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Impact of Nutritional Status on the Somatotropic Axis and Ghrelin in Phocid Seals

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IMPACT OF NUTRITIONAL STATUS ON THE SOMATOTROPIC AXIS AND GHRELIN

IN PHOCID SEALS

by

Rachael Elaine Dailey

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CERTIFICATE OF APPROVAL

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Table of Contents

List of Figures

Chapter 2

Chapter 3

Chapter 4

List of Tables

Chapter 2

Chapter 4

Acknowledgements

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Abstract

Metabolic hormones and their axes, including the target tissues and receptors, regulate the tissue specific utilization of nutrients with in the body. The purpose of this research was to understand the hormonal control of complex nutrient partitioning mechanisms involved in young, growing animals. Specifically, this involved the investigation of metabolic hormones and the regulation of growth in two common species of phocids (true seals): harbor seal (Phoca vitulina) and Northern elephant seal (Mirounga angustirostris) pups. This longitudinal study examines young phocids from nutritional nadir through realimentation (realimentation) to investigate how metabolic hormones involved in both food intake and nutrient partitioning change with respect to nutritional state.

To investigate the role of metabolic hormones during realimentation in a small phocid seal, chapter 2 focuses on the changes in the somatotropic axis and ghrelin during a 10-week period of realimentation following nutritional nadir. Chapter 3 focuses on the application of the results of previous research and the second chapter of this thesis to a specific experimental feeding project. Chapter 4 focuses on the response to changes in nutritional status in the fasting adapted NES. Given the changes in metabolism and priority of nutrient utilization associated with transitioning from a nursing neonate to a fasting adapted juvenile, NES provide a unique opportunity to assess the effect of age on the response to realimentation.

Overall, this research will further expand the understanding of tissue specific demands and the effect on endocrine response to realimentation. By incorporating assessments of metabolic changes based on nutrition as well as age, this study will expound on how metabolic hormones are involved in regulating the trade-off between adipose and lean tissue development in this unique taxon.

Chapter 1

Literature Review and Introduction

Introduction

Metabolic hormones and their axes, including the target tissues and receptors, regulate the tissue specific utilization of nutrients within the body. Concentrations of hormones as well as the availability and location of receptors controls which crucial functions energy is allocated toward: maintenance, growth, storage, or reproduction (Perrin and Sibly 1993). In young animals undergoing rapid growth and development, nutrients are primarily devoted to growth and maintenance of tissues (Perrin and Sibly 1993). After these requirements are supplied, any excess energy is shunted toward storage. In mammals, this storage is generally in the form of adipose tissue (Vanghan et al. 2011).

The purpose of this research is to understand the hormonal control of complex nutrient partitioning mechanisms involved in these young, growing animals. Specifically, this involved the investigation of metabolic hormones and the regulation of growth in two common species of phocids (true seals): harbor seal (*Phoca vitulina*) and Northern elephant seal (*Mirounga angustirostris*) pups. In marine mammals, the period of growth in early life is of particular interest. Marine mammals require large quantities of adipose in the form of blubber to facilitate efficient thermoregulation in an aquatic environment (Schulz and Bowen 2005). Due to this demand for adipose tissue accretion, nutrients may be allocated preferentially to vital adipose stores over other tissues. The

physiological mechanisms regulating this potential metabolic trade-off are not yet well understood in marine mammals.

This longitudinal study examines young phocids from nutritional nadir through realimentation (refeeding) to investigate how metabolic hormones involved in both food intake and nutrient partitioning change with respect to nutritional state. This study focuses on how key metabolic hormones, components of the somatotropic axis, as well as ghrelin, change with respect to nutritional status (adequacy of nutrient intake relative to metabolic demand).

Hormonal Function

The somatotropic axis is the hormonal axis responsible for regulating somatic growth throughout the body tissues (Breier 1999; Renaville et al. 2002; Etherton 2004). The primary components of the axis, growth hormone (GH), insulin-like growth factors (IGF)-I and -II, and the IGF binding proteins (IGFBP; Breier 1999; Etherton 2004) regulate the allocation of nutrients to different tissues (Breier 1999; Renaville et al. 2002; Etherton 2004).

Growth hormone, a 191-amino acid peptide hormone, is secreted in a pulsatile manner by the anterior pituitary in response to two hormones working in opposition (Renaville et al 2002; Tuggle and Trenkle 1996). Secretion is stimulated by Growth Hormone Releasing Hormone (GHRH) and inhibited by somatostatin (Hartman *et al*. 1991; Tuggle and Trenkle 1996). After secretion from the pituitary, GH binds to GH receptors throughout the body, inducing direct metabolic and growth effects (Wang *et al.* 2004). Growth hormone regulates the mobilization of fatty acids from adipose and decreases protein catabolism (Wang *et al.* 2004). Growth hormone also acts indirectly

via the stimulation of IGF-I production and release from the liver (Chung et al. 1985; Buonomo et al. 1987; Reviewed in: Buonomo and Baile 1991; LeRoith *et al.* 2001).

Insulin-like growth factor-I is the peptide hormone primarily responsible for the anabolic effects of the axis, inducing cell proliferation and differentiation throughout the body tissues (Duan *et al.* 2010). GH also stimulates autocrine and paracrine actions of IGF-I in bone and muscle (Wang *et al.* 2004; LeRoith *et al.* 2001). After secretion, the biological action of IGF-I is further modulated by IGFBP, which alter the bioavailability of IGF-I to bind to target tissue receptors (Firth and Baxter 2002).

In times of undernutrition, concentrations of GH generally increase (Breier 1999; Renaville *et al.* 2002; Richmond et al. 2010; Ortiz et al. 2003). This increase in GH concentrations stimulates the mobilization of fatty acids to be utilized as a source of energy when intake is low (Renaville *et al.* 2002, Wang *et al.* 2004). Even though GH concentrations are increased at this nutritional state, IGF-I concentrations do not increase but actually decrease during periods of undernutrition (Breier 1999; Buonomo and Baile 1991; Breier et al. 1986; Richmond et al. 2010; Ortiz et al. 2003). The stimulation of IGF-I by GH is uncoupled during periods of undernutrition through the down-regulation of GH receptors in the liver (Bornfeldt *et al.* 1989; Dauncey et al. 1994). The resulting decrease in IGF-I concentrations reduces somatic growth in favor of mobilizing energy for basic metabolic requirements (Thissen *et al.* 1999).

Ghrelin is a metabolic hormone associated with satiety and the regulation of food intake (Nakazato *et al.* 2001; Korbonits et al 2004). This peptide hormone is expressed primarily in the stomach but is also present in other parts of the digestive system and brain (Date *et al.* 2000). In humans and rodents, ghrelin increases prior to meals and

during periods of fasting, resulting in increases in food intake and stimulation of growth hormone release (Nakazato *et al*. 2001; Toshinai *et al*. 2001; Takaya *et al*. 2000; Strassburg et al 2008). Research suggests that ghrelin is involved in short-term and long-term regulation of nutrient intake relative to energy status of the organism (Nakazato *et al*. 2001; Cummings *et al.* 2002; Tanaka *et al.* 2004). Ghrelin decreases after consumption of individual meals but also exhibits a long-term (1 to 3 months) decrease upon realimentation after extended fasting or anorexia (Nakazato *et al*. 2001; Cummings *et al*. 2002; Tanaka *et al*. 2004). Additionally, upon realimentation ghrelin facilitates lipid synthesis and storage (Sangiao-Alvarellos *et al*. 2011). When ghrelin is experimentally suppressed, fasted rats fail to gain weight upon realimentation (Sangiao-Alvarellos *et al*. 2011). This suggests that ghrelin has a vital role in recuperation of weight, particularly in the form of adipose.

Overall, the increase of ghrelin and GH and the decrease of IGF-I during undernutrition would result in increased lipolysis, decreased energy expenditure, and decreased growth (Buonomo and Baille 1991; Thissen et al. 1999; Ortiz et al. 2003). Upon realimentation, the change in action and concentration of these hormones would again favor adipose deposition and somatic growth. The resultant change in nutrient allocation as a function of these hormones can be assessed through growth of tissues and changes in proportion of tissues (adipose versus lean). This is accomplished through many techniques, including ultrasound and bioelectric impedance analysis (Fanelli and Kuczmarski 1984; Gales et al. 1994). Additionally, measurements of specific metabolites in the blood are assessed, representing changes in metabolism of different types of nutrients and movement of energy between tissues (Mellish and

Iverson 2001; Bossart et al 2001; Castellini and Rea 1992). These metabolites include

blood urea nitrogen (BUN), total protein, and creatinine that are both indicators of

protein metabolism (Castellini and Rea 1992; Bossart et al 2001). Additionally

metabolites, such as triglycerides and glucose indicate the extent of lipid and

carbohydrate metabolism respectively (Bossart et al 2001). These blood metabolites are

also often analyzed for common veterinary procedures, making them an easily

accessible indicator for metabolism of specific nutrients.

Previous Research in Marine Mammals

As part of normal life history, marine mammals generally acquire significant

quantities of adipose that is vital for survival. Phocid, or true seal, pups can exceed 50%

body fat at weaning (approximately one month old; Schulz and Bowen 2004). Increases

in body mass and fat mass at weaning are positively correlated with increase survival

through the first year of life (Muelbert *et al*. 2003, Harding *et al*. 2005). Adipose in the

form of blubber is used for thermoregulation, forming a streamlined body shape, as well

as an energy reserve (Schulz and Bowen 2005; Strandberg et al. 2008). While these

functions are common among mammals, the substantial quantity of adipose required

and rapid growth rate of young animals may elicit a differential response in metabolic

hormones as a result of changes in nutritional status.

Limited research on these hormones in marine mammals has been conducted

using either northern elephant seals (NES) or harbor seals. Research in NES is limited

to fasting (Ortiz et al 2003; Champagne et al. 2012), and has not investigated the

response of these hormones to realimentation. Research in harbor seals has been

conducted on the response of components of the somatotropic axis to realimentation

(Richmond et al. 2008; 2010). However, the understanding of this response can be expanded by incorporating other metabolic hormones that participate in the regulation of nutrient partitioning and satiety.

Ghrelin has been shown to increase during fasting in young NES (Ortiz *et al.* 2003). However, ghrelin has yet to be studied in pinnipeds during realimentation or normal nutrient intake. Given the role of ghrelin in the promotion of adiposity and the unique requirements of adipose accretion in marine mammals, ghrelin may exhibit a differential response to realimentation than what has been observed in other mammals.

Investigations of the somatotropic axis in pinnipeds support that the axis follows patterns common in terrestrial mammals with regard to nutritional status (Richmond et al. 2010; Ortiz et al. 2003). In two phocid seal species, NES and harbor seals, GH increases during times of undernutrition or fasting (Richmond *et al.* 2008, 2010; Ortiz *et al.* 2003). IGF-I exhibits the typical pattern in response to nutrition in harbor and Weddell seals, decreasing during periods of undernutrition and increasing with realimentation (Richmond *et al.* 2008, 2010; Eisert et al. 2005).

Blood metabolites have been extensively used as indicators for specific nutrient metabolism in marine mammals. Blood metabolites are employed to understand fasting metabolism in a variety of species including harbor seals and NES (Castellini and Rea 1992; Trumble et al. 2006; Fadely 1997). These variables have never been investigated with metabolic hormones to assess the metabolic response to realimentation. Blood metabolites are also routinely tested during normal veterinary blood panels for animals in captivity and rehabilitation. However, without the context of other physiological parameters, large reference ranges limit veterinary application (Lander et al. 2003).

Phocid Seals

Phocid seals, including extensively studied NES and harbor seals, generally give birth to relatively precocial pups and have short nursing periods with rapid growth (Bowen 1991; Schulz and Bowen 2004). During the nursing period, harbor seals and NES exhibit a daily increase in body mass of 5-9% and 10% respectively (Cottrell et al 2002). This rapid growth and accretion of adipose is vital for survival for these species, with increases in body mass and fat mass correlated with first year survival (Muelbert et al. 2003; Harding et al. 2005). Reduced intake or fasting follow weaning in these species and the accumulated adipose serves as a source of energy in the form of fatty acids (Noren et al 2003).

Harbor seal pups nurse for approximately 24 to 32 days on energy-dense milk: 40-50% fat and approximately 9% protein, and <1% carbohydrate content (Bowen *et al*. 2001; Muelbert and Bowen 1993). Free-ranging pups are generally born at 8 to 12 kg and gain 0.35 to 0.6 kg/day (Cottrell et al 2002). At weaning, harbor seals are approximately 40% body fat and enter a period of limited nutrient (or caloric) intake while learning to forage efficiently (Bowen 1991).

NES also have a short nursing period, approximately 25 to 28 days with milk composed of 43% fat and 11% protein and <1% carbohydrate (Kretzmann et al 1993; Bowen 1991; Schulz and Bowen 2004). The pups are born at approximately 40 kg and wean at 130 kg, gaining 4 kg/day (Kretzmann et all 1993; Schulz and Bowen 2004). At weaning, NES pups are approximately 50% body fat and then enter into a post-weaning fast of approximately 6-8 weeks (Champagne et al 2012; Bowen 1991; Schulz and Bowen 2004).

Study System and Objectives

Occasionally during the rapid growth period in early life for harbor and northern elephant seals, some animals are abandoned, become undernourished, and are brought to rehabilitation facilities for realimentation treatment (Richmond et al. 2008; 2010; Colegrove et al 2005). Pups enter rehabilitation facilities at a nutritional nadir after not feeding for several days (Richmond et al. 2008). The animals are subsequently refed through a process of realimentation, generally with an artificial milk formula via gastric intubation, or gavage feeding (Townsend and Gage 2001). Throughout normal veterinary care, blood samples are routinely taken for analysis of general chemistry panels, complete blood counts, and blood metabolites. As the animals undergo realimentation, they grow in mass, standard length, and girth. Since the metabolic and growth hormones and blood metabolites in this study are known to change with nutritional status, animals undergoing this veterinary care provide a unique opportunity to investigate the metabolic regulation behind nutrient allocation.

Overall, this study aims to expand the understanding of the hormonal axes that ultimately regulate nutrient allocation in this family of seals by incorporating multiple hormones and blood metabolites using a comparative, longitudinal approach. This study will further the understanding of how these hormones are involved in the regulating the trade-off between adipose and lean tissue development when faced with nutritional challenges.

To investigate the role of metabolic hormones during realimentation in a small phocid seal, chapter 2 focuses on the changes in the somatotropic axis and ghrelin during a 10-week period of realimentation following nutritional nadir. Previous research on the somatotropic axis in this species suggests that patterns in response to fasting

and realimentation are similar to terrestrial mammals (Richmond et al. 2008, 2010). This work expands upon previous research by Richmond et al. (2010) by incorporating measurements of a satiety hormone connected to nutrient allocation (ghrelin) as well as indicators of metabolism in the form of blood metabolites. Incorporation of these other components may suggest unique adaptations that allow marine mammals to rapidly acquire such large quantities of adipose rapidly and without detrimental metabolic effects (Champagne et al. 2012). Additionally, this research will allow for more precise assessments of nutritional status by investigating the longitudinal changes in blood metabolites during realimentation and correlating changes to metabolic hormones.

Chapter 3 focuses on the application of the results of previous research and the second chapter of this thesis to a specific experimental feeding project. Metabolic hormones and morphometrics were used to evaluate the efficacy of a new alternative feeding mechanism for animals in rehabilitation. By utilizing indicators of metabolism in this way, this research may help to increase the success of rehabilitation by providing support for new methods.

Chapter 4 focuses on the response to changes in nutritional status in the fasting adapted NES. While the endocrine response to the post-weaning fast has been studied (Ortiz et al. 2003), the response to realimentation has not been investigated. Due to their extended fasting period (6-8 weeks) directly after weaning, pups of different ages have drastically different priorities for nutrient utilization (Noren et al. 2002). Given the changes in metabolism and priority of nutrient utilization associated with transitioning from a nursing neonate to a fasting adapted juvenile, NES provide a unique opportunity to assess the effect of age on the response to realimentation. To investigate this,

neonate pups, weaning-age pups, and yearlings were compared using metabolic

hormones as well as blood metabolites.

Overall, this research will further expand the understanding of tissue specific

demands and the effect on endocrine response to realimentation. By incorporating

assessments of metabolic changes based on nutrition as well as age, this study will

expound on how metabolic hormones are involved in regulating the trade-off between

adipose and lean tissue development in this unique taxon.

Chapter 2

Response of metabolic hormones and blood metabolites to nutritional status changes in rehabilitated harbor seal (*Phoca vitulina*) pups

Introduction

Blood metabolites are often used to indicate the overall condition and health status of animals and can be an important aspect of determining treatment and care (Bossart et al 2001). Blood metabolites, such as blood urea nitrogen (BUN), total protein, triglycerides, and glucose, also provide valuable information about the nutritional status of an animal based upon the types of energy sources (protein, lipid, carbohydrate) being utilized (Bossart et al 2001; Mellish and Iverson 2004). This utilization and allocation of nutrients within the body is controlled via metabolic hormones. These metabolic hormones include the satiety and adipose promoting hormone ghrelin as well as the components of the somatotropic axis [growth Hormone (GH) and insulin-like growth factor (IGF)-I] responsible for somatic growth (Etherton 2004; Nakazato et al. 2001). Metabolic hormones change predictably with nutritional status in most mammals (Thissen et al. 1999). Ghrelin and GH generally increase during times of undernutrition and slowly decrease upon realimentation (Cummings et al. 2002; Breier 1999; Richmond et al. 2010; Ortiz et al. 2003). Insulin-like growth factor-I decreases during undernutrition and increases upon realimentation (Breier et al 1986; Breier 1999; Richmond et al. 2008; 2010).

The physiological response to realimentation (realimentation) after emaciation or undernutrition in animals with high demands for adipose accretion, such as marine

mammals, may differ from other mammals. Marine mammals require rapid accumulation of large quantities of adipose to maintain the fusiform shape of their body, thermoregulation, and energy storage (Schulz and Bowen 2005; Strandberg et al. 2008). Previous studies in marine mammals suggest that metabolic hormones follow patterns typical of the mammalian response to fasting and realimentation (Richmond et al. 2010; Ortiz et al. 2003). However, little work has been conducted associating these hormones with nutrient utilization during realimentation using blood metabolites as indicators of metabolic fuel use.

Harbor seals (*Phoca vitulina*) are one of the most commonly rehabilitated marine mammals in the U.S. (Gulland et al. 2001). Every year, young harbor seal pups are admitted to rehabilitation facilities due to maternal separation and are undernourished or emaciated. In the wild, harbor seal pups nurse for approximately 24-32 days on very energy-dense milk (40-50% fat and 9% protein; Bowen *et al*. 2001; Schulz and Bowen 2005) gaining 0.35-0.6 kg/day (Cottrell et al 2002). At weaning (4 weeks of age), wild harbor seals are approximately 23-25 kg and 40% body fat (Cottrell et al 2002; Bowen 1991).

Given the normally high rate of gain in the wild, these undernourished pups have to obtain significant weight and large adipose stores rapidly. Due to the need for rapid accumulation of lean and adipose tissue, these animals make excellent models to investigate the physiological mechanisms controlling growth during realimentation. Additionally, correlation of blood metabolites to other metabolic hormones is novel. In harbor seals, reference ranges for many metabolites are wide. Therefore, investigating

the changes in blood metabolites longitudinal in conjunction with metabolic hormones may refine how blood metabolite data is interpreted for these animals.

Methods

Animals and Diet

All animals used in this research were undergoing routine veterinary care for malnutrition at The Marine Mammal Center (TMMC; Sausalito, CA). Pups (n = 12) included in the study met the following criteria: (1) categorized as healthy but abandoned, and (2) were less than 10 days old upon admission to the facility based upon pelage, tooth eruption, and umbilicus status (Bowen *et al*. 1994; Richmond *et al*. 2010).

All animals were initially fed a milk matrix formula (Zoologic 30/55, Pet-Ag, Inc. Hampshire, IL; 31.3% protein, 55.8% fat, and 3.1% carbohydrate) supplemented with fish oil and pinniped multivitamins. Formula was approximately 3700 kcal / L (Richmond et al. 2010) and amount fed was prescribed based upon estimated caloric requirement for growth at three times the resting energy requirements (kcal/day) = 70(Body weight kg)$^{0.75}$; Lavigne et al. 1993). Pups were fed via gastric intubation approximately 5 times per day for 4 weeks (Townsend and Gage 2001). At 4 weeks of age, pups were gradually weaned from formula to whole herring (1500 kcal / kg) fed at 10% of body weight per day (Richmond et al. 2010).

Standard morphometrics (mass, axillary girth, and standard length) were measured every 2 weeks to assess growth and body condition. A body condition index

(BCI) of axial girth/standard length x 100 was calculated to estimate overall body condition (Richmond *et al.* 2010; Fadely 1997).

Blood Analysis

Blood samples (2 to 4 mL) were collected before the first feed of the day via the extradural intravertebral vein (Bossart *et al.* 2001) into serum separator tubes (SST) and Ethylenediaminetetraacetic acid (EDTA) vacutainers. Blood in SST was allowed to clot for 15 minutes then centrifuged at 3,000 x g for 15 minutes to separate serum (Lander et al. 2003). EDTA tubes were centrifuged at 3,000 x g for 15 minutes to separate plasma (Lander et al. 2003). Blood metabolites (blood urea nitrogen (BUN), creatinine, glucose, total protein, cholesterol, triglycerides) were quantified in plasma using Vet Test 8008 (Idexx Laboratories Inc., Westbrooke, Maine, USA; Lander et al. 2003).

Sera were frozen and shipped on dry ice to the University of North Florida and maintained at -80°C until hormone analysis. Growth hormone and IGF-I were quantified in the serum via heterologous radioimmunoassay (RIA) developed and validated for harbor seal serum by Richmond and Zinn (2009). Ghrelin was quantified using a commercially available RIA kit validated for use with harbor seal serum (Millipore, Billerica, MA Cat. #GHRT-89HK). Validations performed on pooled harbor seal serum showed percentage recovery of ghrelin was 97.1 ± 4.4%. Pooled serum averaged 403.9 ± 20.1 pg / ml with intra-assay coefficient of variation of 8.6 and a dilution linearity with $R^2 = 0.93$.

Statistical Analysis

Metabolic hormones, blood metabolites, caloric intake, mass, and morphometrics were compared by week of rehabilitation using a mixed model ANOVA with repeated

measures utilizing Tukey's post-hoc comparisons (SAS Inst. Inc., Cary, NC).

Differences were considered significant if $P \leq 0.05$ and trends if $0.10 \geq P > 0.05$. A step-wise linear regression was used to determine correlation of blood metabolites and body condition with hormone concentrations. Variables were retained in the model if $P < 0.15$.

Results

On average, pups remained in the rehabilitation facility for 11.2 ± 1.2 weeks. Caloric intake increased initially upon realimentation (Figure 1A; $p < 0.001$), with a peak at week 2 when all animals were consuming formula (3543.3 ± 210.7 kcal / day). For the remainder or rehabilitation, caloric intake remained consistent at 2535.2 ± 118.7 kcal / day. Animals gained mass at 0.9 ± 0.14 kg/week and nearly doubled body mass throughout the rehabilitation period (Figure 1B; week 0: 8.1 ± 0.8 kg; week 10: 15.0 ± 1.1 kg; $p < 0.001$). Axial girth (week 0: 49.6 ± 2.3 cm; week 10: 63.2 ± 2.1 cm), standard length (week 0: 69.9 ± 2.4 cm; week 10: 84.7 ± 2.3 cm), and body condition (Figure 1C; week 0: 11.3 ± 0.6; week 10: 18.2 ± 1.0) all increased throughout rehabilitation ($p < 0.001$).

As expected, GH concentrations decreased throughout realimentation (Figure 2A; $p < 0.001$). GH concentrations were highest at week 0 (22.0 ± 2.2 ng / ml) and decreased to 3.5 ± 0.4 ng / ml by week 10. Concentrations of IGF-I increased from below detectable limits of the RIA at week 0 (<40 ng/ml) to 100.1 ± 20.9 ng / ml (week 10; Figure 2B; $p = 0.051$). Surprisingly, the lowest ghrelin concentrations were observed upon admit (196.4 ± 38.1 pg/ml; Figure 2C; $p = 0.002$). Ghrelin concentrations increased between week 0 and 4 and remained elevated through week 10 of

rehabilitation. Ghrelin concentrations were greatest at week 6 of realimentation (351.6 ± 38.1 pg / ml).

During realimentation, BUN (Figure 3A) and total protein (Figure 3B) increased by 27.5 ± 4.5 mg/dl and 2.1 ± 0.3 g/dl respectively ($p < 0.001$). Creatinine was elevated to 0.49 ± 0.03 mg/dl in week 0 (Figure 3C; $p = 0.005$) but declined and remained consistent from week 2 to week 10 at an average of 0.38 ± 0.01 mg/dl. Glucose concentration did not change significantly throughout realimentation (Figure 3D; 156.96 ± 5.06 mg/dl; $p = 0.46$). Cholesterol (Figure 3E) increased from week 2 (236.83 ± 15.27 mg/dl) to week 10 (306.34 ± 17.45 mg/dl; $p = 0.007$). Triglycerides (Figure 3F) exhibited a slight increase in week 4 (282.73 ± 43.66 mg/dl; $p = 0.07$).

Step-wise linear regression yielded significant correlation of metabolic hormones with body mass, BCI, BUN, total protein, creatinine, and glucose (Table 1). Insulin-like growth factor-I and ghrelin were positively correlated with body mass and BCI, explaining 50% of the variability in body mass and 44% of the variability in body condition. Positive ghrelin and IGF-I correlations and negative GH correlations explained 38% of variability in BUN and 53.4% of variability in total protein concentration. Creatinine and glucose concentrations were positively correlated to GH concentrations with a model significance of $p < 0.15$; however, GH explained only 5% and 6% of variability respectively. Triglycerides exhibited no correlation to hormone concentrations ($p > 0.15$).
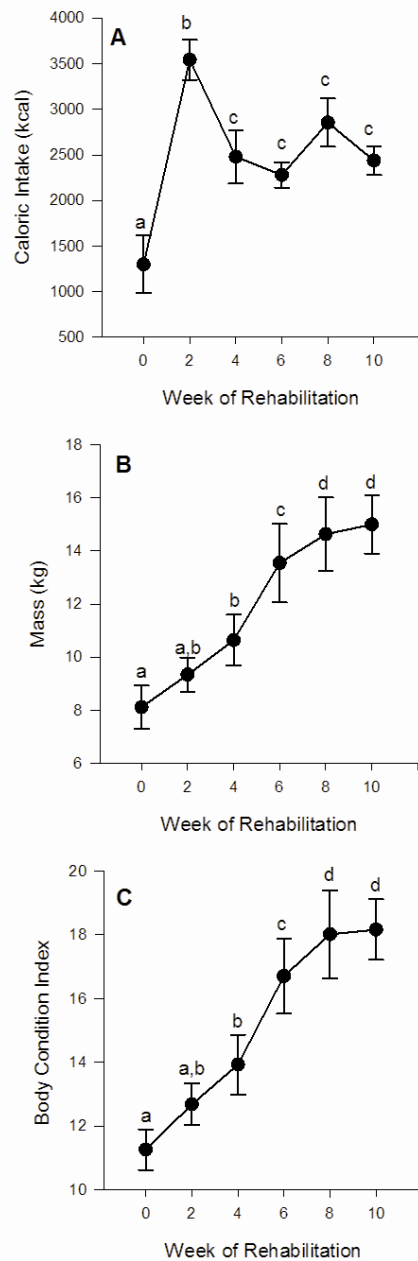
Figure 1. A. Caloric intake (kcal ± SE) by week of rehabilitation. **B.** Body mass (kg ± SE) by week of rehabilitation. **C.** Body Condition Index (± SE) by week of rehabilitation. Different letters indicate statistical difference between weeks (p ≤ 0.05).
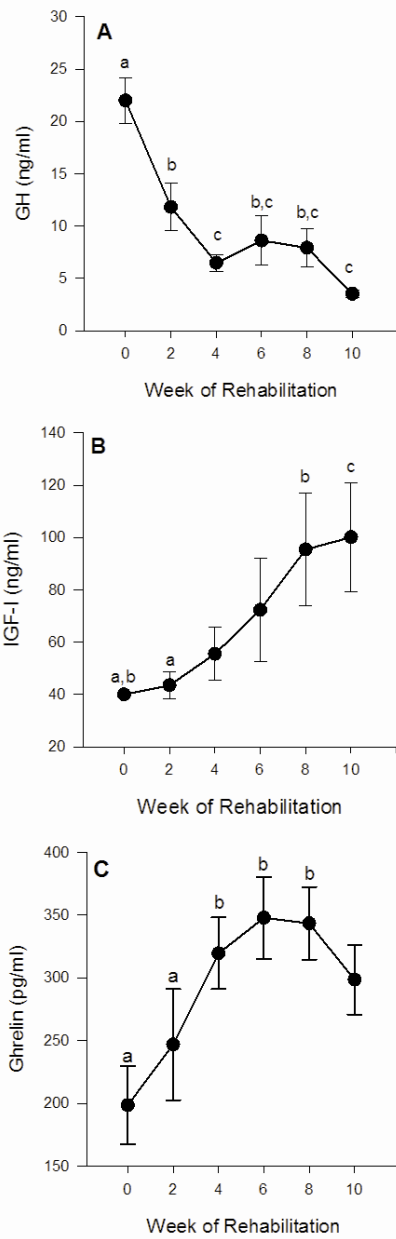
Figure 2. A. Growth Hormone concentration (ng/ml ± SE) by week of rehabilitation. **B.** Insulin-like Growth Factor-I concentration (ng/ml ± SE) by week of rehabilitation. **C.** Ghrelin concentration (pg/ml ± SE) by week of rehabilitation. Different letters indicate statistical difference between weeks (p ≤ 0.05).
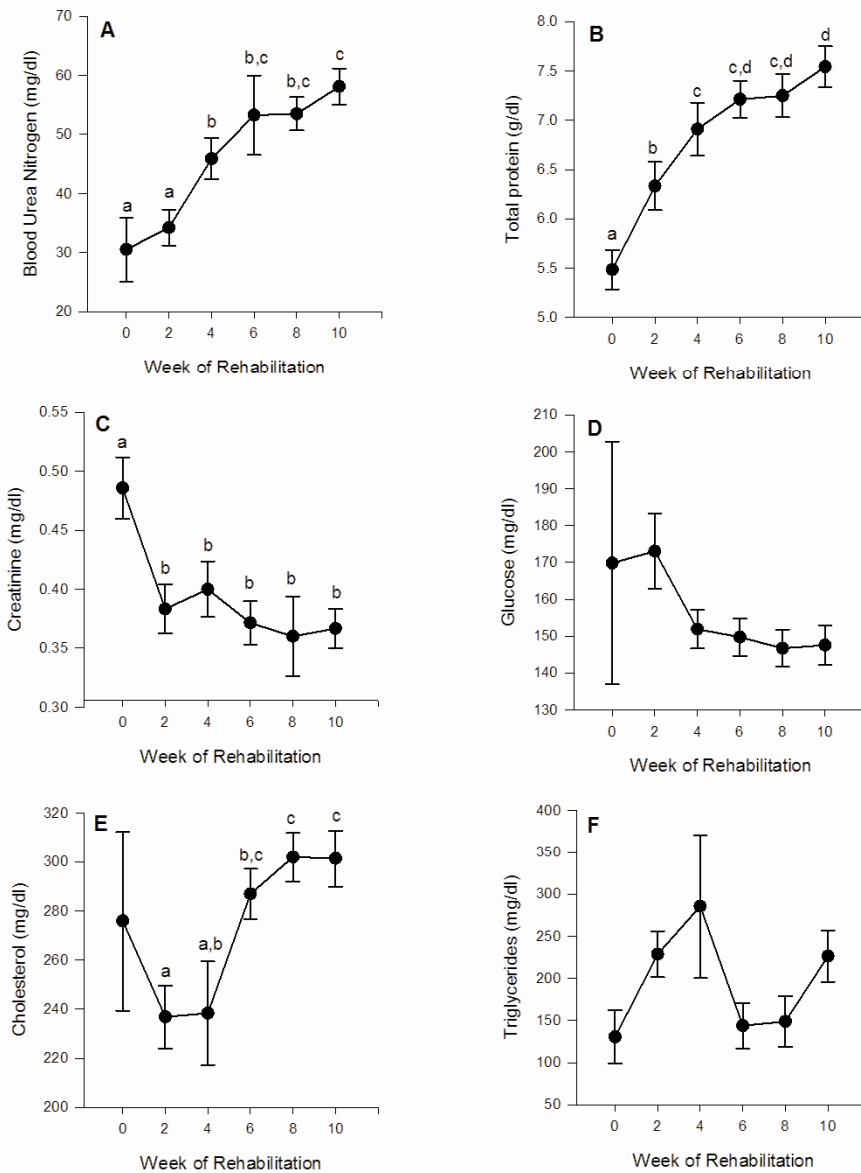
Figure 3. A. Blood Urea Nitrogen concentration (mg/dl ± SE) by week of rehabilitation. **B.** Total serum protein (g/dl ± SE) by week of rehabilitation. **C.** Creatinine (mg/dl ± SE) by week of rehabilitation. **D.** Glucose (mg/dl ± SE) by week of rehabilitation. **E.** Cholesterol (mg/dl ± SE) by week of rehabilitation. **F.** Triglycerides (mg/dl ± SE) by week of rehabilitation. Different letters indicate statistical difference between weeks (p ≤ 0.05).

Table 2. Stepwise Linear Regression Model for Morphometrics and Blood Metabolites

Variable	Model	P value	r^2
Body mass	6.0 + 0.06(IGF) + 0.007(ghrelin)	0.001	0.503
Body Condition Index	9.76 + 0.056(IGF) + 0.006(ghrelin)	0.001	0.444
BUN	35.67 - 0.83(GH) + 0.12(IGF) +0.03(ghrelin)	0.001	0.383
Total Protein	5.60 - 0.04(GH) + 0.01(IGF) + 0.004(ghrelin)	0.001	0.534
Creatinine	0.36 + 0.003(GH)	0.087	0.052
Glucose	140.9 + 1.28(GH)	0.062	0.066
Triglycerides	No correlation	--	--
Cholesterol	306.98 + 0.21(ghrelin)	0.012	0.138

Discussion

Many previous studies of blood metabolites and metabolic hormones in marine mammals have focused on the physiology of fasting adaptation and determination of nutritional status in wild populations for health assessment (Roletto 1993; Mellish and Iverson 2001; Hall 1998; Trumble et al. 2006; Castellini and Rea 1992). However, research has been limited on the response to realimentation in these species. Investigation of the physiology of realimentation after fasting and starvation events provides insight into the metabolic priorities of these unique animals with high demands for adipose accretion.

At the end of the rehabilitation period, animals in this study weighed 15.0 ± 1.1 kg at approximately 10-12 weeks old. Body mass and body lipid at weaning are positively correlated with first year survival in harbor seals (Muelbert et al. 2003; Harding et al. 2005). Free-ranging pups of the same age gain 0.35-0.6 kg/day (Schulz and Bowen 2004). At weaning (4 weeks of age), wild harbor seals are approximately 24 kg (Schulz and Bowen 2004; Bowen 1991). Therefore, pups in this study are underweight for their

age. Improvement in body mass gains and increases in overall body condition in rehabilitation would likely contribute to increased chances of survival post-release (Lander et al 2002).

In previous research on terrestrial mammals, concentrations of GH generally increase with decreases in nutrient intake (Breier 1999; Renaville *et al.* 2002; Buonomo and Baile 1991). Increases in GH concentrations contribute to increased lipolysis (Renaville *et al.* 2002, Wang *et al.* 2004). In periods of adequate nutrition, GH stimulates IGF-I in the liver (Breier 1999; Renaville *et al.* 2002; Buonomo and Baile 1991). Even though GH concentrations are higher at low nutritional states, IGF-I concentrations do not increase but actually decrease during periods of undernutrition due to a down-regulation of GH receptors in the liver (Breier 1999; Buonomo and Baile 1991). The resulting decrease in IGF-I concentrations should halt somatic growth in favor of other metabolic needs (Thissen *et al.* 1999). Upon realimentation, GH concentrations slowly decline while IGF-I concentrations increase (Buonomo and Baile 1991).

In this study, GH concentrations gradually decrease upon realimentation. However, by the end of the 10 week realimentation period, concentrations remain higher than well-fed wild harbor seals of comparable age (approximately 2 ng/ml; Richmond et al. 2008). As expected, IGF-I increased during realimentation; however, determination of differences in early realimentation was limited by the detection limits of the assay (40 ng/ml). By week 10, IGF-I concentrations were still much lower than free-ranging counterparts (approximately 150 ng/ml; Richmond et al. 2008).

Regardless of differences in concentrations from free-ranging animals, these results are consistent with the typical mammalian pattern in response to realimentation. Insulin-like growth factor-I concentrations below 40 ng/ml coupled with high GH concentration at admit indicates a very low plane of nutrition. However, decreasing concentrations of GH and increasing IGF-I concentrations indicate the nutritional status of these animals continually improves throughout realimentation.

In most mammalian species, ghrelin increases during fasting and decreases with realimentation over the short term (1-2 hours) and long term (1-3 months) (Nakazato *et al.* 2001; Cummings *et al.* 2002; Tanaka *et al.* 2004). Contrary to the previously reported responses to fasting and fed states, ghrelin concentrations in this study were actually greater in the fed state than when animals were at nutritional nadir.

While these results were generally surprising, arctic foxes, another species requiring significant accumulation of adipose, also showed atypical responses of ghrelin during fasting and realimentation (Fuglei et al. 2004). Ghrelin in these animals reduced slightly during fasting, and actually increased upon realimentation in seasons with high adipose demand (prior to winter; Fuglei et al. 2004). Given that the normal increase in ghrelin during fasting in rats was correlated with reduced lipolysis and increased fat deposition (Tschop et al. 2000), Fuglei et al. (2004) hypothesized that in arctic fox ghrelin could be reduced during fasting to facilitate fat utilization. In this study, given reduced ghrelin concentrations at nutritional nadir, our data support this hypothesis. Coversely, ghrelin concentrations may be actually increased upon realimentation rather than reduced at nutritional nadir.

Given that ghrelin is vital for lipid synthesis and deposition (Sangiao-Alvarellos et al. 2011), it is possible that the increase in ghrelin concentrations upon realimentation is a mechanism facilitating compensatory accumulation of lipids after periods of nutritional deprivation. This increase in ghrelin may also result in a hyperphagic response allowing increased consumption regardless of energy status (Tanaka et al. 2002). Since accretion of adipose is vital for survival in this species (Muelbert et al. 2003; Harding et al. 2005), it is possible that this mechanism could be facilitating the accretion of adipose over lean tissue upon realimentation. In this study, increases in BCI (axial girth / standard length x 100) suggest that adipose (reflected in axial girth) is being gained preferentially to lean tissue (reflected in standard length; Fadley 1997). Further measurement of specific tissue accretion throughout realimentation is needed to further investigate this hypothesis.

Metabolic hormones IGF-I and ghrelin were highly predictive of body mass and BCI. Previous research has correlated body mass gains with IGF-I concentration (Richmond et al. 2010). Given the promotion of lean and adipose tissue by IGF-I, this gain likely reflects increases in both body tissues. However, ghrelin also promotes adiposity and recuperation of body weight in the form of adipose (Tschop et al. 2000; Sangiao-Alvarellos et al. 2011). Therefore, the correlation of body mass with ghrelin likely corresponds to gains in adipose. This is further supported by measurements of body condition. Increases in axial girth relative to standard length represent increases in mass per unit of length, which suggests a composition of gain favoring adipose accretion (Fadely 1997).

Both BUN and total serum protein were decreased early in rehabilitation consistent with malnourishment in this species (Bossart et al. 2001). Decreased BUN and total protein at admit is also consistent with the protein sparing response of this taxa to the first weeks of fasting during breeding and molting periods (Castellini and Rea 1992). Correlation of BUN and total protein with changes all three metabolic hormones during realimentation indicate that they are well predicted by GH, IGF-I, and ghrelin in this species. Given this correlation, BUN and total protein may be a reliable clinical indicator for nutritional status. Increased creatinine at admit to the facility was correlated to GH concentrations. However, GH explained only 5% of variability in creatinine. Increased creatinine production can be indicative of increased muscle catabolism (Bossart et al. 2001; Perrone et al. 1992), which many animals would be undergoing if entering final phases of starvation. However, since many of the animals enter the facility dehydrated, increased creatinine may also be related to dehydration and the impact on renal filtration rather than increased production of creatinine (Bossart et al 2001; Perrone et al. 1992).

While glucose concentrations at admit were highly variable, no change was seen throughout realimentation. Stable glucose concentrations throughout rehabilitation are not surprising for marine mammals, which generally have relatively stable blood glucose concentrations due to efficient gluconeogenesis (Champagne et al 2012). Total cholesterol increased throughout the realimentation period, associated with concentrations of ghrelin. Ghrelin has been previously correlated with HDL cholesterol but results have been mixed (Purnell et al. 2003). However, it has been suggested that HDL cholesterol may act as a carrier for ghrelin in the blood to prolong half-life (Purnell

et al. 2003). Triglycerides in the blood are often extremely variable in marine mammals, so it is not surprising that it did not differ by week of realimentation and had high variability between animals (Reference value for healthy harbor seal pups: 93 ± 84 mg/dl; Roletto 1993).

Overall, this study expands upon previous research by incorporating metabolite utilization during realimentation in this species. While the response of the somatotropic axis is typical of the mammalian pattern in response to realimentation, the surprising response of ghrelin to realimentation provides evidence for the priority of adipose accretion and a possible mechanism regulating the recuperation of that vital tissue. Correlation of metabolites and body condition to metabolic hormones may also provide refined diagnostic information for animals in rehabilitation regarding improvements in nutritional status.

Chapter 3

Evaluating The Physiologic Response To An Alternative Feeding Mechanism: Metabolic Hormones In Remote-Fed Versus Gavage-Fed Harbor Seal (*Phoca vitulina*) Pups

Introduction

Pinniped pups undergoing rehabilitation are traditionally fed milk-matrix or herring based formula via gavage (Townsend and Gage 2001). Gavage feeding requires restraint of the animal approximately 5 times per day (Townsend and Gage 2001) possibly inducing a state of chronic stress while in the facility. Chronic stress has been shown to decrease food intake and body weight and impair function of the intestinal epithelial barrier in rats (Santos *et al*. 2000).

Metabolic hormones, including components of the somatotropic axis and ghrelin, are known to change with nutrient intake and can indicate nutritional status of an organism (Breier 1999; Nakazato et al. 2001; Renaville et al. 2002; Richmond et al. 2010). The somatotropic axis promotes growth and regulates nutrient partitioning (Breier 1999; Renaville et al. 2002). Ghrelin is generally associated with regulation of food intake, energy balance, and promotion of lipid accumulation during realimentation (Nakazato et al. 2001; Sangiao-Alvarellos et al. 2011). Additionally, cortisol in concert with growth hormone has been shown to increase basal lipolysis and lipolysis stimulated by catecholamines (Ottosson *et al*. 2000). Due to the nature of these metabolic hormones and their relationship to growth and nutrient intake, they are of particular interest to assess the efficacy of alternative feeding mechanisms.

Young pups undergoing rehabilitation need to rapidly recover from nutritional nadir, increasing mass and adipose in order to increase their chances of survival (Muelbert et al. 2003; Harding et al. 2005). Therefore, the aim of this study was to investigate an alternative feeding mechanism, remote feeding via artificial nipple, and evaluate its efficacy based on morphometric and metabolic hormone variables. If similar results can be achieved using remote feeding mechanisms with reduced stress and human contact, growth rates may be improved and rehabilitation success may be increased.

Methods

All animals in this study were undergoing routine veterinary care at rehabilitation facilities. The comparison of gavage (n=3) and remote fed (n=2) animals was completed using age and size matched pups from Vancouver Aquarium (VA, Vancouver, Canada). Pups included in the study met the following criteria: (1) categorized as healthy but abandoned, and (2) were less than 10 days old upon admission to the facility based upon pelage, tooth eruption, and umbilicus status (Bowen *et al*. 1994; Richmond *et al*. 2010).

For this study, both gavage and remote animals were fed milk matrix formula (Zoologic 30/55, PetAg, USA) at 5 feedings/day. After 4 weeks, animals were weaned onto whole fish diets. Animals were fed at 10-11% of body mass/day (approximately 2500 kcal/day). Blood samples were collected prior to release after 8-16 weeks of realimentation. Hormone concentrations (GH, IGF-I, and ghrelin) were analyzed in serum via heterologous radioimmunoassay (Richmond and Zinn 2009; Chapter 2).

Results and Conclusions

When comparing remote-fed with gavage-fed animals, no significant differences were observed in ghrelin, IGF-I, or mass just prior to release. However, concentrations of GH were increased in the remote feeder group (p = 0.02). Pups gained weight at similar rates (135 ± 0.5 g/day) in both the remote feeder and traditional gavage feeding groups (MacRae et al. 2010). Ghrelin concentrations were lower at release in this study (232.1 ± 25.9 pg/ml) than previously reported concentrations for harbor seals (351.6 ± 38.1 pg / ml; Chapter 2). Concentrations of GH were high prior to release (4.9 ± 0.6 ng/ml). However, IGF-I concentrations prior to release (217.7 ± 55.4 ng/ml) were greater than in animals from The Marine Mammal Center (chapter 2). When nutrition is adequate, high IGF-I in conjunction with increased GH is correlated with increased mass gain. While no difference in mass gain was seen, hormone profiles of remote-fed animals suggest a high nutritional status and growth rate. Since these hormones have been shown to accurately predict the nutritional status of rehabilitated harbor seals (Chapter 2), these data support that the remote-feeder mechanism provides similar improvements in nutritional status to gavage feeding. Given this, future investigation into this feeding mechanism as an alternative to gavage is warranted, specifically addressing the possible benefits of reduced stress and human contact. By incorporating measurements of cortisol in future studies, we can determine the level of chronic stress induced during gavage feeding and investigate if reduced stress improves mass gain, growth rate, and nutritional status.
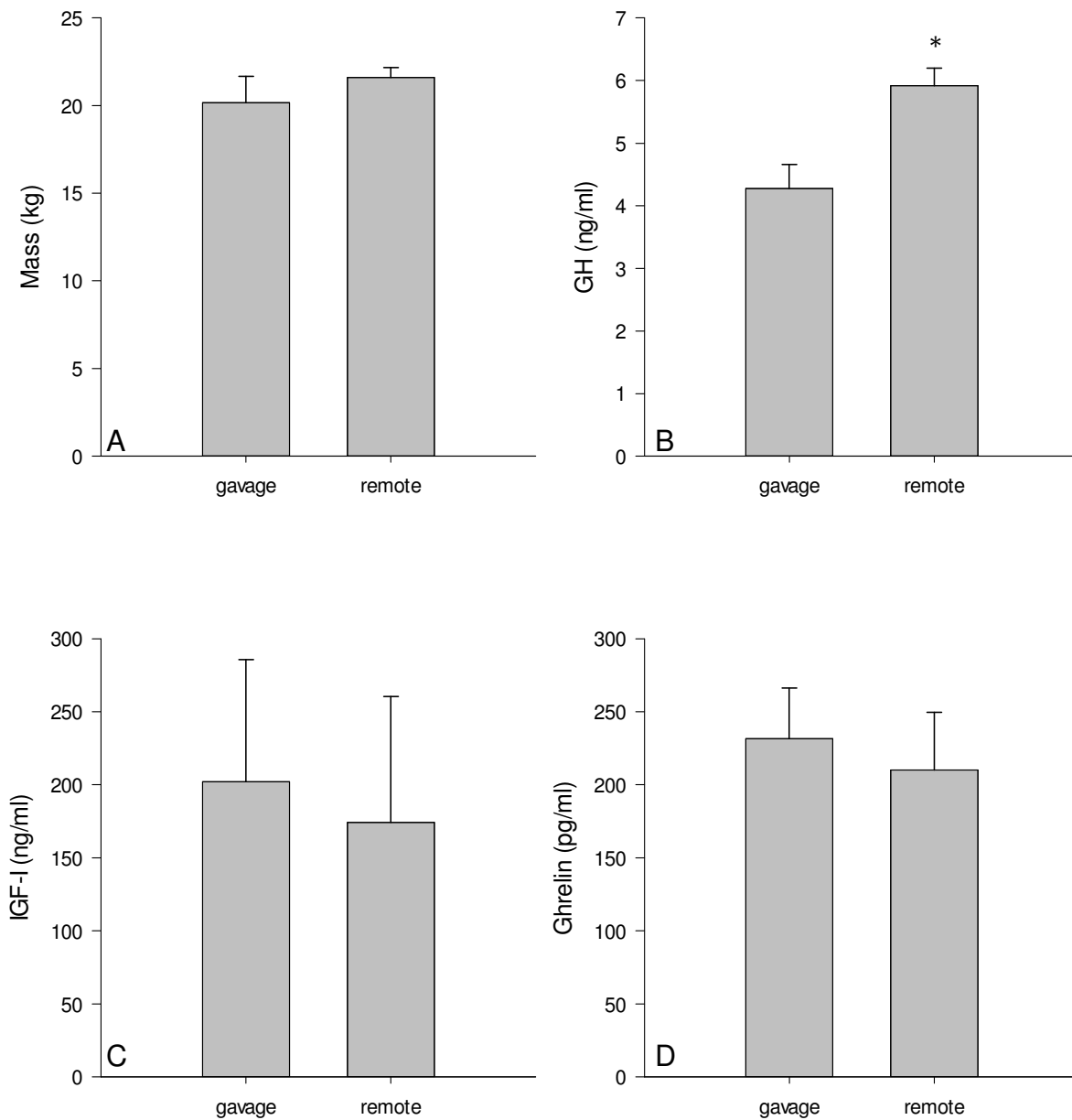
Figure 1. Comparison of gavage fed versus remotely fed harbor seals at time of release. **A.** Mass (kg) **B.** Growth hormone (ng/ml) **C**. Insulin-like growth factor (IGF)-I (ng/ml) **D.** Ghrelin (pg/ml). Significant differences between treatment groups (p < 0.05) indicated with (*). Values presented as mean ± standard error.

Acknowledgements

Chapter 4

Influence of age on the endocrine response to realimentation in young Northern elephant seals (*Mirounga angustirostris*)

Introduction

The priorities of tissue specific growth and maintenance are dependent upon changes in nutrient intake as well as age and developmental stage (Bell et al. 1987). The somatotropic axis is a group of hormones that participates in the regulation of nutrient allocation and tissue specific growth (Breier 1999; Etherton 2004). Components of this axis [growth hormone (GH) and insulin-like growth factor (IGF)-I] regulate how nutrients are distributed and utilized by the tissues and are responsive to the nutritional status and physiological state of an individual (Breier 1999; Thissen et al. 1999). In addition to components of the somatotropic axis, the satiety hormone ghrelin contributes to the partitioning of nutrients through the promotion of feeding and adiposity (Tschop et al 2000; Sangiao-Alvarellos et al. 2011).

As animals age, developmental priorities of nutrient requirements and allocation change (Bell et al. 1987). While ghrelin is not known to change with age, the somatotropic axis contributes to the regulation of age related nutrient priorities, with decreases in GH and increases in IGF-I with age (Skaar et al 1994). When nutrient intake is decreased, such as during a fast, IGF-I concentrations decrease while GH increases (Breier 1999; Richmond et al. 2010; Ortiz et al. 2003). Ghrelin normally exhibits pre-prandial increases as well as increases during fasting (Cummings et al.

2002;). This endocrine response to fasting regulates the nutrient utilization of specific tissues. Increases in GH facilitate lipolysis to mobilize fatty acids from adipose as an energy source (Wang et al. 2004). Decreasing concentrations of IGF-I halt the processes of somatic growth when nutrient intake is low (Thissen et al. 1999). Upon realimentation, the anabolic effects of the somatotropic are restored, facilitating the deposition of fat as well as the growth of lean tissue (Thissen et al. 1999). Ghrelin participates in weight recuperation during realimentation by promoting adiposity and facilitating lipogenesis (Sangiao-Alvarellos et al. 2011; Tschop et al 2001).

Northern elephant seals (NES; *Mirounga angustirostris*) are considered a fasting adapted species, capable of undergoing extended periods of fasting without the negative effects of nutritional deprivation (Castellini and Rea 1992). One such extended fast, the post-weaning fast, occurs at approximately 4 weeks of age concurrent with the first molt (Bowen 1991). Pups are born at approximately 40 kg (Kretzmann et al 1993; Schulz and Bowen 2005). Mass is gained rapidly during the 4-week nursing period and pups weigh approximately 130 kg with 50% body fat (Kretzmann et al 1993; Schulz and Bowen 2005). Over the subsequent 6 to 8 weeks, newly weaned pups fast and may lose up to 50 kg (Noren et al 2003).

Due to this unique developmental milestone, the endocrine response to alterations in nutritional status may differ with age. Therefore, the objective of this study was to investigate if age affects the response of ghrelin, growth hormone (GH), and insulin-like growth factor (IGF)-I from nutritional nadir to realimentation (refeeding).

Methods

Animals and Diet

All animals used in this research were undergoing routine veterinary care for malnutrition at The Marine Mammal Center (TMMC; Sausalito, CA). Animals were categorized upon admittance into one of three age classifications: neonate pups (<4 weeks of age; n = 6), post-molt pups (4-8 weeks of age; n=18), and yearling (approximately 1 one year of age; n=15). Age classifications were based on pelage, umbilical cord status, tooth eruption, size, and time of year in relation to peak (or mean) pupping date (Le Beouf and Laws 1994).

Animals classified as neonate pups (younger than weaning age) were initially fed milk matrix formula (Zoologic 30/55, Pet-Ag, Inc. Hampshire, IL; 31.3% protein, 55.8% fat, and 3.1% carbohydrate) diluted with water and supplemented with fish oil and pinniped multivitamins. Formula was approximately 2100 kcal / L and amount fed was prescribed based upon estimated caloric requirement for growth at three times the resting energy requirements (RER; kcal/day) = 70(Body weight in kg)$^{0.75}$ (Lavigne et al. 1986). Pups were fed via gastric intubation approximately 5 times per day for 4 weeks (Townsend and Gage 2001). At approximately 4 weeks of age, pups were gradually weaned from formula to whole herring (1500 kcal / kg; Richmond et al. 2010), fed at 10% of body weight per day. Animals older than 4 weeks (post-molt and yearling age classifications) were initially fed a fish mash (900 kcal/L) diet via gavage based on RER until transitioning to whole herring (10% of body mass) when able to eat on their own.

Mass and standard length were measured at admit and prior to release to assess growth and body condition. A body condition index of mass/standard length x 100 was

calculated to estimate overall body condition (Fadely 1997).

Blood Analysis

Blood samples (2 to 4 mL) were collected before the first feed of the day via the extradural intravertebral vein (Bossart *et al*. 2001) into serum separator tubes (SST). Blood in SST was allowed to clot for 15 minutes then centrifuged at 3,000 x g for 15 minutes (Lander et al. 2003). Sera were collected, frozen, and shipped on dry ice to the University of North Florida and maintained at -80°C until hormone analyses were completed.

Growth hormone and IGF-I were quantified in the serum via heterologous radioimmunoassay (RIA) developed and validated for use with serum of multiple pinniped species by Richmond and Zinn (2009). Validations performed for NES pooled serum showed percentage recovery of growth hormone at 99.1 ± 8.0%. NES pooled serum averaged 11.0 ± 0.2 ng / ml GH and a dilution linearity with R^2 = 0.94. GH assays had an intra- and inter-assay coefficient of variation (CV) of 4.2 and 14.3 respectively. Insulin-like growth factor concentrations in pooled serum averaged 44.6 ± 1.7 ng / ml with an intra-assay CV of 6.6.

Ghrelin was quantified using a commercially available RIA kit validated for use with NES serum (Millipore, Billerica, MA Cat. #GHRT-89HK). Percentage recovery of ghrelin in pooled NES serum was 92.8 ± 2.1%. NES pooled serum averaged 430.1 ± 26.1 pg / ml ghrelin with intra-assay CV of 9.2. Inter-assay CV across ghrelin assays was 7.9. Pooled serum showed dilution linearity with R^2 = 0.93.

Statistical Analysis

Metabolic hormones, mass, and caloric intake were compared by age class and

week of rehabilitation. Rate of mass gain, standard length and body condition index were compared at admit and release between all age classes. All comparisons were made using a mixed model ANOVA with repeated measures using Tukey's post-hoc comparisons (SAS Inst. Inc., Cary, NC). Differences were considered significant if $P \leq 0.05$ and trends if $0.10 \geq P > 0.05$. A step-wise linear regression was used to determine correlation of mass and body condition with hormone concentrations. Variables were retained in the model if $P < 0.15$.

Results

The average rehabilitation period across all age classes was 8.9 weeks. Daily caloric intake increased across rehabilitation for all age classes ($p < 0.001$; Figure 1A). However, absolute intake was higher in yearlings compared to post-molt and neonate pups ($p < 0.01$). Intake in neonate pups increased between week 0 (1913.4 ± 1087.9 kcal/day) and week 4 (9037.3 ± 2934.5 kcal/day; $p = 0.002$) then remained stable for the rest of realimentation at an average of 9650.9 ± 1645.0 kcal/day. Post-molt pups had reduced intake upon admit (week 0: 3015.1 ± 552.6 kcal/day; $p < 0.02$) but then increased to approximately 7481.9 ±418.6 kcal/day for the remainder of realimentation. Intake in yearling animals increased from week 0 (3219.5 ± 858.9 kcal/day) through week 4 (15875.5 ± 2706.2 kcal/day) then remained stable ($p < 0.001$).

Neonate pups did not gain significant body mass (Figure 1B) until week 8 of realimentation at 102.5 ± 55.5 kg, an increase of roughly 55 kg ($p < 0.001$). Post-molt pups had an earlier increase in mass by week 4 (43.6 ±2.7 kg; $p = 0.05$) and continued to increase through week 8 (54.2 ± 3.9 kg; $p < 0.001$). Yearlings increased in mass by

week 6 (98.0 ± 24.4 kg; p < 0.001) and remained consistent through the remainder of the realimentation period. Rate of gain (kg/day) from admit to release did not differ among age classes (p = 0.96). Standard length increased in post-molt (p = 0.004; admit: 121.7 ± 3.3 cm; release: 136.5 ± 3.9 cm) and yearling (p = 0.02; admit: 150.1 ±4.0 cm; release: 157.7 ± 5.8 cm) age classes but not in pups (p = 0.32). Body condition increased over realimentation in all three of the age classes (Figure 2; p < 0.001).

Growth hormone concentrations (Figure 3A) decreased as expected in post-molt pups and yearlings (p < 0.01). Concentrations of GH in post-molt pups decreased gradually and over the entire realimentation period (p < 0.01), whereas yearlings exhibited a more rapid decline from week 0 (18.0 ± 2.1 ng/ml) to week 4 (11.1 ± 2.2 ng/ml; p = 0.002) with stable concentrations for the remainder of realimentation. Surprisingly, no decrease was observed in neonate pups, despite concentrations at week 8 (4.5 ± 1.8 ng/ml) roughly half of admit concentrations (9.3 ± 2.6 ng/ml; p = 0.43). Additionally, overall concentrations of GH were greater in post-molt pups compared with neonates and yearlings (p < 0.03).

Concentrations of IGF-I were initially low (< 40 ng/ml) and were below 70 ng/ml for the entire realimentation period in all age classes and showed no change in concentration by week (p = 0.45; Figure 3B). Ghrelin concentrations (Figure 3C) did not change significantly by week (p = 0.99) but concentrations differed by age class (p < 0.001), with concentrations increased in post-molt pups (404.6 ± 21.6 pg/ml) compared to neonate pups (272.1 ± 39.4 pg/ml; p = 0.005) and yearlings (208.3 ± 27.8 pg/ml) (p < 0.001). Metabolic hormones were predictive of body mass and BCI as determined by step-wise linear regression (Table 1). Negative correlation with GH explained 12.7% of

variability in BCI. Both GH and ghrelin were negatively correlated with body mass,

explaining 18.5% of variability.
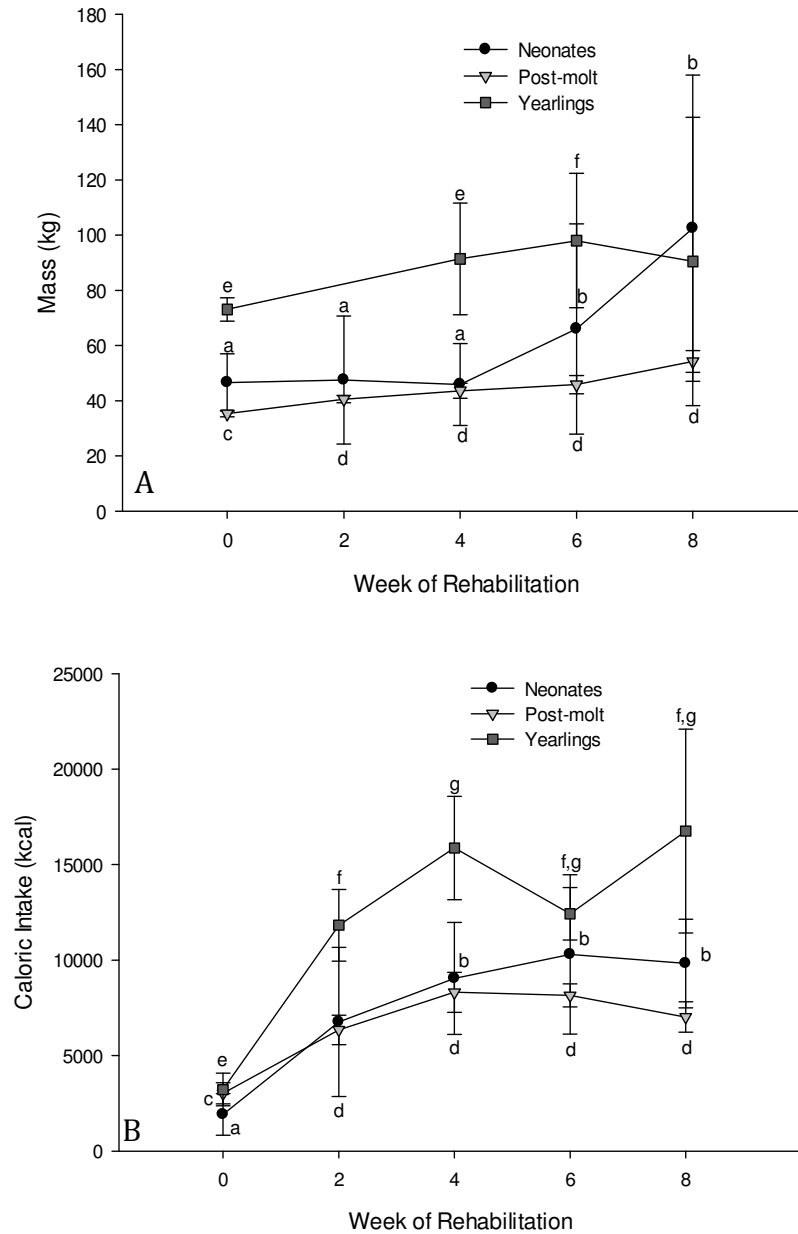
Figure 1. A. Body mass (kg ± SE) by week of rehabilitation. Different letters indicate statistical difference (p ≤ 0.05) between weeks within age class (neonates: a, b; post-molt: c, d; yearling: e, f) **B.** Caloric intake (kcal ± SE) by week of rehabilitation. Different letters indicate statistical difference (p ≤ 0.05) between weeks within age class (neonates: a, b; post-molt: c, d; yearling: e, f, g)
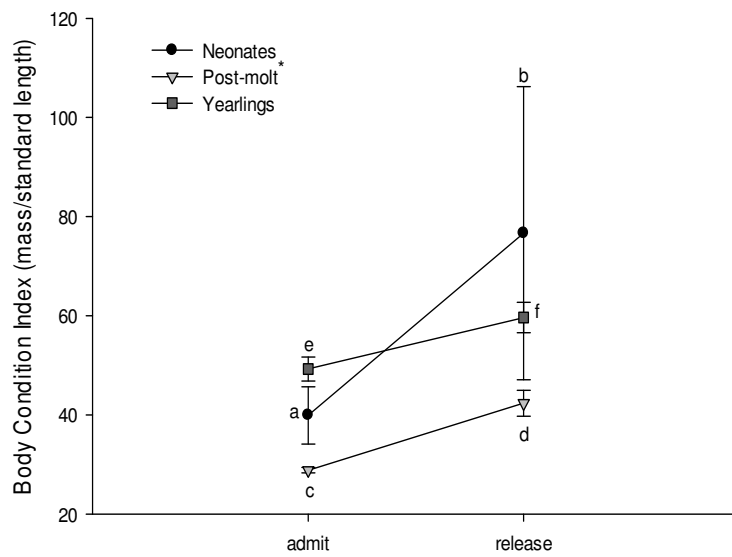
Figure 2. Body Condition Index (± SE) by week of rehabilitation. Different letters indicate statistical difference (p ≤ 0.05) between weeks within age class (neonates: a, b; post-molt: c, d; yearling: e, f)**.** Difference between age classes indicated with (*) in legend.
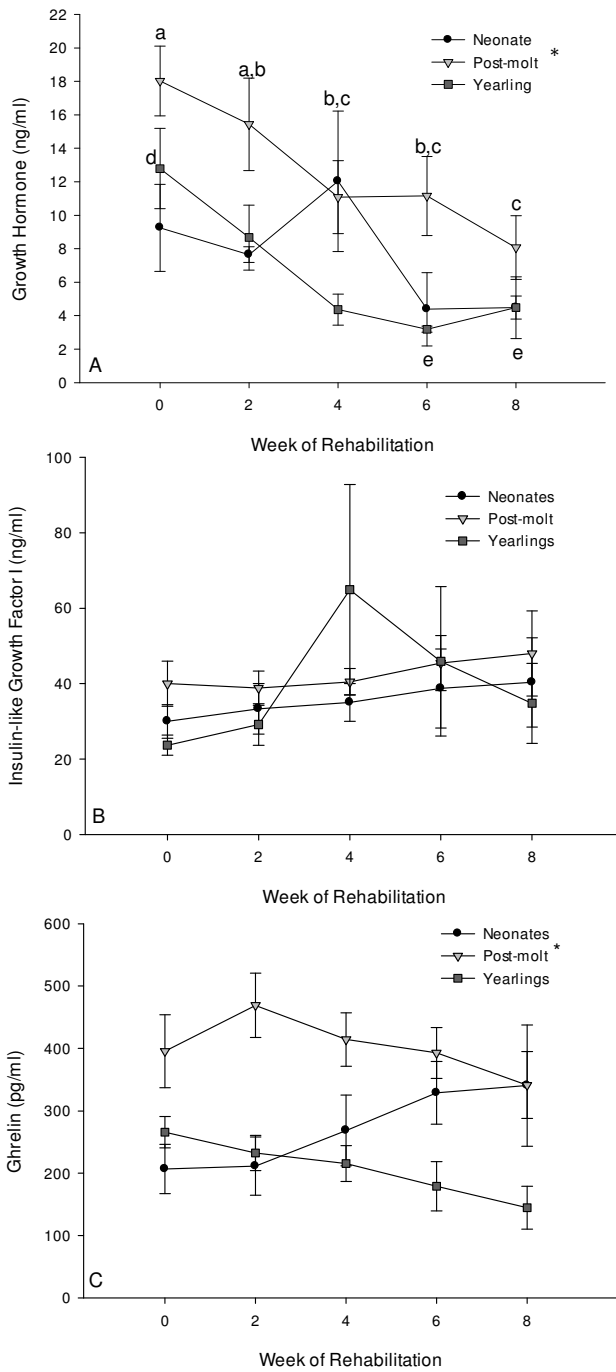
Figure 3. A. Growth Hormone concentration (ng/ml ± SE) by week of rehabilitation and age class. Different letters indicate statistical difference (p ≤ 0.05) between weeks within age class (post-molt: a, b, c; yearling: d, e). Overall differences among age classes indicated with (*) in legend. **B.** Insulin-like Growth Factor-I concentration (ng/ml ± SE) by week of rehabilitation and age class. **C.** Ghrelin concentration (pg/ml ± SE) by week of rehabilitation. Difference between age classes indicated with (*) in legend.

Table 1. Stepwise Linear Regression Model for morphometrics correlation to metabolic hormones

Variable	Model	P value	r^2
Body mass	76.0 - 0.73(GH) - 0.04(Ghrelin)	0.001	0.19
Body Condition Index	51.6 - 0.7(GH)	0.01	0.13

Discussion

Northern elephant seals routinely undergo extended periods of fasting for mating, lactation, and molting, as well as post-weaning (Castellini and Rea 1992; Champagne et al 2012; Schulz and Bowen 2005). Given this unique fasting adaptation, significant research investigating fasting physiology has been conducted in this species. However, the endocrine response to realimentation has not been investigated. Given the changes in metabolism and priority of nutrient utilization associated with transitioning from a nursing neonate to a fasting adapted juvenile, NES provide a unique opportunity to assess the effect of age on the response to realimentation.

While all animals in this study gained mass during realimentation, the body mass throughout rehabilitation was less than expected for free-ranging contemporaries (neonates: 40-130 kg; post-molt pups and yearlings: 80-130 kg; Kretzmann et al. 1993). Post-molt pups exhibited the greatest difference from expected free-ranging contemporary mass. Body mass of this age class (1-3 months) was less than or equal to expected birth mass (approximately 40 kg; Kretzmann et al. 1993). While absolute rate of gain (kg/day) did not differ between age classes, post-molt pups were the smallest age class and, therefore, gained the largest amount of mass per unit of body mass.

In this study, caloric intake increased throughout the realimentation period for all of the age classes investigated. However, caloric intake in a rehabilitation setting is regulated based upon body mass (10% of body mass per day feeding rate), explaining higher intake in larger yearling animals. Animals were realimented with a high fat and energy dense fish, herring. Despite this, mass gains were small, suggesting that food may not have been absorbed or assimilated efficiently. Alternatively, nutrient intake may be low compared to free-ranging animals of similar ages. Caloric intake for neonates in this study was less than the average daily intake in wild nursing harbor seals (approximately 7400 kcal/day; Schulz and Bowen 2004).

Normally, GH and ghrelin decrease with realimentation whereas IGF-I increases (Cummings et al. 2002; Breier 1999; Richmond et al. 2010; Ortiz et al. 2003; Eisert et al. 2005). While GH decreased as expected, the animals in this study did not exhibit predicted patterns with regard to IGF-I and ghrelin. No differences in IGF-I were observed across realimentation for any age class. Given that concentrations of IGF-I were initially below the detectable limit of the RIA (40 ng/ml), any changes early in rehabilitation would be undetectable. However, concentrations were low in all age classes compared to other mammals and well-fed NES (93.2 ± 6.3 ng/ml; Ortiz et al 2003), suggestive of a low plane of nutrition.

Ghrelin generally increases during fasting periods and decreases during realimentation (Nakazato et al 2001; Cummings et al. 2002; Tanaka et al 2004). Surprisingly, this study showed no change over realimentation but a difference between age groups. Due to the stimulatory effect of ghrelin on GH release (Strassburg et al 2008), increases in ghrelin due to nutritional status may be the mechanism responsible

for increased GH in the post-molt age class. Ghrelin is involved in the signaling of nutrient intake to the hypothalamus. Thus, when ghrelin increases due to decreased intake, signaling in the brain may be at least partially responsible for the increase in GH during fasting.

The neonate age class (animals < 4 weeks of age upon admit) exhibited significant mass gains late in rehabilitation. Concentrations of GH in neonates were less than other age classes at admit, suggesting they were less nutritionally compromised at the start of realimentation compared with other age classes. This hormone profile and delayed mass gains suggest a shorter period of nutritional deprivation prior to realimentation. However, the stable GH and IGF-I concentrations throughout realimentation suggest that the low nutritional status did not improve during realimentation and that neonates may recover more slowly from even relatively minor nutritional deprivation.

Post-molt pups were smallest for their age based upon expected mass of free-ranging NES. However, post-molt pups exhibited significant increases in mass earliest in rehabilitation, potentially reflecting a mechanism for catch up (compensatory) growth. However, IGF-I concentrations did not change throughout rehabilitation, despite observed correlations to mass. Ghrelin concentrations were also greater in post-molt pups than other age classes. Ghrelin has been shown to aid in the recuperation of mass after a fast and favors the accretion of adipose (Sangiao-Alvarellos et al 2011; Tschop et al. 2000). Additionally, small for gestational age human infants have elevated ghrelin concentrations through the 3 months of life to facilitate compensatory growth (Fidanci et al. 2010). Therefore, increased ghrelin and may be a mechanism for facilitating

42

recuperation of vital adipose tissue in animals most nutritionally compromised. GH in

post-molt pups remained elevated for the entire 8-week realimentation period relative to

well-fed concentrations of free-ranging counterparts (3 ng/ml; Ortiz et al 2003). Overall,

increased GH and ghrelin concentrations relative to other age classes suggest that the

nutritional compromise and mass loss in post-molt pups may be more severe.

In yearlings, GH concentrations decreased early in rehabilitation. Similarly to

other age classes, IGF-I concentrations did not change across realimentation. While

mass did not increase until week 6, overall metabolic hormone profiles suggest that

yearling animals recovered from nutritional nadir more rapidly than other age groups.

In conclusion, differences between neonates and post-molt pups are primarily

explained by physiological state related to the duration of nutritional deprivation, not

developmental age. However, yearling animals, which are more developed, do appear

to recover from nutritional nadir more rapidly.

Acknowledgements

Chapter 5

Conclusions

Discussion

The allocation of nutrients to specific tissues is a complex and dynamic system under control of multiple hormonal axes. However, the overarching need of animals to acquire sufficient nutrients for maintenance, growth, reproduction, and storage is similar across mammals (Perrin and Sibly 1993). In young rapidly growing animals, it is unusual for adipose accumulation to take priority over growth of lean tissues. However, the accumulation of large stores of adipose is a major priority for young phocid seals during a short nursing period (Schulz and Bowen 2004). Due to the high caloric demands for this rapid accretion, young phocid seals have metabolic demands much greater than many other mammals.

In harbor seals, the components of the somatotropic axis responded similarly to other mammals; however, ghrelin exhibited unexpected increases during realimentation (Chapter 2). This type of response is consistent with more recent ghrelin research regarding its effect on adipose. Initial research investigating ghrelin merely focused on the affect of ghrelin on satiety (Nakazato et al 2001). However, more recent research has elucidated the function of ghrelin in weight recuperation, inhibition of lipolysis, and fat deposition (Sangiao-Alvarellos et al 2011).

While both species in this study are adapted to thermoregulation in the aquatic environment, NES also require adipose as an energy reserve for months of fasting

during very energetically demanding physiological activities (mating, molting, and lactation; Castellini and Rea 1992; Champagne et al 2012; Schulz and Bowen 2005). In NES, based upon measurements of the somatotropic axis, young animals took longer to recover from nutritional nadir than yearlings (Chapter 4). However, all age classes were at a low nutritional state throughout the entire 8-week realimentation period. The surprising differences in ghrelin between age classes rather than week of realimentation suggest that the overall response of ghrelin is based upon nutritional status, not age.

The increase in ghrelin concentrations throughout realimentation in harbor seal pups and the elevated concentrations in post-molt pups may be facilitating compensatory accumulation of lipids after periods of nutritional deprivation. Since accretion of adipose is vital for survival in these species (Muelbert et al. 2003; Harding et al. 2005), it is possible that this mechanism could be facilitating the accretion of adipose over lean tissue upon realimentation. However, to assess the effect of ghrelin on adipose, future studies should incorporate measurements specific tissue accretion throughout realimentation via ultrasound or bioelectrical impedance analysis.

Future Research

Results of this study suggest that the adipose accumulation in young marine mammals is a higher metabolic priority than lean tissue accretion. However, more research is needed to determine the nutrient allocation occurring as a result of metabolic hormone and blood metabolite response to realimentation. In order to more fully assess the role of adipose tissue during realimentation, future experiments could

incorporate binding proteins of the somatotropic axis components, adipose derived metabolic hormones, and tissue specific accretion measurements.

Insulin-Like Growth Factor-I and Binding Proteins

Given the low concentrations of IGF-I in this study, future studies incorporating different assays with lower thresholds of detection would resolve changes in IGF-I early in realimentation. In addition, investigation of IGF-I binding proteins (IGFBP), which modulate the effects of IGF-I in the target tissues (Firth and Baxter 2002), would expand the context with which hormonal concentrations can be interpreted. Although there are at least six IGFBP, IGFBP-2 and IGFBP-3 are closely associated with changes in nutrition (Govoni *et al.* 2003; Richmond *et al.* 2010). IGFBP-2 generally inhibits the action of IGF-I in most peripheral tissues while IGFBP-3 generally facilitates the action of IGF-I (Conover 1992; Reviewed in: Firth and Baxter 2002; Renaville *et al.* 2002; Estavaris and Ziegler 1997). Additionally, IGFBP-2 increases and IGFBP-3 decreases with undernutrition and protein restriction (Smith *et al.* 1995). Given the action of IGFBP-2 and -3 on IGF-I, inhibitory and stimulatory respectively, the changes in their concentrations in nutritional nadir should further impede the action of IGF-I at many target tissues. While IGFBP have been investigated in harbor seals, they have not been quantified in NES. Additionally, adding more components of the somatotropic axis to step-wise linear regression could improve model predictions of blood metabolites and body composition.

Leptin

Leptin is another hormone that participates in the regulation of energy balance. (Reviewed in: Ahima and Flier 2000). It is produced mainly in adipose tissue and, in most mammals, increases proportionally to adipose, resulting in decreased food intake and increased energy expenditure (Maffei *et al.* 1995; Considine *et al.* 1996; Halaas *et al.* 1995; Ahima and Flier 2000). Since leptin alters food intake and energy expenditure relative to quantity of adipose, it is considered to be a key regulator of the maintenance of body weight (Reviewed in: Considine and Caro 1997; Friedman and Halaas 1998).

As adipose stores are utilized as a source of energy during fasting, leptin concentrations also decrease, as expected given their correlation with adipose (Considine *et al.* 1996; Ahima and Flier 2000). However, in short term starvation, leptin decreases disproportionately to decreases in body fat mass (Boden *et al.* 1996; Considine and Caro 1997). Research suggests that the relationship between adipose and leptin may be uncoupled during times of undernutrition as an adaptation to fasting (Ahima and Flier 2000).

The relationship of leptin to adipose may also result in a differential response to nutritional changes in these marine mammals. Some leptin studies seem to indicate that leptin may not be correlated with fat mass in marine mammal species (Ortiz *et al.* 2001; Arnould *et al.* 2002). Given the need for adipose accretion in marine mammals, it would not be advantageous to decrease intake as adiposity increases. To continue to intake nutrients with increasing adiposity, marine mammals may exhibit some degree of leptin resistance (reduced leptin receptors or receptor sensitivity) or reduction in leptin production. To assess this role, future studies would require analysis of leptin

concentrations in serum across varying body composition. Additionally, assessment of leptin receptor mRNA and protein in various tissues would address the question of leptin resistance.

Applications

This study is unique in its broad investigation of realimentation in phocid seals. The use of rehabilitation animals served as an excellent model for the transition from nutritional nadir through realimentation. However, collaboration with rehabilitation facilities also serves another purpose. This study provides valuable information for veterinary assessment regarding the nutritional status of animals. In both NES and harbor seals, metabolic hormones take several weeks to months to return to concentrations comparable to well-fed free-ranging animals, signaling a full recovery from nutritional nadir.

The results of this research, suggesting a slow rate of gain and low nutritional status, provide support for future research into more nutritionally appropriate formulas for realimentation of abandoned pups. While maternal milk in free-ranging phocid seals is usually 40-50% fat, milk replacer formula is only 30% fat by volume (MacRae et al 2010). Additionally, given the slow recuperation of some animals, this study further supports previous suggestions that estimation of resting energy requirements and growth requirements based upon terrestrial mammal data may be underestimated for marine mammals (Williams et al. 2001). This suggests that calculated caloric need for animals in rehabilitation may be an underestimate. Therefore, increasing digestible fat

content of formula and increasing caloric value may improve growth rate and nutritional status across realimentation.

In this study, a combination of body condition indices and blood metabolites showed significant correlation to harbor seal hormonal profiles. These correlations could prove to be a valuable tool for assessing the nutritional status of animals in captivity or rehabilitation. While reference values for blood metabolites in these species often have large ranges (Bossart et al. 2001), correlation with metabolic hormones can refine the use of blood metabolites as indicators of nutritional status in a veterinary setting.

The use of metabolic hormones and blood metabolites as indicators of nutritional status could also aid in management decisions when used in wild populations. Currently, nutritional stress is a leading hypothesis for the decline of some species of pinnipeds, including Steller sea lions in the Western Aleutian Islands of Alaska (Trites et al., 2007). Also, within the last year, starvation and emaciation was a leading cause of stranding in young California sea lions (NOAA Fisheries Unusual Mortality Event Report). Developing indices of nutritional stress in these and other species can provide information to management agencies facilitating the protection of these species.

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Vita

Rachael Dailey received her B.S. in Zoology from the University of Florida in 2010. Her career has been focused on the fields of marine animal research and veterinary sciences. In order to further knowledge in these disciplines, her thesis research focused on metabolic hormone assessments of nutritional status in rehabilitated harbor seals and Northern elephant seals. Rachael's overall career goal is to incorporate education, public outreach, and research to improve population health, rehabilitation success, and animal welfare. Rachael was employed prior to attending UNF as a manatee biologist and an intern at Mote Marine Lab (Sarasota, Florida) in the sea turtle rehabilitation hospital. During her studies at UNF, she completed an internship at Vancouver Aquarium's Marine Mammal Rescue Center in Vancouver, Canada. After graduation, Rachael intends to continue her career in marine mammal biology and publish her research conducted at UNF.