Project Title: Harbor Seal Recovery: Phase III. Effects of Diet on Lipid Metabolism and Health: Completion of Sample Analysis and Manuscript Preparation. Submitted under the BAA

Project Number:	02441
Restoration Category:	Research
Proposer:	Randall Davis, Ph.D., Texas A&M University at Galveston
Lead Trustee Agency:	Alaska Dept. of Fish and Game
Cooperating Agencies:	
Alaska SeaLife Center:	No
Duration:	4th year, 4-year project
Cost FY 04:	$ 20.4
Geographic Area:	Prince William Sound and Alaska SeaLife Center
Injured Resource:	Harbor seals

ABSTRACT

In 1998, we began a three-year study on the effects of diet on lipid metabolism and health in harbor seals (*Phoca vitulina richardsi*). This study was prompted by a decline in the number of harbor seals in Prince William Sound during the past 10-15 years. An underlying hypothesis for the decline is that ecosystem-wide changes in food availability could be affecting harbor seal population recovery. To better understand the results from field studies of harbor seal health, body condition and feeding ecology, we need data for seals on diets that vary in nutritional composition. Working with the Alaska SeaLife Center, we collected blubber and muscle samples from captive harbor seals during controlled diets of herring and pollock that were alternated every four months for two years. These samples will be used to determine how the fatty acid composition changes with diet to better interpret field data for wild harbor seals. Preliminary analysis of available data indicates that the fatty acid signature in the blubber changes in response to an alternating diet of herring and pollock. However, analysis of the remaining blubber samples from captive harbor seals is needed to resolve the temporal scale of the changes. In the proposed work, we will complete the analysis of blubber samples that have already been taken, but could not be completed due to a shortage of funds available to the EVOS Trustee Council in FY2001. In addition, we will prepare a final report and manuscripts. Our results will complement those from other studies of harbor seal dietary preferences, health, body condition and ecology conducted by Frost (Project 001- Harbor seal condition and health status; Project 064- Monitoring habitat use and trophic interactions of harbor seals), Iverson (Project 117-BAA- Harbor seal blubber and lip), and Castellini (Harbor Seal Recovery. Phase II: Controlled Studies of Health and Diet).

INTRODUCTION

Understanding the feeding ecology and nutritional status of harbor seals is an essential component of ecosystem-based research on the recovery of species impacted by the Exxon Valdez oil spill in Prince William Sound. Until recently, determinations of prey preferences for pinnipeds have been based on stomach content and fecal analyses, both of which can only yield information on the most recent meals and may be biased due to differential rates of passage of food items. A new technique using fatty acid profiles of blubber can provide details on cumulative dietary history. It can also, in some cases, be used to determine foraging habitat. In pinnipeds, as with other carnivores and monogastric animals, dietary fatty acids generally remain intact through the digestion process and are deposited in adipose tissue with little or no modification (Iverson 1993). As a result, differences in the fatty acid composition of carnivore blubber can be used to infer dietary differences between individuals or populations and perhaps even species composition of the diet.

Previous research has shown that fatty acid signatures are significantly affected by spatial or temporal heterogeneity in habitat and food webs (Iverson 1993). In a study of harbor seal foraging ecology (Project 117-BAA; Harbor seal blubber and lipids) supported by the Restoration Program, Iverson et al (1997) were able to distinguish individual species of fish using fatty acid signatures. They also found fatty acid composition of these prey items to be correlated with body size as well as location within a study area. Hence, analysis of fatty acids in pinnipeds and their prey should provide details on the spatial scales of foraging and habitat use of both individuals and populations. Evaluating how harbor seal blubber fatty acids change with diet during controlled feeding studies where species composition of diet is known will improve the spatial and temporal interpretation of fatty acid profiles of wild seals whose diet composition is unknown.

With controlled feeding studies of harbor seals at the Alaska SeaLife Center now completed, we are analyzing samples that will provide new information on the effects of diet on fatty acid signatures in blubber and the metabolic function of muscle, especially with regards to lipid. Because of the limited availability of funds, the EVOS Trustee Council deferred part of our support during the third year of this project. As a result, we were unable to complete the analysis of the remaining blubber samples being stored in our freezers. Funds requested in this proposal will enable us to complete the analysis of all of our samples, which will greatly increase spatial and temporal resolution as well as the statistical significance of results. The results will improve our understanding of harbor seal feeding ecology and the effects of diet on health and metabolism.

Status of Sample Analysis

Table 1 shows the number of samples that were obtained for analysis, the number (n) of animals sampled, the number of samples for which analysis will be completed in 2001 (Year 3 of this project), and those samples for which we are seeking additional funds to complete our analyses in 2002. In terms of the number of blubber and food samples collected, 56% will have been analyzed by the end of 2001, leaving the remaining 44% for 2002.

Table 1

Tissue/Sample	***Type of Analysis***	***n***	***No. of Samples***	***Samples Completed in 2001***	***To Be Completed in 2002***
Blubber[1]	Fatty acid analysis	8	331	168	163
Dietary fish samples[1]	Fatty acid analysis		41	41	0
Total			***372***	***209***	***163***

[1] Samples taken from captive harbor seals on a controlled diet at the Alaska SeaLife Center

Preliminary Results

Analysis of blubber fatty acids in captive harbor seals on a controlled diet of herring and pollock. We have completed the analysis of about half of the blubber samples and all of the dietary samples (Table 1). These blubber samples were taken at the beginning and end (i.e., every four months) of each dietary regime (see Table 2 in the Methods for details). CART analysis of the data indicates that the fatty acid signature in the blubber changes in response to an alternating diet of herring and pollock. However, the temporal resolution will be greatly enhanced and the statistical significance improved by analyzing the samples taken at the mid-point of each dietary regime. These were some of the samples that had to be deferred due to a lack of funds in FY 2001. If this proposal is funded, these latter samples will be analyzed in October and November of 2001 (i.e., early FY 2002).

Mitochondrial volume density and lipid droplet density in the muscles of captive harbor seals on a controlled diet. In a preliminary study with free-ranging harbor seals (Kanatous et al., 1999), we observed that the volume density of mitochondria, myoglobin concentration, volume density of lipid droplets and citrate synthase activity in the swimming muscles of harbor seals were elevated relative to terrestrial mammals of comparable size and appeared to be an adaptation to maintain aerobic metabolism during diving. However the results of the same study indicated diminished lipid stores in Prince William Sound harbor seals compared to other species of non-breeding Alaskan pinnipeds ($0.2 \pm 0.1\%$ in harbor seals and $1.1 \pm 0.3\%$ in Northern fur seals). These diminished lipid stores may have been a result of nutritional stress faced by these animals. Preliminary results from this study show a slightly higher volume density of lipid droplets ($0.3 \pm 0.08\%$ vs. $0.2 \pm 0.1\%$), but a significantly lower volume density of mitochondria in the skeletal muscles of captive harbor seals as compared to our previous values found in free-ranging harbor seals ($3.7 \pm 0.3\%$ and $9.3 \pm 0.2\%$). The lower volume density of mitochondria may be due to the effects of captivity (e.g., less activity and shorter dive durations). There also appears to be no effect of changes in diet on the volume density of lipid droplets or mitochondria in the skeletal muscles ($p=0.05$).

Analysis of enzyme activity in the muscles of captive harbor seals on a controlled diet and wild harbor seals. Assays for the three enzymes (citrate synthase, B-hydroxyacyl CoA dehydrogenase and lactate dehydrogenase) have been completed for 140 muscle biopsies from captive harbor seals and 500 samples from wild seals taken by Native hunters (Table 1). A total of 5,760 enzyme assays were run (640 muscle samples assayed for three enzymes in triplicate). Contour maps of the enzyme activities for the wild seals are being prepared (Surfer, Golden Software,

Inc., Colorado). Preliminary analysis of transverse sections of the swimming muscle (*Longissimus dorsi*) shows considerable heterogeneity for all three enzymes (i.e., a gradient in concentration is very apparent). Citrate synthase and lactate dehydrogenase both have higher activities toward the exterior of the muscle and lower activities toward the interior of the muscle closest to the attachment to the spine. β-hydroxyacyl CoA dehydrogenase has a higher activity in the dorsal portion of the muscle, and the activity decreases ventrally. Analysis and interpretation of the entire data set will be completed in 2001.

Fiber typing in the muscles of wild harbor seals.
Preliminary data from fiber typing of the swimming muscle (*Longissimus dorsi*) of wild harbor seals indicates that the muscle is comprised of Type I fibers (slow-twitch oxidative) and Type IIa fibers (fast-twitch oxidative), with Type IIb fibers (fast-twitch glycolytic) conspicuously absent. Fibers were counted only if they showed good staining specificity for the appropriate myosin heavy chain isoform. The average percentages of Type I fibers for the anterior, medial, and posterior cross-sections of the muscle were 47.0%, 47.0%, and 48.4%, respectively. The average percentages of Type IIa fibers for the anterior, medial, and posterior cross-sections were 52.0%, 51.7%, and 52.1%, respectively. No Type IIb fibers were detected in any of the muscle sections. These results differ from previous results of fiber typing using traditional histochemical techniques. The published data on fiber typing of harbor seal *Longissimus dorsi* indicates a high percentage of Type I fibers (approximately 45-47%), few (<10%) Type IIa fibers, and a high percentage (approximately 45-47%) of Type IIb fibers (Reed et al., 1994; Hochachka and Foreman, 1993). The difference in our results and those of previous studies probably results from the extreme specificity inherent in the immunohistochemical procedure used in our study. Further analysis will elucidate whether the majority of harbor seal swimming muscle is comprised of either oxidative muscle fibers or a mixture of oxidative and glycolytic fibers. If most of the fibers turn out to be Type I and Type IIa, our results will confirm the oxidative poise of harbor seal skeletal muscle and its ability to maintain lipid metabolism during diving. The results from mitochondrial volume density and lipid droplet density for matching muscle sections will further confirm this oxidative, lipid-based metabolism.

Mitochondrial volume density and lipid droplet density in the heart, liver, kidneys and small intestine of wild harbor seals. Volume densities of total mitochondria in the liver of the dog, rat, and harbor seal were 15.8%, 14.4%, and 22.9%, respectively. Volume densities of lipid droplets for the dog, rat, and harbor seal were 0.21%, 0.02%, and 1.3%, respectively. A previous study in our lab showed that pinnipeds have an elevated mitochondrial volume density and lipid droplet density in their swimming muscles as an adaptation for fat-based energy metabolism during diving hypoxia (Kanatous et al. 1999). This is the first study to examine other organs for similar adaptations. These preliminary data suggest that liver tissue, in addition to skeletal muscle, may have an enhanced aerobic capacity for fatty acid metabolism. Assays will also be run for citrate synthase, B-hydroxyacyl CoA dehydrogenase and lactate dehydrogenase. These analyses will be finished in FY 2001.

NEED FOR THE PROJECT

A. Statement of Problem

The Restoration Program has supported three harbor seal studies in Prince William Sound

(Project 001- Harbor seal condition and health status; Project 064- Monitoring habitat use and trophic interactions of harbor seals; Project 117-BAA- Harbor seal blubber and lipids). One objective of these studies was to measure health and body condition indices related to metabolic alterations that might occur in animals that were food deprived. Although these studies collected much useful information, some researchers realized that controlled dietary studies were needed to better interpret field data. In 1997, the Restoration Program funded a captive study (Harbor Seal Recovery. Phase II: Controlled Studies of Health and Diet) at the Alaska SeaLife Center designed to quantify the nutritional value of several key Alaskan fish species for harbor seals and to follow health indices over time in both healthy and rehabilitation animals. That project, which was successfully completed in October 2000 at the Alaska SeaLife Center, fed controlled diets of fish to harbor seals to examine changes in body condition, health, assimilation efficiency and blood chemistry biomarkers. We participated in these controlled feeding studies and took blubber biopsies every two months for fatty acid analysis and muscle biopsies every four months for mitochondrial volume density and lipid droplet density from eight harbor seals on the diets that alternated between herring and pollock. This resulted in 331 blubber samples, 41 dietary samples, and 140 muscle samples from eight harbor seals during the two year feeding trial (see (Table 1 above for details). In addition, we collected 500 muscle samples for enzyme and myoglobin analysis, 250 muscle samples for fiber typing and 40 organ samples for mitochondrial volume density and enzyme analysis from 10 wild harbor seals as part of the BIOSAMPLING Program in Prince William Sound. The analysis of this very large set of samples has occupied our lab for he past 18 months. However, additional funds will be necessary to complete analysis of the blubber samples. We requested these funds during the third year of this project, but the EVOS Trustee Council deferred our request. This proposal will enable us to complete the analysis of our samples and incorporate the results into the final report and manuscripts. This important work will augment previously funded investigations of diet and health to provide a more in depth understanding of the nutritional role of dietary fat for harbor seals.

B. Rationale

The harbor seal population in Prince William Sound has not recovered and may continue to decline. An underlying hypothesis is that ecosystem wide changes in food availability could be affecting harbor seal population recovery. To better understand the behavioral and physiological results obtained from field studies of harbor seal health, body condition and feeding ecology supported by the Restoration Program, we need comparable data for seals on diets that vary in nutritional composition. In 1998, a captive study was begun at the Alaska SeaLife Center to quantify the health effects of feeding several key Alaskan fish species to harbor seals. We collected extensive tissue samples to study changes in fatty acid profiles in seal blubber and muscle lipid content during controlled feeding studies where fish species composition was known. In addition, we collected muscle samples from harbor seals in the controlled feeding study and from wild animals in Prince William Sound to quantify the aerobic capacity and activities of enzymes that are crucial for muscle lipid metabolism and which may be affected by nutritional stress. Although most of these samples have been analyzed, additional funds (which were deferred in Year 3 of this project), are needed to complete the analyses. When completed, this will be the most extensive study of it's kind on the effects of diet on lipid metabolism in harbor seals.

C. Location

The blubber samples, which have already been obtained and are currently stored at -70^{o} C, will be analyzed at Texas A&M University.

COMMUNITY INVOLVEMENT AND TRADITIONAL ECOLOGICAL KNOWLEDGE

Monica Riedel at the Alaska Native Harbor Seal Commission in Cordova was instrumental in arranging for us to obtain blubber and muscle samples from wild harbor seals as part of the BIOSAMPLING program (Project 96244). The cooperation of the Native community was excellent in giving us access to animals within six hours of death, which was critical for this study. As a result, we have a large sample size that will enhance the statistical significance of our results if we can complete the analysis. Copies of the final report and published manuscripts will be provided to the Alaska Native Harbor Seal Commission.

PROJECT DESIGN

A. Objectives

1. Complete analysis of blubber samples taken from captive harbor seals during controlled diets of herring and pollock.

B. Methods

1. Hypotheses to be Tested.

1. Null hypothesis: Fatty acid profiles in the blubber of harbor seals are not affected by the fatty acid composition of the diet.

   Alternative hypothesis: Fatty acid profiles in the blubber of harbor seals will be directly affected by the fatty acid composition of the diet and will change as the diet is altered.

   Methodology: Feed controlled diets of different fish species to captive harbor seals. Assess temporal changes in the fatty acid composition of the blubber by taking serial biopsies.

2. Harbor Seal Feeding Trials Conducted at the Alaska SeaLife Center (ASLC).

Animals. Eight harbor seals were acquired by the ASLC for the feeding trials that began in September 1998. During the staggered feeding trials, the diet was changed every four months. During these dietary manipulations, we obtained serial blubber samples every two months and muscle biopsies every four months from two sites on each animal. Blubber and muscle biopsies were taken from the same incisions located above the *L. dorsi* muscle in the dorsal lumbar region and above the pectoralis muscle on the animal's ventral thorax.

Design for Feeding Trials. The procedure used a crossover repeated measures approach that will allow statistical comparisons within any one group of seals between diet and season (Table 2).

Table 2. Crossover Repeated Measures ANOVA Feeding Trials for harbor seals

Period	Herring	Pollock	Condition
Sept-Dec 1998	Seals A,B,C,D	Seals E,F,G,H	Molting
Jan-April 1999	E,F,G,H	A,B,C,D	Spring
May-Aug 1999	A,B,C,D	E,F,G,H	Breeding
Sept-Dec 1999	E,F,G,H	A,B,C,D	Molting
Jan-April 2000	A,B,C,D	E,F,G,H	Spring
May-Aug 2000	E,F,G,H	A,B,C,D	Breeding

This feeding matrix allowed each group of seals to experience a different diet at similar physiologically relevant times of the year. Seals A,B,C,D for example, received a herring diet during the molting season in Year 1 and a high pollock diet in Year 2. A problem with crossover ANOVA designs is that residual or carry-over effects from previous treatments can complicate the analysis. We corrected for this with long test periods and phased crossovers. That is, since each feeding trial lasted for four months, several weeks of diet switching were allowed. This will provide the additional advantage of allowing us to study the impact of the phased switch on blubber and muscle lipid content and composition, and on muscle lipid metabolism.

Blubber Biopsies. Blubber samples were obtained through the full depth of blubber layer with a 6-mm punch biopsy inserted through a small incision in the skin. Each sample was then divided along its length to give an inner and outer sample. Samples were immediately transferred to liquid nitrogen and stored at -70°C until analysis. Total lipids will be extracted in chloroform according to Folch et al. (1957) as modified by Iverson (1988). Fatty acid methyl esters (FAME) will be prepared from the purified lipid extracts using the Hilditch reagent (0.5 N H_2SO_4 in methanol). FAME for fish in the controlled diets were obtained similarly from homogenates of individual food items. The methyl esters will be analyzed by temperature-programmed capillary gas-liquid chromatography. FAME will be identified and quantified using a combination of standard mixtures, including those identified using chromatography and an ion-trap mass detector. Individual fatty acids, expressed as weight percent of the total fatty acids, will be analyzed using classification and regression trees (CART) in S-plus (StatSci, Seattle), a non-parametric multivariate technique for classifying data. CART uses a series of algorithms to split data into groups as differently as possible, based on measures of deviance; the splitting continues in a tree-like form until a classification is made at a terminal node.

Statistical Analysis. Results will be expressed as the mean ± one standard error. We will use a crossover repeated measures approach that will allow statistical comparisons within any one group of seals between diet and season. Statistical software (SYSTAT) will be used to analyze

the crossover method. The relative proportions of fatty acids from blubber samples of seals in the controlled feeding study will be used as a basis for generating tree-based models (using S-Plus; StatSci, Seattle) of groups or classes of samples such that new samples can be compared with the modeled classes to decide their membership, i.e. obtain a classification of their "diet". Similarly, classification and regression trees will be used to screen the set of prey fatty acids and choose a subset of those fatty acids which can be used to classify the "diets" of seals based the patterns of fatty acid proportions in their blubber.

SCHEDULE

Measurable Project Tasks for FY 02

2001

Sept-Dec	Analyze remaining blubber samples.

2002

Jan-Mar	Statistical analysis and integration of data, including health and body condition results from Dr. Michael Castellini (Harbor Seal Recovery. Phase II: Controlled Studies of Health and Diet).
Apr-June	Prepare Final Report and begin manuscripts.
June-Aug	Complete manuscripts and submit to peer-reviewed journals. Five manuscripts are anticipated at this time.

B. Completion Date

This project will finish on September 30, 2002.

PUBLICATIONS AND REPORTS

Since this is a new project, there are no current publications. We anticipate at least five publications by 2002 on the effects of diet on fatty acids in blubber and the aerobic capacity and lipid metabolism in harbor seal muscle. The manuscripts are tentatively entitled:

Manuscript 1: Effects of diet on the fatty acid signature in the blubber of harbor seals.

Manuscript 2. Effects of diet on the aerobic capacity and lipid content of harbor seal muscle.

Manuscript 3: Spatial distribution of aerobic enzymes for lipid metabolism in the muscles of harbor seals.

Manuscript 4: The skeletal muscles of harbor seals are composed solely of oxidative fibers:

implications for lipid metabolism during exercise and diving.

Manuscript 5: Aerobic capacity and lipid droplet density in the heart, liver, kidneys and small intestine of harbor seals.

PROFESSIONAL CONFERENCES

The PI requests funds to attend a scientific meeting, most likely the American Physiological Society, to present the results from this research. Four papers/posters will be submitted entitled: 1) "Spatial distribution of aerobic enzymes for lipid metabolism in the muscles of harbor seals", 2) "The skeletal muscles of harbor seals are composed solely of oxidative fibers: implications for lipid metabolism during exercise and diving", 3) "Aerobic capacity and lipid droplet density in the heart, liver, kidneys and small intestine of harbor seals", and 4) "Effect of diet on the fatty acid signature in the blubber of harbor seals".

COORDINATION AND INTEGRATION OF RESTORATION EFFORT

We are working in close coordination with Dr. Michael Castellini (PI on Harbor Seal Recovery. Phase II: Controlled Studies of Health and Diet) on data interpretation and preparation of the final report and manuscripts.

EXPLANATION OF CHANGES IN CONTINUING PROJECTS

As stated above, we have collected 331 blubber samples and 41 dietary samples from eight harbor seals during the two year feeding trial (see Table 1 above for details). Additional funds will be necessary to complete the analysis of blubber samples currently stored at -70^{o} C. We requested these funds during the third year of this project, but the EVOS Trustee Council deferred our request. This proposal will enable us to complete the analysis of our samples and incorporate the results into the final report and manuscripts.

PROPOSED PRINCIPAL INVESTIGATOR

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PRINCIPAL INVESTIGATOR

Randall Davis, Ph.D., specializes in the physiology and metabolism of marine mammals. He is a Professor of Marine Biology at Texas A&M University and has worked in this field for over 24 years. In 1989, Dr. Davis was the Project Leader for Exxon's Oiled Sea Otter Rehabilitation Program in Prince William Sound.

Publications by Dr. Randall Davis relevant to the proposed research:

Kanatous SB, Davis RW, DiMichele LV, Cowan DF. (1999) High aerobic capacities in the skeletal muscles of seals, sea lions and fur seals: An adaptation to diving hypoxia. Journal of Applied Physiology 86:1247-1256

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OTHER KEY PERSONNEL

Dr. Tammy Adams is currently working for the National Marine Fisheries Service in Silver Springs, Washington, D.C. She has conducted research on the fatty acid composition of marine mammal blubber and how it is affected by diet. Her role will be to analyze the fatty acid data from blubber and dietary samples and to prepare this section of the draft final report. She will also be a co-author on the manuscript dealing with this part of the study

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EVOS 2002 Revised Budget

Personnel Costs

Name	Position Description	Months	Monthly Cost	Proposed FY 2002
R. Davis	Principal Investigator	0.5	$7,520.00	$3,760.00
Total Personnel Costs				$3,760.00
Fringe Benefits				
R. Davis	Principal Investigator	0.5	$1,577.60	$788.80
Total Fringe Benefits				$788.80

Travel Costs

Description	Ticket Price	Round Trips	Total Days	Per Diem	Proposed
Attend American Physiological Society meeting to present results	$700.00	1	3	$155.00	$1,16
Total Travel					$1,16

Contractual Cost

Description	Proposed FY 2002	
Gas chromatograph Analysis of blubber fatty acids	$6,000.00	
Interpretation of fatty acid data	$2,500.00	Subcontract to Tammy Adams for intrepretation
Total Contract Costs	$8,500.00	

Commodities Costs

Description	Proposed FY 2002
Expendable supplies and chemicals	$1,000.00
Pulblication and page charges	$500.00
Total Commodities Costs	$1,500.00

Telephone

Proposed FY 2002

$500

Grand Total Direct Costs	**$16,213.80**
Indirect Costs	**$4,053.45**
Grand Total	**$20,267.25**

l FY 2002
65.00
65.00

of fatty acid