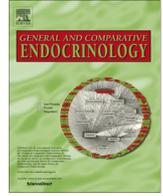




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## Endocrine response to realimentation in young northern elephant seals (*Mirounga angustirostris*): Indications for development of fasting adaptation



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### ABSTRACT

Most organisms undergo changes in their environment, both predictably and unpredictably, which require them to alter priorities in nutrient allocation with regards to food availability. Species that more predictably encounter extended periods of limited food resources or intake while mitigating the negative effects of starvation are considered to be fasting adapted. Northern elephant seals (NES) are one such species and routinely undergo extended periods of fasting for breeding, molting, as well as a post-weaning fast at 6–8 weeks of age. However, during unusual times of nutritional deprivation, animals may enter stage III fasting. While fasting and foraging in this species has been extensively studied, realimentation following fasting beyond normal life history parameters has not been investigated. In this study, changes in ghrelin, growth hormone (GH), and insulin-like growth factor (IGF)-I were compared across 8 weeks of realimentation following emaciation in three age classes: neonates, post-molt pups, and yearlings. Longitudinal changes in hormone profiles indicate that neonate and post-molt pups are slow to recover mass and positive energy balance despite an energy dense diet fed at 10% body mass. In addition, ghrelin and GH concentrations remained elevated in post-molt pups compared to other age classes. Changes in hormone concentrations early in realimentation indicate that yearling animals recover more rapidly from periods of nutritional deprivation than do younger animals. Overall, this suggests that the ability to regulate metabolic homeostasis with regards to nutrient allocation may develop over time, even in a species that is considered to be fasting adapted.

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### 1. Introduction

Throughout life, many organisms experience alterations in the need and ability to respond to physiological stressors. This may include altering patterns of nutrient utilization and allocation to cope with changes in tissues specific requirements such as reproduction, molting, seasonal adaptation, and periods of reduced nutrient intake. The somatotrophic axis is a group of hormones that participates in the regulation of nutrient utilization and allocation (Breier, 1999; Etherton, 2004). Components of this axis [growth hormone (GH) and insulin-like growth factor (IGF)-I] are responsive to the nutritional status and physiological state of an individual (Breier, 1999; Thissen et al., 1999). In addition, the satiety hormone ghrelin contributes to the partitioning of

nutrients through the promotion of feeding and adiposity (Sangiao-Alvarellos et al., 2011; Tschöp et al., 2000).

Models developed in domestic species have primarily defined the actions of these hormones with respect to changes in concentration and cellular action during changes in nutrient intake as well as with age. When nutrient intake is decreased, such as during a fast, IGF-I concentrations decrease while GH increases (Breier, 1999). Decreasing concentrations of IGF-I halt the processes of somatic growth when nutrient intake is low (Thissen et al., 1999). Increases in GH facilitate lipolysis to mobilize fatty acids from adipose as an energy source (Wang et al., 2004). Ghrelin normally exhibits pre-prandial increases as well as increases during fasting (Cummings et al., 2002). Upon realimentation, the anabolic effects of the somatotrophic axis 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; Tschöp et al., 2000). While ghrelin

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is not known to change with age, the somatotrophic 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).

In addition to the domestic animal model, GH, IGF-I and ghrelin have been studied in species considered to be fasting adapted. These species require mechanisms to maintain metabolic homeostasis to accomplish this extension of the fasting period without negative effects on health and survival. Fasting adaptation requires the ability to transition from the first (primary carbohydrate utilization) and extend the second phase (primary lipid utilization) of fasting, postponing protein utilization and organ damage for extended periods of time (Castellini and Rea, 1992; McCue, 2010). Northern elephant seals (NES) are one such species, routinely experiencing extended periods of fasting as a part of their normal life-history patterns. Unlike some other fasting adapted species, NES couple periods of fasting with energetically demanding activities such as mating (including defense of territory), molting, and lactation (Castellini and Rea, 1992; Champagne et al., 2012; Riedman, 1991). Given this unique fasting adaptation, significant research investigating fasting physiology has been conducted in this species. However, the endocrine response to realimentation (re-feeding following fasting) has not been investigated nor the impact of developmental stage on this response.

NES pups are born at approximately 40 kg (Kretzmann et al., 1993; Schulz and Bowen, 2005) and gain mass rapidly during the nursing period. Upon weaning at approximately 4 weeks old, pups weigh approximately 130 kg with 50% body fat (Kretzmann et al., 1993; Schulz and Bowen, 2005). The first natural extended fasting period for a NES pup is the post-weaning fast, which begins at approximately the same time as the first molt and continues for 6–8 weeks (Bowen, 1991; Kretzmann et al., 1993).

However, some pups are prematurely abandoned by their mothers and often experience a loss of homeostatic control over the fasting response, become emaciated, and have lower survival through the post-weaning period (Rea and Costa, 1992; Houser and Costa, 2003). Additionally, animals experience varying degrees of foraging success after the post-weaning fast through the first year (Le Boeuf et al., 1994). These animals are often the subject of human intervention and taken into rehabilitation facilities for controlled realimentation. This subset of animals, which experience extreme fasting beyond normal life history patterns, provide a unique opportunity to investigate the physiological response to realimentation following late stage fasting or whether the ability to recover may be influenced by developmental stage. Therefore, the objective of this study was to investigate the ability to regain homeostatic regulation of nutrient utilization and allocation via the response of ghrelin, GH, and IGF-I from nutritional nadir through realimentation (refeeding) in three age classes representing different developmental stages: neonates (less than 4 weeks of age), post-molt pups (>4–8 weeks of age), and yearlings (approximately 1 year of age).

## 2. Methods

### 2.1. Animals and diet

All animals used in this study had undergone routine veterinary care for malnutrition at The Marine Mammal Center (TMMC; Sausalito, CA) between 1996 and 2013. 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 (status of molt), umbilical cord status, tooth eruption, size, and time of year in relation to peak (or mean) pupping date (Le Boeuf et al., 1994).

Animals were included in the study if they had greater than three blood sampling events during the rehabilitation period. The TMMC veterinary staff determined time of release depending on overall health condition and body mass of the animals. Only blood samples taken at or before 8 weeks were included in the analysis based on the average length of rehabilitation at 8.9 weeks. Thus, some animals included in the study were in rehabilitation longer than the 8 weeks of the study period.

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 by veterinary staff at TMMC based upon estimated caloric requirement for growth at three times the resting energy requirements (RER; kcal/day) =  $70 \times (\text{Body weight in kg})^{0.75}$  (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), fed at 10% of body weight per day (Richmond et al., 2010). 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. Approximate daily caloric intake (kcal) was calculated based upon recorded actual intake of prescribed diet (mL and/or kg) and caloric content of formula (3700 kcal L<sup>-1</sup>) and fish (1500 kcal kg<sup>-1</sup>) (Townsend and Gage, 2001; Richmond et al., 2010).

Mass was measured to the nearest 0.5 kg, at minimum, at admit and release, with most animals weighed at each blood collection (approximately every 2 weeks). Standard length (SL) was measured to the nearest centimeter at admit and prior to release to assess growth and body condition. A body condition index of mass/standard length \* 100 was calculated for admit and release to estimate overall body condition (Fadely, 1997).

### 2.2. Blood analysis

Blood samples (2–4 mL) were collected approximately every 2 weeks depending on clinical requirements. Samples were collected before the first feed of the day after an overnight fast. Animals were manually restrained and blood was collected via the extradural intravertebral vein (Bossart et al., 2001) into serum separator tubes (SST). Blood in SST was allowed to clot for 15 min then centrifuged at 3000×g for 10 min. 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. Due to differences in date of blood samples between animals, samples were grouped into two week periods based on the number of days since admittance: week 0 (<7 days since admit), week 2 (7–20 days), week 4 (21–34 days), week 6 (35–48 days), week 8 (49–63 days).

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  $99.1 \pm 8.0\%$ . NES pooled serum averaged  $11.0 \pm 0.2$  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-I concentrations in pooled serum averaged  $44.6 \pm 1.7$  ng/ml with a dilution linearity of  $R^2 = 0.94$ , an intra-assay CV of 6.6 and an inter-assay CV of 15.8. IGF-I assays had a detection limit of 20 ng/ml. Samples with concentrations below detection limits were included for statistical analysis at 10 ng/ml.

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  $92.8 \pm 2.1\%$ . NES pooled serum averaged  $430.1 \pm 26.1$  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  $R^2 = 0.93$ .

### 2.3. Statistical analysis

Metabolic hormones, mass, and caloric intake were evaluated 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. Statistical analyses were completed using SAS (SAS Inst. Inc., Cary, NC) software package. To determine if age influenced the response to realimentation a mixed model ANOVA with repeated measures using Compound Symmetry (CS) covariance structure was completed. Minimal AIC value and data structure were used to determine appropriateness of this model. Sex was included in the initial statistical analysis; however, there was no effect of sex on any variable measured, therefore data were combined in the final analysis. To evaluate differences among age classes at individual time points least significant difference comparisons were performed. Differences were considered significant if  $P \leq 0.05$  and trends if  $0.10 \geq P > 0.05$ . All values are reported as  $\pm$ SEM.

### 3. Results

Daily caloric intake increased across rehabilitation for all age classes ( $F_{2, 44} = 9.54$ ;  $p < 0.001$ ; Fig. 1A). However, absolute intake was higher in yearlings compared to post-molt and neonate pups ( $F_{8, 89} = 2.44$ ;  $p < 0.01$ ). Intake in neonate pups increased between week 0 ( $1913.4 \pm 1087.9$  kcal/day) and week 4 ( $9037.3 \pm 2934.5$  kcal/day;  $F_{8, 89} = 10.17$ ;  $p = 0.002$ ) then remained stable for the rest of realimentation at an average of  $9650.9 \pm 1645.0$  kcal/day. Post-molt pups had reduced intake upon admit (week 0:  $3015.1 \pm 552.6$  kcal/day;  $F_{8, 89} = 6.05$ ;  $p < 0.02$ ) but then increased to approximately  $7481.9 \pm 418.6$  kcal/day for the remainder of realimentation. Intake in yearling animals increased from week 0 ( $3219.5 \pm 858.9$  kcal/day) through week 4 ( $15875.5 \pm 2706.2$  kcal/day) then remained stable ( $F_{8, 89} = 71.57$ ;  $p < 0.001$ ).

Neonate pups did not gain significant body mass (Fig. 1B) until after week 6 of realimentation at  $66 \pm 38.1$  kg, an increase of

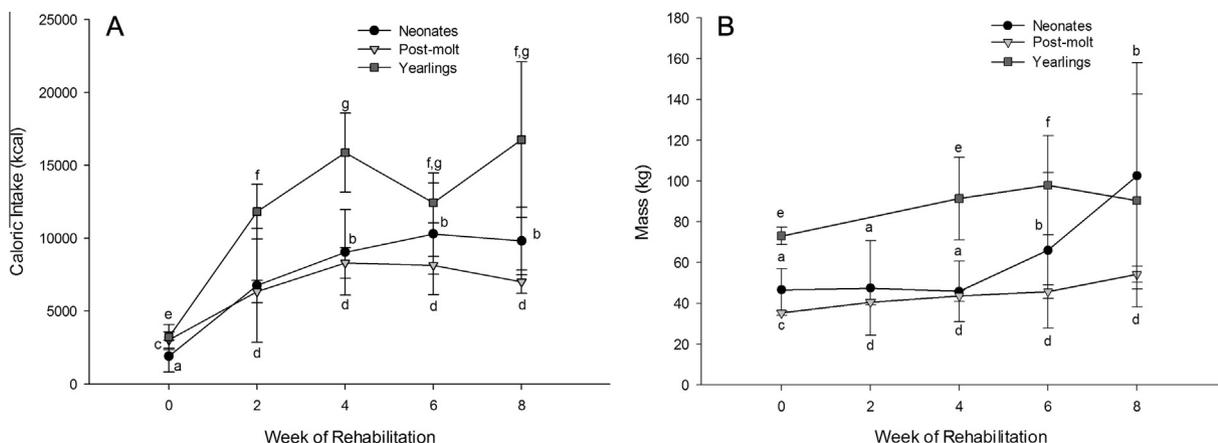
roughly 20 kg ( $F_{7, 61} = 20.25$ ;  $p < 0.001$ ). Post-molt pups had an earlier increase in mass by week 2 ( $40.5 \pm 1.4$  kg;  $F_{7, 61} = 3.80$ ;  $p = 0.05$ ) and remained stable through week 8 ( $54 \pm 3.9$  kg;  $F_{7, 61} = 20.25$ ;  $p < 0.001$ ). Yearlings increased in mass by week 6 ( $98 \pm 24.4$  kg;  $F_{7, 61} = 23.62$ ;  $p < 0.001$ ) 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 ( $F_{2, 31} = 0.39$ ;  $p = 0.96$ ). Standard length increased in post-molt ( $F_{2, 27} = 9.92$ ;  $p = 0.004$ ; admit:  $122 \pm 3.3$  cm; release:  $137 \pm 3.9$  cm) and yearling ( $F_{2, 27} = 2.85$ ;  $p = 0.02$ ; admit:  $150.1 \pm 4.0$  cm; release:  $158 \pm 5.8$  cm) age classes but not in pups ( $F_{2, 27} = 1.02$ ;  $p = 0.32$ ). Body condition index increased over realimentation in all three of the age classes (neonate: admit  $39.9 \pm 4.2$ , release  $76.3 \pm 7.2$ ; post-molt: admit  $28.9 \pm 2.5$ , release  $42.4 \pm 3.2$ ; yearling: admit  $49.3 \pm 2.8$ , release  $59.6 \pm 2.9$ ;  $F_{1, 38} = 35.85$ ;  $p < 0.001$ ).

Growth hormone concentrations (Fig. 2A) decreased as expected in post-molt pups and yearlings. Concentrations of GH in post-molt pups decreased gradually and over the entire realimentation period ( $F_{8, 91} = 12.75$ ;  $p < 0.01$ ), whereas yearlings exhibited a greater decline from week 0 ( $18.0 \pm 2.1$  ng/ml) to week 4 ( $11.1 \pm 2.2$  ng/ml;  $F_{8, 91} = 9.73$ ;  $p = 0.002$ ) with stable concentrations for the remainder of realimentation. Surprisingly, no decrease was observed in neonate pups, despite concentrations at week 8 ( $4.5 \pm 1.8$  ng/ml) roughly half of admit concentrations ( $9.3 \pm 2.6$  ng/ml;  $F_{8, 91} = 0.64$ ;  $p = 0.43$ ). Additionally, average concentrations of GH across all 8 weeks were greater in post-molt pups compared with neonates and yearlings ( $F_{8, 91} = 4.80$ ;  $p < 0.03$ ).

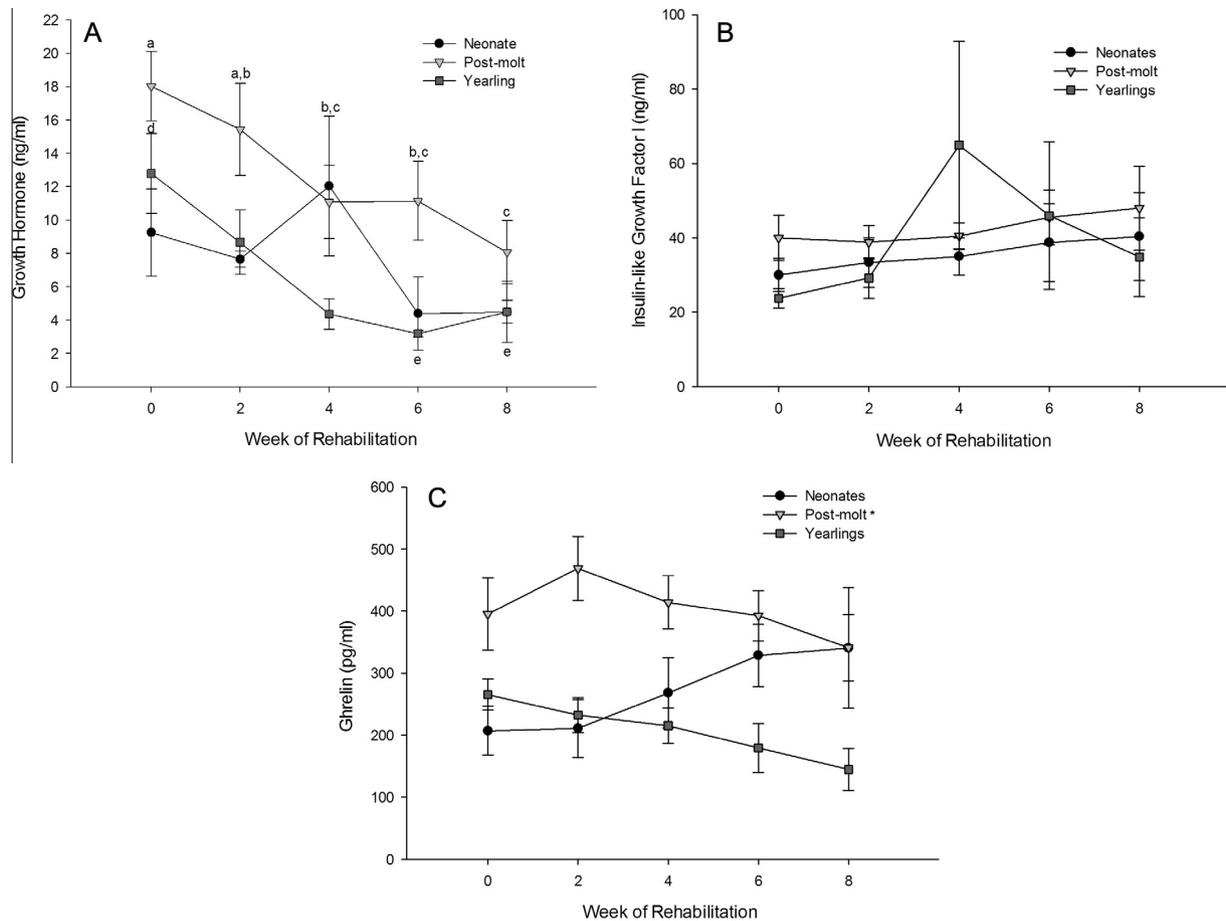
Concentrations of IGF-I were initially low ( $< 40$  ng/ml) and were below 70 ng/ml for the entire realimentation period in all age classes and showed no change in concentration by week ( $F_{4, 87} = 0.93$ ;  $p = 0.45$ ; Fig. 2B). Ghrelin concentrations (Fig. 2C) did not change significantly by week ( $F_{4, 96} = 0.07$ ;  $p = 0.99$ ) but average concentrations across all 8 weeks differed by age class ( $F_{2, 45} = 16.43$ ;  $p < 0.001$ ), with concentrations increased in post-molt pups ( $404.6 \pm 21.6$  pg/ml) compared to neonate pups ( $272.1 \pm 39.4$  pg/ml;  $p = 0.005$ ) and yearlings ( $208.3 \pm 27.8$  pg/ml;  $p < 0.001$ ).

### 4. Discussion

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 prescribed based upon body



**Fig. 1.** (A) Body mass (kg  $\pm$  SE) by week of rehabilitation. Different letters indicate statistical difference ( $p \leq 0.05$ ) between weeks within age class (neonates: a, b; post-molt: c, d; yearling: e, f) (B) Caloric intake (kcal  $\pm$  SE) by week of rehabilitation. Different letters indicate statistical difference ( $p \leq 0.05$ ) between weeks within age class (neonates: a, b; post-molt: c, d; yearling: e, f, g).



**Fig. 2.** (A) Growth Hormone concentration (ng/ml  $\pm$  SE) by week of rehabilitation and age class. Different letters indicate statistical difference ( $p \leq 0.05$ ) 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  $\pm$  SE) by week of rehabilitation and age class. (C) Ghrelin concentration (pg/ml  $\pm$  SE) by week of rehabilitation. Difference between age classes indicated with (\*) in legend.

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 herring. Despite this, mass gains were small, suggesting that food may not have been absorbed or assimilated efficiently. Alternatively, nutrient intake may simply 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 NES (approximately 70 MJ/day, 16,700 kcal/day; Kretzmann et al., 1993; Costa et al., 1986).

While all animals in this study gained mass during realimentation, body mass was consistently less than expected for free-ranging contemporaries (neonates: 40–130 kg; post-molt pups and yearlings: 80–130 kg; Kretzmann et al., 1993; Noren et al., 2003; Oliver et al., 1998). 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 rate of gain (kg/day) did not differ between age classes, post-molt pups were the smallest age class compared to neonates and yearlings and, therefore, gained the largest amount of mass per unit of body mass.

In domestic species, GH and ghrelin decrease with realimentation whereas IGF-I increases (Breier, 1999; Cummings et al., 2002; Eisert et al., 2005). For GH, and IGF-I this pattern has also been observed in neonate harbor seals (Richmond et al., 2010). However, relationship of hormones and food intake are equivocal in NES, with no change in IGF-I or ghrelin in adult males during fasting (Crocker et al., 2012), decreases of GH during fasting in

juveniles (Kelso et al., 2012), and increases of GH and ghrelin during fasting in post-weaned fasting pups (Ortiz et al., 2003). While GH decreased as expected based on the domestic animal model, 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 (20 ng/ml), any changes early in rehabilitation would be undetectable. However, concentrations were low in all age classes compared to newly weaned NES pups of similar age ( $93.2 \pm 6.3$  ng/ml; Ortiz et al., 2003), suggestive of a low plane of nutrition.

Ghrelin generally increases during fasting periods and decreases during realimentation (Cummings et al., 2002; Nakazato et al., 2001; Tanaka et al., 2004). Ghrelin has also been previously shown to be responsive to nutrient intake in NES, increasing during the post-weaning fast (Ortiz et al., 2003) but did not change during the fast in adult males (Crocker et al., 2012). This study showed no change over realimentation but a difference between age groups on average. Ghrelin is one gut-derived hormone which signals nutrient intake to the hypothalamus (Cowley et al., 2003) and is also the endogenous ligand for the growth hormone secretagogue receptor (GHS-R; Kojima et al., 2001). If ghrelin increases in this species due to decreased intake, signaling in the brain could be partially responsible for the increase in GH during fasting (Muller et al., 2002). Due to the stimulatory effect of ghrelin on GH release (Strassburg et al., 2008), increases in ghrelin due to nutritional status could be a mechanism responsible for increased GH in the post-molt age class. However, no

direct correlation was seen between GH and ghrelin concentrations ( $R = 0.10$ ) in this study. Alternatively, increases in ghrelin in the post-molt weaned animals in this study as well as Ortiz et al., 2003 could be related to age or development. Further investigations could be focused on determining possible relationship to age and the signaling to begin foraging after the post-weaning fast.

The neonate age class (animals < 4 weeks of age upon admit) exhibited significant mass gains late in realimentation. Concentrations of GH in neonates were less than other age classes at admit, suggesting a higher nutritional plane at the start of realimentation compared with other age classes. Despite gains in mass late in realimentation, the stable GH and IGF-I concentrations throughout realimentation suggest that the low nutritional status did not improve during realimentation. This hormone profile and delayed mass gains suggest 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. 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; Tschöp et al., 2000). Additionally, human infants that are small for their gestational age have elevated ghrelin concentrations through the first 3 months of life to facilitate compensatory growth (Fidanci et al., 2010). Therefore, increased ghrelin may be a mechanism for facilitating 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). However, IGF-I concentrations did not change throughout rehabilitation. 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. Overall, metabolic hormone profiles returning to concentrations expected in well-fed animals earlier in rehabilitation suggests that yearling animals recovered from nutritional nadir more readily than other age groups.

## 5. 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 fasting beyond normal limits more rapidly. This difference in the response to realimentation between the younger age classes and yearlings suggests that the ability to recuperate from periods of nutritional deprivation may develop over time even in fasting adapted species.

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