DIETARY EFFECTS ON PROTEIN TURNOVER IN THREE PINNIPED SPECIES, *EUMETOPIAS JUBATUS, PHOCA VITULINA, AND LEPTONYCHOTES WEDDELLII*

By

Susan D. Inglis, MS

A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

in

Marine Biology

University of Alaska Fairbanks

May 2019

APPROVED:

Michael Castellini, Committee Chair
Shannon Atkinson, Committee Member
Perry Barboza, Committee Member
James Carpenter, Committee Member
Lorrie Rea, Committee Member
Matthew Wooller, Chair
Department of Marine Biology
Bradley S. Moran, Dean
College of Fisheries and Ocean Sciences
Michael Castellini, *Dean of the Graduate School*

Abstract

The role of dietary protein in pinniped (seal and sea lion) nutrition is poorly understood. Although these marine mammals derive the majority of their daily energetic needs from lipid, lipids cannot supply essential amino acids which have to come from protein fractions of the diet. Protein regulation is vital for cellular maintenance, molt, fasting metabolism, exercise and development. Proteins are composed of linked amino acids (AA), and net protein turnover is the balance between protein synthesis from component AA, and degradation back to AA. Protein regulation is influenced by dietary intake and quality, as well as physiological and metabolic requirements.

In this work, pinniped diet quality was assessed through comparisons of amino acid profiles between maternal milk, blood serum, and seasonal prey of wild juvenile Steller sea lions (*Eumetopias jubatus*) in Southcentral Alaska. Both Pacific herring (*Clupei pallasi*) and walleye pollock (*Gadus chalcogramma*) showed similar patterns to milk in essential and branched chain amino acid content. Serum amino acid profiles suggest the juvenile sea lions were not in protein deficit at the time of capture.

Protein metabolism in the blood and urine was assessed through turnover studies using amino acid tracers. The turnover kinetics of ^{15}N-labelled glycine in the blood amino acid and protein pool, red blood cells, and urine urea were measured in wild adult female Weddell seals (*Leptonychotes weddellii*) in the Antarctic. Labelled glycine moved quickly into serum protein and red blood cells (1-2 hours) and urinary urea (2-4 hours). The turnover rates in the blood amino acid and urine urea pools demonstrated a reduced turnover rate associated with molting.

Lastly, whole body protein turnover experiments using a single bolus ^{15}N-labelled glycine tracer method with endproduct collection of blood, feces and urine were conducted on 2 Cohort groups of captive Alaskan harbor seals over 2 years. Season was found to have the greatest effect on whole body protein turnover, which increased during the winter and decreased in the summer molt. Conversely, protein intake decreased during the winter and increased in the summer molt. This pattern corresponded with an increase in mass and protein synthesis in the winter, while mass decreased and protein degradation rates increased in molting seals. Weaning also influenced the patterns with reduced protein turnover in newly weaned animals that had recently transitioned from milk to a fish diet.

This project presents results on whole body protein turnover rates in nonfasting pinnipeds and reveals that protein turnover is strongly regulated by developmental and seasonal physiological and metabolic demands.

Dedication

This work is dedicated to my father, who taught me that a curious mind was a wonderful thing and was always there to catch me when I stumbled.

Table of Contents

List of Figures

Page

List of Tables

Page

Acknowledgements

This dissertation would not have been possible without the contribution of many individuals. First and foremost, I would like to sincerely thank my graduate advisor Michael Castellini for allowing me the opportunity to learn from his vast experience. Mike had an open door policy and was always there to help me work through scientific questions and provide encouragement when small traumas and concerns cropped up. He taught me the valuable lesson of how to think through problems. Working in his lab with Leslie Cornick, Steve Trumble, Tami Mau, Vicki Steagall, Lying Zhao, and Shawn Harper was a fun and supportive learning experience. In particular, I would like to thank Maggie Castellini for her help and advice through all components of my project, and for letting me make their home my second home when I was in Fairbanks.

I would also like to acknowledge and thank my graduate committee; Shannon Atkinson, Lorrie Rea, Perry Barboza and James Carpenter. This dissertation has been a long and winding journey and I always felt the support and encouragement of my committee, and I will forever be grateful. Thank you to Shannon Atkinson, James Carpenter and Lorrie Rea for sharing data with me from their projects and helping me with all the sample collection during the protein turnover trials. A special thank you to Perry Barboza, who trained me in many of the laboratory techniques I needed to complete this project. I also appreciated all the help and support from his students Dave Gustine and Jennifer Addison.

I was fortunate enough to travel to some wonderful locations to collect data and I owe a huge thanks to the Antarctic Field Team: Michael Castellini, Lorrie Rea, Maggie Castellini, Tami Haase, Shawn Harper and Vicki Stegall. It was an experience of a lifetime for me!

This dissertation required research support from many individuals and institutions including McMurdo Station in Antarctica, University of Alaska Soil Science Laboratory, and Tim Howe and Norma Haubenstock with the Alaska Stable Isotope Facility. In particular, I would like to thank the support and staff of the Alaska SeaLife Center. There were many long days and late nights collecting samples that required the help of many people.

Financial support for this project was provided by Alaska Sea Grant, the Alaska SeaLife Center Research Program with funds from National Marine Fisheries Service, and the National Science Foundation, Office of Polar Programs Grant 01-30417. Marine mammal tissue collection protocols were approved by the UAF Institutional Animal Care and Use Committee (protocol Nos. 01-46, 01-06, 03-009 and 06-006), the Alaska Department of Fish and Game Animal Care and Use Committee (protocol NO. 03-00002) and Alaska SeaLife Center Animal Care and Use Committee under the following NMFS permits No. 358-1564, NMFS permit 14335 and the Marine Mammal Protection Act Permit # 1033-1683.

Finally, I would like to thank the support of my family and friends over the years.

Chapter 1
General Introduction

Dietary adaptation in mammals is based on the type of food they consume and the flexibility of the digestive and metabolic processes (Singer 2003). Most pinnipeds such as seals and sea lions are considered opportunistic piscivores and have developed unique strategies to survive in an energetically challenging environment (Brown and Mate 1983). It is assumed that a piscivore feeding strategy would require some degree of digestive and metabolic flexibility to be successful, as the nutritional constituents in fish may vary both temporally and spatially. Changing environmental conditions associated with alterations in trophic food webs has led to nutritional stress hypotheses for the decline in several Alaska pinniped populations of Steller sea lions (*Eumetopias jubatus*) and harbor seals (*Phoca vitulina*). Most of the studies conducted to address nutritional stress hypotheses have focused on the caloric value of prey.

Our understanding of how nutritional constituents vary in prey items and how that impacts pinnipeds is still not well understood. For example, studies on harbor seals demonstrated digestive flexibility when maintained on different diets (Stanberry 2003; Trumble et al. 2003). Trumble et al. (2003) found that harbor seals maintained and gained weight when placed on a single source low fat (walleye pollock) diet through digestive adaptations including increased feed assimilation efficiency, gut fill and intake. In both studies, high fat content significantly decreased the total intake of fish consumed by harbor seals. Seasonal components were also found to be significant. A study conducted concurrently with the Trumble et al. (2003) experiment measured plasma amino acid levels and found evidence of differing protein metabolic pathways corresponding to herring (*Clupei pallasii*) and pollock (*Gadus chalcogramma*) diets (Zhao 2002). There was a decrease in the free amino acid pool in harbor

seals maintained on the pollock diet. These findings suggest that protein turnover and regulation were being altered based on diet type.

Many other studies have been conducted on captive pinnipeds to address questions of nutritional physiology (Ashwell-Erickson 1981; Keiver et al. 1984; Krockenberger and Bryden 1994; Rosen and Trites 2000). These studies primarily focused on the lipid biochemistry and overall caloric benefits of prey.

However, a diet that provides adequate calories can be inadequate with regard to nutrients for growth and building and maintaining cellular and metabolic requirements (Schmidt-Nielsen 1997). Amino acids (AA) are the functional building blocks that make up protein and most carnivores require a dietary source of ten essential amino acids; arginine (ARG), isoleucine (ILE), leucine (LEU), lysine (LYS), methionine (MET), phenylalanine (PHE), threonine (THR), tryptophan (TRP), histidine (HIS) and valine (VAL), (Robbins 1983; Barboza et al. 2009). The importance of protein in muscle structure, enzymes, hormones, membrane transport, and immune functions underscores the need to study impacts on protein metabolism and potential consequences to nutritional physiology and metabolic regulation (El-Khoury 1999).

Pinnipeds will spare protein and preferentially use lipid stores for energy when dietary sources are restricted, but must also retain blubber for insulation, buoyancy, hydrodynamic streamlining, and as a reservoir of energy and metabolic water during fasting (Worthy and Lavigne 1987; Webb et al. 1998). However, unlike lipid, protein is stored in muscle tissue and mammals can generally not tolerate a loss in lean body mass greater than 20-30 % (Cahill 1978; Pernia 1984). Small irregularities in protein metabolism would not be relevant from a caloric perspective but could have significance consequences to animal health.

Few studies have focused on the role of protein in pinniped nutrition, even though these carnivores have life strategies that utilize protein in unique ways including annual molts, where pelage is replaced, and fasts during breeding and lactation (Castellini and Rea 1992; Champagne et al. 2012). As fish contain almost negligible quantities of carbohydrate (Bando 2002), protein can also be an important source of glucose during life history fasts for the central nervous system and erythrocytes through gluconeogenesis (Champagne et al. 2012). In gluconeogenesis, the transamination or deamination of amino acids frees the carbon skeleton for use to generate glucose (MacDonald et al. 1984, Barboza et al. 2009). Understanding the metabolism and allocation of available dietary protein is thus an important component in assessing the effect of diet on pinniped health. Net protein turnover is the balance between protein synthesis and degradation, and is influenced by dietary intake and quality, as well as physiological and metabolic requirements (Boren et al. 1996; Waterlow 2006; Barboza et al. 2009).

Protein quality is evaluated by the amount of nitrogen absorbed from the diet and a comparison of the proportions of amino acids that cannot be synthesized by the body (essential amino acids, EAA) in the food, with those in the tissues of the consumer (Robbins 1983; Barboza et al. 2009). Nitrogen is a constituent of protein and the biological value (BV) is a measurement of how well the body can utilize protein from the diet; or the nitrogen absorbed versus excreted, and is often used as a general measurement of protein quality (Robbins 1983; Barboza et al. 2009). The BV of a protein is often determined by the most limiting AAs. For example, if an animal has a high daily requirement of phenylalanine and the protein source is low in this EAA, it will have a low BV since it will only utilize a portion of the protein and will need to consume other protein to meet its daily requirement of this AA. As the requirements for certain AAs can change for an animal, so can the BV of a protein. The closer the dietary AA profile reflects the animal tissues,

the higher the value. High quality dietary protein such as muscle tissue, milk and eggs have the highest BV of ~1 (Robbins 1983; Waterlow 1999; Barboza et al. 2009). Knowledge of the AA composition of the diet is necessary to evaluate quality for the consumer.

As discussed above, protein quality is a measurement of the nitrogen incorporated from the diet in the form of amino acids. Serum amino acid profiles represent a balance between the AA intake from the diet and rate of use in protein synthesis, catabolism and/or oxidation. Protein deficient diets will lead to different serum AA kinetics and concentration ratios than seen in animals with sufficient dietary protein. Ratios of different groups of serum AAs have been used successfully in different species to evaluate protein status in animals (Whitehead and Dean 1964; Arroyave 1970; Davis et al. 1995; Sarwar et al. 1998; Schaafsma 2000). In general, the % EAA in blood serum decreases (most notability in the branched-chain group; LEU, ILE and VAL that are specifically required for protein synthesis) and serum non-essential amino acids (NEAA) increases in protein poor diets due to the consequent breakdown of body muscle proteins. The recommended indices of animal condition based on AA composition are total serum concentrations of sulfur AA and the ratios of BCAA: NBCAA (nonbranched chain AA), and EAA:NEAA (Boren et al. 1996; Sarwar et al. 1998).

As marine mammals, pinnipeds experience life history metabolic challenges which include weaning, fasting, molting, thermoregulation and hydrodynamic costs associated with living in a marine environment. Protein turnover experiments can study how these metabolic challenges are met through protein intake and metabolism.

The dynamics of protein turnover within the body include two opposite processes: protein synthesis and degradation. Protein turnover is the rate at which protein is synthesized and

degraded. Tracer methods provide a technique to quantify the summed kinetics of these opposite processes (Waterlow et al. 1978; Huntley et al. 1987; Wolfe 1992; Fouillet et al. 2002; Jackson 2009). The principle of these methods involves introducing a labelled compound that is biochemically similar, but distinct enough to detect the compound of interest. Isotopic tracers are created by substituting an atom in a specific location with an isotope that is less abundant (Waterlow et al. 1978; Huntley et al. 1987; Wolfe 1992). For example, ^{14}N, which is the most common isotopic form of nitrogen in the environment (~99.636 %) and ^{15}N (also a stable isotope), a less abundant isotopic form of nitrogen in the environment and can be used to label specific amino acids (e.g., ^{15}N labelled glycine). Some examples of compounds studied using this technique are amino acids, urea, fatty acids or glycerol.

The amino acid stable isotope tracer ^{15}N labelled glycine followed by blood and/or urine endproduct collection is another common tracer that has been successfully used to study protein turnover in both human and wildlife studies (Waterlow et al. 1978; Freudenberger and Nolan 1993; Barboza et al. 1997; Castellini 2001; Russell et al. 2003). Glycine was chosen specifically as the tracer for this study because it is active in protein synthesis and its metabolic pathway includes excretion through urinary urea which is a noninvasive method for endproduct collection (Fern et al. 1985, Waterlow 2006). Since this tracer is regularly used to quantify protein turnover in human studies (Waterlow 2006) and has been used broadly in other mammals including bears *Ursus americanus* and *U. arctos* (Barboza et al. 1997), honey possum (*Tarsipes rostratus*), kangaroos (*Macropus robustus robustus* and *M. erubenscens*), feral goats (*Capra hircus*) (Freudenberger and Nolan 1993), and cats (*Felis silvestris catus*) (Russell et al. 2003) it also provides comparative studies for the interpretation of our results.

Protein kinetics models (Picou and Taylor-Roberts 1969; Waterlow et al. 1978; Huntley et al. 1987; Waterlow 2006; Jackson 2009; Wolf et al. 2009) assume that there are two nitrogen pools in the body, a free amino acid pool and a protein pool. The free amino acid pool is generally the connection between dietary substrates and protein metabolism. This pool supplies the substrates derived from the diet and protein degradation to the protein pool for protein synthesis and oxidation (Waterlow 2006; Wolf et al. 2009).

Whole body protein turnover is the sum of all turnover activities of individual proteins in the body (El-Khoury 1999) and provides an indication of nutritional protein requirements and metabolic flexibility (Freudenberger and Nolan 1993; Barboza et al. 1997; Russell et al. 2003). By measuring whole body turnover, we can test the effects of dietary intake and metabolic demands such as growth and season on the rate of nitrogen flux as well as how available nitrogen is partitioned (synthesis or degradation) to accommodate these demands.

Several of these different types of physiological tracers have been used to report protein turnover in animals. In fasting northern elephant seal (*Mirounga angustriostris*) (Pernia 1984; Crocker et al. 1998; Houser and Costa 2001) and Antarctic fur seal (*Arctocephalus gazelle*) (Arnould and Hindell 2001) studies, a single bolus injection dose of ^{14}C labelled urea was used as a physiological tracer with the labelled urea collected in blood and urine endproducts.

As pinniped populations are experiencing increasing competition with anthropogenic demands on ocean resources through fisheries interactions, contaminants and climate change, shifts in prey availability and the ability of pinnipeds to accommodate these changes becomes imperative in management.

The goal of this dissertation is to improve understanding of how dietary protein is metabolized and allocated to meet physiological and metabolic demands experienced by pinnipeds. The three primary objectives of this study are:

(1) evaluate the protein quality in pinniped diets. As protein intake and quality effect protein metabolism, we must first assess the protein quality and seasonal changes in the quantity and/or quality of protein in the diet (Chapter 2),

(2) using the amino acid tracer (^{15}N-labelled glycine) report on turnover kinetics in the serum amino acid and protein pools, red blood cells, and urinary urea to understand the metabolic pathways of potential tracers (Chapter 3), and

(3) conduct a 2 year whole body protein turnover experiment to understand the how growth, age, season, and protein intake affect protein turnover and allocation in a non-fasting pinniped (Chapter 4).

Chapter 2

Protein Quality in the Diet of Steller Sea Lions, *Eumetopias jubatus,* in Southcentral Alaska[1]

Abstract

Seasonal differences in protein content and quality of Steller sea lion (SSL; *Eumetopias jubatus*) prey were determined for the Chiswell Island, Alaska SSL rookery and haulout sites using proximate and amino acid analysis. We found no seasonal differences in the protein quality of prey, suggesting that SSL have access to consistent protein quality throughout the year at this site. This study also examined amino acid (AA) profiles and specific AA ratios of prey, milk and serum to assess dietary protein quality and evaluate short term protein nutritional status in juvenile SSL. Amino acid profiles and ratios were compared between four sets of samples: walleye pollock (*Theragra chalcogramma*), Pacific herring (*Clupei pallasi*), SSL milk from pups and juvenile SSL serum (ages 11.5 and 22-35 months). The closer the prey AA profile matches the essential amino acid requirements of the animal, as represented by serum and milk values, the higher the dietary protein quality. There were significant differences in the AA composition in all four sample categories (Kruskal-Wallis; $p<0.001$). Sea lion milk had the highest branched chain amino acid: nonbranched chain amino acid ratio (0.3) followed by serum (0.25), herring (0.23) and pollock (0.19). For nonessential amino acid ratios, sea lion milk was again highest (0.9), followed by juvenile SSL serum and herring at 0.8, and pollock lowest at 0.6. These results suggest that herring provides a slightly higher quality protein than pollock for juvenile SSL during the weaning process, but both prey species contain high quality protein. Further, serum AA profiles indicated that young of the year SSL were consuming a diet similar in protein quality to yearling SSL and did not show any sign of protein deficiency.

[1]Inglis, S.D., Rea, L.D, Atkinson, S. and M.A. Castellini. Protein Quality in the Diet of Steller Sea Lions, *Eumetopias jubatus,* in Southcentral Alaska. Prepared for submission to Physiological and Biochemical Zoology.

Introduction

The western population of Steller sea lions (SSL; *Eumetopias jubatus*) in Alaska experienced a dramatic population decline beginning in the 1960s (Estes et al. 2009). A leading theory for the cause of this decline has been that a regime shift in available prey caused chronic nutritional stress that increased juvenile mortality, decreased reproductive success and impaired health (Alverson 1992; Castellini 2001). The nutritional stress hypothesis has led to numerous studies conducted on captive and wild pinnipeds to address questions on the effect of diet on nutritional physiology (Rosen and Trites 2000b; Castellini 2001; Anza 2002; Bando 2002; Kitts et al. 2004; Atkinson et al. 2008; du Dot et al. 2009; Rosen 2009; Calkins et al. 2013). However, most studies have primarily focused on the relative caloric benefits of different SSL prey items as a function of lipid content. Protein is composed of many linked amino acids (AA) and its production and degradation into its AA components provides a view of its metabolic turnover. While protein metabolism is important for growth, muscle, neurological, and hormonal function (Allison 1958; Waterlow 1999), only three studies have examined AA profiles in Alaskan pinniped diets. Anza (2002) and Kitts et al. (2004) found that the essential amino acid (EAA) content of walleye pollock compared favorably with other high quality protein sources and a third study reported significant differences in the free AA composition of Pacific herring (*Clupea pallasi*) and walleye pollock (*Gadus chalcogrammus*) with associated changes in harbor seal (*Phoca vitulina*) serum AA composition (Zhao et al. 2006).

Protein synthesis in animals requires all 20 common AA and are categorized into essential (isoleucine (ILE), leucine (LEU), lysine (LYS), methionine (MET), phenylalanine (PHE), threonine (THR), tryptophan (TRP), histidine (HIS) and valine (VAL), non-essential (arginine (ARG), alanine (ALA), asparagine (ASN) aspartate (ASP), cysteine (CYS), glutamate (GLU),

glutamine (GLN), glycine (GLY), proline (PRO), serine (SER), and tyrosine (TYR)), conditionally essential (ARG, CYS, GLU, PRO, TYR) and branched-chained (LEU, ILE, and VAL) and nonbranched-chained (all other AA) (Robbins 1983; Barboza et al. 2009). When the body cannot synthesize an AA using the pool of available nitrogen and carbon, the AA must be supplied in the diet and is considered an EAA. Although the AA requirements for pinnipeds have not yet been determined, most carnivores require a dietary source of ten EAAs; (ARG), (ILE), (LEU), (LYS), (MET), (PHE), (THR), (TRP), (HIS) and (VAL); (Barboza et al. 2009, Eisert 2011).

Protein quality is generally evaluated by measuring the nitrogen absorbed from the diet and comparing the proportions of EAA in the food with those in the tissues of the consumer (Robbins 1983; Barboza et al. 2009). The ratio of metabolizable to digestible nitrogen intake is defined as the biological value (BV) of the diet. The closer the dietary AA profile reflects the animal tissues, the higher the biological value. For example, high quality dietary protein such as muscle tissue, milk and eggs have the highest BV of ~1 (Robbins 1983; Waterlow 1999; Barboza et al.. 2009). Therefore, knowledge of the AA composition of the diet is necessary to evaluate protein quality for the consumer.

If the pool of amino nitrogen and carbon in the body is not large enough to meet metabolic demands, the amino acid is considered conditionally essential or essential under special conditions. For example, HIS is a conditionally essential AA in young, growing animals. Arginine is also considered conditionally essential for urea synthesis when there are high nitrogen loads (Waterlow 1999; Barboza et al. 2009).

The branched chain amino acids (BCAA) are EAA that include the proteinogenic AA LEU, ILE, and VAL. Proteinogenic AAs are incorporated into proteins during translation and thus help build proteins within the body. They account for 35 % of the EAA in muscle proteins and 40 % of the preformed AA required by mammals (Arroyave 1970; Waterlow 1999; Zhao 2002; Barboza et al. 2009). For this study we categorized nine AA as essential: ARG, THR, VAL, MET, ILE, LEU, LYS, PHE and HIS. Non-essential amino acids (NEAA) are those that can be synthesized at a rate that will meet metabolic demands and for this study these include ALA, ASP, CYS, GLU, GLN, GLY, PRO, SER, and TYR. The AA TRP is not included in this analysis as quantification requires a specialized analytical process that we were unable to accommodate for this project.

Amino acid profiles represent a balance between the AA intake from the diet and rate of use in protein synthesis, catabolism and/or oxidation. Protein deficient diets will lead to different AA kinetics and concentration ratios than seen in animals with sufficient dietary protein. Ratios of different groups of AA have been used to evaluate protein status in animals (Whitehead and Dean 1964; Arroyave 1970; Davis et al. 1995; Sarwar et al. 1998; Schaafsma 2000). In general, the total % EAA in serum decreases (most notability in the branched-chain group; LEU, ILE, and VAL that are specifically required for protein synthesis) and serum NEAA increases in protein poor diets due to the consequent breakdown of body muscle proteins. The recommended indices of animal condition based on AA composition are total serum concentrations of sulfur AA (CYS, MET) and the ratios of BCAA: NBCAA (nonbranched chain AA), and EAA:NEAA (Boren et al. 1996; Sarwar et al. 1998).

Marine mammal milk is a high quality dietary protein source and is distinct from other mammals in that it is low in water and high in protein and fat content (Ashworth et al. 1966; Lauer and

Baker 1977; Davis et al. 1995). In sea lions and fur seals (otariids), the fat content of milk is about 20-30 % and the protein content about 10 % by volume. A study by Davis et al. (1995) compared the AA composition of milk in four pinniped species: northern elephant seal (*Mirounga angustirostris*), Antarctic fur seal (*Arctocephalus gazella*) California sea lion (*Zalophus californianus*) and Australian sea lion (*Neophoca cinerea*). Glutamate, PRO and LEU were the most abundant AA in milk protein of all four species, with EAA making up 40%, BCAA 20 % and sulfur AA 4% of the AA pool. This composition did not change significantly during the lactation period (Davis et al. 1995). The AA composition of SSL milk has not been previously reported.

Juveniles are thought to be the most vulnerable life stage in SSL populations as young animals transition from a diet of mother's milk to catching, digesting and assimilating whole fish (Merrick et al. 1997; Merrick and Loughlin 1997; Fritz and Hinckley 2005; Rosen and Trites 2005; Frid et al. 2009; Calkins et al. 2013). Most SSL pups in Alaska remain on a solely milk diet for their first year of life. The weaning process onto a fish diet is prolonged in this species and by age two, most juveniles have transitioned entirely from milk to a fish diet (Mamaev and Burkanov 2004; Trites et al. 2006). The quality of prey available to juveniles is important for a successful dietary transition.

Measurements of body mass and composition, and in particular lipid stores, are all techniques commonly used to assess the nutritional status and general health of pinnipeds (Rea et al. 1997; Trumble et al. 2003; Mellish et al. 2011; Champagne et al. 2012). These techniques, however, are not sensitive to short term changes in protein status that can impact health. Few studies have focused on the role of protein in pinniped nutrition, even though these carnivores have life strategies that utilize protein in unique ways. For example, in carnivores NEAA not only provide

the precursors for essential biochemical compounds such as hormones and neurotransmitters, but because pinnipeds do not consume preformed glucose, they also likely play an important role in providing carbon for gluconeogenesis (Eisert 2011, Champagne et al. 2012, Eisert et al. 2013).

The present study evaluated dietary protein quality in SSL by determining the seasonal composition of available prey adjacent to a SSL rookery and haul outs and measuring seasonal differences in prey quality. A theory for the decline in the SSL western distinct population segment has focused on the change in prey availably from higher lipid herring to walleye pollock, both which are common in SSL diets (Trumble et al. 2003; Rosen and Trites 2004, 2005). The quality of these two fish species as of a source of protein for growing juvenile SSL was evaluated by comparing the AA composition of herring and walleye pollock from a SSL rookery and haul out with the tissue AA composition in milk and serum from juvenile SSL. The use of specific AA ratios as indices of protein nutrition and animal condition were investigated.

Methods

Collection and analysis of prey samples

To determine seasonal variation in protein content in SSL diets, whole fish were collected from the Gulf of Alaska Chiswell Island hydro-acoustic trawl surveys (Adams et al. 2008) and evaluated for seasonal nutritional quality. Fish were collected within 10 nautical miles of the Chiswell Island SSL rookery and haulout sites in April 2003, August 2003, and November 2003 (Figure 2.1). The sampling periods were timed to parallel key metabolic periods for the SSL (pupping/breeding, post breeding /molting, winter). Prey items were collected at night between evening and morning civil twilights hours using a midwater trawl fitted with a 1.9 cm mesh cod end liner and with vertical and horizontal openings of 12 and 22 m. A random sub sample of fish (n=20; if available for the species) from a larger trawl sample (n=50) were frozen at -20 °C at sea

and transported to the laboratory at the University of Alaska Fairbanks for nutrient analysis. Once at the laboratory, the samples were vacuum sealed and held in -60 ºC freezers prior to analysis. The sub samples were assumed representative of the trawl sample based on length/weight frequencies. The species collected for analysis were walleye pollock, herring, prowfish (*Zaprora silenus)*, sablefish (*Anoplopoma fimbria*), eulachon (*Thaleichthys pacificus*) and capelin (*Mallotus villosus*). Several size categories of walleye pollock were collected and were partitioned into the following age classes (Shima et al. 2002): age 0<15 cm, age 1=15-23 cm, age 2=24-33 cm, and adult >33 cm. Nutritional quality of prey was assessed by the following parameters: energy density, proximate analysis, AA profile and specific AA ratios. Proximate analysis was performed to provide the percent moisture, ash, crude protein and crude fat of the prey using standard methods of the Association of Official Analytical Chemists (AOAC 1990). The carbohydrate component in fish is considered negligible and is therefore not recorded (Sidwell et al. 1974; Bando 2002). Each macronutrient measured was analyzed in duplicate.

Frozen fish samples from the trawl collection were measured to the nearest cm (standard length), weighed (g) and sexed. The fish were cut into smaller pieces while frozen and homogenized in a food blender. Duplicate 10 g samples of the homogenate were collected and frozen at -80 ºC and freeze-dried to constant mass over 48-72 hours (*i.e.* less than 0.001 g weight change) under vacuum (VirTis Freeze Dryer Model 5463, VirTis, Gardiner, New York). The water content of each sample was calculated using the difference in mass following lyophilization which freeze dries the tissue and expressed as a percent of total mass (gm). The dry homogenate was ground to a fine powder using a food mill.

The crude fat or lipid content of the dried, homogenized sample was determined using a modified ether extract Soxhlet procedure (Soxtec system, model HT6, Foss North America (Tercator), Silver Spring, Maryland). Analysis of crude protein content was determined using the Duma method (AOAC 1990) and conducted at the Soils Laboratory at the University of Alaska Fairbanks. Nitrogen values were obtained from samples using an elemental analyzer (Model # CNS 2000, LECO, St. Joseph, Michigan) and expressed as % crude protein assuming that 100 g crude protein contained 16 g nitrogen (Robbins 1983).

$$\textit{Protein content (\%)} = \textit{nitrogen content (\%)} \times 6.25 \qquad \text{(Equation 1)}$$

Ash (mineral) content was quantified as the weight of material after 0.5 g of dried homogenate was combusted in a muffle furnace at 550 ^{o}C for 24 hours and represented as a percentage of the original dry mass (AOAC, 1990). The energy density of the prey was determined using an adiabatic bomb calorimeter (Parr Instrument Co. Moline, IL) loaded with approximately 0.5 g of dry homogenate. The change in heat was converted to dry matter chemical energy (calories) expressed as kcal g $^{-1}$ and converted to kJ (1 kcal = 4.184 kJ). All analyses were conducted on the dry tissue samples and were subsequently converted to a wet mass value using the following equation (Watt and Merrill 1975).

$$\textit{Wet mass} = \textit{dry mass} \div \textit{(1-water content of prey item)} \qquad \text{(Equation 2)}$$

Collection of SSL samples

Serum samples (n= 8) were collected from juvenile SSL ranging in age from 11.5 months (young of the year; YOY) (n=4) to 22-35 months (yearling) (n=4) at Glacier Island, Prince William Sound (PWS) in July 2001 (samples TJ17, TJ18, TJ19, TJ20) and May 2002 (samples TJ28, TJ29, TJ30, TJ31) as described in (Mellish et al. 2006) Samples were collected shortly after capture and thus it is assumed that they are representative of animals on a natural diet. Blood samples were collected from the caudal gluteal vein, centrifuged and the serum separated and then frozen at -80 °C until analysis. Age of individuals was estimated by body mass, degree of canine tooth eruption (King et al. 2007), and mean birth date of the population (Pitcher et al. 2001; Holmes and York 2003).

Ingested milk samples (n=9) were collected from the stomachs of 5 to 10 month old SSL in PWS using a 14 French foal feeding tube inserted into the esophagus while juveniles were under anesthesia (Stegall et al. 2008). When milk was present, a subsample was extracted using a large feeding syringe, and then frozen in cryovials on dry ice. Samples were stored at -75 °C until analysis. These samples were not curdled and thus considered "fresh" (consumed < 3 hours prior) and representative of the milk diet.

Amino acid analysis

Amino acid profiles of potential SSL prey were produced from the dry homogenate samples of walleye pollock (age 0, 1, 2 and adult), adult Pacific herring, adult prowfish, juvenile sablefish, adult eulachon and adult capelin using a Hitachi L8900 Amino Acid Analyzer with post-column, ninhydrin derivatization (AAA Service Laboratory 14865 SE Regner Terrace Drive Damascus,

OR*)*. Results were provided as % mole composition and mg AA per gram of product for the following AA; (ALA, ARG, ASP, CYS, GLU, GLY, HIS, ILE, LEU, LYS, MET, PHE, PRO, SER, THR, TYR, VAL and hydroxyproline (HYP)). Only adult Pacific herring and walleye pollock had samples available for each season and were analyzed to test for statistical seasonal differences.

Protein quality in the diet of juvenile SSL was evaluated using the measured AA profiles of two important prey species, walleye pollock and Pacific herring (n=10) collected in the month of April and using published protein digestibility values (Rosen and Trites 2000a). The April survey samples were selected to coincide with the collection period of SSL samples (serum and milk). Whole adult pollock and herring homogenates, juvenile SSL serum and SSL milk were analyzed for AA composition with the results provided as mean % mole composition and mg AA per gram of product. Amino acid profiles from dry homogenized prey samples and from frozen serum and milk samples were compared on % mole (± SD) basis to accommodate the different type of samples. Prey AA profiles were then correlated with AA profiles of serum and ingested milk from juvenile SSL. Amino acid concentration indices for protein condition MET:CYS; EAA:NEAA; and BCAA:NBCAA were used to assess the condition of the wild caught juvenile SSL that provided serum samples for this study.

Statistical analysis

Significant differences in prey proximate composition and energy density between seasons were statistically tested using analysis of variance (ANOVA) followed by Tukey's post hoc test on treatment means (CoreTeam 2013). Kruskal-Wallis and Mann-Whitney tests were used on results requiring nonparametric analysis (Zar 1984).

Differences in AA concentrations among the four tissue groups and seasonal walleye pollock and herring samples were compared using a Kruskal–Wallis test because the data were not normally distributed even after log transformation (Sokal and Rohlf 1995); post hoc comparisons were conducted with the methods described in (Siegel and Castellan 1988) using the function kruskalmc in the R package (CoreTeam 2013). A significance level of $p \leq 0.05$ was used for all statistical tests. Means are reported ± SD.

Results

Seasonal Variability in Prey Constituents

Walleye pollock and Pacific herring were the major components of the available prey base in the seasonal Chiswell Island mid water trawl surveys making up 62 % and 36 % (April), 41 % and 56 % (August) and 73 % and 8 % (November), respectively, of the total catch provided for analysis. Other fish species present in the total catch were eulachon, sable fish, and prowfish. These results are in agreement with biomass estimates reported in Adams et al. (2008) showing adult Pacific herring and walleye pollock as the major components of the SSL prey base and an increase in species diversity in the fall.

The energy density based on wet mass of the prey samples collected ranged from 2.84 ±0.03 kcal g^{-1} in eulachon (November) to only 0.61 kcal g^{-1} in the prowfish sample. Energy density was greatest in the fall and lowest in the spring (Kruskal-Wallis; $p < 0.05$) (Figure 2.2). All fish species in this survey exhibited seasonal changes in energy content, with herring having the largest seasonal changes (*i.e.*, 1.38 kcal g^{-1}) greater in fall than in spring; ($p<0.05$) and pollock (of all age classes) the least seasonal variability (less than 0.46 kcal g^{-1} difference between seasons and only a significant difference between April and November sampling periods ($p<0.05$; Figure 2.2). The seasonal energy densities of adult pollock from the Gulf of Alaska in

this study were compared with values reported in the Bering Sea (Kitts et al. 2004). There were no differences in the energy density (kJ g^{-1}dry mass) in the fall pollock but small differences were observed between the spring and summer energy density values. Chiswell Island adult pollock had slightly lower values (19.60 ±0.14 kcal g^{-1} *vs* 20.78±0.40 kcal g^{-1}, respectively; p=0.06) compared to the same season in Bering Sea pollock.

The proximate analysis of prey species (Figure 2.3) reflected seasonal changes in water and lipid content but little variability in percent protein and ash (mineral) content. For example, the lipid content of Pacific herring and adult pollock increased from 6 % and 3 % wet mass, respectively in April to 23 % and 6 % respectively in November ($p<0.001$; $p<0.05$). The mean wet mass protein contents of pollock (13.48 % ± 0.46) and herring (12.70 % ±0.58) were constant among the different seasonal life history stages $p>0.05$ (e.g. spawning and over-wintering).

Seasonal differences in the individual AA composition in herring and pollock were detected between April and November (Kruskall–Wallis, df =3, $p<0.001$; Tables 2.1A, B). In contrast, no significant differences were found in the % EAA, NEAA, BCAA, or NBCAA values among the collection periods.

Protein Quality

Comparison of amino acid profiles

Protein content of serum samples ranged from 5.48 %-6.47 % (w/v) and there were no significant differences in the serum free AA composition or EAA:NEAA and BCAA:NBCAA ratios in juvenile SSL samples collected in 2001(n=4) and 2002 (n=4) (Figure 2.4) (Kruskal-Wallis, $P<0.05$). However, the % mean mole of CYS and MET:CYS ratio were statistically different (student t test, p=0.04; p=0.036) different between the 2 groups with YOY juveniles (TJ

17-20) collected in 2001 having a higher average MET:CYS ratio (0.44 ± 0.05) and lower % mole CYS (2.849± 0.324) than the 2002 yearling juveniles (TJ 28-31) (0.37±0.02) which showed a reciprocal increased level of % CYS (3.289± 0.117). There were no consistent differences seen with age and the statistical difference between the MET:CYS ratios was associated with an outlier (TJ 19) that had a MET:CYS of 0.50. Therefore, the two age groups were combined for the subsequent AA ratio analysis.

The quality of available protein in prey as measured by the % mole AA composition of adult herring and adult pollock was compared with the AA composition of juvenile SSL serum and milk in the stomach of pups (Table 2.2). The EEA, NEAA, BCAA, and NBCAA composition and associated ratios of these AA groups for each tissue type (Figures 2.5 and 2.6) showed a consistent pattern of AA composition between the four tissues. However, results from nonparametric Kruskal–Wallis tests were significant ($df = 3$, $p<0.001$) for the median of all 19 AA analyzed. Post hoc comparisons revealed statistically significant differences in % mole concentration of individual AA among the 4 tissue groups (Table 2.3). Within the 4 tissue groups, SSL milk was highest in the AA GLU, PRO, ILE, LEU and HIS ($df=3$, $p<0.001$) and juvenile SSL serum contained the highest concentration of the AA CYS, THR, SER, VAL, TYR and PHE ($df=3$, $p<0.001$). The AA content in whole herring was highest in the AA ASP, MET and LYS ($df=3$, $p<0.001$). Pollock was highest in GLY, ALA, and ARG, and was the only tissue that contained significant quantities of HYP ($df=3$, $p<0.001$).

These results were consistent with observed ratios of EAA:NEAA and BCAA:NBCAA (Figure 2.5). Pollock had a significantly lower EAA:NEAA ratio of 0.63±0.01 compared to herring 0.80±0.01, juvenile SSL serum 0.81±0.02, and SSL milk 0.88±0.00 ($p<0.05$). The BCAA:NBCAA ratio was similar with pollock values significantly lower at 0.19±0.01, herring

0.23±0.00, juvenile SSL serum 0.25±0.00 and SSL milk 0.30±0.01($p<0.05$). Levels of CYS in juvenile SSL serum were higher than in milk and fish tissues, but the MET:CYS ratio was lower than in the other tissues (Table 2.2).

The AA composition of SSL milk was different from the AA milk profiles of four pinniped species (Northern elephant seal, Antarctic fur seal, California sea lion and Australian sea lion) previously reported (Davis et al. 1995). SSL milk had high ratios of EAA:NEAA (0.09) and BCAA:NBCAA (0.30) (Table 2.4).

Discussion

Results on seasonal changes in available prey composition at Chiswell Island, AK sites are consistent with the findings from other studies on SSL prey from the Bering Sea and SSL sites in AK (Anza 2002; Bando 2002; Kitts et al. 2004). The percent protein in SSL prey did not vary seasonally and remained consistent between the prey species and, in the case of walleye pollock between age classes. Thus, unlike lipid, the quantity of available protein in the prey is consistent for SSL at Chiswell Island haulouts year round.

Seasonality was observed in some individual AA in adult pollock and adult herring, particularly between April and November collection periods, but EAA:NEAA and BCAA:NBCAA ratios did not differ, suggesting that protein quality also does not show seasonality in SSL diets. The sample sizes of other prey species collected from the Chiswell Island site were not large enough to statistically test for differences in AA profiles. However, these limited results imply that, as seen between adult pollock and herring, there may be AA composition differences between species and these distinctions should be further investigated as the abundance of different prey species varies seasonally.

The AA composition of milk provides all required AAs for growth in neonates and thus represents the highest quality diet (Davis et al. 1995; Barboza et al. 2009; Eisert et al. 2013). The AA composition of herring and pollock closely reflected SSL milk AA composition suggesting that both species are a good source of high quality protein. The mole percent AA composition in whole herring was highest in the EAA MET and LYS and the NEAA ASP. The EAA MET is one of the proteinogenic sulfur AA and fish are generally considered a good source for this AA (Sidwell et al. 1974). Aspartate is the NEAA precursor for several EAA such as MET, THR, ILE, LYS. It is also a metabolite in the urea cycle, plays a role in gluconeogenesis, and is metabolized to produce acetyl CoA in mammals. Pollock was significantly lower in LYS (an important EAA and principal component of collagen) than herring but higher in concentration than found in SSL milk. Pollock was highest in the NEAA GLY, ALA, ARG, and HYP. Hydroxyproline is a nonproteinogenic AA and plays a major role with PRO in collagen synthesis (Arroyave 1970). Although ALA is a NEAA, it is the primary AA in gluconeogenesis (Zhao 2002; Houser and Crocker 2003; Champagne et al. 2012) and GLY is an important precursor for proteins.

Classically ARG is considered a NEAA but we have categorized it as essential due to its role in urea synthesis which removes ammonia from the body. Ammonia is a by-product of protein metabolism and is toxic in high concentrations. Pinnipeds are carnivores and consume high quantities of nitrogen in their diet, and it is essential that they are able to adequately remove ammonia. Thus, although there are differences in the amount of some individual amino acids, which may be partially explained by the active metabolic pathways and condition of the fish when captured, generally both herring and pollock contain amino acid profiles consistent with high quality protein.

The quality of protein in a diet is also influenced by bioavailability. Dry-matter digestibility (DMD; 90.1 % ± 1.8 for herring and 86.5 % ± 3.4 for pollock) and digestive efficiency (DE; herring 95.4 % ± 0.7 and pollock 93.9 % ± 1.4) is high for both pollock and herring (Rosen and Trites 2000a). Herring contained a significantly higher % composition of EAA and BCAA than pollock suggesting that herring would statistically be considered a higher quality prey for protein. However, in comparison with SSL tissue AA composition, combined with the high DMD and DE for herring and pollock, both species can be considered a source of high quality protein.

Juvenile SSL transition from milk to fish diets within the first two years of life and thus provide a good model for studying protein changes in diet (Mamaev and Burkanov 2004). Results from this study confirm the designation of maternal milk as the highest quality dietary protein and provides the first report of amino acid profiles for SSL milk. SSL milk had the highest concentration of EAA and BCAA of the four tissues studied. Milk was especially high in the EAA HIS, LEU, ILE which are important precursors for protein synthesis. Milk was also high in GLU and PRO compared to serum and prey tissues. PRO is the only cyclic AA and plays an important role in folding proteins and thus the structure and function of proteins (Boren et al. 1996). Glutamine, the most abundant AA in all four tissues, is important for many biochemical processes, including protein synthesis, the production of ammonium for acid-base regulation in the kidney, nitrogen donation for anabolic processes and carbon donations for the citric acid cycle. Most importantly, it is responsible for the nontoxic transport of ammonia in the blood (Arroyave 1970; Boren et al. 1996; Houser and Crocker 2003; Barboza et al. 2009) which is particularly important in carnivores.

Steller sea lion milk is not only a high quality protein source but, as with other pinniped milk, a highly concentrated energy source with a high fat and protein content and low water and carbohydrate content (Ashworth et al. 1966; Oftedal et al. 1987; Oftedal 1999; Houser and Crocker 2003; Eisert et al. 2013). Pinniped milk composition generally reflects the maternal investment of the species. In short maternal investment periods associated with phocid species (up to 5 weeks), milk fat content can be as high as 60 % in contrast with 20-35 % fat content in milk of otariids that spend one to two years alternating foraging trips with onshore nursing of young. The protein content is high (approximately 10 %, with the highest levels of free AA) in both these pinniped groups compared to other mammalian milk (Ashworth et al. 1966; Davis et al. 1995).

The AA composition of pinniped milk is also quite distinct from other mammals. In an analysis of milk AA composition in mammalian species, California sea lion and Antarctic fur seal milk was clustered in one group and northern elephant seal and Australian sea lion in another group based on taurine and HIS levels (Sarwar et al. 1998). Differences were found comparing the AA ratios of SSL milk with the four other pinniped species in the above study. In a comparison of EAA:NEAA, BCAA: NBCAA and MET:CYS ratios in the northern elephant seal, Antarctic fur seal, California sea lion and Australian sea lion, SSL milk had higher ratios of EAA:NEAA (0.09) and BCAA:NBCAA (0.30) compared to the other pinniped species. This difference in the AA composition of milk between the pinniped species may be partially explained by differences in the proportion of whey and casein, as the AA profiles in these two types of protein differ (Lauer and Baker 1977; Heine et al. 1991). The proportion of whey and casein in SSL milk was not determined in this study due to the milk sample collection methodology. Future studies,

using samples collected directly from lactating female SSL, should include the percent content and AA profiles for each of these two proteins in SSL milk.

The AA profile of serum in juvenile SSL is dynamic and reflects the absorbed amino acids from the diet and the metabolic requirements of the individual animal at the time of sample collection. Serum levels of CYS in juvenile SSL serum were higher than in milk and fish tissues but the MET:CYS ratio was lowest due to a combination of high CYS and lower circulating MET in the serum. In northern elephant seals, plasma CYS levels increased during the postweaning fast (Houser and Crocker 2003). The yearling juvenile SSL in this study had a slightly higher % mean mole CYS level than the YOY juveniles which would be consistent with the weaning process. However, the EAA:NEAA ratio and BCAA levels of the individual juvenile SSL serum samples did not indicate long-term fasting. Circulating levels of LYS, which produces acetyl CoA, essential for fat and carbohydrate metabolism and energy production were higher in juvenile SSL serum than in milk. Juvenile SSL are actively growing, suggesting a high requirement for BCAA. The BCAA, and especially LEU, which are important for muscle growth and protein synthesis, were also found in high concentrations in the serum of these juvenile SSL. In human adults the LEU pool is small but highly utilized; suggesting that this AA could limit protein synthesis (Gibson et al. 1996). All of the AA indices tested indicated that the juvenile SSL sampled for this study were not experiencing protein deficiency in their diet or nutritional stress at the time of collection. These findings are consistent with condition indices from morphometric and blood chemistries collected at the time of capture (Mellish et al. 2006). Although the sample size of SSL for these analyses was small (n=9), the results support the potential of this methodology for assessing the nutritional condition of SSL. Controlled laboratory experiments are recommended to further investigate the efficacy of this technique.

It is also noteworthy that even though the samples came from juvenile SSL of different ages and sexes, the AA compositions suggested that when the blood samples were taken the animals were feeding on a diet of similar protein quality. Studies on the influence of diet on AA composition in rats (Johnson and Anderson 1982; Peters and Harper 1985) have found that BCAA concentrations reflect those found in the diet regardless of the quantity or source of protein. The mean mole % BCAA concentrations in the juvenile SSL serum were almost identical among individuals (19.97±0.21) and more closely reflected the BCAA composition of the prey (herring) than milk tissue. The serum sample from TJ20 had a slightly higher BCAA value (20.47) than the other individuals. If you remove this one animal's value, the mean BCAA becomes (19.89 ±0.04). In 5 of the 6 AA groups and indices (BCAA, NBCAA, sulfur AA, EAA:NEAA, BCAA:NBCAA) juvenile SSL serum was closer in composition to a fish than milk diet. However, results from whisker isotope analysis conducted on the SSL (Stricker et al. 2015) suggest that the YOY (2001) in our study were likely not weaned at sample collection. Female SSL actively forage during the weaning process and thus their amino acid pool is influenced more strongly by their diet and less by the recycling of amino acids. In pinnipeds that fast during the weaning process, the amino acid pool would be influenced by the breakdown of muscle and recycling of amino acids. This may explain why the AA profile of juvenile SSL serum seemed to suggest influence from a fish diet. Also, rats are omnivores, and thus caution is required in extrapolating these results to pinnipeds. A controlled diet study is required to confirm if the BCAA dietary signature is valid for pinnipeds.

The results from the present study found that fish provide high quality protein in the diet of SSL and that protein quantity and quality have low seasonality in the prey species we evaluated. The question of protein quality in the prey of SSL is therefore more a question of how pinnipeds

utilize available protein to fulfill metabolic requirements. Our findings suggest that values for specific serum AA such as sulfur AA and ratios such as EAA:NEAA and BCAA:NBCAA can provide some insight into nitrogen balance and metabolic health in pinnipeds. However, nitrogen balance is a combination of the metabolic demands for growth, reproduction, and other metabolic challenges. Amino acid profiles only represent a "snapshot" of conditions when the samples were collected and they do not explain the kinetics of those AA. Limitation of AA profiles to assess long term nutritional status is confirmed in the laboratory finding that harbor seals made metabolic adjustments to accommodate different quality in prey (Zhao et al. 2006). However, we maintain that the use of AA ratios in combination with other blood metabolite concentrations and techniques such as stable isotope analysis of tissues can provide a useful index for SSL condition as well as evaluating the protein quality of their prey.

Acknowledgments

We would like to thank Dr. Charles Adams for providing the prey species for this study, Vicki Stegall and the Alaska Department of Fish Game Steller sea lion research team for collecting the milk samples, and Dr. Jo-Ann Mellish and Alaska SeaLife Center for collecting serum samples from juvenile Steller sea lions. Support for this project was provided by the Alaska SeaLife Center Steller Sea Lion Research Program with funds from National Marine Fisheries Service. Marine mammal tissue collection protocols were approved by the Alaska Department of Fish and Game Animal Care and Use Committee (protocol NO. 03-00002) and Alaska SeaLife Center Animal Care and Use Committee under the following NMFS permits No. 358-1564 and 14335.

Literature Cited

Adams, C.F., R.J. Foy, D.S. Johnson and K.O. Coyle. 2008. Seasonal changes in pelagic fish biomass around the Chiswell Island Steller sea lion rookery in 2003. Fisheries Research 93: 179-185.

Allison, J.B. 1958. Calories and protein nutrition. Annals of the New York Academy of Sciences 69: 1009-1024. New York.

Alverson, D.L. 1992. A review of commercial fisheries and the Steller sea lion (*Eumetopias jubatus*)-the conflict arena. Reviews in Aquatic Sciences 6: 203-256.

Anza, C.D. 2002. Seasonal variation in nutrient composition of Alaska walleye pollock (*Theragra chalcogramma*) and its effect on the nutritional status of Steller sea lions (*Eumetopias jubatus*). MSc Thesis. Department of Food Science. University of British Columbia, Vancouver. 112pp.

Asssociation of Official Analytical Chemists (AOAC). 1990. Official Methods of Analysis. AOAC, 15th edition: 2, Arlington, Virgina.

Arroyave, G. 1970. Comparative sensitivity of specific amino acid ratios versus "essential to nonessential" amino acid ratio. The American Journal of Clinical Nutrition 23: 703-706.

Ashworth, U., G. Ramaiah and M. Keyes. 1966. Species difference in the composition of milk with special reference to the Northern fur seal. Journal Dairy Science 49: 1206-1211.

Atkinson, S., D. Calkins, V. Burkanov, M. Castellini, D. Hennen and S. Inglis. 2008. Impact of changing diet regimes on Steller sea lion body condition. Marine Mammal Science 24: 276-289.

Bando, M. 2002. Comparing the nutritional quality of Steller sea lion (*Eumetopias jubatus*) diets. MS Thesis. School of Fisheries and Ocean Science. Univeristy of Alaska Fairbanks, Fairbanks. 108pp.

Barboza, P., K. Parker and I. Hume. 2009. Integrative Wildlife Nutrition. Springer-Verlag, Berlin.

Boren, J., R. Lochmiller and D. Leslie, Jr. 1996. Relation of serum and muscle free amino acids to dietary protein in the Northern Bobwhite. Proceedings of the Oklahoma Academy of Science 76: 55-65.

Calkins, D.G., S. Atkinson, J.E. Mellish, J.N. Waite and J.R. Carpenter. 2013. The pollock paradox: Juvenile Steller sea lions experience rapid growth on pollock diets in fall and spring. Journal of Experimental Marine Biology and Ecology 441: 55-61.

Castellini, M. 2001. Captive studies with Steller sea lions at the Alaska SeaLife Center. in D. DeMaster and S. Atkinson, eds. *Steller sea lion decline: Is It Food II.*, University of Alaska Sea Grant, Fairbanks, Alaska. p. 80.

Champagne, C.D., D.E. Crocker, M.A. Fowler and D.S. Houser. 2012. Fasting physiology of the pinnipeds: the challenges of fasting while maintaining high energy expenditure and nutrient delivery for lactation. in M. McCue, ed. *Comparative Physiology of Fasting, Starvation, and Food Limitation.* Springer, Berlin, Heidelberg. 309-336.

CoreTeam. 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, ed, Vienna, Austria. http://www.R-project.org/.

Davis, T.A., H.V. Nguyen, D.P. Costa and P.J. Reeds. 1995. Amino acid composition of pinniped milk. Comparative Biochemistry and Physiology 110B: 633-639.

du Dot, T.J., D.A.S. Rosen and A.W. Trites. 2009. Energy reallocation during and after periods of nutritional stress in Steller sea lions: low quality diet reduces capacity for physiological adjustments. Physiological and Biochemical Zoology 82: 516-530.

Eisert, R. 2011. Hypercarnivory and the brain: protein requirements of cats reconsidered. Journal of Comparative Physiology B 181:1-17.

Eisert, R., O.T. Oftedal and G.K. Barrell. 2013. Milk composition in the Weddell seal *Leptonychotes weddellii*: evidence for a functional role of milk carbohydrates in pinnipeds. Physiological and Biochemical Zoology 86: 159-175.

Estes, J.A., Doak, D.G., Springer, A.M. and T.M. Williams. 2009. Causes and consequences of marine mammal populations declines in southwest Alaska: a food-web perspective. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 364 (1524): 1647-58.

Frid, A., J. Burns, G.G. Baker and R.E. Thorne. 2009. Predicting synergistic effects of resources and predators on foraging decisions by juvenile Steller sea lions. Oecologia 158: 775-786.

Fritz, L.W., and S. Hinckley. 2005. A critical review of the regime shift-"Junk Food" nutritional stress hypothesis for the decline of the Western stock of Steller sea lion. Marine Mammal Science 21: 476-518.

Gibson, N.R., A. Fereday, M. Cox, D. Halliday, P.J. Pacy and D.J. Millward. 1996. Influences of dietary energy and protein on leucine kinetics during feeding in healthy adults. American Journal of Physiology-Endocrinology and Metabolism 270: E282-E291.

Heine, W.E., P.D. Klein and P.J. Reeds. 1991. The importance of lactalbumin in infant nutrition. The Journal of Nutrition 121: 277-283.

Holmes, E.E., and A.E. York. 2003. Using age structure to detect impacts on threatened populations: a case study with Steller sea lions. Conservation Biology 17: 1794-1806.

Houser, D.S., and D.E. Crocker. 2003. Age, sex, and reproductive state influence free amino acid concentrations in the fasting elephant seal. Physiological and Biochemical Zoology 77: 838-846.

Johnson, D.J., and G.H. Anderson. 1982. Prediction of plasma amino acid concentration from diet amino acid content. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 243: R99-R103.

King, J.C., T.S. Gelatt, K.W. Pitcher and G.W. Pendleton. 2007. A field based method for estimating age in free ranging Steller sea lions (*Eumetopias jubatus*) less than twenty four months of age. Marine Mammal Science 23: 262-271.

Kitts, D., M. Huynh, C. Hu and A. Trites. 2004. Seasonal variation in nutrient composition of Alaskan walleye pollock. Canadian Journal of Zoology 82: 1408-1415.

Lauer, B.H., and B.E. Baker. 1977. Amino acid composition of casein isolated from the milks of different species. Canadian Journal of Zoology 55: 231-236.

Mamaev, E.G., and V.N. Burkanov. 2004. How long do Steller sea lions drink milk? From Sea Lions of the World. *Conservation and Research in the 21st century*. 22nd Wakefield Fisheries Symposium, p.7, Anchorage, Alaska, USA.

Mellish, J.-A.E., A.G. Hindle and M. Horning. 2011. Health and condition in the adult Weddell seal of McMurdo Sound, Antarctica. Zoology 114: 177-183.

Mellish, J.E., D.G. Calkins, D.R. Christen, M. Horning, L.D. Rea and S.K. Atkinson. 2006. Temporary captivity as a research tool: comprehensive study of wild pinnipeds under controlled conditions. Aquatic Mammals 32: 58-65.

Merrick, R.L., M.K. Chumbley and G.V. Byrd. 1997. Diet diversity of Steller sea lions (*Eumetopias jubatus*) and their population decline in Alaska: a potential relationship. Canadian Journal of Fisheries and Aquatic Sciences 54: 1342-1348.

Merrick, R.L., and T.R. Loughlin. 1997. Foraging behavior of adult female and young-of-the-year Steller sea lions in Alaskan waters. Canadian Journal of Zoology 75: 776-786.

Oftedal, O. 1999. Use of maternal reserves as a lactation strategy in large mammals.. Proceedings of the Nutrition Society, Animal Nutrition and Metabolism Group Symposium on "regulation of maternal reserves and effects on lactation and the nutrition of young animals", University of Glasgow. 99-106.

Oftedal, O., D. Boness and R. Tedmann. 1987. The Behavior, Physiology, and Anatomy of Lacation in the Pinnipedia. Plenum Press, New York, New York.

Peters, J.C., and A.E. Harper. 1985. Adaptation of rats to diets containing different levels of protein: effects on food intake, plasma and brain amino acid concentrations and brain neurotransmitter metabolism. The Journal of Nutrition 115: 382-398.

Pitcher, K.W., V.N. Burkanov, D.G. Calkins, B.J. Le Boeuf, E.G. Mamaev, R.L. Merrick and G.W. Pendleton. 2001. Spatial and temporal variation in the timing of births of Steller sea lions. Journal of Mammalogy 82: 1047-1053.

Rea, L., R. Groscolas, E. Mioskowski and M. Castellini. 1997. Changes in plasma fatty acids indicate change in nutritional status in developing Weddell seal pups. Polar Biology 18: 351-357.

Robbins, C.T. 1983. Wildlife Feeding and Nutrition. Academic Press, New York, NY.

Rosen, D.A., and A.W. Trites. 2000a. Digestive efficiency and dry-matter digestibility in Steller sea lions fed herring, pollock, squid, and salmon. Canadian Journal of Zoology 78: 234-239.

Rosen, D.A.S., and A.W. Trites. 2000b. Pollock and the decline of Steller sea lions: testing the junk-food hypothesis. Canadian Journal of Zoology 78: 1243-1250.

Rosen, D.A., and A.W. Trites. 2004. Satiation and compensation for short-term changes in food quality and availability in young Steller sea lions (*Eumetopias jubatus*). Canadian Journal of Zoology 82: 1061-1069.

Rosen, D.A., and A.W. Trites. 2005. Examining the potential for nutritional stress in young Steller sea lions: physiological effets of prey composition. Journal of Comparative Physiology B 175: 265-273.

Rosen, D.A.S. 2009. Steller sea lions (*Eumetopias jubatus)* and nutritional stess: evidence from captive studies. Mammal Review 39: 284-306.

Sarwar, G., H.G. Botting, T.A. Davis, P. Darling and P.B. Pencharz. 1998. Free amino acids in milks of human subjects, other primates and non-primates. British Journal of Nutrition 79: 129-131.

Schaafsma, G. 2000. The protein digestibility-corrected amino acid score. The Journal of Nutrition 130: 1865S-1867S.

Shima, M., A.B. Hollowed and G.R. Van Blaricom. 2002. Changes over time in the spatial distribution of walleye pollock (*Theragra chalcogramma*) in the Gulf of Alaska, 1984-1996. Fishery Bulletin 100: 307-323.

Sidwell, V., P. Foncannon, N. Morre and J. Bonnet. 1974. Composition of the edible portion of raw (fresh or frozen) crustaceans, finfish and mollusks. I. Protein, fat, moisture, ash, carbohydrate, energy value, and cholesterol. Marine Fisheries Review 36: 21-35.

Siegel, S., and J.N. Castellan. 1988. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill.New York.

Sokal, R., and F. Rohlf. 1995. Biometry: The Principles and Practice of Statistics in Biological Research. W.H. Freemann, New York.

Stegall, V.K., S.D. Farley, L.D. Rea, K.W. Pitcher, R.O. Rye, C.L. Kester, C.A. Stricker and C.R. Bern. 2008. Discrimination of carbon and nitrogen isotopes from milk to serum and vibrissae in Alaska Steller sea lions (*Eumetopias jubatus*). Canadian Journal of Zoology 86: 17-23.

Trites, A.W., B.P. Porter, V.B. Deecke, A.P. Coombs, M.L. Marcotte and D.A.S. Rosen. 2006. Insights into the timing of weaning and the attendance patterns of lactating Steller sea lions (*Eumetopias jubatus*) in Alaska during winter, spring, and summer. Aquatic Mammals 32: 85.

Trumble, S.J., P.S. Barboza and M.A. Castellini. 2003. Digestive constraints on an aquatic carnivore: effects of feeding frequency and prey composition on harbor seals. Journal of Comparative Physiology B 173: 501-509.

Waterlow, J.C. 1999. The mysteries of nitrogen balance. Nutrition Research Reviews 12: 25-54.

Watt, B.K., and A.L. Merrill. 1975. Handbook of the Nutritional Contents of Foods. U.S. Department of Agriculture. Dover Publications, New York, NY.

Whitehead, R.G., and R.F.A. Dean. 1964. Serum amino acids in kwashiorkor II. an abbreviated method of estimation and its application. The American Journal of Clinical Nutrition 14: 320-330.

Zar, J. 1984. Biostatistical analysis. Prentice-Hall, Englewood Cliffs, New Jersey.

Zhao, L. 2002. Tracing amino acid metabolism of harbor seal *(Phoca vitulina)* using stable isotope techniques. PhD Thesis. University of Alaska Fairbanks, Fairbanks, AK. 211pp.

Zhao, L., M.A. Castellini and D.M. Schell. 2006. Metabolic adjustments to varying protein intake in harbor seals (*Phoca vitulina*): evidence from serum free amino acids. Physiological and Biochemical Zoology 79: 965-976.

Tables

Table 2.1A. Seasonal amino acid values (mean ± SD) for whole adult walleye pollock captured from Chiswell Island, Alaska. Values are presented as the mole percentage of total amino acids. An asterisk (*) denotes significant differences between seasonal values (a, b, c) from Kruskall-Wallis ranked sum test post-hoc comparisons.

Season	April a	August b	November c	
				H Value
Pollock (n)	10	9	10	
Essential amino acids (EAA)	**37.95±1.45**	**38.49±1.02**	**38.79±1.04**	**5.20**
Valine	5.11 ± 0.08	5.05 ±0.40	5.26 ±0.27	9.80
Methionine *(a,c) ; (b,c)	2.56 ± 0.06	2.61 ±0.11	2.31 ± 0.09	22.30
Isoleucine	3.64 ± 0.28	3.76 ± 0.33	3.62 ± 0.31	7.60
Leucine	6.82 ± 0.27	6.56 ± 0.29	6.74 ± 0.40	5.90
Threonine *(a,b) ; (a,c)	4.39 ± 0.17	4.81 ± 0.20	4.82 ± 0.29	19.82
Phenylalanine	2.73 ± 0.11	2.71 ± 0.17	2.95 ± 0.18	6.30
Histadine * (a,b) ; (a,c) ; (b,c)	1.55 ± 0.23	1.82 ± 0.14	1.74 ± 0.21	24.95
Lysine	6.31 ± 0.25	6.51 ± 0.37	6.57 ± 0.46	9.40
Arginine	4.82 ± 0.26	4.61 ± 0.21	4.79 ± 0.43	7.30
Nonessential amino acids (NEAA)	**60.43±1.45**	**60.35±1.02**	**60.42±1.04**	**5.10**
Cysteine * (a,b) ; (a,c) ; (b,c)	1.76 ± 0.02	1.84 ± 0.07	1.52 ± 0.28	25.00
Aspartate *(a,c) ; (b,c)	9.02 ± 0.12	8.99 ± 0.14	9.79 ± 0.41	23.99
Tyrosine	2.08 ± 0.59	2.24 ± 0.53	2.37 ±0.37	5.60
Serine	5.83 ± 0.60	5.82 ± 0.35	5.95 ± 0.37	6.00
Glutamine *(a,c) ; (b,c)	11.95±0.38	11.65±0.31	13.12±0.42	23.07
Proline	5.52 ± 0.18	5.22 ± 0.30	5.14 ± 0.22	8.40
Glycine *(a,b) ; (a,c) ; (b,c)	15.15±0.07	15.41±0.31	13.85 ± 0.6	24.90
Alanine *(a,c) ; (b,c)	9.14 ± 0.15	9.29 ± 0.23	8.68 ± 0.54	22.80
Amino Acid Indices				
EAA:NEAA	0.63±0.01	0.64±0.01	0.64±0.01	
Branched-chain amino acids (BCAA)	15.59±0.38	15.37±0.55	15.61±0.68	5.60
Nonbranched-chain amino acids (NBCAA)	82.78±0.27	83.48±0.56	83.60±0.60	7.40
BCAA:NBCAA	0.19±0.01	0.18±0.01	0.19±0.01	
Sulfur amino acids * (a,b) ; (a,c) ; (b,c)	4.31±0.06	4.45±0.04	3.84±0.02	14.27
Methionine: Cysteine	1.45±0.05	1.42±0.03	1.52±0.03	

Table 2.1B. Seasonal amino acid values (mean ± SD) for whole adult Pacific herring captured from Chiswell Island, Alaska. Values presented as the mole percentage of total amino acids. An asterisk (*) denotes significant differences between seasonal values (a, b, c) from Kruskall-Wallis ranked sum test post-hoc comparisons.

Season	April a	August b	November c	
				H value
Herring (n)	8	8	9	
Essential amino acids (EAA)	**44.40±1.20**	**44.74±1.14**	**44.71±1.14**	**5.65**
Valine	6.19 ± 0.33	6.15 ±0.31	6.52 ±0.29	7.43
Methionine	2.86 ± 0.14	2.87 ±0.09	2.85 ±0.12	5.10
Isoleucine *(a,c) ; (b,c)	4.17 ± 0.06	4.11 ±0.25	4.91 ±0.40	18.35
Leucine	7.99 ± 0.22	8.02 ±0.21	8.11 ±0.35	6.81
Threonine *(a,c) ; (b,c)	5.39 ± 0.10	5.52 ±0.18	4.43 ±0.42	20.20
Phenylalanine	3.21 ± 0.40	3.26 ±0.37	3.48 ±0.39	9.47
Histadine *(a,c) ; (b,c)	2.32 ± 0.16	2.52 ±0.13	2.02 ±0.20	20.87
Lysine	7.70 ± 0.29	7.71 ±0.40	7.61 ±0.43	5.61
Arginine	4.56 ± 0.42	4.57 ±0.32	4.78 ±0.44	5.85
Nonessential amino acids (NEAA)	**55.23± 1.22**	**55.23±1.13**	**55.29±1.02**	**5.73**
Cysteine * (a,c) ; (b,c)	1.55 ± 0.06	1.54 ±0.09	2.25 ±0.20	16.91
Aspartate * (a,c) ; (b,c)	9.52 ± 0.21	9.53 ±0.32	9.94 ±0.65	17.11
Tyrosine	2.37 ± 0.42	2.32 ±0.44	2.67 ±0.51	8.60
Serine *(a,c) ; (b,c)	5.48 ± 0.12	5.44 ±0.10	3.75 ±0.30	19.61
Glutamine *(a,c) ; (b,c)	11.60 ± 0.29	11.41±0.38	12.83±0.29	17.76
Proline *(a,c) ; (b,c)	5.17 ± 0.31	5.46 ±0.34	4.25 ±0.56	17.39
Glycine	10.73 ± 0.92	10.74±0.53	10.26±0.83	9.71
Alanine	8.87 ± 0.18	8.77 ±0.33	9.32 ±0.52	9.80
Amino Acid Indices				
EAA:NEAA	0.80±0.01	0.81±0.04	0.81±0.02	
Branched-chain amino acids (BCAA)	18.35±0.11	18.28±0.42	19.54±0.38	14.61
Nonbranched-chain amino acids (NBCAA)	81.27±0.22	81.69±0.42	80.44±0.35	7.33
BCAA:NBCAA	0.23±0.00	0.22±0.01	0.24±0.01	
Sulfur amino acids *(a,c) ; (b,c)	4.41±0.02	4.42±0.02	5.10±0.02	16.01
Methionine: Cysteine *(a,c) ; (b,c)	1.84±0.04	1.86±0.04	1.26±0.01	18.46

Table 2.2. Mole percent composition of amino acids (%, mean ± SD) in Steller sea lion (SSL) milk, juvenile SLL serum, adult walleye pollock, and adult Pacific herring samples collected in spring (April). Statistical comparison between the mole % amino acid concentrations and the biological samples are presented in Table 3.

	SSL milk	SSL serum	Herring	Pollock
	(n=9)	(n=8)	(n=8)	(n=10)
Essential Amino Acids (EAA)	**46.82±0.42**	**44.61±0.66**	**44.40±1.20**	**37.95±1.45**
Valine	7.84 ± 0.13	7.96 ± 0.15	6.19 ± 0.33	5.11 ± 0.08
Methionine	2.43 ± 0.06	1.23 ± 0.02	2.86 ± 0.14	2.56 ± 0.06
Isoleucine	4.86 ± 0.13	2.40 ± 0.07	4.17 ± 0.06	3.64 ± 0.28
Leucine	10.39 ± 0.25	9.61 ± 0.13	7.99 ± 0.22	6.82 ± 0.27
Threonine	5.11 ± 0.18	5.63 ± 0.13	5.39 ± 0.10	4.39 ± 0.17
Phenylalanine	3.77 ± 0.06	4.17 ± 0.06	3.21 ± 0.40	2.73 ± 0.11
Histadine	2.59 ± 0.07	2.25 ± 0.02	2.32 ± 0.16	1.55 ± 0.23
Lysine	5.61 ± 0.03	7.14 ± 0.94	7.70 ± 0.29	6.31 ± 0.25
Arginine	4.24 ± 0.14	4.24 ± 0.09	4.56 ± 0.42	4.82 ± 0.26
Nonessential amino acids (NEAA)	**53.18±0.42**	**55.39±0.66**	**55.23±1.22**	**60.43±1.45**
Cysteine	1.70 ± 0.59	3.07 ± 0.33	1.55 ± 0.06	1.76 ± 0.02
Aspartate	8.75 ± 0.10	8.89 ± 0.07	9.52 ± 0.21	9.02 ± 0.12
Tyrosine	3.62 ± 0.10	3.63 ± 0.04	2.37 ± 0.42	2.08 ± 0.59
Serine	4.24 ± 0.24	6.92 ± 0.28	5.48 ± 0.12	5.83 ± 0.60
Glutamine	15.60 ± 0.67	12.68 ± 0.17	11.60 ± 0.29	11.95±0.38
Proline	9.49 ± 0.83	6.32 ± 0.17	5.17 ± 0.31	5.52 ± 0.18
Glycine	3.29 ± 0.68	5.73 ± 0.40	10.73 ± 0.92	15.15±0.07
Alanine	6.48 ±0.31	8.14 ± 0.12	8.87 ± 0.18	9.14 ± 0.15
Amino Acid Indices:				
EAA:NEAA	0.88±0.00	0.81±0.02	0.80±0.01	0.63±0.01
Branched-chain amino acids (BCAA)	23.08±0.43	19.97±0.21	18.35±0.11	15.59±0.38
Nonbranched-chain amino acids (NBCAA)	76.92±0.43	80.03±0.21	81.27±0.22	82.78±0.27
BCAA:NBCAA	0.30±0.01	0.25±0.00	0.23±0.00	0.19±0.01
Sulfur amino acids	4.13±0.62	4.30±0.31	4.41±0.02	4.31±0.06
Methionine: Cysteine	1.58±0.50	0.40±0.05	1.84±0.04	1.45±0.05

Table 2.3. Kruskal-Wallis ranks sum test results (H value) and post hoc comparisons between the mole % amino acid concentrations in the four biological samples (April caught pollock and herring, serum, milk) tested. Hydroxyproline was only found in significant concentrations in pollock tissue.

Amino Acid	Kruskal Wallis rank sum test (H value)	Significant Post -hoc Comparisons
Cysteine	22.40	serum>herring; serum>milk
Aspartate	29.27	herring>milk; herring>serum; pollock>milk
Threonine	30.51	serum>milk; serum>pollock; herring>pollock;
Serine	29.43	serum>milk; serum>herring; pollock>milk
Glutamine	30.78	milk>herring; milk>pollock; serum>herring
Proline	31.00	milk>pollock; milk>herring; serum>herring
Glycine	31.85	pollock>serum; pollock>milk; herring>milk
Alanine	30.91	pollock>serum; pollock>milk
Valine	29.34	serum>herring; serum>pollock; milk>pollock
Methionine	31.63	herring>serum; pollock>serum
Isoleucine	31.15	milk>pollock; milk>serum; herring>serum
Leucine	31.90	milk>herring; milk>pollock; serum>pollock
Tyrosine	28.46	serum>pollock; milk>pollock
Phenylalanine	31.87	serum>herring; serum>pollock; milk>pollock
Histadine	29.98	milk>serum; milk>pollock; herring>pollock
Lysine	27.62	herring>pollock; herring>milk; serum>milk
Arginine	28.26	pollock>milk; pollock>serum
Hydroxyproline	31.30	pollock>milk; pollock>serum

Table 2.4. A comparison of the amino acid composition of Steller sea lion milk reported in the present study with four other pinniped species from Davis *et al.* (1995). We have used amino acid ratio indices of % mole amino acid content. The indices are total essential amino acids: nonessential amino acids (EAA:NEAA), branched-chain amino acids: nonbranched-chain amino acids (BCAA:NBCAA) and methionine:cysteine (MET:CYS).

	EAA:NEAA	BCAA:NBCAA	MET:CYS
Steller sea lion (n=9) (This Study)	0.90	0.30	1.43
Northern elephant seal (n=3)	0.89	0.24	0.95
Antarctic fur seal (n=5)	0.82	0.23	3.10
California sea lion (n=5)	0.88	0.25	1.73
Australian sea lion (n=6)	0.79	0.24	2.25

Figure Legends

Figure 2.1. Sites of seasonal SSL prey sample collection in mid-water trawls. Samples were collected in the months of April (●), August (■) and November (▲) from (Adams et al. 2007). The open circles represent non-prey sample collection locations.

Figure 2.2. The mean energy density (kcal g^{-1} ±SD) of potential Steller sea lion prey species per collection period based on wet mass. The number next to species name is the sample number (n=). All fish exhibited seasonal changes in energy content, with herring having the largest seasonal changes (*i.e.* 1.38 kcal g^{-1} greater in fall than in spring; $p<0.05$) and pollock (of all age classes) the least seasonal variability (less than 0.46 kcal g^{-1} difference between each season and only a significant difference between April and November sampling periods $p<0.05$).

Figure 2.3. Mean proximate analysis (% wet mass ±SD) of potential Steller sea lion prey species per sample period where a is April, b is August and c is November. Each prey species was measured for water (■), lipid (■), protein (■) and ash (■) content as a percentage of wet mass of homogenized fish tissue. The sample size for each species is presented beside the species name. Seasonal differences (Kruskall Wallis; $p<0.05$) are indicated above the bar. Lipid and water content have an inverse relationship in most fish species and showed the greatest seasonal difference.

Figure 2.4. The ratio of essential to non-essential amino acids (EAA: NEAA) and branched-chain amino acids to non-branched-chain amino acids (BCAA:NBCAA) for each juvenile Steller sea lion serum sample. Serum samples from young of the year (YOY) TJ17, TJ18, TJ19, and TJ20 were collected from Glacier Island, PWS Alaska in July, 2001 and yearlings TJ28, TJ29,

TJ30 and TJ31 were collected from the same location in May, 2002 sample. Asterisks identify the young of the year (YOY) samples.

Figure 2.5. The concentration of total essential amino acids (EAA), nonessential amino acids (NEAA), branched-chain amino acids (BCAA), nonbranched-chain amino acids (NBCAA) and sulfur amino acids (Sulfur AA) (mean mole % ±SD) measured in juvenile Steller sea lion serum and milk (extracted from the stomach of Steller sea lion pups) as well as two Steller sea lion prey species (whole adult pollock and herring). There were significant differences in the protein indices between all tissues except within sulfur AA values and the NEAA and NBCAA values between serum and herring tissue.

Figure 2.6. The ratio (unitless ± SD) of essential to non-essential amino acids (EAA:NEAA) and branched-chain amino acids to non-branched-chain amino acids (BCAA:NBCAA) and the amino acids methionine and cysteine (MET:CYS) measured in juvenile Steller sea lion serum and milk (extracted from the stomach of Steller sea lion pups) and in two Steller sea lion prey species (whole adult pollock and herring). There were significant differences in the protein indices between all tissues except the EAA:NEAA values between serum and herring tissue.

Figures

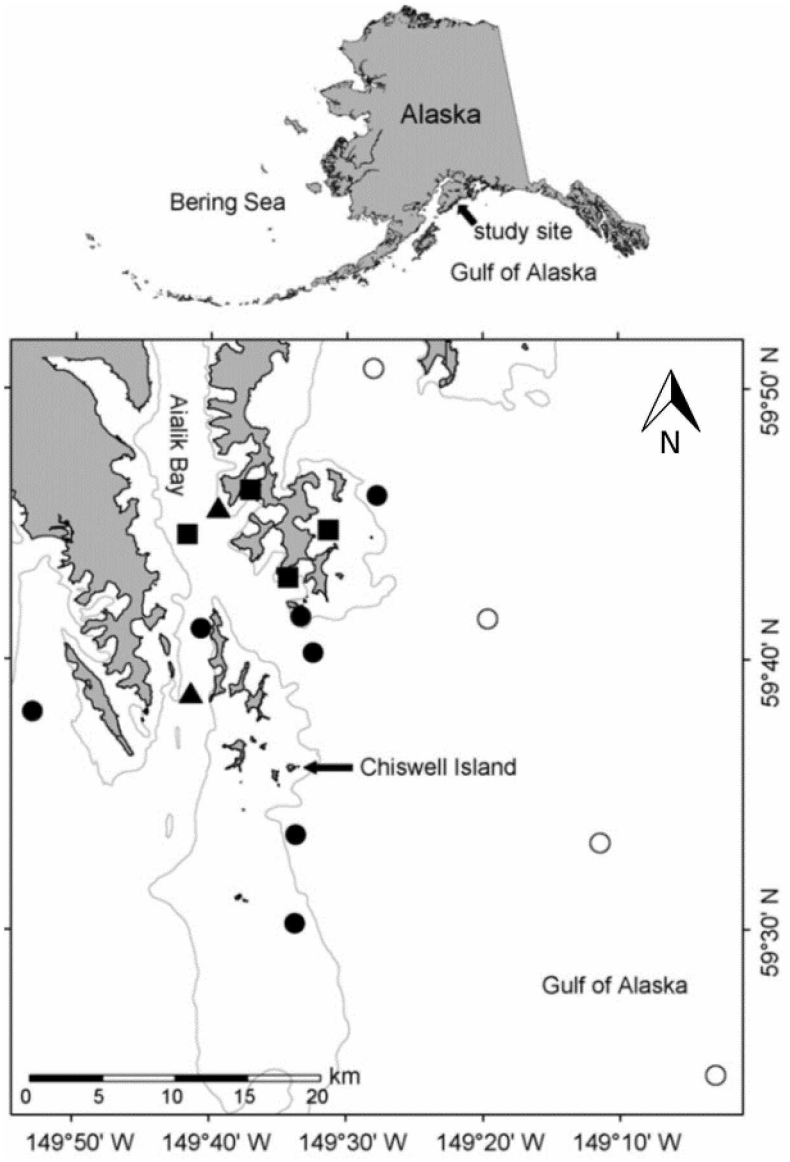


Figure 2.1.

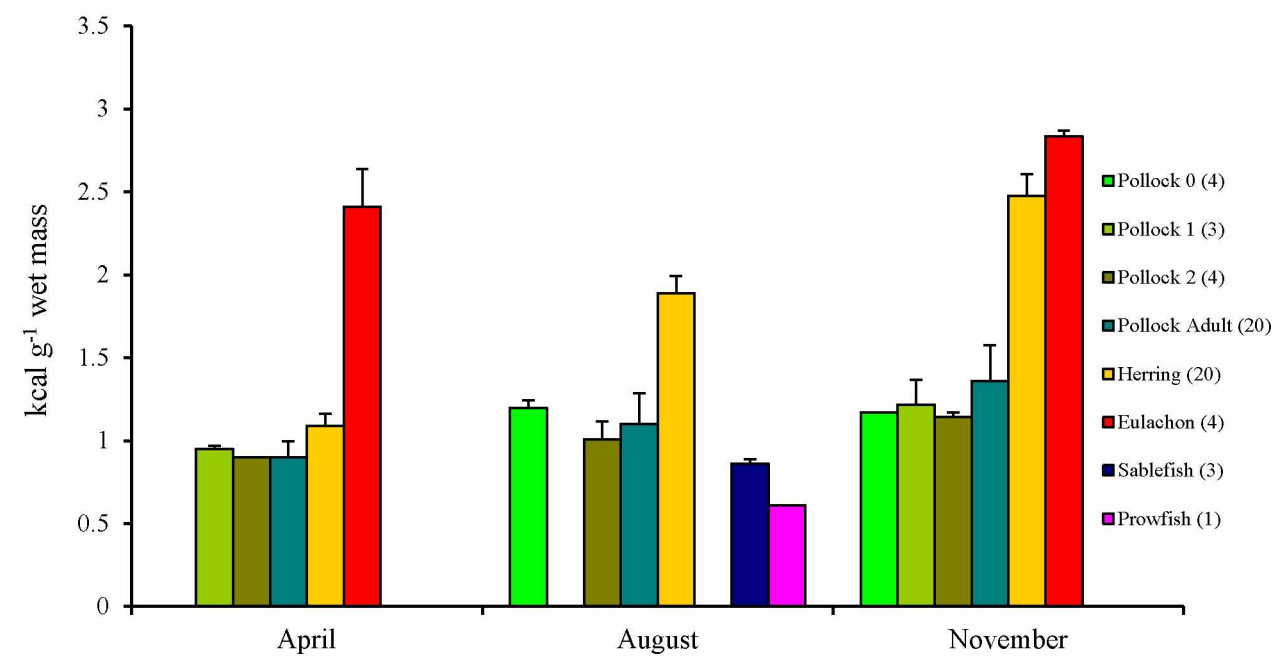

3.5
3
2.5
2
1.5
1
0.5
0
kcal g-1 wet mass
April
August
November
Pollock 0 (4)
Pollock 1 (3)
Pollock 2 (4)
Pollock Adult (20)
Herring (20)
Eulachon (4)
Sablefish (3)
Prowfish (1)

Figure 2.2.

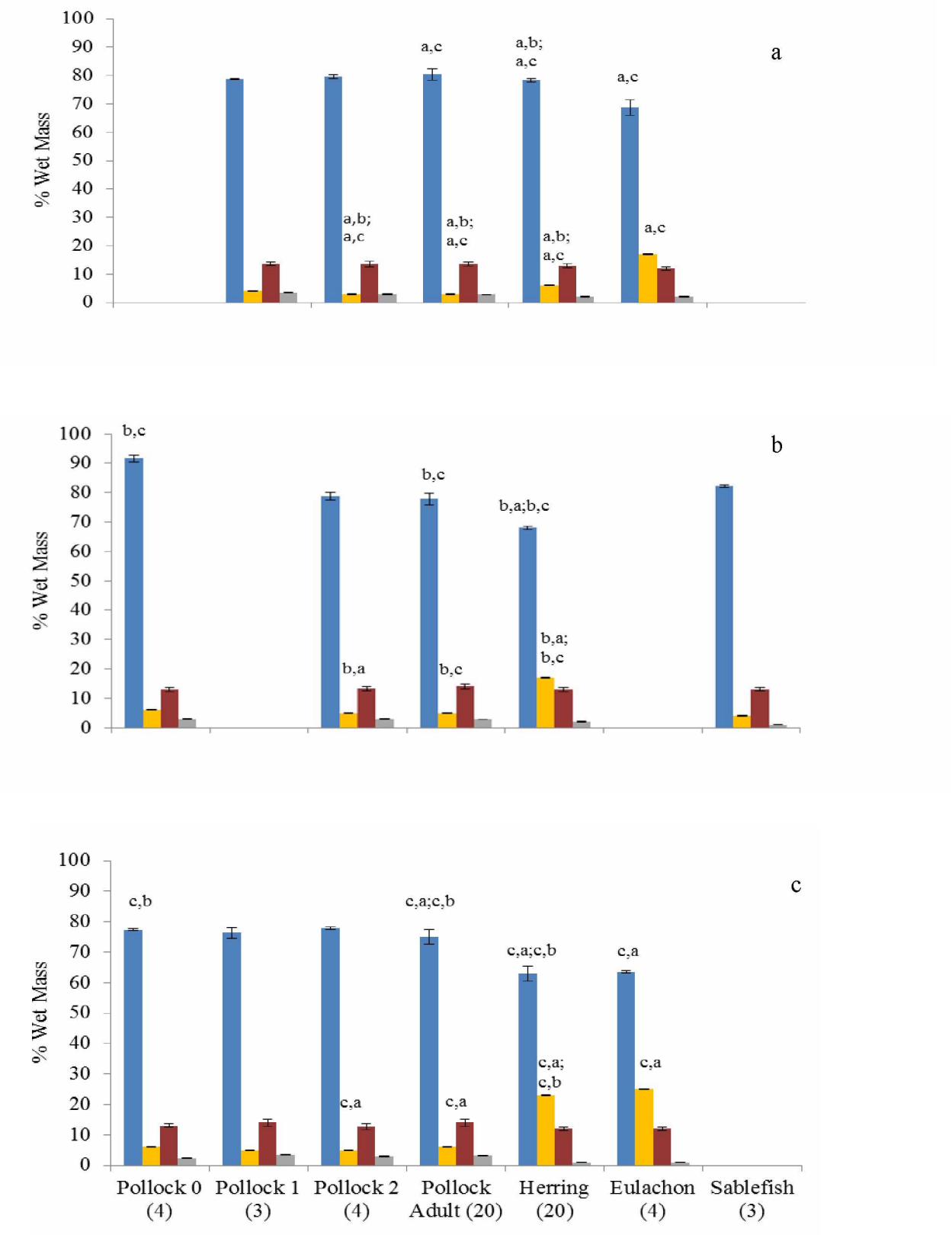

a
b
c
% Wet Mass
100
90
80
70
60
50
40
30
20
10
0
a,c
a,b;
a,c
a,b;
a,c
a,b;
a,c
a,c
a,b;
a,c
a,c
b,c
b,a
b,c
b,a;b,c
b,a;
b,c
b,c
c,b
c,a
c,a
c,a;c,b
c,a;
c,b
c,a;c,b
c,a
c,a
Pollock 0 (4)
Pollock 1 (3)
Pollock 2 (4)
Pollock Adult (20)
Herring (20)
Eulachon (4)
Sablefish (3)

Figure 2.3.

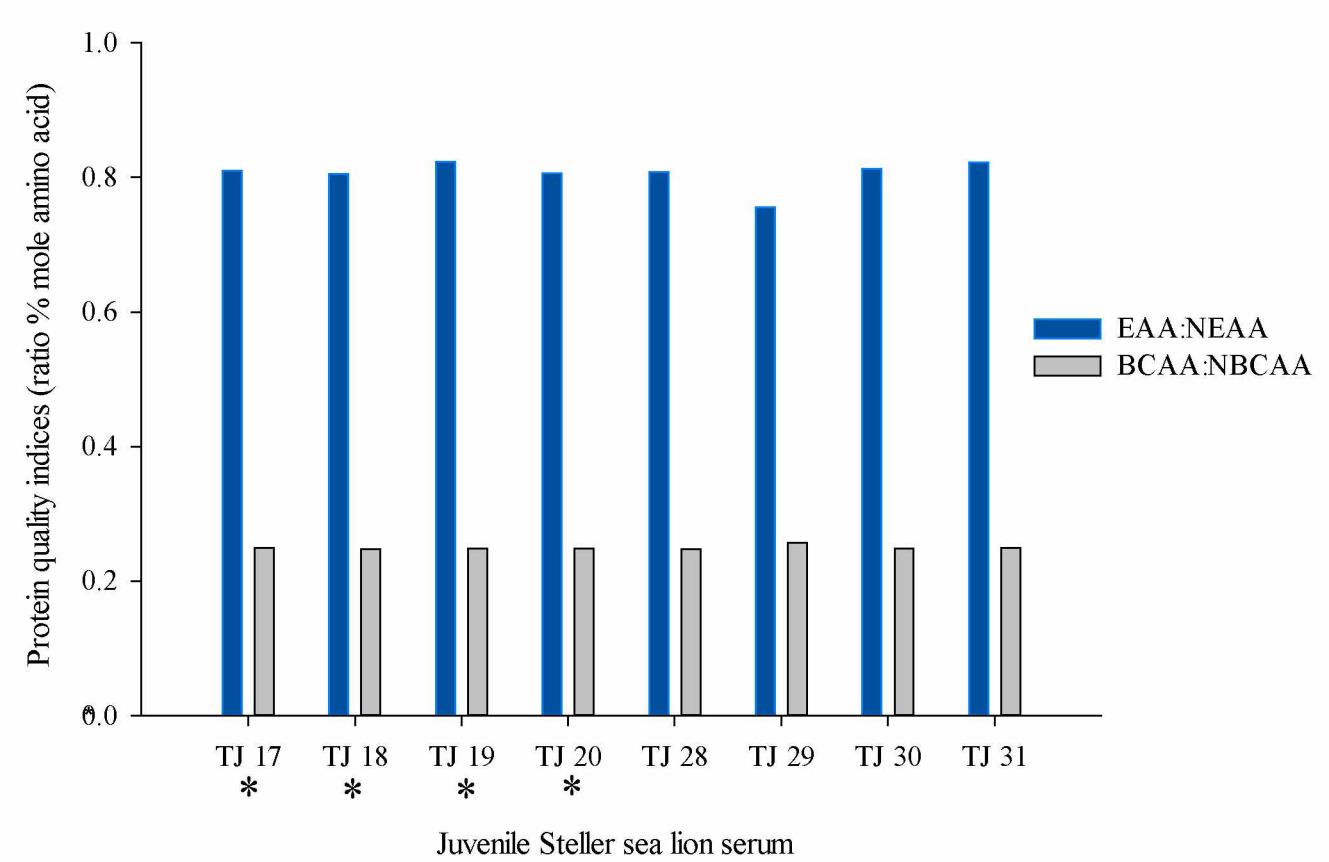

1.0
0.8
0.6
0.4
0.2
0.0
Protein quality indices (ratio % mole amino acid)
EAA:NEAA
BCAA:NBCAA
TJ 17
TJ 18
TJ 19
TJ 20
TJ 28
TJ 29
TJ 30
TJ 31
*
*
*
*
Juvenile Steller sea lion serum

Figure 2.4.

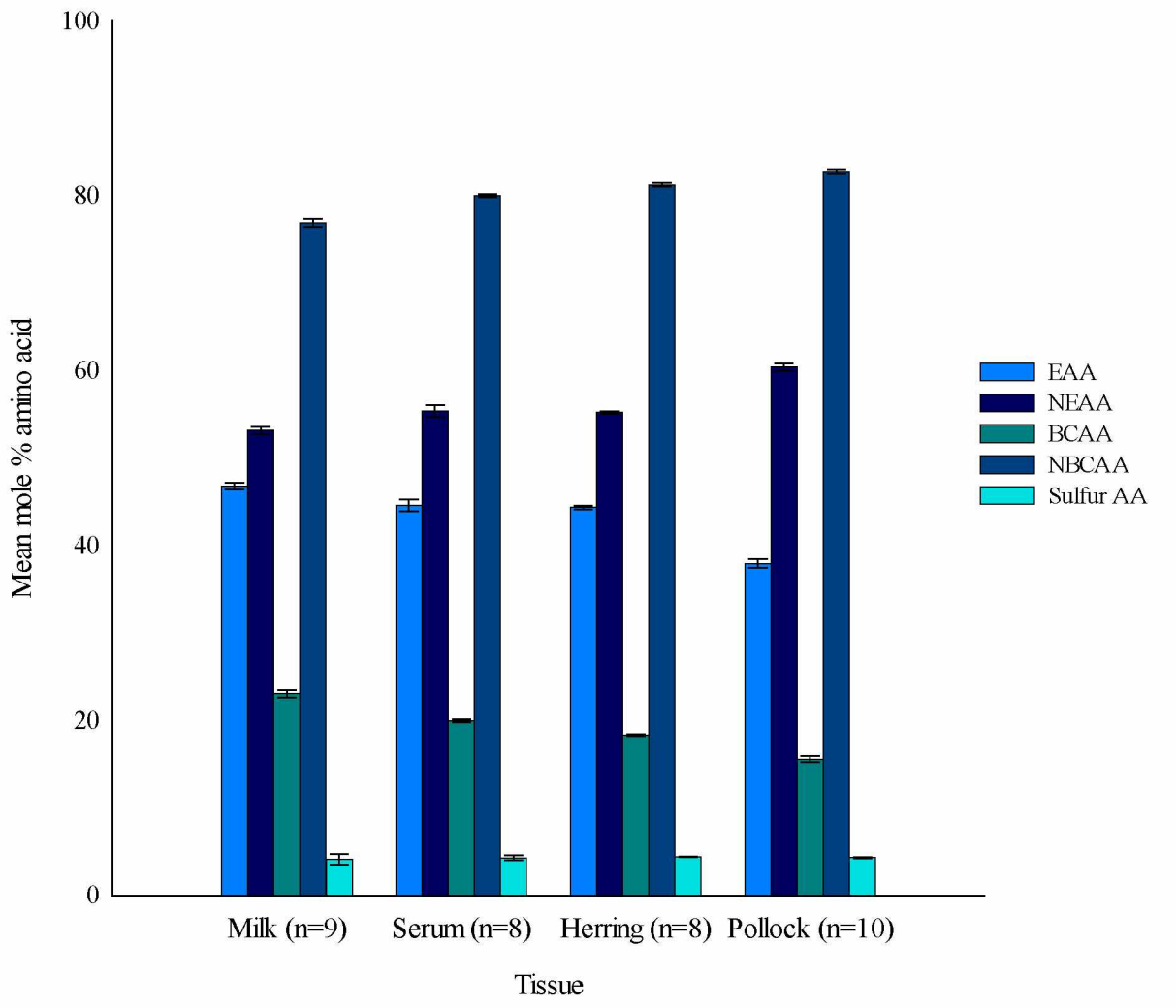

100
80
60
40
20
0
Mean mole % amino acid
EAA
NEAA
BCAA
NBCAA
Sulfur AA
Milk (n=9)
Serum (n=8)
Herring (n=8)
Pollock (n=10)
Tissue

Figure 2.5.

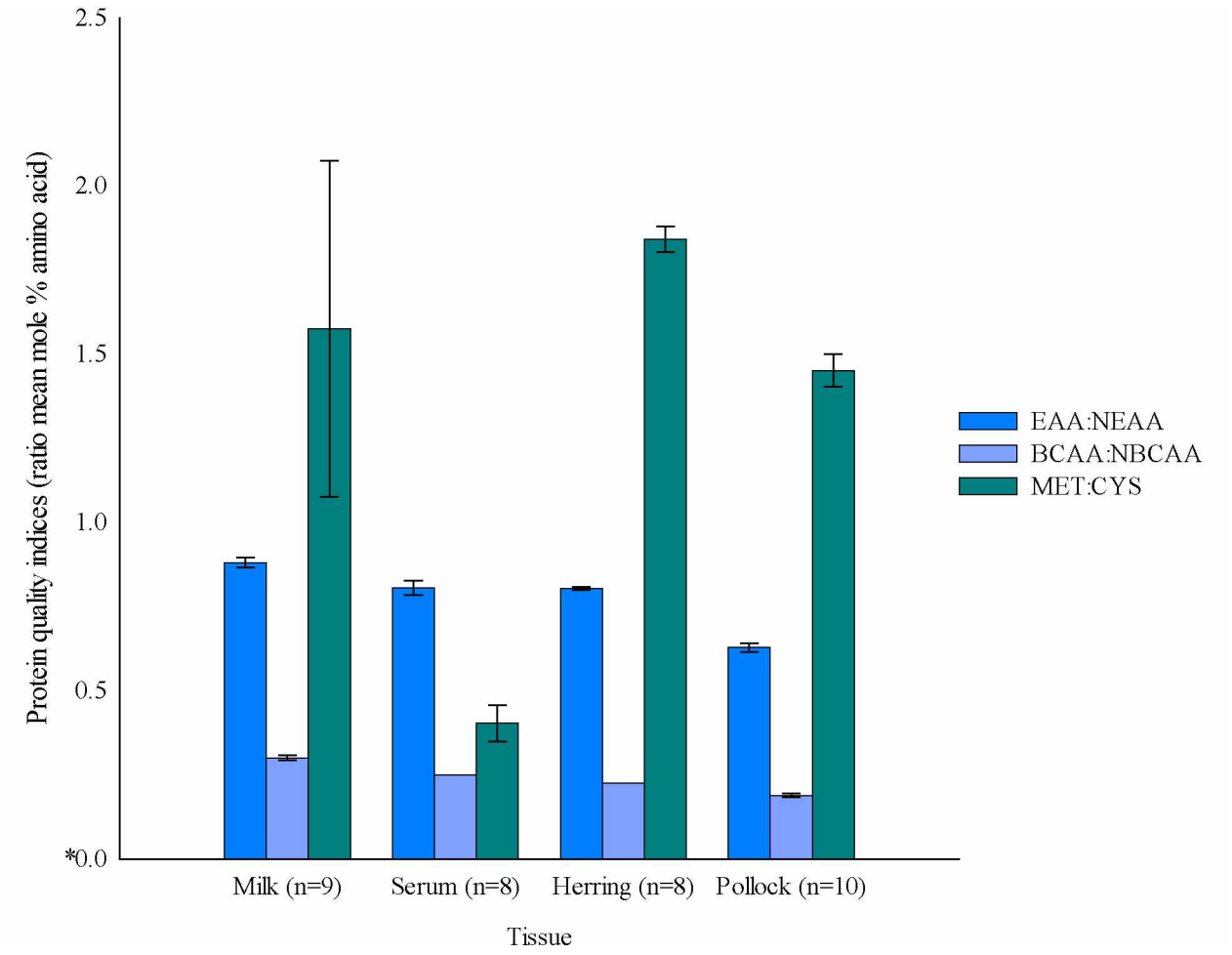

2.5
2.0
1.5
1.0
0.5
*0.0
Protein quality indices (ratio mean mole % amino acid)
Milk (n=9)
Serum (n=8)
Herring (n=8)
Pollock (n=10)
Tissue
EAA:NEAA
BCAA:NBCAA
MET:CYS

Figure 2.6.

Appendix

Table A1. Mean mole percent (± SD) composition of amino acids for whole fish species captured from Chiswell Island, Alaska in April, August and November 2003. The number of samples per species is designated in brackets (n=).

Prey	Pollock (4)	Pollock (3)	Pollock (4)	Pollock (3)	Prowfish (1)	Eulechon (3)	Eulechon (4)	Sablefish (3)
Age	0	1	2	1	adult	adult	adult	juvenile
Season	November	November	November	April	August	April	November	August
Essential amino acids (EAA):	40.43±1.06	39.50±1.08	40.11±1.11	41.33±1.03	44.92	43.12±0.98	44.77±0.87	44.77±1.01
Valine	5.56±0.28	5.27±0.16	5.44±0.18	5.79±0.14	6.40	6.00 ±0.12	5.93±0.11	6.16±0.24
Methionine	2.72±0.13	2.68±0.09	2.70±0.07	2.72±0.11	2.81	2.84±0.14	2.94±0.18	2.85±0.10
Isoleucine	3.92±0.14	3.77±0.20	3.86±0.16	4.58±0.20	5.16	4.21±0.19	4.23±0.15	4.53±0.23
Leucine	7.37±0.39	7.13±0.14	7.32±0.22	7.55±0.23	8.17	8.15±0.20	8.28±0.30	8.24±0.20
Threonine	4.71±0.11	4.55±0.13	4.60±0.27	4.16±0.16	4.54	5.15±0.10	5.79±0.23	5.28±0.17
Phenylalanine	2.96 ±0.16	2.88±0.19	3.05 ±0.25	3.20±0.09	3.48	2.97±0.07	3.11±0.10	3.16±0.14
Histadine	1.61±0.09	1.84±0.17	1.63±0.15	1.73±0.41	2.04	1.88±0.18	1.91±0.16	2.04 ±0.13
Lysine	6.83±0.25	6.61±0.2	6.75±0.30	6.66±0.19	7.33	7.27±0.28	7.88±0.34	7.76±0.37
Arginine	4.76±0.28	4.76±0.1	4.76±0.21	4.95 ±0.24	4.98	4.65±0.23	4.70±0.25	4.75±0.21
Nonessential amino acids (NEAA):	58.60±1.08	59.31±1.07	58.37±1.11	57.78±1.03	55.08	56.88±0.97	55.23±0.88	55.23±1.00
Cysteine	1.64±0.07	1.47±0.11	1.52±0.10	2.71±0.20	1.29	1.82±0.08	1.35±0.06	1.46±0.11
Aspartate	9.59±0.41	9.41±0.28	9.48±0.21	9.67±0.34	10.01	9.43±0.14	9.61±0.23	10.12±0.30
Tyrosine	2.30±0.19	2.20±0.10	2.30±0.16	2.54±0.39	2.78	2.40±0.17	2.47±0.13	2.61±0.15
Serine	5.92±0.27	5.96±0.24	5.95±0.11	4.08±0.30	3.97	6.05±0.30	5.56±0.28	5.79±0.21
Glutamine	12.69±0.37	12.45±0.27	12.71±0.20	12.68±0.31	12.78	13.22±0.29	12.02±0.41	13.23±0.37
Proline	5.42±0.16	5.51±0.31	5.30±0.17	4.56±0.34	4.39	5.20±0.31	4.82±0.28	4.52±0.26
Glycine	12.14±0.40	13.34±0.36	12.57±0.34	12.23±0.40	10.74	9.87±0.31	10.69±0.45	8.94±0.21
Alanine	8.91±0.14	8.98±0.22	8.84±0.24	9.32±0.29	9.13	8.88±0.20	8.71±0.23	8.55±0.23

Table A1 continued.

Amino Acid Indices:								
EAA:NEAA	0.69±0.01	0.67±0.02	0.68±0.01	0.72±0.01	0.82	0.76±0.00	0.81±0.01	0.81±0.01
BCAA Pool	16.85±0.15	16.17±0.12	16.62±0.14	17.92±0.18	19.74	18.36±0.24	18.44±0.19	18.93±0.22
NBCAA Pool	82.18±0.21	82.64±0.22	82.16±0.31	81.20±0.20	80.26	81.64±0.21	81.56±0.30	81.07±0.26
BCAA:NBCAA	0.21±0.01	0.20±0.00	0.20±0.00	0.22±0.01	0.25	0.22±0.01	0.23±0.01	0.23±0.01
Sulfur AA	4.36±0.01	4.15±0.03	4.22±0.02	5.42±0.02	4.10	4.66±0.02	4.29±0.01	4.31±0.01
MET:CYS	1.65±0.01	1.83±0.01	1.78±0.00	1.00±0.01	2.17	1.56±0.01	2.18±0.01	1.96±0.01

Chapter 3
Tracking Protein Turnover in Adult Female Weddell Seals (*Leptonychotes weddellii)* Using ^{15}N-labelled Glycine [1]

Abstract

The protein metabolism of wild, naturally feeding seals is poorly understood and is technically challenging. As carnivores, protein is important in the seal diet and they will spare protein when they fast. How they process protein when they are not fasting was the basis for this study on Weddell seals (*Leptonychotes weddellii)* in Antarctica. Eight non-pregnant adult females were caught near breeding sites on the sea ice, held at a sea-ice camp during experimental trials, and then released. Animal condition was assessed prior to experimental treatment through morphometric and blood chemistry values. The amino acid and protein analysis of several prey species of fish were tested and showed good quality protein with a high content of essential and branched chained amino acids. Serum amino acid profiles collected at capture and then repeated at time of release suggested differences in time since last meal between the individuals. The amino acid glycine, which had been isotopically labeled with ^{15}N (99.5 atm % in 8 % saline), was injected into the blood as a tracer of protein turnover in serum amino acid and protein pools, red blood cells and urinary urea. Blood and urine samples were serially collected for up to 35 hours and then opportunistically up to 31 days post-dose injection. The appearance/disappearance of ^{15}N from glycine in these pools were assessed to determine protein turnover rate. The serum amino acid pool decay curves had fractional turnover rates that were consistent with expected rates for large mammals and ranged from 12.5 to 25.6 (h^{-1}) and were trial and animal specific. The ^{15}N label increased in the serum protein pool and reached equilibrium by 5-8 hours post glycine injection. Animals sampled 5 and 10 days post release still exhibited elevated ^{15}N levels in the serum protein pool. Molting resulted in a slower urinary urea turnover rates and was likely associated with hormone regulation of protein metabolism during

this process. Blood cells retained the label for up to a month, but changes in isotopic enrichment were masked by a residual signal from the protein pool in the clot. In a single experiment using ^{15}N-labelled (99.5 atm % in 8 % saline) phenylalanine (an essential amino acid in mammals), the label was only detected in serum amino acids and suggests different metabolic pathways between these amino acid tracers. This study presents results from protein turnover trials conducted on non-fasting seals under natural conditions. Our findings demonstrate regulation of protein to accommodate metabolic challenges and provide baseline tissue turnover rates in Weddell seals for future isotopic studies.

[1]Inglis, S.D., Castellini, M.A, Rea, L.D, and P. Barboza. Tracking protein turnover in adult female Weddell seals (*Leptonychotes weddellii)* using ^{15}N-labelled glycine. Prepared for submission to Frontiers in Marine Science:Aquatic Physiology.

Introduction

Weddell seals (*Leptonychotes weddellii*) are one of the few mammals to inhabit the fast-ice of the Ross Sea off the Antarctic continent (Laws 1977; Ainley 1985). They congregate in McMurdo Sound during the austral spring (September-November) to give birth and breed, completing their annual molt in January before leaving the breeding colony to forage over the austral winter (Stirling 1969). Weddell seals are piscivores and many studies have been conducted on how these animals nutritionally meet the environmental challenges of living on the sea ice (Costa 1991; Rea et al. 1997; Burns et al. 1998; Williams et al. 2004; Eisert and Oftedal 2009; Trumble et al. 2010; Goetz et al. 2017). However, most studies have focused on the relative caloric benefits of different prey items as a function of lipid content. The importance of protein in growth, enzyme, hormone and immune functions, as well as pelage regeneration in molting underscores the need to study nutritional and metabolic impacts on protein metabolism (El-Khoury 1999). Small alterations in protein metabolism may not be relevant from a caloric perspective but can have significant consequences to animal health.

Tracer methods provide a simple technique to quantify the kinetics of protein metabolism (Waterlow 1984; Huntley et al. 1987; Wolfe 1992; Fouillet et al. 2002). Several different types of physiological tracers have been used to report protein turnover in animals. In northern elephant seal (*Mirounga angustirostris*; Pernia 1984; Costa 1991; Houser and Costa 2001) and Antarctic fur seal (*Arctocephalus gazelle*) (Arnould and Hindell 2001) studies a single bolus dose of ^{14}C-labelled urea was used as a physiological tracer with blood and urine endproduct collection. The amino acid stable isotope tracer ^{15}N-labelled glycine followed by blood and/or urine endproduct collection is another common tracer used to study protein turnover in both

human and wildlife studies (Waterlow et al. 1978; Freudenberger and Nolan 1993; Barboza et al. 1997; Russell et al. 2003; Jackson 2009).

Protein kinetics using endproduct methods assumes that there are two nitrogen pools in the body, a free amino acid pool and a protein pool and that there is no re-entry of the isotope from the protein pool into the free amino acid pool within the trial period (Picou and Taylor-Roberts 1969; Waterlow 2006; Jackson 2009) (Figure 3.1). Since amino acids and proteins are not stored in the body, there is a constant turnover of protein. The rate of turnover is different between different protein pools with blood turning over more quickly than muscle (Waterlow 2006). The free amino acid pool is generally the connection between dietary substrates and protein metabolism. This pool supplies the substrates derived from the diet and protein degradation to the protein pool for protein synthesis and oxidation (Waterlow 2006).

Plasma and red blood cells are free amino acid pools in the blood. Amino acid pool kinetics can be determined by calculating the apparent rate coefficient k′ from the decay curves following enrichment from a single dose of tracer. Thus, k′ represents the disposal rate as free amino acids are used for protein synthesis or oxidation (Waterlow et al. 1978; Waterlow 2006). When amino acids are catabolized, they produce energy and ammonia. As in most mammals, the ammonia in marine mammals is then converted to urea and excreted, in urine. Thus, urinary urea is an endproduct of protein metabolism and a measurement of the protein flux (Q).

In the present study we measured amino acid and protein turnover in adult (nonlactating) female Weddell seals. The tissues used for these analyses were serum, red blood cells, and urine. We used the non-essential amino acid glycine (that had been labeled with ^{15}N above natural abundance levels) as the primary tracer method for this study and tracked the tracer from the

amino acid pool to the protein pool in serum. Glycine was chosen as the tracer for this study because it is active in protein synthesis and its metabolic pathway includes excretion through urinary urea (Waterlow 2006). It is also used in the synthesis of the protoporphyrin component of hemoglobin in red blood cells. Since heme is not reutilized after the red blood cell (RBC) disintegrates, the appearance-disappearance of the label can provide information on the lifespan of red blood cells in that species (Shemin and Rittenberg 1946; London et al. 1949). The metabolic pathway of glycine to urea is illustrated in Figure 3.2. The tracer ^{15}N-labelled phenylalanine (EAA) was tested in a single animal and provided insight into potential differences between the kinetics of an essential vs non-essential amino acid.

Body condition, dietary protein intake and metabolic processes such as molting have significant effects on protein metabolism. Prey species were collected for amino acid (AA) analysis to assess protein quality in Weddell seal diet. Morphometrics and body composition (total body water (TBW) and extra cellular water (ECW) were measured prior to the experiment to assess the nutritional status of the animal. Changes in serum amino acid profiles and blood metabolites before and after the experimental period were also measured. Whole body protein turnover studies using the endproduct technique are usually conducted in controlled laboratory conditions to facilitate the collection of urine. We modified an isolated dive hole experimental design (Castellini et al. 1988; Castellini et al. 1992; Burns and Castellini 1996) to accommodate a urine collection device for field use on sea ice. The animals were held in confinement for 24-36 hours for urine collection.

The present study reports on protein turnover in a freely foraging pinniped. Our objectives were to use a ^{15}N-labelled amino acid tracer to calculate turnover rates in serum free amino acid pools, and protein flux in urea urine. Results from this study provided new insight into protein

metabolism in Weddell seals and produced baseline tissue turnover rates for future isotope studies.

Methods

An isolated dive hole field camp was set up in McMurdo Sound in the Ross sea, Antarctica (latitude and longitude 77 46.466S; 166 26.310E). A small heated portable hut was placed over a large (4 ft. diameter) hole drilled into the sea ice. For the protein turnover experiments an aluminum pan was placed within the ice hole and a Chemgrate© panel, that could support the weight of an adult Weddell seal, placed over the surface of the hole in the floor of the hut. This provided a recessed pan under the animal for urine collection.

Animal and sample collection

A total of eight adult female Weddell seals (non-lactating) were captured from colonies on the Ross Sea Ice Shelf in McMurdo Sound Antarctica during the austral spring (September-December 2004). Baseline blood samples were collected from the vein of a hind flipper and opportunistic urine samples were collected prior to the animal being transported by sled to the laboratory field hut. Once in the hut, the seals were chemically anesthetized with a mixture of ketamine (2.0 mg kg^{-1}) and valium (0.0025 mg kg^{-1}) given intramuscularly. Morphometric measurements were recorded and the seal weighed in a canvas sling suspended by a tripod. Two blood sampling catheters were inserted percutaneously in the intra-vertebral extradural vein for collection of serial blood samples. The seal was positioned on the Chemgrate© panel so that urine could be collected from the pan below. Total body composition (% lean and % fat tissue) was assessed using deuterium oxide (D_2O) at a dose of 100mg kg^{-1} BW; 99.9 % Sigma-Aldrich, St. Louis, MO, USA to measure total body water (Metabolic Solutions Technical Paper #913)

(Bowen and Iverson 1998; Mellish et al. 1999). To determine the amount of water external to the cells, sodium bromide (NaBr) salt (Cambridge Isotope Laboratories, Inc.) diluted in a sterile saline solution and at a dose of 0.1ml kg^{-1} BW was administered intravenously (IV) at the same time as the D_2O injection. Whole blood samples for D_2O and NaBr analyses were collected (IV) into a serum separator tube two and a half hours post injection and extracellular body water was calculated (Miller et al. 1989; Wong et al. 1989).

Baseline blood and urine samples were collected prior to injection of the ^{15}N-labelled glycine tracer (^{15}N 99.5 atm. % (atom percent) diluted to a 8 % solution in sterile saline solution (Cambridge Isotope Laboratories, Inc.) via IV catheter at a dose of 5.0 mg kg^{-1} body weight (BW) followed by a 10 ml saline flush. The ^{15}N-labelled phenylalanine (^{15}N 99.5 atm. % (atom percent) diluted to a 8 % solution in sterile saline solution (Cambridge Isotope Laboratories, Inc.) dose was 1.0 mg kg^{-1} BW. All dose syringes were weighed (±0.000) prior to and following the injection and the change in weight recorded to calculate the actual dose. Whole blood was collected in red-top vacutainers and green top heparin tubes every hour for isotope analysis of RBC, serum and body water. The red-top vacutainers were allowed to clot and then centrifuged at 1,000–2,000 x g for 10 minutes. The supernatant (serum) was removed using a pipette and transferred to a 0.5 ml aliquot and then frozen and stored at -80 °C. The blood clot was collected and frozen for RBC analysis. Green top vacutainers were centrifuged at 1,000–2,000 x g for 10 minutes and the plasma removed using a pipette and transferred to a 0.5 ml aliquot and frozen for body water analysis.

Urine samples were collected immediately from the collection pan drain and the time and approximate volume of the sample recorded. The urine samples were poured from a collection beaker into a graduated cylinder with a funnel and gauze filter to remove any debris and the

volume once again recorded (ml). The "clean" urine samples were preserved with 5M HCl at a dilution of 1 ml 5M HCl per 100 ml urine to prevent bacterial ureolysis and loss of ammonia. Samples were stored in 1-2 liter Nalgene polycarbonate bottles and stored at -20 °C for subsequent analyses.

The duration of the protein turnover trials ranged from 2 to 24 hours depending on animal condition at capture and other experimental protocols. At the end of the trial the seals were returned to the capture location. All the seals were previously flipper tagged from participation in a long term ecological study allowing identification and recapture for collection of subsequent blood samples for isotope analysis.

Weddell seal prey species were collected for diet analysis. The pelagic bald notothen, (*Pagothenia borchgrevinki*) was collected via hook and line and benthic fish such as emerald rockcod (*Trematomus bernacchii*), striped rockcod (*Trematomus hansoni*), and ploughfish (*Gymnodraco acutceps*) were collected using scuba dive capture. Samples of whole Antarctic silver fish (*Pleuragramma antarcticum*) and Antarctic toothfish (*Dissostichus mawsoni*) muscle tissue were donated by another project conducting Weddell seal studies. The samples were frozen at -20 °C for subsequent amino acid analysis to evaluate protein quality.

Laboratory Analyses

Body Composition

Total body water (TBW) samples were analyzed and calculated following the methods of Bowen and Iverson (1998) and converted to total body fat and protein based on empirical calculations for grey seals (Reilly and Fedak 1990).

Extra cellular water (ECW) was calculated by measuring the bromide space as described below. Intracellular water was calculated as:

$$ICW\ (l) = TBW(l) - ECW(l)$$

Extra Cellular Water:

Whole blood samples were centrifuged for 15 minutes and the resultant serum extracted into cyrovials and initially frozen at -20 °C and then moved to a -80 °C freezer for storage prior to analysis. Samples were shipped to Metabolic Solutions Inc. Amherst, MA for analysis of the bromide dilution space NaBr (umol l^{-1}). The results were modified to gm/l using the molecular weight of NaBr (102.89 gm). The bromide space was calculated as the volume of bromide distribution (V_{Br}) using the equation from Fielding et al. 2003:

$$V_{Br} = Br\ dose / ([Br]_{eq} - [Br]_{zero})$$

Where Br dose is the dose administered and $[Br]_{eq}$ and $[Br]_{zero}$ are the bromide concentrations at equilibrium (post plasma sample) and prior to NaBr injection (pre plasma sample) respectively. The dose was calculated for 8 % NaBr in physiological saline solutions using a specific gravity for NaBr of 1.277 gm l^{-1}.

Extra cellular water (EWC) was the corrected bromide space (CBS) using the following equation:

$$ECW\ (L) = CBS = V_{Br} \times 0.90 \times 0.95 \times 0.94$$

Where 0.90 is a correction for the non-extracellular distribution of bromide in the red blood cells, 0.95 is the Donnan equilibrium factor and 0.94 is the proportion of water in the plasma. The

bromide loss in urine is typically ~ 1.5 % and has a negligible effect on the measurement of CBS (Metabolic Solutions Technical Paper 915).

Prey species

Fish species were measured and then individually homogenized. Duplicate 10 gm samples of the homogenate were collected and frozen at -80 ºC and then freeze-dried to constant mass over 48-72 hours (*i.e.* less than 0.001 g weight change) under vacuum (VirTis Freeze Dryer Model 5463, VirTis, Gardiner, New York). Amino acid profiles of were produced from dry homogenate samples using a Hitachi L8900 Amino Acid Analyzer with post-column, ninhydrin derivatization *(*AAA Service Laboratory 14865 SE Regner Terrace Drive Damascus, OR). Results were provided as % mole composition and mg AA per gram of product for the following amino acids AA; (alanine (ALA), arginine (ARG), aspartic acid (ASP), glutamine (GLU), glycine (GLY), histadine (HIS), isoleucine (ILE), leucine (LEU), lysine (LYS), methionine (MET), phenylalanine (PHE), proline (PRO), serine (SER), Threonine (THR), Tyrosine (TYR), and valine (VAL).

Blood samples

Samples collected for isotope enrichment and amino acid analysis were centrifuged for 15 minutes and the resultant serum extracted into cryovials and initially stored at -20 °C and then moved to a -80 °C freezer prior to analysis. The condition of the serum was recorded (e.g. amount of hemolyzation). Excess serum was decanted from clotted blood to leave a clot mainly comprised of RBCs and fibrin. The clots were extracted and stored at -20 ^{0}C and then moved to a -80 ^{0}C freezer. Serum samples collected at the start and completion of the protein turnover experiments were submitted for amino acid analysis as described above.

Deproteinization of Serum samples

Serum protein was deproteinized with sodium tungstate ($Na_2 WO_4$) into a protein precipitate (protein pool) and a supernatant containing amino acid (Nolan and Leng 1972; Gustine et al. 2010). The supernatant was then run through an ion-exchange column conditioned with methanol and the binding buffer KH_2PO_4 pH=2 to remove urea and small peptides. The remaining fraction was eluded with a releasing buffer KH_2PO_4, pH=12 to collect the amino acids. fraction.

The RBC, protein precipitate and amino acid supernatant were freeze-dried under vacuum (VirTis Freeze Dryer Model 5463, CirTis, Gardiner, New York) for 48 hours. The dried samples were weighed (~0.300 mg) into tin (Sn) capsules for isotope analysis using an isotope ratio mass spectrometer (Finnigan Delta Plus XP). The isotopic compositions of samples were expressed in delta notation (i.e., $\delta^{15}N$ values) and reported relative to the isotopic composition of atmospheric nitrogen. Stable isotope ratios were expressed in δ notation as parts per thousand (‰) according to:

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 1{,}000 \text{ where } X = {}^{15}N \text{ and } R_{sample} = {}^{15}N/{}^{14}N$$

Urine samples

Urea labelled with ^{15}N was isolated from 2 ml of whole urine for analysis using a double steam distillation process (Barboza et al. 1997) based on the method of Nolan and Leng 1972. Distillation procedures took place in an alkalized environment using an ammonium sulfate (NH_4) SO_4 standard. During the first distillation (pre-distillation) all ammonia-N was removed. The receiving flask with the remaining urea was fitted with a rubber stopper and incubated overnight

with the enzyme urease (jack bean in 0.2 M phosphate buffer at pH7; Sigma Chemicals, St. Louis, Missouri) to hydrolyze urea to produce ammonium (NH_4^+).

During the second distillation, ammonia from the urea was captured in a solution of 4 % boric acid containing a color indicator (Kjelsorb, Fisher Chemicals). The distillate was then titrated with hydrochloric acid (HCl) to quantify the amount of NH_4^+ and represent complete recovery of ^{15}N from urea. The distillate was then dried in a drying oven at 100 ^0C to provide solid ammonium borate for subsequent ^{15}N analysis. Isotope enrichment was determined using an isotope ratio mass spectrometer (Finnigan Delta Plus XP). A sample weight of ~0.300 mg was used for this analysis.

Calculations

Protein turnover was calculated using ^{15}N kinetics expressed as isotopic enrichment (IE) above the background reading following methods of Waterlow 2006 and Barboza 1997. The IE in stable isotope studies represents a similar measurement and is analogous to specific activity (SA) associated with radioisotope tracer studies. Isotopic enrichment was calculated from the fractional isotope abundance (F) using the following equations:

$$F = ((\delta^{15}N /1000)+1/((1/0.003677)+(\delta^{15}N /1000)+1))$$

$$IE = F - (\text{predose } F)$$

The IE was log transformed (LN) and the regression of LN(IE) with time (t) from time of entry of injected ^{15}N into the protein pool to the end of the trial (~ 24 hours) after dosing provided IE_0 and the fractional turnover rate (k).

Nitrogen flux (Q) was calculated for the urine urea endproduct using the following equation:

Q= total N excreted in urea x (dose of ^{15}N given / total ^{15}N excreted in urea)

$$Q = \int_0^{48} IE_0 \cdot e^{kt} dt$$

Protein kinetics were based on a pool of amino acid N connected with a large protein pool (Picou and Taylor-Roberts 1969; Barboza et al. 1997).

Statistical Analysis

Significant differences in the body fluid compartments, blood chemistries, protein turnover (Q) and (K) between individuals were statistically tested using analysis of variance (ANOVA) followed by Tukey's post hoc test on treatment means. Kruskal-Wallis and Mann-Whitney tests were used on results requiring nonparametric analysis (Zar 1984). Statistical testing was conducted using R software (CoreTeam 2013).

Results

Protein quality of prey

The quality of available protein in prey as measured by the % mole AA composition is reported in Table 3.1. These values were compared to AA values reported on Pacific herring (*Clupea pallasi*) and walleye pollock (*Theragra chalcogramma*) (Zhao 2002). The mean % mole EEA, NEAA and BCAA and EAA:NEAA ratios of *D. mawsoni, P. borchgrevinki, P. antarcticum, T. bernacchii and T. pennellii* were statistically similar (student T-test, $p<0.05$) to values observed in Pacific herring with *G. acuticeps* similar to walleye pollock (student T-test, $p<0.05$).

Animal condition

Morphometrics and water compartment and composition results at intake are presented in Table 3.2A. The values for ECW and ECW:TWB ratios are significantly higher than those reported in

humans and horses (Miller et al. 1989; Fielding et al. 2003) but is consistent with previous measurements on Weddell seals (Castellini et al. 2003). Changes were reported in two blood metabolites associate with protein metabolism and serum amino acid profiles prior to glycine injection and at the end of the protein turnover experiment. There was a significant decrease in the mean BUN value ($n=4$, $p<0.05$) by the end of the experiment but only a weakly significant change in mean creatinine level ($n=4$, $p=0.05$) Table 3.2B. Serum amino acid profiles changed in individual amino acid values and there was a reduction in EAA and NEAA values by the end of the experiment. The largest decrease was observed in animals that participated in the experiment for the longest duration. However, BCAA remained positive in all animals Table 3.3 and Figure 3.3.

Turnover kinetics

Blood amino acid pools

We were successfully able to track the movement of the labelled amino acid into the protein pool and RBC. Blood cells retained the label for up to a month, but the blood clots were not washed prior to analysis and thus changes in enrichment were masked by a residual signal from the serum remaining in the clot. The ^{15}N-labelled glycine decay curves for each animal is presented in Figures 3.4AB, 3.5AB, and 3.6AB. The equilibrium and retention times and fractional turnover rates (k) $h^{-1}$are reported in Table 3.4. Although only one animal was tested with ^{15}N-labelled phenylalanine, there were apparent differences observed in the kinetics of the nonessential AA glycine and essential AA phenylalanine tracer. Both glycine and phenylalanine labels were detected in the serum amino acid pool in our first sample post injection. Serum protein pool curves increased and reached equilibrium with the amino acid label 5-8 hours post

injection for glycine but failed to reach equilibrium with phenylalanine. The serum amino acid pool decay curves had fractional turnover rates of 2.93-25.56 hours and were trial and animal specific. The longer the animal was in captivity and fasting, the slower the turnover rate. Animals resampled 5 and 10 days post release still exhibited elevated ^{15}N levels in the serum protein pool. The glycine label was assimilated into red blood cells between 1-2 hours post injection and remained high in animals recapture 31 days later. Labelled phenylalanine was not observed in red blood cells.

Urine urea protein turnover

We were only able to calculate fractional turnover rates (k)h^{-1} in two of the five animals tested (WSO45 and WS047) and the protein flux expressed as mmol N•24 h^{-1} (Q) in only one animal (WS045). The decay curves for WS044-7 are presented in Figure 3.7 and the turnover rates in Table 3.4. Tracer recycling was observed in WS044 and more significantly in WS046. Seal WS047 was in full molt during the experiment and had a lower (k) value (3.1 h^{-1}) than seal WS045(48.1 h^{-1}). The protein flux Q for seal WS045 was 9.3 mmol N•24 h^{-1}. This value is comparable to those reported from captive subadult harbor seals (Chapter 4).

Discussion

The present study provides protein turnover values for Weddell seals. We have reported on fractional turnover rates of a labelled amino acid in the serum amino acid pool, entry and equilibrium rates of the protein pool and entry into RBC. We were also able to obtain fractional turnover rates from the urine urea endproduct for two seals and a protein flux rate (Q) mmolN•24h^{-1} from one seal.

Although both glycine and phenylalanine labels immediately entered the serum amino acid pool their kinetics were very different and reflected the different roles in protein synthesis. The serum protein pool curves increased and reached equilibrium with the amino acid label 5-8 hours post injection for glycine but failed to reach equilibrium with phenylalanine. We suggest that the quick disappearance of the phenylalanine label may be due to its role as an essential amino acid and hydroxylation to tyrosine (Waterlow 2006).

The serum amino acid pool decay curves produced a large range of fractional turnover rates of 2.93-25.56 hours and were animal specific. The longer the animal was in captivity and fasting, the slower the turnover rate. Animals re-sampled 5 and 10 days post release still exhibited elevated ^{15}N levels in the serum protein pool suggesting high recycling of the glycine label.

Glycine is used in the synthesis of the protoporphyrin component of hemoglobin in red blood cells. The glycine label was assimilated into red blood cells between 1-2 hours post injection and remained in high in animals recapture 31 days later. However, as blood clots were not washed prior to analysis changes in enrichment were masked by a residual signal from the serum protein pool remaining in the clot. Thus, in this study we were unable to interpret heme kinetics and study the RBC life cycle in Weddell seals. The average life span of RBCs is 115-120 days in dogs and humans (London et al. 1949).

As these data are from one the few protein turnover studies collected on wild caught pinnipeds, it is important to consider some of the nutritional and metabolic conditions that may have influenced the results in this uncontrolled state.

Protein quality and intake is known to effect protein turnover rates (Waterlow et al. 1978; Boren et al. 1996; Barboza et al. 1997). Protein quality of Weddell seal prey species we tested had high

levels of both essential amino acids (EAA) and branch chained amino acids (BCAA). High % mole content of EAA, BCAA and ratios of EAA: nonessential amino acids (NEAA) are signatures of a high quality protein in a diet (Whitehead and Dean 1964; Arroyave 1970; Boren et al. 1996; Sarwar et al. 1998). Also, the Antarctic fish AA acid profiles were very similar to Pacific herring and walleye pollock that are both considered high quality protein prey for Alaskan harbor seals (*Phoca vitulin*a) and Steller sea lions (*Eumetopias jubatus*) (Castellini 2001; Zhao et al. 2006; Atkinson et al. 2008).

As the seals participating in this experiment were wild caught, the time of their last meal was unknown and we could not measure the effect of protein intake on our results. We initially assessed serum lipemia from samples collected at intake as a measure of having recently fed. However, in several seals serum lipemia fluctuated over the experimental period and thus we could not ultimately categorize the seals into recently fed and not fed experimental groups. However, body condition measurements of seals in the experiment were comparable to previously published values for adult female nonlactating Weddell seals (Mellish et al. 2011). This is only the second study that reports extra cellular water values for Weddell seals and our results are comparable to previously reported values (Castellini et al. 2003). Compared to humans, Weddell seal ECW values were high and the change may be attributed to the large blubber component. The ECW:TBW values for Seal WS048 was higher than the other seals. This seal exhibited diarrhea during the protein turnover trial which may have affected the body water results.

Another variable that can affect protein metabolism in pinnipeds is molting. The seal (WS047) that was molting during the experiment had different kinetic and body composition values than the other seals. Molting in pinnipeds is regulated by hormones "turning on and off" protein

synthesis and breakdown. (Ashwell-Erickson 1981). Metabolic adjustments are also made with seals reducing their metabolic rates. Serum cortisol levels increase prior to the pelage molt inhibiting protein synthesis and increasing protein degradation (McGrath and Goldspink 1982; Kershaw and Hall 2016). Once the animal is in full molt, cortisol levels decrease and thyroid hormones increase which is thought to be associated with an increase in hair growth to replace the pelage (Ashwell-Erickson 1981; Kershaw and Hall 2016). However, the actual role of thyroid hormones in hair growth is under debate and it may be that the role of thyroid hormones in the molt process is confined to adjusting metabolic rates (Renouf and Brotea 1991). Weddell seals usually molt from January-February (Stirling 1969) and seal WS047 was in full molt when captured in December.

The potential effects of both intake and molt can be observed in the changes in serum AA profiles during the experiment. Individual AA levels were lower in seals that participated longer in the protein turnover experiment, but there was considerable individual variability that may be attributed to whether the animal had recently fed prior to capture. The mean % mole value of AAs in seal WS047 were significantly lower at the end of the approximately 24 hour experiment, reflecting the energetic demands associated with molting. The BUN and creatinine levels in this animal were also significantly lower than the other seals tested. Thus, the low fractional turnover rate observed in this animal's urine urea value may be attributed to the agnostic effect cortisol has on protein synthesis with increase in protein breakdown (McGrath and Goldspink 1982).

Collecting urine as an endproduct for whole body protein turnover is logistically difficult to accomplish in the field. However, we were able to successfully collect urine endproducts samples following ^{15}N-labelled glycine injection using the modified hut arrangement. We found that the animals that were sampled for the longest duration produced the best results, allowing

for the label to enter and leave the urinary urea pool. Thus, we recommend that when conducting a single dose ^{15}N-labelled glycine tracer method with an endproduct collection of urine that the samples should be collected for a minimum of 24 hours.

In summary, we successfully tracked a nonessential ^{15}N-labelled glycine tracer from the serum amino acid pool into the protein pool, and to an endproduct of protein metabolism (urine urea). We quantified tissue turnover rates and amino acid kinetics for the blood fractions of the amino acid and protein pools, and measured protein turnover using the urine urea endproduct method. Ecological trophic dietary studies using the stable isotope approach rely on accurate tissue turnover rates to design sampling collection protocols and interpret results (Del Rio and Carleton 2012). Results from this study will provide important baseline tissue turnover rates for future isotope studies on Weddell seals.

Acknowledgments

This research was supported by a grant from the National Science Foundation, Office of Polar Programs 01-30417. The research was carried out under Marine Mammal Protection Act. Permit #1033-1683. Experimental protocols for the study were reviewed and approved by the UAF Institutional Animal Care and Use Committee (protocols 01-46 for fish collection and 01-06 for marine mammal collections).

We would like thank research support NSF McMurdo Station for logistical help, assistance in the field and fabricating the equipment needed to conduct the urine for the protein turnover experiment. Special thanks to the rest of the project team; Maggie Castellini, Tami Haase, Shawn Harper and Vicki Stegall who assisted with sample collection and processing in the field.

Literature Cited

Ainley, D.G. 1985. Biomass of Birds and Mammals in the Ross Sea. Springer Berlin Heidelberg, Berlin, Heidelberg. 498-515.

Arnould, J.P., and M.A. Hindell. 2001. Dive behaviour, foraging locations, and maternal-attendance patterns of Australian fur seals (*Arctocephalus pusillus doriferus*). Canadian Journal of Zoology 79: 35-48.

Arroyave, G. 1970. Comparative sensitivity of specific amino acid ratios versus "essential to nonessential" amino acid ratio. The American Journal of Clinical Nutrition 23: 703-706.

Ashwell-Erickson, S.M. 1981. The energy cost of free existence for Bering Sea harbor and spotted seals. PhD Thesis. University of Alaska Fairbanks. 207pp.

Atkinson, S., D. Calkins, V. Burkanov, M. Castellini, D. Hennen and S. Inglis. 2008. Impact of changing diet regimes on Steller sea lion body condition. Marine Mammal Science 24: 276-289.

Barboza, P.S., S.D. Farley and C.T. Robbins. 1997. Whole-body urea cycling and protein turnover during hyperphagia and dormancy in growing bears (*Ursus americanus* and *U. arctos*). Canadian Journal of Zoology 75: 2129-2136.

Boren, J., R. Lochmiller and D. Leslie, Jr. 1996. Relation of serum and muscle free amino acids to dietary protein in the Northern Bobwhite. Proceedings of the Oklahoma Academy of Science 76: 55-65.

Bowen, W.D., and S.J. Iverson. 1998. Estimation of total body water in pinnipeds using hydrogen-isotope dilution. Physiological Zoology 71: 329-332.

Burns, J., and M. Castellini. 1996. Physiological and behavioral determinants of the aerobic dive limit in Weddell seal (*Leptonychotes weddellii*) pups. Journal of Comparative Physiology B 166: 473-483.

Burns, J., S. Trumble, M. Castellini and J. Testa. 1998. The diet of Weddell seals in McMurdo Sound, Antarctica as determined from scat collections and stable isotope analysis. Polar Biology 19: 272-282.

Castellini, M. 2001. Captive studies with Steller sea lions at the Alaska SeaLife Center. p. 80 in D. DeMaster and S. Atkinson, eds. *Steller sea lion decline: Is It Food II*. University of Alaska Sea Grant, Fairbanks, Alaska. p. 80.

Castellini, M.A., G.L. Kooyman and P.J. Ponganis. 1992. Metabolic rates of freely diving Weddell seals: correlations with oxygen stores, swim velocity and diving duration. Journal of Experimental Biology 165: 181-194.

Castellini, M.J., Zhao, L, and M.A. Castellini. 2003. Estimation of extracellular water volume in Antarctic Weddell seals: A comparative study of bromide assay methods. Society of Marine Mammalogy Abstract 15th Biennial Conference, Greensboro, NC.

CoreTeam. 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, ed, Vienna, Austria. http://www.R-project.org/.

Costa, D.P. 1991. Reproductive and foraging energetics of high latitude penguins, albatrosses and pinnipeds: implications for life history patterns. American Zoologist 31: 111-130.

Del Rio, C.M., and S.A. Carleton. 2012. How fast and how faithful: the dynamics of isotopic incorporation into animal tissues. Journal of Mammalogy 93: 353-359.

Eisert, R., & Oftedal, O. T. 2009. Capital expenditure and income (foraging) during pinniped lactation: The example of the Weddell seal (*Leptonychotes weddellii*). In I. Krupnik, M. A. Lang, & S. E. Miller (Eds.), *Smithsonian at the poles: Contributions to international polar year science (IPY)*, 335–346. Washington, DC: Smithsonian Institution Scholarly Press. https://doi.org/10.5479/si.097884601X.0

El-Khoury, A.E. 1999. Methods for Investigation of Amino Acid and Protein Metabolism. CRC Press, Florida, USA.

Fielding, C.L., K.G. Magdesian, D.A. Elliott, A.L. Craigmill, W.D. Wilson and G.P. Carlson. 2003. Pharmacokinetics and clinical utility of sodium bromide (NaBr) as an estimator of extracellular fluid volume in horses. Journal of Veterinary Internal Medicine 17: 213-217.

Fouillet, H., C Bos, C. Gaudichon and D. Tomé. 2002. Approaches to quantifying protein metabolism in response to nutrient ingestion. Journal of Nutrition 132: 3208S-3218S.

Freudenberger, D., and J. Nolan. 1993. Protein turnover in two kangaroo sub-species (*Macropus robustus robustus* and *M. r. erubescens*) from divergent habitats and the sympatric feral goat (*Capra hircus*). Comparative Biochemistry and Physiology Part A: Physiology 105: 443-448.

Goetz, K.T., J.M. Burns, L.A. Hückstädt, M.R. Shero and D.P. Costa. 2017. Temporal variation in isotopic composition and diet of Weddell seals in the western Ross Sea. Deep Sea Research Part II: Topical Studies in Oceanography 140: 36-44.

Gustine, D.D., P.S. Barboza and J.P. Lawler. 2010. Dynamics of body protein and the implications for reproduction in captive muskoxen (*Ovibos moschatus*) during winter. Physiological and Biochemical Zoology 83: 687-697.

Houser, D., and D. Costa. 2001. Protein catabolism in suckling and fasting northern elephant seal pups (*Mirounga angustirostris*). Journal of Comparative Physiology B 171: 635-642.

Huntley, A., D. Costa, G. Worthy and M. Castellini. 1987. Approaches to Marine Mammal Energetics.Society for Marine Mammalogy, Special Publication 1. 253pp.

Jackson, A.A. 2009. Protein Turnover. European Journal Of Clinical Nutrition 63: 153.

Kershaw, J.L., and A.J. Hall. 2016. Seasonal variation in harbour seal (*Phoca vitulina*) blubber cortisol - A novel indicator of physiological state? Scientific Reports 6, 21889:1-9.

Laws, R. 1977. Seals and whales of the Southern Ocean. Philosophical Transactions of the Royal Society of London B: Biological Sciences 279: 81-96.

London, I.M., D. Shemin, R. West and D. Rittenberg. 1949. Heme synthesis and red blood cell dynamics in normal humans and in subjects with polycythemia vera, sickle-cell anemia, and pernicious anemia. Journal of Biological Chemistry 179: p. 463.

McGrath, J.A., and D.F. Goldspink. 1982. Glucocorticoid action on protein synthesis and protein breakdown in isolated skeletal muscles. Biochemical Journal 206: 641-645.

Mellish, J.-A.E., A.G. Hindle and M. Horning. 2011. Health and condition in the adult Weddell seal of McMurdo Sound, Antarctica. Zoology 114: 177-183.

Mellish, J.-A.E., S.J. Iverson and W.D. Bowen. 1999. Variation in milk production and lactation performance in grey seals and consequences for pup growth and weaning characteristics. Physiological and Biochemical Zoology 72: 677-690.

Miller, M.E., J. Cosgriff and G.B. Forbes. 1989. Bromide space determination using anion-exchange chromatography for measurement of bromide. The American Journal of Clinical Nutrition 50: 168-171.

Nolan, J., and R. Leng. 1972. Dynamic aspects of ammonia and urea metabolism in sheep. British Journal of Nutrition 27: 177-194.

Pernia, S.D. 1984. Protein turnover and nitrogen metabolism during long term fasting in northern elephant seal pups.PhD Thesis. University of California, Santa Cruz,California.

Picou, D., and T. Taylor-Roberts. 1969. The measurement of total protein synthesis and catabolism and nitrogen turnover in infants in different nutritional states and receiving different amounts of dietary protein. Clinical Science 36: 283-296.

Rea, L., R. Groscolas, E. Mioskowski and M. Castellini. 1997. Changes in plasma fatty acids indicate change in nutritional status in developing Weddell seal pups. Polar Biology 18: 351-357.

Reilly, J.J., and M.A. Fedak. 1990. Measurement of the body composition of living gray seals by hydrogen isotope dilution. Journal of Applied Physiology 69: 885-891.

Renouf, D., and G. Brotea. 1991. Thyroid hormone concentrations in harbour seals (*Phoca vitulina*): no evidence of involvement in the moult. Comparative Biochemistry and Physiology Part A: Physiology 99: 185-194.

Russell, K., G. Lobley and D. Millward. 2003. Whole-body protein turnover of a carnivore, *Felis silvestris catus*. British Journal of Nutrition 89: 29-37.

Sarwar, G., H.G. Botting, T.A. Davis, P. Darling and P.B. Pencharz. 1998. Free amino acids in milks of human subjects, other primates and non-primates. British Journal of Nutrition 79: 129-131.

Shemin, D., and D. Rittenberg. 1946. The life span of the human red blood cell. Journal of Biological Chemistry 166: 627-636.

Stirling, I. 1969. Ecology of the Weddell seal in McMurdo Sound, Antarctica. Ecology 50: 573-586.

Trumble, S.J., S.R. Noren, L.A. Cornick, T.J. Hawke and S.B. Kanatous. 2010. Age-related differences in skeletal muscle lipid profiles of Weddell seals: clues to developmental changes. Journal of Experimental Biology 213: 1676-1684.

Waterlow, J. 1984. Protein turnover with special reference to man. Quarterly Journal of Experimental Physiology 69: 409-438.

Waterlow, J.C. 2006. Protein Turnover. CABI Publishing, Wallingford, Oxfordshire.

Waterlow, J.C., P.J. Garlick and D. Millward. 1978. Protein Turnover in Mammalian Tissues and in the Whole Body. Elsevier/North-Holland Publishing Co., Amsterdam, The Netherlands.

Whitehead, R.G., and R.F.A. Dean. 1964. Serum amino acids in kwashiorkor II. an abbreviated method of estimation and its application. The American Journal of Clinical Nutrition 14: 320-330.

Williams, T.M., L.A. Fuiman, M. Horning and R.W. Davis. 2004. The cost of foraging by a marine predator, the Weddell seal *Leptonychotes weddellii*: pricing by the stroke. Journal of Experimental Biology 207: 973-982.

Wolfe, R. 1992. Radioactive and Stable Isotope Tracers in Biomedicine: Principles and Practice of Kinetic Analysis. Wiley, New York, NY.

Wong, W.W., H.-P. Sheng, J.C. Morkeberg, J.L. Kosanovich, L.L. Clarke and P.D. Klein. 1989. Measurement of extracellular water volume by bromide ion chromatography. The American Journal of Clinical Nutrition 50: 1290-1294.

Zar, J. 1984. Biostatistical analysis. Prentice-Hall, Englewood Cliffs, New Jersey.

Zhao, L. 2002. Tracing amino acid metabolism of harbor seal *(Phoca vitulina)* using stable isotope techniques. PhD Thesis. University of Alaska Fairbanks, Fairbanks, AK. 211pp.

Zhao, L., D.M. Schell and M.A. Castellini. 2006. Dietary macronutrients influence ^{13}C and ^{15}N signatures of pinnipeds: Captive feeding studies with harbor seals (*Phoca vitulina*). Comparative Biochemistry and Physiology, Part A 143: 469-478.

Table 3.1. Amino acid profiles reported as % mole (mean ±SD) of Weddell seal prey species. The values represent whole fish samples except for D. mawsoni (*) that is based on muscle tissue only. All species provide high quality protein with high essential amino acid (EAA) content.

Species	D. *mawsoni*	P. *borchgrevinki*	P. *antarcticum*	T. *bernacchii*	*T. pennellii*	*G. acuticeps*
N	1*	5	2	5	4	3
% protein	22.38	39.98± 0.41	27.97±0.16	44.17±0.53	42.35±0.68	43.48±0.49
Essential amino acid pool (EAA)	**45.8**	**44.2±1.21**	**45.7±0.11**	**42.0±0.28**	**40.4±1.13**	**39.3±1.09**
Valine	5.9	5.3±0.06	5.4±0.01	5.8±0.21	6.4±0.15	6.0 ±0.14
Methionine	3.0	2.7±0.18	2.7±0.01	2.7±027	2.8±0.23	2.8±0.15
Isoleucine	4.7	3.8±0.15	3.9±0.03	4.6±0.09	5.2±0.26	4.2±0.07
Leucine	8.5	7.1±0.14	7.3±0.01	7.5±0.13	8.2±0.18	8.1±0.09
Threonine	5.4	4.6±0.24	4.6±0.02	4.2±0.24	4.5±0.20	5.2±0.12
Phenylalanine	3.1	2.9±0.27	3.0 ±0.02	3.2±0.19	3.5±0.27	3.0±0.10
Histidine	1.6	1.8±0.19	1.6±0.01	1.7±0.14	2.0±0.18	1.9±0.06
Lysine	8.8	6.6±0.24	6.7±0.01	6.7±0.20	7.3±0.09	7.3±0.11
Arginine	4.8	4.8±0.18	4.8±0.02	5.0 ±0.17	5.0±0.21	4.6±0.03
Nonessential amino acid pool (NEAA)	**54.2**	**55.8±1.23**	**54.3±0.06**	**58.0±1.22**	**59. 6±1.19**	**60.7±1.11**
Aspartate	10.2	9.4±0.08	9.5±0.01	9.7±0.13	10.0±0.16	9.4±0.11
Tyrosine	2.8-	2.2±0.07	2.3±0.03	2.5±0.18	2.8±0.20	2.4±0.04
Serine	5.5	6.0±0.21	5.9±0.02	4.1±0.21	4.0±0.19	6.0±0.05
Glutamine	13.5	12.5±0.22	12.7±0.02	12.7±0.25	12.8±0.26	13.2±0.01
Proline	4.2	5.5±0.18	5.3±0.01	4.6±0.14	4.4±0.13	5.2±0.03
Glycine	8.6	13.3±0.06	12.6±0.02	12.2±0.24	10.7±0.15	9.9±0.12
Alanine	9.4	9.0±0.13	8.8±0.03	9.3±0.16	9.1±0.20	8.9±0.09
Amino Acid Indices						
EAA:NEAA	0.8	0.8±0.01	0.8±0.01	0.7±0.02	0.7 ±0.02	0.7±0.01
Branched-chain amino acids (BCAA)	19.1	18.3±0.12	19.5±0.05	17.6±0.09	16.3±0.10	16.3±0.04
Nonbranched-chain amino acids (NBCAA)	80.9	81.7±0.14	80.5±0.03	82.4±0.16	83.7±0.09	83.7±0.10
BCAA:NBCAA	0.2	0.2±0.01	0.2±0.00	0.2±0.01	0.2±0.00	0.2±0.00

Table 3.2A. Summary of animal intake measurements on body condition. Not all measurements were taken for each animal due to sample collection schedules for different experiments. Animals WS041 and 2 participated in an isolated dive hole experiment and were in captivity for approximately 5 hours.

Animal ID	Mass (kg)	Standard Length (cm)	Total Body Water (TBW)(l)	Excellular Water (ECW)(l)	ECW/TBW	Lean (kg)	% Lean	Fat (kg)	% Fat
WS041	328.0	236							
WS042	317.3	214							
WS043	211.2	207	115.8	67.41	0.58	153.6	72.7	57.6	27.3
WS044	367.6	220							
WS045	375.4	229	186.9	106.85	0.57	244.4	65.1	131.0	34.9
WS046	409.2	236	170.6	114.53	0.67	216.5	52.9	192.7	47.1
WS047	396.0	230	182	124.96	0.69	235.0	59.3	161.0	40.7
WS048	391.9		189.2	121.47	0.80	246.2	62.8	145.7	37.2

Table 3.2B. Summary of animal blood chemistries prior to and at the conclusion of the experiment. Not all measurements were taken for each animal due to sample collection schedules for different experiments. Animals WS041 and 2 participated in an isolated dive hole experiment and were in captivity for approximately 5 hours.

Animal ID	Mass (kg)	Duration (hrs)	Blood urea nitrogen (BUN) (mg/dL)		Creatinine (mg/dL)	
			Intake	Release	Intake	Release
WS041	328.0	2.08				
WS042	317.3	5.55				
WS043	211.2	15.85				
WS044	367.6	12.85				
WS045	375.4	12.88	23	7	1.6	1.6
WS046	409.2	22.03	22	13	1.3	1.6
WS047	396.0	23.80	18	9	1.2	1.3
WS048	391.9	23.22	32	13	1.5	1.6

Table 3.3. Changes in serum protein (mg protein gm^{-1}) and amino acid content (mg aa gm^{-1}) in adult female Weddell seals over the sample collection period. The lower protein content is reflected in the proportional decrease in individual amino acids and does not infer differences in amino acid kinetics between animals. Branched-chain amino acid content remains positive even with a decrease in total protein content.

Animal ID	WS041	WS042	WS043	WS044	WS045	WS046	WS047	WS048
mg protein $gram^{-1}$	-1.2	4.5	-0.6	-4.1	1.2	-8.7	-16.6	1.2
Time (hours)	2.08	5.55	15.85	12.88	12.88	22.03	23.8	23.22
Essential amino acids (EAA):	**-0.54**	**2.28**	**-0.35**	**-2.13**	**0.65**	**-4.29**	**-8.59**	**0.62**
Valine	-0.08	0.34	-0.06	-0.33	0.08	-0.64	-1.30	0.08
Methionine	-0.02	0.07	-0.01	-0.07	0.01	-0.13	-0.26	0.01
Isoleucine	-0.03	0.11	-0.02	-0.11	0.02	-0.21	-0.42	0.03
Leucine	-0.11	0.44	-0.06	-0.43	0.14	-0.90	-1.69	0.10
Threonine	-0.06	0.28	-0.05	-0.26	0.06	-0.52	-1.03	0.10
Phenylalanine	-0.06	0.24	-0.04	-0.23	0.08	-0.22	-0.96	0.06
Histidine	-0.05	0.11	-0.03	-0.11	0.03	-0.24	-0.45	0.01
Lysine	-0.09	0.44	-0.04	-0.34	0.18	-0.90	-1.48	0.13
Arginine	-0.06	0.25	-0.04	-0.24	0.05	-0.53	-1.00	0.10
Nonessential amino acids (NEAA):	**-0.75**	**2.19**	**-0.30**	**-2.01**	**0.52**	**-4.41**	**-7.95**	**0.54**
Aspartate	-0.10	0.40	-0.05	-0.38	0.11	-0.79	-1.54	0.09
Tyrosine	-0.08	0.26	-0.06	-0.23	0.06	-0.49	-0.95	0.08
Serine	-0.06	0.26	-0.08	-0.28	0.05	-0.49	-1.10	0.18
Glutamine	-0.15	0.64	-0.10	-0.55	0.17	-0.96	-2.10	0.13
Proline	-0.28	0.28	0.03	-0.22	0.03	-0.86	-0.66	-0.09
Glycine	-0.03	0.14	-0.02	-0.14	0.03	-0.39	-0.72	0.10
Alanine	-0.05	0.22	-0.02	-0.22	0.07	-0.44	-0.88	0.06
Branched-chain amino acids (BCAA)	**0.73**	**1.04**	**1.17**	**1.06**	**1.24**	**0.97**	**1.08**	**1.15**
Nonbranched-chain amino acids (NBCAA)	-0.22	0.89	-0.13	-0.87	0.23	-1.75	-3.41	0.21
Branched-chain amino acids (BCAA)	-1.07	3.58	-0.51	-3.26	0.93	-6.95	-13.12	0.95
Nonbranched-chain amino acids (NBCAA)	0.21	0.25	0.26	0.27	0.25	0.25	0.26	0.22

Table 3.4. The kinetics of different tissues for adult female Weddell seals labelled with ^{15}N-labelled glycine or ^{15}N-labelled phenylalanine. The value (k) represents the fractional turnover rate of the labeled amino acid pool (h^{-1}). Other values report the hours post injection that the label is first observed in red blood cells and where the protein pool reaches equilibrium. Animal WS047 was in molt at intake for the experiment.

Animal ID	Tracer	Amino Acid	Protein Pool	Red Blood Cells	Urine Urea	Urine urea flux Q
		k (h^{-1})	Equilibrium (h)	Tracer present (h)	k (h^{-1})	Mmol N•24 h^{-1}
WS041	^{15}N-labelled glycine	2.9	6	1.6		
WS042	^{15}N-labelled glycine	3.0	5	2		
WS043	^{15}N-labelled glycine	13.6	8	2		
WS044	^{15}N-labelled glycine	12.5	6	3.2		
WS045	^{15}N-labelled glycine	22.0	7	1	48.1	9.3
WS046	^{15}N-labelled glycine	24.9	8	1		
WS047	^{15}N-labelled glycine	21.2	8	1	3.2	
WS048	^{15}N-labelled phenylalanine	25.6	No	No		

Figure Captions

Figure 3.1. Schematic of protein kinetics using end-product which assumes that there are two nitrogen pools in the body, a free amino acid pool and a protein pool and that there is no re-entry of the isotope from the protein pool into the free amino acid pool within the trial period.

Figure 3.2. Schematic of glycine metabolic pathway.

Figure 3.3A. Serum amino acid profiles of Weddell seals WS044-1-4 at the start of the sample collection period prior to tracer injection (first bar) and at the end of the experiment (second bar). The total time elapsed between the first and last sample is reported in hours. The * symbol represents the amino acid used for the ^{15}N tracer study.

Figure 3.3B. Serum amino acid profiles of Weddell seals WS045-9-8 at the start of the sample collection period prior to tracer injection (first bar) and at the end of the experiment (second bar). The total time elapsed between the first and last sample is reported in hours. The * symbol represents the amino acid used for the ^{15}N tracer study.

Figure 3.4A. ^{15}N-labelled glycine decay curves in the serum amino acid (●) and protein (△) pools of adult female Weddell seals WS041-4 including recapture data.

Figure 3.4B. ^{15}N-labelled glycine decay curves in the serum amino acid (●) and protein (△) pools of adult female Weddell seals WS045-7 including recapture data. WS048 was administered the essential amino acid ^{15}N-labelled phenylalanine as the tracer.

Figure 3.5A. ^{15}N-labelled glycine decay curves in the red blood cells of adult female Weddell seals WS041-4 including recapture data. The values are the mean (± SD). The amino acid glycine is incorporated into the heme component of red blood cells.

Figure 3.5B. ^{15}N-labelled glycine decay curves in the red blood cells of adult female Weddell seals WS045-7 including recapture data. The values are the mean (± SD). The amino acid glycine is incorporated into the heme component of red blood cells. WS048 was administered the essential amino acid ^{15}N-labelled phenylalanine as the tracer.

Figure 3.6A. ^{15}N-labelled glycine decay curves in the serum amino acid and protein pools and red blood cells of adult female Weddell seals WS041-4 during the experimental collection period.

Figure 3.6B. ^{15}N-labelled glycine decay curves in the serum amino acid and protein pools and red blood cells in adult female Weddell seals WS045-7 during the experimental collection period. WS048 was administered the essential amino acid ^{15}N-labelled phenylalanine and does not appear to enter the protein pool during the collection period.

Figure 3.7. ^{15}N-labelled glycine decay curves in urinary urea endproduct in Weddell seals WS044-7. The data were graphed using a natural logarithmic transformation (Ln). The data were log transformed and then the slope (k)h^{-1} calculated for the fractional turnover rate. Both WS044 and WS046 showed signs of tracer recycling and animal WS047 was in full molt.

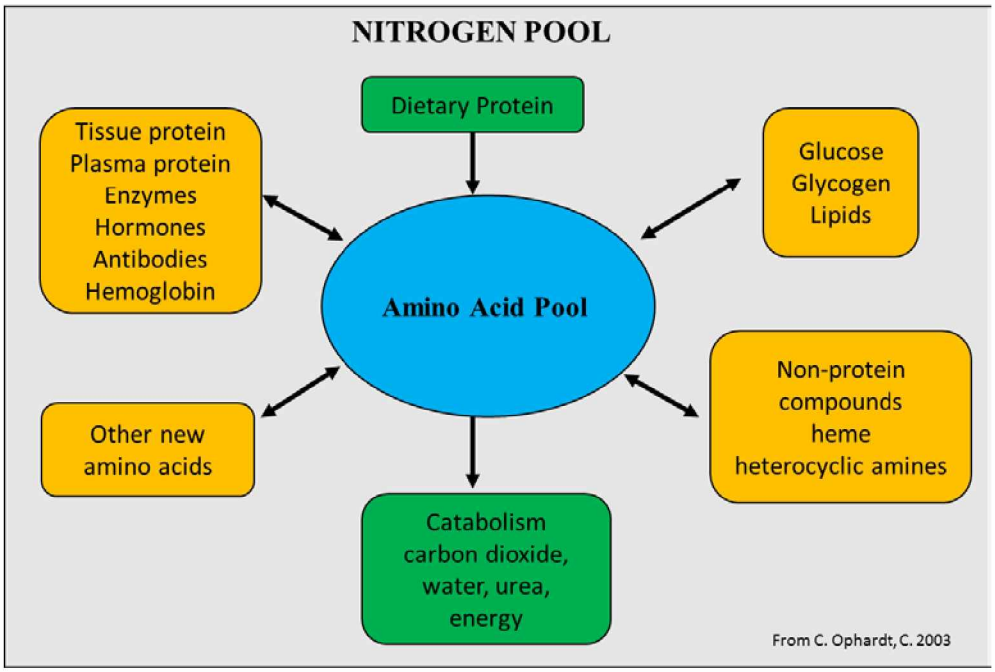


Figure 3.1

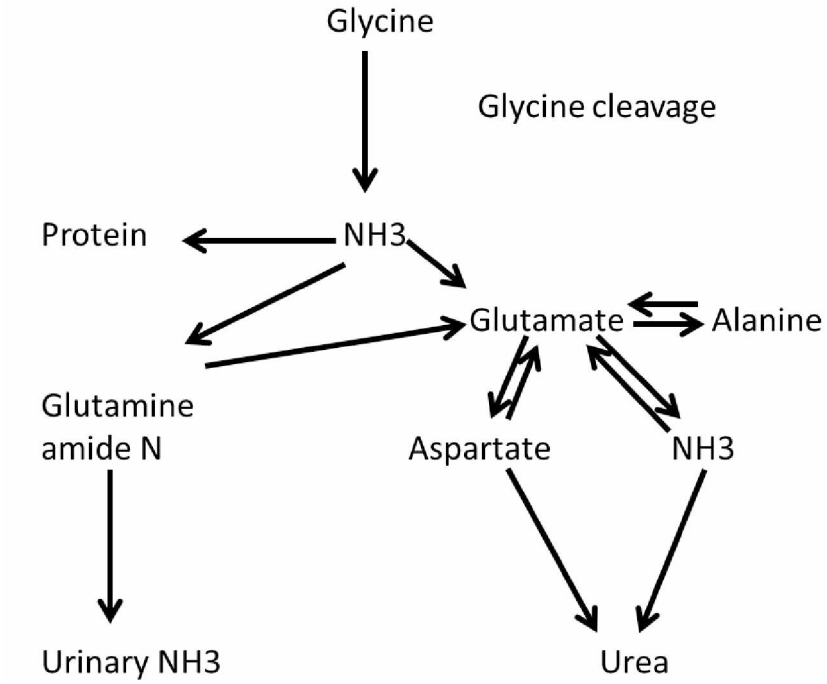


Figure 3.2.

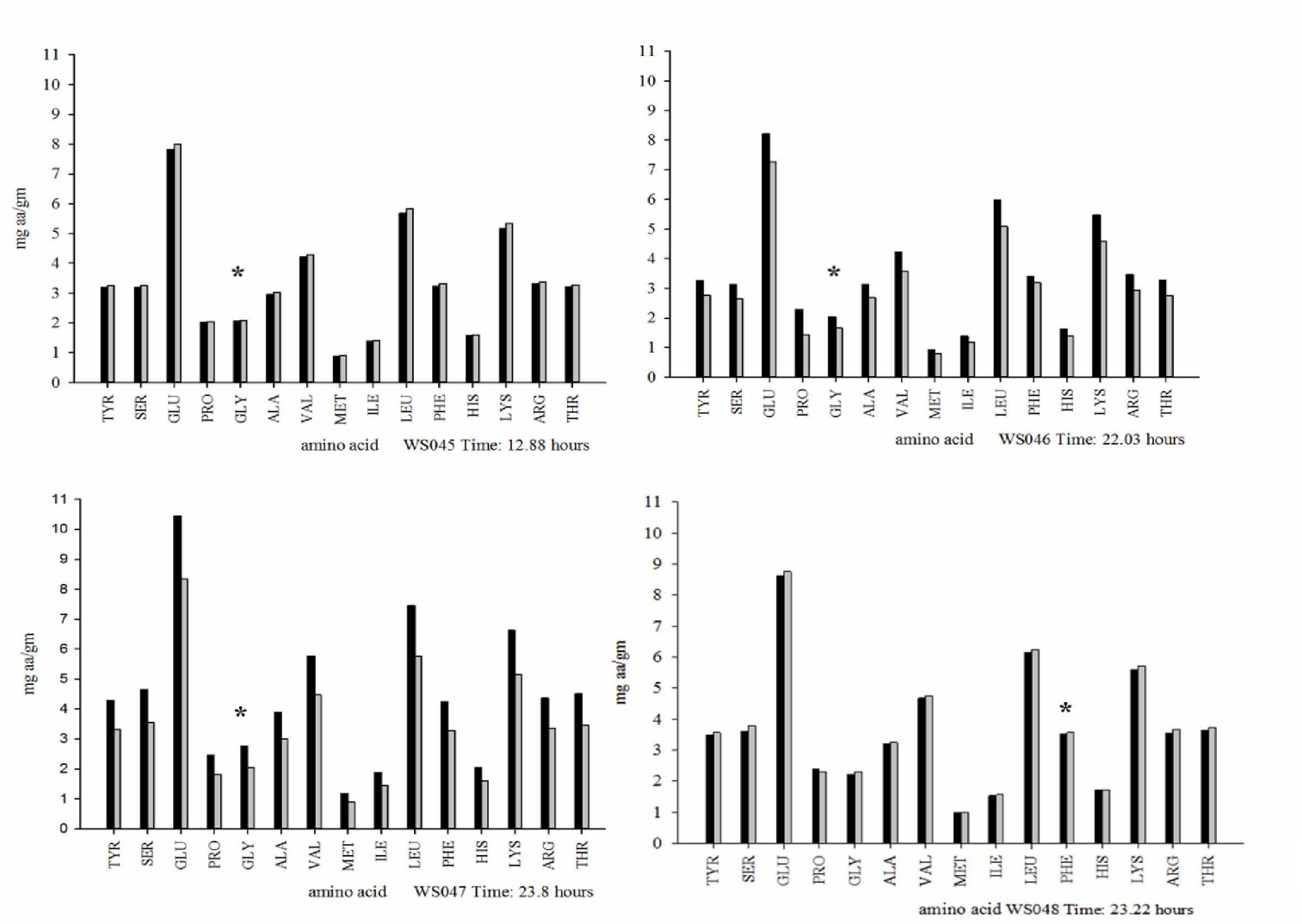

mg aa/gm
amino acid
WS045 Time: 12.88 hours
WS046 Time: 22.03 hours
WS047 Time: 23.8 hours
amino acid WS048 Time: 23.22 hours
TYR SER GLU PRO GLY ALA VAL MET ILE LEU PHE HIS LYS ARG THR

Figure 3.3A

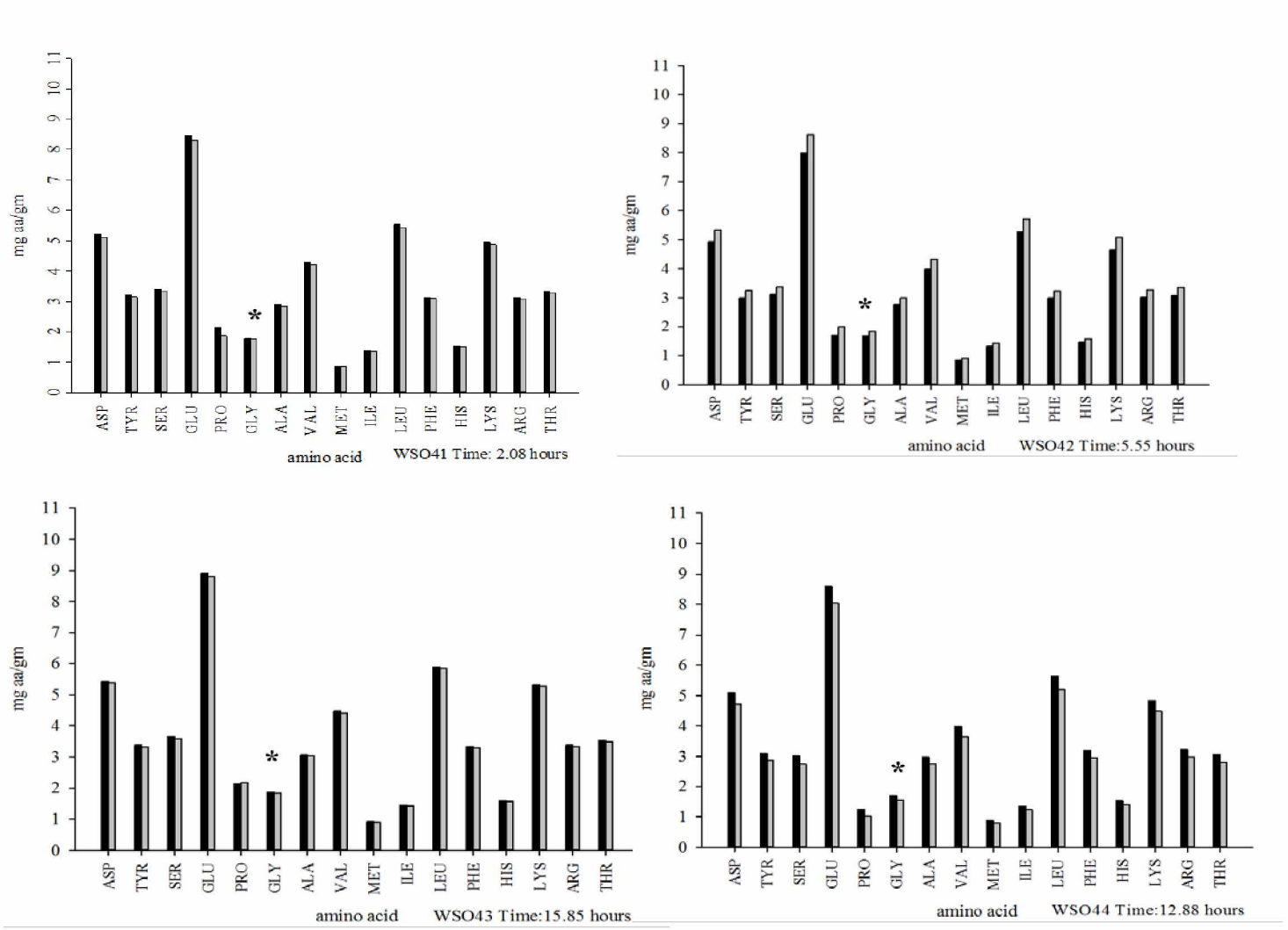

mg aa/gm
0 1 2 3 4 5 6 7 8 9 10 11
ASP TYR SER GLU PRO GLY ALA VAL MET ILE LEU PHE HIS LYS ARG THR
*
amino acid
WSO41 Time: 2.08 hours
mg aa/gm
0 1 2 3 4 5 6 7 8 9 10 11
ASP TYR SER GLU PRO GLY ALA VAL MET ILE LEU PHE HIS LYS ARG THR
*
amino acid
WSO42 Time:5.55 hours
mg aa/gm
0 1 2 3 4 5 6 7 8 9 10 11
ASP TYR SER GLU PRO GLY ALA VAL MET ILE LEU PHE HIS LYS ARG THR
*
amino acid
WSO43 Time:15.85 hours
mg aa/gm
0 1 2 3 4 5 6 7 8 9 10 11
ASP TYR SER GLU PRO GLY ALA VAL MET ILE LEU PHE HIS LYS ARG THR
*
amino acid
WSO44 Time:12.88 hours

Figure 3.3B

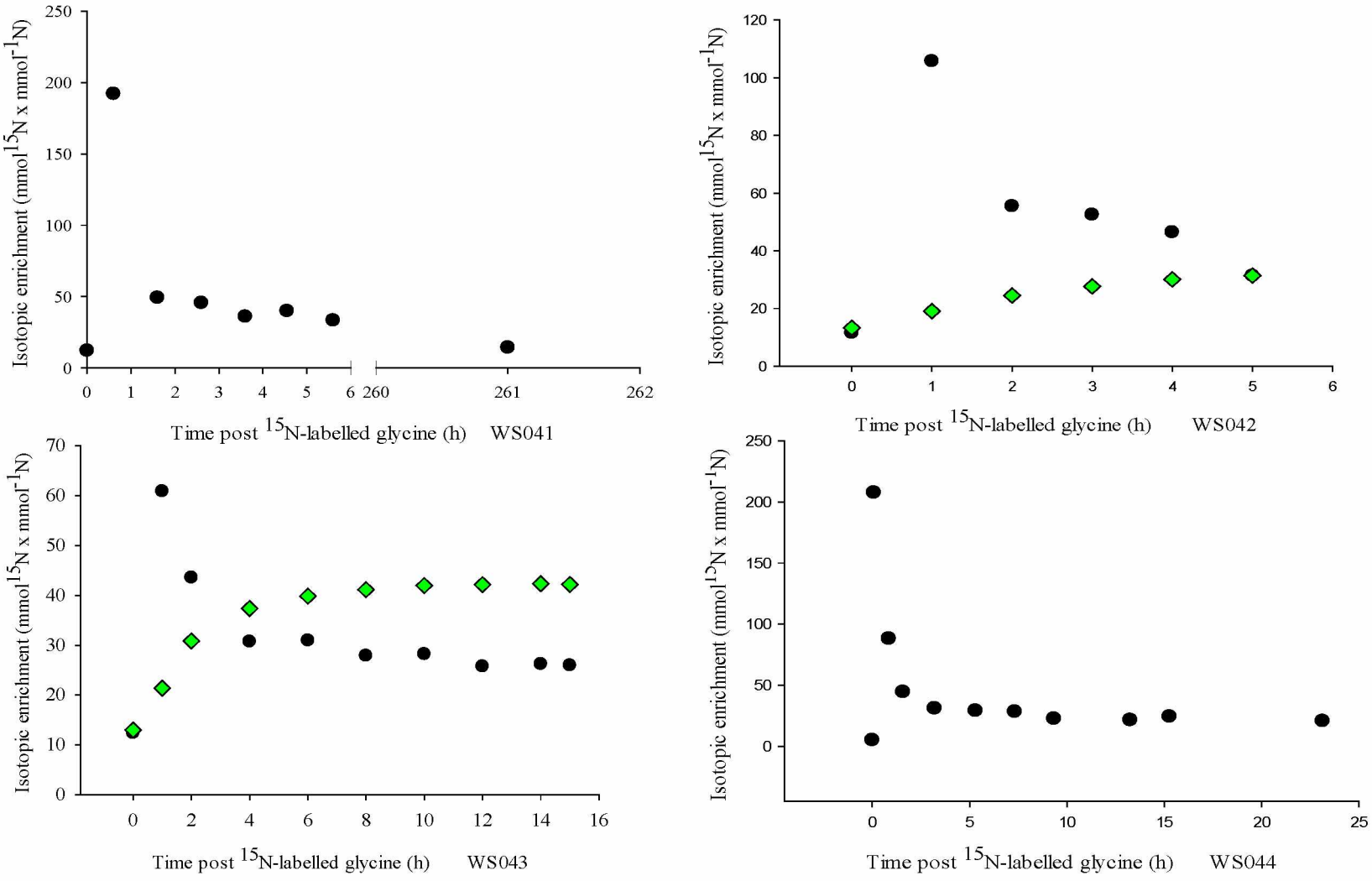

Isotopic enrichment (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled glycine (h) WS041
Isotopic enrichment (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled glycine (h) WS042
Isotopic enrichment (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled glycine (h) WS043
Isotopic enrichment (mmol ^{12}N x mmol^{-1}N)
Time post ^{15}N-labelled glycine (h) WS044

Figure 3.4A

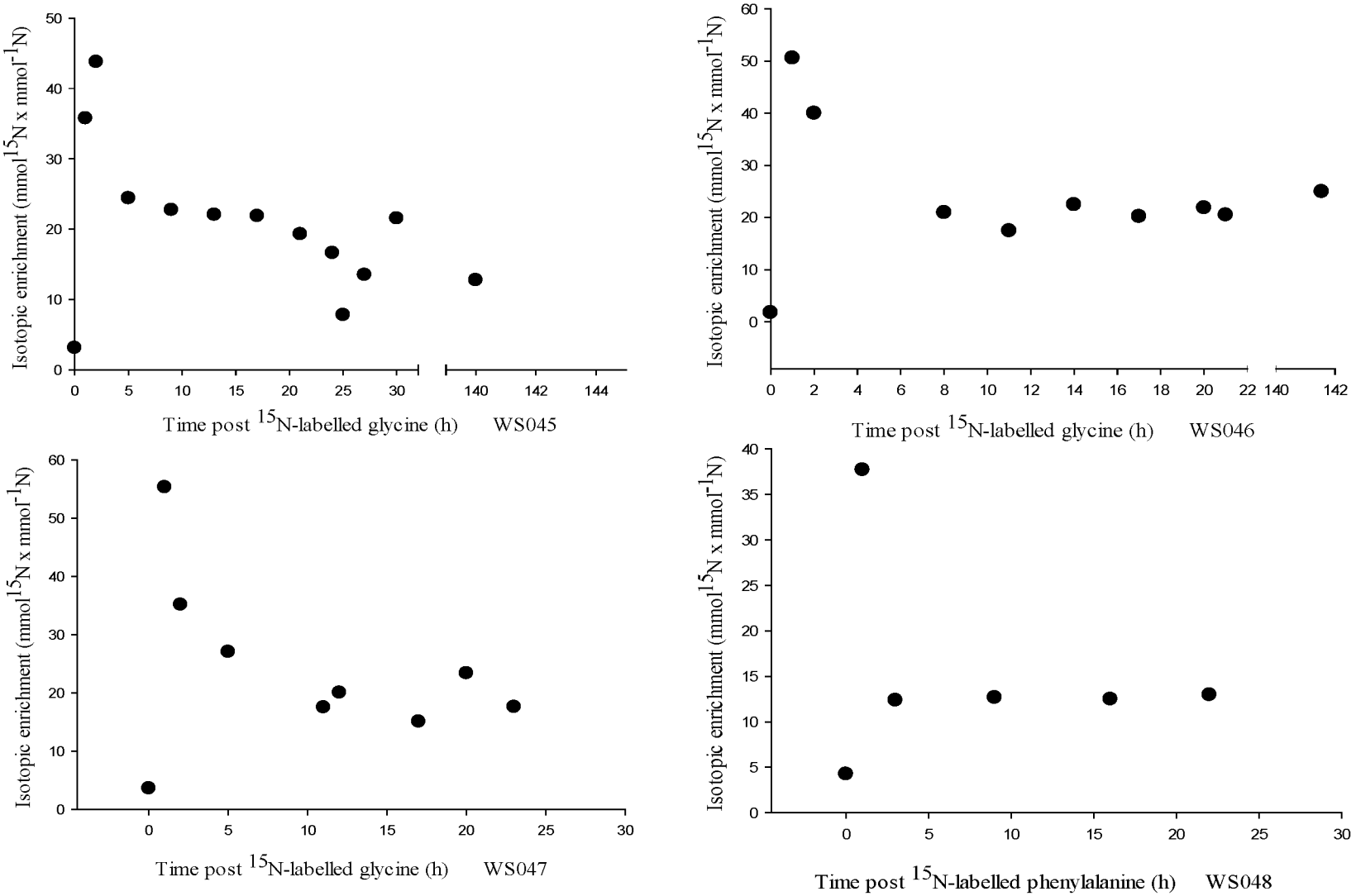

Isotopic enrichment (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled glycine (h) WS045
Isotopic enrichment (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled glycine (h) WS046
Isotopic enrichment (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled glycine (h) WS047
Isotopic enrichment (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled phenylalanine (h) WS048

Figure 3.4B

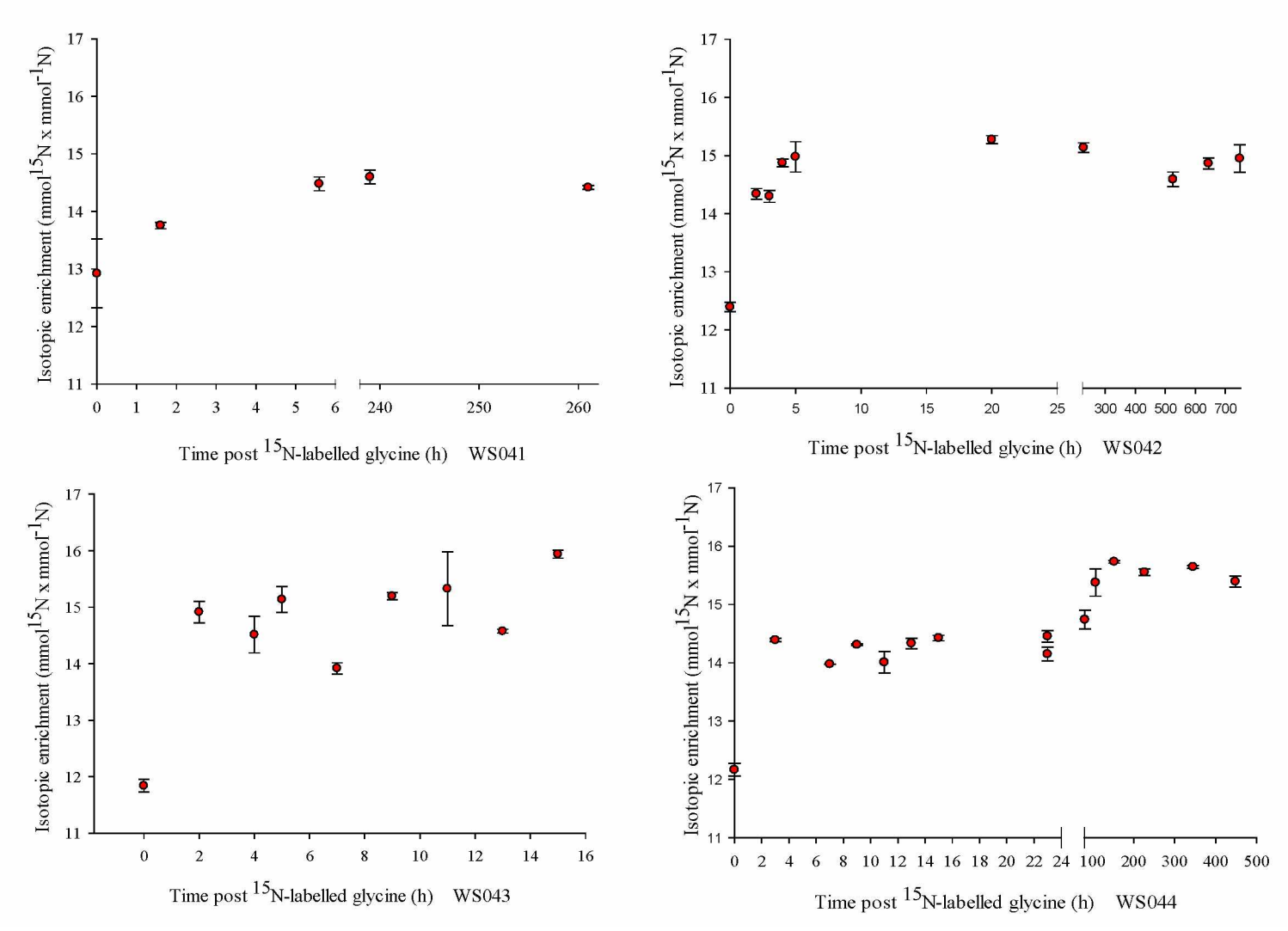

Isotopic enrichment (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled glycine (h) WS041
Time post ^{15}N-labelled glycine (h) WS042
Time post ^{15}N-labelled glycine (h) WS043
Time post ^{15}N-labelled glycine (h) WS044

Figure 3.5A

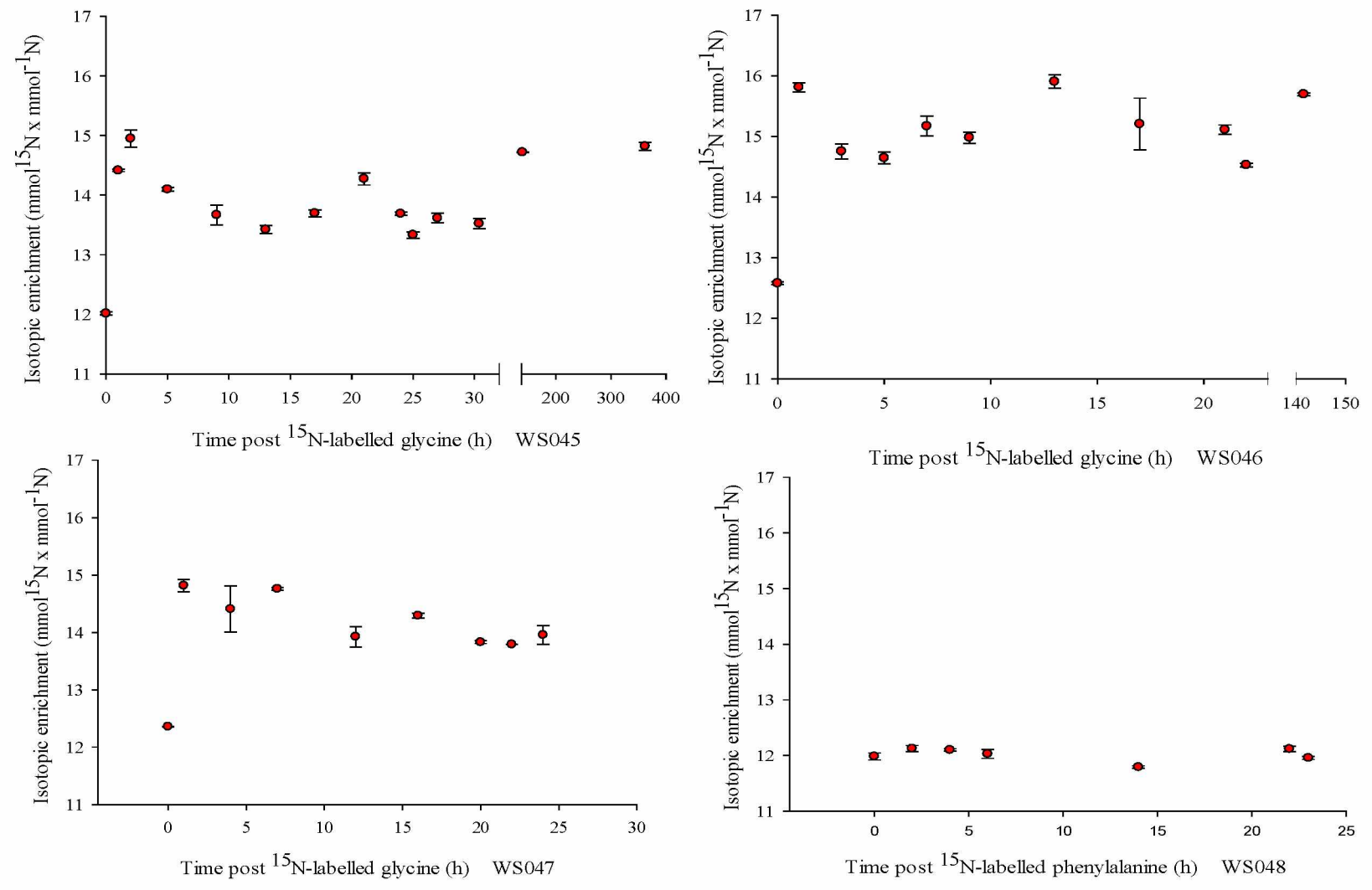

Isotopic enrichment (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled glycine (h) WS045
Time post ^{15}N-labelled glycine (h) WS046
Time post ^{15}N-labelled glycine (h) WS047
Time post ^{15}N-labelled phenylalanine (h) WS048

Figure 3.5B

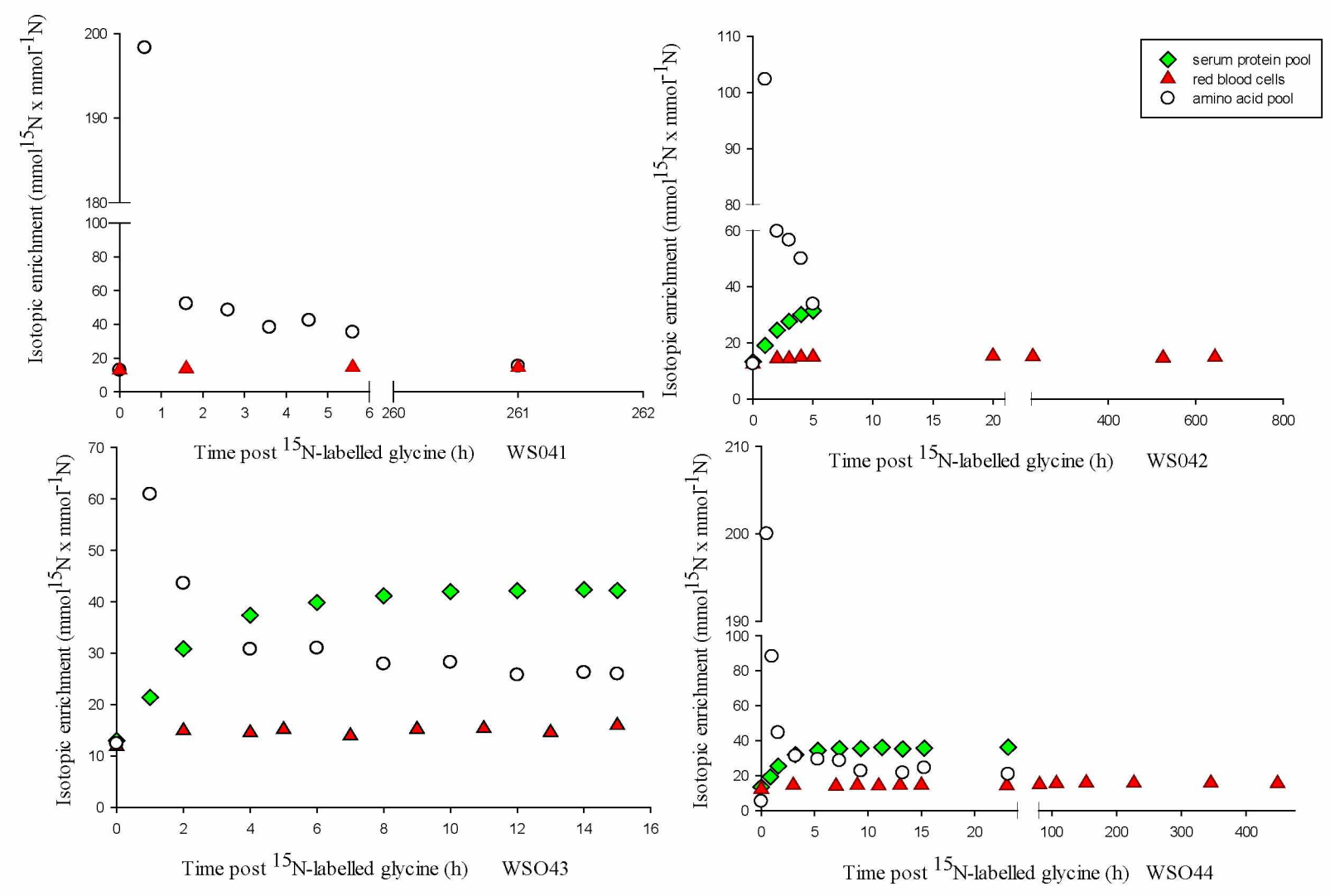

Isotopic enrichment (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled glycine (h)
WS041
WS042
WSO43
WSO44
serum protein pool
red blood cells
amino acid pool

Figure 3.6A

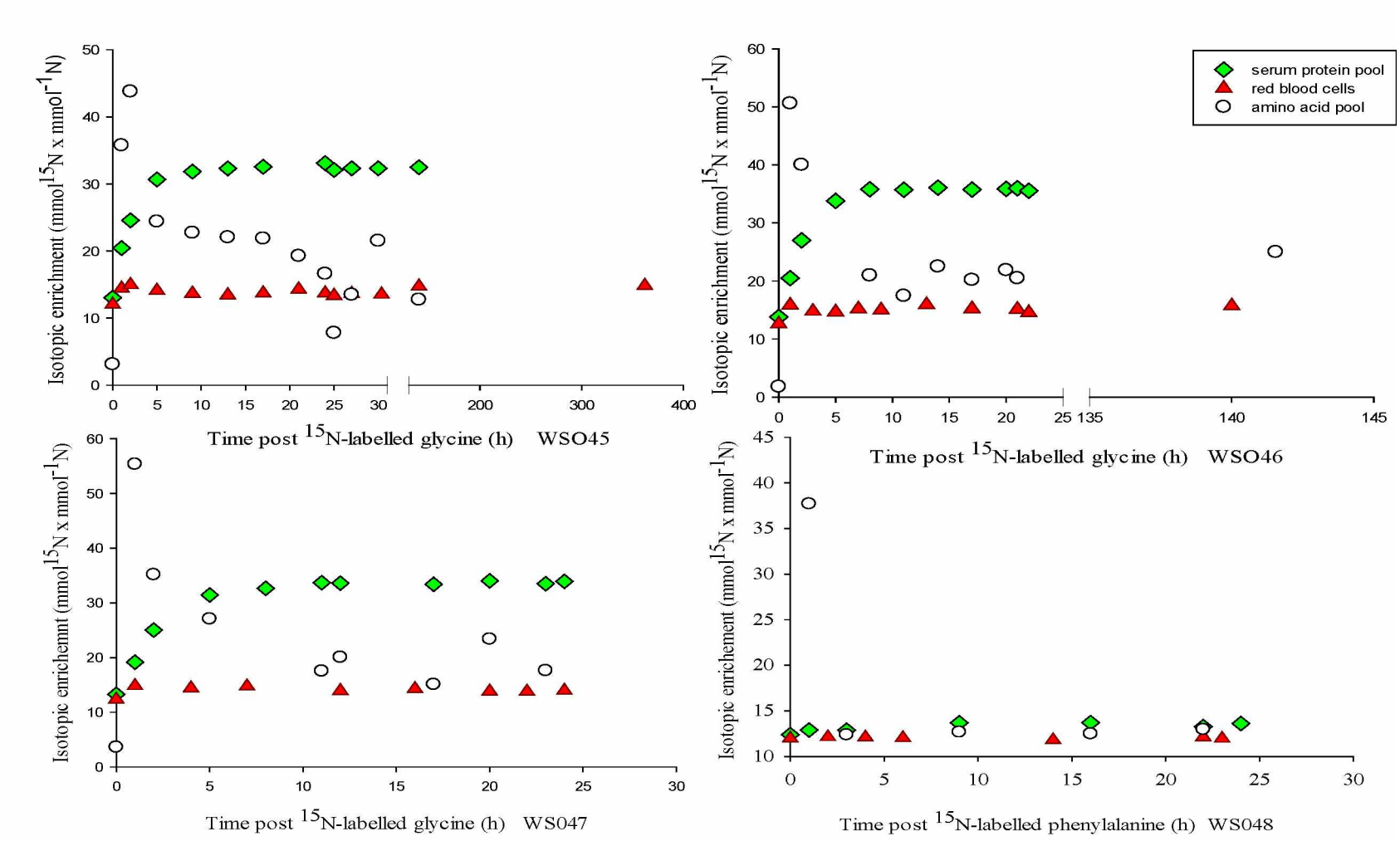

Isotopic enrichment (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled glycine (h) WSO45
Isotopic enrichemnt (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled glycine (h) WS047
Isotopic enrichment (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled glycine (h) WSO46
Isotopic enrichement (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled phenylalanine (h) WS048
serum protein pool
red blood cells
amino acid pool

Figure 3.6B

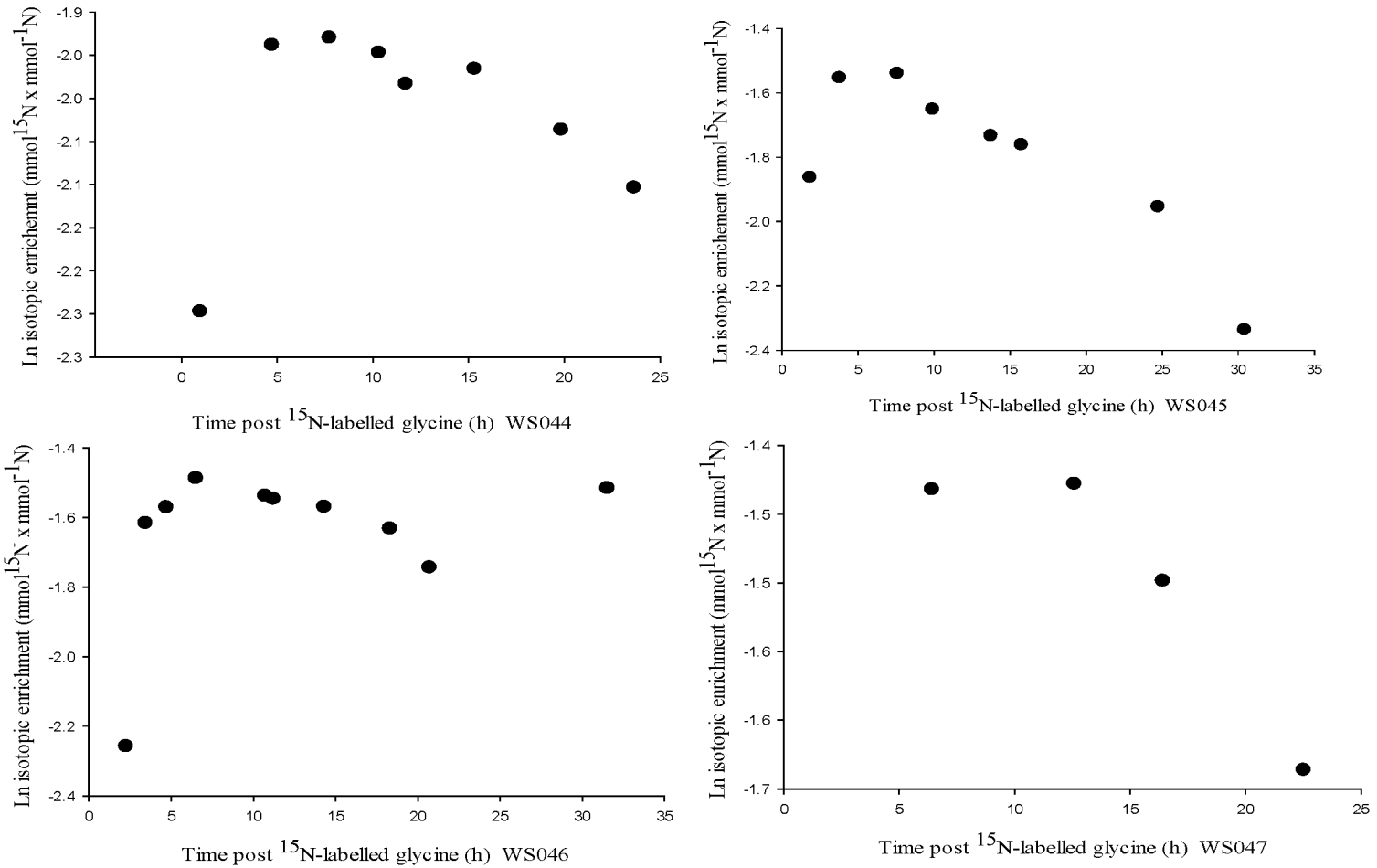

Ln isotopic enrichemnt (mmol15N x mmol-1N)
Time post 15N-labelled glycine (h) WS044
Ln isotopic enrichement (mmol15N x mmol-1N)
Time post 15N-labelled glycine (h) WS045
Ln isotopic enrichment (mmol15N x mmol-1N)
Time post 15N-labelled glycine (h) WS046
Ln isotopic enrichment (mmol15N x mmol-1N)
Time post 15N-labelled glycine (h) WS047

Figure 3.7

Appendix 1

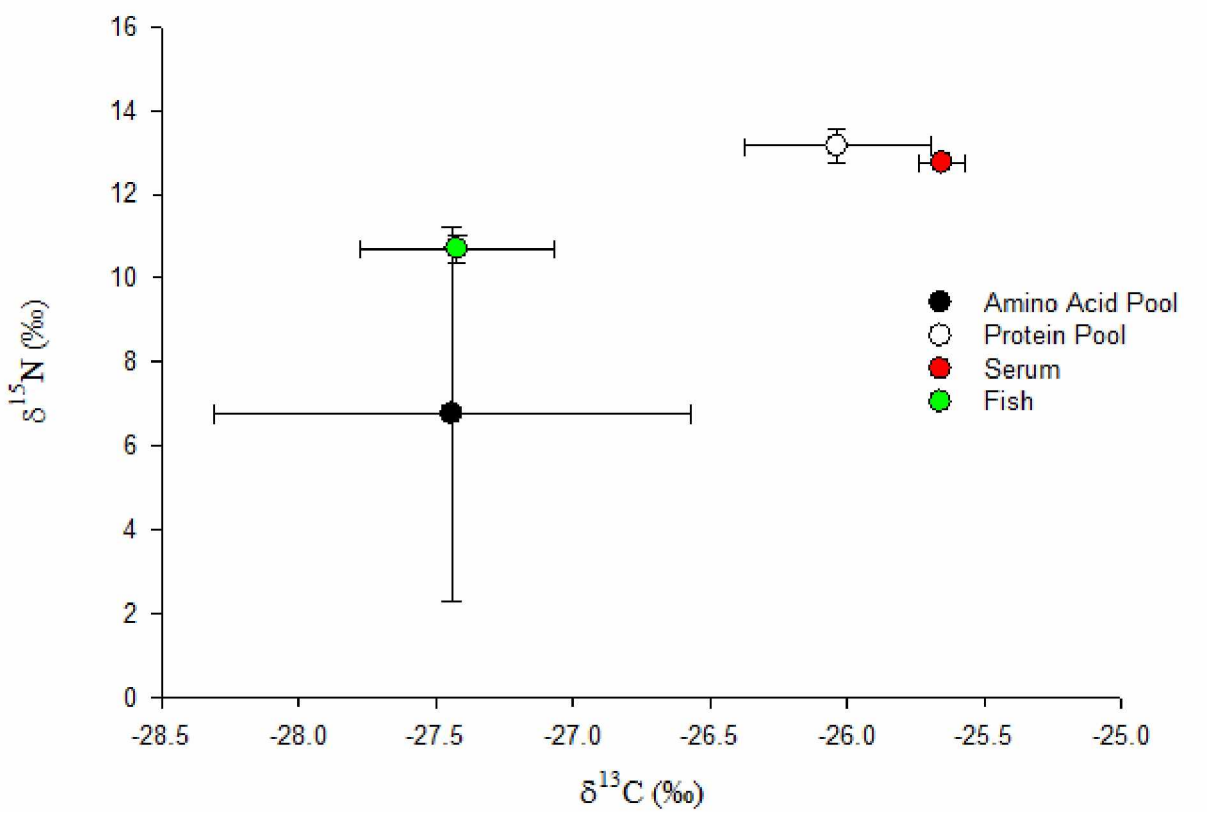


Figure A1. The stable isotopic (C and N) signatures of pooled prey species of fish and Weddell seal serum, serum amino acid and protein pool for WS041-8 ±SD. The amino acid pool is similar to the fish isotopic signature, while the protein and serum signatures overlap.

Chapter 4
Whole Body Protein Turnover in Subadult Alaskan Harbor Seals (*Phoca vitulina*)[1]

Abstract

The role of dietary protein in marine mammal nutrition is poorly understood. Although these mammals derive most of their daily energetic needs from lipid, protein regulation is vital for cellular maintenance, fasting metabolism, exercise and development. We measured whole body protein turnover in 2 groups (Cohorts) of harbor seals fed a mixture of squid and fish to test the effects of dietary protein, season and age on protein turnover. The study was conducted twice a year during the summer molt and winter from when the seals were 2 months to ~3 years of age. Body composition was monitored using deuterium oxide and sodium bromide dilution to monitor changes in body water and extra cellular space. We followed a single dose of ^{15}N-labelled glycine tracer in blood and urinary urea over 48 hours to calculate protein turnover. Rates of protein turnover increased during the winter and decreased in the summer, while dietary protein intake increased during the summer and decreased in the winter. This pattern corresponded with an increase in mass observed in the animals in the winter trial with protein allocation favoring protein synthesis. During the summer trials animals were in different stages of the molt and this process was associated with decreased rates of synthesis and increased rates of protein breakdown. The increased rate of protein breakdown and high individual variability in protein turnover observed was likely due to the hormonal influence on protein metabolism. Age did not appear to influence protein turnover rates except in newly weaned animals, suggesting that there is a difference in the way animals that are newly weaned process dietary protein. Together, these results suggest a complex regulation of dietary protein allocation to balance growth with seasonal metabolic costs.

[1]Inglis, S.D., Barboza, P., Carpenter, J., Atkinson, S. and M.A. Castellini. Whole Body Protein Turnover in subadult Alaskan Harbor Seals (*Phoca vitulina*). Prepared for Submission to Physiological and Biochemical Zoology

Introduction

Pinnipeds are aquatic carnivores that have developed unique strategies to survive in an energetically challenging aquatic environment. Over the last three decades several harbor seal (*Phoca vitulin*a) populations have dramatically declined in Alaska (Boveng et al. 2003). Although nutritional stress has been proposed as a factor in the population decline (Alverson 1992; Spies 2007), harbor seals are carnivores that consume a diverse diet of fish (piscivores) that often varies with the season availability of prey. Because dietary adaptation is based on the type of food consumed and the flexibility of digestive and metabolic processes (Singer 2003), a flexible digestive and metabolic response to dietary substrate would accommodate the unpredictability of prey type and abundance (Geiger et al. 2013). Further, this might reduce the cost associated with increased foraging bouts to meet energetic prey requirements.

Captive harbor seals maintained on herring (*Clupea pallasii*) and walleye pollock, (*Theragra chalcogramma*) were found to maintain and gain weight when placed on a single source low fat (walleye pollock) diet through digestive adaptations including increased feed assimilation efficiency, gut fill and intake (Trumble et al. 2003). Plasma amino acid levels measured during that experiment found evidence of differing protein metabolic pathways corresponding to herring and pollock diets and a decrease in the free amino acid pool in harbor seals maintained on the pollock diet (Zhao et al. 2006). These findings suggest that protein turnover and regulation were being altered. Similar results were observed in adult Steller sea lions, *Eumetopias jubatus* (Castellini 2001). Other studies (Stanberry 2003; Tsugawa 2009) have also shown that captive harbor seals exhibit digestive flexibility with respect to different prey type (e.g. high fat vs. low fat diets). However, the metabolic flexibility of these processes and in particular the role of protein regulation in response to metabolic requirements is not well understood.

Subadult phocids experience rapid somatic (body) growth accompanied by seasonal energetic requirements for molting and "over-wintering" that affect both their energy requirements and patterns of energy use (Field et al. 2005). These metabolic requirements are particularly challenging in the first year of life when young animals learn to forage for prey. In Alaska, harbor seal pups are born in May-mid July, weaned after approximately one month, and attain sexually mature at 3-7 years of age in males and approximately 2-6 years of age in females (Bigg 1981; Hutchinson et al. 2016). Harbor seals usually molt in late summer but the timing of the molt depends upon sex and age class (Daniel et al. 2003). Several studies have confirmed seasonal changes in the physiology and metabolism of Alaskan harbor seals (Fadely et al. 1998). Thus, harbor seals exhibit age class and seasonal body mass and composition variations (Pitcher 1986, 1990; Chabot and Stenson 2002; Tsugawa 2009). Pitcher (1986) identified three energetically different seasons for harbor seals; summer (May - July), molt (August-September), and winter (October-April).

The importance of protein in muscle structure, enzymes structure and function, membrane transport, immune functions and hair regeneration in molting underscores the need to study nutritional and metabolic impacts on protein regulation (El-Khoury 1999). Small alterations in protein metabolism may not be relevant from a caloric perspective but could have significant consequences to animal health. This is particularly true in young animals that have an energetic demand for the production of new tissue for growth and development, and yet may be vulnerable to periods of nutritional stress during weaning (Robbins 1983; Schmidt-Nielsen 1997). Thus, subadult animals must develop energetic strategies to accommodate changing physiological and environment conditions (Weiser 1994).

Phocids will spare protein and preferentially use lipid stores for energy when food sources are restricted, but must also retain blubber for metabolic water, thermoregulation, buoyancy, and hydrodynamic streamlining (Worthy and Lavigne 1987; Webb et al. 1998). However, unlike lipid, protein is stored in muscle tissue and mammals can generally not tolerate a loss in lean body mass greater than 20-30 % (Cahill 1978; Pernia 1984). Biochemically, approximately 1 gram of nitrogen is required to synthesize 6 grams of protein (Waterlow 1999). Thus, amino acid conservation and nitrogen sparing is a critical component in fasting physiology (Castellini and Rea 1992). For example, at the onset of the post weaning fast in the northern elephant seal (*Mirounga angustirostris*) 4 % of their energetic needs are provided by protein catabolism, but that value reduces to ~1 % by the end of the fast (Adams and Costa 1993). Since northern elephant seals fast during energetically challenging conditions (post weaning, lactation, molting) several protein turnover studies have been conducted on these animals representing a fasting pinniped model for protein metabolism (Pernia 1984; Crocker et al. 1998; Houser and Costa 2001).

The dynamics of protein turnover within the body include two opposite processes: protein synthesis and protein breakdown or degradation. Whole body protein turnover is the rate at which protein is synthesized and degraded. Tracer methods provide a simple technique to quantify the summed kinetics of these opposite processes (Waterlow 1984; Huntley et al. 1987; Wolfe 1992; Fouillet et al. 2002). Several different types of physiological tracers have been used to measure protein turnover in animals. In northern elephant seal (Pernia 1984; Crocker et al. 1998; Houser and Costa 2001) and Antarctic fur seal (*Arctocephalus gazelle*) (Arnould and Hindell 2001) studies a single bolus dose of ^{14}C-labelled urea was used to trace urea turnover with blood and urine endproduct collection.

The amino acid stable isotope tracer ^{15}N-labelled glycine followed by blood and/or urine endproduct collection is another common tracer used to study protein turnover. The non-essential amino acid glycine is active in protein synthesis and its metabolic pathway includes excretion through urinary urea (Fern et al. 1985; Waterlow 2006). This tracer is regularly used in human studies (Waterlow 2006) and has been used broadly in other mammals including bears *Ursus americanus* and *U. arctos* (Barboza et al. 1997), honey possum (*Tarsipes rostratus*), kangaroos (*Macropus robustus robustus* and *M. erubenscens*), feral goats (*Capra hircus*) (Freudenberger and Nolan 1993), and cats (*Felis silvestris catus*) (Russell et al. 2003).

Whole body protein turnover is the sum of all turnover activities of individual proteins in the body (El-Khoury 1999) and provides an indication of nutritional protein requirements and metabolic flexibility (Barboza et al. 1997; Russell et al. 2003). By measuring whole body protein turnover, we can test the effects of dietary intake and metabolic demands such as growth and season on the rate of nitrogen flux as well as how available nitrogen is partitioned to accommodate these demands. This study examines the effects of age, season and dietary protein intake on protein turnover rate and protein allocation in subadult harbor seals.

Methods

Animals and Experimental Design

Protein turnover experiments were conducted on eight newly weaned female harbor seals housed at the Alaska SeaLife Center (ASLC) in Seward, AK. Four animals were collected in July 2004 and four other animals July 2005 from the Prince William Sound/ Kenai Fjords seal populations that have shown historical declines. Animals were housed in salt water pools with access to natural photoperiods and temperatures. Daily feeding records were used to measure intake. The

animals were divided into two groups of four based on the year of collection (Cohort) and fed a mixed diet of herring, pollock, capelin (*Mallotus villosus*) and squid (*Loligo spp.*) (Tsugawa 2009) (Table 4.1). The diet produced differences in crude protein (g d^{-1}) intake per individual. The Cohorts (2004 and 2005) were grouped by age with season and protein intake as co-variables. Protein turnover was measured during harbor seal molt in August (summer) and January (winter) trials (Pitcher 1986), starting in January 2005 and concluding in January 2007.

Isotopic tracer methods using ^{15}N-labelled glycine were used to determine the effect of season and age on protein turnover. The amino acid glycine was chosen as it is a non-essential amino acid that is involved in protein synthesis and includes urinary urea in its metabolic pathway (El-Khoury 1999). Whole-body protein kinetics studies were conducted via endproduct methods (Picou and Taylor-Roberts 1969; Waterlow et al. 1978) using a modified single dose of ^{15}N-labelled glycine (Picou and Taylor-Roberts 1969; Barboza et al. 1997; Russell et al. 2003; Waterlow 2006). This method assumes that there are two nitrogen pools in the body, a free amino acid pool and a protein pool and that there is no re-entry of the isotope from the protein pool into the free amino acid pool within the trial period (Figure 4.1). It also assumes that the isotope label excreted in the urine urea endproduct is the same as the contribution of the unlabeled N. The injected ^{15}N-labelled glycine signal enters the free amino acid pool by transamination. Amino acid nitrogen (N) leaves the free pool and enters the protein pool via protein synthesis; excess amino acid nitrogen in the form of ammonia is converted to urea and excreted (Waterlow 2006).

Animals were trained to rest on a platform scale for measures of body weight (mean ± 0.2 kg). We used individual metabolic cages to collect feces and urine for 72 hours (Figure 4.2). During the trial period the animals were maintained on their assigned diet regime and their daily intake

recorded. Fecal samples were collected hourly, with the time of collection recorded, the sample weighed and frozen for subsequent analyses. Urine samples were collected at hourly intervals into a beaker that was cooled with ice packs. We recorded time of collection before filtering the urine sample to remove debris and to measure volume (in ml). We added 1ml of 5M HCl per 100 ml urine to prevent bacterial ureolysis and loss of ammonia. Samples were stored in polycarbonate bottles at -20 ^{0}C for subsequent analyses.

After 24 hours of urine and fecal baseline sample collection, the animals were weighed again and a pre ^{15}N-labelled glycine blood sample (7 ml whole blood) was collected intravenously (IV) into a serum separator tube. Blood was allowed to coagulate and the serum was separated by centrifugation and then stored initially at -20 ^{0}C and then moved to a -80 ^{0}C freezer prior to analysis. The average volume of serum collected was 2.5 ml. All IV procedures were conducted via the extradural intravertebral venous sinus with the animals under behavioral constraint. The animal was injected with ^{15}N-labelled glycine (99.5 atom %) (Cambridge Isotope Laboratories, Inc.) and diluted in sterile saline solution (100 mg ml^{-1} in 0.9% saline) via IV catheter at a dose of 5.0 mg kg^{-1} body mass (BM) followed by a 10 ml saline flush and returned to the metabolic cage. The syringe was weighed (± 0.000) prior to and following the injection and the change in weight recorded to calculate the actual ^{15}N-labelled glycine dose. Fecal and urine samples continued to be collected for another 48 hours. Following the 48 hour period the animals were weighed again and a post ^{15}N-labelled glycine blood sample collected for serum analysis (7 ml whole blood).

Total body composition (% lean and % fat tissue) was assessed using deuterium oxide (D_2O) at a dose of 100 mg kg^{-1} BW to measure total body water (Metabolic Solutions Technical Paper #913). Total body water results cannot distinguish between water in tissues (muscle and fat) and

water that is extracellular. Calculation of lean tissue and fat assumes that extracellular water stays the same and the only change is in fat or lean tissue. Studies have shown in other animals that extracellular water volumes can change due to poor nutrition, growth or disease. To determine the amount of water external to the cells, sodium bromide (NaBr) salt (Cambridge Isotope Laboratories, Inc.) diluted (8 %) in a sterile saline solution and at a dose of 40 mg kg^{-1} BW was administered intravenously (IV) at the same time as the D_2O injection. When NaBr is injected into blood it distributes into the whole body ECW compartment (Fielding et al. 2003). The syringe was weighed (±0.000) prior to and following the injection and the change in weight recorded to calculate the actual NaBr dose. Whole blood samples (7 ml whole blood) for the D_2O and NaBr analyses were collected (IV) into a serum separator tube two and a half hours post injection. These samples were sent to Metabolic Solutions for isotope analysis and the results were used to calculate total body (Reilly and Fedak 1990) and extracellular body water values (Miller et al. 1989; Wong et al. 1989).

Values on animal weight (kg), length (cm), body composition, prey analyses, assimilation efficiency, protein intake (Tsugawa 2009), and blood chemistry (Allard 2006) were collected concurrently with the protein turnover trials, and were used in the body water, nitrogen balance, and nitrogen flux calculations. All experiments and handling procedures were approved by the ASLC and University of Alaska Institutional Animal Care and Use Committee IACUC (Protocol # 03-009 and 06-006), and under the NMFS permit # 881-1710.

Laboratory Analysis

Serum Enrichment

All blood samples were centrifuged for 15 minutes following blood draw, the serum extracted into cryovials, initially stored at -20 ^{0}C and then moved to a -80 ^{0}C freezer prior to analysis. The condition of the serum was recorded (e.g. presence/absence of hemolyzation). Frozen 2 ml subsamples were then freeze-dried under vacuum (VirTis Freeze Dryer Model 5463, CirTis, Gardiner, New York) for 48 hours. Dried serum samples were weighed (~0.300 mg) into tin (Sn) capsules for stable isotope signature analysis using an isotope ratio mass spectrometer (Finnigan Delta Plus XP). The isotopic compositions of $\delta^{15}N$ was reported relative to air. Stable isotope ratios were expressed in δ notation as parts per thousand (‰) according to $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1{,}000$ where $X = {}^{15}N$ and $R_{sample} = {}^{15}N/{}^{14}N$. The change in $\delta^{15}N$ pre and post the ^{15}N-labelled glycine injection was calculated ($\Delta^{15}N$).

Extraction of Urine Urea:

Urea labelled with ^{15}N was isolated from 2 ml of whole urine for analysis using a double steam distillation process adapted by Barboza et al. (1997) and based on the method of (Nolan and Leng 1972). Distillation procedures took place in an alkalized environment using an ammonium sulfate (NH_4SO_4) standard. During the first distillation all ammonia-N was removed. The receiving flask with the remaining urea was fitted with a rubber stopper and incubated overnight with the enzyme urease (Jack Bean in 0.2 M phosphate buffer at pH7; Sigma Chemicals, St. Louis, Missouri) to hydrolyze urea to produce ammonium (NH_4^+).

During the second distillation, ammonia from the urea was captured in a solution of 4 % boric acid containing a color indicator (Kjelsorb, Fisher Chemicals). This distillate represents complete

recovery of ^{15}N from urea and titrated with 0.01N hydrochloric acid (HCl) to quantify the amount of NH_4^+. The distillate was dried in a drying oven at 100 ^{0}C to provide solid ammonium borate for subsequent isotopic analysis. Isotope enrichment was determined using an isotope ratio mass spectrometer (Finnigan Delta Plus XP) (see Blood Sample section for details). A sample weight of ~0.300 mg was used for this analysis.

Protein Turnover Calculations

Protein turnover was calculated using ^{15}N urine urea kinetics expressed as isotopic enrichment following methods of Barboza 1997. Isotopic enrichment (IE) was calculated from the fractional isotope abundance (F) using the following equations:

$$F = ((\delta^{15}N /1000)+1/((1/0.003677)+(\delta^{15}N /1000)+1))$$

$$IE= F - (\text{predose F})$$

The IE was log transformed (LN) and the regression of LN(IE) with time (t) from time of entry of injected ^{15}N into the protein pool to the end of the trial (~ 48 hours) after dosing provided IE_0 and the fractional turnover rate (k).

Nitrogen flux (Q) was calculated using the following equation:

Q= total N excreted in urea x (dose of ^{15}N given / total ^{15}N excreted in urea)

$$Q = \int_0^{48} IE_0 \cdot e^{kt}\, dt$$

Protein kinetics were based on a pool of amino acid N connected with a large protein pool (Picou and Taylor-Roberts 1969; Wolfe 1992). The results were expressed as mmol N/48 hours and

converted to crude protein using the conversion factor 6.25 based on 0.16 g nitrogen per g protein (Robbins 1983; Barboza et al. 2009).

Rates of protein synthesis and breakdown were calculated from the following equation:

$$Q=E+Z=I+B$$

where Q is the nitrogen urea flux (units), E is the total N excretion (urine and feces) (units), Z is the rate of whole body synthesis (units), I is the rate of dietary intake of N and B is the rate of whole-body protein breakdown (Barboza et al. 1997; Russell et al. 2003). Rates were reported based on metabolic mass as g kg^{-75} day^{-1}. Values for dietary intake (I) and fecal output (E) were collected concurrently with the protein turnover experiments (Tsugawa 2009). Urine samples for each day of the trial were pooled and a 25 ml composite sample collected for nitrogen content (E). These samples were weighed and freeze dried to a constant weight to determine the water weight (g). The N content of the dried samples were determined by the Dumas method using an automated Leco FP428 analyzer (The Leco Corporation, Saint Joseph, MI). The values were then converted from nitrogen (g) dry weight to nitrogen (g) wet weight using the water weight to provide a total N content to the volume (ml) of urine collected during the protein turnover experiment (N excreted).

Body Water

Total body water (TBW) values from Tsugawa (2009) were collected during the protein turnover experiments and used for the analysis into body water dynamics during the trial periods. Extra cellular water (ECW) was calculated by measuring the bromide space as described below. Intracellular water was calculated as:

$$ICW\ (l) = TBW(l) - ECW(l)$$

Extra Cellular Water

Whole blood samples were centrifuged for 15 minutes and the resultant serum extracted into cyrovials and initially frozen at -20 ^{0}C and then moved to a -80 ^{0}C freezer for storage prior to analysis. Samples were shipped to Metabolic Solutions Inc. Amherst, MA for analysis of the bromide dilution space NaBr (umol l^{-1}). The results were modified to g l^{-1} using the molecular weight of NaBr (102.89 g). The bromide space was calculated as the volume of bromide distribution (V_{Br}) using the equation from Fielding et al. 2003:

$$V_{Br}=\text{Br dose}/([\text{Br}]_{eq} - [\text{Br}]_{zero})$$

Where Br dose is the dose administered and $[\text{Br}]_{eq}$ and $[\text{Br}]_{zero}$ are the bromide concentrations at equilibrium (post plasma sample) and prior to NaBr injection (pre plasma sample) respectively. The dose was calculated for 8 % NaBr in physiological saline solutions using a specific gravity for NaBr of 1.277 gm l^{-1}.

Extra cellular water (EWC) was the corrected bromide space (CBS) using the following equation:

$$\text{ECW (L)} = \text{CBS} = V_{Br} \times 0.90 \times 0.95 \times 0.94$$

Where 0.90 is a correction for the non-extracellular distribution of bromide in the red blood cells, 0.95 is the Donnan equilibrium factor and 0.94 is the proportion of water in the plasma (Metabolic Solutions Technical Paper 915). The bromide loss in urine is typically ~ 1.5 % and has a negligible effect on the measurement of CBS.

Statistical Analysis

Significant differences between the body fluid compartments and blood chemistries by trial were statistically tested using analysis of variance (ANOVA) with age, season, and cohort as co-

variates followed by Tukey's post hoc test on treatment means. Kruskal-Wallis and Mann-Whitney tests were used on results requiring nonparametric analysis (Zar 1984). Comparisons of protein turnover (Q) and fractional turnover rates (k) and protein allocation were conducted using ANCOVA tests with protein intake per metabolic weight (mass $^{-0.75}$) as the covariate. A significance level of 0.05 was used for all statistical tests. Means are reported with the standard deviation (SD) of the sample. Statistical testing was conducted using R software.

Results

Body Composition

The mean changes in the body weight (BW) and composition of each Cohort using D_2O methods are presented in Figure 4.3 using data from Tsugawa (2009). Changes in BW, % fat, % lean, and % TBW were statistically tested with individual and trial variables. In the 2004 (04) Cohorts, there were significant differences found in BW, % lean mass, and % TBS between individuals but no significant difference in % lean mass (ANOVA, $p<0.05$). Within the 2005 (05) cohorts, the only significant difference between individuals was in % fat mass. In comparisons between trials, significant differences were found in all variables tested (BW, % fat, % lean, and % TBW) within the 04 Cohort. Within the 05 Cohorts, significance was found in BW, % fat, and a weak significance in % lean mass ($p= 0.04$, $p= 0.03$ in 2 of 6 trial comparisons; ANOVA, $p<0.05$). There was no significant difference in % TBW between trials.

Results from the analysis on the mean (± SD) changes in the body water compartments (TBW, total body water; ICW, intracellular water; ECW, extracellular water and ECW:TBW ratio) with trial for each Cohort group are presented in Figure 4.4. Several significant differences were observed between individuals within the 04 Cohort group as well. In the 04 Cohort group Sustina had significantly different ICW values than the other cohorts (Miki $p=0.003$, Atuun $p= 0.01$ and

Qilak p=0.05). While the TBW values for Qilak were significantly different from all the other animals (Qilak-Miki p=0.002; Qilak-Susitna p=0.004; Qilak-Atuun p=0.0005). There was no significant effect of individual on the body water analyses results in the 05 Cohort group. The effect of trial was highly significant ($p<0.001$) on the TBW values in the 04 Cohort group but not in the 05 Cohort group. Overall, 04 Cohort group showed more significant effects in the body water analyses both within the group and per trial.

Blood Chemistry

Albumin (ALB), blood urea nitrogen (BUN), creatinine (CREAT) and total protein (TP) are often used as diagnostic values that reflect changes in protein metabolism. Significant changes were observed in several of these blood chemistry parameters both within each Cohort group and per trial period (Table 4.2). The TP values showed the most individual variability, but only in the 05 Cohort group. Significant differences were observed between Tikanni and both Siku and Anya ($p= 0.004$ and $p= 0.006$ respectively). In the 04 Cohort group the BUN values differed between individuals but only between two animals (Qilak and Sustina with $p=0.03$). In both Cohort groups BUN and CREAT values were affected by trial ($p<0.05$). However, in the 05 Cohort group there were highly significant differences ($p<0.001$) in TP with all Trial tests except between winter 2006 (W06) and summer 2006 (S06). All the results were found to be within the normal pooled blood parameter range of wild Alaska harbor seals (Trumble and Castellini 2002).

Whole Body Protein Turnover

Dietary Protein Intake

Dietary protein intake was not constant in these experiments and thus functioned as a covariate in the protein turnover analyses. The animal's metabolic weight was used to calculate both

fractional turnover rate of tracer ^{15}N-labelled glycine (k) and whole body protein turnover (Q). Therefore, protein intake was reported as grams protein /metabolic body weight (protein/BW kg $^{-0.75}$) Table 4.3. There were no significant changes in the animals' weight within the trial period and in only 3 out of 35 trials did an animal's dietary intake significantly decrease during the experiment (designated with * in Table 4.2). Significance was set at $p < 0.05$ (ANOVA, post hoc Tukey tests).

Removing the first summer trial results (S05) for the 05 Cohorts from analysis as an outlier due to an age covariate associated with newly weaned animals, both Cohorts followed a significant seasonal pattern with respect to body weight (kg $^{-0.75}$) and protein intake (kg $^{-0.75}$) (ANOVA, $p<0.001$). Animals exhibited an increase in dietary protein intake per metabolic weight during the summer trials and a decrease during the winter (Table 4.3). These findings corresponded to an increase in body weight (kg $^{-0.75}$) in the winter followed by a reduction in body weight during the summer trials.

Serum enrichment

The change in δ^{15}N or Δ^{15}N between the control (pre ^{15}N-labelled glycine injection) and at the end of the trial indicates that the serum pool was still enriched at the end of the trial regardless of season or Cohort group (Figure 4.5). The larger the Δ^{15}N value the slower the elimination of the labeled isotope from the amino acid pool. Once again, the 04 Cohorts exhibited the greatest within group variability with Atuun as the outlier in both the W05 and S05 trials and both Atuun and Qilak contributing to the upper limit in the W06 trial. The significant difference ($p<0.001$) in the values from the SO5, 05 Cohorts trial suggests that age is contributing to the higher Δ^{15}N value as these animals were 2 months old and newly weaned. Subsequent trials between both Cohorts did not show any significant effects of season or age.

Protein Turnover

Results of the fractional turnover rate (k) of the ^{15}N tracer in the urine urea pool are presented in Figure 4.6 and Table 4.4. The flux of protein (Q) through the urea pool is presented in Table 4.4. The results of ANCOVA analysis on the effect of individual, Cohort group, pooled Cohort groups, trial and season on the protein flux (Q) found season ($p=0.006$) and protein intake ($p=0.04$) to have the greatest effects on this variable. The fractional turnover rate however, was highly significant to the intake effect $p=0.001$, but only weakly impacted by season ($p=0.06$).

Separating the results into Cohort groups using the covariate effects of individual, trial and season on protein turnover, protein intake had a significant effect on the fractional turnover rate ($p= 0.032$) in the 05 Cohort group but not on the overall protein flux. In the 04 Cohort group the individual influenced the protein flux $p=0.05$, but not on the fractional turnover rate. The trial effect that includes both age and season variables did not have any significant effect on the protein turnover values (Q and k) but was nearly significant within the 05 Cohort group $p=0.07$. The 05 Cohort group included very young, newly weaned animals and the isotopic enrichment (Figure 4.6) of the O5 Cohorts in the summer trial of 2005 (05 Cohort S05) is reflected in a regression line that is higher than the other trials.

The effect of intake and season on protein turnover is most evident in the O4 Cohort group. For example, harbor seal Atunn's Q value during the W06 trial was significantly lower than her Cohorts' Q values (Tables 4.3 and 4.4) and this corresponded to a significantly reduced mean protein intake for that trial. Another animal in this Cohort group, Miki also exhibited significantly lower Q values during the summer months, however with increased protein intake. All the animals were in different stages of molt when the summer trials were conducted. In

general, the 04 Cohort group exhibited more individual variability in nitrogen flux dynamics than the 05 Cohorts.

The rates of protein synthesis (Z) and breakdown (B) reported as g $kg^{-0.75}$ day^{-1} for each individual animal are presented in Table 4.5. The values for protein synthesis and breakdown were highly significant for season $p<0.001$ (ANOVA, Tukey tests). Trials where rates of protein breakdown were greater than protein synthesis are highlighted in Table 4.5 and occurred almost exclusively during the summer trials that were associated with increased intake, reduction in mass from the previous trial, and animals at different stages of the molt process. Results from the S05 trial of the 05 Cohort group are consistent with this pattern but may be confounded by the animals being nutritionally challenged as they transitioned to a fish only diet. Both protein synthesis allocation and rates of protein synthesis based on metabolic weight were higher in the winter trials and corresponded to an increase in body mass from the last trial suggesting protein allocated to growth. However, winter trials also had a lower protein intake based on metabolic weight than the summer trials. Once again, 04 Cohorts exhibited the most individual effect on the results.

Discussion

This study presents results on whole body protein turnover dynamics in a nonfasting pinniped. Our results are within ranges reported for growing bears (Barboza et al. 1997), kangaroo and goat (Freudenberger and Nolan 1993) and eutherians (Waterlow 1984), but lower than those reported by Russell et al (2003) on adult domestic cats. Russell et al. (2003) attributed the difference in their results from other studies on omnivores and herbivores to an increased need for specific essential amino acids rather than non-essential N. Although pinnipeds are also carnivores, they have very different seasonal metabolic demands than those in the cat model, and

there can be significant differences in protein kinetics even between subspecies (Freudenberger and Nolan 1993). The present study on harbor seals included the physiological and seasonal covariates; protein intake, age (newly weaned), growth, over-wintering and molt.

A significant confounding variable in this study was differences between individuals, particularly in the 04 Cohort group. Within Cohort group differences were especially evident in the body water analysis. Changes in body water are often associated with changes in BW. In both Cohort groups, BW were higher at the start of winter trials and reduced at the beginning of the summer trials. The 04 Cohort group experienced significant changes in body water values (TBW, ECW and ICW) between trials with the exception of the ECW:TBW ratio. These dynamics were not observed in 05 Cohort group where body water compartments remained surprisingly constant between the summer and winter trials, even though there were reported changes in body mass and protein intake. The changes in mass and intake associated with the 05 Cohort group were not as large as those observed in the 04 Cohort group. Thus, it may be that the changes in mass and intake were not significant enough or sustained long enough to effect body water compartments in this group. The changes in body water observed in the 04 Cohort group were consistent with the changes in mass and intake associated with this group. Only one other study has reported ECW values for a pinniped species (Castellini et al. 2003) and our harbor seal values are consistent with their findings. Based on human medical standards (Hamadeh et al. 1999), the values from this current study do not indicate any nutritional or health concerns; with the ICW:ECW ratio approximately 3:2 and the mean TBW:ECW generally within the range of 0.360 to 0.390. However, the harbor seal values from this study are at the higher range of the TBW:ECW ratio and may be indicative of higher fat composition in the seals, as values closer to 0.360 are associated with higher lean body mass in humans (Lichtenbelt and Fogelholm 1999).

Protein turnover in both groups were affected by intake and season but these variables were not independent from each other. Season had the most consistent effect on protein turnover. As described above, Cohort groups BW were higher at the start of winter trials and reduced at the beginning of the summer trials, however protein intake was lowest during winter trials and greatest during the summer. These findings are consistent with other captive harbor seal studies (Renouf and Brotea 1991; Rosen and Renouf 1995) that found captive harbor seals gained body mass during the winter despite a decrease in their feed intake. Thus, our expectation that protein turnover would increase with protein intake was confounded by the seasonal effects of growth and molt.

Another variable to consider in these protein turnover results is the digestibility of protein in the diet. The animals were on a mixed diet of herring, pollock, capelin and squid in this study and the digestibility of the protein from the different species was considered equal. However, protein digestibility is lower than lipid and there is an effect of total lipid intake on protein digestibility in mixed diets (Trumble et al. 2003). Also, the locations and seasons that the prey species were collected from for the diet changed over the study period. Therefore, we acknowledge that changes in protein digestibility may be a variable not considered in our interpretation of the protein turnover results.

We expected protein turnover rates to decrease in the winter due to a metabolic response to conserve energy (Renouf and Brotea 1991). However, the increased BW, protein turnover and protein synthesis rates are consistent with the hypothesis that protein was being allocated to growth during this period. Although within normal values for wild harbor seals (Trumble and Castellini 2002), creatinine levels were higher in the winter months than summer, particularly in the 04 Cohort group which also had higher protein turnover and synthesis rates. The blood

chemistry results may be an adaptive response to the metabolic demands of growth combined with reduced dietary intake.

The protein turnover rates were lower during the summer trials with increased dietary protein intake. All individuals in the Cohort groups were in different stages of molt during the summer trials and there was significant variability in the protein turnover rates. Protein turnover over rates and synthesis were reduced and protein breakdown rates exceeded protein synthesis in (McGrath and Goldspink 1982) several of the animals. Blood urea nitrogen levels were elevated during the summer trials reflecting increased protein kinetics (Markussen et al. 1989; Houser and Costa 2001; Trumble and Castellini 2002; Trumble et al. 2006). Molting in harbor seals is regulated by hormones "turning off and on" protein synthesis and breakdown and the entire process may take between 120-170 days (Ashwell-Erickson et al. 1986). Metabolic adjustments are also made with harbor seals reducing their metabolic rates an average of 18.6 % during the molting process. Serum cortisol levels increase prior to the pelage molt inhibiting protein synthesis and increasing protein degradation (McGrath and Goldspink 1982; Kershaw and Hall 2016). Once the animal is in full molt, cortisol levels decrease and thyroid hormones increase which is thought to be associated with an increase in hair growth to replace the pelage (Ashwell-Erickson et al. 1986; Kershaw and Hall 2016). However, the actual role of thyroid hormones in hair growth is under debate and it may be that the role of thyroid hormones in the molt process is confined to adjusting metabolic rates (Renouf and Brotea 1991). The individual variability we observed in protein turnover rates and allocation were probably dependent on at what stage each seal was in the molt process; with seals at the start of the molt showing increased protein breakdown and the rate leveling off as seals entered the hair growth stage.

It is important to mention that urea pool size values were only available for the end of each protein turnover trial. Protein allocation calculations do not include a correction of the urea pool size (Fern et al. 1985) and caution is advised when interpreting the results as absolute values. However, during the study period, only 3 animals out of 35 trials exhibited a reduced dietary intake during the cage trials and none of the animals lost mass during the tracer experiment. The estimates of protein synthesis and degradation from this study have considerable comparative value and are instructive on how harbor seals regulate protein metabolism with seasonal metabolic demands.

Subadult harbor seals must balance cumulative and sometimes competing metabolic demands. In particular, they must regulate dietary protein allocation to balance growth with seasonal metabolic costs. The seasonal changes in protein metabolism that were observed in this study suggest the benefit of future studies that serially monitor thyroid and corticosteroid hormone levels during protein turnover trials. Results from this type of study would elucidate the hormonal effects on protein turnover and allocation.

This experiment is one of a few that employs an amino acid stable isotope tracer (^{15}N-labelled glycine) urine endproduct method to study protein turnover and allocation in a pinniped species. The results from this study confirm the efficacy of this technique and emphasize the complex effects protein intake, growth and season have on protein turnover and allocation.

Acknowledgments

We would like to thank the Alaska SeaLife Center (ASLC) marine mammal and research staff for their assistance with this project. In particular, we thank Stephanie Tsugawa for assistance collecting samples and sharing her data. Support for this project was provided by the ASLC with

funds from National Marine Fisheries Service and Alaska Sea Grant. All experiments and handling procedures were approved by the ASLC and University of Alaska Institutional Animal Care and Use Committee IACUC (Protocol # 03-009 and 06-006), and under the NMFS permit # 881-1710.

Literature Cited

Adams, S., and D. Costa. 1993. Water conservation and protein metabolism in northern elephant seal pups during the postweaning fast. Journal of Comparative Physiology B 163: 367-373.

Allard, M. 2006. A Comparison of the Imapct of Environmental Stressors on Blood Parameters of Captive Harbor Seals (*Phoca vitulina*). Senior Honors Thesis, University of Rhode Island.

Alverson, D.L. 1992. A review of commercial fisheries and the Steller sea lion (*Eumetopias jubatus*)-the conflict arena. Reviews in Aquatic Sciences 6: 203-256.

Arnould, J.P., and M.A. Hindell. 2001. Dive behaviour, foraging locations, and maternal-attendance patterns of Australian fur seals (*Arctocephalus pusillus doriferus*). Canadian Journal of Zoology 79: 35-48.

Ashwell-Erickson, S., F.H. Fay, R. Elsner and D. Wartzok. 1986. Metabolic and hormonal correlates of molting and regeneration of pelage in Alaskan harbor and spotted seals (*Phoca vitulina* and *Phoca largha*). Canadian Journal of Zoology 64: 1086-1094.

Barboza, P., K. Parker and I. Hume. 2009. Integrative Wildlife Nutrition. Springer-Verlag, Berlin.

Barboza, P.S., S.D. Farley and C.T. Robbins. 1997. Whole-body urea cycling and protein turnover during hyperphagia and dormancy in growing bears (*Ursus americanus* and *U. arctos*). Canadian Journal of Zoology 75: 2129-2136.

Bigg MA (1981) Harbour seal -*Phoca vitulina* and *P. largha*. In: Ridgway SH, Harrison RJ, editors. Handbook of marine mammals Vol. 2. London: Academic Press. pp. 1–27.

Boveng, P.L, Bengston, J.L.,Withrow, D.E., Cesarone, J.C., Simpkins, M.A., Frost, K.J and J. J. Burns. 2003. The abundance of harbor seal in the Gulf of Alaska. Marine Mammal Science 19:111-127.

Cahill, G.F. 1978. Physiology of acute starvation in man. Ecology of Food and Nutrition 6: 221-230.

Castellini, M. 2001. Captive studies with Steller sea lions at the Alaska SeaLife Center. in D. DeMaster and S. Atkinson, eds. *Steller sea lion decline: Is It Food II*. University of Alaska Sea Grant, Fairbanks, Alaska. p. 80.

Castellini, M., and L. Rea. 1992. The biochemistry of natural fasting at its limits. Cellular and Molecular Life Sciences 48: 575-582.

Castellini, M.J., Zhao, L, and M.A. Castellini. 2003. Estimation of extracellular water volume in Antarctic Weddell seals: A comparative study of bromide assay methods. Society of Marine Mammalogy Abstract 15th Biennial Conference, Greensboro, NC.

Chabot, D., and G.B. Stenson. 2002. Growth and seasonal fluctuations in size and condition of male Northwest Atlantic harp seals *Phoca groenlandica*: an analysis using sequential growth curves. Marine Ecology Progress Series 227: 25-42.

Crocker, D.E., P.M. Webb, D.P. Costa and B.J. Le Boeuf. 1998. Protein catabolism and renal function in lactating northern elephant seals. Physiological Zoology 71: 485-491.

Daniel, R.G., L.A. Jemison, G.W. Pendleton and S.M. Crowley. 2003. Molting phenology of harbor seals on Tugidak Island, Alaska. Marine Mammal Science 19: 128-140.

El-Khoury, A.E. 1999. Methods for Investigation of Amino Acid and Protein Metabolism. CRC Press, Florida, USA.

Fadely, B.S., J.M. Castellini and M.A. Castellini. 1998. Recovery of harbor seals from EVOS: condition and health status. Exxon Valdez Oil Spill Trustee Council.Anchorage, Alaska.

Fedak, M.A., and S.S. Anderson. 1987. Estimating the energy requirements of seals from weight changes. Approaches to Marine Mammal Energetics Vol 1. *Society for Marine Mammalogy Special Publication.* Allen Press, Lawrence, KS, p 205-226.

Fern, E.B., P.J. Garlick and J.C. Waterlow. 1985. Apparent compartmentation of body nitrogen in one human subject: its consequences in measuring the rate of whole-body protein synthesis with ^{15}N. Clinical Science 68: 271-282.

Field, I.C., C.J. Bradshaw, H.R. Burton, M.D. Sumner and M.A. Hindell. 2005. Resource partitioning through oceanic segregation of foraging juvenile southern elephant seals (*Mirounga leonina*). Oecologia 142: 127-135.

Fielding, C.L., K.G. Magdesian, D.A. Elliott, A.L. Craigmill, W.D. Wilson and G.P. Carlson. 2003. Pharmacokinetics and clinical utility of sodium bromide (NaBr) as an estimator of extracellular fluid volume in horses. Journal of Veterinary Internal Medicine 17: 213-217.

Fouillet, H., C. Bos, C. Gaudichon and D. Tomé. 2002. Approaches to quantifying protein metabolism in response to nutrient ingestion. The Journal of Nutrition 132: 3208S-3218S.

Freudenberger, D., and J. Nolan. 1993. Protein turnover in two kangaroo sub-species (*Macropus robustus robustus* and *M. r. erubescens*) from divergent habitats and the sympatric feral goat (*Capra hircus*). Comparative Biochemistry and Physiology Part A: Physiology 105: 443-448.

Geiger, G.L., S. Atkinson, J.N. Waite, G.M. Blundell, J.R. Carpenter and K. Wynne. 2013. A new method to evaluate the nutritional composition of marine mammal diets from scats applied to harbor seals in the Gulf of Alaska. Journal of Experimental Marine Biology and Ecology 449: 118-128.

Hamadeh, M.J., L. Robitaille, D. Boismenu, P. Hongsprabhas, O.A. Mamer and L.J. Hoffer. 1999. Human extracellular water volume can be measured using the stable isotope $Na_2{}^{34}SO_4$. The Journal of Nutrition 129: 722-727.

Houser, D., and D. Costa. 2001. Protein catabolism in suckling and fasting northern elephant seal pups (*Mirounga angustirostris*). Journal of Comparative Physiology B 171: 635-642.

Hutchinson, E., Atkinson, S and A. Hoover-Miller. 2016. Growh and reproductive tracts from fetal to adult harbor seasl in the Gulf of Alaska. Marine Ecology Progress Series 557: 277-288

Huntley, A., D. Costa, G. Worthy and M. Castellini. 1987. Approaches to Marine Mammal Energetics. Vol 1. *Society for Marine Mammalogy Special Publication.* Allen Press, Lawrence, KS. 253pp.

Kershaw, J.L., and A.J. Hall. 2016. Seasonal variation in harbour seal (*Phoca vitulina*) blubber cortisol - A novel indicator of physiological state? Scientific Reports 6, 21889:1-9.

Lichtenbelt, W.D.V.M., and M. Fogelholm. 1999. Increased extracellular water compartment, relative to intracellular water compartment, after weight reduction. Journal of Applied Physiology 87: 294-298.

Markussen, N., A. Bjørge and N. Øritsland. 1989. Growth in harbour seals (*Phoca vitulina*) on the Norwegian coast. Journal of Zoology 219: 433-440.

McGrath, J.A., and D.F. Goldspink. 1982. Glucocorticoid action on protein synthesis and protein breakdown in isolated skeletal muscles. Biochemical Journal 206: 641-645.

Miller, M.E., J. Cosgriff and G.B. Forbes. 1989. Bromide space determination using anion-exchange chromatography for measurement of bromide. The American Journal of Clinical Nutrition 50: 168-171.

Nolan, J., and R. Leng. 1972. Dynamic aspects of ammonia and urea metabolism in sheep. British Journal of Nutrition 27: 177-194.

Pernia, S.D. 1984. Protein turnover and nitrogen metabolism during long term fasting in northern elephant seal pups. PhD Thesis.University of California, Santa Cruz, California.

Picou, D., and T. Taylor-Roberts. 1969. The measurement of total protein synthesis and catabolism and nitrogen turnover in infants in different nutritional states and receiving different amounts of dietary protein. Clinical Science 36: 283-296.

Pitcher, K.W. 1986. Variation in blubber thickness of harbor seals in southern Alaska. The Journal of Wildlife Management 50: 463-466.

Reilly, J.J., and M.A. Fedak. 1990. Measurement of the body composition of living gray seals by hydrogen isotope dilution. Journal of Applied Physiology 69: 885-891.

Renouf, D., and G. Brotea. 1991. Thyroid hormone concentrations in harbour seals (*Phoca vitulina*): no evidence of involvement in the moult. Comparative Biochemistry and Physiology Part A: Physiology 99: 185-194.

Robbins, C.T. 1983. Wildlife Feeding and Nutrition. Academic Press, New York, NY.

Rosen, D., and D. Renouf. 1995. Variation in the metabolic rates of captive harbour seals. Developments in Marine Biology 4: 393-399.

Russell, K., G. Lobley and D. Millward. 2003. Whole-body protein turnover of a carnivore, *Felis silvestris catus*. British Journal of Nutrition 89: 29-37.

Schmidt-Nielsen, K. 1997. Animal Physiology: Adaptation and Environment. Cambridge University Press, New York, New York.

Singer, M.A. 2003. Do mammals, birds, reptiles and fish have similar nitrogen conserving systems? Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 134: 543-558.

Spies, R.B. 2007. Long-term ecological change in the Northern Gulf of Alaska. Elsevier, Amsterdam, The Netherlands.

Stanberry, K. 2003. The effect of changes in dietary fat level on body composition, blood metabolites and hormones, rate of passage, and nutrient assimilation efficiency in harbor seals. Animal Science. MS Thesis. University of Hawaii. 109 pp.

Trumble, S.J., P.S. Barboza and M.A. Castellini. 2003. Digestive constraints on an aquatic carnivore: effects of feeding frequency and prey composition on harbor seals. Journal of Comparative Physiology B 173: 501-509.

Trumble, S.J., and M.A. Castellini. 2002. Blood chemistry, hematology, and morphology of wild harbor seal pups in Alaska. The Journal of Wildlife Management 66: 1197-1207.

Trumble, S.J., M.A. Castellini, T.L. Mau and J.M. Castellini. 2006. Dietary and seasonal influences on blood chemistry and hematology in captive harbor seals. Marine Mammal Science 22: 104-123.

Tsugawa, S.K. 2009. Factors affecting intake and the effects of diet and season on juvenile harbor seal body condition. MS Thesis. Animal Science. University of Hawaii.113pp.

Waterlow, J. 1984. Protein turnover with special reference to man. Quarterly Journal of Experimental Physiology 69: 409-438.

Waterlow, J.C. 1999. The mysteries of nitrogen balance. Nutrition Research Reviews 12: 25-54.

Waterlow, J.C. 2006. Protein Turnover. CABI Publishing, Wallingford, Oxfordshire.

Waterlow, J.C., P.J. Garlick and D. Millward. 1978. Protein Turnover in Mammalian Tissues and in the Whole Body. Elsevier/North-Holland Publishing Co., Amsterdam, The Netherlands.

Webb, P., D. Crocker, S. Blackwell, D. Costa and B. Boeuf. 1998. Effects of buoyancy on the diving behavior of northern elephant seals. Journal of Experimental Biology 201: 2349-2358.

Weiser, W. 1994. Cost of growth in cells and organisms: general rules and comparative aspects. Biological Reviews 69: 1-33.

Wolfe, R. 1992. Radioactive and Stable Isotope Tracers in Biomedicine: Principles and Practice of Kinetic Analysis. Wiley, New York, NY.

Wong, W.W., H.-P. Sheng, J.C. Morkeberg, J.L. Kosanovich, L.L. Clarke and P.D. Klein. 1989. Measurement of extracellular water volume by bromide ion chromatography. The American Journal of Clinical Nutrition 50: 1290-1294.

Worthy, G.A., and D. Lavigne. 1987. Mass loss, metabolic rate, and energy utilization by harp and gray seal pups during the postweaning fast. Physiological Zoology 60: 352-364.

Zar, J. 1984. Biostatistical analysis. Prentice-Hall, Englewood Cliffs, New Jersey.

Zhao, L., M.A. Castellini and D.M. Schell. 2006. Metabolic adjustments to varying protein intake in harbor seals (*Phoca vitulina*): evidence from serum free amino acids. Physiological and Biochemical Zoology 79: 965-976.

Tables

Table 4.1. The initial capture weight (kg) and cohort designation for each of the eight harbor seals participating in the protein turnover trials. The seal Miki (PV04012) died prior to the completion of this study (Fall 2006).

Seal	Identification Number	Cohort	Initial Mass (kg)
Atuun	PV04009	2004	26.5
Miki	PV04012	2004	25
Qilak	PV04010	2004	22.5
Susitna	PV04011	2004	30
Anya	PV05016	2005	21
Shila	PV05013	2005	28.7
Siku	PV05015	2005	25.7
Tikanni	PV05014	2005	22.5

Table 4.2. The mean (± SD) serum values for albumin (ALB), blood urea nitrogen (BUN), creatinine (CREAT), and total protein (TP). These values are not outside the range of results for wild caught harbor seals (Trumble and Castellini 2002). Significance is $p<0.05$. An asterisk * represents a significance of $p<0.001$ (ANOVA, Tukey tests). The trial abbreviations are season and year; winter (W) or summer (S), 2005 (05), 2006 (06) and 2007 (07).

O4 Cohort	ALB (g dl^{-1})	BUN (mg $dl^{-1)}$	CREAT (mg dl^{-1})	TP (g dl^{-1})
Trial	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
W05	3.27 ± 0.2	40.90 ± 1.6[a]	0.90 ± 0.1	6.33 ± 0.2
S05	3.13 ± 0.3	29.25 ± 2.2[a,b,c]	0.73 ± 0.1[a]	6.68 ± 0.5
W06	3.45 ± 0.1	40.50 ± 6.0[b]	1.00 ± 0.2[a]	6.78 ± 0.3
S06	3.05 ± 0.5	30.00 ± 5.2[a,c]	0.73 ± 0.1[a]	6.50 ± 0.4
W07	2.93 ± 0.2	43.33 ± 1.5[c]	1.00 ± 0.1	6.53 ± 0.4
05 Cohort				
Trial				
S05	3.43 ± 0.1[a*]	47.25 ± 8.3[a]	0.78 ± 0.1[a]	6.93 ± 0.1[a*,b*,c]
W06	3.30 ± 0.1[b]	39.25 ± 6.6	0.80 ± 0.1	6.28 ± 0.2[b*]
S06	2.75 ± 0.2[a*]	32.50 ± 8.7[a]	0.53 ± 0.1[a,b]	6.30 ± 0.3[a*d]
W07	2.88 ± 0.1[b]	38.25 ± 5.2	0.73 ± 0.2[b]	6.60 ± 0.1[c,d]

Table 4.3. The mean (± SD) dietary protein intake of individual animal per protein turnover trial. The mean value represents the intake the day prior to the animal being placed in the metabolic cage and during the protein turnover trial (n=3 days). An asterisk * signifies that the animal's dietary intake significantly decreased during the trial.

Cohort 04										
	Body Weight ($kg.^{-0.75}$)	Mean Intake (Protein g /BW $kg^{-0.75}$)	Body Weight ($kg.^{-0.75}$)	Mean Intake (Protein g /BW $kg^{-0.75}$)	Body Weight ($kg.^{-0.75}$)	Mean Intake (Protein g /BW $kg^{-0.75}$)	Body Weight ($kg.^{-0.75}$)	Mean Intake (Protein g /BW $kg^{-0.75}$)	Body Weight ($kg.^{-0.75}$)	Mean Intake (Protein g /BW $kg^{-0.75}$)
Trial	W05	W05	S05	S05	W06	W06	S06	S06	W07	W07
Age (months)	9	9	16	16	21	21	28	28	33	33
Miki	11.98	28.78±0.0	11.68	39.87±1.7	15.43	28.18±1.2	12.43	38.08±1.1		
Susitna	13.30	27.91±0.1	13.96	39.52±1.0	17.63	28.81±0.70	13.39	39.68±0.4	17.00	30.53±0.9
Atuun	12.79	13.40±0.3	11.98	28.40±14.0*	15.09	11.44±14.4*	13.96	29.82±0.6	15.34	32.80±0.0
Qilak	11.51	25.97±0.5	11.28	36.96±4.0	13.39	23.97±6.5	12.79	32.54±0.6	13.64	27.62±5.61
Cohort 05										
	Body Weight ($kg.^{-0.75}$)	Mean Intake (Protein g /BW $kg^{-0.75}$)	Body Weight ($kg.^{-0.75}$)	Mean Intake (Protein g /BW $kg^{-0.75}$)	Body Weight ($kg.^{-0.75}$)	Mean Intake (Protein g /BW $kg^{-0.75}$)	Body Weight ($kg.^{-0.75}$)	Mean Intake (Protein g /BW $kg^{-0.75}$)	Body Weight ($kg.^{-0.75}$)	Mean Intake (Protein g /BW $kg^{-0.75}$)
Trial	W05	W05	S05	S05	W06	W06	S06	S06	W07	W07
Age (months)			2	2	9	9	16	16	21	21
Anya			12.14	10.86±0.0	12.20	23.43±6.6	10.78	32.72±8.0	12.37	33.27±2.7
Shila			13.86	10.97±1.2	13.11	23.43±6.6	10.71	42.53±0.9	11.91	36.07±7.0
Siku			13.41	8.11±5.5	12.63	26.69±8.4*	11.41	32.55±0.6	13.04	27.92±0.0
Tikanni			12.06	8.79±5.9	11.41	23.67±5.6	10.05	42.30±1.3	12.88	38.08±1.1

Table 4.4. Results from the protein turnover trials conducted on subadult harbor seals during summer (S) and winter (W) trials 2005-2007. Q represents the flux of protein through the urine urea pool per day (mmol N•24 h^{-1}) and k represents the fractional turnover rate of the labeled urine urea pool (h^{-1}) for each seal and trial.

Cohort 04										
	k	Q	k	Q	k	Q	k	Q	k	Q
Trial	W05	W05	S05	S05	W06	W06	S06	S06	W07	W07
Age (months)	9	9	16	16	21	21	28	28	33	33
Miki	11.06	19.50	14.67	4.72	12.55	18.80	12.94	3.30		
Susitna	10.89	20.39	12.89	9.35	10.52	33.94	13.27	11.77	11.32	21.53
Atuun	17.11	14.55	13.84	10.86	58.16	6.15	14.98	7.50	14.75	10.11
Qilak	11.47	20.58	8.16	23.48	12.34	25.99	17.03	7.87	14.08	11.40
Cohort 05										
	k	Q	k	Q	k	Q	k	Q	k	Q
Trial	W05	W05	S05	S05	W06	W06	S06	S06	W07	W07
Age (months)			2	2	9	9	16	16	21	21
Anya			16.22	14.24	14.16	13.99	13.76	10.66	12.69	12.19
Shila			26.37	8.95	14.67	13.88	12.85	10.83	11.71	13.54
Siku			32.72	6.53	16.57	9.68	12.87	13.97	14.00	12.91
Tikanni			28.31	6.40	16.65	8.57	10.97	13.54	12.55	12.59

Table 4.5. Comparative results on rates of urea nitrogen turnover and protein metabolism in subadult harbor seals during 2005-2007. Values for protein synthesis and breakdown were highly significant for season $p<0.001$ (ANOVA, Tukey tests). Rate of protein breakdown that exceeded protein synthesis are highlighted.

Animal	Cohort/ Trial	Urea nitrogen turnover mgN day^{-1} (Q)	Dietary intake g N day^{-1} (I)	Excreation g N day^{-1} (E)	Nitrogen synthesis mg N day^{-1} (Z=Q-E)	Protein synthesis mg day^{-1}	Protein synthesis g kg$^{-0.75}$ day^{-1}	Protein breakdown g kg$^{-0.75}$ day^{-1}
Qilak	04/W05	90.8	48.0	3.5	87.2	545.2	47.4	23.2
Atuun		36.8	27.7	2.6	34.2	213.9	16.7	4.5
Miki		95.2	55.1	4.4	90.9	567.8	47.4	20.9
Sustina		102.5	59.3	4.8	97.7	610.8	45.9	20.3
Qilak	04/S05	233.8	62.1	4.0	229.9	1436.7	20.4	15.2
Atuun		36.2	31.3	2.6	33.6	209.8	2.8	0.4
Miki		12.1	72.6	6.3	5.8	36.3	0.5	5.2
Sustina		36.9	87.4	7.1	29.8	186.4	2.1	3.6
Anya	05/S05	32.0	25.9	1.0	31.0	193.8	16.0	3.1
Shila		14.8	25.7	0.9	13.9	86.7	6.3	4.9
Siku		8.5	22.2	0.7	7.8	48.8	3.6	6.4
Tikanni		10.1	23.5	0.8	9.3	58.0	4.8	6.9
Qilak	04/W06	104.1	43.1	3.7	100.4	627.5	46.9	4.6
Atuun		7.7	5.4	0.7	7.1	44.2	2.9	0.2
Miki		73.0	69.3	8.5	64.5	403.0	26.1	0.2
Sustina		183.3	81.8	8.0	175.3	1095.8	62.1	5.8
Anya	05/W06	41.0	44.1	3.4	37.7	235.3	19.3	1.6
Shila		41.1	39.9	3.3	37.8	236.2	18.0	0.5
Siku		23.8	45.5	2.8	21.0	131.1	10.4	10.8
Tikanni		22.0	38.0	2.6	19.4	121.4	10.6	8.8
Qilak	04/S06	19.3	67.1	7.4	11.9	74.1	5.8	3.7
Atuun		22.1	67.1	4.8	17.3	107.9	7.7	3.2
Miki		12.4	85.4	10.7	1.7	10.9	0.9	5.9
Sustina		39.4	85.4	5.5	33.9	211.7	15.8	3.4
Anya	05/S06	33.2	63.5	11.7	21.4	134.0	12.4	17.6
Shila		37.3	72.6	8.1	29.2	182.5	17.0	20.6
Siku		48.4	58.9	5.5	42.9	268.1	23.5	5.7
Tikanni		64.0	67.1	8.5	55.5	346.7	34.5	1.9
Qilak	04/W07	34.6	66.3	6.7	28.0	174.8	12.8	2.3
Atuun		30.2	80.5	6.3	23.9	149.1	9.7	3.3
Sustina		86.1	84.0	7.4	78.6	491.4	28.9	0.1
Anya	05/W07	44.7	68.5	5.1	39.6	247.7	20.0	12.0
Shila		58.6	62.9	11.3	47.3	295.5	24.8	2.2
Siku		42.3	58.3	4.5	37.8	236.5	18.1	7.6
Tikanni		57.5	78.2	7.1	50.4	315.1	24.5	10.0

Figure Legends

Figure 4.1. Model for whole-body amino acid metabolism. From the free amino acid pool, amino acids are assumed to have only two fates: 1. oxidation (carbon to expired air CO_2 and nitrogen primarily to urinary urea and ammonia, secondarily feces). 2. protein synthesis. Protein degradation is assumed to be negligible over the period tested in this study (72 hours). (Adapted from El-Khoury 2000).

Figure 4.2. Metabolic cage designed for urine collection during protein turnover trials (bottom). Seals were individually housed in the cage (top). The angle and creases in the collecting pan directed urine to the drain (A) that emptied into a beaker on ice packs (B) for collection.

Figure 4.3. The mean changes in weight with body composition (±SD) of each Cohort group (n=4) per trial (WO5-winter 05; S05-summer 05; W06-winter 06; S06-summer 06; W07-winter 07) (Adapted from Tsugawa 2009). As the animals were already weaned prior to capture, the age estimate assumes that the pups were born in the month of May. The * denotes significant differences between trials within Cohort group (ANOVA, $p<0.05$).

Figure 4.4. Mean (± SD) body water compartments (TBW, total body water, ICW, intracellular water, ECW, extracellular water, and ECW:TBW ratio) within each Cohort group per protein turnover trial. Significance was set at $P< 0.05$ (ANOVA, post hoc Tukey tests).

Figure 4.5. Boxplot of the change in ^{15}N enrichment in serum samples taken before the injection of the ^{15}N-labelled glycine tracer and at the completion of the trial. Results from the 04 Cohorts are on the left and 05 Cohorts on the right.

Figure 4.6. Seasonal differences in the isotopic enrichment of ^{15}N during protein turnover trials. The individual data was pooled per Cohort and graphed using a natural logarithmic transformation (Ln). The data was then fitted with a regression line and the slope (k) calculated.

Figures

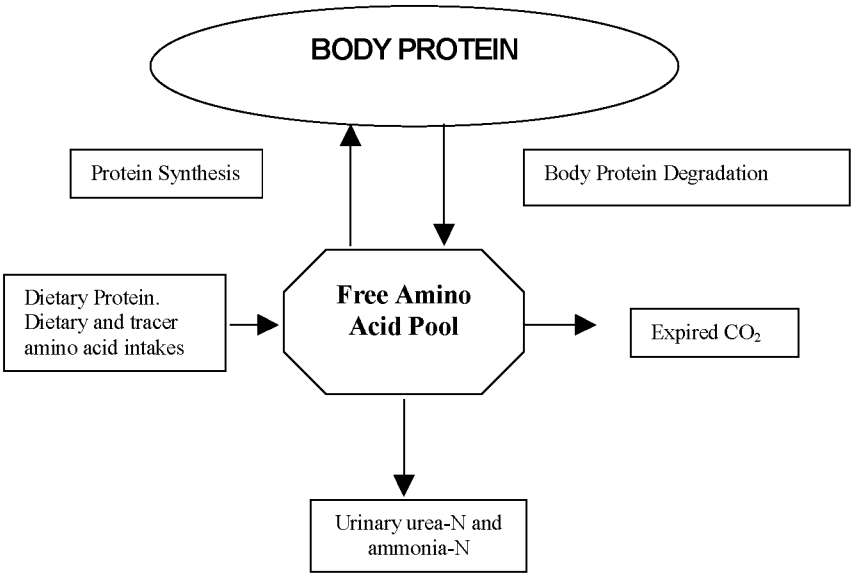


Figure 4.1.

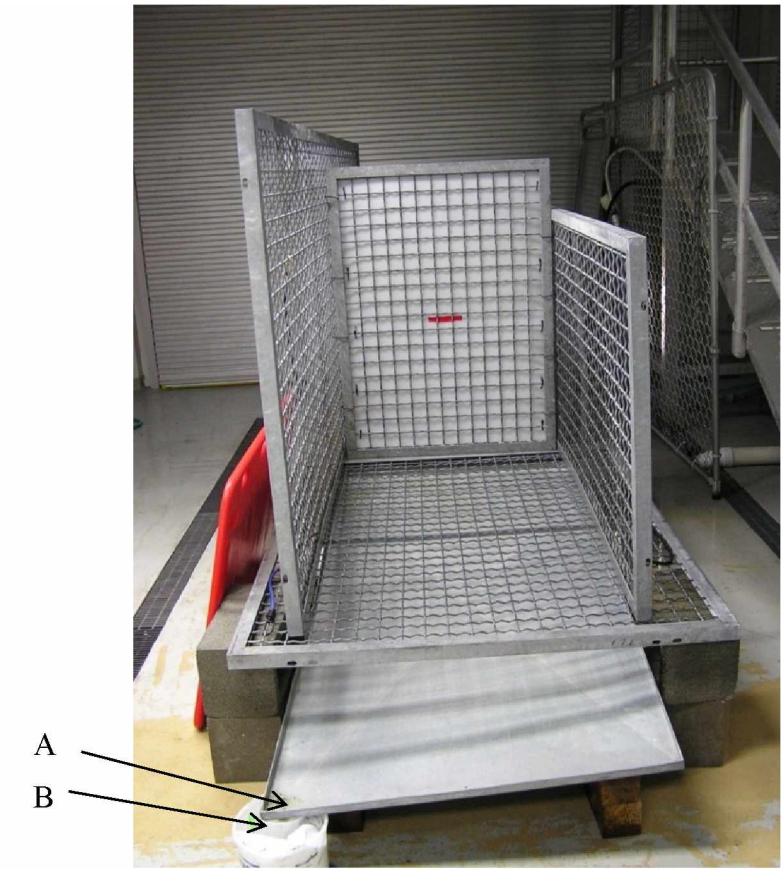
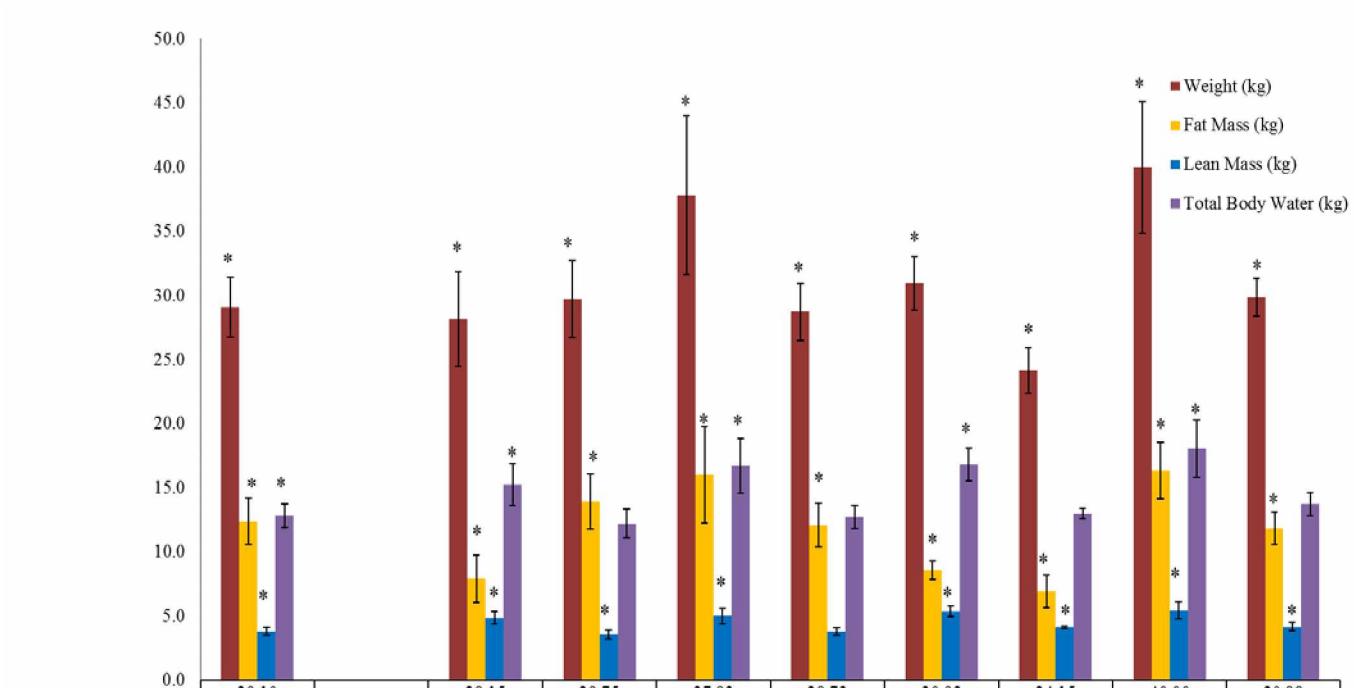


Figure 4.2



	29.10 W05 04 cohort 9	W05 05 cohort -	28.15 S05 04 cohort 16	29.75 S05 05 cohort 2	37.83 W06 04 cohort 21	28.73 W06 05 cohort 9	30.93 S06 04 cohort 28	24.15 S06 05 cohort 16	40.00 W07 04 cohort 33	29.88 W07 05 cohort 21
	Animal Age (months)									
Weight (kg)	29.10		28.15	29.75	37.83	28.73	30.93	24.15	40.00	29.88
Fat Mass (kg)	12.36		7.90	13.90	15.99	12.08	8.57	6.91	16.36	11.86
Lean Mass (kg)	3.82		4.86	3.55	4.99	3.81	5.37	4.13	5.44	4.16
Total Body Water (kg)	12.80		15.24	12.19	16.70	12.73	16.81	12.98	18.05	13.73

Figure 4.3.

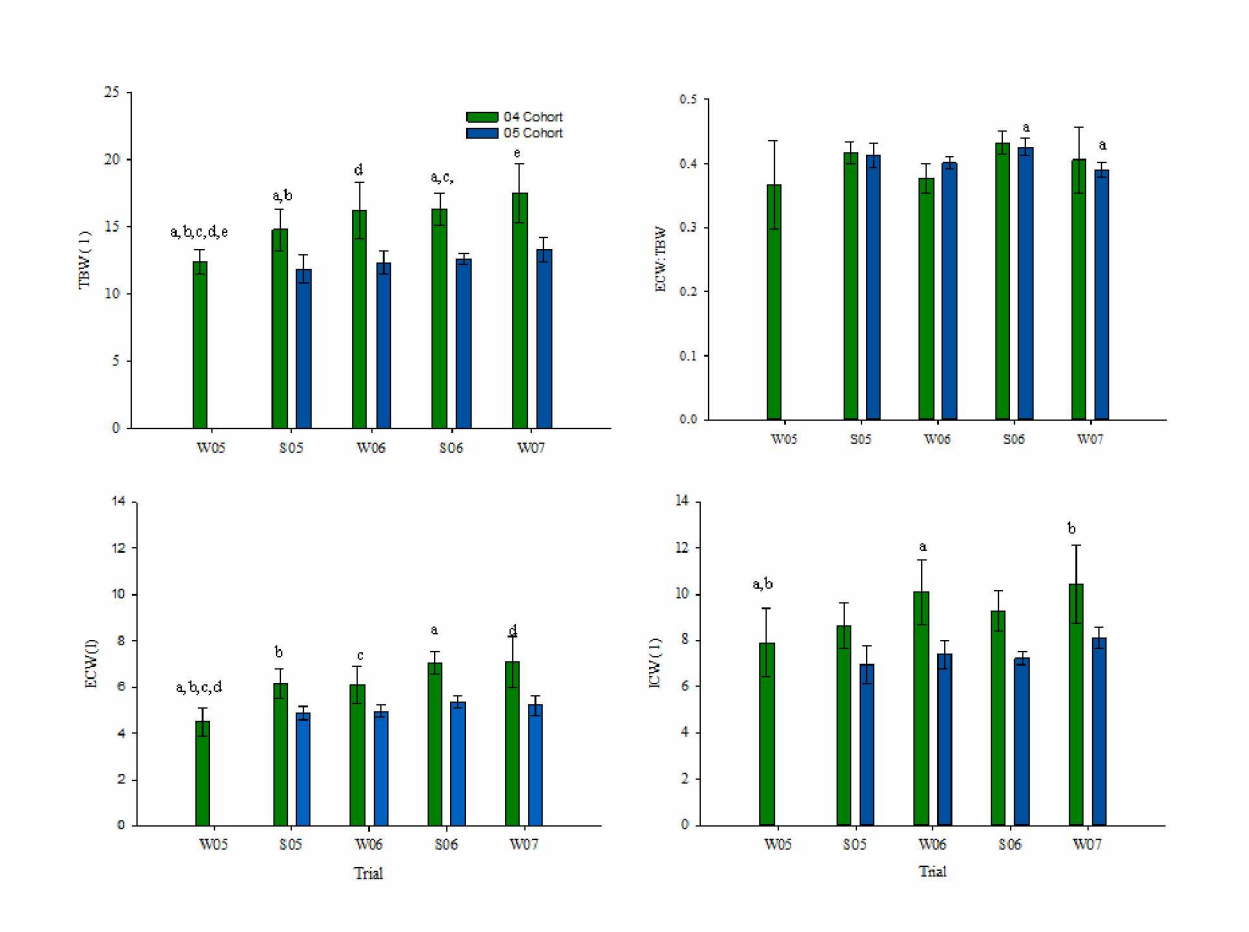

04 Cohort
05 Cohort
TBW (l)
ECW: TBW
ECW (l)
ICW (l)
a,b,c,d,e
a,b
d
a,c,
e
a
a
b
c
a
d
a,b,c,d
a,b
a
b
W05
S05
W06
S06
W07
Trial

Figure 4.4.

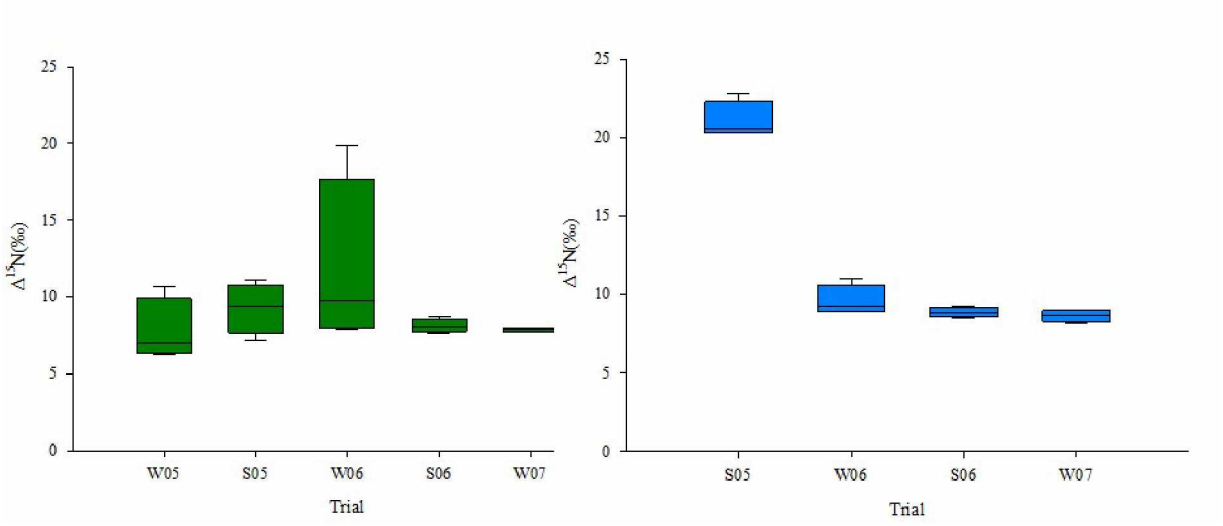

25
20
15
10
5
0
Δ15N(‰)
W05
S05
W06
S06
W07
Trial
25
20
15
10
5
0
Δ15N(‰)
S05
W06
S06
W07
Trial

Figure 4.5.

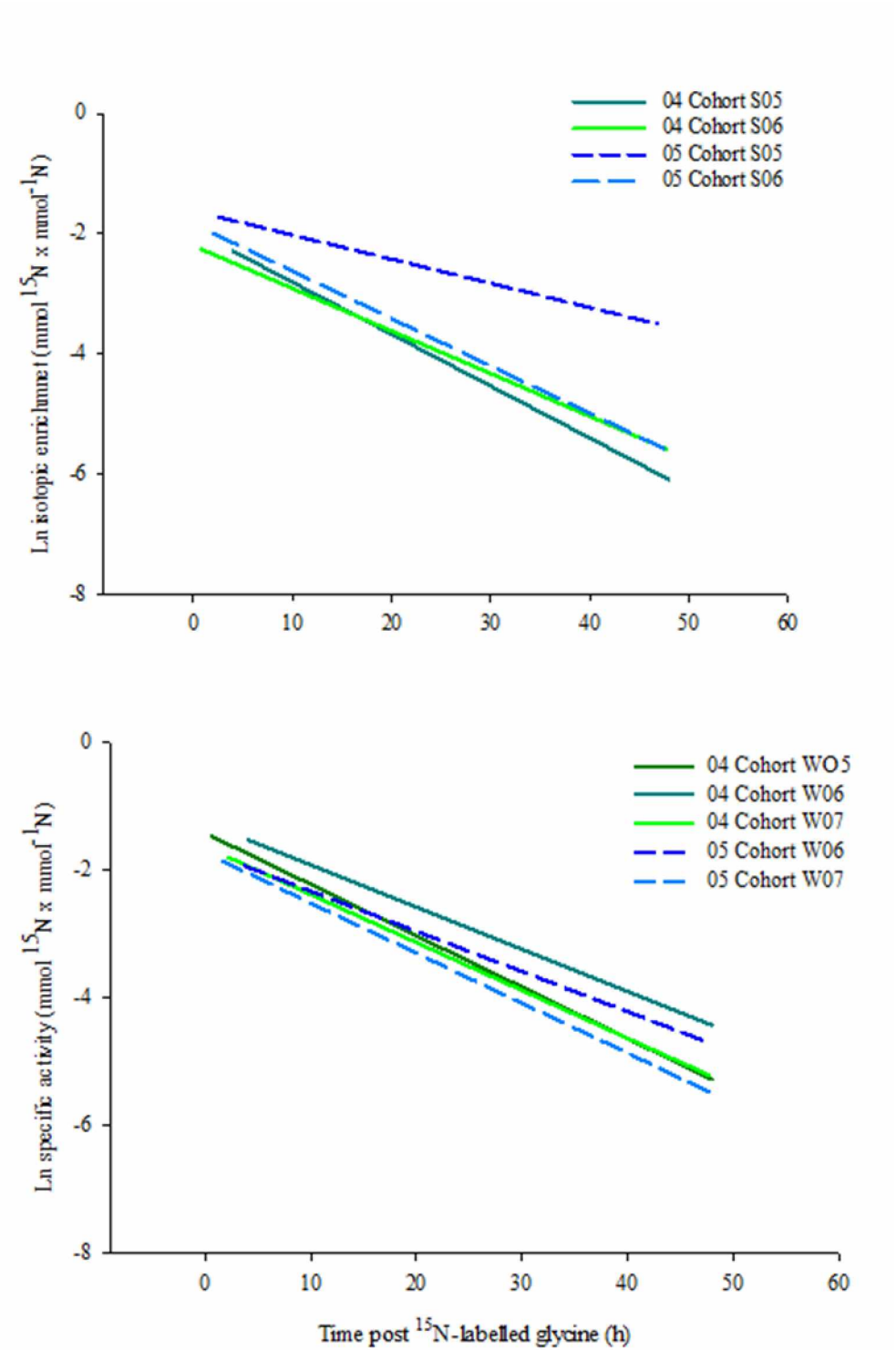

04 Cohort S05
04 Cohort S06
05 Cohort S05
05 Cohort S06
Ln isotopic enrichment (mmol ^{15}N x mmol^{-1}N)
0
-2
-4
-6
-8
0
10
20
30
40
50
60
04 Cohort WO5
04 Cohort W06
04 Cohort W07
05 Cohort W06
05 Cohort W07
Ln specific activity (mmol ^{15}N x mmol^{-1}N)
Time post ^{15}N-labelled glycine (h)

Figure 4.6

Chapter 5
General Conclusions

Although pinnipeds derive much of their daily energetic needs from lipid, protein regulation is vital for growth, cellular maintenance, hormone and immune functions, as well as molting and fasting biology. This emphasizes the need to study nutritional and metabolic impacts on protein metabolism (El-Khoury 1999). Net protein turnover is the balance between protein synthesis and degradation, and is influenced by dietary intake and quality, as well as physiological and metabolic requirements (Boren et al. 1996; Waterlow 2006; Barboza et al. 2009). In this work, dietary protein quality is assessed and new information is presented on the influence of protein intake and physiological and metabolic demands on whole body turnover in nonfasting pinnipeds.

The quality of protein in pinniped diets is evaluated in the paper "Protein Quality in the Diet of Steller sea lions, *Eumetopias jubatus,* in Southcentral Alaska" (Chapter 2). Protein quality is generally evaluated by the amount of nitrogen absorbed from the diet and comparing the proportions of EAA in the food with those in the tissues of the consumer (Robbins 1983; Barboza et al. 2009). In this study, diet quality was assessed through comparisons of amino acid (AA) profiles in the maternal milk with serum and seasonal prey of wild juvenile Steller sea lions (*Eumetopias jubatus*) in Southcentral Alaska. Both Pacific herring (*Clupei pallasi*) and walleye pollock (*Theragra chalcogramma*) are common prey for Steller sea lions and were the focus of this assessment. Maternal milk has a high biological value in animal diets and both Pacific herring and walleye pollock compared positively with milk in essential and branched chained amino acid content. The protein content in the prey tested did not change seasonally and although there were differences in individual amino acid content, the essential amino acid (EAA):nonessential amino acid (NEAA) and branched chain amino acid (BCAA):nonbranched

chain amino acids (NBCAA) ratios did not differ seasonally. Thus, Steller sea lions feed on a seasonally consistent high quality protein diet.

Specific ratios of amino acid groups such as EAA:NEAA and BCAA:NBCAA are often used as indicators of protein nutrition and animal condition (Whitehead and Dean 1964; Arroyave 1970; Boren et al. 1996). Using these ratios, the serum profiles of juvenile Steller sea lions suggest the juvenile animals tested were not in nutritional stress at the time of capture and these results were consistent with intake health evaluations. Although AA profiles only represent a "snap shot" of the condition when the sample is collected, they can elucidate short term changes in protein status that can impact health. However, the serum amino acid pool is very dynamic and controlled laboratory experiments are required to understand how intake, digestibility, and physiological and metabolic conditions affect these amino acid ratios if they are to be used as indicators of nutritional status in pinnipeds.

It is also important to note that the essentiality of different amino acids have not been experimentally tested in pinnipeds, as they have in domestic and farm animals. Dietary protein quality in pinnipeds is therefore based on our knowledge of amino acid essentiality in other carnivores combined with assumptions on requirements for growth, reproduction and molt. However, even among carnivores there are significant differences in amino acid requirements. For, example taurine is essential in cats but not in mink (MacDonald et al. 1984; Eisert 2011). Several tissues, including the nervous system and erythrocytes require glucose as fuel and recent studies on pinnipeds have focused on the importance of amino acids and lipids as precursors for glucose in a carbohydrate poor diet (Champagne et al. 2004; Eisert 2011; Champagne et al. 2012; Eisert et al. 2013).

In the second paper, "Tracking Protein Turnover in Adult Female Weddell Seals (*Leptonychotes weddellii)* Using ^{15}N-labelled Glycine" (Chapter 3) the turnover kinetics of the nonessential amino acid ^{15}N-labelled glycine in the serum amino acid and protein pool, red blood cells, and urinary urea were measured in wild adult female Weddell seals (*Leptonychotes weddellii*) in the Antarctic. We also tested the essential amino acid ^{15}N phenylalanine on one animal. There appeared to be differences in the kinetics between the two tracers based on the tissue tested and their metabolic pathways. Labelled glycine moved quickly into serum protein (4-5 hours) and into red blood cells (1-2 hours) and urine urea (2-4 hours). The label persisted in the protein pool for 10-15 days and was still observed in red bloods cells 31 days post injection. Labelled phenylalanine rapidly disappeared from serum protein and was not detected in red blood cells. Glycine is used in the synthesis of the protoporphyrin component of hemoglobin in red blood cells but in this study blood clots were not washed of unquantified residual serum prior to analysis so RBC results reflected enrichment from the serum protein pool as well. However, since RBC do not recycle, if the blood clot had been properly washed of residual serum, N^{15}-labelled glycine may be a good label to estimate the life span of red blood cells in this species.

The turnover rates in the blood amino acid and urine urea pools were animal specific with a reduced rate associated with molting. Molting is hormonally regulated through fluctuating cortisol and thyroid levels (Ashwell-Erickson et al. 1986; Oki and Atkinson 2004) which influences protein synthesis and degradation rates. Future studies that combine hormonal analysis of serum and urine samples with protein turnover calculations would provide insight into hormonal regulation in protein metabolism.

The apparent differences between the non-essential and essential amino acid label observed in our results recommends future studies with an essential amino acid as the primary label. Glycine

was used in our study due to its published efficacy as a whole body protein turnover label (Freudenberger, and Nolan 1993; Barboza et al. 1997 and Russell et al. 2003) and its metabolic pathway ending in urine urea, however, an essential amino acid may provide greater insight into the question of diet and metabolism. The results from this study show the potential of stable isotope labelled amino acids have to address different protein metabolism questions in wild animals.

In the final paper, "Whole Body Protein Turnover in subadult Alaskan Harbor Seals (*Phoca vitulin*a)" (Chapter 4), whole body protein turnover experiments using a single bolus ^{15}N-labelled glycine tracer method with endproduct collection of blood, feces and urine were conducted on 2 Cohort groups of subadult harbor seals over 2 years. Season was found to have the greatest effect on whole body protein turnover. Seasonally, protein turnover rates increased during the winter and decreased in the summer molt, with protein intake following an opposite trend. This pattern corresponded with an increase in mass and protein synthesis in the winter, while mass decreased, and protein degradation rates increased in molting seals. In molting seals, protein degradation was sometimes greater than protein synthesis resulting in a net loss of body protein. This reduction in protein synthesis combined with an increase in protein degradation is likely due to the influence of hormonal regulation in the molting process.

In this study we used urine urea as the endproduct collection to calculate protein turnover and allocation, but it is important to note that another endproduct from protein metabolism is expired CO_2. The use of a metabolic chamber to capture expired CO_2 following the injection of a labelled amino acid would be another method for calculating protein turnover.

This study reports results from one of the few whole body protein turnover rates in a nonfasting pinniped model and confirmed the efficacy of the single bolus ^{15}N-labelled glycine tracer method with endproduct collection for studying whole body protein turnover in pinnipeds. Pinnipeds consume high quality protein diets and protein turnover is strongly regulated by developmental, seasonal, physiological and metabolic demands. The strong seasonal influence observed on protein regulation recommends that further studies be conducted seasonally that combine protein turnover methods with hormone sample collection. The use of stable isotope labelled amino acids as tracers to look at whole body protein turnover and protein allocation has great efficacy for future studies.

Anthropogenic demands on ocean resources continue to exert stress on pinniped populations through fisheries interactions, contaminants and climate change. Together these studies provide insight and techniques on how to evaluate dietary protein quality and test the ability of pinnipeds to accommodate to these changes.

Literature Cited

Arnould, J.P., and M.A. Hindell. 2001. Dive behaviour, foraging locations, and maternal-attendance patterns of Australian fur seals (*Arctocephalus pusillus doriferus*). Canadian Journal of Zoology 79: 35-48.

Arroyave, G. 1970. Comparative sensitivity of specific amino acid ratios versus "essential to nonessential" amino acid ratio. The American Journal of Clinical Nutrition 23: 703-706.

Ashwell-Erickson, S.M. 1981. The energy cost of free existence for Bering Sea harbor and spotted seals. PhD Thesis. University of Alaska Fairbanks. 207pp

Ashwell-Erickson, S., F.H. Fay, R. Elsner and D. Wartzok. 1986. Metabolic and hormonal correlates of molting and regeneration of pelage in Alaskan harbor and spotted seals (*Phoca vitulina* and *Phoca largha*). Canadian Journal of Zoology 64: 1086-1094.

Barboza, P., K. Parker and I. Hume. 2009. Integrative Wildlife Nutrition. Springer-Verlag, Berlin.

Barboza, P.S., S.D. Farley and C.T. Robbins. 1997. Whole-body urea cycling and protein turnover during hyperphagia and dormancy in growing bears (*Ursus americanus* and *U. arctos*). Canadian Journal of Zoology 75: 2129-2136.

Boren, J., R. Lochmiller and D. Leslie, Jr. 1996. Relation of serum and muscle free amino acids to dietary protein in the Northern Bobwhite. Proceedings of the Oklahoma Academy of Science 76: 55-65.

Brown, R.F., and B.R. Mate. 1983. Abundance, movements, and feeding habits of harbor seals, *Phoca vitulina*, at Netarts and Tillamook Bays, Oregon. Fishery Bulletin 81: 291-301.

Cahill Jr, G.F. 1978. Physiology of acute starvation in man. Ecology of Food and Nutrition 6: 221-230.

Castellini, M. 2001. Captive studies with Steller sea lions at the Alaska SeaLife Center. in D. DeMaster and S. Atkinson, eds. *Steller sea lion decline: Is It Food II.* University of Alaska Sea Grant, Fairbanks, Alaska. p. 80

Castellini, M., and L. Rea. 1992. The biochemistry of natural fasting at its limits. Experientia 48: 575-582.

Champagne, C.D., Houser, D.S and D.E. Crocker. 2004. Glucose production and substrate cycle actvity in a fasting adapted animal, the northern elephant seal. Journal of Experimental Biology 208:859-868.

Champagne, C.D., D.E. Crocker, M.A. Fowler and D.S. Houser. 2012. Fasting physiology of the pinnipeds: the challenges of fasting while maintaining high energy expenditure and nutrient delivery for lactation. in M. McCue, ed. *Comparative Physiology of Fasting, Starvation, and Food Limitation.* Springer, Berlin, Heidelberg. 309-336.

Crocker, D.E., P.M. Webb, D.P. Costa and B.J. Le Boeuf. 1998. Protein catabolism and renal function in lactating northern elephant seals. Physiological Zoology 71: 485-491.

Davis, T.A., H.V. Nguyen, D.P. Costa and P.J. Reeds. 1995. Amino acid composition of pinniped milk. Comparative Biochemistry and Physiology 110B: 633-639.

Eisert, R. 2011. Hypercarnivory and the brain: protein requirements of cats reconsidered. Journal of Comparative Physiology B 181:1-17.

Eisert, R., O.T. Oftedal and G.K. Barrell. 2013. Milk composition in the Weddell seal *Leptonychotes weddellii*: evidence for a functional role of milk carbohydrates in pinnipeds. Physiological and Biochemical Zoology 86: 159-175.

El-Khoury, A.E. 1999. Methods for Investigation of Amino Acid and Protein Metabolism. CRC Press, Florida, USA.

Fouillet, H., C Bos, C. Gaudichon and D. Tomé. 2002. Approaches to quantifying protein metabolism in response to nutrient ingestion. Journal of Nutrition 132: 3208S-3218S.

Freudenberger, D., and J. Nolan. 1993. Protein turnover in two kangaroo sub-species (*Macropus robustus robustus* and *M. r. erubescens*) from divergent habitats and the sympatric feral goat (*Capra hircus*). Comparative Biochemistry and Physiology Part A: Physiology 105: 443-448.

Houser, D., and D. Costa. 2001. Protein catabolism in suckling and fasting northern elephant seal pups (*Mirounga angustirostris*). Journal of Comparative Physiology B 171: 635-642.

Huntley, A., D. Costa, G. Worthy and M. Castellini. 1987 Approaches to Marine Mammal Energetics Vol 1. *Society for Marine Mammalogy Special Publication.* Allen Press, Lawrence, KS.253p.

Jackson, A.A. 2009. Protein Turnover. European Journal Of Clinical Nutrition 63: 153.

Keiver, K., K. Ronald and F. Beamish. 1984. Metabolizable energy requirements for maintenance and faecal and urinary losses of juvenile harp seals (*Phoca groenlandica*). Canadian Journal of Zoology 62: 769-776.

Krockenberger, M., and M. Bryden. 1994. Rate of passage of digesta through the alimentary tract of southern elephant seals (*Mirounga leonina*)(Carnivora: Phocidae). Journal of Zoology 234: 229-237.

MacDonald, M.L., Rogers, Q.R. and J.G. Morris. 1984. Nutrtion of the domestic cat, a mammalian carnivore. Annual Review of Nutrition 4:521-562.

Oki, C., and S. Atkinson. 2004. Diurnal patterns of cortisol and thyroid hormones in the Harbor seal (*Phoca vitulina*) during summer and winter seasons. General and Comparative Endocrinology 136: 289-297.

Pernia, S.D. 1984. Protein turnover and nitrogen metabolism during long term fasting in northern elephant seal pups. PhD Thesis. University of California, Santa Cruz,California.

Picou, D., and T. Taylor-Roberts. 1969. The measurement of total protein synthesis and catabolism and nitrogen turnover in infants in different nutritional states and receiving different amounts of dietary protein. Clinical Science 36: 283-296.

Robbins, C.T. 1983. Wildlife Feeding and Nutrition. Academic Press, New York, NY.

Rosen, D.A., and A.W. Trites. 2000. Digestive efficiency and dry-matter digestibility in Steller sea lions fed herring, pollock, squid, and salmon. Canadian Journal of Zoology 78: 234-239.

Russell, K., G. Lobley and D. Millward. 2003. Whole-body protein turnover of a carnivore, *Felis silvestris catus*. British Journal of Nutrition 89: 29-37.

Sarwar, G., H.G. Botting, T.A. Davis, P. Darling and P.B. Pencharz. 1998. Free amino acids in milks of human subjects, other primates and non-primates. British Journal of Nutrition 79: 129-131.

Schaafsma, G. 2000. The protein digestibility-corrected amino acid score. The Journal of Nutrition 130: 1865S-1867S.

Schmidt-Nielsen, K. 1997. Animal Physiology: Adaptation and Environment. Cambridge University Press, New York, New York.

Singer, M.A. 2003. Do mammals, birds, reptiles and fish have similar nitrogen conserving systems? Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 134: 543-558.

Stanberry, K. 2003. The effect of changes in dietary fat level on body composition, blood metabolites and hormones, rate of passage, and nutrient assimilation efficiency in harbor seals. Animal Science. MS Thesis. University of Hawaii. 109pp.

Trumble, S.J., P.S. Barboza and M.A. Castellini. 2003. Digestive constraints on an aquatic carnivore: effects of feeding frequency and prey composition on harbor seals. Journal of Comparative Physiology B 173: 501-509.

Waterlow, J.C. 1999. The mysteries of nitrogen balance. Nutrition Research Reviews 12: 25-54.

Waterlow, J.C. 2006. Protein Turnover. CABI Publishing, Wallingford, Oxfordshire.

Waterlow, J.C., P.J. Garlick and D. Millward. 1978. Protein Turnover in Mammalian Tissues and in the Whole Body. Elsevier/North-Holland Publishing Co., Amsterdam, The Netherlands.

Webb, P., D. Crocker, S. Blackwell, D. Costa and B. Boeuf. 1998. Effects of buoyancy on the diving behavior of northern elephant seals. Journal of Experimental Biology 201: 2349-2358.

Whitehead, R.G., and R.F.A. Dean. 1964. Serum amino acids in kwashiorkor II. an abbreviated method of estimation and its application. American Journal of Clinical Nutrition 14: 320-330.

Wolf, N., S.A. Carleton and C.M. del Rio. 2009. Ten years of experimental animal isotopic ecology. Functional Ecology 23: 17-26.

Wolfe, R. 1992. Radioactive and Stable Isotope Tracers in Biomedicine: Principles and Practice of Kinetic Analysis. Wiley, New York, NY.

Worthy, G.A., and D. Lavigne. 1987. Mass loss, metabolic rate, and energy utilization by harp and gray seal pups during the postweaning fast. Physiological Zoology 60: 352-364.

Zhao, L. 2002. Tracing amino acid metabolism of harbor seal *(Phoca vitulina)* using stable isotope techniques. PhD Thesis. University of Alaska Fairbanks, Fairbanks, AK. 211pp.