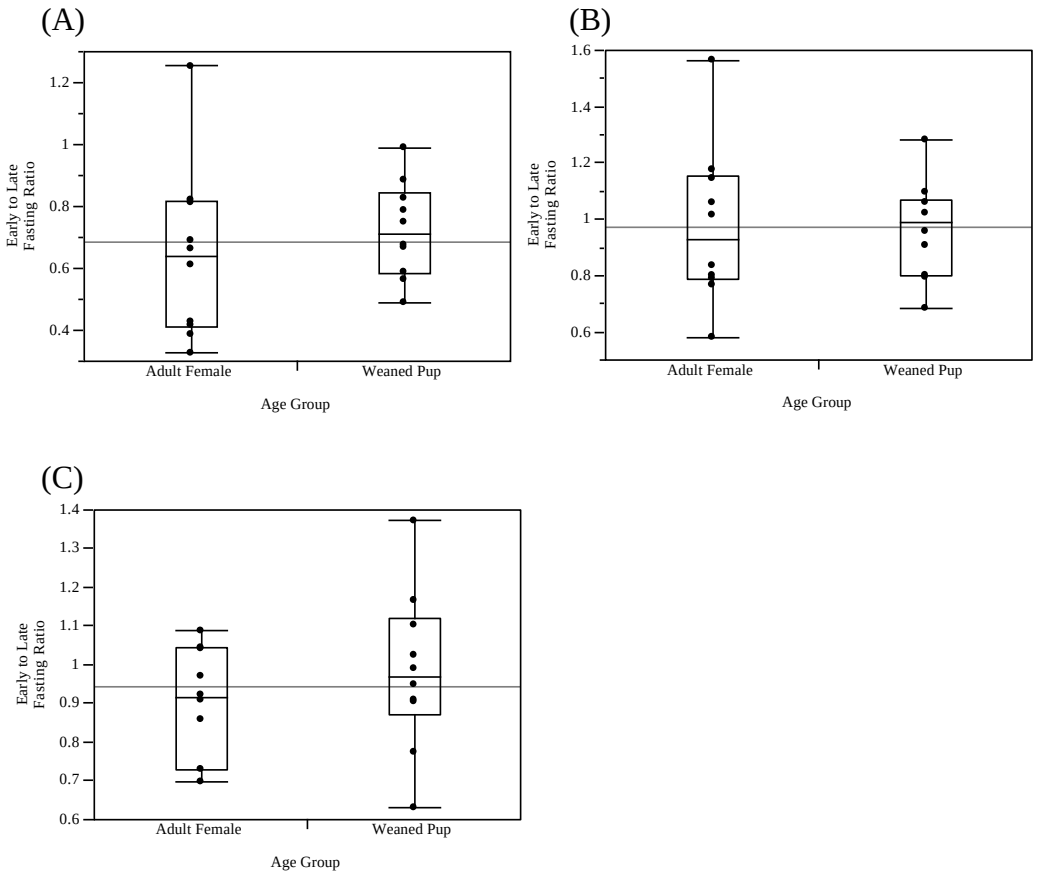

Figure 4. Comparison of the utilization of saturated fatty acid species between the two age groups (adult female EL:LL *vs.* weaned pups EF:LF). (A) Myristate (14:0) (B) Palmitate (16:0) (C) Stearate (18:0). EF = early post-wean fast, LF = late post-wean fast, EL = early lactation fast, LL = late lactation fast. Horizontal line representative of grand mean.

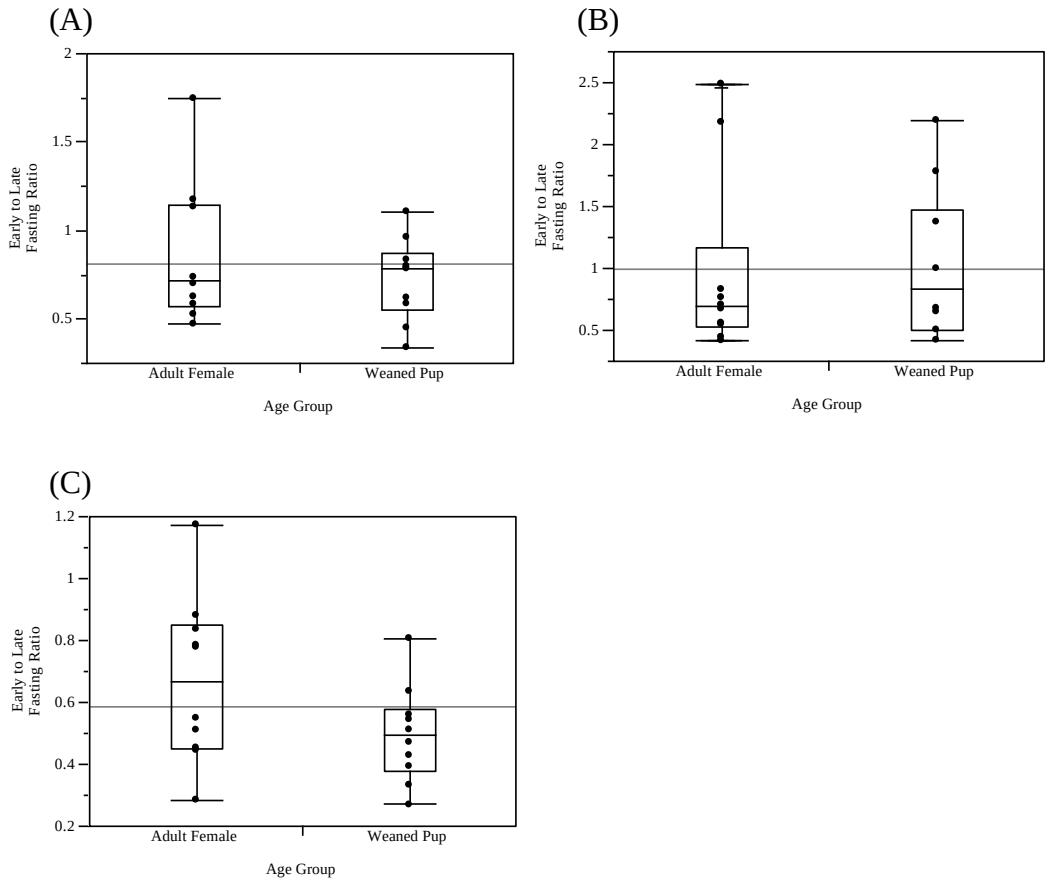

Figure 5. Comparison of the utilization of monosaturated fatty acid species between the two age groups (adult female EL:LL *vs.* weaned pups EF:LF). (A) Palmitoleate (16:1n7) (B) Cis-Vaccenate (18:1n7) (C) Oleate (18:1n9)*. EF = early post-wean fast, LF = late post-wean fast, EL = early lactation fast, LL = late lactation fast. Horizontal line representative of grand mean. Asterisk indicative of significant difference between the adult female and weaned pup groups.

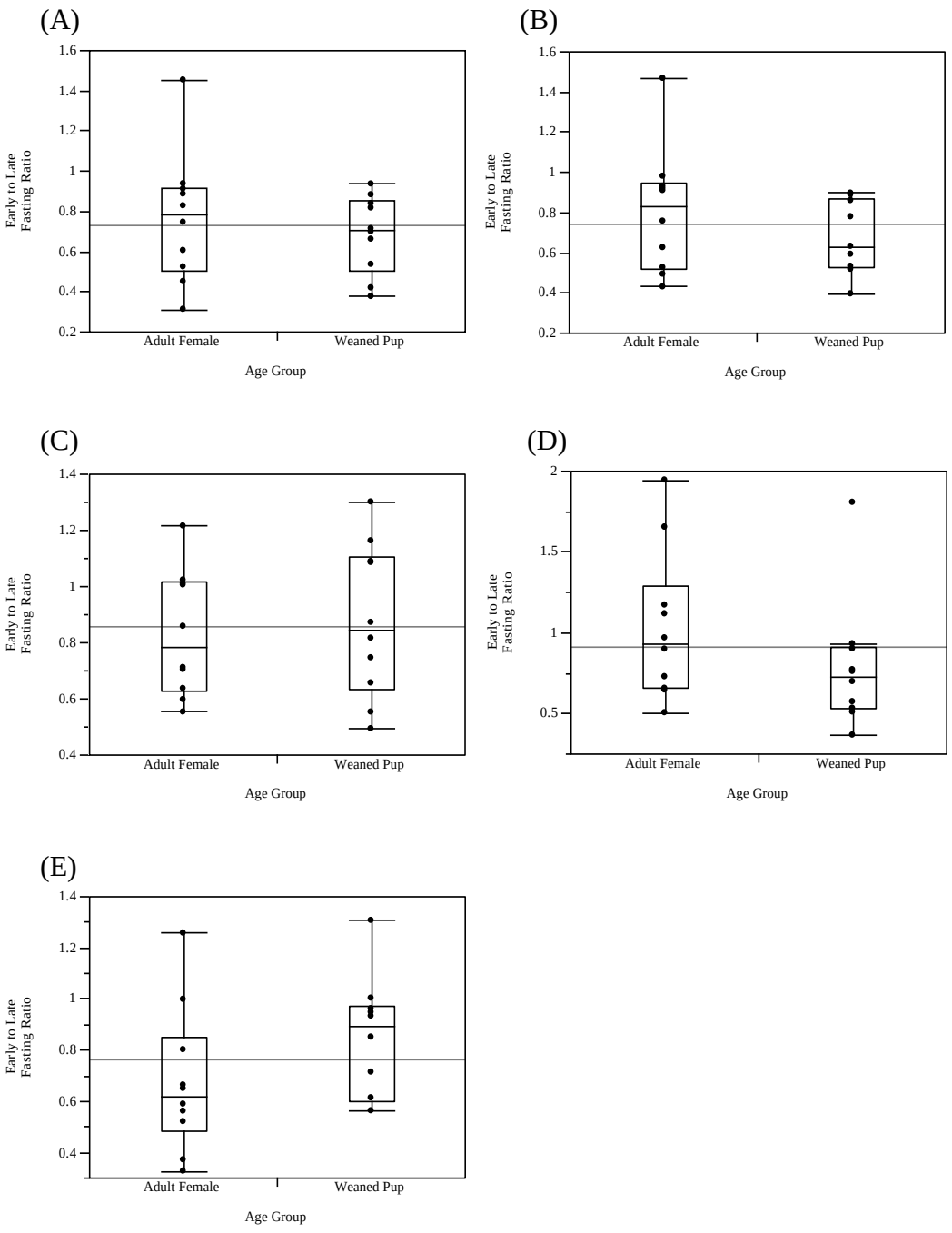

Figure 6. Comparison of the utilization of polyunsaturated fatty acid species between the two age groups (adult female EL:LL *vs.* weaned pups EF:LF). (A) Linoleate (18:2n6) (B) Linolenate, α or γ (18:3n3 or 18:3n6) (C) Aracidonate (20:4n6) (D) EPA (20:5n3) (E) DHA (22:6n3). EF = early post-wean fast, LF = late post-wean fast, EL = early lactation fast, LL = late lactation fast, DHA = docosahexaenoate, EPA = eicosapentaenoate. Horizontal line representative of grand mean.

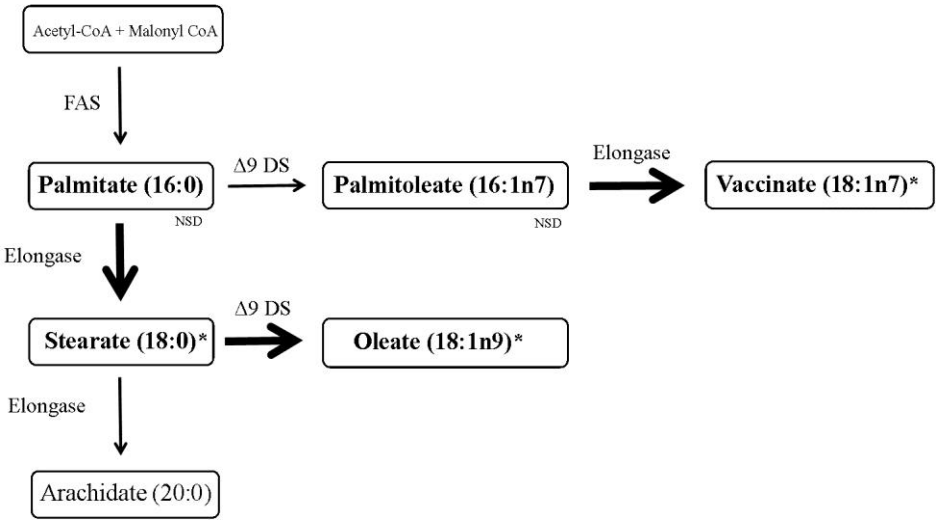

Figure 7. General endogenous saturated and monounsaturated fatty acid synthesis and transformation pathway in adult female NES during lactation. Bolded fatty acids are those measured in this study. Increases in circulating fatty acid appearance over lactation indicated by bolded arrows. Significant difference indicated by asterisk. DS = desaturase, NSD = no significant difference.

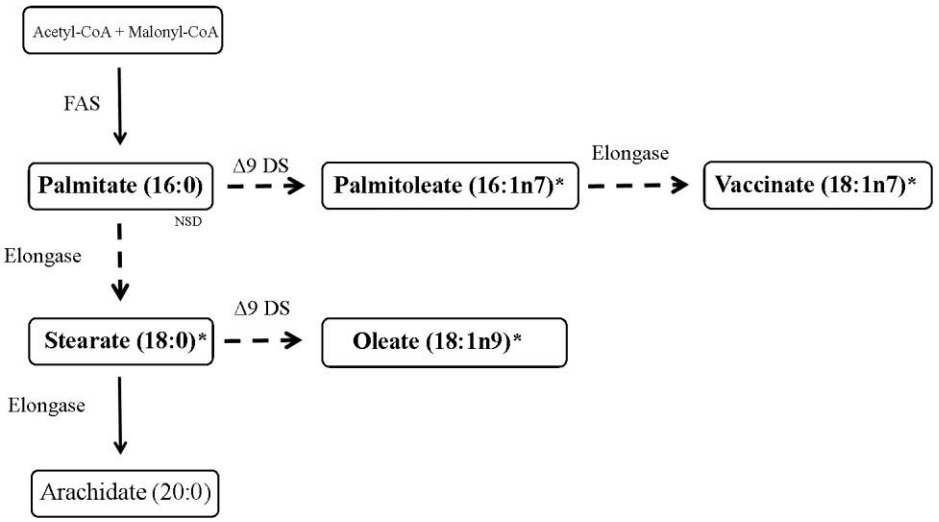

Figure 8. General endogenous saturated and monounsaturated fatty acid synthesis and transformation pathway in post-wean fasted NES pups. Bolded fatty acids are those measured in this study. Decrease in circulating fatty acid appearance over the fast indicated by dashed arrows. Significant difference indicated by asterisk. DS = desaturase, NSD = no significant difference.

Discussion

Fatty Acid Utilization

Saturated fatty acids, more recognized for their negative effects on obesity and insulin sensitivity than their resistance to reactive oxygen species damage (ROS), comprise a large percentage of prey and the second-most prevalent fatty acid in milk (Riedman and Ortiz 1979; Takama et al.1999). Interestingly, pups only displayed an increase in utilization of only one SFA, stearate, over the course of the fast (Figure 1). However, this does not suggest that the other two SFAs were not substantially utilized over the course of the fast, as the other two SFAs, particularly palmitate, are important precursors and can be endogenously synthesized. Rather, this illustrates the ability of both pups and adult females to maintain circulating levels of palmitate while fasting. While we are unable to determine the final fate of stearate, oxidation of stearate is corroborated by the decreased prevalence in the intramuscular lipids (IML) and intramuscular triglycerides (IMTG) in comparable species (Trumble et al. 2010). The utilization of SFA for fuel is supported by data indicating SFAs comprise a small percentage of pup IML and IMTG compared to total unsaturated fatty acids (Calder 1996; Trumble et al. 2010). From the decrease in appearance of circulating fatty acids in the pups, we can infer an increase in utilization (Figure 8). However, due to the limited nature of this study, we cannot ascertain whether these fatty acids were used for oxidation or developmental purposes. Conversely, lactating females which are experiencing the simultaneous stressors of fasting and lactation, displayed an increase in appearance of all SFAs, and significantly so in 14:0 and 18:0 (Figure 1). This is in accordance with the current knowledge of comparable species, which SFAs and MUFAs comprise a

significantly greater percentage of membrane fatty acids than PUFAs. Additionally, intermembranous storage of SFAs would be beneficial for adult females because SFAs have a higher resistance to damage by ROS (Trumble and Kanatous 2012). These benefits in combination with those of utilizing PUFAs, present an interesting hypothesis by which adult females may mitigate some of the negative physiological effects of simultaneous lactation and fasting. An increase in appearance of circulating fatty acids in the adult females is indicative of increased production and transformation or a decrease in utilization (Figure 7). Given the energetically expensive nature of lactation, the increase in appearance is most likely due to an increase in production and transformation of these fatty acids, as there was no significant difference in circulating palmitate during lactation. However similar to the pups, we are unable to confirm the final destination of these fatty acids, whether incorporated into milk or utilized for energy. Despite the differences in fatty acid dynamics over the course of the fast within each age group, there was no significant difference in utilization of SFA between lactating adult females and pups (Figure 4A-C).

Monounsaturated fatty acids play a considerable role in the fuel stores of pinnipeds, with MUFAs comprising the vast majority of adipose stores in the NES and Weddell seals, in addition to the intramusclular lipid stores (IML) of the latter species (Trumble et al. 2010; Wheatley et al. 2008). Although the females displayed no difference in palmitoleate throughout the fast, there was a significant decrease in palmitoleate appearance in pups (Figure 2, Figure 8). Previous studies in mammals found this fatty acid substantially increases the activity of carnitine palmitoyltransferase I (CPT I), which affects the rate of β-oxidation, compared to other fatty acids, however

palmitoleate composition in pups and females differ markedly between seal species, with palmitoleate being more prevalent in the Antarctic-based Weddell seal and less so in the northern elephant seal (Hulbert et al. 2007; Power and Newsholme 1997; Wheatley et al. 2008). The difference in mass-specific thermoregulatory challenges, the environment, and prey species of these two seal species may provide insight into the difference in the usage of this fatty acid as well. Overall, there was not a substantial difference in utilization of MUFAs by lactating females in comparison to pups (Figure 5A, B), however there was a significant difference in oleate utilization between age groups, with females significantly decreasing their utilization and pups experiencing significant increase (Figures 3 and 5C). There has been considerable debate about the role of an oleate laden diet may have on hepatic lipoprotein dynamics and the effect of this fatty acid on β-oxidation in skeletal muscle in mice. (Coll et al. 2008; Reaven et al. 1993; Reaven et al. 1991). Most of these studies have been performed *in vivo* or in cultured cells of terrestrial mammals, like *M. musculus*, therefore it is unknown how this may affect a fasting mammal with a fatty acid based metabolism.

While PUFAs comprise the smallest percentage of fatty acid species in NES and Weddell seal blubber and Weddell IML, where they comprise the majority of fatty acids in the cell membrane of Weddell seal pups. Incorporation of PUFAs into cellular membranes has shown to be more thermogenically beneficial for developing Weddell seal pups, however whether these pups are incorporating these fatty acids, and the degree of their unsaturation, warrants further investigation (Trumble and Kanatous 2012). PUFAs play an important role in regulation of immunity and inflammation and cellular structure (Calder 1996; 2001). Eicosapentanoic acid (EPA) and docosahexanoic acid

(DHA), both α-linolenic derivatives, were increasingly prevalent in circulating fatty acids of adult females over the course of the fast (Figure 3). The anti-inflammatory properties of this fatty acid may be important in mitigating the metabolically stressful effects of fasting and lactation. Consistent incorporation of these developmentally important fatty acids into milk over the course of lactation is also another explanation for elevated EPA and DHA levels. This is consistent with the measurements of milk DHA and EPA concentrations in both the NES and Weddell seal (Eisert et al. 2013; Puppione et al. 1996; Riedman and Ortiz 1979; Wheatley et al. 2008). Another anticipated result, pups exhibited a significant increase in prevalence of these two essential fatty acids (Figure 3). While this may be indicative of maternal transfer, the ability of pups to endogenously transform these fatty acids confounds any definite conclusions regarding maternal transfer. Synthesis of DHA and EPA by weaned pups over the course of the fast is supported by our observation in an increase in utilization of the essential FA linolenate throughout the fast (Figure 3). A recent study elucidating the blubber fatty acids in NES pups over the post wean fast observed considerable variability between sampling season, however in both sampling seasons the EPA percentage within the blubber significantly decreased (Noren et al. 2013). Decreasing concentrations of this fatty acid within the blubber may reflect its increasing circulation during the fast observed in this study. PUFA, specifically DHA and EPA, play a critical role in brain and sensory development in humans and other mammals, and it would be advantageous for lactating females to incorporate both essential PUFA and their developmentally important derivatives. Due to the essential nature of linolenate, it is logical that there is a limit on the synthesis of its derivatives in a fasting organism. Therefore, we were unable to generate fatty acid

pathways based on our data because we are not able to isolate the contribution of EPA, DHA, and their precursors from the mothers of the fasting pups.

Metabolomics analysis of these fatty acids in both the age groups revealed a few of the main biological process we would expect to see in a fasting mammal: fatty acid oxidation, carbohydrate metabolism, formation of lipid droplets, and lipid transport. It is important to note that one of the limitations of this study it the ability to monitor only circulating fatty acids and the destination of these circulating fatty acids, whether for oxidation, storage, or incorporation into cellular structure, is unknown. However, for a simultaneously growing and fasting pup, it would be reproductively advantageous for adult females to incorporate the developmentally important fatty acid derivatives, like DHA and EPA, in addition to their essential precursors. Additionally, while the adult females and pups sampled in this study were unrelated, we are able to draw parallels from the increase in appearance of specific fatty acids (i.e. stearate, oleate, and vaccinate) in the adult female with corresponding decreases in appearance over the course of the post-wean fast in pups. Based on these observations, we can infer that these specific fatty acids do play an important developmental, reproductive, and energetic role in fasting pups and adult females. Future studies employing metabolomics analysis should be performed in conjunction with blubber and muscle sample analysis to provide a more complete picture of how these fatty acids are being utilized.

CHAPTER THREE

Qualification of Fatty Acid Transporter and Binding Proteins in the Northern Elephant Seal

Introduction

There is an abundance of evidence supporting the role lipids play in regulating metabolism and maintaining cell structure in mammals (Corcoran 2007; Hulbert et al. 2007; Power et al. 1997). In deep diving phocids, which primarily have a lipid-based metabolism, recent studies suggest a more intrinsically vital role of lipids in these diving mammals, from oxygen conservation to the utilization of specific fatty acids based on life stage (Trumble et al. 2010; Trumble and Kanatous 2012). While we are aware how fatty acids can play a developmental role in marine mammals, it is less lucid how this is mediated. Fatty acid transport proteins (FATPs) and fatty acid binding proteins (FABPs) are proteins involved in intercellular and cytosolic transport of fatty acids. There are currently 9 membrane transport proteins elucidated, with six of those entitled FATP1 – 6, and the remaining three being fatty acid translocase/CD36, caveolin-1, and plasma membrane fatty acid binding protein (FABPpm) (Glatz et al. 2010). FATPs range in size from 70 – 80 kDa and specificity varies with cell and tissue type. Studies evaluating fatty acid transport proteins (FATPs) in murine models indicate that these proteins are tissue specific and can be used to determine the fatty acid oxidation capacity of different tissues. FATPs play a large role in the development of obesity and other obesity-related metabolic disorders, however insulin sensitivity of FATPs is highly debated, and varies with animal and tissue type (Glatz et al. 2010; Stefanyk et al. 2012). The FABPs are a

family of at least 13 ~15 kDa proteins responsible for fatty acid transport within the cell. FABPs are expressed in multiple tissues and have been correlated to the lipid metabolizing capacity of the tissue (Hagberg 2013; Makowski 2004). While various FATPs and FABPs are present in multiple tissues, CD36, FATP 1, 4, and 6 have been primarily associated with the skeletal muscle and FATP 1 and 4 are major transport proteins in adipose tissue (Glatz et al. 2010). FABP3 has been found in mammalian muscle and heart tissue, while FABP4 and FABP5 have been associated with adipose tissue and epidermal cells, respectively (Hulbert et al. 2007; Makowski and Hotamisligil 2004; Smathers and Petersen 2011; Viscarra et al. 2012).

There has been little work done investigating the presence of FATP and FABP in marine mammals, and it is of interest to evaluate the presence and role of FATP and FABP in the northern elephant seal, an insulin resistant animal equipped to mitigate the negative effects of a lipid based diet (Viscarra et al. 2011; Viscarra et al. 2012). While fatty acid binding and transporter proteins are evolutionarily conserved across different mammalian species, accurate qualification of tissue specific proteins are necessary to investigate the role of various physiological factors, such as development, hypoxic condition and fasting state (Hagberg et al. 2013). During this study, we will investigate the activity of binding proteins and transporter proteins expressed in the two main tissues associated with fatty acid metabolism: adipose tissue and skeletal muscle. In this study, we are evaluating the presence of CD36, FATP1, FATP4, FABP4 and FABP3 in all age classes of the NES. I hypothesize that we will be able to find all of these FATPs and FABPs in these age classes, primarily due to the evolutionarily conserved nature of these proteins and the reliance of the NES on a fatty acid based metabolism. Due to the

simultaneous challenges of lactation and fasting, we expect lactating females to display the highest prevalence of all FATPs and FABPs. Secondly, we also expect weaned pups to display an intermediately high expression of FATP and FABPs because of the physiological challenges of fasting and development. Lastly, adult males will exhibit the least expression of FATP/FABPs because of the absence of the combinatory effects experienced by the other age classes, and adult males exhibiting the primary challenge of fasting. Although the majority of these proteins have yet to be detected in a marine mammal, the evolutionarily conserved nature of the proteins supports the hypothesis that these transporter and binding proteins will be present in all age classes. However, the lifestyles and physiological challenges and needs of each age class are considerably different, and the upregulation or down regulation of fatty acid transporter and binding proteins may reflect these differences. This study will provide insight into the unique dynamics of utilization, storage, and transportation of fatty acids during consistent metabolic stress in a naturally obese mammal.

Materials and Methods

Site Selection and Sample Collection

Adult male samples for this portion of the study were collected in July 2013 in the Año Nuevo state reserve in San Mateo County, CA. Of the individuals sampled, four adult males were chosen for this study due to their similar adult status, state of molting, and sampling site. All males were sampled from North Point and ranged in molting status from 50-65%. Post-weaned pups and lactating adult females were collected at the same sample site in February 2014. Of the pups and females sampled, four post-weaned pups

(2 male and 2 female), and one adult female was selected for this study. Selected animals were immobilized with an induction dose of Telazol and given maintenance doses of diazepam and ketamine as needed (Fort Dodge Laboratories, Ft. Dodge, IA, USA). Administration was performed as previously described (Boaz et al. 2012). Fifteen minutes after the Telazol injection, the biopsy site was sterilized with iodine and a 1 mL subcutaneous injection of lidocaine was administered. Five minutes after the injection, the sample site was punctured with a scalpel and muscle biopsies were taken from the longissimus dorsi muscle with a skeletal muscle biopsy needle (4.5mm, Bignell Surgical Instruments, Ltd., Essex, UK). Immediately after collection, samples were placed in liquid nitrogen and later stored in – 80 °C until further analysis. After the biopsy procedure was completed, animals were given a topical antibiotic and monitored until fully awaken from the anesthesia. Mouse (*Mus musculus*) muscle samples were obtained to utilize as protein standards (The Jackson Laboratory, Bar Harbor, ME).

Western Blotting

Samples were homogenized in CelLytic MT Cell lysis buffer with a protease inhibitor cocktail (PIC, Sigma). Homogenates were centrifuged at 14,000 rpm for 10 minutes at 4 °C and the aqueous supernatants were transferred. Extracted total protein concentration was determined through the Bradford Assay (Bio-Rad Laboratories, Hercules, CA, USA) and was utilized to normalize the quantity of protein loaded into gel wells for electrophoretic analysis. Fifty micrograms of total protein were resolved in 4-20% SDS TEO-HCl gradient gels (CBS Scientific, Del Mar, CA). Proteins were electroblotted using the Bio-Rad Mini Protean Transfer apparatus onto 0.20 μm nitrocellulose membrane. Membranes were blocked overnight at 4 °C using 1% casein

protein blocking solution (G-Biosciences, St. Louis, MO). Blocked membranes were incubated overnight at 4 °C with primary antibodies against CD36, ACSVL5 (FATP1), ACSVL4 (FATP4), FABP3, and FABP4 with dilutions ranging from 1:400 – 1:1000 (Thermoscientific, Waltham, MA; Santa Cruz Biotechnologies, Santa Cruz, CA; GeneTex, Irvine, CA). Membranes were washed and incubated in an HRP-conjugated secondary antibody, followed by washing with a 0.001% Tween-20 TBS solution, and developed utilizing the LumiGLO Reserve Chemiluminescent Substrate kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Membranes were visualized using a GE ImageQuant LAS 4000 digital imaging system (GE Healthcare, Pittsburg, PA) and qualified using ImageJ Software (U. S. National Institutes of Health, Bethesda, Maryland, USA).

Results

Adult Male Group

Previous studies have established the presence of FATP1 and CD36 in post-weaned NES pups, but their expression and their quantification has yet to be elucidated in other age classes (Crocker et al. 2014; Viscarra et al. 2012). CD36 was consistently expressed at ~75 kDa in all individuals, as well as FATP1 at ~65 kDa. Although FATP4 is well documented as an insulin-independent transporter in the skeletal muscle of terrestrial mammals, to our knowledge this is the first recorded expression of FATP4 in a marine mammal (Glatz et al. 2010; Jeppesen et al. 2012; Stahl et al. 2001; Wu et al. 2006). FATP4 was clearly observed across all individuals at ~72 kDa. The detection of

binding proteins FABP3 and FABP4, while faint, could not be distinctly distinguished from other potential conflicting sources of binding.

Pup and Adult Female Groups

Similar to the adult male group, CD36 was readily detected at ~75 kDa for all pups sampled and the adult female (Figure 8). The expression of CD36 over the course of the post-wean fast has been evaluated in NES pups, but it has not been documented in NES adult females until this study. FATP4 was also ubiquitously expressed across pups and the adult female, corroborating its importance in insulin-independent fatty acid transport in skeletal muscle during fasting conditions (Figure 8). To our knowledge, this is the first documented expression of FATP4 in post-wean pups and adult female NES. As seen in adult males, FATP1 was consistently observed across pups and the adult female. Lastly, although FABP3 activity was unobservable in adult males, it was consistently observed in both post-weaned pups and adult females at ~15 kDa (Figure 8). As a major binding protein in cardiac and skeletal muscle, the expression of FABP3 in the fasting NES is logical. However, similar to adult males, FABP4 was indistinguishable in fasting pups and adult females and therefore is not included in our results.

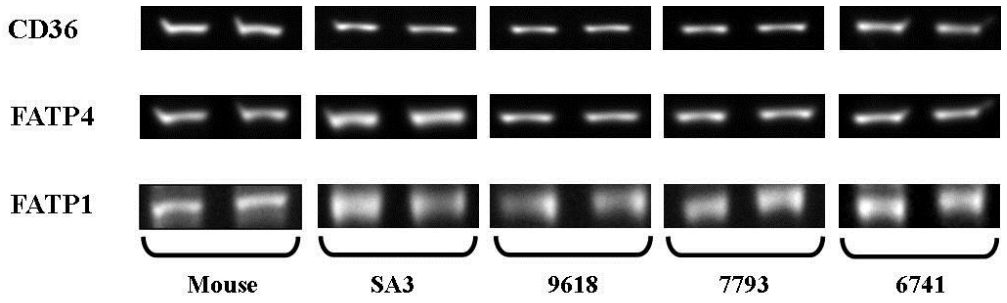

Figure 7. Representative Western blots of fatty acid transporter proteins CD36, FATP4 (SLC27A4), and FATP1 (ACSVL5) in *M. musculus* and four adult male northern elephant seals.

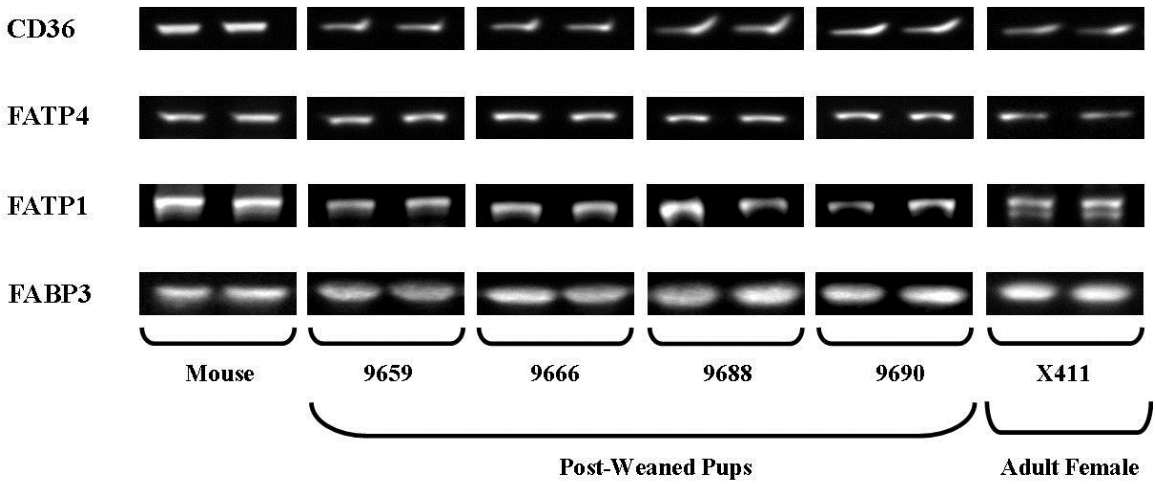

Figure 8. Representative Western blots of fatty acid transporter proteins CD36, FATP4 (SLC27A4), FATP1 (ACSLV5), and FABP3 in *M. musculus* and four post-weaned pups and one adult female.

Discussion

FATPs and FABPs are an integral part of fatty acid delivery to and within the cell, and therefore are of particular importance to animals depending primarily on fatty acid oxidation for fuel. This is the most comprehensive investigation of FATPs and FABPs in a marine mammal. We were able to successfully identify FAT/CD36, FATP1, and FATP4 in the skeletal muscle of all age classes, and also identified FABP3 in adult females and post-weaned pups. While FABP3 was unable to be identified in adult males, further confirmation of its absence is warranted. FABP3 plays an important cytosolic role in transportation of fatty acids in skeletal muscle, and its absence in active adult males would suggest another FABP might supplement in the absence of FABP3. FABP4 was also unable to be positively identified in any age class, however due to the documented primary role of FABP4 within adipocytes, our observation of its absence in skeletal muscle is not surprising (Makowski and Hotamisligil 2004; Smathers and Petersen 2011). Previous investigations positively confirming the presence of transporter proteins (FATP1 and CD36) in marine mammals are localized to NES adipose tissue, but utilization of fatty acids as a critical substrate for β-oxidation in skeletal muscle throughout fasting in the NES, thereby underscoring the importance of identifying these proteins in other tissues (Viscarra et al. 2012).

FAT/CD36

Among the currently known FATPs, CD36 is the most prominent and best characterized transporter protein (Glatz et al. 2010; Stahl et al. 2001). It has been characterized in cardiac muscle, skeletal muscle, white and brown adipose tissue, and

within numerous other tissues where it acts as a saturable membrane transporter of LCFA (Glatz et al. 2010; Jeukendrup 2002; Stahl et al. 2001). In addition to transporting fatty acids, CD36 plays a wide variety of roles outside of fatty acid transport in numerous tissues, but it plays a particularly critical role in fatty acid supplementation in skeletal muscle (Stahl et al. 2001). Within skeletal muscle, CD36 has been identified on the outer mitochondrial membrane, the sarcolemma, and within t-tubules (Brennan et al. 2011; Holloway et al. 2006; Sefanyk et al. 2012). However, in response to exercise in humans and rodents, CD36 has been observed to primarily translocate to both the sarcolemma and the outer mitochondrial membrane, supporting its importance for LCFA transport into the cell and for increased rates of oxidation in mammals (Brennan et al. 2011; Glatz et al. 2010; Holloway et al. 2006; Stefanyk et al. 2012). Indeed, CD36-null murine muscles and CD36 knockdowns were observed to have a substantial reduction in FA oxidation and IMTG formation (Glatz et al. 2010). Therefore, our results of consistently identifying CD36 in the primary locomotor muscle in the NES, a mammal heavily reliant on FA oxidation for energy, underscore its importance in numerous tissues in the NES (Viscarra et al. 2012). While CD36 plays an integral role in fatty acid transport in a variety of tissues, lack of substantial difference in chemiluminescence across age classes during the fast suggest that it may not be the most dynamic FATP utilized, and that it may be providing a basal level of fatty acid transport regardless of nutritional state.

FATP1 and FATP4

While FAT/CD36 is a more cosmopolitan transport protein in its distribution, FATP1 and FATP4 are more localized to specific tissue types, with strongest expression occurring in cardiac muscle, skeletal muscle, and adipose tissue (Glatz et al. 2010; Jeppesen et al. 2012; Stahl et al. 2001; Wu et al. 2006). An important distinction between these two transporters is found in their response to insulin stimulation. FATP1 is an insulin sensitive transporter that has been reported to facilitate TG biosynthesis (Hatch et al. 2002; Kim et al. 2004; Wu et al. 2006). Conversely, FATP4 is an insulin-independent transporter that has been observed to have a significant increase in expression in human skeletal muscle after exercise training and is one of the main fatty acid transporters in the main locomotor muscles in mice (Jeppesen et al. 2012; Nickerson et al. 2009). A study evaluating the upregulation of FATPs in response to exercise in human skeletal muscle observed a significant decrease in FATP1 expression and a concurrent significant increase in FATP4 and FABPpm (fatty acid binding protein plasma membrane) after an eight week training period. While these results suggest a shift in the mosaic of FATPs concurrent with exercise and muscle development, FATP1 and FATP4 play arguably important roles within skeletal muscle in a variety of metabolic states (Hagberg et al. 2013; Jeppesen et al. 2012)

Prior to this study, FATP1 had been the only FATP, other than CD36, to be elucidated in a marine mammal (Viscarra et al. 2012). Investigation of the role of FATP1 in adipose tissue of fasting post-weaned NES pups revealed a decrease in expression of FATP1 and CD36 over the course of the fast (Viscarra et al. 2012). This is a compelling observation, which I believe is indicative of two possible explanations: the first being that

the decreased expression of these FATPs in adipose tissue is logical because NES are not actively building their adipose stores and are increasingly insulin resistant over the course of the fast (Crocker et al. 2014; Houser et al. 2013; Viscarra and Ortiz 2013; Viscarra et al. 2011). It is important to note that the influx of fatty acids into adipocytes is understood to be mediated by a variety of protein complexes and transport proteins, free fatty acid efflux out of the adipocyte after lipolysis is still somewhat mysterious, but is suggested to be actively mediated by an unknown transport protein (Kampf et al. 2007). The second potential explanation of decreased expression of FATP1 and CD36 in adipocytes, in addition to decreased FFA storage, is that FATP1 and CD36 are not the transporter proteins responsible for FFA transport out of the adipocyte. During the NES fast, elevated circulating NEFA levels provide a readily available substrate for tissues that rely on fatty acids, such as muscle, or for incorporation into milk in lactating adult females (Houser et al. 2007).

In conclusion, the consistent expression of FATP4 and FATP1 in all age classes, as illustrated in this study, was expected. Worth noting is the degree of nutritional stages represented by the sampled individuals in this study: the female was sampled at the end of lactation and fasting, pups were sampled at the beginning of the post-wean fast, and the adult males were sampled during the middle of their "catastrophic" molt and therefore fasting. Although our data is strictly qualitative in nature, it was surprising to observe what appeared to be a lower expression of FATP4 in the adult female than in the other age classes. Given the insulin independent role of FATP4 in skeletal muscle, we expected to see an upregulation of FATP4 in the adult females, especially during an energetically demanding season such as lactation. However, due to the small sample size of adult

females (n = 1), additional samples must be obtained and quantified before arriving at conclusions regarding the role of FATP4 in lactating adult females. In addition, samples of the same individual at multiple time points are critical to evaluating the dynamics of FATP1 and FATP4 over the course of the fast.

FABP3 and FABP4

FABPs are small, cytosolic proteins responsible for transport of fatty acids throughout the cell. FABP3, a 15 kDa protein found primarily in cardiac and skeletal muscle, was consistently identified in the post-weaned pup and the adult female. One of the primary functions of FABP3 is to shuttle FAs towards the mitochondria for β-oxidation (Makowski and Hotamisligil 2004; Smathers and Petersen 2011). Therefore the expression of FABP3 in the NES during fasting is not surprising. FABP4 is the primary FABP4 found in white adipose tissue, brown adipose tissue, and macrophages. However, FABP4 has been observed to be expressed in skeletal muscle, but to a much lesser extent than FABP3 (Fischer et al. 2006). The complete absence of FABP4 in all age classes is indicative of the variability of expression of FABP4 in skeletal muscle. Either FABP in NES skeletal muscle is either below our detectable limit or FABP4 is not the primary binding protein involved in intracellular fatty acid transport. Interestingly, FABP4 is becoming an ideal target as an indicator of obesity. It has been correlated positively with waist circumference and fasting insulin, and significantly higher concentrations of FABP4 have been detected in the serum of obese individuals compared to lean individuals (Xu et al. 2006). Future research should evaluate the activity of FABP4 in the serum of the NES, a naturally obese mammal.

While there has been observed to be a baseline concentration of FATPs and FABPs in the cell regardless of nutritional statushe novelty of FATPs and FABPs is found in the ability to quickly upregulate these proteins in response to a variety of stimuli (i.e. fasting, exercise, hormones, feeding, etc.) (Wu et al. 2006). However, when interpreting quantified data involving FATPs and FABPs, nutritional status of the individual is particularly important to account for, as it directly affects FATP and FABP activity (Nickerson et al. 2009). NES appear to possess a mosaic of FATPs and FABPs within their skeletal muscle and adipose tissue, and further investigation of the dynamics of these proteins over the course of the fast is warranted. Additionally, I believe there are still FATPs which may play a substantial role in FA transport within NES skeletal muscle, such as FABPpm, that have yet to be elucidated.

CHAPTER FOUR

Fatty Acid Utilization in the Northern Elephant Seal: Future Directions

Future Directions

Intramuscular Lipid Availability

In order to understand the significance of FATPs and FABPs, we must first understand the source of the fatty acids destined for utilization by the muscle, either from endogenous sources, intramuscular triglycerides (IMTG) and intramuscular lipids (IML), or derived from the products of lipolysis within the blubber adipocytes. Studies evaluating blood triglyceride levels in post-weaned fasting NES pups concluded that blood triglyceride content in nursing pups is higher than in post-weaned fasting pups (Houser et al. 2013). This is expected due to the intake of the extremely high fat content of the milk by nursing pups contrasted with the simultaneous developmental metabolic stress experienced by fasting pups. Measurements of circulating NEFA levels in lactating females indicated a reduction of uptake by adipose stores and decreased re-esterification, and transport for utilization by muscle or for milk production (Houser et al. 2007). Our findings corroborate this observation, as the general trend for lactating females was having higher circulating fatty acids at the end of the lactation, available for incorporation into milk or skeletal muscle. Whether circulating NEFAs in fasting pups and females are destined for skeletal muscle are directed towards oxidation or IMTG storage is unknown at this time.

While IMTG and IML have been characterized in the Weddell seal (*Leptonychotes weddellii*), they have yet to be elucidated in the NES. IMTG and IML stores in the Weddell seal accurately reflect the fatty acid composition of the blubber, with MUFAs as the most prevalent fatty acids, followed by SFAs and PUFAs, respectively (Trumble and Kanatous 2012; Trumble et al. 2010; Wheatley et al. 2008). Only recently has the blubber fatty acid composition of the NES been reported (Noren et al. 2013). Due to the similar blubber fatty acid composition of the Weddell seal and the NES, which is ultimately the result of their marine-based fatty diet, the IMTG and IML stores of the NES will most likely reflect this similarity.

IMTGs have been getting particular attention over the past decade due to the correlation of IMTG with insulin resistance. However, studies of endurance-trained athletes, which express higher IMTG yet maintain high insulin sensitivity, challenges the assumption of a positive correlation between IMTG levels and insulin resistance. Endurance trained athletes exhibit a significantly higher IML content than more sedentary individuals and those with type II diabetes mellitus (T2DM) (Dubé et al. 2008). Additionally, this training is coupled with a significant increase in the number of slow oxidative type I fibers and an increased capacity to oxidize fatty acids (Corcoran et al. 2007; Dubé et al. 2008; Jeppesen et al. 2012; Simoneau et al. 1999; Thyfault et al. 2010; Wang et al. 2004) Conversely, obese individuals exhibited a decreased oxidative capacity and a decrease in type I fibers (Hickey et al. 1995; Tanner et al. 2002).

As NES develop, they gain a higher prevalence of type I fibers (Moore et al. , unpublished data). This fiber transformation during development has also been documented in Weddell seals, in addition to a higher accumulation of IML in type I fibers

(Kanatous et al. 2002; Trumble et al. 2010). I hypothesize that as NES develop, they increasingly gain the oxidative capacity for diving through transformation of muscle fiber type and therefore the ability to readily accumulate and utilize IMTG and IML stores, similar to an endurance trained athlete. This places particular importance on the development of pups during the post-wean fast. As the fast proceeds, they increasingly rely on fatty acids derived from adipose tissue and deposited into muscle. The importance of fatty acid deposition in muscle is seen in trained humans, where lipid droplets are typically located adjacent to the mitochondria and provide an immediate fuel source. When fiber type development and increased IMTG are coupled with the increased mitochondrial biogenesis that occurs with training, this increases the oxidative capacity of the muscle (Jeppesen et al. 2012).

Peroxisome Proliferator-Activated Receptors

Over the past decade, peroxisome proliferator-activated receptors (PPAR) have gained increasing attention due to their role in regulating fuel stores. For the NES, of particular interest are PPARδ and PPARγ. While PPARδ is expressed in many tissues, PPARγ is primarily expressed in adipose and liver tissues. PPARγ is known to be involved with adipocyte differentiation and lipid accumulation in mature adipocytes (Wang et al. 2003). A study of PPARγ activation in the NES over the course of the fast in post-wean pups indicated a decreased activation of PPARγ, which is logical for a fasting mammal (Viscarra et al. 2011). While PPARγ expression has been associated with lipid storage, PPARδ has been associated with lipid oxidation. Indeed, a interesting study which overexpressed PPARδ in adipose tissue of high fat fed mice resulted in a significant depletion of serum FFA and TG and activated a variety of genes involved in

fatty acid oxidation (Wang et al. 2003). A later study from the same lab evaluated genetically modified mice overexpressing PPARδ in skeletal muscle. Over expression resulted in a significantly higher level of type I fibers, oxidative enzymes, and mitochondrial biogenesis even in the absence of physical activity. One of the most striking observations was the treated mouse was able to run an hour longer and a kilometer further than its wild-type equally weighted littermate. In addition, PPARδ-null mice were able to run for significantly less time and distance than their wild-type littermates (Wang et al. 2004). These results and the ability of PPARδ to upregulate fat metabolism in skeletal muscle provide compelling evidence for a role for PPARδ in transformation into a more trained, aerobic type I fiber without physical activity. PPARδ activation can occur by the presence of LCFAs, both saturated and unsaturated, and prostacyclins, and is often activating in conjunction with PGC-1α (Evans et al. 2004; Luquet et al. 2005; Russell et al. 2003). As discussed in earlier chapters, NES milk is one of the most fatty acid rich milks in the animal kingdom. Given the inactive nature of developing NES pups, in conjunction with their high fatty acid diet and elevated circulating NEFA, investigating a potential overexpression of PPARδ in the skeletal muscle of the NES may provide insight on the mechanism behind the development of muscle stores in preparation for diving.

FATP/FABP

FATP and FABP have been established to play important roles in FA delivery and oxidation (Jeppesen et al. 2012). Further, some transporter proteins appear to be insulin sensitive, while others are independently upregulated (Wu et al. 2006). FATP1 and FATP4 are of particular interest in skeletal muscle due to the observed upregulation of

FATP4 and simultaneous decrease in FATP1 in endurance athletes during training (Jeppesen et al. 2012; Nickerson et al. 2009). Ultimately, this increased the basal amounts of insulin independent transporters, which is conducive to an individual with increasing oxidative efficiency. For the NES, FATP1 has been observed to decrease in adipose tissue over the course of the fast. Decreased insulin signaling and an increase in insulin resistance over the course of the fast may clarify the reduced expression of FATP1 in adipose tissue, and reflect the NES increasing reliance on insulin independent pathways for fuel. As of this study, FATP1, FATP4, FABP3, and CD36 have been identified in NES skeletal muscle and FATP1 and CD36 in adipose tissue (Viscarra et al. 2012). However, these are only a few of the proteins that may play a role in the mosaic of FATP and FABP in the cell. Other FATPs, particularly FABPpm, may also play important roles in shuttling fatty acids in skeletal muscle over the fast. Cultured NES myocytes may also provide an interesting avenue to study FATP and FABP dynamics. Through cell culture, we are able to manipulate fatty acid substrates to further investigate the association of fatty acid species with specific FATP and FABP, and the development of IMTG (Hatch et al. 2002; Wu et al. 2006). Therefore, future studies should elucidate and quantify these transporters in skeletal muscle over the fast to give a more complete picture of fatty acid delivery within and to the cell during periods of metabolic stress.

Conclusions

Currently, much of the focus on the metabolic dynamics and development in the fasting NES is on their insulin resistance and associated hormones, which has been hypothesized to be part of an altered metabolic syndrome, akin to a modified T2DM (Crocker et al. 2014; Houser et al. 2007; 2013; Viscarra et al. 2011). However, I believe

our current knowledge of the "athlete's paradox" provides a viable alternative hypothesis. NES are well-established to be one of the animal kingdom's most elite divers, and therefore oxidative capacity and muscle development while fasting is critical. With the exception of lactating females who contribute a significant amount of their body protein into milk, protein catabolism is not the primary source of energy for NES during the fast (Adams and Costa 1993; Crocker et al. 1998). The abundant type I fiber distribution, increased fatty acid oxidation, and increased mitochondrial biogenesis observed in the NES also support this view. However we are still left with two interesting paradoxes: the first, that insulin resistance observed in the NES that is altogether absent in endurance-trained athletes; and secondly, the higher conservation of muscle protein in the NES contrasted with muscle atrophy in patients with metabolic syndrome. The activity of proteolytic pathways in NES skeletal muscle is an ideal target to address the latter. While further investigation is necessary, I believe alterations to glucose metabolism in the NES may be related to a protective mechanism against protein catabolism, and prevent an exacerbated futile cycle of development and degradation. To expand our current knowledge of the fatty acid based metabolism of the NES and to address this hypothesis, we must confirm and quantify the presence of other FATPs in skeletal muscle, quantify IML and IMTG, and investigate the activity of PPARδ and PPARγ in skeletal muscle throughout fasting. Doing so will provide an enormous amount of insight into the biochemical pathways involved in the metabolism and development of the NES, in addition to the adaptations to muscle development during simultaneous metabolic stress in a mammal.

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