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REGULATION OF LIPID METABOLISM AND MILK LIPID CONTENT IN NORTHERN ELEPHANT SEALS

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By

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ABSTRACT

Melinda A. Fowler

REGULATION OF LIPID METABOLISM AND MILK LIPID CONTENT IN NORTHERN ELEPHANT SEALS

Animals that fast depend on mobilizing lipid stores to power metabolism. Northern elephant seals (*Mirounga angustirostris*) incorporate extended fasting into several life history stages: post weaning development, molting, breeding and lactation. The mobilization and subsequent utilization of lipid stores could have consequences for the current and future survival and reproduction. Despite the importance of lipid metabolism to elephant seals little is known about the regulation of lipid mobilization and the physiological drivers of milk lipid content. This thesis focused on three aspects of lipid reserve rationing in adult female elephant seals. Chapter 1 investigated the mobilization of specific fatty acids from blubber and the partitioning of fatty acids between maternal metabolism and milk synthesis in northern elephant seals. In Chapter 2 I examined the hormonal regulation of lipid mobilization and milk lipid content and in Chapter 3 I investigated the activity of lipolytic enzymes and their relationship to milk lipid content. To examine these topics, fatty acid signatures were compared between the blubber and milk in early and late lactation. Additionally, lipid metabolites and milk lipid content were assessed in relationship to circulating levels of growth hormone, cortisol and insulin. Milk lipid content was also assessed relative to the activity of two lipolytic enzymes in the

blubber, hormone sensitive lipase and adipose triglyceride lipase. Results indicate that elephant seals mobilize specific fatty acids in a similar fashion to many other mammals and birds. Additionally, the milk is enriched in long chain monounsaturated fatty acids in late lactation. Mass and adiposity were significant ($p<0.05$) predictors of milk lipid content. Other important predictors of milk lipid content were circulating non-esterified fatty acid and triglyceride levels, cortisol and insulin. Unexpectedly I found that growth hormone was uncoupled from both circulating lipid metabolite concentrations and milk lipid content. Hormone sensitive lipase was found to be negligible in northern elephant seal blubber, another unexpected result. Adipose triglyceride lipase protein content was higher in late lactation relative to early lactation, but activity levels were stable across lactation and unrelated to milk lipid content.

Molting seals regulate lipolysis differently than lactating seals, with insulin mediating an even greater release of lipid stores in lactating females, to facilitate availability for milk production. There was a more positive relationship between triglycerides and milk lipid content than expected, but there was strong evidence supporting the hypothesis that milk lipid content is regulated at the level of mobilization of milk precursors from lipid stores. Contrary to previous hypotheses, hormone sensitive lipase was not the primary lipolytic enzyme and adipose triglyceride lipase assumed a more prominent role in lipid mobilization. Additionally, the specific regulation of these enzymes may differ among age classes and species. Despite their phenomenal lipid mobilization ability, mechanisms of the

lipid mobilization of specific fatty acids are conserved among northern elephant seals and other mammals. Conversely, the hormonal and enzymatic regulation of lipid mobilization exhibit differences between elephant seals and other mammals.

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INTRODUCTION

During times of low nutrient availability, animals must ration their stored reserves. Many animals, typically small mammals, undergo periods of fasting while dramatically lowering their metabolic rates, either by entering torpor or hibernation (Florant and Healy 2012). Other animals experience nutrient deprivation during times of high energetic expenditure as part of their life history strategy, such as breeding or growth (Cherel et al. 1987; Castellini and Rea 1992; Costa et al. 1986). When high metabolic demands are paired with times of nutrient restriction the animal faces a conflict of how to allocate fuel towards competing physiological demands.

The northern elephant seal (*Mirounga angustirostris*), a large phocid, displays a life history pattern that includes several extended periods of fasting. Extended periods of fasting enables the separation of marine foraging and terrestrial breeding, which is the hallmark of the reproductive pattern of elephant seals and many other phocids (Costa 1993). Fuel stores are built up over two long foraging trips thousands of kilometers out to sea (Le Boeuf et al. 2000; Robinson et al. 2012). When elephant seals are ashore, they fast for several consecutive weeks or months, not only during breeding (Costa et al. 1986; Deutsch et al. 1990), but also during postweaning development, (Ortiz et al. 1978) and while molting (Worthy et al. 1992).

Fasting while carrying out tissue reorganization or shuttling tremendous amounts of energy to the pup during lactation requires finely tuned metabolic control. Fuel stores accumulated over the foraging trip at sea must be rationed accordingly. Catabolizing fuel stores during fasting to levels that are difficult to recoup when

foraging could affect future survival and reproduction (Arnbom et al. 1997; McMahon et al. 2000). The catastrophic molt, a process which involves a 3-4 week fast onshore while all skin and hair is sloughed and regrown from onboard reserves is an uncommon strategy displayed by only a few species (northern and southern elephant seals and Hawaiian and Mediterranean monk seals and Ross seals) (Bengtson et al. 2011; Le Boeuf and Laws 1994; Boyd et al. 1993; Badosa et al. 2006; Kenyon and Rice 1959). Northern elephant seals fast throughout this time of tissue reorganization (Worthy et al. 1992; Le Boeuf and Laws 1994).

Of all reproductive costs, lactation demands the most energy (Gittleman and Thompson 1988); the combination of fasting and lactation is a rare life history strategy involving conflicting metabolic demands. Despite these challenges, the evolution of simultaneous fasting and lactation has occurred in phocid seals, mysticete whales and bears. Fasting and lactating animals must synthesize milk from only precursors stored in the body. This is extremely unusual, as most lactating mammals obtain the majority of precursors for milk synthesis from dietary input (Neville and Picciano 1997). The pairing of fasting and lactation necessitates metabolic alterations of milk production as compared to mammals that feed during lactation.

In lactating seals the demands of maintaining maternal stores for future reproduction competes with delivering a large amount of lipid to the pup to prepare it for the post-weaning fast. While lactation and fasting have conflicting demands, they both rely on lipid as an energy source because it contains more energy per gram than

either carbohydrate or protein (Frayn 2010). Due to its high energy density, fat is the most efficient fuel to transfer milk energy quickly. Like other phocids, elephant seals have a very short, intense lactation period that is enabled by the rapid transfer of energy in lipid rich milk. Female elephant seals give birth to a single offspring, and lactate while fasting for ~26 days while producing lipid rich milk (20-55% lipid) (Crocker et al. 2001; Costa et al. 1986). The combination of fasting and lactation has driven the composition of pinniped milk (i.e. high fat, low water, almost no carbohydrate) (Oftedal 1993; Costa 1991).

The transfer of milk energy ultimately impacts the ability of the pup to successfully forage. Elephant seal pups undergo an extended period of fasting after weaning, during which time their diving ability develops (Thorson and Le Boeuf 1994; Tift et al. 2012). The fat accumulated during suckling provides fuel all activities and processes, including the development of diving capacity during the 6-8 week post-weaning fast. High fat milk is important for lipid storage in pups, which impacts the duration of the postweaning fast, where fatter pups fast longer (Noren et al. 2003). Therefore, an ample store of fat reserves to remain ashore in order to develop adequate diving abilities is important.

Utilizing lipid stores for maintenance metabolism and milk production includes mobilization from the lipid depot (blubber) and subsequent uptake by tissue from the bloodstream, either mammary gland or oxidation by other tissue. Stored lipids are released from the blubber into the blood stream as non-esterified fatty acids (NEFA). NEFA may eventually be taken up by the liver or other tissues and re-

esterified into triacylglycerol (TAG) bound to lipoproteins (Diraison and Beylot 1998). Lipolysis rates in fasting and lactating elephant seals are very high relative to other mammals, but remain stable across lactation; while lipolysis in late molting seals was found to be considerably lower than lactating seals. By-products of lipolysis (NEFA) increase over lactation, despite the stable rates of lipolysis (Houser et al. 2007). The increase of by-products of lipolysis concomitant with stable lipolysis rates suggests re-esterification rates of mobilized lipid decrease across lactation.

Milk lipid production is dependent on both the supply and the uptake of milk precursors, either of which may be limiting. Does the mammary gland regulate the uptake of fatty acids from the blood and subsequent secretion, or does the supply of fatty acids determine milk lipid secretion? Circulating NEFA can diffuse passively across membrane borders down a concentration gradient, or be facilitated by transport proteins (Glatz et al. 2010), while TAG must be hydrolyzed before entering tissue. Lipoprotein lipase (LPL) is the primary enzyme enabling tissues to uptake circulating lipoproteins to metabolize (Frayn et al. 1995). Therefore, in order for TAG to be utilized for milk lipid production, it must be hydrolyzed by mammary LPL to facilitate entry. The interplay between TAG and NEFA availability can affect the patterns of mammary utilization. When plasma TAG are low and plasma NEFA are high, the NEFA are used directly by the mammary gland for synthesis of milk lipids (Neville and Picciano 1997).

In hooded and grey seals, the activity of mammary LPL increases and was postulated to be the driving factor in milk lipid content, potentially affecting the uptake of lipid from the bloodstream into the mammary gland (Mellish et al. 1999b; Iverson et al. 1995a). In elephant seals, however, mammary LPL was not related to milk lipid content (McDonald and Crocker 2006), suggesting potential regulatory roles at the level of release from lipid stores through lipolytic enzymes or other regulators of lipolysis. The lack of a role for mammary LPL in regulating milk lipid content suggests that circulating NEFA and regulators of fatty acid release from adipocytes may play a crucial role in regulating milk lipid content.

Fasting animals are constrained by finite energy reserves and must ration reserves accordingly over time. Given the dependence on lipid in several life history stages of elephant seals, inability to appropriately ration lipid stores to prepare for reproduction and manage delivery to the pup could have consequences. Depletion of lipid stores to a level that is difficult to recover from could affect both the future breeding attempts by the female and the inadequate provisioning of lipid to the pup could impact its survival. The goal of this thesis was to investigate lipid metabolism in fasting seals; both during tissue reorganization and during the added demand of lactation. I also aimed to elucidate aspects of how the lipid content of milk is regulated in fasting, lactating seals.

CHAPTER 1

LIPID MOBILIZATION AND COMPARISON TO MILK LIPID CONTENT IN NORTHERN ELEPHANT SEALS

Abstract

A fundamental feature of the life history of baleen whales, bears and true seals is lactation while fasting. This study examined the mobilization of fatty acids from blubber and their subsequent partitioning maternal metabolism and milk production in northern elephant seals (*Mirounga angustirostris*). The fatty acid composition of blubber and milk were measured in both early and late lactation. Proportions of fatty acids in milk and blubber were found to display a high degree of similarity both early and late in lactation. Seals mobilized an enormous amount of lipid (~66 kg in 17 days), but thermoregulatory fatty acids, those that remain fluid at low temperatures, were conserved in the outer blubber layer. Despite the stratification, the pattern of mobilization of specific fatty acids conforms to biochemical predictions. The relative proportions of fatty acids in milk and blubber were quite similar. Long chain (>20C) monounsaturated fatty acids (MUFA) were the least mobilized from blubber and the only class of fatty acids that increased in milk in late lactation. Polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA) were more mobilized from the blubber, but both proportions decreased in milk at late lactation. These data suggest that of the long chain MUFA mobilized, the majority is directed to milk synthesis. The mother may preferentially use PUFA and SFA for her own metabolism, decreasing the

availability for deposition into milk. The potential impact of milk fatty acid delivery on pup diving development and thermoregulation are exciting avenues for exploration.

Introduction

Fasting and lactation is a rare life history strategy involving conflicting metabolic demands. Lactation involves the mobilization of reserves while fasting is a time when many species decrease metabolic expenditure in order to conserve nutrient stores (Florant and Healy 2012; Castellini and Rea 1992). Northern elephant seal females lose ~40% of their initial mass and deplete ~58% of their lipid stores without any intake of food or water (Costa et al. 1986; Crocker et al. 2001). Fasting and lactating animals must synthesize milk from only precursors stored in the body. This is extremely unusual, as most lactating mammals obtain the majority of precursors for milk synthesis from dietary input (Neville and Picciano 1997).

The utilization of lipid stores to support maintenance metabolism and milk production consists of mobilization from the lipid depot (blubber) and uptake by tissue, either mammary gland or by other tissue for oxidation. In fasting seals, lipids to be directed to milk production can only come from stored lipid or via *de novo* synthesis in the mammary gland. The mother's diet will impact the fatty acid distribution in her blubber available to be mobilized during fasting (Iverson et al. 2004). The chain length of milk fatty acids can provide some insight into their source. Short and medium chain fatty acids (≤ 16 carbons) can be synthesized *de novo* in the mammary gland (Dils 1983). Seal milk studied previously has shown

chain lengths of 12 carbons to be the shortest chain fatty acid present, with small amounts of 14 carbon fatty acids (Iverson et al. 1992; Debier et al. 1999; Riedman and Ortiz 1979; Wheatly et al. 2008). C16:0 can be synthesized by the mammary gland in several species, including ruminants, (Grummer 1991). While previous studies have assumed that *de novo* synthesis in seal mammary glands is minimal (Iverson 1993), the presence of significant amounts of C16:0 in seal milk suggests the possibility of *de novo* synthesis.

Fatty acids can be divided into broad classes including saturated fatty acids (SFAs), containing no double bonds, monounsaturated fatty acids (MUFAs), containing one double bond, and polyunsaturated fatty acids (PUFAs), containing more than one double bond. In laboratory studies, for a given number of double bonds, shorter chain fatty acids are more readily mobilized from the storage depot; but for a given chain length, fatty acids are more readily mobilized as the degree of unsaturation increases (Conner et al. 1996; Raclot 2003; Raclot et al. 1995b). Additionally, the mobilization of each fatty acid is generally unaffected by the relative prevalence of each fatty acid, until the exhaustion of a particular fatty acid (Raclot and Groscolas 1995; Raclot et al. 1995b).

The lipid composition of milk is critical to the developing pup. The weaned pup will subsist mainly on stored lipid for the duration of the postweaning fast (2-2.5 months) (Noren et al. 2003). In addition to providing energy, fatty acids serve multiple purposes in a developing pup; they are involved in sensory systems,

particularly vision, immune function and development, among many other functions (Jump 2002).

Not only do fatty acids function in fuel and cell signaling, marine mammals utilize fatty acids for thermal insulation. Phocid seals store their fat in a subcutaneous blubber layer, rather than in scattered internal depots. It has been reported in many different species of marine mammals that blubber layers are stratified from inner to outer layers (Best et al. 2003; Strandberg et al. 2008; Wheatley et al. 2007; Koopman et al. 1996). External layers have a higher proportion of medium chain ($\leq$ 18C) MUFA and are more prevalent in the exterior possibly as a homeoviscous adaptation (Sinensky 1974), i.e. for the purpose of maintaining membrane fluidity at low temperatures encountered at sea. Interior layers in phocid blubber were more enriched in saturated fatty acids, long chain ($\geq$ 20 C) MUFA and were more heavily metabolized (Koopman et al. 1996; Koopman et al. 2002; Strandberg et al. 2008). In Weddell seal pups, the distribution of MUFA and PUFA may affect the development of thermoregulatory capabilities and oxidative capacity for diving (Wheatly et al. 2008; Trumble et al. 2010).

For the mother, differing proportions of MUFA, PUFA or SFA mobilized from blubber may affect plasma cholesterol (Kris-Etherton and Yu 1997) and triglycerides (Harris 1997), as well as stimulate lipolysis (Guo et al. 2005). Additionally, females must sequester adequate amounts of the appropriate fatty acid in the outer, "thermoregulatory" layer of blubber for their return to sea. The

mobilization and subsequent utilization of specific fatty acids is likely to have consequences for both the mother and pup.

The dynamics of movement of lipid transfer from blubber to milk have been investigated in only a few species, including hooded seals (Iverson et al. 1995b), grey seals (Grahl-Nielsen et al. 2000; Arriola Ortiz 2010) and Weddell seals (Wheatly et al. 2008). Our goals in this study were 1) to quantify how northern elephant seals mobilize lipid stores relative to other mammals and 2) to understand partitioning of fatty acids between maternal metabolic use and milk production in northern elephant seals under controlled fasting and lactation durations.

Methods

Study Site and Subjects

This study was carried out at Año Nuevo State Reserve, San Mateo County, CA during the 2005 breeding season (Jan-Feb). To facilitate identification, adult female seals were marked with hair dye (Lady Clairol, Stamford, CT), shortly after arrival on land. Parturition dates were established by daily observations and considered to be the first day a marked female was observed with a pup, provided she had been observed without a pup the previous day. Twenty-two mother/pup pairs were captured early in lactation (day 5 post-partum) and 19 of these recaptured late in lactation (day 22 post-partum). From these animals, blubber was obtained from 15 mothers early in lactation and 17at late lactation. Fourteen individuals were blubber sampled in both early and late lactation. Milk was obtained from 22 animals in early lactation and 19 in late lactation, with 19 individuals sampled in both. Within early

lactation 15 animals were sampled for both blubber and milk. Within late lactation, 17 individuals were sampled for both blubber and milk.

Body composition measurements were made using the truncated cones method (Crocker et al., 2001; Gales and Burton, 1987). This method calculates the proportion of mass due to adipose and lean tissue and has been validated in elephant seals using isotopic dilution (Webb et al., 1998). Dorsal, lateral and ventral blubber depth measurements were made using a portable ultrasound (Ithaca Scanprobe, Ithaca, NY) at each of six locations along the seal. Lengths and girths were taken at these six points, as well as total curved length. Mass was measured using a tripod, canvas sling and scale (± 1 kg) MSI, Seattle, WA).

Sample Collection and Processing

Females were initially immobilized with Telazol (tiletamine/zolazepam HCl, Fort Dodge Labs, Ft. Dodge, IA) at a dosage of ~1mg/kg, administrated intramuscularly. Continued immobilization was maintained with ~100 mg bolus intravenous injections of ketamine via the extradural vein. At both captures milk and blubber samples were collected from the mothers. A blubber biopsy extending the full depth of the blubber layer was taken laterally, several centimeters anterior to the pelvis using a 6mm biopsy punch (Uni-Punch, Premier Medical, Plymouth, PA, USA). Milk was collected from the teat using a clean cut-off syringe after a intramuscular injection of 40 IU of oxytocin (American Pharmaceuticals Partners, Los Angeles, CA, USA) near the mammary gland. Samples were placed on ice for transport to the lab. The blubber core was separated into inner and outer cores and

stored and analyzed separately. All samples were stored at -80°C in Nunc tubes until analysis. Work was conducted under NMFS Marine Mammal permit #87-1463 and all procedures were approved by the Sonoma State University Institutional Animal Care and Use Committee.

Fatty acid quantification

Fatty acid profiles were obtained by gas-liquid chromatography of the fatty acid methyl ester derivatives. Lipids were extracted by using the method of Folch modified by Christie (1982) for blubber and by using an adaptation of the method of Radin (1981) and Schweigert and Stobo (1994) for milk (see (Debier et al. 1999) for more details). Fatty acids from the lipid extract were methylated in a solution of KOH in methanol (0.1 mol/L) at 70 ◦C for 60 min, then in a solution of HCl in methanol (1.2 mol/L) at 70 ºC for 20 min. The fatty acid methyl esters (FAME) were then extracted with hexane and separated and quantified with a gas–liquid chromatograph (GC Trace ThermoQuest, ThermoFinnigan, Milan, Italy) equipped with a flame ionization detector, an automatic injector and a fused silica capillary column (100 m * 0.25 mm internal diameter) coated with a 0.2μm film of biscyanopropyl polysiloxane (Rt-2560, Restek, Bellefonte, PA, USA) (Dang Van et al. 2011). Each peak was identified and quantified by comparison of retention times with pure FAME standards.

Fatty acids are expressed as percent of total fatty acids within an individual sample. Data for both outer and inner layers are presented here and the differences statistically evaluated, but we focus on the inner layer for the comparisons to milk, as

the inner layer is metabolically more active (Best et al. 2003; Koopman et al. 1996; Strandberg et al. 2008).

Mass of each fatty acid was calculated by first using body composition measurements to calculate the mass of blubber stores of the animal. Previous studies have shown that elephant seal adipose tissue is ~90% lipid both early and late in lactation (Crocker et al. 2001), TAG makes up 99.9% of the lipid in phocid blubber and 95% of the mass of TAG is due to fatty acid (Wheatly et al. 2008). Total kilograms of adipose stores were calculated by multiplying the proportion of adipose tissue by mass of the animal. The adipose mass was multiplied by 0.90 to obtain the lipid mass of adipose tissue. The fatty acid mass was calculated by multiplying lipid mass by 0.95 to get total mass of the fatty acid. Because blubber samples were split equally into inner and outer portions and the fatty acid composition analyzed separately, the total mass of the fatty acid was multiplied by 0.5 and then by the proportion of the fatty acid in inner and outer, respectively. The total mass of each blubber fatty acid (KG_B) is the sum of the inner and outer blubber compositions.

Mobilization of individual fatty acids was assessed relative to how much was initially available by assessing the amount of fatty acid that remained in late lactation relative to the amount of fatty acid that was available in early lactation. Proportion of each fatty acid mobilized (pmFA) was calculated for matched early and late samples (n=14) as in (Arriola Ortiz 2010):

$$pmFA=1-(FA_{kgL} /FA_{kgE})$$

Where FA_{kgE} represents kilograms of fatty acid in early lactation and FA_{kgL} represents kilograms of fatty acid in late lactation.

Statistical analyses

Statistical analyses were performed using the software R (Version 2.13.1, R Development Core Team, www.R-project.org). Packages `AED` (Zuur 2009) and `nlme` (Pinheiro et al. 2009) were used to assess normality, homogeneity of variance and perform mixed effects modeling. Post hoc tests were carried out using the `multcomp` package (Hothorn et al. 2008).

Due to a mixture of paired and non-paired samples, a linear mixed model fitted using REML was used to evaluate significant differences from early to late lactation. To account for longitudinal sampling, subject was included as a random effect in the models. Variances were found to be unequal among classes for both double bonds and chain length, thus a variance component structure (varIdent) was used in the analysis. Paired t-tests were run to investigate differences between inner and outer blubber core fatty acids. Statistical significance was considered at $p<0.05$.

The similarity among the fatty acids in the blubber and those in the milk, was assessed using two similarity indices. A percent similarity index was calculated as:

$$\%\text{Sim}=\sum_i minimum\ (p1i, p2i)$$

Where $p1i$ represents the proportion of fatty acid i in blubber and $p2i$ is the proportion of fatty acid i in milk. (Krebs 1999).

A cosine similarity index (Petraitis 1981; Yin et al. 2011) was calculated as:

$$Cosine\theta = B \cdot M / ||B||\ ||M||$$

Where B equals the vector of the inner blubber fatty acid proportion values and M is vector of the milk fatty acid proportion values. This value varies between 1 and 0, with 1 representing identical vectors and 0 representing completely different vectors.

Results

Mass and body composition

Mass decreased significantly from 446 (SD=68) kg to 326 (SD=47) kg ($F_{1,16}$=471.5; p<0.001) with seals losing 120±23 kg or 27% of body mass over 16 days. The proportion of adipose tissue decreased significantly from 37.2 (SD=2.0) to 29.4 (SD=2.0) % ($F_{1,15}$=193.5; p<0.001) over the study period losing an average of 70 kg or 42% of their adipose tissue reserves. The mean total mass of fatty acids lost from the blubber depot was 66 (SD=15) kg.

Blubber fatty acid signatures and mobilization

A total of 20 different fatty acids were quantified in blubber and milk (Table 1.1), ranging in chain length from 12 to 24 carbons. In both early and late lactation the majority of the inner blubber core was made up of 18:1n-9 (oleic acid). The second and third most abundant fatty acids were 16:0 and 20:1n-11. Similarly, outer blubber was made up of primarily 18:1n-9, 16:0 and 20:1n-11 in both early and late lactation.

Fatty acids were categorized into saturated (SFA), monounsaturated (MUFA) or polyunsaturated (PUFA) (Table 1.1), and investigated with respect to outer versus inner blubber (in both early and late lactation), as well as between classes within each layer from early to late lactation.

All classes of fatty acids were different from inner to outer portion of the blubber except for late lactation PUFA (Table 1.2; Figure 1.1). MUFA were further subdivided into medium chain (≤ 18 carbons) or long chain (≥ 20 carbons) in keep with previous studies regarding stratification in marine mammals (Strandberg et al. 2008; Best et al. 2003; Koopman et al. 1996; Wheatley et al. 2007). In early lactation there were fewer MC MUFA in the inner layer relative to the outer blubber layer; while LC MUFA, PUFA and SFA were higher in the inner layer than in outer blubber (Figure 1.1). In late lactation there were fewer MC MUFA in the inner layer relative to the outer blubber layer; more LC MUFA and SFA in inner blubber compared to outer blubber. PUFA levels remained stable between inner and outer layers in late lactation.

MC MUFA remained stable in the outer blubber from early to late lactation, while it declined in inner blubber layer (Table 1.2). In contrast LC MUFA increased in inner and outer layers from early to late lactation. PUFA levels decreased in the inner and outer layers from early to late lactation, as did SFA (Table 1.2, Figure 1.1).

The mobilization of fatty acids from the blubber (pmFA) was calculated using the change in mass of each fatty acid in the entire blubber core from early to late lactation. This calculation indicated that the three most mobilized fatty acids were PUFAs C20:5n-3, C20:3n-3 and C18:3n-3 (Table 1.3). This mobilization index takes into account how much the mass of a particular fatty acid decreased, relative to how much was initially present. For example, although C18:1n-9 is present in high

proportions in both blubber and milk in early and late lactation, when viewed from the mass lost in the blubber, it is only the 9th most mobilized fatty acid.

The proportion of fatty acids mobilized from the blubber was analyzed according to class (MC MUFA, LC MUFA, PUFA, SFA). Different classes were mobilized at different rates ($F_{3, 261}$=43.0; p<0.001; Figure 1.2). PUFAs were the most mobilized class, (0.50; SD=0.1), followed by SFAs (0.41; SD=0.07) and MC MUFA (0.42; SD=0.11) and then by LC MUFAs (0.28; SD=0.09). Additionally, the number of double bonds ($F_{6, 252}$=104.7, p<0.001) and chain length ($F_{6, 252}$=29.9, p<0.001) were significant predictors of proportion mobilized, with greater mobilization as double bonds increased and chain length decreased.

Milk fatty acids

Milk fatty acids from early and late lactation were composed primarily of 18:1n-9, 16:0 and 20:1n-11, similar to blubber (Table 1.1). When fatty acids were divided into the classes of saturated, monounsaturated or polyunsaturated, the proportion of all categories changed significantly from early to late lactation (Table 1.2, Figure 1.1). PUFA and SFA in milk both decreased across lactation as did the proportion of MC MUFA, while LC MUFA was the only class to significantly increase in milk.

Fatty acid signatures between blubber and milk

Inner layer blubber fatty acid patterns relative to milk fatty acid were compared by looking at the difference between each specific fatty acid. The greatest difference between milk and blubber ($\%FA_{milk}$ - $\%FA_{blubber}$) were from C20:1n-11,

with a difference of 5.9% between milk and inner blubber in early lactation, with levels of C20:1n-11 making up a larger proportion of blubber than milk (Figure 1.3). Both C20:1n-9 and C20:1n-11 comprised higher proportions of blubber than milk; however, these two LC MUFA exhibited the largest increases in milk from early to late lactation. As shown in Table 1.3, these are among the least mobilized of FA. C18:1n-9, C18:1n-11, and C16:0 make up larger proportions of the milk than blubber, while C18:1n-9 and C16:0 had the two largest absolute decreases in milk over lactation (Figure 1.3). C18:1n-9 and C16:0 are in the middle range of mobilization, when compared to all fatty acids.

The patterns between blubber and milk appear to be quite similar at early and late lactation. However, proportions of C22:6n-3 were lower in milk relative to blubber in early lactation, but not at late lactation, while proportions in milk increased over lactation (Figure 1.3). Additionally, the proportion of C20:5n-3 in the milk decreased from early to late lactation.

Both similarity metrics indicate that blubber FA signatures are very similar to milk FA, in both early and late lactation, with %Sim values near 90% similar (Table 1.4). These two metrics were calculated using the proportion of FA in inner blubber and milk FA proportions in early and late lactation, as well as between milk samples, early and late. Data are used from individuals for whom matched samples are available for early and late blubber and milk.

Discussion

The inner and outer blubber layer of elephant seals retained the stratification favorable for thermoregulation in cold water despite prolonged fasting and the sheer magnitude of lipid mobilized (~ 66 kg). The mechanism of mobilization of specific fatty acids appears to be conserved among multiple species of mammals and birds, following biochemical predictions. Relative to its' mobilization, LC MUFA appears to be deposited in milk at a higher rate than other classes of FA; possibly due to differential oxidation by maternal tissues.

Fatty Acid Mobilization From Blubber

Our results were highly similar to previously published patterns in other species (Figure 1.4). *In vivo* and *in vitro* studies have shown the pattern of fatty acid mobilization primarily depends on the structure of the fatty acid (Raclot et al. 1995a; Conner et al. 1996; Raclot et al. 1995b; Price et al. 2008). Fatty acid mobilization is affected by chain length and the number of double bonds. Mobilization of a fatty acid for a given chain length increases with unsaturation and for a given unsaturation, shorter chain length fatty acids are mobilized faster (Raclot and Groscolas 1993). This relationship is largely independent of the relative amounts of fatty acid present until extreme depletion (Raclot et al. 1995b; Raclot and Groscolas 1995). Chain length and number of double bonds were significant predictors of mobilization in the current study, agreement with previous studies. C20:5n-3 was the most mobilized fatty acid, which is consistent with laboratory studies with rats (Conner et al. 1996; Raclot and Groscolas 1995). However, there were a few small deviations from the

typical pattern of mammalian fatty acid mobilization. The few fatty acids that diverged slightly from expected patterns were: C16:0 that was slightly more mobilized than expected and C18:1n-11 and C20:4n-6, which were slightly less mobilized than expected (Figure 1.4).

On the whole, these patterns of mobilization are very similar to other studies in rabbits, rats, minks and birds (Raclot et al. 1995a; Raclot et al. 1995b; Conner et al. 1996; Price et al. 2008; Raclot and Groscolas 1995; Nieminen et al. 2006). This is reassuring as different methods were used in calculating relative mobilization. In this study, mobilization was based on mass change in the blubber depot of individual fatty acids across several weeks of fasting; in Raclot et al (1995b), fatty acid release was stimulated *in vitro* from excised adipose tissue and in Connor et al (1996) plasma NEFA were compared to adipose after lipolytic stimulation *in vivo*.

When the current fatty acid data are compared to other seal fatty acid patterns, there are many similarities. Although seals with different diets will have different fatty acid in their blubber (Iverson et al. 2004), the proportion mobilized can be calculated and used to compare with Weddell and grey seals. The blubber fatty acids of Weddell (Wheatly et al. 2008) and elephant seals are mobilized in similarly manner. However, in grey seals, several fatty acids showed slightly different mobilization patterns. When rank order of mobilization was considered, C20:3n-3 was less mobilized in grey seals (Arriola-Ortiz 2010). When the mobilization index calculated with mass of fatty acids are considered, C24:1n-9 was more mobilized in the current study than in Arriola-Ortiz (2010). In both the Wheatley study and the

Arriola-Oritz study, 20:5n-3 was highly mobilized in fasting and lactation, similar to this study. Hooded seals also show high depletion of C20:5n-3 over lactation (Iverson et al. 1995b).

Studies of free ranging animals demonstrate agreement with laboratory sudies. Starved mink (Nieminen et al. 2006) and captive studies of migratory passerines also demonstrate a general agreement with expected patterns of lipid mobilization (Price et al. 2008). Differences from the expected patterns are physiologically possible For example hibernating marmots preferentially mobilize saturated fatty acids (C14:0-C18:0) and retain C18:2 (Florant et al. 1990). Hibernating echidnas primarily mobilize MUFA (C16:1-C18:1) during the hibernation period, while SFA (C12:0-C18:0) and PUFA (C18:2) increased in the fat depot, (Falkenstein et al. 2001). A mechanism by which some fatty acids accumulate in the blubber depot could be selective re-esterification (Xia et al. 1993).

In the outer layer, MC MUFA was higher and remained stable from early to late lactation. MC MUFA has been hypothesized to contribute the most to insulative thermoregulatory capacity, because of the ability to remain fluid at lower temperatures (termed homeoviscous adaptation) (Sinensky 1974). It appears that these proportions are maintained at a stable level in the outer layer of female blubber, despite high levels of overall mobilization.

Fatty Acids in Blubber Compared to Milk

Subsequent to mobilization, fatty acids are utilized by other tissues, including the mammary gland for milk synthesis (Figure 1.5). When the proportion of blubber

and milk fatty acid compositions were compared, the metrics used in this study indicate a high degree of similarity (Table 1.4). Although there is a high degree of similarity, the data show that overall, LC MUFA are mobilized least from the blubber and LC MUFA proportions increase in the milk. When the %Sim are compared, there is approximately 10% difference between blubber and milk (Table 1.4). These differences, albeit small, could be accounted for by differences in utilization by the mother for energy use versus milk production and variations in mammary gland uptake. Two LC MUFA; C20:1n-11 and C20:1n-9 show higher levels in blubber than milk suggesting less mobilization from blubber (also seen in the mobilization index), but the proportions increase in the milk. MC MUFAs (C18:1n-9 and C18:1n-11) and a SFA (C16:0) show higher proportions in milk than in blubber, suggesting higher mobilization and deposition in milk or potentially *de novo* synthesis in the mammary gland (Figure 1.3). However, MC MUFA proportions in milk decrease across the fast. The differences in milk distribution of FA across lactation seem to be driven by the decrease in MC MUFA and increase of LC MUFA in the milk. Potential explanations may include some *de novo* synthesis in the mammary gland, or the utilization by the mother, such that some fatty acids mobilized from the blubber are not available to the mammary gland, thus affecting the distribution in the milk. Preferential uptake by the mammary gland or selective re-esterification of mobilized fatty acids are other potential causes for patterns that differ across lactation.

In ruminants, about half of C16:0 can come from *de novo* synthesis in the mammary gland (Grummer 1991), due to elongation of shorter chain fatty acids

synthesized endogenously from acetyl CoA (Mepham 1987; Akers 2002). Mammary gland fatty acid synthase activities have not been quantified in pinnipeds, but it seems possible that they could possess the biochemical machinery to produce C16:0 in the mammary gland. C16:0 is higher in milk than in blubber in this study, a pattern also seen in hooded seals (Iverson et al. 1995b) and grey seals (Grahl-Nielsen et al. 2000; Arriola Ortiz 2010). The higher levels support the possibility of *de novo* synthesis in pinniped mammary gland, although it is likely minimal.

In addition to *de novo* synthesis of fatty acids, desaturases add double bonds and elongases lengthen fatty acids. For example, C18:0 may be turned into C18:1n-9 by Δ^9 desaturase. Desaturase activity has been detected in pinniped blubber (Strandberg et al. 2008; Budge et al. 2004), but has not been investigated in mammary tissue. Proportions of milk C18:1n-9 are indeed higher in milk than blubber, but, given the low levels of precursor C18:0 in both blubber and milk, it seems unlikely that desaturase enzymes are playing a large role in milk production.

The pattern of increasing LC MUFA in the milk despite the lower mobilization rate from blubber could be affected by maternal fatty acid oxidation rates. The oxidation of fatty acids has been shown to be affected by structure, where oxidation increases with unsaturation and decreases with chain length in mammals and birds (DeLany et al. 2000; Price et al. 2011). Long chain fatty acids with only one double bond (C20:1) have the potential to be oxidized by maternal tissue slower than other shorter chain or more unsaturated fatty acids. Thus, maternal tissue may

not utilize and oxidize each type of fatty acid equally, affecting the availability of mobilized fatty acid for milk production.

Selective mechanisms of re-esterification of fatty acids (synthesizing TAG from liberated NEFA) have been hypothesized for hibernating animals' mobilization patterns that differ from expectations projected based on fatty acid distribution (Xia et al. 1993). TAG synthesis is a process that occurs normally in adipose, liver or other tissue (Nye et al. 2008; Diraison and Beylot 1998). Re-esterification rates have not been quantified in northern elephant seals, but plasma NEFA increases across lactation while whole body lipolysis rates remain stable, suggesting the possibility of lower re-esterification rates later in lactation (Houser et al. 2007). If selective re-esterification of specific fatty acids occurs in the adipose tissue, an accumulation of specific fatty acids in the blubber would result, and we do not see evidence of particular fatty acids being sequestered in the blubber more than expected. TAG synthesis by the liver could result in circulating TAG with a pattern of specific fatty acids. The fatty acids selectively deposited in circulating TAG could appear in milk via pickup by lipoprotein lipase (LPL) in the mammary gland (Figure 1.5). McDonald and Crocker (2006) found LPL to be minor in contributing to milk lipid content. It is quite probable that re-esterification rates are dynamic in fasting elephant seals (Champagne et al. 2012; Houser et al. 2007), but the selective re-esterification of fatty acids does not seem to be driving the patterns seen in this study.

Milk fatty acid composition varies greatly between species, differing both because of dietary intake, as well as differential mechanisms of *de novo* lipid

synthesis (Neville and Picciano 1997) (Table 1.5). The milk content of animals often varies with diet, with animals that consume more fish producing milk higher in fatty acids ≥20 carbons, or diets high in terrestrials sources of PUFA consisting of C18:2 or C18:3. Ruminant milks tend to have high levels of SFA (Rodríguez-Alcalá et al. 2009) as a result of microbial activity, while human milk has higher levels of C18-PUFA than seal milk (Neville and Picciano 1997; Koletzko et al. 2001). Two other terrestrial animals are presented for comparison, a carnivore (domestic cat) (Jacobsen et al. 2004) and omnivore (black bear) (Iverson et al. 2001). Polar bears, a species known to forage on a marine diet have higher levels of marine source PUFA (≥20C) than black bears, which have a terrestrial diet (Iverson et al. 2001; Hedberg et al. 2011). Elephant seals, with a marine diet, not surprisingly produce milk with higher ≥20C PUFA than terrestrial animals. Polar bear milk shows similar longer chain PUFA levels to seals (~9% in seals vs ~8% in polar bears) (Hedberg et al. 2011).

The high proportions of milk MUFA found in this study agree with previous work in fasting and lactating pinnipeds. Proportions of LC MUFA were seen to increase in milk across lactation in a previous elephant seal study (Riedman and Ortiz 1979). In hooded and harp seal milk, C20:1 was the only FA to clearly increase in milk across lactation (Debier et al. 1999). Similar to the current study, LC MUFA tend to increase in milk across lactation in Weddell and grey seals (Wheatley et al. 2008; Arriola-Ortiz 2010). However, the proportional increase is higher in elephant seals and Weddell seals than in grey seals, which also exhibit a slight increase in milk

MC MUFA proportions. Comparisons with Weddell seal milk should be made cautiously, as the seals were likely foraging late in lactation (Wheatly et al. 2008).

Health Consequences for the Pup

Increased milk LC MUFA in late lactation raises the question: is there a benefit to the pup of increased milk LC MUFA? Fuel for postweaning fasting, thermoregulation and diving capability are all potential benefits that could be impacted by fatty acid distribution. It is unlikely that the increase in LC MUFA in the milk in this study would provide a preferential fuel for oxidation in during the postweaning fast, as LC MUFA is typically mobilized slower and oxidized slower than other fatty acids (DeLany et al. 2000; Astrup et al. 2010; Price et al. 2011).

Many marine mammals, including elephant seals, have displayed a stratification of blubber, where MC MUFA accumulates in the outer layer of the blubber, presumably for homeoviscous adaptation (Koopman et al. 1996; Koopman et al. 2002; Strandberg et al. 2008). Weddell seal milk displayed a pattern of increasing LC MUFA, although with lower absolute proportions, and pups were shown to store high proportions of MC MUFA and low proportions of LC MUFA (Wheatley et al. 2008), although blubber layers were not differentiated. In elephant seal pups, although the LC MUFA milk proportions increase across lactation, the predominant FA class in milk is still MC MUFA. MC MUFA stored in the pup blubber may contribute to insulative thermoregulatory benefits via deposition in the outer layer for maintaining fluidity at low temperatures, but increasing LC MUFA likely isn't driven

by a need to confer homeoviscous benefits unless there is modification prior to deposition in the pup blubber layer.

Trumble et al (2010) found that PUFA accumulated in the muscle of Weddell seal pups and hypothesized that PUFA were directed to the muscle for increased aerobic capacity for diving and non-shivering thermoregulatory benefits (Noren et al. 2008; Kanatous et al. 2008). Trumble et al (2010) also put forth the hypothesis that the availability of stored MUFA to oxidize could be important in order to spare PUFA for muscle development and non-shivering thermogenesis. It is unknown if this occurs in elephant seal pups, but would be topic for further investigation.

Aside from diving development and thermoregulation, fatty acids serve other metabolic roles in pups. In particular, long chain (>20 C) PUFA have been shown to be very important in neonatal development for humans, including neuronal development (Makrides et al. 1995; Larque et al. 2002). Long chain PUFA are typically concentrated in early lactation milk and decrease thereafter. C20:5n-3 displayed the typical mammalian pattern of decreasing across lactation. The lack of decrease of C22:6n-3 (docosahexaenoic acid; DHA) in milk from early to late lactation is a pattern that has been shown in other lactating phocids (Wheatly et al. 2008; Debier et al. 1999; Riedman and Ortiz 1979; Arriola Ortiz 2010). Seals in this study were sampled 5 days after birth, so any early spike in milk DHA levels may have been missed. Additionally, seal milk contains considerably higher levels of DHA compared to a recommended human milk level of 0.35% (seal milk contains ~

5%) (Larque et al. 2002), possibly negating the need for an early increase (Debier et al. 1999).

C20:5n-3 was the most efficiently mobilized and milk values reflect blubber values fairly closely (Figure 1.3). The proportion of milk C20:5n-3 decreases across lactation (Table 1.1), similar to Weddell seals (Wheatley et al. 2008) and grey seals (Arriola Ortiz 2010). Other pinniped studies have postulated selective mobilization of C20:5n-3 (Iverson et al. 1995b) and preferential deposition into milk (Wheatley et al. 2008). However, in this study, blubber proportions matched milk proportions rather closely and given the very high mobilization rate of this fatty acid and the fact that it is present in such excess amount relative to human milk (Fleith and Clandinin 2005) it is difficult to make a confident assertion that there is preferential deposition in the milk for the benefit of the pup.

Hypotheses regarding milk fatty acid distribution to thermoregulatory, sensory, metabolic and aerobic capacity development in pups warrant further investigation. Sampling the deposition of fatty acids into the different layers of pup blubber and the subsequent decline over the post-weaning fast as well as the relationship of the blubber fatty acids to pup muscle fatty acids and aerobic performance with the development of diving capability would inform the question of if a particular distribution of fatty acids in elephant seal milk may directly benefit pups.

Conclusion

In an animal the mobilizes an enormous proportion of its lipid stores over a very short period, elephant seal lipid mobilization in fasting and lactating seals conform to previously described patterns attributed to biochemical properties that govern the mobilization of fatty acids. This agreement with other animals demonstrates a conservation of lipid mobilization patterns across several genera in the face of very different energy demands and life history patterns. Fatty acids conferring a homeoviscous advantage were preserved in the outer layer, despite heavy mobilization of lipid stores. Blubber and milk fatty acid distributions were found to be very similar, implying very little *de novo* synthesis by the mammary gland. The pattern of fatty acid mobilization compared with the distribution in the milk did elucidate a few discrepancies, however. LC MUFA were least mobilized from the blubber, but increased in milk across the fast. LC MUFA may be more available for milk production late in lactation due to differential partitioning among maternal tissues. The way in which high MUFA content affects the metabolism and development of the pup and are avenues for future investigation to understand the potential adaptive significance of the observed patterns.

Tables

Table 1. 1: Proportion of fatty acids in inner and outer blubber layers and m ilk samples in early and late lactation in northern elephant seals. Values are expressed as mean percent (SD=standard deviation). MC MUFA= $\leq$ 18C monounsaturated fatty acids; LC MUFA= $\geq$ 20C monounsaturated fatty acids; PUFA=polyunsaturated fatty acid; SFA=saturated fatty acid

	Inner Blubber Early Lactation	SD	Inner Blubber Late Lactation	SD	Outer Blubber Early Lactation	SD	Outer Blubber Late Lactation	SD	Milk Early Lactation	SD	Milk Late Lactation	SD
C12:0	0.06	0.01	0.06	0.01	0.08	0.01	0.07	0.01	0.07	0.01	0.06	0.01
C14:0	3.85	0.44	3.91	0.71	2.56	0.18	2.45	0.20	3.06	0.34	3.29	0.51
C14:1n-5	0.11	0.04	0.12	0.04	0.22	0.03	0.22	0.03	0.09	0.02	0.09	0.03
C16:0	14.46	0.81	11.36	1.34	11.10	0.73	10.73	0.90	17.85	0.97	14.64	1.50
C16:1n-7	4.99	0.90	3.44	0.95	7.96	0.89	7.75	0.99	5.74	1.00	4.39	1.06
C18:0	3.73	0.21	3.86	0.24	2.72	0.26	2.62	0.24	3.62	0.35	3.78	0.24
C18:1cisn-11	2.53	0.53	3.27	0.46	3.88	0.84	4.49	0.62	5.83	0.78	5.51	0.80
C18:1cisn-7	3.83	1.01	3.74	1.26	4.73	0.68	4.76	0.56	4.67	0.88	4.52	1.01
C18:1cisn-9	30.99	2.07	29.77	3.13	39.91	1.81	40.18	1.87	35.13	2.70	32.87	2.42
C18:2cisn-6	1.69	0.10	1.76	0.13	2.21	0.15	2.20	0.13	1.79	0.11	1.75	0.09
C18:3cisn-3	0.50	0.05	0.37	0.05	0.50	0.05	0.47	0.06	0.60	0.06	0.57	0.15
C20:0	0.30	0.03	0.40	0.07	0.16	0.03	0.15	0.02	0.10	0.01	0.19	0.03
C20:1n-11	12.53	1.73	16.76	2.91	8.52	1.01	8.79	1.22	6.66	1.26	10.80	2.26
C20:1n-9	8.87	1.28	11.17	2.06	6.86	0.64	7.12	0.71	4.89	0.91	8.02	1.40
C20:3 n-3	0.19	0.06	0.15	0.03	0.12	0.03	0.10	0.03	0.16	0.03	0.22	0.04
C20:4 n-6	0.57	0.07	0.39	0.06	0.63	0.10	0.64	0.10	0.88	0.08	0.73	0.08
C20:5 n-3	2.22	0.88	0.74	0.42	1.30	0.36	1.09	0.39	2.80	0.99	1.40	0.81
C22:5 n-3	1.53	0.36	1.75	0.55	1.57	0.23	1.52	0.29	1.08	0.25	1.26	0.33
C22:6 n-3	6.38	0.96	5.93	1.21	4.76	0.65	4.46	0.75	4.89	0.80	5.64	0.88
C24:1 n-9	0.68	0.09	1.04	0.26	0.20	0.06	0.19	0.05	0.10	0.02	0.27	0.07
∑MC MUFA	42.45	2.40	40.35	3.41	56.71	1.96	57.40	1.50	51.45	1.85	47.37	1.92
∑LC MUFA	22.08	2.56	28.97	4.58	15.59	1.65	16.10	1.92	11.65	3.28	19.10	2.04
∑PUFA	13.09	1.71	11.16	1.98	11.09	1.19	10.48	1.44	12.21	1.87	11.69	1.81
∑SFA	22.39	1.09	19.41	1.55	16.61	0.80	16.02	0.99	24.70	1.38	21.88	1.94

Table 1. 2: Statistical comparison of fatty acids in inner and outer blubber cores and in milk in early and late lactation.
MC MUFA= ≤ 18C monounsaturated fatty acids; LC MUFA= ≥ 20C monounsaturated fatty acids PUFA=polyunsaturated fatty acid; SFA=saturated fatty acid. Italics highlight significant results.

	BLUBBER			
	MC MUFA	**LC MUFA**	**PUFA**	**SFA**
Early vs Late lactation (Inner blubber)	*$F_{(1,13)}$=14.2, p=0.002*	*$F_{(1,13)}$=69.3 p<0.001*	*$F_{(1,13)}$=42.7; p<0.001*	*$F_{(1,13)}$=54.9; p<0.001*
Early vs Late lactation (Outer blubber)	$F_{(1,13)}$=1.7, p=0.22	*$F_{(1,13)}$=7.9, p=0.01*	*$F_{(1,13)}$=4.8, p=0.045*	*$F_{(1,13)}$=8.4, p=0.01*
Inner versus outer (Early Lactation)	*t=-23.4, df=14; p<0.001*	*t=8.6, df=14, p<0.001*	*t=5.1, df=14, p<0.001*	*t=16.0, df=14, p<0.001*
Inner versus outer (Late Lactation)	*t=-20.9, df=16, p<0.001*	*t=11.0, df=16, p<0.001*	t=1.8, df=16, p=0.09	*t=9.0, df=16, p<0.001*

	MILK			
	MC MUFA	**LC MUFA**	**PUFA**	**SFA**
Early vs Late lactation	*$F_{(1,18)}$=246.2; p<0.001*	*$F_{(1,18)}$=375.0; p<0.001*	*$F_{(1,18)}$=4.9, p=0.04*	*$F_{(1,18)}$=135.8, p<0.001*

Table 1. 3: Proportion of fatty acid mobilized from blubber early to late lactation. Values are ordered from highest to lowest mobilization. See Methods for calculation details.

	Mean	Standard Deviation
C20:5 n-3	0.70	0.06
C20:3 n-3	0.53	0.10
C18:3cis n-3	0.52	0.04
C16:1n-7	0.51	0.05
C16:0	0.50	0.05
C20:4 n-6	0.49	0.14
C22:6 n-3	0.44	0.08
C18:1cis n-7	0.44	0.05
C18:1cis n-9	0.44	0.05
C14:0	0.43	0.05
C14:1n-5	0.43	0.07
C18:0	0.42	0.06
C18:2cis n-6	0.42	0.05
C12:0	0.39	0.14
C22:5 n-3	0.39	0.06
C20:1n-9	0.33	0.05
C20:0	0.31	0.08
C20:1n-11	0.30	0.08
C18:1cis n-11	0.28	0.13
C24:1 n-9	0.18	0.15

Table 1. 4: Similarity metrics comparing proportions of all fatty acids in inner blubber to all fatty acids milk in northern elephant seal early and late lactation. Metrics are expressed as means (standard deviation).

	Cosine	Percent
Early Lactation Blubber to Milk	0.967 (0.01)	86.05 (1.36)
Late Lactation Blubber to Milk	0.972 (0.01)	87.91 (3.1)
Early to Late Milk to Milk	0.98 (0.01)	89.98 (1.66)

Table 1. 5: Interspecies milk fatty acid composition comparisons

Species	% ≤ 18C MUFA	% ≥20C MUFA	% ≤18C PUFA	% ≥ 20C PUFA	% SFA	Reference
Human*	20-37	--	9-11	1-3	40-50	Neville and Picciano 1997; Koletzko et al 2001
Bovine*	33	--	5	<1	61	Rodriguez-Alcala et al 2009
Domestic cat*	< 1	44	21	3	32	Jacobsen et al 2004
Black bear*	25-73	<1	20-44	<1	30-15	Iverson et al 2001
Elephant seal	47-52	12-20	2	9	22-25	this study

*terrestrial forager

MUFA= monounsaturated fatty acids; MUFA= monounsaturated fatty acids; PUFA=polyunsaturated fatty acid; SFA=saturated fatty acid

Figures

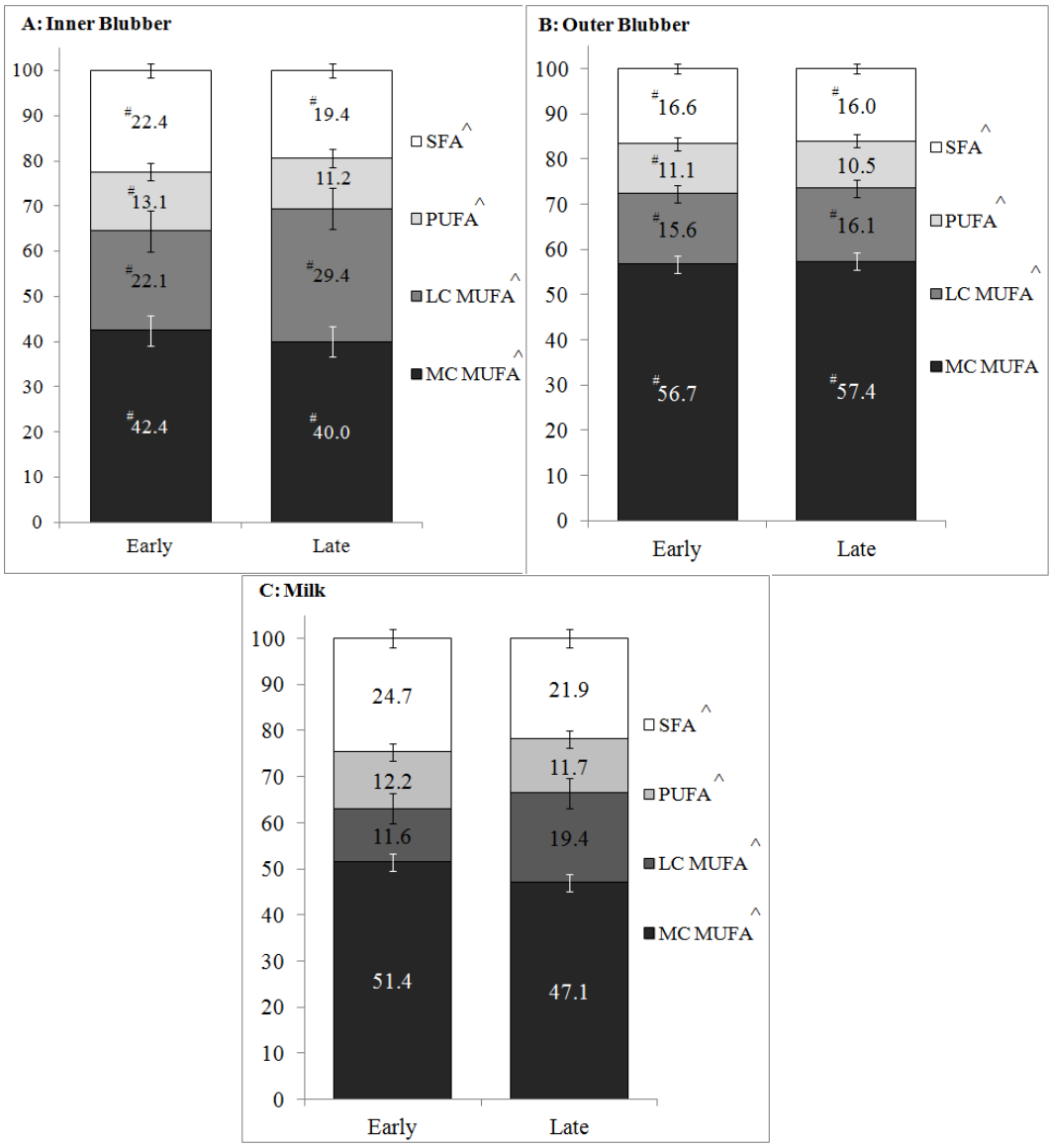

Figure 1. 1: Change in proportion of fatty acid classes in inner blubber (A), outer blubber (B), and milk (C) across lactation in northern elephant seals. Bars are standard deviations. ^=significantly different between stages; # =significantly different between blubber layers; SFA=saturated fatty acid; PUFA=polyunsaturated fatty acid, LC MUFA=long chain monounsaturated fatty acid; MC MUFA=medium chain monounsaturated fatty acid

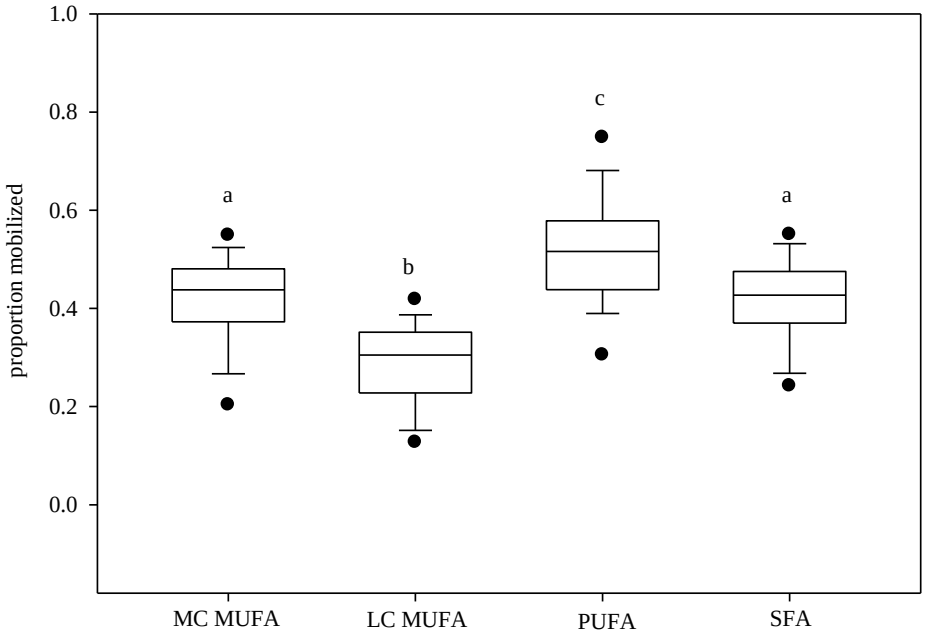

Figure 1. 2: Boxplot of proportion mobilized in different classes of lipid across lactation. Box boundaries are 25th and 75th percentiles, the line indicates the median and the whiskers denote 5th and 95th percentiles. Different letters indicate significantly different mobilization ($p<0.05$). MC MUFA=medium chain monounsaturated fatty; LC MUFA=long chain monounsaturated fatty acid; PUFA=polyunsaturated fatty acid; SFA=saturated fatty acid

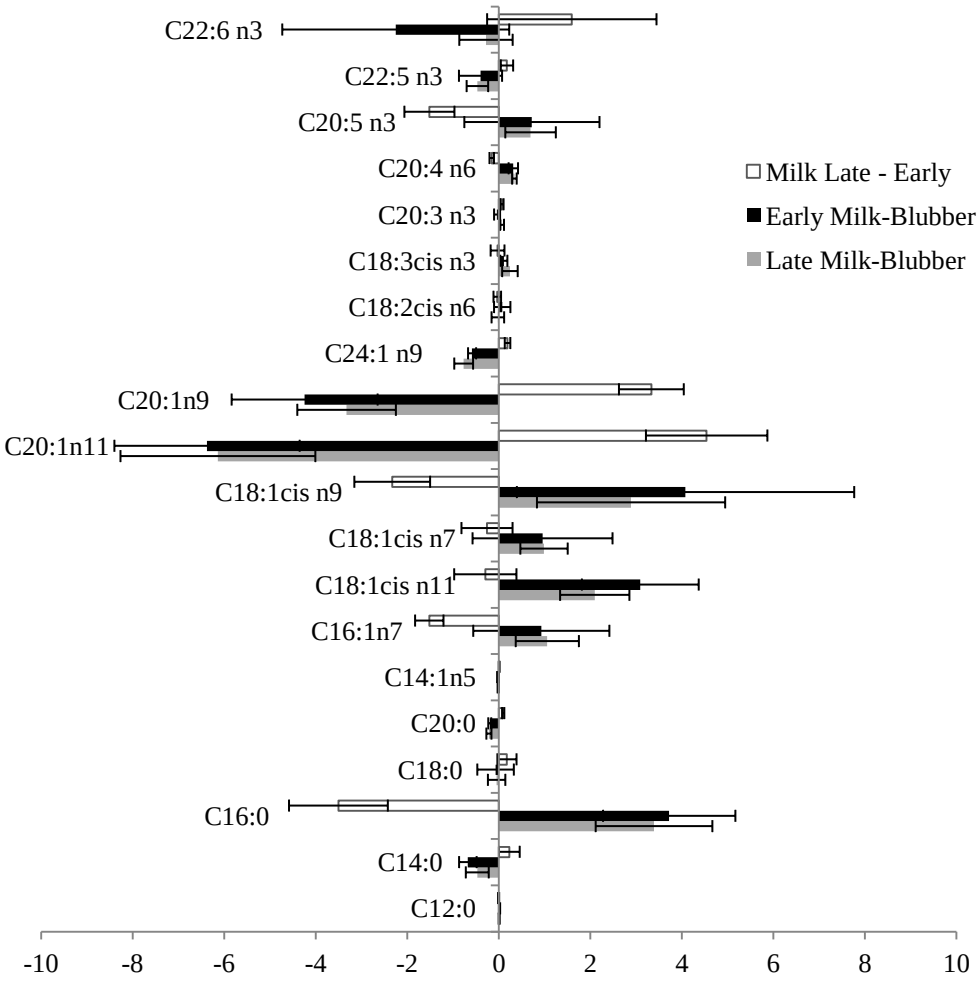

Figure 1. 3: Proportional differences in fatty acids from blubber to milk, in early and late lactation. Values are calculated by subtracting $\%FA_{milk}$-$\%FA_{blubber.}$ Negative values indicate a higher proportion in blubber than in milk. Milk FA values are $\%FA_{Late}$-$\%FA_{Early}$. Negative values indicate a decrease in milk proportions across lactation. Fatty acids are ordered vertically, with SFA at the bottom, ascending to MUFA and then PUFA. Blubber values are reflective of the inner layer.

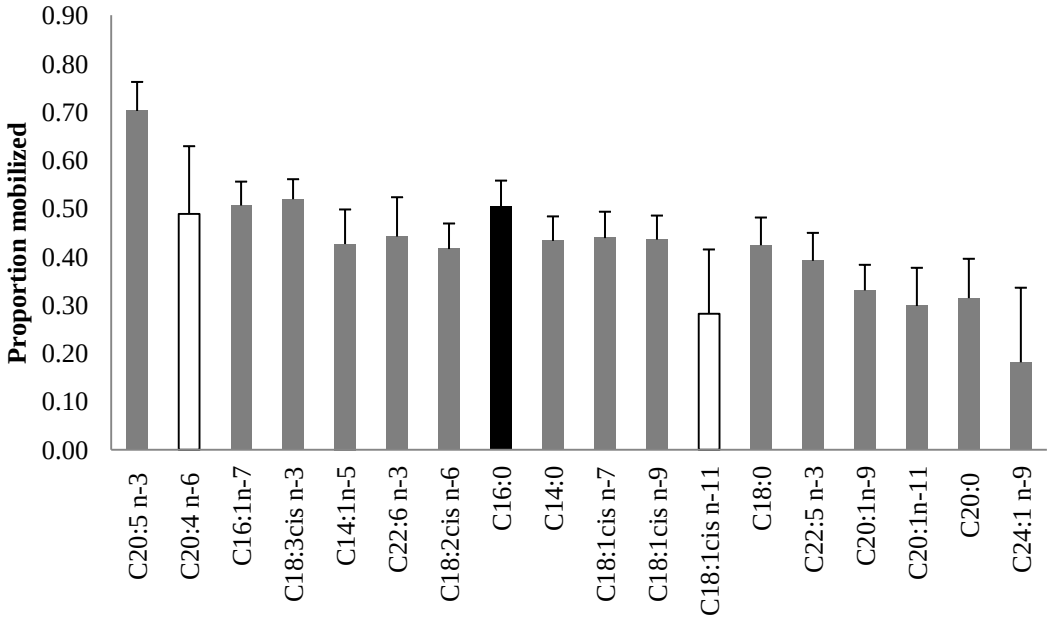

Figure 1. 4: The proportional mobilization of fatty acids across lactation in northern elephant seals. The fatty acids are ordered from left to right by decreasing expected order of mobilization (Raclot 03). Light bars reflect fatty acids that are slightly less mobilized than expected and dark bars are fatty acids that are somewhat more mobilized than expected. Error bars represent standard deviations. Estimates for C12:0 and C20:3n-3 were not available based on Raclot 03 and are not included in the figure.

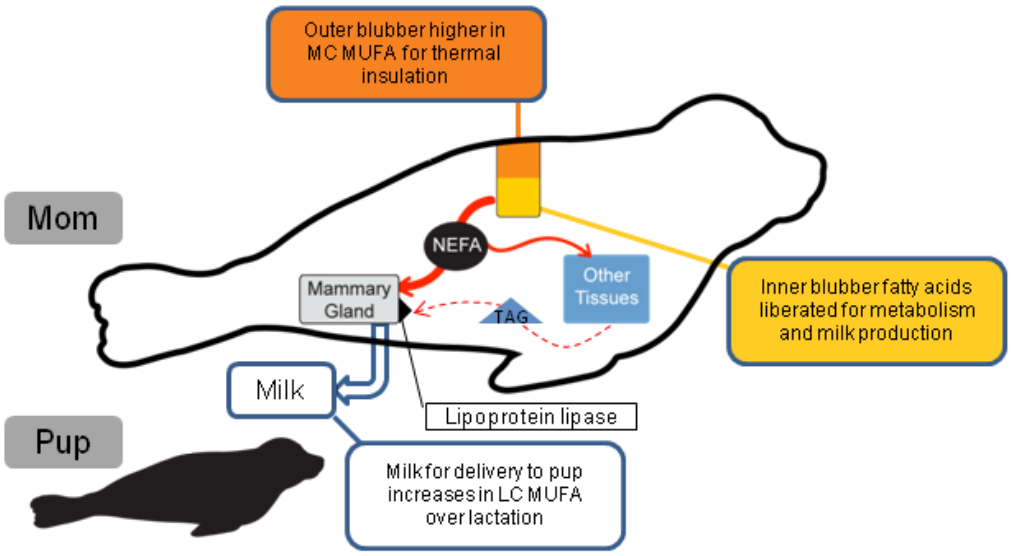

Figure 1. 5 Conceptual summary of mobilization of lipid from maternal blubber to non-esterified fatty acids (NEFA) in the bloodstream, potential re-esterfication by liver or other tissues and delivery to mammary gland for milk production or other tissues for oxidation. MC MUFA=medium chain monounsaturated fatty acids; LC MUFA=long chain fatty acids.

CHAPTER 2

THE DEMANDS OF LACTATION PROMOTE DIFFERENTIAL REGULATION OF LIPID STORES IN FASTING ELEPHANT SEALS

Abstract

Fasting animals must ration stored reserves appropriately for metabolic demands. Animals that experience fasting concomitant with other metabolically demanding activities are presented with conflicting demands of energy savings and energy expenditure. This study aimed to understand whether fasting, molting northern elephant seals and fasting, lactating northern elephant seals differentially regulate the mobilization of lipid reserves. Additionally, I aimed to understand how milk lipid content was regulated in lactating elephant seals. Thirty-six early lactation females and 39 late lactation females were sampled, while eight early molt females and six late molt females were sampled. Mass and adiposity were measured, as well as circulating non-esterified fatty acids (NEFA), triglycerides (TAG), cortisol, insulin and growth hormone levels. Milk was collected from lactating females and milk lipid content increased from 31% in early to 51% in late lactation ($p<0.05$). In lactating females increasing cortisol and decreasing insulin were significantly related to NEFA levels ($p<0.05$), but in molting seals, only variation in cortisol was related to NEFA ($p<0.05$). Milk lipid content varied with mass, adiposity, NEFA, TAG, cortisol and insulin ($p<0.05$). Growth hormone was not related to metabolites or milk lipid. Decreasing insulin appears to be the differential regulator of lipolysis in lactating seals versus molting seals, facilitating the additional liberation of stored lipids

required for milk synthesis. Milk lipid was strongly impacted by the supply of substrate to the mammary gland, indicating regulation at the level of mobilization of lipid reserves.

Introduction

Northern elephant seals experience fasting concomitant with energetically expensive activities in many life history stages, including post-weaning development (Ortiz et al. 1978), molting (Worthy et al. 1992) and breeding (Costa et al. 1986; Deutsch et al. 1990). Due to the combination of fasting with energetically expensive activities, fasting seals undergo extended fasts without the remarkable depression of metabolic rate seen in other fasting animals (Florant and Healy 2012). For example, the average daily metabolic rate of molting seals and lactating seals (without the inclusion of milk energy expenditure) are 2.0-2.4 times higher than the predicted metabolic rate for a similarly sized animal (Worthy et al. 1992; Costa et al. 1986; Crocker et al. 2001). The energy devoted to maintenance metabolism in both stages is fairly similar, but the inclusion of the remarkable amount of energy delivered to the pup in the milk lipid highlights the difference in adipose store depletion between molting and lactation. The adipose stores of the lactating females decreases more than the fasting, molting seal (Costa et al. 1986; Worthy et al. 1992; Crocker et al. 2001), in order to provide substrate for milk lipid. These differences in energy expenditure and nutrient delivery suggest the possibility of differential regulation of lipid metabolism between life history stages.

The focus of this study was to investigate how stored reserves are partitioned during the different fasting stages in the northern elephant seal (molting and lactation). I compared the effects of hormones on lipid mobilization to seals that are not experiencing the demand of milk production, but are fasting and molting. I also aimed to evaluate the effects of hormones on lipid mobilization and subsequent allocation to milk in fasting and lactating seals.

Growth hormone (GH), cortisol and insulin are three hormones that can affect the mobilization and use of fuel stores. GH and cortisol can increase lipolysis (Djurhuus et al. 2004), while insulin inhibits lipid breakdown (Frayn et al. 1994). GH and cortisol have opposite effects on protein metabolism; GH promotes lean tissue accretion (Norrelund et al. 2001), while cortisol promotes protein catabolism (Brillon et al. 1995). Elephant seals undergoing fasts in different life history stages experience different challenges regarding the partitioning of their lipid and protein reserves and hormones likely play a large role in managing fuel catabolism.

Lactating elephant seals mobilize large quantities of stored lipid and deplete their protein stores more than any other age class (Crocker et al. 1998; Crocker et al. 2001; Houser and Costa 2001), while molting females deplete less of their lipid stores, but still have the protein requirements of synthesizing new skin and pelage (Worthy et al. 1992). Post weaning fasting pups must develop muscle and blood volume, among other physiological adjustments, to prepare for their first foraging trip (Thorson and Le Boeuf 1994; Tift et al. 2012). Adult breeding males fast for up to 3 months, sustain high metabolic rates while actively competing for females and yet

catabolize very little of the protein stores (Crocker et al. 2012a). Juvenile fasting male elephant seals spare protein stores more effectively than juvenile females (Kelso et al. 2012).

Increases in GH while fasting is a typical response in other species (Ho et al. 1988; Eigenmann et al. 1985; Webster et al. 1999). Fasting pups (Ortiz et al. 2003) and lactating females (McDonald 2003), show an increase in GH across the fasting duration. GH decreases over ~25-44 days of fasting in juvenile and adult males elephant seals, even though these groups exhibit efficient protein sparing (Crocker et al. 2012b; Kelso et al. 2012). The differences among age classes suggest either potential differential action of GH, or the importance of other hormones to lipolysis. GH has also been shown to affect milk lipid content in domestic species (Eppard et al. 1985; Bitman et al. 1984), which makes it a primary candidate for enacting large effects in fasting and lactating seals.

Cortisol levels increased in fasting weaned pups (Champagne et al. 2005) and both northern and southern lactating female elephant seals (Engelhard et al. 2002; Champagne et al. 2006), but cortisol levels remained stable in fasting juveniles (Kelso et al. 2012) and adult males (Crocker et al. 2012b), again raising the question of differential actions in different age classes. GH and cortisol were thought to act synergistically to promote lipolysis in weaned pups (Ortiz et al. 2003). Lipolysis was not related to blood cortisol concentrations in lactating seals (Houser et al. 2007), but the combined effect of these hormones on lipid stores is unclear in molting and

lactating, fasting seals. The combined effect of lipolytic hormones on milk lipid content is also unclear.

Insulin, an anti-lipolytic hormone, also likely affects various age classes of fasting seals differently. Insulin remained stable in fasting juveniles (Kelso et al. 2012) and adult males (Crocker et al. 2012b) over several weeks of fasting. During the postweaning fast, both stable (Champagne et al. 2005) and decreasing (Viscarra et al. 2011b) basal insulin levels were observed. The importance of reducing circulating insulin level in lactation was highlighted by the abolishment of the insulin response to a glucose challenge by late lactation (Fowler et al. 2008), suggesting that facilitating lipid mobilization with low insulin levels supersedes the need to regulate an increase in circulating glucose levels in females late in lactation. Reducing circulating levels of insulin may be important to maintaining appropriate lipid mobilization for the demands of lipid rich milk production, but this has not been directly assessed.

Given the multiple effects of these hormones and the variation between lipolysis rates, lipid metabolites and hormones in different fasting stages in elephant seals, our understanding of how these hormones interact and affect lipid mobilization and milk lipid content remains superficial. I hypothesized that GH and cortisol, both lipolytic hormones, would be positively related to NEFA, as well as to milk lipid content. Insulin (an anti-lipolytic hormone) was expected to be negatively related to NEFA and milk lipid. Circulating NEFA was also hypothesized to be positively related to milk lipid content, while TAG levels were not expected to be positively related to milk lipid content.

The mechanisms underlying nutrient mobilization from reserves and delivery to the mammary gland are crucial in linking foraging success at sea to parental investment on land in numerous pinniped species. Variation in nutrient mobilization potentially affect both the magnitude and composition of milk production and the resulting level of parental investment in offspring. Similarly, the factors influencing the mobilization and use of body reserves may impact the physiological state of the female, influencing the fitness costs of reproduction and future survival.

Methods

Study Site and Individual Animals

This study was carried out at Año Nuevo State Reserve, San Mateo County, during the 2005 and 2010 breeding seasons (Jan-Feb) and 2009 molting period (Apr-May). Soon after arrival on land, adult female seals were marked with hair dye (Lady Clairol, Stamford, CT) to facilitate identification. Parturition dates were established by daily observations and considered to be the first day a marked female was observed with a pup, provided she had been observed without a pup the previous day. In 2005, 18 females were sampled in early lactation (day 5 post-partum) and all females were recaptured late in lactation (day 22 post partum) plus one additional female and the sampling procedures repeated. In 2010, 18 females were sampled in early lactation (day 5-7) and 20 females captured in late lactation (day 21-25), seven of which were repeated individuals from early lactation. Eight early molt females were captured within five days of arriving on land and six late molt study females were selected on the basis of fully molted pelage, ensuring ~3 weeks of fasting (Le

Boeuf and Laws 1994). Sampling was conducted under NMFS permit 87-1743-06 and all procedures were approved by the Sonoma State University and University of California Santa Cruz IACUC.

Sample Collection and Processing

Females were initially immobilized with Telazol (tiletamine/zolazepam HCl, Fort Dodge Labs, Ft. Dodge, IA) at a dosage of ~1mg/kg, administrated intramuscularly. Continued immobilization was maintained with ~100 mg bolus intravenous injections of ketamine. Milk (~5 mL) was collected after 40 units of oxytocin were administered intramuscularly. Serum and plasma were collected into chilled vacutainers (heparinized tubes for plasma samples) within 20 minutes of sedation. Samples were immediately placed on ice and transported back to laboratory within 2-3 hours. Samples were centrifuged at 4°C, and frozen at -80ºC until further analysis.

Body composition measurements were made using the truncated cones method (Crocker et al. 2001; Gales and Burton 1987). Dorsal, lateral and ventral blubber depth measurements were made using a portable ultrasound (Ithaca Scanprobe, Ithaca, NY) at each of six locations along the seal. Lengths and girths were taken at these six points, as well as total curved length. The blubber and lean tissue volumes were calculated, using assumed densities of blubber (0.94 g/ml) and lean tissue (1.1 g/ml; Webb et al. 1998). Mass was measured using a tripod, canvas sling and scale (± 1 kg) MSI, Seattle, WA).

NEFA was measured in triplicate in plasma (2010 and 2009) and serum (2005) samples using a commercially available kit (Wako Diagnostics, Richmond, VA). Mean %CV of the assay was 3.65%. For 2009 molt samples and 2010 breeding samples, serum TAG was measured in duplicate after the methods of Tift et al (2011), using a Cholestech LDX Analyzer (Cholestech, Hayward, CA). The lower detection limit was 45 mg/dL. Sixteen of the 53 samples measured were below the detectable limit and so were assigned the value of 45 mg/dL. The average %CV was 4.34%. Samples from 2005 were analyzed using commercially available kits (Wako Diagnostics, Richmond, VA).

For 2010 samples, milk lipid was extracted in duplicate by the Roese-Gottleib method (McDonald and Crocker 2006), including six washes with 20 mL ethyl ether and 20 mL petroleum ether to ensure complete extraction. Milk samples from 2005 were analyzed after (Debier et al. 2003). Briefly, milk lipids were extracted using an accelerated solvent extractor (Dionex ASE 2000, Dionex Corporation) with a mixture of hexane, dichloromethane and methanol at 80°C under a pressure of 1500 Psi. The fat content of milk samples was then determined gravimetrically.

Hormones were assayed in duplicate in serum samples from molt season 2009 and breeding season 2010. Insulin was assayed using radioimmunoassay (Sensitive Rat, Millipore, Catalog #SRI-13K). This kit has been previously validated for northern elephant seals (Champagne et al. 2005). The mean %CV for the assay was 1.88%.

GH in 2005 was measured with by radioimmunoassay (Linco, catalog # PGH-46HK) and has been validated previously (Ortiz et al. 2003). GH in 2010 was measured using rat/mouse growth hormone ELISA assay (Millipore, catalog # EZRMGH-45K). The kit was validated by assessing the slope of the standard curve to serially diluted elephant seal plasma samples. The slopes were 0.042 and 0.048 in the standard and seal serum, respectively. The mean %CV was 7.22%.

Cortisol was measured by radioimmunoassay (Siemens, Catalog #TKCO2). This kit has previously been validated for elephant seals (Champagne et al. 2005; Ortiz et al. 2001). The mean %CV was 2.57%.

Statistics

Statistical analyses were performed using the software R (Version 2.14.1, R Development Core Team, www.R-project.org) and JMP v10 (SAS Institute, Cary, NC). Multicollinearity among all variables was assessed using a variance inflation factor (VIF) calculated in R with the `car` package (Fox and Weisberg 2011). A cut off of 5 was assigned to the VIF to determine undue influence of multicollinearity and no variables were found to be above 3. Variables found to deviate from a normal distribution were normalized by a $\log_{(10)}$ transformation. Milk lipid proportions were square root arcsin transformed. Four fasting stages were analyzed, early lactation, late lactation, early molt and late molt. Due to the paired nature of some samples, a linear mixed model was used to assess differences between fasting stages, with individual seal included as a random effect. No interactions were found to be significant in any model and so they were excluded. Tukey's HSD was used to

evaluate pairwise comparisons following a significant mixed model. Least squares linear regression was used to look at the relationship between variables for analyses that did not involve matched individuals. The significance of all results was considered at α=0.05.

Results

Differences among fasting stages

Mass declined significantly from 475 (SD=65) kg in early lactation to 347 (SD=47) kg in late lactation ($F_{3,39}$=361.6, p<0.001, Table 2.1). Early lactation females were the largest seals followed by early molt females at 425 (SD=32) kg. Late lactation and late molt (304, SD=30 kg) were similar, as the groups with the least mass ($F_{3,39}$=361.6, p<0.001) (Figure 2.1). Despite the differences in mass, only early lactation females had significantly more adipose stores than other stages of fasting ($F_{3,73}$=57.3, p<0.001) (Table 2.1; Figure 2.2). Milk lipid increased significantly from 31.01 (SD=5.43)% in early lactation to 50.61 (SD=5.45)% in late lactation ($F_{1,44}$=291.24, p<0.001) (Table 2.1).

Cortisol displayed the largest magnitude of change among fasting stages, increasing tenfold across the molt fast. Early molt levels were significantly lower than all other stages at 0.84 (SD=0.18) µg/dL, and levels increased over both the molt fast and lactation fast to display equivalent values in late molt and late lactation ($F_{3,73}$=53.65, p<0.001) (Table 2.1; Figure 2.3). Insulin decreased across the lactation fast, but levels were not different among late lactation and early and late molt ($F_{3,71}$=19.56, p<0.001) (Figure 2.4). Additionally, NEFA levels increased across

lactation, to a peak of 2.59 (SD=1.12) mmol/L in late lactation, but early and late molt were found to be equivalent to early lactation levels ($F_{3,73}$=25.33, p<0.001) (Figure 2.5). Although TAG differed between early (67.45 (SD=26.40) mg/dL) and late lactation (82.23 (SD=26.43) mg/dL), molt values were not different from either lactation stage ($F_{3,68}$=4.5, p<0.006) (Figure 2.6). GH displayed a similar pattern, with an increase over lactation from 2.5 (SD=1.01) ng/mL early to 3.03 (SD=1.17) ng/mL late, but molt values were not significantly different from either lactation stage ($F_{3,57}$=3.6, p=0.02) (Figure 2.7).

Hormone-Metabolite Relationships

When the cumulative effects of hormones on circulating NEFA were assessed in lactating seals, insulin ($F_{1,70}$=6.65, p=0.01) and cortisol ($F_{1,71}$=29.65, p<0.001) were found to affect NEFA, while GH did not ($F_{1,62}$=1.31, p=0.22). Interestingly, when lactation stages were separated, and the effect of individual hormones on NEFA analyzed within early or late lactation, the positive relationship between cortisol and NEFA in late lactation (R^2=0.14, p=0.02;) was the only detectable effect. The full model including all hormones and both lactation stages highlights the importance of the decrease in insulin and the increase in cortisol across lactation to circulating NEFA. Addressing all three hormones in molting animals, only the significant effect of cortisol ($F_{1,10}$=9.8, p=0.01) was detected; insulin and GH were not significantly related to NEFA levels. Even when hormones in molting seals were assessed separately relative to NEFA, rather than in the full model, only cortisol significantly affected NEFA (Figure 2.8).

A mixed model approach was also applied to assess the effect of hormones on TAG. In lactating animals only cortisol significantly affected TAG ($F_{1,68}$=8.34, p=0.005), while in molting seals none of the hormones significantly affected TAG (p>0.05).

Milk Lipid Variation

Decreasing mass ($F_{1,52}$=6.95, p=0.01), decreasing adiposity ($F_{1,70}$=23.62, p<0.001), increasing NEFA ($F_{1,70}$=8.34, p=0.005), and increasing TAG ($F_{1,70}$=5.92, p=0.02) were significantly related to milk lipid across lactation. The cumulative effect of hormones was also assessed relative to milk lipid content. Increasing cortisol levels ($F_{1,69}$=22.36, p<0.001) and decreasing insulin ($F_{1,65}$=24.27, p<0.001) were significantly related to milk lipid levels, while GH levels did not affect milk lipid (p>0.05).

Discussion

Hormonal Effects on Lipid Metabolites

The release of lipid stores is regulated differently in fasting and molting seals compared to fasting and lactating seals. Increased cortisol and decreased insulin facilitated the release of NEFA from lipid stores in lactating females, while cortisol was the main effector of circulating NEFA levels in molting seals. Both cortisol and GH have been shown to stimulate lipolysis in other studies (Djurhuus et al. 2004; Xu et al. 2009), while insulin inhibits lipolysis (Frayn et al. 1994). My hypothesis was that cortisol, a lipolytic hormone would be positively related to NEFA levels and insulin would be negatively related. The results followed my hypothesis, but the

effect of insulin on lipid release was different between molt and lactation. The removal of lipolytic inhibition, promoted by the decrease in insulin, is key in facilitating the accelerated release of NEFA during lactation, relative to the molting females (Figure 2.9).

The additional effect of decreasing insulin on lipid release differentiates lactating seals from molting seals, indicating they are regulating lipid release differently. Indeed, molting seals lose less of their blubber stores (Figure 2.2) and while the average daily metabolic rates (excluding milk energy) are similar between fasting and molting seals, lactating seals incorporate ~ 4 kg of lipid into milk daily, expending almost 50% of their total energy reserves (Costa et al. 1986; Worthy et al. 1992; Crocker et al. 2001). These two different life history stages have vastly different energy demands the demand for lipid release may not be as high in molting seals. This study found that insulin is an important regulator differentiating the rates of lipid mobilization between lactation and molting.

Insulin plays an important role in multiple aspects of both carbohydrate and lipid metabolism. The inhibition of lipolysis is an important property of insulin in fasting animals, which rely on the mobilization lipid stores. Fasting northern elephant seals pups display insulin resistance in adipose tissue have decreased insulin signaling in adipose tissue across the fast (Viscarra et al. 2011b; Viscarra et al. 2011a). The magnitude of insulin response to a glucose challenge was abolished across the fasting duration in lactating elephant seals. Maintaining very low levels of insulin, an anti-lipolytic hormone, demonstrate the importance of maintaining lipid release, even at

the expense of glucose disposal (Fowler et al. 2008). The current study agrees with previous assertions that a decrease in insulin is important in fasting elephant seals.

In addition to insulin's effect on lipid metabolism, it plays an important role in carbohydrate metabolism. Fasting elephant seals display increased plasma glucose levels, as well as high endogenous glucose production (Champagne et al. 2005; Champagne et al. 2006). In weaned pups, recycling glucose through 3-carbon intermediates such as lactate contributes to the high rates of glucose production (Tavoni et al. 2012; Champagne et al. 2012) and it is likely that females utilize a similar mechanism. Insulin was positively related to both lactate turnover and glucose disposal in fasting weaned elephant seal pups (Houser et al. 2012; Tavoni et al. 2012).

The influence of insulin on glucose metabolism may affect lipid metabolism. Fasting elephant seals exhibit high rates of lipid mobilization and subsequent beta-oxidation, resulting in large amounts of acetyl-CoA production. Acetyl-CoA acceptance into the TCA cycle depends on an adequate pool of TCA cycle intermediates (namely oxaloacetate). If sufficient TCA cycle substrates are not available, excess acetyl-CoA molecules are converted into ketones; at high levels these may lead to metabolic ketoacidosis. The high rates of TCA cycle activity required in fasting elephant seals necessitate high levels of TCA cycle intermediates; these may be supplied by high rates of glucose cycling. In elephant seals, high rates of TCA cycle have been measured, with intermediates supplied by high glucose cycling rates (Champagne et al. 2012). Fasting elephant seals have very low levels of

ketones, despite their high rates of lipid oxidation (Champagne et al. 2005; Houser et al. 2007). The supply of TCA cycle intermediates from glucose cycling has been hypothesized to contribute to high TCA cycle activity in elephant seals, facilitating the entry of acetyl-CoA into the TCA cycle, alleviating ketone accumulation.

Both GH and cortisol were predicted to be related to circulating lipid metabolites, given that both of these hormones stimulate lipid breakdown (Djurhuus et al. 2004). Despite GH's lipolytic actions in other studies, GH's influence on lipid mobilization is contrary to that expected in lactating elephant seals. Ortiz et al. (2003) found that NEFA increased significantly with both cortisol and GH in fasting elephant seal weanlings and suggested that cortisol and GH may act synergistically to promote lipolysis. In adult, lactating elephant seals, no effect of GH was detected on lipid metabolites. In fasting adult male elephant seals GH and lipid metabolism appear uncoupled (Crocker et al. 2012b), with a decrease of GH across the fast. Thus, despite the differing patterns of baseline level change across the fast between adult males and females, the pattern of uncoupling GH from lipid catabolism appears consistent. GH patterns in fasting seals appear to diverge from typical mammalian patterns. Effects of GH are likely complex and dependent on specific demands during the fasting stage (i.e. growth or protein catabolism) (Kelso et al. 2012; Crocker et al. 2012b; Ortiz et al. 2003). The demand for maintenance of protein stores may differ between juveniles, males and females.

Cortisol was positively related to circulating TAG levels during lactation in the current study. Circulating NEFA may be removed from the bloodstream and re-

esterified to TAG. The newly resynthesized TAG is then incorporated into a lipoprotein complex and secreted into the bloodstream (Figure 2.9). The liver is traditionally viewed as the major source NEFA re-esterification and TAG release, but in humans, extrahepatic tissues have been shown to contribute about half of all re-esterification (Diraison and Beylot 1998). Houser et al (2007) found stable lipolysis rates across lactation in northern elephant seals, but plasma NEFA levels increased over the same interval. This combination of stable rates of lipolysis with increased plasma NEFA suggests that re-esterification rates decreased across lactation. However, while there is evidence that cortisol stimulates enzymes involved in re-esterification, there are not conclusive patterns on the effect of cortisol on the secretion of TAG by the liver (Dolinsky et al. 2004; Glenny and Brindley 1978). Thus, the mechanism by which cortisol affects TAG is unclear.

Milk Lipid Content

Lipid released from reserves travel in the bloodstream to be utilized by other tissues, and in the case of milk production, the mammary gland. NEFA bound to albumin and TAG bound to lipoproteins are the two predominant forms of lipid in the bloodstream. Circulating NEFA can diffuse passively across membrane borders down a concentration gradient, or be facilitated by transport proteins (Glatz et al. 2010), while TAG must be hydrolyzed before entering tissue. Lipoprotein lipase (LPL) is the primary enzyme enabling tissues to uptake circulating lipoproteins to metabolize (Frayn et al. 1995). Therefore, in order for TAG to be utilized for milk lipid production, it must be hydrolyzed by mammary LPL to facilitate entry. In this

study, the positive relationship of TAG to milk lipid content was unexpected given the low mammary LPL levels observed by McDonald and Crocker (2006) Additionally, McDonald and Crocker (2006) did not detect a relationship between LPL and milk lipid content. The relationship of TAG to milk lipid suggests that it is being removed from the bloodstream by LPL and contributing to milk lipid. My hypothesis in this study was that NEFA would be positively related to milk lipid, and while that was upheld, it appears that TAG is also contributing to milk lipid.

Mass and adiposity significantly affected milk lipid, indicating that the amount of endogenous resources a female has available is an important predictor of the amount of lipid she deposits into her milk. The strong effect of NEFA on milk lipid supports the hypothesis that there is regulation of milk lipid at the level of supply of lipid substrate, rather than dictated purely by uptake by the mammary gland. Crocker et al (2001) also found mass and body composition influence milk energy output in northern elephant seals. Interestingly, in another northern elephant seal study, mass and milk lipid were positively related early in lactation, but neither NEFA nor TAG were related to milk lipid (McDonald 2003). Clearly the release of substrate from lipid stores is an important factor. The dominant metabolic process determining milk lipid content, however, may be either lipid release from adipose tissue or uptake by the mammary tissue and might, in fact, be species dependent. In both grey and hooded seals, two related phocid species hypothesized to regulate milk lipid at the level of the mammary gland (Mellish et al. 1999b; Iverson et al. 1995a), NEFA significantly correlated with milk lipid (Mellish and Iverson 2001). In grey

seals, neither mass nor adiposity explained milk fat content (Mellish et al. 1999a). While NEFA seems to imply in that lipid supply is important to milk content in both these species, the lack of relationship of adiposity and mass to milk lipid suggests the mammary gland has tighter control of milk lipid content in hooded and grey seals, contrasting with the current study, indicating that the regulation at the level of release from lipid stores may play a large role in milk lipid content in elephant seals.

In cattle, lipolysis rates have been shown to be positively related to milk fat output (McNamara and Hillers 1986a), and thus in an animal as dependent on lipid mobilization as an elephant seal, I predicted that either lipolytic hormones or by-products of lipid breakdown may be related to milk lipid content. GH, which has been shown to affect milk lipid content in domestic species (Eppard et al. 1985; Bitman et al. 1984), as well as lipolysis (Djurhuus et al. 2004) was not related to milk lipid content in this study. Both the present study and McDonald (2003) failed to detect an association between GH and milk fat content.

Cortisol and insulin affected milk lipid content, and given their significant effect on NEFA, the mechanism by which they are affecting milk lipid is likely through liberating fat stores to increase availability of NEFA to the mammary gland (Figure 2.9). Cortisol affects the mammary gland in several ways, as a transcription factor for many milk proteins (Casey and Plaut 2007) as well as lipogenesis (Dils et al. 1976). However, in elephant seal mammary gland, there is presumably little *de novo* lipogenesis (see Chapter 1 of this thesis and (Iverson et al. 2004). The effect of cortisol would thus likely be on adipose tissue lipid breakdown, rather than mammary

gland lipogenic stimulation. Although cortisol may stimulate mammary LPL (Flint et al. 1984), this is unlikely in elephant seals because mammary LPL activity is low (McDonald and Crocker 2006). The relationship of cortisol to both NEFA and milk lipid indicates that cortisol is probably affecting milk lipid content via increased lipolysis to provide substrates to the mammary gland.

Insulin affects many tissues throughout the body. Insulin stimulates the ability of tissues to uptake circulating TAG via LPL (Kraemer et al. 1998) and elevated insulin can decrease milk fat yield and NEFA availability (Corl et al. 2006). In several species, insulin levels decrease during lactation and tissue insulin sensitivity increases (humans, (Tigas et al. 2002), sheep (Hatfield et al. 1999), cattle (Sartin et al. 1985), goats (Debras et al. 1989), and rats (Burnol et al. 1986). Based on the inhibitory effect of insulin on lipolysis (Frayn et al. 1994) and the lack of milk lipid regulation via LPL (McDonald and Crocker 2006), I hypothesized that insulin would have a negative relationship to milk lipid content, which was supported. This relationship was only apparent when concomitant cortisol levels were included in the analysis. The negative relationship between insulin and NEFA release in lactating females indicates that insulin is likely affecting milk lipid content via the provision of NEFA; the removal of the inhibition of insulin on lipolysis is critical to the supply of NEFA to the mammary gland.

Conclusions

This work assessed how fasting animals regulate lipid stores during energetically costly life history stages, molting and lactation. I elucidated the actions

of three hormones, cortisol, GH and insulin on lipid mobilization during two life history stages, molting and lactation. Additionally, I investigated how hormones, lipid metabolites and body reserves impacted milk lipid content in lactating seals. The lipolytic actions of cortisol combined with the reduction of the anti-lipolyic hormone insulin facilitate the mobilization of ~ 60% (Crocker et al. 2001) of lipid stores (~70 kg) across lactation. Molting seals experience less demand for lipid mobilization; insulin did not decline during molting as it did during fasting. Insulin therefore, contributes to differential lipid store regulation in lactating versus molting seals. GH was surprisingly not found to be related to lipid mobilization or milk lipid content in elephant seals, and appears uncoupled from lipid mobilization as in adult males (Crocker et al. 2012b). While the contribution of TAG to milk lipid levels indicates that mammary LPL may also be involved in facilitating the entry of lipid into the mammary gland, there was still strong evidence for regulation at the level of release of lipid stores. Significant effects of mass and adiposity, along with the contribution of NEFA to milk lipid content, suggests that there is considerable regulation of milk lipid content at the level of the release of lipid rather than at entry to the mammary gland.

Tables

Table 2. 1: Hormone, metabolite and mass changes during fasting and lactation and fasting and molting northern elephant seals. Values are expressed as means (standard deviation)

	Lactation		**Molt**	
	Early Lactation (n=36)	Late Lactation (n=39)	Early Molt (n=8)	Late Molt (n=6)
Mass (kg)	475 (64)[a]	347 (47)[b]	425 (32)[c]	304 (30)[b]
Adiposity (proportion)	0.36 (0.03)[a]	0.30 (0.02)[b]	0.32 (0.02)[b]	0.30 (0.01)[b]
TAG (mg/dL)	67.45 (26.40)[a]	82.23 (26.43)[b]	57.81 (15.20)[ab]	71.61 (21.87)[ab]
NEFA (mmol/L)	1.27 (0.44)[a]	2.59 (1.12)[b]	1.00 (0.02)[a]	1.52 (0.47)[a]
Milk lipid (% composition)	31.01 (5.43)[a]	50.61 (5.45)[b]	NA	NA
Cortisol (µg/dL)	6.48 (1.87)[a]	9.93 (2.18)[b]	0.84 (0.18)[c]	9.23 (2.66)[b]
Insulin (pg/mL)	82.05 (25.98)[a]	44.43 (22.83)[b]	42.03 (22.08)[b]	47.08 (21.4)[b]
GH (ng/mL)	2.5 (1.01)[a]	3.03 (1.17)[b]	2.98 (1.31)[ab]	3.60 (1.89)[ab]

NEFA=non esterified fatty acids

TAG= triacyglycerol

Values that do not share superscripts within rows that are significantly different (Tukey's $p<0.05$).

Figures

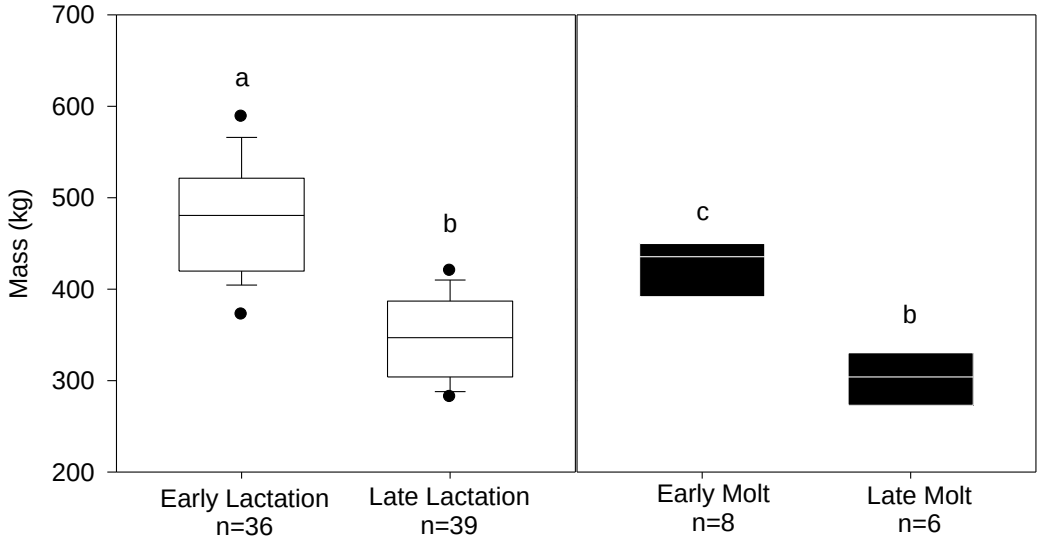

Figure 2. 1: Mass changes in 2005 and 2010 breeding elephant seals compared to 2010 molting elephant seals. Box boundaries are 25th and 75th percentiles. 5th and 95th percentiles are shown by black dots. Boxes that do not share a letter are significantly different between stages (Tukey's HSD $p<0.05$).

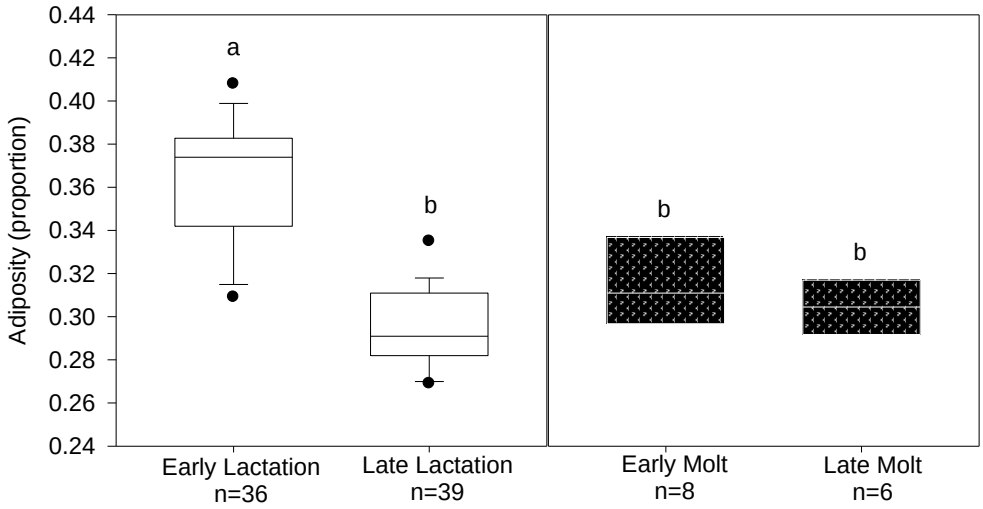

Figure 2. 2: Adiposity changes in 2005 and 2010 breeding elephant seals compared to 2010 molting elephant seals. Box boundaries are 25th and 75th percentiles. 5th and 95th percentiles are shown by black dots. Boxes that do not share a letter are significantly different between stages (Tukey's HSD $p<0.05$).

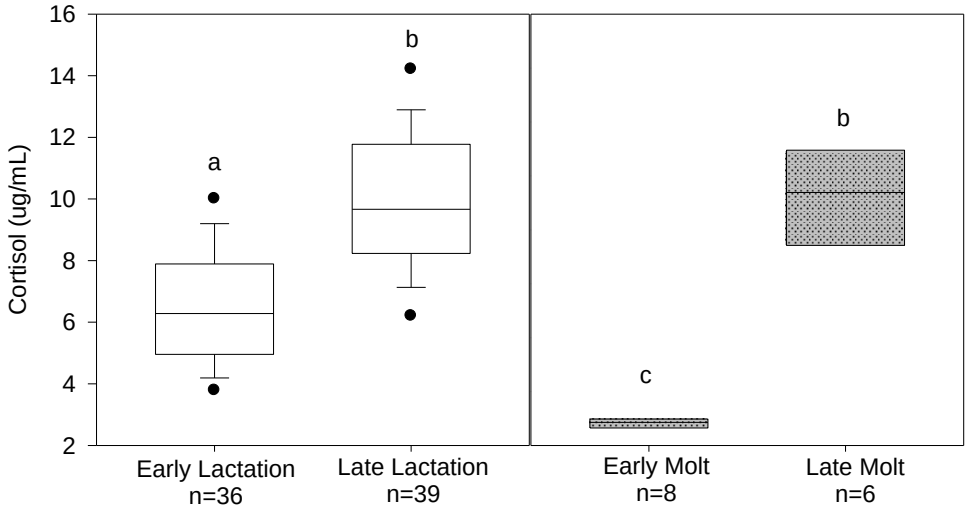

Figure 2. 3: Cortisol changes in 2005 and 2010 breeding elephant seals compared to 2010 molting elephant seals. Box boundaries are 25th and 75th percentiles. 5th and 95th percentiles are shown by black dots. Boxes that do not share a letter are significantly different between stages (Tukey's HSD $p<0.05$).

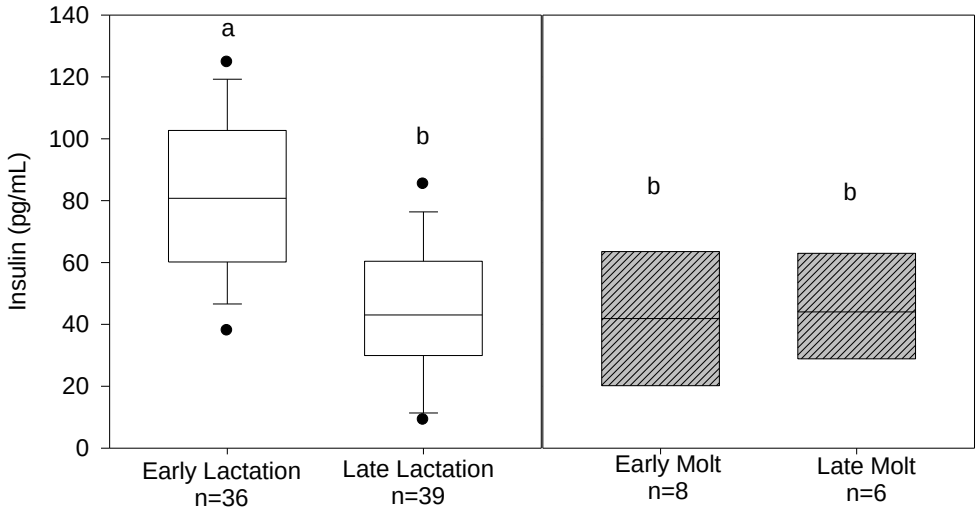

Figure 2. 4: Insulin changes in 2005 and 2010 breeding elephant seals compared to 2010 molting elephant seals. Box boundaries are 25th and 75th percentiles. 5th and 95th percentiles are shown by black dots. Boxes that do not share a letter are significantly different between stages (Tukey's HSD $p<0.05$).

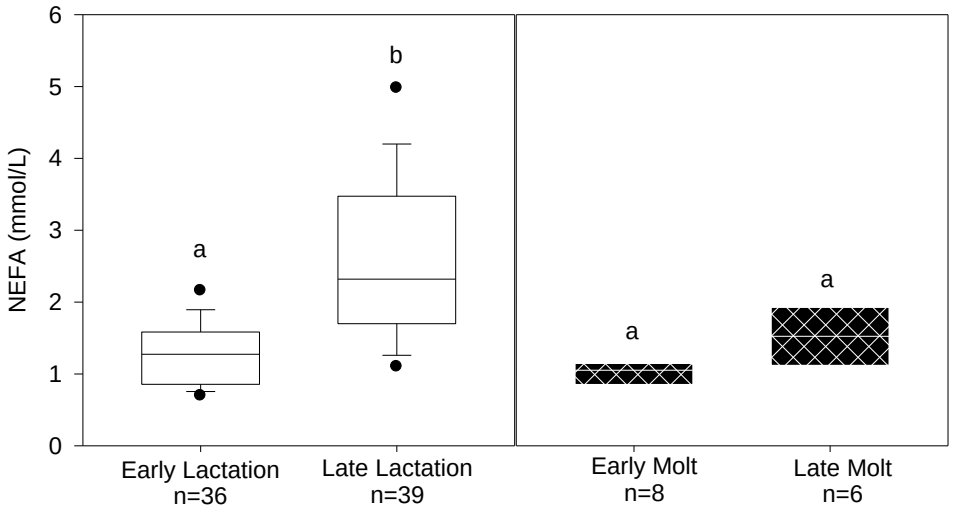

Figure 2. 5: Circulating NEFA (non-esterified fatty acid) changes in 2005 and 2010 breeding elephant seals compared to 2010 molting elephant seals. Box boundaries are 25th and 75th percentiles. 5th and 95th percentiles are shown by black dots. Boxes that do not share a letter are significantly different between stages (Tukey's HSD $p<0.05$).

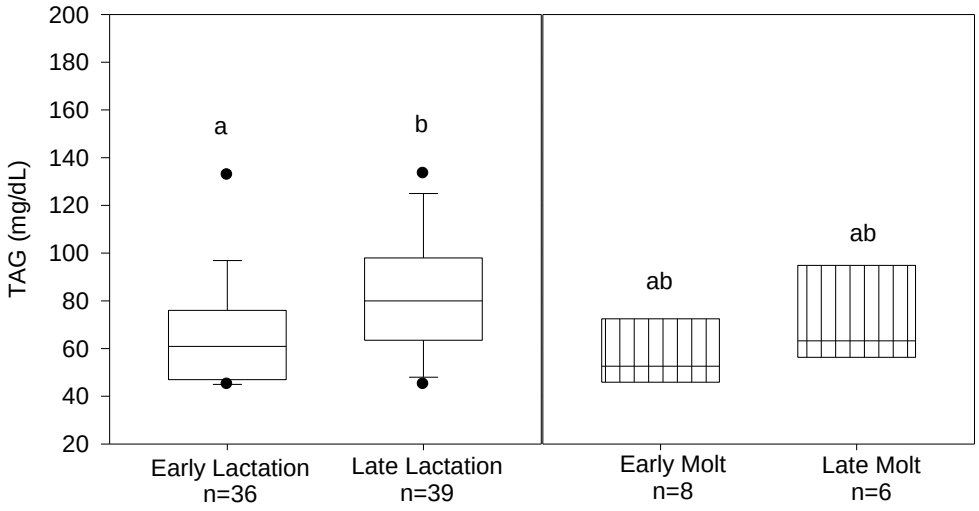

Figure 2. 6: Circulating TAG (triacylglycerol) changes in 2005 and 2010 breeding elephant seals compared to 2010 molting elephant seals. Box boundaries are 25th and 75th percentiles. 5th and 95th percentiles are shown by black dots. Boxes that do not share a letter are significantly different between stages (Tukey's HSD $p<0.05$).

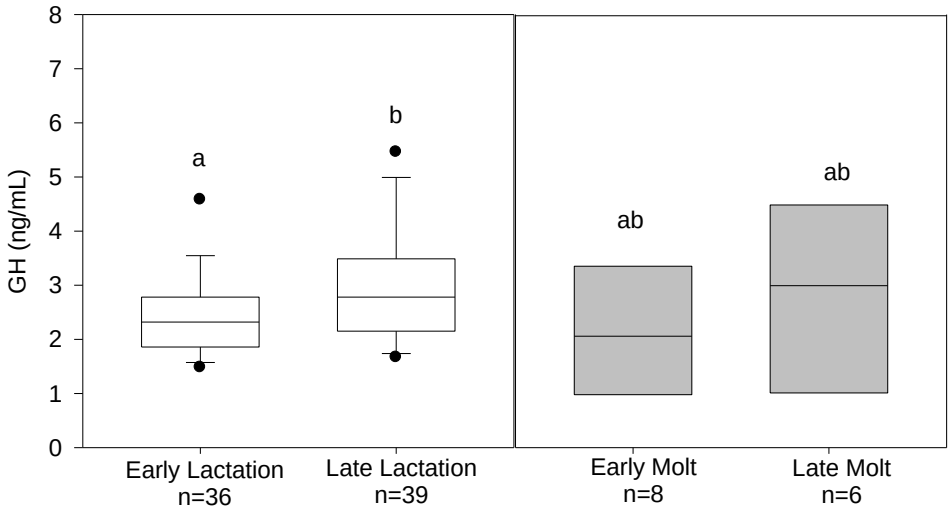

Figure 2. 7: Growth hormone (GH) changes in 2005 and 2010 breeding elephant seals compared to 2010 molting elephant seals. Box boundaries are 25th and 75th percentiles. 5th and 95th percentiles are shown by black dots. Boxes that do not share a letter are significantly different between stages (Tukey's HSD $p<0.05$).

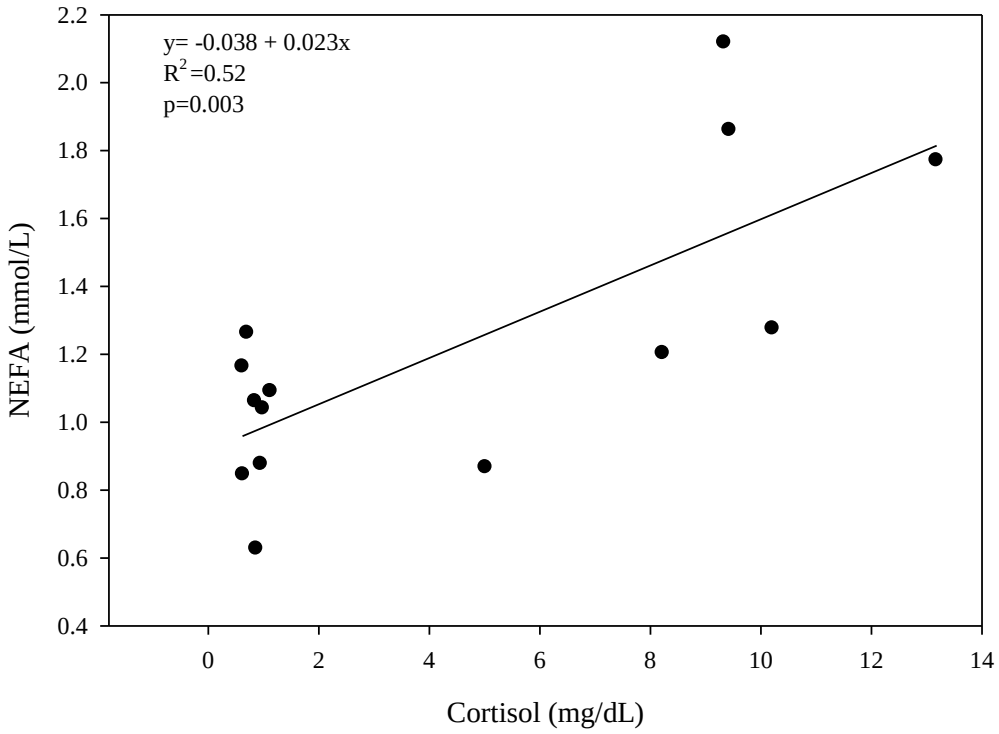

Figure 2. 8: Serum cortisol level are significantly related to plasma NEFA (non-esterified fatty acid) in early and late molting adult female northern elephant seals (n=14).

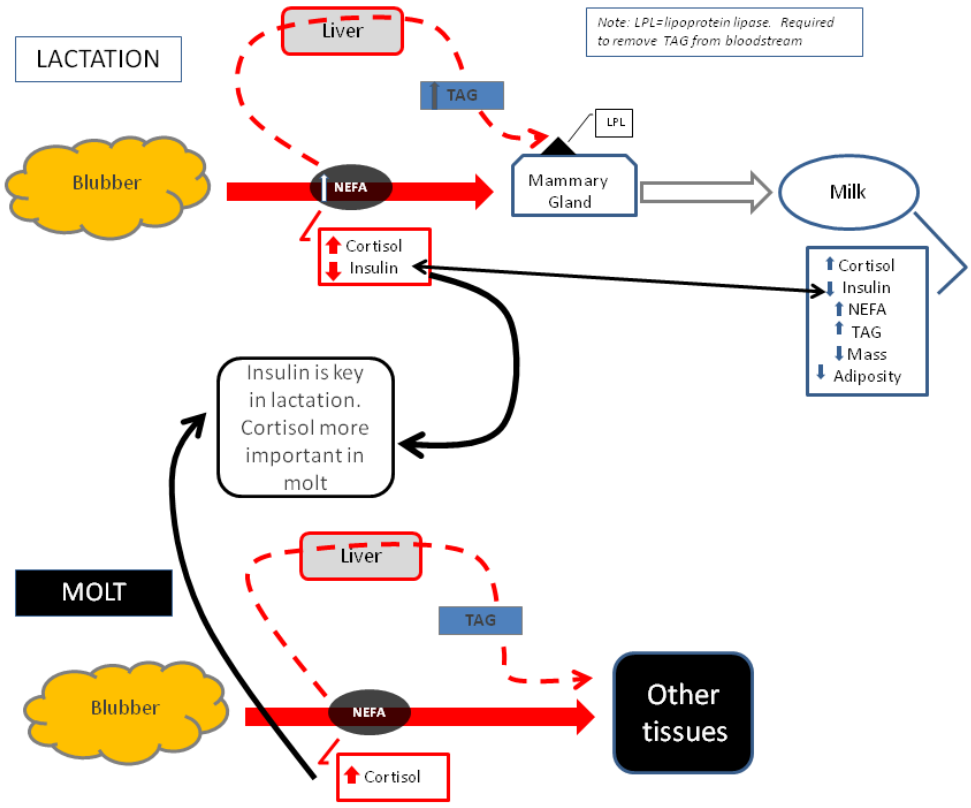

Figure 2. 9: Summary of hormonal effects on lipid release and milk production in fasting elephant seals. Insulin differentially regulates lipid release in lactation vs molt.

CHAPTER 3

ADIPOSE TRIGLYCERIDE LIPASE, NOT HORMONE SENSITIVE LIPASE, IS THE PRIMARY LIPOLYTIC ENZYME IN FASTING ELEPHANT SEALS

Abstract

Northern elephant seals (*Mirounga angustirostris*) fast while on land to breed and molt and fat reserves are mobilized rapidly to fuel their own metabolism and create high fat milk for the pup. Maternal rationing of lipid stores both for both maternal metabolism and milk production has physiological consequences for the mother as well as impacts the growth and survival of the pup. In mammals, there are 2 primary enzymes that release fatty acids from stored triglycerides. Hormone sensitive lipase (HSL) has been hypothesized to be an important lipolytic enzyme in fasting seals, but the activity of HSL and adipose triglyceride lipase (ATGL) have not been quantified in fasting seals, nor has their relationship to milk lipid content been assessed. The objective of this study was to 1) quantify HSL and ATGL lipolytic activity, as well as protein expression levels in fasting, molting seals and fasting, lactating seals and 2) assess the relationship of lipolytic enzymes to milk lipid content in fasting, lactating seals. Seventeen early lactation and 21 late lactation females were captured and blubber and milk samples obtained. Four early molting and four late molting seals' blubber lipolytic enzyme activity were measured. HSL activity was found to be negligible in seal blubber at all fasting stages and was not detectable with antibodies developed for mice. ATGL activity was stable ($p>0.05$) among early lactation (10.00 SD=2.74 nmol/mg protein), late lactation (10.40 SD=2.63 nmol/mg protein) and early

molt (9.93 SD=1.83 nmol/mg protein), but increased ($p<0.05$) in late molting seals (16.59 SD=2.82 nmol/mg protein). Expression of ATGL protein increased ($p<0.05$) across fasting, but neither activity nor protein levels were related to circulating non-esterified fatty acids or milk lipid content, suggesting the possibility of other regulatory pathways relating lipolytic activity and milk lipid deposition. The results of this study are an exciting demonstration that fasting, lactating seals regulate lipolysis differently than previously thought. HSL is not the primary lipolytic enzyme in fasting adult female seals and its contribution to lipase activity was much lower than expected; ATGL contributes more to lipolysis than HSL.

Introduction

Fasting animals must carefully regulate their reserves and in animals that undergo energetically expensive activities concomitant with fasting, different physiological processes may compete for fuel or substrates. For example, in the fasting, lactating northern elephant seal, individuals must partition nutrients between maternal energy demands and mammary gland needs for milk production. Fasting and lactating animals must synthesize milk from only precursors stored in the body. The goal of this study was to investigate the regulation of lipid stores and how that impacts milk lipid production. Does the mammary gland regulate the uptake of fatty acids from the blood and subsequent secretion, or does the supply of fatty acids determine milk lipid secretion? Previous studies on pinnipeds put forth that the mammary gland should direct uptake of milk lipid precursors (Iverson et al. 1995a; Mellish et al. 1999b). The rational cited was founded on studies performed on fed

ruminants, which aside from the differences in fatty acid synthesis due to rumination, are also not depending as heavily on body stores (Annison et al. 1967; Moore and Christie 1979). It is unclear whether this relationship should hold true for fasting pinnipeds for whom milk lipid synthesis is likely quite different. The mammary gland can synthesize fatty acids *de novo,* which typically results in fatty acids with chains lengths of 16 or less (Mepham 1987). All seal species studied to date, including elephant seals, lack or have negligible amounts of both short and medium chain fatty acids (6-12 carbons) in their milk, but there are appreciable amounts of C16 fatty acids (Iverson et al. 1992; Debier et al. 1999; Riedman and Ortiz 1979; Wheatly et al. 2008). *De novo* lipid synthesis in the pinniped mammary gland is thought to be minimal, but may contribute to milk lipid synthesis.

In goats, mammary gland uptake of fatty acids is determined by arterial supply, rather than mammary synthetic activity (Nielsen and Jakobsen 1994). These and other studies from rabbits and rats suggest that mammary utilization of fatty acids is affected by the supply of the substrate (Corl et al. 2006). When long chain fatty acids represent a large portion of substrate supply to the mammary gland, ATP utilization by the mammary gland is depressed because *de novo* synthesis is inhibited (Davis and Collier 1985). A decrease in ATP utilization by the mammary gland for *de novo* fatty acid synthesis could represent an energy savings for a fasting animal.

Fatty acids can be transported in the bloodstream as non- esterified fatty acids (NEFA), taken up by tissues from the bloodstream and the non-utilized NEFA are re-incorporated back into triacylglycerol (TAG) bound to lipoproteins in the liver. The

interplay of TAG and NEFA availability can affect the patterns of mammary utilization. When plasma TAG are low and plasma NEFA are high, the NEFA are used directly by the mammary gland for large quantities of milk lipid synthesis (Neville and Picciano 1997). Circulating NEFA can diffuse across membrane borders down a concentration gradient, or be facilitated by transport proteins (Glatz et al. 2010). In order for the mammary gland to make use of circulating TAG bound to lipoproteins, lipoprotein lipase (LPL) must hydrolyze the TAG/lipoprotein complex to facilitate entry into the mammary gland. LPL is the primary enzyme enabling tissues to uptake circulating lipoproteins to metabolize (Frayn et al. 1995). During lactation in many species LPL activity increases in mammary tissue to enable uptake of circulating TAG bound to lipoproteins, while it decreases in adipose tissue to facilitate the release of lipid stores (Robinson 1963; McBride and Korn 1963; Hamosh et al. 1970). However, when lactating mice are fasted, mammary LPL activity decreases (Jensen et al. 1994). Insulin is an activator of LPL (Da Costa and Williamson 1994) and shown to decrease milk lipid yield as well as NEFA availability (Corl et al. 2006). Insulin has the effect of not only stimulating LPL, but also inhibiting the release of stored fatty acids (Frayn et al. 1994). The differential effect of insulin on adipose versus mammary tissue may be mediated by prolactin in other species (Ros et al. 1990). The strategy of fasting during lactation is rare and many species do not fast during lactation and thus do not depend as heavily on mobilizing lipid reserves as elephant seals; thus there may be metabolic alterations in animals pairing fasting and lactation. Insulin has been shown to decrease across

lactation and is a significant effector of milk lipid content in northern elephant seals (Chapter 2, this thesis).

In hooded and grey seals LPL activity in the mammary gland has been found to increase across lactation (Mellish et al. 1999b; Mellish et al. 2000) and LPL activity correlates with milk lipid increase across lactation in grey seals (Iverson et al. 1995a) suggesting that LPL could be important in directing lipids to milk. McDonald and Crocker (2006) found low and stable mammary LPL activity in elephant seals across lactation, and found no relationship to milk lipid.

If mammary uptake of milk lipid precursors via LPL hydrolysis of TAG is not regulating milk lipid in elephant seals, it is possible that the regulation of the release of precursors on the side of the adipose stores is involved. Houser et at (2007) found very high, but unchanging, rates of lipolysis across lactation in lactating northern elephant seals while milk lipid is seen to increase dramatically during lactation (McDonald and Crocker 2006). Several enzymes are key players in regulating adipose stores. Within the adipocyte TAG is hydrolyzed, resulting in NEFA. The enzymes hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) are responsible for 95% of lipolytic activity in mouse (Schweiger et al. 2006). The activity of these lipolytic enzymes liberating NEFA into the bloodstream may be a regulator of the supply of milk lipid precursors and thus milk lipid content.

HSL and ATGL have never been quantified in fasting and molting or fasting and lactating elephant seal adipose tissue, nor has their relationship to milk lipid been investigated. Chapter 2 of this thesis showed a relationship of increasing TAG to

milk lipid content in elephant seals. Therefore, how mammary gland regulation versus adipose tissue regulation differentially affects milk lipid content in elephant seals remains unclear.

This study aimed to quantify HSL and ATGL in the blubber of fasting seals. In order to understand if there was differential regulation due to the additional demand for milk lipid precursors in lactating seals, I compared fasting, molting to fasting, lactating seals. To further understand the regulation of competing physiological demands during fasting, I assessed lipolytic enzyme activity importance to milk lipid content in lactating northern elephant seals. I hypothesized that lipolytic activity in the blubber would be lower in molting seals and related to both the circulatory NEFA and milk lipid content.

Fasting animals are constrained by finite energy reserves and must ration reserves accordingly over time. Given the dependence on lipid in several life history stages of elephant seals, inability to appropriately ration lipid stores and manage delivery to the pup could have consequences. Depletion of lipid stores to a level that is difficult to recover from could affect both the future breeding attempts by the female and the inadequate provisioning of lipid to the pup could impact its survival.

Methods

Study Site and Individual Animals

This study was carried out at Año Nuevo State Reserve, San Mateo County, during the 2010 breeding season (Jan-Feb) and 2009 molting period (Apr-May). Soon after arrival on land, adult female seals were marked with hair dye (Lady

Clairol, Stamford, CT) to facilitate identification. Parturition dates were established by daily observations and considered to be the first day a marked female was observed with a pup, provided she had been observed without a pup the previous day. In 2010, 17 females were sampled in early lactation (day 5-7) and 21 females captured in late lactation (day 21-25), six of which were repeated individuals from early lactation. Early molt females (n=5) were captured within 5 days of arriving on land and late molt study females (n=5) were selected on the basis of fully molted pelage, ensuring ~3 weeks of fasting (Le Boeuf and Laws 1994). This work was approved under NMFS permit 87-1743-06 and University of California Santa Cruz IACUC Costd0809.

Sample Collection and Processing

Females were initially immobilized with Telazol (tiletamine/zolazepam HCl, Fort Dodge Labs, Ft. Dodge, IA) at a dosage of ~1mg/kg, administrated intramuscularly. Continued immobilization was maintained with ~100 mg bolus intravenous injections of ketamine. Milk (~5 mL) was collected after 40 units of oxytocin were administered intramuscularly. Serum and plasma was collected into chilled vacutainers within 20 minutes of sedation. Samples were immediately placed on ice and transported back to laboratory within 2-3 hours. Samples were centrifuged at 4°C, and frozen at -80ºC until further analysis.

Subsequent to betadine scrubbing and the administration of a local anesthetic (lidocaine), a blubber biopsy extending the full depth of the blubber layer was taken laterally, several centimeters anterior to the pelvis using a 6mm biopsy punch (Uni-

Punch, Premier Medical, Plymouth, PA, USA). Blubber was stored in foil, inner and outer portion labeled and put on ice for transport back to the lab, where it was frozen at -80°C until further analyses. Mass was measured using a tripod, canvas sling and scale (± 1 kg) MSI, Seattle, WA).

Laboratory Analyses

Chemicals and Reagents: Reagents were obtained from the following sources: Bovine serum albumin (fraction V) from Sigma, St. Louis, MO; ECL Western blotting detection reagents, Bradford protein assay kits from Bio-Rad, Inc.; Hercules, CA; BCA protein kits from Pierce Biotechnology, Inc., plasma NEFA kits from Wako Diagnostics, Richmond, VA; Rockford, IL; nitrocellulose paper from Schleicher and Schuell, Keene, NH; organic solvents were from J.T. Baker, Phillipsburg, NJ; mouse anti-ATGL antibodies, from Cell Signaling Technology, Danvers, MA, mouse anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) from Ambion, Grand Island, NY; Antimouse full-lengthHSL IgG was prepared as described previously (Wang et al. 2005). Odyssey blocking buffer, goat antimouse IgG-IR dye 800cw, and goat antirabbit IgG-IR dye 680cw from Li-Cor Biosciences, Lincoln, NE. Eletrophoresis gels from Lonza, Rockland, ME. Glycerol-tri-[9,10-3H(*n*)] oleate from Perkin Elmer Life and Analytical Sciences, Boston, MA. Phosphatidylcholine from Avanti Polar Lipids, Alabaster, AL. All other reagents were from Sigma, St. Louis, MO, unless otherwise noted.

Plasma NEFA was measured in triplicate. Mean %CV of the assay was 3.65%. Milk lipid was extracted by the Roese-Gottleib method (McDonald and

Crocker 2006), including six washes with 20 mL ethyl ether and 20 mL petroleum ether to ensure complete extraction.

Lipolytic Enzyme Activity assay: HSL activity was measured via methods in Shen et al (1998). Briefly, inner blubber cores, the half of the blubber core closest to the interior of the animal, were homogenized in 20mM Tris EDTA sucrose buffer pH 7.4 with 1 unit/mL protease inhibitor leupeptin. Inner versus outer cores were tested for patterns regarding activity, but no clear patterns emerged. The inner value was used to be as consistent as possible. The resulting homogenate was centrifuged for 20 min at 4 ° C at 14000*g*. The supernatant, containing the cytosolic fraction, was removed and used for subsequent analysis. Protein levels were measured by Bradford protein assay using bovine serum albumin standards. Protein in the fat cake was extracted with cold acetone and total protein quantified via bicinchoninic acid (BCA) assay.

Measurement of HSL activity was performed using cholesteryl ester as substrate (Shen et al. 1998). Briefly, aliquots of supernatant protein adjusted to 100 *u*L with buffer were mixed with 140 *u*L of 0.05% bovine serum albumin in 100 mM potassium phosphate, pH 7.0. After the addition of 10 *u*L of substrate (100*u*M), the assay was carried out at 37 °C for 60 min. The reaction was stopped by addition of chloroform:methanol:heptane (250:230:180). After the addition of borate/carbonate buffer (0.1 M, pH 10.5), the tubes were vortexed and centrifuged, and aliquots of the upper phase were taken for liquid scintillation counting in a Beckman scintillation

counter. A sample of mouse adipose tissue prepared identically was included as a positive control in all assays. 3-96 µg of protein were used in the HSL assays.

Measurement of ATGL was performed using a triacylglycerol substrate. Substrate for the triacylglycerol assay was prepared after Holm et al (2001), with the substitution of glycerol tri[9,10(*n*)-3H]-oleate and trioleolylglycerol for cholesteryl [1-14C]oleate and cholesteryl oleate, respectively. Triglyceride lipase activity was assayed in triplicate using an emulsified substrate containing 0.8 mM triolein, 7.5 µCi of [^{3}H-triolein], 0.1 M potassium phosphate buffer and 20% BSA. Aliquots of supernatant protein were adjusted to 100 *u*L with buffer (20mM potassium phosphate, 1mM EDTA, 1mM dithioerythritol, 0.02% fatty acid free-BSA, ph 7.0). 100 *u*L of substrate (835*u*M in final assay volume) was added to the sample/buffer mixture and incubated for 30 minutes at 37°C. The reaction was stopped by addition of chloroform:methanol:heptane (250:230:180). After the addition of borate/carbonate buffer (0.1 M, pH 10.5), the tubes were vortexed and centrifuged, and aliquots of the upper phase were taken for liquid scintillation counting in a Beckman scintillation counter. The results were normalized with the amount of protein and expressed in nmol of cholesteryl oleate or trioleolylglycerol or hydrolyzed/mg of protein. Mean intrassay variability for positive control mouse adipose samples was 9.1% while mean intrassay variability for seal samples was 15.9%. Interassay variability for positive control mouse samples was 25.3% and for seal samples it was 31.4%.

To assess optimal temperature and pH for seal ATGL activity, trials of varying temperature (5°C, 23°C, 30°C, 40°C) and pH (4.5, 5.2, 6.25, 7, and 8.97)

were performed. It was determined that 37°C and pH 7 were the appropriate values for the assay. Linearity for the assay relative to quantity of protein added was assessed and it was concluded that between 5 and 10 *u*g was the optimal amount to maintain linearity of the ATGL activity assay.

Immunoblotting: Immunoblotting was performed as described previously (Shen et al. 1999; Shen et al. 2010). Approximately 40-50 μg proteins were resolved by 4-20% gradient SDS-PAGE gel and blotted onto nitrocellulose membranes. Membranes visualizing ATGL were blocked with 5% nonfat milk in 0.1% PBS (0.1% Tween 20), for 60 minutes at room temperature and then incubated with the primary antibody in 2.5% nonfat milk in 0.1% PBS (0.1% Tween 20), over night at 4° C. Membranes visualizing HSL were blocked with Li-Cor blocking buffer for 30 minutes at room temperature and primary antibodies were incubated in Li-Cor blocking buffer for 1 hour at room temperature. HSL competition experiments were carried out by incubating a membrane with 30*u*g recombinant HSL concomitant with the anti-HSL antibodies. Membranes and were incubated with primary antibodies at the following dilutions: anti-ATGL: 1: 1000, anti-HSL: 1:5000, anti-GAPDH: (as a loading control) 1:1000. Membranes were incubated with the appropriate secondary antibody conjugated to infrared dye (goat antimouse IgG-IR dye 800cw, goat antirabbit IgG-IR dye 680cw) at room temperature for 1 h, washed 3 times with PBS (0.1% Tween 20), rinsed with PBS, then detected by an Odyssey Infrared Fluorescent Imaging System (Li-Cor Biosciences, Lincoln, NE). The image was analyzed using Odyssey V3.0 software to quantify the average intensity of the bands.

Statistical Analyses

Linear mixed effect models were employed to analyze the relationship of lipase activity to metabolites and milk lipid content and seal identity was included as a random effect. Statistical analyses were performed using the software R (Version 2.14.1, R Development Core Team, www.R-project.org). Packages AED (Zuur 2009) and nlme (Pinheiro et al. 2009) were used to assess normality, homogeneity of variance and perform mixed effects modeling. To maintain normality, ATGL:GAPDH band intensity ratio (corresponding to ATGL protein measures) and ATGL activity values were $\log_{10}$ transformed. Milk lipid proportions were square root arcsine transformed. When variable relationships were assessed within stage, least squares linear regression was used. T-tests were utilized to assess differences in ATGL protein content across molt fasting stage while paired t-tests were used when analyzing only matched individuals from early and late lactation. Western blot data relationship to other variables was analyzed with Pearson's product moment correlation.

Results

Seals lost an average of 138 kg over lactation, and milk lipid content increased from 34.2% (SD=3.3) to 54.3% (SD=3.0) across 17 days of lactation (see Chapter 2, Table 2.1). NEFA levels increased significantly from 1.2 (SD=0.4) to 2.5 (SD=1.2) across lactation ($F_{3,44}$= 13.3, $p<0.001$), but molt NEFA levels remained stable (early molt: 1.0 (SD=0.2) late molt: 1.5 (SD=0.5); $p>0.05$).

HSL activity

HSL activity was found to be extremely low in elephant seals. As this is the first time this enzyme has ever been measured in a pinniped, I initially tried a range of 8.5-98 *u*g in 2 molting seals (1 early molt and 1 late molt). The activity levels were 4-6% of positive controls. Seal HSL activity was1.2-2.3 nmol/mg protein while the positive control values (mouse fat) were 30-35 nmol/mg protein. To control for possible degradation due to field preservation, two samples were collected from juvenile seals and immediately put into liquid nitrogen in the field. These samples also showed very low HSL values, only 3-12% of the positive mouse fat controls in the assay, leading me to conclude that preservation methods were not to blame for the low activity values.

HSL activity was quantified from four early lactation seals and four late lactation seals. Early lactation seals' mean activity level was 6.38 (SD=3.01); range = 3.9-10.7 nmol/mg protein. Late lactation seal HSL activity ranged from 0-9.5 nmol/mg protein, with a mean of 3.8 (SD=4.04). These values were 7-15% of the positive control values.

In an effort to test if HSL were bound to the lipid droplet, acetone extractions of the fat cake were performed. Fat cake extraction proved to have even lower HSL activity (0.45-0.47 nmol/mg protein) than cytosolic fractions. Due to the extremely low activity levels I concluded that seals have very little HSL activity in blubber and pursued ATGL for the remaining samples.

HSL Immunoblotting

Bands were visualized in seal blubber using anti-HSL antibodies (Figure 3.1). However, the size of the seal bands were not at the expected molecular size (84kDa) (Kraemer and Shen 2002), and so a competition experiment was performed. After the addition of recombinant HSL to compete with the anti-HSL binding, the mouse band disappeared, but the seal bands remained (Figure 3.2). This result indicated that the antibody was detecting non-specific proteins rather than HSL in the seal bands.

ATGL activity

In order to attempt to optimize the triglyceride lipase assay, differing temperatures and pH were tested. It was found that the highest activity was at pH 7 and 40°C (Figure 3.3). The results led me to conclude that the same conditions for the assay developed for mice (pH 7 and 37° C) were appropriate for seal samples.

Inner (closest to the internal environment) and outer (closest to the skin) portions of the blubber were tested for different activities (n=12). When expressed as a percentage differences, the outer blubber core was 16.6% lower than the inner core. However, the standard deviation was 32%, twice the average percent difference. Thus, no clear patterns were observed and subsequent analyses were performed on inner blubber cores.

As the large amounts of protein in these early assays may have been saturating the assay, a linearity experiment was conducted to ascertain over what range of total protein the assay showed a linear response. Values from 5 to 10 *u*g showed the best

linear increase in counts per minute and thus 6 *u*g total protein were used in assays for lactating seals.

ATGL activity was significantly higher in late molting animals ($F_{3,9}$ =4.44, p=0.04), but all other stages were equivalent (p>0.05). Early lactation mean ATGL activity was 10.00 (SD 2.74) (n=17) nmol/mg protein and equivalent (p>0.05) to late lactation mean ATGL activity: 10.40 (SD 2.63) (n=21) nmol/mg protein and early molt mean ATGL activity: 9.93 (SD 1.83) (n=4) nmol/mg protein. Late molt mean ATGL activity: 16.59 (SD 2.82) (n=4) nmol/mg protein was significantly higher (p<0.05) than all other stages.

ATGL activity in the lipid droplet was tested, but found to be approximately half of the cytosolic fraction (n=2 seals). In mouse fat droplets the ATGL activity was ~15% of the cytosolic fraction. ATGL activity was significantly related to mass ($F_{1,8}$= 5.8, p=0.04), with larger animals having lower ATGL activity. (Figure 3.4). Plasma NEFA levels were not related to blubber ATGL activity ($F_{1,8}$ =0.2; p=0.67). When both lactation stages were analyzed together, ATGL activity did not predict milk lipid content (p<0.05). Milk lipid levels were not related to ATGL activity when lactation stages were separated: early (R^2= 0.01, p=0.80) or late lactation R^2= 0.001, p=0.89).

ATGL Immunoblotting

ATGL bands were visualized in elephant seal blubber cytosolic fractions and were seen to be a good match to the size of mouse ATGL (Figure 3.5). Only matched pair early and late lactation samples were analyzed (n=6). When the intensity of the

bands for ATGL and GAPDH were quantified and expressed as a ratio, there was significantly less ATGL protein in early lactation samples than in late lactation samples (paired t=-2.2, df=5, p=0.04). Early molt seals (n=3) have lower ATGL protein levels than late molt seals (n=3) (t=3.54, p=0.03) (Figure 3.6).

When all samples were pooled (n=17), there was a significant correlation between NEFA and ATGL protein quantified by Western blot (r^2=0.55, p=0.02). However, the large difference in NEFA from early to late lactation should be noted when interpreting correlations. When only molt samples (n=6) are considered, there is no correlation between NEFA and ATGL protein (r^2=0.39, p>0.05) and within lactating seals there is not a significant correlation between NEFA and ATGL protein (r^2=0.54, p>0.05).

All pooled samples (n=17) showed that the intensity of ATGL:GAPDH bands quantified during Western blot did not correlate well with ATGL activity levels (r^2=0.10). When molting seals were considered, ATGL protein abundance and ATGL activity were highly correlated (r^2=0.89), however, the very high late molt activity values concomitant with a very small sample size may should be noted. All lactation seals considered together show no correlation between ATGL protein and ATGL activity (r^2=0.26).

When samples were pooled, there was a significant correlation between the intensity of ATGL:GAPDH bands and milk lipid proportions (r^2=0.9, p<0.001), but the large increase in milk lipid may have a large influence. When samples were

divided into early and late lactation, no significant correlations were seen in either early (r^2= 0.20) or late lactation (r^2= 0.31) ATGL:GAPDH ratios and milk lipid.

Discussion

This is the first study to attempt to delineate HSL and ATGL activity in elephant seal adipocytes and elucidate their relationship to milk lipid content. The regulation of lipid stores is crucial in fasting phocid seals, in multiple life history phases and only a few studies have measured lipase activity in these animals. Lipoprotein lipase (LPL), a lipolytic enzyme located in the capillary endothelium of adipose tissue, hydrolyzes circulating TAG bound to lipoproteins (Frayn et al. 1995; Watt and Steinberg 2008) while HSL and ATGL work within the adipocyte to hydrolyze TAG in the lipid droplet (Frayn et al. 1995; Watt and Steinberg 2008). LPL in blubber 'pick ups' circulating TAG for storage inside the adipocyte, which results in lipogenesis, or the preservation of lipid stores. Due to the need for liberating lipid stores, rather than conserve them, fasting seals have very low deposition of lipid in blubber via LPL (McDonald and Crocker 2006; Mellish et al. 1999b). Lipolytic enzymes such as HSL and ATGL serve to liberate the stored reserves, a critical step in making stored lipids available to other tissues for oxidation or milk lipid production. Despite the importance of lipolysis in seals, only two studies have quantified lipase activity other than LPL. Total lipase activity in Weddell seal muscle (Kanatous et al. 2008) and northern elephant seal weaned pup blubber (Viscarra et al. 2012) has been reported, but lipolytic enzyme activities were not differentiated.

HSL has often been hypothesized to be the important lipolytic enzyme to mobilize blubber in fasting seals (Debier et al. 1999; Iverson et al. 1995a; McDonald and Crocker 2006) and I have shown that this enzyme has negligible activity in fasting elephant seals. I utilized a substrate for which HSL has demonstrated a high specificity (cholesteryl ester (Kraemer and Shen 2002)) and found very, very low activity levels in relation to mouse samples. The presence of ATGL was confirmed by Western blot and while the substrate used to investigate ATGL activity may be acted upon by other lipases (LPL or monoacylglyceride lipase (Watt and Steinberg 2008), blubber LPL in fasting seals is very low (McDonald and Crocker 2006; Mellish et al. 1999b). Monoacylglyceride lipase was not investigated in this study. HSL and ATGL are responsible for 95% of lipolytic activity in mouse (Schweiger et al. 2006), lending support to the idea that the majority of the lipase activity observed is ATGL and not HSL or monoacylglyceride lipase. Low HSL activity was corroborated by competition experiments using Western Blot that revealed bands seen in seal samples are likely non-specific binding, rather than HSL. Interestingly, Viscarra et al (2012) report the detection of stable HSL protein expression in fasting, weaned pups. This study demonstrates that fasting, lactating seals regulate lipolysis differently than previously thought, and indeed, differently than other age classes of elephant seals.

HSL and ATGL in lactation

In previous studies in lactating cattle, HSL activity increased significantly until day 60 of lactation and then began to decrease toward, but not reaching, non-

lactating levels (Smith and McNamara 1990; McNamara et al. 1987). In lactating cattle, lipolysis rates were shown to be positively related to milk fat output (McNamara and Hillers 1986b). The responsiveness of adipocytes to lipolytic stimulation via HSL has been shown to vary throughout lactation stage in cattle (McNamara 1994). Koltes and Spurlock (2011) found no change in HSL protein abundance across lactation in cattle, but did detect an increasing trend in phosphorylated HSL protein during midlactation and the phosphorylated HSL was significantly correlated with total milk yield. Little has been done relating ATGL activity and protein levels to milk content. The abundance of ATGL protein in cattle blubber decreased early in lactation and increased slightly at 150 days into lactation, but was not related to milk yield (Koltes and Spurlock 2011) and activity was not quantified.

ATGL activity was stable across lactation, which supports Houser et al (2007), where lipolysis rates were stable across lactation in fasting, lactating elephant seals. Lactating seals liberate lipids at a similar rate in early and late lactation, despite the high demand for lipid for milk production in late lactation. Houser et al (2007) found that although plasma NEFA increases across lactation in northern elephant seals, lipolysis rates remain stable; raising the question of changing re-esterification rates throughout lactation, with the possibility of decreased re-esterification rates later in lactation. Stable ATGL activity across lactation suggests, as did Houser et al (2007) that there may be additional mechanisms that regulate the availability of NEFA to other tissues, such as increased re-esterification in early

lactation. Jenkins et al (2004) showed that ATGL has transacylase activity, resulting in the production of TAG from mono-or di-acylglycerol, which could affect TAG futile cycling. The increase in ATGL protein in blubber across lactation concurrent with stable ATGL lipolytic capacity doesn't seem to implicate ATGL in decreased TAG cycling in elephant seals across lactation, but would be an interesting avenue for future research. It's likely transacyclase activity of ATGL is minimally involved in re-esterification, but there may be other mechanisms in adipocytes or hepatocytes that influence re-esterification rates over lactation.

The results of this study, that ATGL activity and neither plasma NEFA nor milk lipid were related, were unexpected. My hypothesis of milk lipid regulation via blubber lipolytic enzyme activity was not supported, but raises interesting questions regarding potential mechanisms of lipolytic enzyme regulation (see below). Seal mammary gland lipogenesis is likely minimal (Chapter 1 of this thesis) and although TAG varied with milk lipid in Chapter 2 of this thesis, mammary LPL activity has been shown to be low and stable across lactation, with no relationship to milk fat (McDonald and Crocker 2006). Fatty acid transfer proteins or fatty acid binding proteins in the mammary gland likely facilitate the movement of fatty acids into mammary gland (Clegg et al. 2001). The regulation and abundance of these transport and binding proteins are unknown in elephant seal mammary glands, but fatty acid binding proteins and fatty acid transport proteins could be an additional factor in regulating the entry of fatty acids into the mammary gland.

Regulation of HSL and ATGL

HSL is regulated by post-translational control. It is activated by a cascade of steps including: increases in cAMP which activates protein kinase A (PKA), whereby PKA phosphoryates both HSL and a protein called perilipin, which is located on the lipid droplet and may restrict access of lipolytic enzymes until phosphorylated (Watt and Steinberg 2008; Jaworski et al. 2007). Regulators of ATGL include the chaperon protein CGI-58, which is required for full activation (Lass et al. 2006). CGI-58 also interacts with perilipin on the surface of the intracellular lipid droplet (Subramanian et al. 2004) to facilitate access of lipases to the lipid droplet. In this study ATGL and HSL activity in the lipid droplet was tested, but found to be much lower than that in the cytosolic fraction.

In laboratory animals such as rats, HSL activity was seen to increase during fasting. However, this change was quite slow for a small, non-fasting adapted animal, increasing after 3-5 days (Sztalryd and Kraemer 1994; Bertile et al. 2003). These results suggested that there might be post-translational mechanisms affecting the activity of HSL in the first 3 days of fasting, and pretranslational effects may occur after 3-5 days. Lactating seals had been fasting for a minimum of 5 days, depending on how long they were on the beach before giving birth, typically ~ 6 days (Le Boeuf et al. 1972). Early molting seals were fasting for a minimum of 1 day, ranging up to 6 days.

ATGL mRNA levels have been seen to increase in as little as 12 hours of fasting (Villena et al. 2004) and seen to remain elevated as long as 4 days in rats

(Bertile and Raclot 2011). HSL expression also increased over fasting and both ATGL and HSL were significantly related to plasma NEFA in rats (Bertile and Raclot 2011). In this study, neither protein levels nor activity levels showed a relationship to plasma NEFA.

Human subcutaneous adipose tissue HSL protein levels are downregulated in obese subjects, while ATGL levels are not affected (Rydén et al. 2007; Mairal et al. 2006). Given the large amounts of stored lipid in elephant seals, they may be considered obese. The low levels of HSL activity and expression observed in elephant seals could possibly be due to the 'obese' nature of elephant seals. However, rapid weight loss across lactation does not seem to translate into higher HSL activity in elephant seals. Viscarra et al (2012) found that HSL expression was stable in fasting elephant seals during the postweaning fast, but differentiation between lipolytic activities of HSL and ATGL was not performed.

HSL activity is stimulated by epinephrine, glucagon and growth hormone and expression has been shown to be increased by glucocorticoids (Slavin et al. 1994) and inhibited by insulin (Frayn et al. 1994). *In vitro* studies of rat preadipocytes found that ATGL expression was increased after treatment with glucocorticoids (Villena et al. 2004). Glucocortcoids increase in fasting elephant seals (Chapter 2 of this thesis) and the increase in amount of ATGL protein in lactating seals, concomitant with increased cortisol, suggests that the increased cortisol may be related to an increase in the expression of ATGL, but this appears unrelated to ATGL or HSL activity. Increased insulin levels caused a decrease in ATGL expression in murine adipose

tissue (Kershaw et al. 2006). Elephant seals display a decrease in insulin across lactation (Chapter 2 of this thesis). This would presumably relieve any inhibition on ATGL activity. The presence of ATGL protein increases across lactation, but given the unchanging levels of activity over lactation observed in the present study, it is unknown how insulin may be affecting ATGL.

In fasting weaned pups, Viscarra et al (2012) found increasing ATGL protein expression in the presence of decreasing insulin (Viscarra et al. 2011a). The increased ATGL protein expression occurred concomitant with decreased lipase activity, but the contribution of HSL versus ATGL to lipase activity in weaned pups was not quantified (Viscarra et al. 2012). In both lactating females and weaned pups ATGL expression increased with decreasing insulin levels over the fasting duration; although in the current study, blubber lipase activity was stable across the lactation fast, while blubber lipase activity decreased in weaned pups (Viscarra et al. 2012). These results indicate that there are likely different regulators of lipase activity between lactating females and weaned pups.

The significant relationship between ATGL activity and mass presents an opportunity to consider allometry of enzyme activities. Muscle oxidative enzyme activities in 10 mammalian species were seen to scale negatively with body size and glycolytic enzymes scale positively with body mass (Emmett and Hochachka 1981). Pond and Mattacks (1989) examined glycolytic enzymes in adipose tissue in 17 carnivorous mammals and found that the metabolic capacities of adipose tissue does not scale to body mass. Their rationale was that in many species adipose tissue is a

relatively inert tissue, one which can vary dramatically between individuals and within individuals over time and as such, the metabolism must be under more direct neural or endocrine control. In this study, larger animals had lower ATGL activity (Figure 3.4), similar to the trend of oxidative enzymes found in Emmett and Hochachka (1981). The activity in the current study is calculated per mg of protein in the assay, rather than per mass of tissue as in Emmett and Hochachka (1981) and Pond and Mattacks (1989). The different normalization should be considered when interpreting these relationships. The results of higher ATGL activity in smaller seals is consistent with a study showing that the mass of a lactating seal affects the maintenance metabolic costs, with larger seals expending less energy than smaller seals (Crocker et al. 2001).

Molting seals have much lower whole body lipolysis rates than lactating seals (Houser et al. 2007), as well as lose less adipose tissue during fasting (Chapter 2, Table 2.2). Thus, the higher levels of ATGL activity in late molting seals is intriguing. The small sample size of seals analyzed for protein levels show an increase across the molting fast, but the magnitude is not as large as the increase from early to late lactation (Figure 3.5).

The result of increasing abundance of ATGL protein in blubber, concomitant with stable ATGL activity in lactating seals reveals the possibility of other regulatory pathways. Lipolytic activity does not match the amount of protein expressed; seals may be altering the relationship between activity and expression in unique ways. There could be differing *in vivo* post-translational regulation of ATGL in early versus

late lactation or molt, via the action of perilipin or CGI-58. An additional potential regulatory mechanism includes tumor necrosis factor alpha (TNF-α), which has been shown to increase lipolysis by decreasing an inhibitory of ATGL activity (GS02) (Yang et al. 2011). TNF-α also facilitates lipolysis by downregulating the expression of perilipin (Souza et al. 1998). The possibility of the alternation in intracellular signaling during the fast is an exciting avenue to be explored in intact cell cultures, and may shed light on how seals regulate the interplay between expression of lipolytic enzymes and their action.

Methodological considerations include antibodies and lipase substrate selection. While the antibodies developed for mouse ATGL may have differing specificity for seal comparisons among seal samples should still be informative. The selection of triolein as a substrate for total lipase activity is appropriate, given that oleic acid has been shown to be the most predominant fatty acid in elephant seal blubber tissue (Chapter 1 of this thesis).

This study provided insight into the physiological mechanisms that mother seals utilize to partition nutrients from blubber stores into use for maintenance metabolism and milk production. The consequences to the mother for inadequately rationing her blubber stores may include sacrificing subsequent breeding events. No adult female returning for the breeding season has been measured with an adipose proportion less than 26%, indicating a threshold for survival. Additionally, a female's probability of producing a pup declines with lower fat mass before leaving for the post-molt trip and lower fat mass gain during the post-molt trip (Schwarz,

pers. comm. Therefore, if the mother depletes her blubber stores too low when creating lipid rich milk for the pup during one breeding season, she may not regain the proper blubber stores for the next years' breeding attempt, and the probability of surviving and pupping drops. On the other hand, the consequence of not providing the pup with adequate lipid in the milk could affect the duration of the postweaning fast (Noren et al. 2003), and thus, constrain the development of diving capabilities in the pup (Thorson and Le Boeuf 1994). Given the importance of partitioning lipid, there should be selection for tight control of body reserves and direction to milk. Thus, understanding the mechanisms behind these physiological processes may help us understand how they affect nutrient partitioning between mother and pup. The lipolytic enzymes supplying substrate to the mammary gland do not seem to be driving milk lipid in a direct fashion in this study, which raises interesting new avenues for novel regulatory mechanisms in an animal for which mobilizing lipid is of critical importance.

Future Directions

As this is one of the first studies to attempt to differentiate between lipolytic enzymes in seals, there is exciting work ahead to continue to investigate physiological adaptations. Molecular methods have been developed in the lab using rodents and there is ample opportunity to apply these methods to fasting seal samples. A baseline for feeding seals' adipose lipolytic enzyme activities has not yet been established and is important to complete the understanding of how the activities of these enzymes are changing with metabolic status. Additionally, information on the protein sequence of

the elephant seal ATGL protein would inform the question of how conserved these genes are between species. The development of elephant seal specific anti-ATGL and anti-HSL antibodies would refine the immunoblotting quantification. The culture of adipocytes would begin to enable the pursuit of questions related to regulation via TNF-α, perilipin and CGI-58. On the mammary side of the system, the quantification of FATP and FABP in seal mammary tissue would further our understanding of how lipid is transported into the mammary gland and help further our understanding of the relationship between lipolytic enzyme regulation of supply to the mammary gland and subsequent deposition of lipid into the milk.

Conclusion

The results of this study are a demonstration that fasting, lactating seals regulate lipolysis via different enzymes than previously thought, and indeed, may regulate these enzymes differently than fasting weaned elephant seal pups. HSL is not the primary lipolytic enzyme in fasting adult female seals and its contribution to lipase activity was much lower than expected; ATGL contributes more to lipolysis than HSL. ATGL protein levels increase across both the lactation and molting fast, but this increase is not mirrored in the lipolysis rates, except for a interesting dramatic increase in late molt. ATGL protein did not show conclusive evidence of relationship to milk lipid or plasma NEFA; nor did the ATGL activity levels drive plasma NEFA or milk lipid content. The action of lipolytic enzymes does not directly drive milk lipid content, but highlights that there may be regulation occurring at the cellular level within the adipocyte or the mammary gland. Regulation of lipid stores plays an

important role in fasting elephant seals and I have demonstrated a development in the understanding of lipid regulation, that ATGL is more important than previously thought, while HSL contributes very little to lipolysis.

Figures

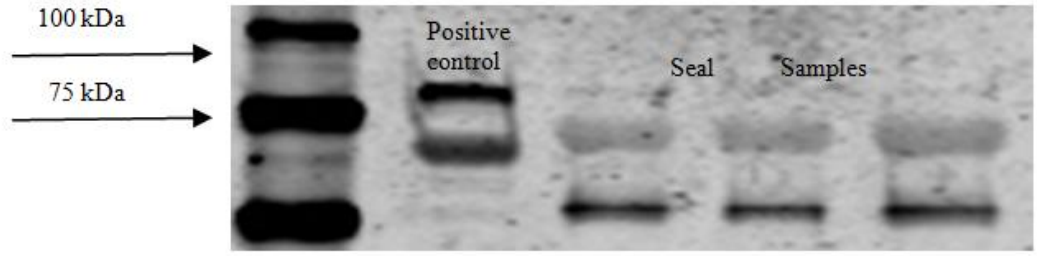

Figure 3. 1: Western blot of mouse (20 µg) and molting seal adipose tissue (50 µg). Blots were incubated with Anti-HSL anti-body (HSL = 84kDa).
Positive control (mouse white adipose tissue) in lane 2, lanes 3-5 seal blubber samples.

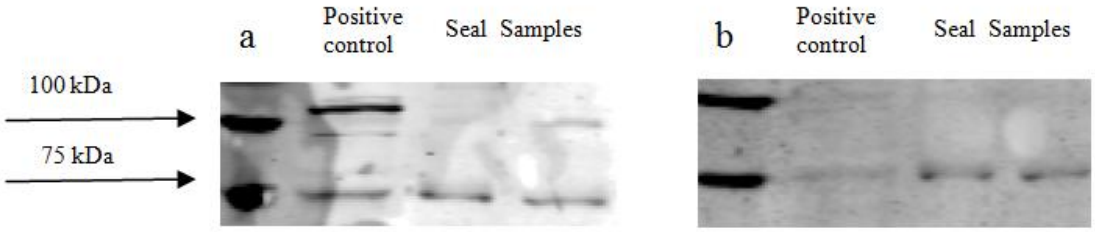

Figure 3. 2: Western blot competition experiment. a) Lane 1: molecular weight marker, lane 2: positive control mouse white adipose tissue (20µg), lanes 3 and 4: seal blubber samples (50 µg). Blots were incubated with full length anti-HSL antibody.
b) Lane 1: molecular weight marker, lane 2: positive control (20µg), lanes 3 and 4: seal blubber samples (50 µg). Full length anti-HSL antibody plus 30 *u*g recombinant HSL. Positive control bands disappear, confirming the success of the competition, but seal bands remain the same, indicating non-specific binding by the anti-HSL antibody.

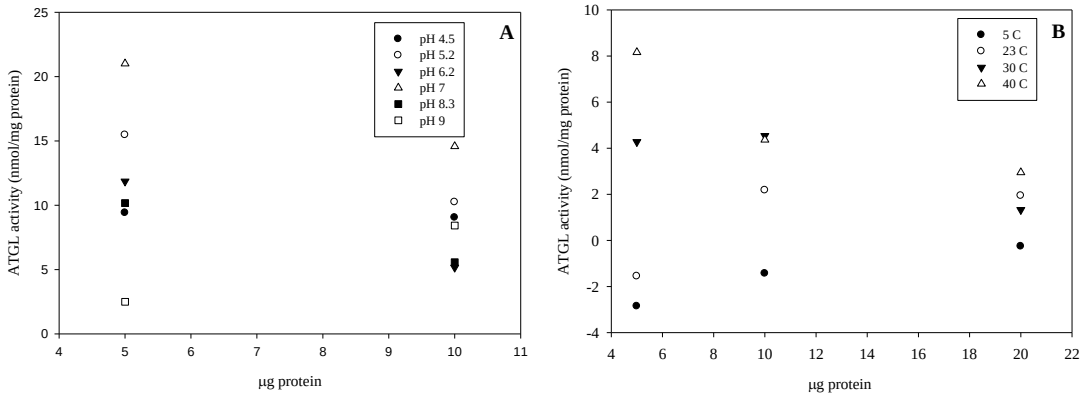

Figure 3. 3: Elephant seal blubber ATGL activity at varying pH (A) and varying temperature (B).

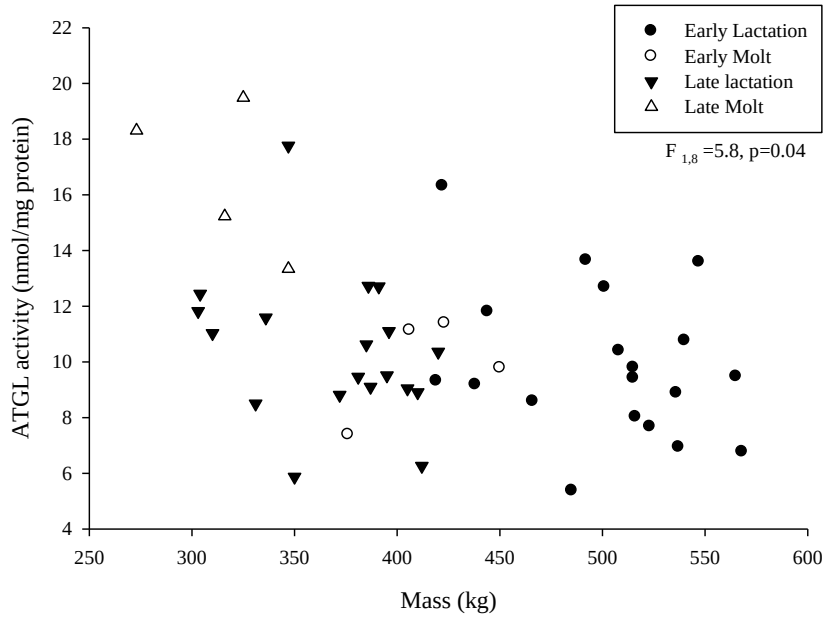

Figure 3. 4: Blubber ATGL activity and mass in all seals, early and late lactation, early and late molt. Significant results obtained with linear mixed effect model with individual seal as a random effect.

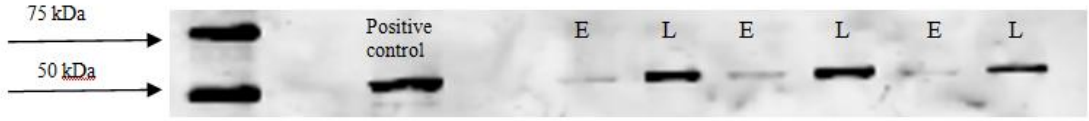

Figure 3. 5: Western blot of mouse and lactating seal adipose tissue (40 µg protein). Blots were incubated with anti-ATGL antibody. (ATGL =55 kDa)
Positive control= mouse white adipose tissue. E=early lactation seal blubber, L=late lactation seal blubber.

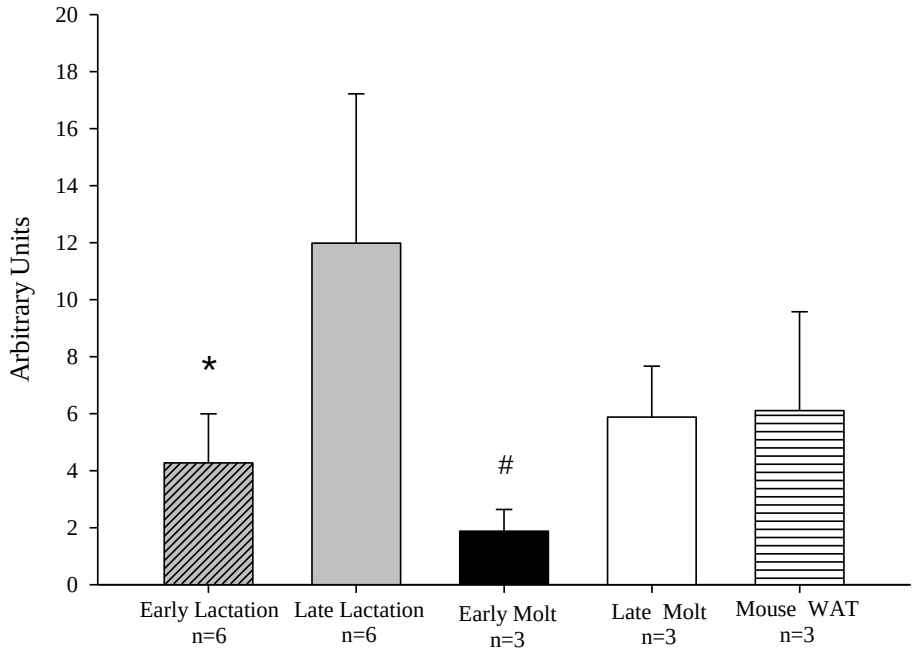

Figure 3. 6: Early and late lactation and molt elephant seal blubber ATGL:GAPDH protein levels from Western blot. WAT= white adipose tissue. * =Early lactation significantly lower than late lactation. # = Early molt significantly lower than late molt.

DISSERTATION CONCLUSION

Lipid has been shown to be the predominant source of energy in fasting elephant seals, including weaned pups (Noren et al. 2003), breeding males (Deutsch et al. 1990), lactating females (Costa et al. 1986) and molting females (Worthy et al. 1992). Our understanding of the regulation of partitioning lipid reserves remains superficial, however. The overarching themes of this thesis were: a) how is lipid mobilization regulated in fasting seals and b) does the release of lipid precursors dictate milk lipid content, or does the mammary gland regulate milk lipid content? This study investigated which specific fatty acids are mobilized from lipid reserves and which are partitioned into milk (Chapter 1). I assessed the relationship among state variables, hormones and lipid metabolites in fasting seals and how lipid metabolites and hormones are related to milk lipid (Chapter 2). In Chapter 3 I quantified lipolytic enzymes in blubber and assessed how these change according to fasting stage, as well as their relationship to milk lipid.

Fasting, lactating elephant seals mobilize large amounts of their lipid stores for milk, but are able to maintain a thermoregulatory lipid layer near the skin. The pattern of mobilization of specific fatty acids conforms to many other species, illustrating that while elephant seals are capable of maintaining stratification, the mechanism for mobilizing specific fatty acids is conserved, and is dictated by biochemical mechanisms. Milk fatty acid profiles and lipid fatty acid profiles match closely, but long-chain monounsaturated fatty acids are preferentially deposited in

milk. A potential mechanism is that availability of long chain monounsaturated fatty acids for milk production remains high due to low oxidation by the mother, implying that supply to the mammary gland dictates milk lipid content. Milk lipid content is also affected by the amount of stored reserves a female has available to milk, as well hormones (increasing cortisol and decreasing insulin) that facilitate lipid release. Decreasing insulin was found to important in facilitating the release of lipids in lactating seals, but not molting seals, likely due to the increased demand of milk synthesis. Growth hormone was found to be uncoupled from both lipid mobilization and milk lipid. Hormone sensitive lipase was much lower expected in fasting seal blubber, while adipose triglyceride lipase was found to contribute more to lipolysis. Adipose triglyceride lipase protein content increased while activity remained stable. Lipolytic enzyme activity wasn't related to milk lipid content, but understanding the action of regulatory molecules, both at the lipid/enzyme interface, as well as re-esterification rates, may further our knowledge of the interaction between lipid release and milk lipid.

Overall, this thesis showed that while specific fatty acids are released in a predictable manner from lipid stores, the hormonal regulation of this release differs among fasting stages, and the enzymes responsible for mobilization is different than previously thought, as is their regulation. Molting seals regulate release of lipid stores differently than lactating seals, primarily through differences in insulin and cortisol. I also showed that regulation at the level of supply of substrate to the mammary gland is an important factor dictating milk lipid content. This was the first

study delineating the activity of two lipolytic enzymes in seal blubber and I showed that one of these enzymes (hormone sensitive lipase) did not contribute to lipolytic activity, while adipose triglyceride lipase is more prominent.

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