Exxon Valdez Oil Spill
Restoration Project Annual Report

Effects of Harbor Seal Metabolism on Stable Isotope Ratio Tracers

Restoration Project 99371
Annual Report

This annual report has been prepared for peer review as part of the *Exxon Valdez* Oil Spill Trustee Council restoration program for the purpose of assessing project progress. Peer review comments have not been addressed in this annual report.

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Study History: The use of stable isotope ratios as natural tracers of food webs is a powerful tool if the food sources are limited and there are distinct and separable isotopic signatures. One complicating factor present in the analysis of isotope ratios in top consumers is the possibility of isotopic fractionation or the smoothing of isotopic signals through metabolic processes. Often, in complex ecosystems, there is sufficient overlap among isotope ratios in prey species that interpretation of consumer isotope ratios is severely compromised. This project seeks to determine the presence or absence of biomarker molecules useful in food web tracing in harbor seals. Although there have been studies indicating the presence of fatty acids in grey seals that are derived solely from dietary prey, there are very little data on amino acids in harbor seals and no data regarding the environmental sources or degree of conservative behavior in passing up the trophic ladder. In this study, we are adding stable isotope labels to captive harbor seals at the Alaska SeaLife Center and following their incorporation into other amino acids and the reverse process in which the catabolism rates of essential amino acids is determined. The goal is to identify conservative biomarkers that would be useful with wild populations to identify feeding habitats and specific prey using a nonlethal sampling such hair, claw or blood with little lasting perturbation to the seal.

Abstract: This project seeks to identify biomarker molecules via controlled isotopic labeling of amino acids in seals at the Alaska SeaLife Center (ASLC). The past 18 months have been spent developing protocols for amino acid separation and in optimizing continuous flow mass spectrometry for the analysis of very small samples. The first seal labeling experiment and subsequent blood sampling has been accomplished. The amino acid standards have been run successfully and the initial serum hydrolyzates using derivatized amino acids separated. Samples of prey species have been acquired and are being readied for sampling. Amino acid labeling of three seals was undertaken at the ASLC in December 1998 and subsequent blood sampling has been conducted throughout the feeding protocol, ending in September 1999. Samples are now being processed and the procedures for the next experiment with essential amino acids beginning in May 2000 are being finalized.

Key Words: *Exxon Valdez* oil spill, food webs, harbor seals, $\delta^{13}C$, $\delta^{15}N$, isotope ratios, *Phoca vitulina*, Prince William Sound.

Project Data: (will be addressed in the final report)

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EXECUTIVE SUMMARY

This project seeks to determine the presence or absence of biomarker molecules useful in food web tracing in harbor seals. Although there have been studies indicating the presence of fatty acid abundances in grey seals that are derived solely from dietary prey, there are very little data on harbor seals and no data regarding the environmental sources or degree of conservative behavior in passing specific fatty acids or amino acids up the trophic ladder. In this study, we are adding stable isotope labeled essential and nonessential amino acids to captive harbor seals at the Alaska SeaLife Center and following their incorporation and metabolism. The goal is to identify conservative biomarkers that would be useful with wild populations to identify feeding habitats and specific prey using only a biopsy sampling or hair clip with little disturbance to the seal. To date, work has consisted of developing the techniques for amino acid analysis and obtaining the isotope ratios from each amino acid. Initial separations using HPLC were successful, but requires collection of eluate containing each amino acid peak and subsequent tedious processing before mass spectrometer. An alternate technique was tried consisting of derivatization of the amino acids into volatile esters that can be separated by gas chromatography. Separation is followed by direct combustion and stable isotope analysis. We have also purchased a new HPLC with greater size capability in order to allow isolation of amino acids present in low percentages.

Three harbor seals at the ASLC were infused with ^{15}N –labeled glycine on 16 December and are currently being monitored for isotope labels in blood serum. Each seal is on a different diet so that dietary effects can be assessed along with monitoring of the isotope distribution over time. The application of label was sufficient to allow the easy identification of any essential amino acids that did not translocate appreciable ^{15}N into synthesis of specific amino acids.

This report describes experiments underway or completed at the Alaska SeaLife Center (ASLC) and at the University of Alaska Fairbanks (UAF) to provide calibration and more detailed information on stable isotope transfers and fractionation in marine mammals. This will enable better interpretation of natural abundance isotope data acquired in Prince William Sound and the adjacent Gulf of Alaska. Coordination with the studies of Dr. M. Castellini who is conducting feeding experiments and dietary studies at ASLC will lead to a thorough integration of efforts and optimization of the use of animal subjects in all years of the study. Years 1 and 2 have consisted of the refinement of analytical techniques isolating amino acids and testing for the presence of essential amino acids in harbor seals at ASLC. This final year will see completion of the analytical work and the synthesis of the results.

INTRODUCTION

This report summarizes the progress to date on restoration project 99371. After 18 months we have completed a major suite of labeling experiments and developed the analytical procedures for isolation and isotopic analysis of the individual amino acids in harbor seal blood. As described below, this has required more time than anticipated

through the complexity of the amino acid isolation procedure and some extensive machine repair problems. These difficulties are mostly resolved and the experimental work is now underway.

Stable isotope ratios have become an essential tool in the study of living organisms and their physiology. The hazards of handling radioisotopes and severe protocol requirements when using live organisms have resulted in a steadily increasing shift to the use of stable isotopes as tracers for both human and animal subjects. Some usages, such as the detection of *Helicobacter pylori* infections in ulcer patients, are now routine and bringing stable isotope analysis to many hospitals as a standard method. In contrast to the employment of natural abundance techniques in the marine environment, most physiology experiments employ compounds enriched with ^{13}C or ^{15}N to enhance detectability and to follow the transfers to different metabolites within the organism. Improved lower limits of detectability and smaller sample size requirements now allow the use of stable isotopes where only radioisotopes would have worked in the past.

Over the past two decades, isotope ratio analysis has emerged as a powerful tool in ecosystem research, both on the process scale and as a validation technique for large-scale ecosystem models (Michener and Schell, 1994). In relevant applications to this study, Saupe et al (1989) and Schell et al. (1989) described a geographic gradient in isotope ratios in biota across the Alaskan Beaufort Sea and the Bering–Chukchi seas and showed that this gradient could be applied to describing bowhead whale natural history. The isotopic gradient arises from the primary producers in the ecosystem (Laws et al. 1995) and is passed up food chains to label consumers up to the top predators. Within each biome, there is reasonable fidelity to the $\delta^{13}C$ observed in the primary producers and a predictable increase in the $\delta^{15}N$ with each known increase in trophic level. However, among individuals of each taxon analyzed there are often large ranges in values, especially in the carbon isotope ratios.

A fundamental assumption in the employment of isotope ratios as natural tracers is that the amount of isotopic fractionation in the process of metabolizing food is known during the incorporation of assimilated components into the consumer. For marine mammals, these data are scarce and most of the ongoing work is based on the findings derived from terrestrial bird and mammal studies. The accurate interpretation of isotope ratio data on food webs and marine mammals depends completely on knowledge of fractionation effects arising from dietary sufficiency and composition. To date, we do not have this knowledge because it has become evident that there exist marked geographic gradients in isotope ratios in Prince William Sound and the Gulf of Alaska. This project is thus aimed at the goal of identifying specific biomarker molecules and acquiring accurate isotope fractionation data on harbor seals through controlled feeding and laboratory experiments. This project is being integrated with ongoing research on harbor seal diets at the ASLC and is complementary to the physiological research projects in progress.

A. Statement of Problem

Harbor seals were undergoing an unexplained decline in numbers before the oil spill and the decline was further accelerated by the disaster. Since that time the population has not recovered and is still at a low level, although now perhaps finally stabilized. No definitive cause and effect relationships have been found for the decline or failure to recover. It is becoming increasingly evident, however, that change in the marine environment in the past two decades has altered the carrying capacity downward in the northern Gulf of Alaska and the effects are being felt to top of the food chains. Carbon isotope ratios in biota of the northern Pacific Ocean appear to have been declining for nearly twenty years (Schell, 2000) and imply that a major decrease in productivity has occurred. Isotope ratios from wild seals also show changes over time in the isotope ratios but the interpretation requires knowledge of both the fractionation that occurs during assimilation and the natural variations arising from migratory movements. If one or more essential amino acids can be identified in the diet of seals, these would allow a conservative tracer independent of isotope fractionation effects. There are almost no data regarding marine mammals on this subject and none on harbor seals. This study will undertake to follow both the "whole animal" carbon and nitrogen isotopic fractionation and the determination of specific biomarkers arising from diet that would allow clearer insight into dietary dependencies.

B. Rationale/Link to Restoration

Carbon isotope ratios serve as conservative tracers of energy supply between trophic levels (phytoplankton to zooplankton to fishes to top consumers). Seals, cetaceans, birds, etc. acquire the isotope ratios in proportion to the amount of food derived from each differing source. This, in turn, is reflected in the composition of body tissues and in keratinous tissues (claws, feathers, baleen, whiskers) as a temporal record when multiple sources of food are consumed over time and space (Schell and Saupe 1993). Comparison of source and consumer isotope ratios allows the discerning of important habitats and food resources in animals such as harbor seals that seasonally migrate or undergo periods of hyper- and hypotrophy. Little is known, however, of the internal fractionation of isotopes that occurs in mammals during fasting and/or extended periods of suboptimal diets. The experiments conducted on the effects of differing diets on captive harbor seals at the ASLC provide an ideal opportunity to enhance the physiological data gained by investigating the efficiency of amino acid transfers in diets and the presence of essential amino in pinnipeds.

Nitrogen isotope ratios reflect both the food sources and the trophic status of that animal. As nitrogen in food is consumed and assimilated by a consumer, the heavy isotope is enriched by approximately 3‰, with accompanying loss of the lighter isotope through excretion. The enrichment occurs with each trophic step and thus allows the construction of conceptual models and food webs and the assignment of relative trophic status to species for which dietary data are sparse. Hobson and Welch (1992) used isotope ratios to describe the trophic relationships of birds and mammals to the available prey species in the Canadian Arctic. Further extension to benthos by Dunton et al. (1991) and to fishes

(Vinette, 1992) has confirmed that the isotopic trends are evident across the entire food web. During fasting or starvation, nitrogen isotopes may be fractionated during transamination reactions leading to overall shifts in the average isotope ratios of the whole animal. Best and Schell (1996) observed, for example, that ^{15}N enrichment in southern right whales evidenced during winter breeding season in South African waters when carbon isotope ratios revealed that very little feeding occurred. Detailed interpretation of data from samples taken from wild seals requires that these effects be known.

C. Location

The research effort will be conducted at the Alaska SeaLife Center and the University of Alaska Fairbanks. The instrumental analyses such as HPLC and gas chromatography-mass spectrometry are being undertaken at UAF on samples collected during the dietary studies and sampling at ASLC by Dr. Castellini's group. In year 3 more of the effort will be focused on amino acid composition in prey and the isotope ratios of the essential amino acids in mammals.

OBJECTIVES

The null hypotheses tested are as follows:

1. The isotope ratios of harbor seals accurately reflect diet under all conditions. Increased fractionation does not occur during periods of fasting or suboptimal feeding and does not affect either carbon or nitrogen isotope ratios in harbor seals.

2. There are no essential amino acids in harbor seals and their prey that can act as conservative markers of specific habitats of food sources or of specific prey species.

The objectives over the past 18 months and ahead are divided into three elements:

1. Year 1 consists of developing methods and protocols for the isolation of metabolites from harbor seal blood and tissue samples to be employed during the following controlled diet studies. This objective required considerably more time than anticipated as detailed below in Methods and Preliminary Results.

2. The second component is a study of the effects of "suboptimal" versus optimal diet on the fractionation of carbon and nitrogen isotopes in harbor seals. Diets of known amount and composition (isotopic and energetic) are being fed to the seals at ASLC and blood protein amino acids will be monitored for composition and isotope ratios. This research has been closely coordinated with studies of controlled diet/assimilation efficiencies in harbor seals by Dr. M. Castellini so that minimal animal handling and sampling will be necessary.

3. The third component was to determine source prey for isotopically distinct fatty acids or other metabolites. The identification of specific fatty acids that carry a conservative signal to top consumers (birds, cetaceans, fissipeds) would yield an valuable tool to follow food web transfers or to identify specific habitat importance. This was to be accomplished by the analysis of lipids in prey species from locations around the study areas and from seals. However, it became apparent that this aspect was also the focus of Project 99441 and that the amino acid study was requiring full time effort. As a result our revised objective for FY01 is to determine the individual amino acid compositions and isotope ratios of proteins in Gulf of Alaska harbor seal prey species. Many of the prey specie samples are already archived and analysis will be conducted over the next year.

METHODS

Isotope Ratios in Harbor Seal Amino Acids

The initial feeding experiments with pollock and herring commenced in September 1998. After the new diets were underway, on 2 December 1998, three seals were infused with ^{15}N –labeled glycine. One seal (Poco) was fed a diet of pollock from September onward; the second (Pender), a diet of herring and the third (Snapper) was fed a 50-50 mix of pollock and herring. Before infusion, blood samples were taken to establish the natural isotope ratio distributions in serum protein amino acids and red blood cells. After intravenous infusion of 500 mg of ^{15}N-glycine, the seals were resampled after four hours to determine the initial blood concentrations of label. Blood sampling was repeated after 24 hours, 3 days, 15 days, 27 days, and 31 days and continued at two-week intervals until 16 September 1999. Each sample was spun down to separate serum and cells and the subsamples held frozen at -70^{o}. Red blood cells were not washed so an initial serum contamination in cell samples was expected in the early samples. A whisker was also collected at the start of the experiment to allow additional data collection on the growth characteristics of vibrissae and to follow the incorporation of label into keratin amino acids. These samples are now in Fairbanks and analysis is underway. Preliminary results are shown below.

Amino acid analysis

The analytical procedures employed identify both free amino acids in serum and the total amino acids present in both free and polymerized forms. In order to facilitate HPLC analysis, sulfosalicylic Acid (SSA) is used initially to precipitate proteins in serum following centrifugation of red blood cells. Samples are brought to room temperature from storage at -80C. A 1.0ml sample is transferred to a microcentrifuge tube followed by the addition of 100µl 20% SSA with vigorous mixing. After cooling in ice for 10 minutes, the SSA extract is centrifuged to pellet the precipitated protein. At this point the supernatant contains only the free amino acids. The supernatant is filtered through a 0.2 µm Teflon filter before injection onto the HPLC column. Free amino acids are measured using precolumn OPA/2-mercaptoethanol derivatization method with a

Gilson automated HPLC system. Separation is performed on an Allsphere ODS-25 Micron (250×4.6 mm, 0.5 μm particle size). UV detection of eluted amino acids is run at 340 nm. The mobile phase gradient progresses from an initial solvent containing 15% methanol in 0.025 M phosphate buffer to 80% methanol using a linear gradient over 25 minutes, holding for 5 minutes, then returning instantly to initial solvent composition and flushing 5 minutes before next injection. Quantitative concentrations of individual amino acids are determined by comparison of sample peak areas with those of standard amino acid mixtures. This procedure is used primarily for quantification of amino acid distributions and will be used to assess essential amino acid abundance in both harbor seal tissues and in prey species.

Total amino acids

In order to measure total amino acids in protein hydrolyzates of blood samples, initial acid hydrolysis is employed to liberate amino acids into solution. The hydrolysis procedure is as follows: Both RBC and serum samples are freeze-dried before the addition of HCl. After weighing 20 mg of sample into 5 ml ampoules, 2 ml 6 N HCl is added. Nitrogen is used to flush traces of oxygen for 2 minutes before sealing the ampoules under nitrogen. Hydrolysis is accomplished at 110C for 22 hours. Before injection onto the HPLC system, hydrolyzates are neutralized with 6 N NaOH and filtered through 0.2 μm Millipore filters. As noted above, the current analytical system employs derivatized amino acids for increased sensitivity in the determination of individual amino acid abundances. Alternate techniques will be required to provide eluted amino acids suitable for mass spectrometry.

Future efforts will be directed toward the use of inorganic buffer streams with underivatized samples to allow direct use of both carbon and nitrogen isotope ratios in the eluted amino acids. This however, will require new columns and machine capability. We are currently purchasing the needed equipment and hope to be online in early summer 2000.

Mass Spectrometry

The samples obtained are dried and powdered for homogeneity and the isotope ratios of carbon and nitrogen determined with a Europa 20/20 mass spectrometer system. The sample is combusted at high temperature and the nitrogen and carbon dioxide gases separated and purified by gas chromatography. These are subsequently led into the mass spectrometer by capillary and the isotope ratios determined. Results are reported in the standard $\delta^{13}C$ and $\delta^{15}N$ notation.

PRELIMINARY RESULTS AND CONCLUSIONS

Free amino acids in serum.

The isolation and separation of free amino acids present in serum showed that concentrations were very low. This indicates that almost all of the amino nitrogen was

present in the form of proteinaceous polymers, which would include both peptides and proteins. Our initial analyses showed 8 peaks of individual amino acids (serine, glycine, tyrosine, phenylalanine, valine, isoleucine, leucine, and lysine) at levels below ranges for accurate quantification. This work is being repeated with higher concentrations to establish relative amounts and to determine the fraction of total nitrogen present in free amino acids. Similarly, red blood cell hydrolyzates are currently being run from each seal to establish relative abundances and the range of variability arising from specific diets.

Labeling of serum and red blood cells

The initial results on the seal blood samples showed that $\delta^{15}N$ values in serum increased immediately after ^{15}N- enriched glycine injection, then decreased exponentially with half-lives of about 10 days for all three seals (Figure 1a,b) during the first 30 days. Initial $\delta^{15}N$ values reached over 80 ‰ but dropped quickly over the next few days as metabolic processes consumed the glycine via transamination, incorporation into proteins and respiration. No attempt was made to measure losses via excretion. The blood sampling at 4 hr after infusion was assumed to occur at the point where loading of label had reached equilibrium throughout the seal blood volume. After metabolic equilibrium had occurred, $\delta^{15}N$ values continued to decrease gradually in response to dilution from the unlabeled diet. Subsequent replacement of blood proteins became slower with average half-lives of approximately 40 days.

In contrast, $\delta^{15}N$ values in red blood cells (RBC), shown in Figure 2 were much lower throughout the sampling period than serum, indicating that the appearance of label depended upon the synthesis of new red blood cells from a much less labeled pool. Initial high values within a few hours of infusion, shown in Figure 2 are due to serum contamination with a much higher level of label. Cells were not washed after centrifugation and the high initial serum label biases the first few samples. Within 15 days, however, the RBC show an average label of approximately 4‰ above the zero-time values indicating significant incorporation into newly formed cells. The label is then slowly lost over the following 170 days and then drops more sharply at the time of diet change. Red blood cells synthesized during the period when high levels of label were in the seal persist for over 120 days. However, all seals then underwent a faster turnover of red blood cells beginning near day 130 and continuing to about day 200. Although the diet was changed during this period, the shift was independent of diet in that the control seal with constant diet showed a very similar pattern. Over time $\delta^{15}N$ values continued to decline slowly in seals as the label was replaced with unlabeled amino acids.

Natural abundance shifts in response to diet and physiology

The carbon in diet represents both the structural components and energy source for the organism. Although this experiment used only elevated levels of ^{15}N, the natural abundance differences in the fishes used in the diet offer insight into the turnover rates of blood amino acids and other metabolites. The $\delta^{13}C$ values in serum of harbor seal blood tracked the diet switches almost exactly (Figure 3). Relatively enriched $\delta^{13}C$ values of pollock as a diet ($\delta^{13}C$ = -19.8‰, n = 7) led to an increase in serum $\delta^{13}C$ values of the

seal Poco in fall 1998. After switching the seal diet to herring in December 1998, the lower values for herring ($\delta^{13}C$ = -22.34‰, n =10) led to a decrease in the total serum protein isotope ratios. Upon switching back to pollock in Spring 1999, the began to increase and after approximately 50 days were near the same range as prior to switching

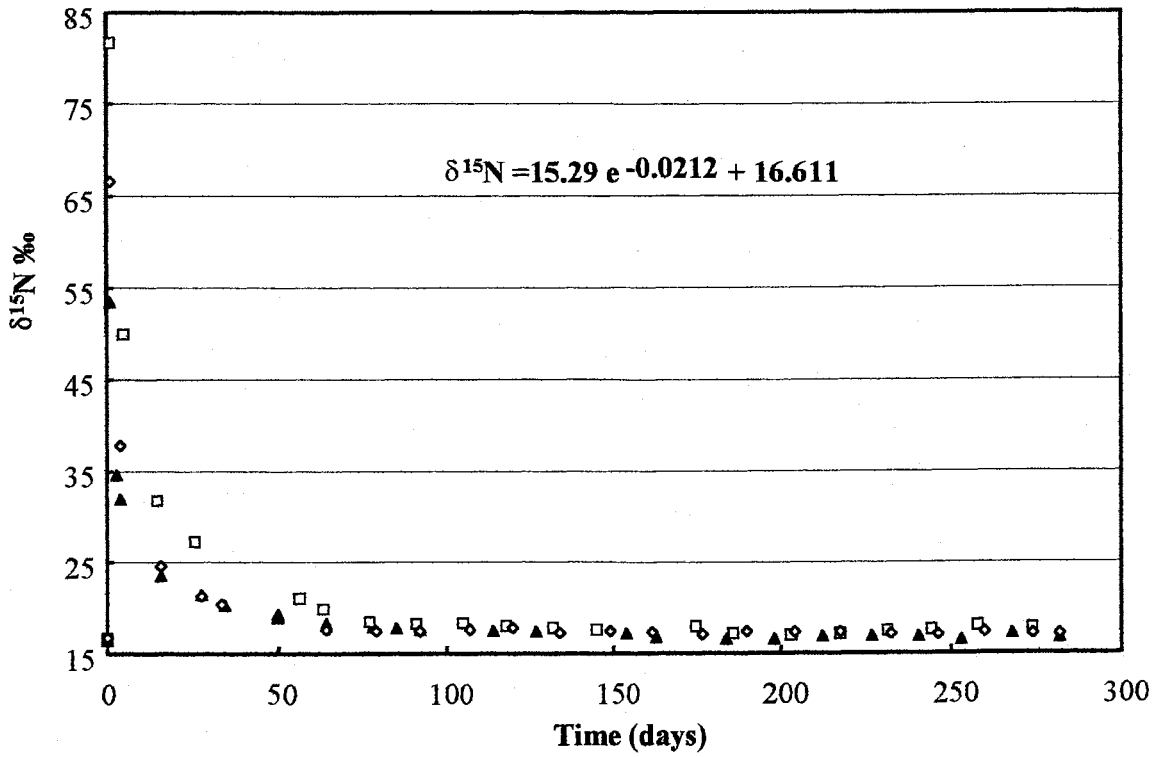

Figure 1a. Nitrogen isotope ratios in harbor seals prior to and following infusion of 500 mg ^{15}N-glycine. Initial labeled samples were taken approximately 5 hrs following infusion. Seals are: ◇ Poco; □ Pender and ▲ Snapper (control diet). See text.

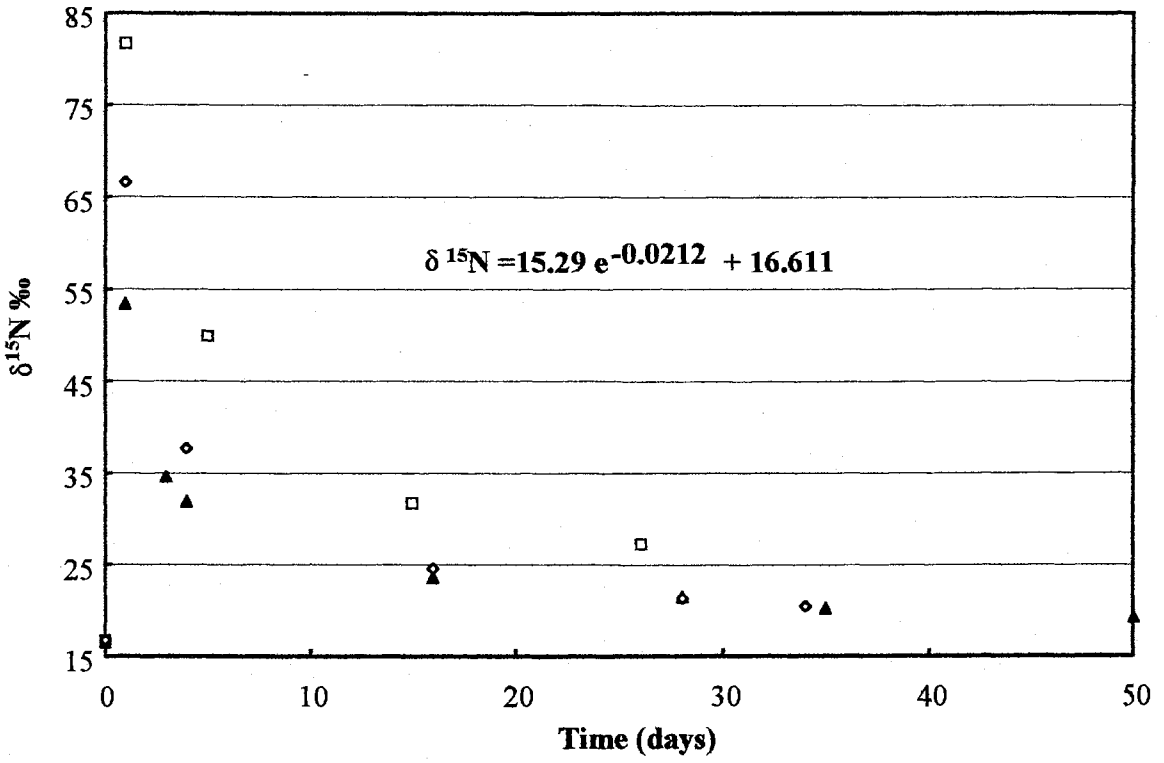

Figure 1b. Expanded version of Figure1a. Seals are: ◇Poco; □Pender and ▲Snapper (control diet). See text.

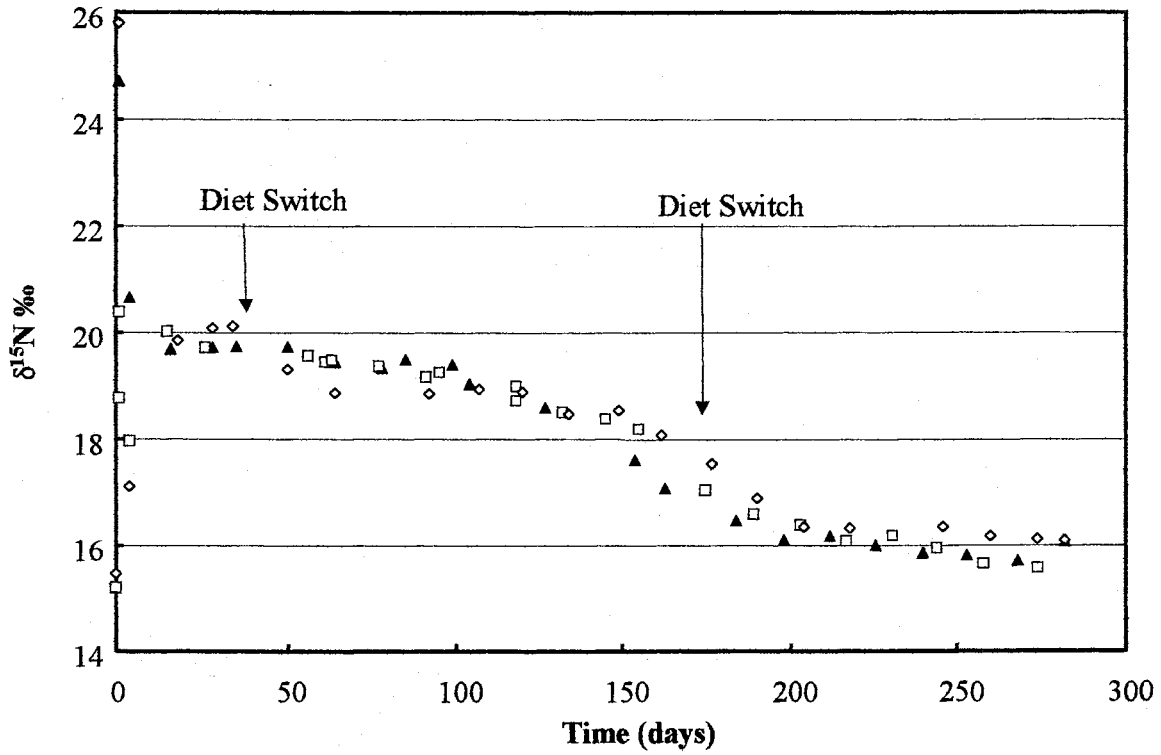

Figure 2. Nitrogen isotope ratios in harbor seal red and white blood cells. Initial values greater than $\delta^{15}N = 22$ ‰ are assumed due to serum contamination as cells were not washed. The inflection near day 150 is physiological and not induced by diet switch as all seals including the control animal (no diet change) followed a similar pattern. Seals are: ◇ Poco; □ Pender and ▲ Snapper (control diet). See text.

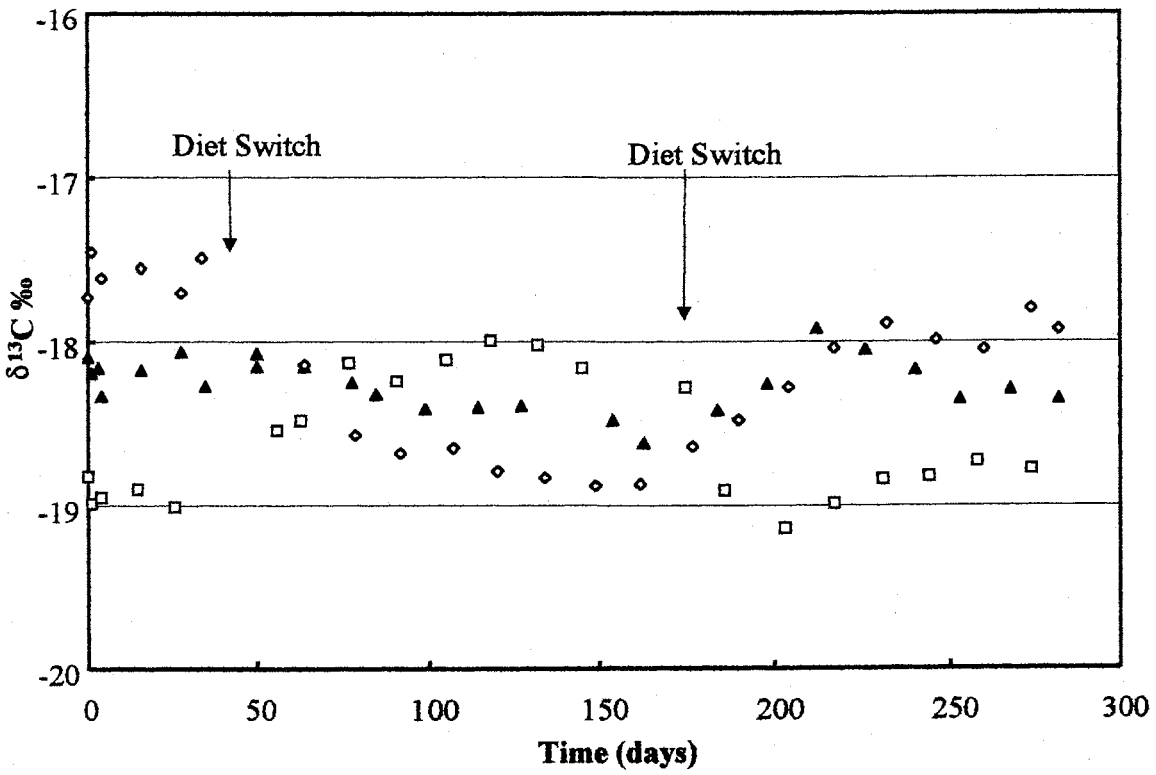

Figure 3. Natural carbon isotope ratio changes in response to diet in harbor seal serum. Poco (◇) diet switched from pollock to herring and back to pollock; Pender (□) diet switched from herring to pollock and back to herring; and Snapper (▲) on a constant control diet of 50% pollock and 50% herring.

to herring. The inverse is evident for the seal Pender who began with a herring diet and was switched to pollock and then back to herring.

In contrast, $\delta^{13}C$ values in blood cells did not reflect the dietary changes over the course of experiment to nearly the same extent (Figure 4). This is ascribed to the much longer mean lifetime of individual cells in the blood with a consequent insufficient time to reach equilibrium with the new diet. Turnover times calculated for the blood cells is similar to that estimated from the nitrogen labeling.

Future Work

This project is currently completing the amino acid analyses on prey species and on the seal proteins to establish the essential amino acid distributions and abundances in diet and consumer. Simultaneously we are developing the analytical capability for isolating individual amino acids for mass spectrometric analysis to determine rates of cross-labeling following infusion and metabolism of essential amino acids in the seals. This experiment is planned for the next dietary change scheduled for May 2000 and will complete the experimental portion of this study. The acquired samples will provide a full suite of amino acid data giving good indication of conservative biomarkers useful in feeding studies. Synthesis of the data and manuscript preparation will ensue over the following months.

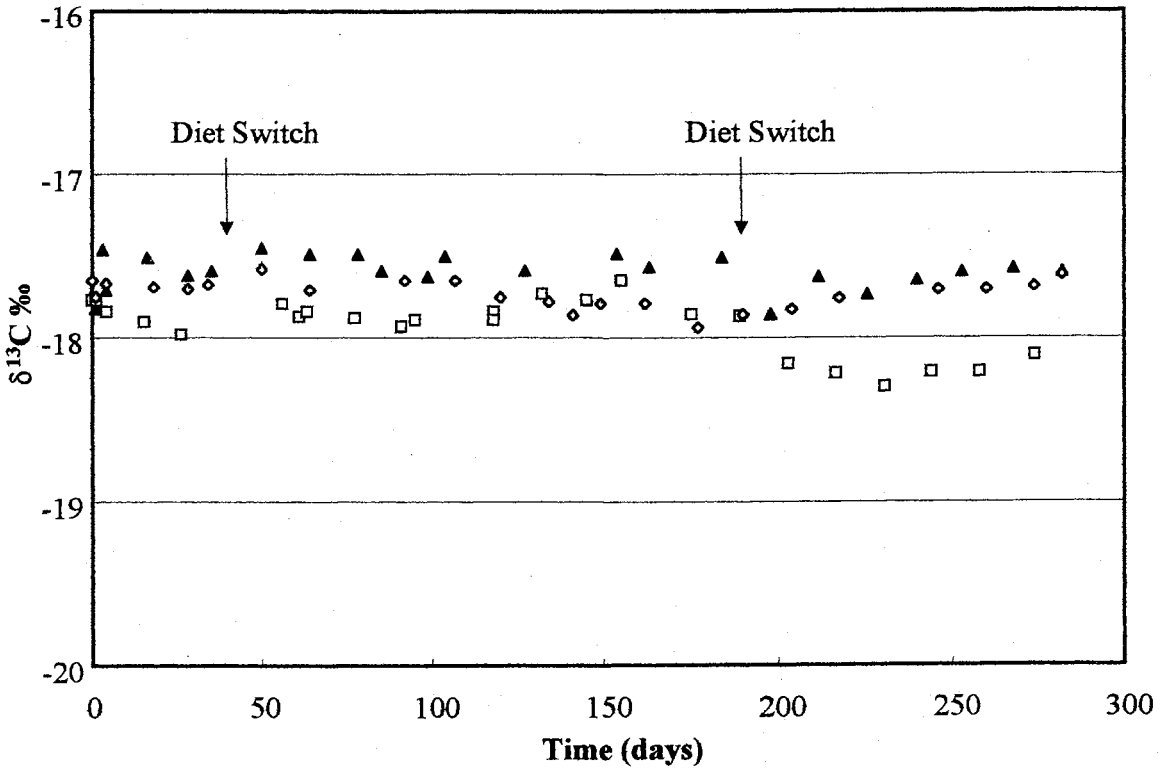

Figure 4. Natural carbon isotope ratios in red and white blood cells over the course of the experiment. Seals are: ◇ Poco; □ Pender and ▲ Snapper (control diet).

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