The demands of lactation promote differential regulation of lipid stores in fasting elephant seals

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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 conservation and expenditure. Our objective was to understand how fasting northern elephant seals regulate the mobilization of lipid reserves and subsequently milk lipid content during lactation. We sampled 36 females early and 39 at the end of lactation. To determine the separate influences of lactation from fasting, we also sampled fasting but non-lactating females early and late (8 and 6 seals, respectively) in their molting fasting period. Mass and adiposity were measured, as well as circulating non-esterified fatty acid (NEFA), triacylglycerol (TAG), cortisol, insulin and growth hormone levels. Milk was collected from lactating females. Milk lipid content increased from 31% in early to 51% in late lactation. In lactating females plasma NEFA was positively related to cortisol and negatively related to insulin, but in molting seals, only variation in cortisol was related to NEFA. Milk lipid content varied with mass, adiposity, NEFA, TAG, cortisol and insulin. Surprisingly, growth hormone concentration was not related to lipid metabolites or milk lipid. Suppression of insulin release appears to be the differential regulator of lipolysis in lactating versus molting seals, facilitating mobilization of stored lipids and maintenance of high NEFA concentrations 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.

Keywords: cortisol, growth hormone, insulin, lipolysis, milk, marine mammal
Abbreviations: NEFA: non esterified fatty acids; TAG: triacylglycerol; GH: growth hormone; LPL: lipoprotein lipase

1. Introduction

Periods of nutrient restriction are common for most free ranging animals (McCue, 2010). For some animals this reduction in food availability occurs seasonally (e.g. winter or migration) (Battley et al., 2001; Florant and Healy, 2012). Animals that routinely fast as part of their life-history often display physiological adaptations that involve a reduction in energy expenditure such as hibernation, torpor or adaptations in nutrient allocation (Castellini and Rea, 1992; Florant and Healy, 2012; Tøien et al., 2011). Some animals, however, combine natural periods of nutrient restriction with activities that impose large nutrient demands.

Northern elephant seals (*Mirounga angustirostris*) experience fasting concomitant with energetically expensive activities in several life history stages. Fuel stores are accumulated over two long foraging trips thousands of kilometers out to sea in each age class (Le Boeuf et al., 2000; Robinson et al., 2012). Adult males forage for ~ four months for both the post breeding and post molting trips to sea (Le Boeuf et al., 2000). The post breeding foraging trip for adult females lasts 2.5 to 3 months, while the post molting trip lasts approximately seven months (Robinson et al., 2012). When elephant seals are ashore, they fast for several consecutive weeks or months. Adult males and females employ a capital breeding strategy, subsisting on stored reserves during breeding (Costa et al., 1986; Deutsch et al., 1990). Pregnant female elephant seals begin to arrive on shore in late December/early January (Le Boeuf and Laws, 1994). A single pup is born several days after arrival and the mother fasts while nursing the pup for approximately 26 days. Lactation ends when the mother returns to the ocean to forage, leaving the newly weaned pup onshore. Breeding occurs in synchrony, with the majority of the pups born before the end of January and most pups weaned by early March (Le Boeuf and Laws, 1994). Elephant seals also fast during 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, with adult females beginning their molt in late April (Worthy et al., 1992). Additionally, pups fast during postweaning development, after they are weaned by their mothers, but before going to sea for their first foraging trip (Ortiz et al., 1978).

Due to conflicting metabolic demands, the combination of fasting and lactation is especially rare, occurring in only three groups, phocid seals, mysticete whales and bears. Of all reproductive costs, lactation demands the most energy; with many lactating mammals increasing their energy intake by 60-200% (Gittleman and Thompson, 1988). Animals such as the elephant seal that lactate while fasting must synthesize milk exclusively from nutrients mobilized from body reserves. This is extremely unusual, as in contrast, 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.

Both fasting and lactation prioritize use of stored lipids for metabolism. In contrast to other large mammals that lactate for months or years, phocid seals lactate for four weeks or less, produce very energy rich milk and transfer a large amount of energy to the offspring in the short nursing period (Costa et al., 1986). The rapid, high intensity lactation of phocid seals is enabled by having one of the highest milk fat contents among taxa (Costa, 1991). On average elephant seal milk approaches 55% fat content by the end of lactation (Crocker et al., 2001). Phocid milk is very high fat, very low carbohydrate and has a relatively high protein content (Oftedal, 2000). Females use lipid oxidation to fuel about 90% of their energy needs during fasting and lactation (Crocker et al., 2001), while balancing the preservation of vital protein stores with the necessity to provide amino acid precursors to the mammary gland (Crocker et al., 1998, and see

Champagne et al., 2012b; Crocker et al., 2014a for a more comprehensive review of the metabolic alterations during fasting). Thus, the combination of the demands of fasting with the composition of milk require that females must deliver large amounts of lipid and protein substrates to the mammary gland for milk synthesis, while supporting their own maintenance metabolism with fatty acid oxidation, and minimizing oxidation of carbohydrates and proteins.

To support the fat based metabolism exhibited by fasting mammals, lipid must be mobilized from storage molecules (triacylglycerol, TAG) in adipose/blubber and released into the bloodstream as non-esterified fatty acids (NEFA) for uptake and use by other tissues; notably, the mammary gland during lactation. Circulating NEFA may also be taken up by the liver, incorporated into TAG/lipoprotein complexes and secreted back into the bloodstream. Circulating NEFA can diffuse passively across membrane borders or be facilitated by transport proteins (Glatz et al., 2010), while TAG/lipoprotein complexes must be hydrolyzed before entering tissue. Lipoprotein lipase (LPL) is the primary enzyme enabling tissues to hydrolyze circulating TAG/lipoproteins (Frayn et al., 1995). In some species, mammary LPL facilitates milk lipid production by hydrolyzing TAG/lipoprotein complexes so that the lipids are available for uptake by the mammary gland. McDonald and Crocker (2006), however, found low and stable mammary LPL activity in northern elephant seals across lactation, and found no relationship to milk lipid, which suggests that circulating NEFA may contribute more to milk lipid than TAG. Maternal lipid reserves directly influence the milk energy delivered to pups (Crocker et al., 2001) and the ability of the female to spare body protein during lactation (Crocker et al., 1998). Catabolizing maternal 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).

Elephant seals undergoing fasts in different life history stages experience different challenges regarding the partitioning of their lipid and protein reserves. Due to the large role hormones play in managing fuel catabolism, there is the possibility of differential hormonal regulation of lipid metabolism between life history stages. Growth hormone (GH), cortisol and insulin can affect the mobilization and use of fuel stores. While both GH and cortisol can increase lipolysis (Djurhuus et al., 2004), they have opposing effects on protein metabolism; GH promotes lean tissue accretion (Norrelund et al., 2001), while cortisol promotes protein catabolism (Brillon et al., 1995).

Given the varied fasting metabolic demands of different life history stages in elephant seals (e.g. lactation/development/molting), it is not surprising there are different patterns of GH levels in different age classes (Crocker et al., 2012a; Kelso et al., 2012; McDonald, 2003; Ortiz et al., 2003). These differences in baseline GH with changing metabolic demands suggest either potentially differential action of GH between life history stages, or the importance of other hormones to lipolysis. The impact of GH on lipolysis and milk production in fasting, lactating elephant seals has not been clearly elucidated. GH has been shown to affect milk lipid content in domestic cattle (Bitman et al., 1984; Eppard et al., 1985), which makes it a primary candidate for enacting large effects in fasting and lactating seals. Similarly, elephant seals exhibit differences in fasting baseline cortisol levels in different life history stages (Champagne et al., 2005; Champagne et al., 2006; Crocker et al., 2012b; Engelhard et al., 2002; Kelso et al., 2012), again raising the question of differential action in different age classes. The combined effect of lipolytic hormones on milk lipid content is also unclear.

Insulin, an anti-lipolytic hormone (Frayn et al., 1994), also likely affects various age classes of fasting seals differently. The importance of reducing circulating insulin levels in

lactating elephant seals 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 late in lactation. Similarly, low insulin levels may facilitate high levels of circulating NEFA by reducing re-esterification of fatty acids in adipose tissue (Crocker et al., 2014a). Low levels of insulin may be important to maintaining high rates of lipid mobilization for the demands of milk synthesis, but this has not been directly assessed.

Given the diverse effects of these regulatory hormones and their wide variation among northern elephant seal life-history stages, our understanding of metabolic regulation during lactation remains superficial. 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 capital breeding phocids. Variation in nutrient mobilization potentially affects 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.

Our objective was to investigate how stored reserves are partitioned during simultaneous fasting and lactation in the northern elephant seal. We evaluated the effects of hormones on lipid mobilization and subsequent allocation of lipid to milk in fasting and lactating seals. For comparison, we examined the effect of hormones on lipid mobilization in seals that are fasting and molting, but not lactating. We hypothesized that GH and cortisol, both lipolytic hormones, would be positively related to NEFA, as well as to milk lipid content. Insulin was expected to be negatively related to NEFA and milk lipid. Circulating NEFA was also hypothesized to be

158 positively related to milk lipid content, while TAG levels were not expected to be related to milk
159 lipid content.

160 ## 2. Methods

161 ### 2.1. Study Site and Individual Animals

162 We conducted measurements in four study groups—at the beginning and end of each of
163 breeding/lactation (early and late lactation, respectively) and molting periods (early and late
164 molting). This study was carried out at Año Nuevo State Reserve, San Mateo County, during two
165 breeding seasons (Jan-Feb, 2005 and 2010) and the spring molting period (Apr-May 2010). Soon
166 after arrival on land, adult female seals were marked with hair dye (Lady Clairol, Stamford, CT)
167 to facilitate identification. Parturition dates were established by daily observations and
168 considered to be the first day a marked female was observed with a pup, provided she had been
169 observed without a pup the previous day; early lactation samples were collected on day five post-
170 partum and late lactation samples on day 22. Over two years 36 seals were sampled in early
171 lactation and 39 in late lactation, 25 of which were paired. Eight early molt females were
172 captured within five days of arriving on land and six late molt study females were selected on the
173 basis of fully molted pelage, ensuring ~3 weeks of fasting (Le Boeuf and Laws, 1994). Sampling
174 was conducted under NMFS permit 87-1743-06, and all procedures were approved by the
175 Sonoma State University and University of California Santa Cruz IACUC.

176 ### 2.2. Sample Collection and Processing

177 Females were immobilized as previously described (Fowler et al., 2008). Briefly,
178 immobilization was induced with an intramuscular injection of Telazol (tiletamine/zolazepam
179 HCl, Fort Dodge Labs, Ft. Dodge, IA) at a dosage of ~1mg/kg and maintained with ~100 mg
180 bolus intravenous injections of ketamine. Milk (~5 mL) was collected after 40 units of oxytocin

were administered intramuscularly. Blood 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. Serum and plasma were centrifuged at 4°C, and frozen at -80ºC until further analysis.

Body composition measurements (percent adiposity) 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 (Scanoprobe II, Ithaca Scanco, 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 or serum samples using a commercially available kit (Wako Diagnostics, Richmond, VA, lower detection limit: 0.0014 mmol/L). Serum TAG was measured in duplicate using commercially available kits (Wako Diagnostics, Richmond, VA) or after the methods of Tift et al (2011), using a Cholestech LDX Analyzer (Cholestech, Hayward, CA). The lower detection limit was 0.51 mmol/L. Sixteen of the 53 samples measured were below the detectable limit and so were assigned the value of 0.51 mmol/L. Milk lipid content (% total) was determined gravimetrically after solvent extraction (Debier et al., 2003; McDonald and Crocker, 2006).

Hormones were assayed in duplicate in serum samples. Cortisol and insulin were assayed using radioimmunoassay (Siemens, Catalog #TKCO2; lower detection limit: 0.2µg/dl, Sensitive Rat, Millipore, Catalog #SRI-13K lower detection limit: 0.02 ng/ml). Both kits have been

203 previously validated for northern elephant seals (Champagne et al., 2005; Ortiz et al., 2001). The
204 mean intra and inter-assay %CV were 2.6% and 3.4% for cortisol and 1.9 and 4.2%, for insulin.
205 GH in 2005 was measured by radioimmunoassay (Linco, catalog # PGH-46HK; lower
206 detection limit: 1 ng/ml) and has been validated previously (Ortiz et al., 2003). The GH
207 radioimmunoassay kit was discontinued, so GH in 2010 was measured using rat/mouse growth
208 hormone ELISA assay (Millipore, catalog # EZRMGH-45K; lower detection limit: 0.07 ng/ml).
209 The assay demonstrated parallelism of the standard curve to serially diluted elephant seal serum
210 samples. The mean intra-assay %CV was 7.2 %. A subsample of 11 plasma samples was assayed
211 using both assay platforms. The %CV between values from the 2 assay platforms was 7.1% and
212 showed no directional bias.
213 **2.3. Statistics**
214 Statistical analyses were performed using the software R (Version 3.0.1, R Development
215 Core Team, www.R-project.org). Multicollinearity among model explanatory variables was
216 assessed using a variance inflation factor (VIF) calculated in R with the car package (Fox and
217 Weisberg, 2011). No factors had VIF > 3.5 indicating a lack of multicollinearity. Study groups
218 were comprised of four fasting stages: early lactation, late lactation, early molt and late molt.
219 Due to repeated sampling of some individuals, linear mixed effect (LME) models were used to
220 assess differences between fasting stages, with individual seal and Year included as random
221 effects. Tukey's HSD post-hoc tests were used to evaluate differences among fasting stages
222 following a significant overall model (package multcomp, Hothorn et al., 2008). Similarly,
223 associations between regulatory hormones and metabolites or milk content were assessed using
224 LME models. LME models were fit with the lme4 package (Bates et al., 2013). F statistics and p
225 values were generated using df with the Kenward-Rogers approximation and the lmerTest

package (Kuznetsova et al., 2013). Model residuals were assessed for approximate normality and homoscedasticity. Response variables were log transformed when necessary (NEFA, TAG and adiposity variables). In models with continuous response and predictor variables, an R^2 for mixed models was calculated for significant fixed effects (Edwards et al., 2008). No interactions were found to be significant in any model and so they were excluded. The significance of all results was considered at $\alpha = 0.05$. Changes (increases or decreases) are described relative to fasting stages.

3. Results

3.1. Differences among fasting stages

The statistical significance of post-hoc tests is reflected in Table 1. As expected, mass declined significantly during fasting, both in lactation and during molting ($F_{3,38.2} = 361.1$, $p < 0.001$, Table 1). Early lactation females had significantly higher adipose stores than all other groups and early molt females had higher adipose stores than late lactation females ($F_{3,68.9} = 56.3$, $p < 0.001$; Table 1). Milk lipid content increased across lactation ($F_{1,44.8} = 460.1$, $p < 0.001$;Table 1).

Cortisol displayed the largest magnitude of change among fasting stages, with mean levels increasing tenfold across the molt fast ($F_{3,70.2} = 50.2$, $p < 0.001$; Table 1). Post-hoc testing revealed early molt levels were significantly lower than all other stages at 8.4 (SD = 1.8) ng/mL, and mean levels increased over both the molt fast and lactation fast (Table 1). Insulin levels varied among the study groups ($F_{3,70.4} = 18.4$, $p < 0.001$, Table 1) decreasing from early to late lactation but levels were not different among late lactation and early and late molt. Mean GH displayed an increasing trend over lactation from early to late ($p = 0.05$), but values were not significantly different among any fasting stage ($p > 0.05$; Table 1). NEFA levels varied among

fasting stage ($F_{3,59.2}$ = 24.2, $p < 0.001$) with the highest concentrations in late lactation, 2.59 (SD = 1.12) mmol/L (Table 1). TAG concentrations varied among fasting stages ($F_{3,67}$ = 4.5, p 0.006), increasing from early to late lactation (Table 1) while molt values were not different from either lactation stage.

3.2. Hormone-Metabolite Relationships

The cumulative effects of hormones on circulating NEFA were assessed in lactating seals as a mixed effect model with insulin, cortisol and GH as predictors and seal ID and year as random effects (% variance due to seal ID < 0.1%). Insulin had a significant negative relationship (slope = - 0.002, R^2 = 0.22, $F_{1,56.4}$ = 5.2, p=0.03) and cortisol a significant positive relationship (slope = 0.05, R^2 = 0.56, $F_{1,67}$ = 28.6, p<0.001) with circulating plasma NEFA, while GH was not related ($F_{1,66.8}$ = 1.9, p = 0.18). 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 (slope = 0.02, R^2 = 0.74, $F_{1,10}$ = 9.5, p = 0.01) was detected; insulin and GH were not related to NEFA levels (p > 0.05). The same mixed model approach was also applied to assess the effect of hormones on TAG. In lactating animals cortisol was positively related to TAG (slope = 0.02, $F_{1,67.2}$ = 6.4, p = 0.01), while in molting seals none of the hormones significantly affected TAG (p > 0.05) (% variance due to seal ID 12.9%).

3.3. Milk Lipid Variation

The cumulative effect of hormones was assessed relative to milk lipid content, with insulin, cortisol and GH as predictors and seal ID and year as random effects (% variance due to seal ID < 0.01%). Increasing cortisol levels (slope = 0.02, R^2= 0.69, $F_{1,68.4}$ = 34.0, p<0.001) and decreasing insulin (slope = - 0.001, R^2= 0.42, $F_{1,67.1}$ = 16.3, p < 0.001) were significantly related

to milk lipid levels, while GH levels did not affect milk lipid ($p > 0.05$) (Figure 1). Relationships among hormones and milk lipid were only apparent across lactation; within stages there were no significant relationships of hormones to milk lipid content. The contribution of mass, adiposity and circulating metabolites (NEFA and TAG) to milk lipid variation was assessed with a linear mixed effects model including seal ID and year as random effects (% variance due to seal ID < 0.01%). Mass (slope: - 0.05, $R^2 = 0.67$, $F_{1,55.7} = 29.3$, $p < 0.001$) and adiposity (slope: -95.1, $R^2 = 0.53$, $F_{1,68.3} = 19.5$, $p < 0.001$) were negatively related to milk lipid, NEFA (slope: 2.7, $R^2 = 0.34$, $F_{1,68.0} = 8.8$, $p = 0.004$) was positively related to milk lipid content across lactation, while TAG did not contribute significantly ($p > 0.05$).

4. Discussion

4.1 Hormonal Effects on Lipid Metabolites

The release of lipid stores during fasting was regulated differently in lactating and molting seals. During lactation NEFA release was facilitated by increased cortisol and decreased insulin. The level of insulin suppression in lactating females predicted NEFA concentrations. No such relationship was evident in molting females, despite low insulin values that were similar to those at the end of lactation. The difference in the apparent regulatory impact of insulin on circulating NEFA may reflect the dramatic differences in nutrient demands for NEFA delivery to the mammary gland during lactation. This difference may also reflect the role that insulin plays in influencing reuptake and re-esterification of NEFA by adipocytes (Crocker et al., 2014a). Seals lose far less of their blubber stores during molting than during lactation (Table 1). While the average daily metabolic rates (excluding milk energy) are similar between lactating 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; Crocker et al., 2001; Worthy et al., 1992). Rates

of lipolysis late in the molt are about 1/3 that during lactation (Houser et al., 2007). The role of insulin in regulating net lipolysis may be accentuated under the higher rates of fatty acid mobilization and maintenance of higher plasma fatty acid concentrations during lactation. When challenged with an exogenous glucose load, lactating females responded with an insulin release early in lactation but this response was abolished at the end of lactation, in direct relation to the loss of adipose tissue reserves (Fowler et al., 2008). Furthermore, fasting northern elephant seal pups develop insulin resistance in adipose tissue over the post-weaning fast (Viscarra et al., 2011a; Viscarra et al., 2011b). This loss of adipocyte sensitivity to insulin may help reduce reuptake and re-esterification of fatty acids. Low insulin levels during lactation may play important roles in reducing re-esterification during lactation and lead to the dramatic decoupling of plasma NEFA from whole-body lipolysis (Crocker et al., 2014a; Houser et al., 2007).

In addition to insulin's effect on lipid metabolism, it plays an important role in carbohydrate metabolism. Fasting elephant seals display relatively high plasma glucose levels, as well as high rates of 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 these high rates of glucose production (Champagne et al., 2012a; Tavoni et al., 2012) and it is likely that females use 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). Insulin promotes *de novo* synthesis of lipids from glucose by the mammary gland (Robinson et al., 1978), but seals are likely not synthesizing milk lipids *de novo* (Fowler et al., 2014). Low insulin levels during lactation may also suppress glucose uptake and disposal by the mammary gland and peripheral tissues.

317 The influence of insulin on glucose metabolism may affect lipid metabolism. Fasting
318 elephant seals exhibit high rates of lipid mobilization and subsequent beta-oxidation, resulting in
319 large amounts of acetyl-CoA production. Acetyl-CoA acceptance into the tricarboxylic acid
320 (TCA) cycle depends on an adequate pool of TCA cycle intermediates (namely oxaloacetate). If
321 sufficient TCA cycle substrates are not available, excess acetyl-CoA molecules are converted
322 into ketones and at high levels these may lead to metabolic ketoacidosis. The high rates of TCA
323 cycle activity required in fasting elephant seals necessitate high levels of TCA cycle
324 intermediates; these may be supplied by high rates of glucose recycling. In elephant seals, high
325 rates of TCA cycle have been measured, with intermediates likely supplied by high rates of
326 glucose recycling (Champagne et al., 2012a). Fasting elephant seals maintain very low levels of
327 ketones, despite their high rates of lipid oxidation (Champagne et al., 2005; Houser et al., 2007).
328 The supply of TCA cycle intermediates from active glucose recycling has been hypothesized to
329 support the high TCA cycle activity in elephant seals, facilitating the entry of acetyl-CoA into
330 the TCA cycle and thus alleviating ketone accumulation (Houser et al., 2012).
331 Both GH and cortisol were predicted to be related to circulating NEFA, given that both of
332 these hormones stimulate lipid breakdown (Djurhuus et al., 2004). We did not however, detect a
333 relationship between circulating lipid metabolites and GH. Ortiz et al. (2003) found that NEFA
334 increased significantly with both cortisol and GH in fasting elephant seal weanlings and
335 suggested that cortisol and GH may act synergistically to promote lipolysis. Despite GH's
336 lipolytic actions in other studies, GH's influence on lipid mobilization was contrary to our
337 expectations in the present study. However, in fasting adult male and juvenile northern elephant
338 seals GH concentrations and NEFA availability did appear to be uncoupled (Crocker et al.,
339 2012b), with GH decreasing during fasting. High NEFA levels have been shown to have a

340 negative feedback on GH secretion (Dieguez and Casanueva, 1995). The inhibitory effects of
341 increasing NEFA may keep GH from increasing unchecked over the fast, but due to the
342 demonstrated lipolytic effect of GH, we would still expect a positive relationship between NEFA
343 and GH. Varied GH patterns in fasting elephant seals remains unexplained. Effects of GH are
344 likely complex and dependent on specific demands during the fasting stage (i.e. growth or need
345 to minimize protein catabolism) (Crocker et al., 2012b; Kelso et al., 2012; Ortiz et al., 2003).

346 **4.2. Milk Lipid Content**

347 The strong effect of NEFA on milk lipid supports the hypothesis that milk energy content
348 is regulated by fatty acid delivery to the mammary gland. Interestingly, a previous investigation
349 in northern elephant seals also found a positive relationship between mass and milk lipid early in
350 lactation, but failed to detect a relationship between either NEFA or TAG and milk lipid content
351 (McDonald, 2003). The much larger sample size of the current study (over twice as many
352 measurements as McDonald (2003) may have strengthened our ability to detect a pattern.

353 In this study, we did not detect a relationship between TAG and milk lipid, which is
354 expected given the low mammary LPL activity and lack of relationship between LPL activity
355 and milk lipid (McDonald and Crocker, 2006). The lack of influence of circulating TAG is
356 consistent with a reduced role for mammary LPL in facilitating milk synthesis in northern
357 elephant seals. In both grey and hooded seals, two capital breeding phocid species hypothesized
358 to regulate milk lipid at the level of the mammary gland, milk lipid content increased in parallel
359 with mammary LPL concentrations as well as circulating NEFA concentrations, implying
360 mammary use of both circulating TAG and NEFA for milk synthesis (Iverson et al., 1995;
361 Mellish et al., 1999b). NEFA significantly correlated with milk lipid in grey seals, (Mellish and
362 Iverson, 2001) but neither mass nor adiposity explained milk fat content (Mellish et al., 1999a).

While the correlations between NEFA and milk lipid content seem to imply that lipid supply is an important factor influencing milk content in both these species, the lack of relationship of adiposity and mass to milk lipid suggests that the mammary gland may have tighter control of milk lipid content in hooded and grey seals. Differential tissue regulation among phocids appears possible, where regulation of lipid release from storage depots, rather than at the level of the mammary gland via LPL, may play a large role in milk lipid content in elephant seals. These significant differences in mammary gland regulation in closely related species suggest that lactation efficiency and mammary function may be under strong selection in phocid seals. In comparison to other mammals, goat 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 (e.g fatty acids) (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.

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 increasing availability of NEFA to the mammary gland. Cortisol affects the mammary gland in several ways, as a transcription factor for many milk proteins (Casey and Plaut, 2007) as well as promoting lipogenesis (Dils et al., 1976). In elephant seal mammary gland, however, there is presumably little *de novo* lipogenesis (Fowler et al., 2014; Iverson et al., 2004). The effect of cortisol would thus likely be on lipid mobilization within adipose tissue, rather than lipogenic

386 stimulation within the mammary gland. Although cortisol may stimulate mammary LPL (Flint et
387 al., 1984), this is unlikely in elephant seals because mammary LPL activity remains low
388 throughout lactation (McDonald and Crocker, 2006). The relationship of cortisol to both NEFA
389 and milk lipid indicates that cortisol is probably affecting milk lipid content by increasing
390 circulating NEFA availability (by increased lipolysis, decreased fatty acid re-esterification, or a
391 combination of the two; see Crocker et al., 2014a; Houser et al., 2007), thus increasing substrate
392 supply to the mammary gland.

393 Insulin affects many tissues; it stimulates the uptake of circulating TAG via LPL
394 (Kraemer et al., 1998) and elevated insulin can decrease milk fat yield and NEFA availability
395 (Corl et al., 2006). In several species, insulin levels decrease during lactation and tissue insulin
396 sensitivity increases (humans: Tigas et al., 2002, sheep: Hatfield et al., 1999, cattle: Sartin et al.,
397 1985, goats: Debras et al., 1989, and rats: Burnol et al., 1986), while elephant seals display
398 insulin insensitivity during lactation (Fowler et al., 2008). Based on the inhibitory effect of
399 insulin on lipolysis (Frayn et al., 1994) and the lack of milk lipid regulation via LPL (McDonald
400 and Crocker, 2006), we hypothesized that insulin would have a negative relationship to milk
401 lipid content. This hypothesis was supported, but the relationship was only apparent when
402 concomitant cortisol levels were included in the analysis. The negative relationship between
403 insulin and NEFA release in lactating females indicates that insulin is likely affecting milk lipid
404 content via the provisioning of NEFA; these data suggest that the removal of the inhibition of
405 insulin on lipolysis is critical to the supply of NEFA to the mammary gland.

406 GH is most consistently associated with an increase in milk yield in dairy cattle via IGF-1
407 signaling in the mammary gland (Hoshino et al., 1991), but has also been associated with an
408 increase in milk lipid (Bitman et al., 1984; Eppard et al., 1985). Although the time frame for

lactating cattle is quite different, given that they lactate for many months, GH increases shortly after parturition and is highest during peak milk production times in cattle (the first month), but decreases thereafter (Hoshino et al., 1991).While we did not measure yield in this study, there is potential for GH to exert control via IGF-1.

As lactation progresses females lose mass and catabolize lipid reserves while milk lipid content increases, resulting in smaller/leaner females producing more lipid rich milk in late lactation, thus the negative relationship in this study. Females lose insulin sensitivity and ability to secrete insulin in direct relation to the depletion of adipose reserves (Fowler et al., 2008). In response to a glucagon challenge, females at the end of lactation (ie smaller, leaner females) display an attenuated insulin response, while the lipolytic response increases (Crocker et al., 2014b), but whole body lipolysis rates are stable as females become leaner from early to late lactation (Houser et al., 2007). All of these responses suggest a potential role for adipose-derived hormones in regulating metabolism in a way that maximizes lipolysis and NEFA availability as reserves are depleted. Indeed, circulating NEFA and milk fat are highest when individual females are leanest and smallest, at the end of lactation. In addition larger females have higher rates of metabolic water production (Costa et al. 1986) and this may facilitate higher milk water content. These mechanisms may allow smaller, leaner females to compensate for lower overall rates of milk production (Crocker et al., 2001) with increased lipid content and may be critical to facilitating high milk lipid content when females have depleted the bulk of their adipose reserves.

4.3. Conclusions

Two hormones, cortisol and insulin, appear to exert the predominant regulatory control on NEFA availability to the mammary gland in fasting, lactating northern elephant seals. Surprisingly, there was no evidence of the impact of GH on either NEFA availability or milk

lipid content. A comparison to molting females, fasting for similar durations, showed that the regulatory impacts of insulin were specific to lactation. The substantial increases in plasma cortisol that occurred during fasting have implications for the interpretation of baseline cortisol levels in breeding phocids. The importance of low insulin levels during lactation supports the adaptive significance of hypoinsulinemia and insulin insensitivity in fasting seals.

NEFA availability had important impacts on milk lipid content, supporting the ability of the mammary gland to uptake fatty acids is the primary determinant of milk energy delivery in northern elephant seals. Body size and composition did not influence NEFA levels and had inverse relationships to milk lipid content. This result is consistent with previous suggestions that NEFA availability results from complex regulation of both lipolysis and rates of re-esterification and that metabolic responses to regulatory hormones are altered by changing adiposity. This decoupling of plasma NEFA availability from the magnitude of fat reserves may facilitate the dramatic increase in milk lipid content late in lactation despite significant depletion of adipose reserves.

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Figures Legends

Figure 1: Relationship of insulin and cortisol to milk lipid content. Regression parameters were extracted from a mixed model with 'seal ID' and 'Year' as random effects. A) Insulin was negatively related to milk lipid content across lactation (R^2= 0.42, $F_{1,67.1}$ = 16.3, $p < 0.001$). B) Cortisol was positively related to milk lipid content across lactation (R^2= 0.69, $F_{1,68.4}$ = 34.0, $p<0.001$). Open circles are late lactation, closed circles are early lactation.

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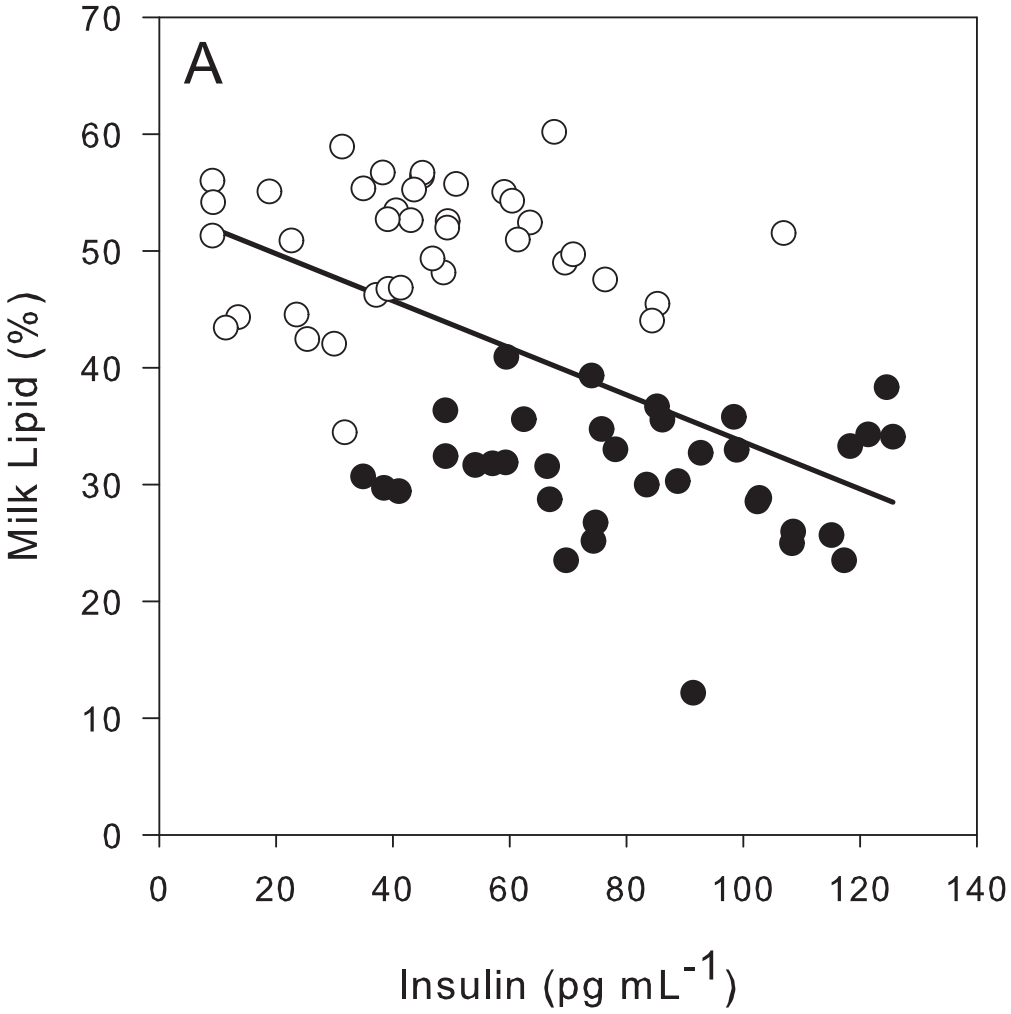
Tables

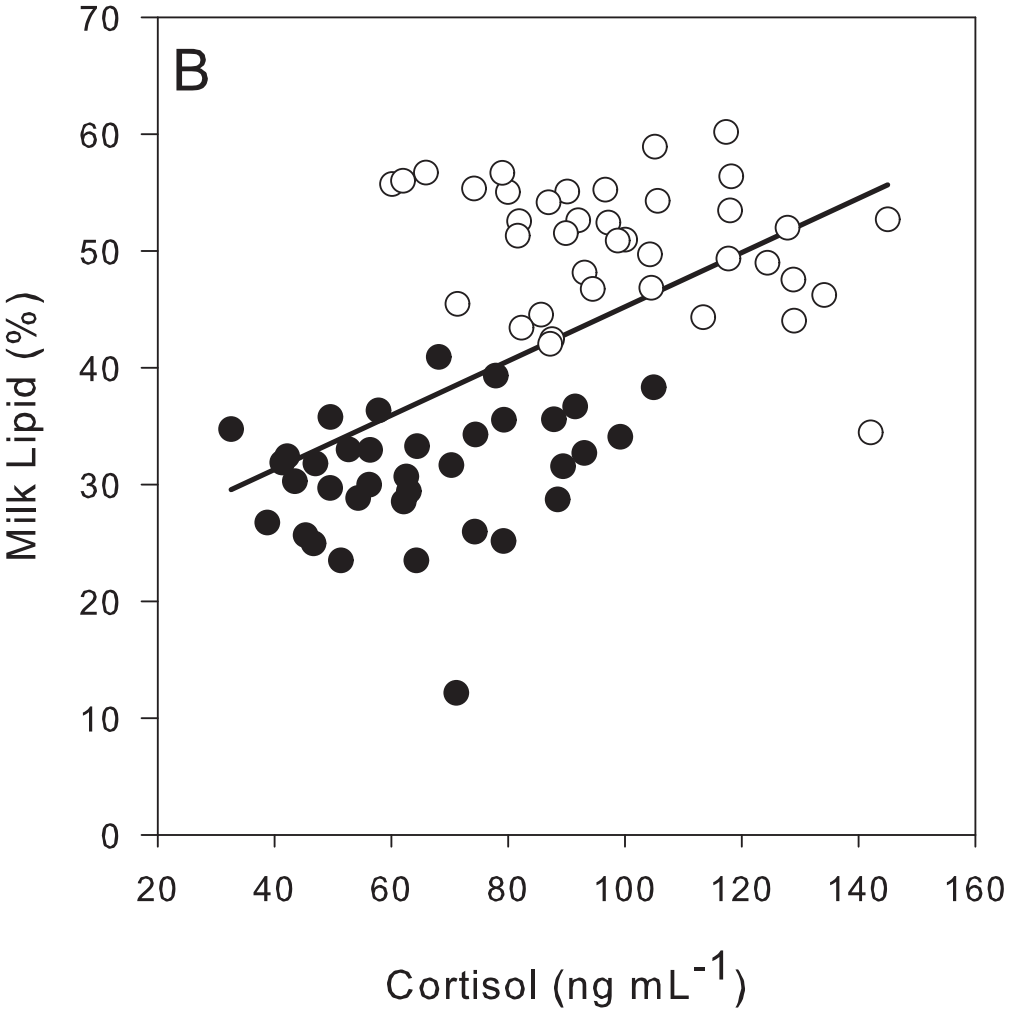
Table 1: Hormone, metabolite and mass changes during fasting and lactation and fasting and molting northern elephant seals. Values are expressed as means (standard deviation).

	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)^{c}	0.30 (0.01)^{bc}
TAG (mM)	0.76 (0.30)^{a}	0.93 (0.30)^{b}	0.65 (0.17)^{ab}	0.81 (0.25)^{ab}
NEFA (mM)	1.27 (0.44)^{a}	2.59 (1.12)^{b}	1.00 (0.02)^{a}	1.52 (0.47)^{ac}
Milk lipid (%)	31.0 (5.4)^{a}	50.6 (5.4)^{b}	NA	NA
Cortisol (ng/mL)	64.8 (18.7)^{a}	99.3 (21.8)^{b}	8.4 (1.8)^{c}	92.3 (26.6)^{b}
Insulin (pg/mL)	82.1 (26.0)^{a}	44.4 (22.8)^{b}	42.0 (22.1)^{b}	47.1 (21.4)^{b}
GH (ng/mL)	2.5 (1.01)	3.03 (1.17)	2.98 (1.31)	3.60 (1.89)

NEFA=circulating non esterified fatty acids
TAG= circulating triacylglycerol
Values that do not share similar superscripts within rows are significantly different (LMM, followed by Tukey's HSD test, $p<0.05$).

Figures


A
Milk Lipid (%)
Insulin (pg mL-1)
0
10
20
30
40
50
60
70
0
20
40
60
80
100
120
140


B
Milk Lipid (%)
Cortisol (ng mL-1)
0
10
20
30
40
50
60
70
20
40
60
80
100
120
140
160