Exxon Valdez Oil Spill
Restoration Project Final Report

Harbor Seal Recovery. Phase III: Effects of Diet on Lipid Metabolism and Health

Restoration Project 01441
Final Report

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Study History: 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 results from field studies of harbor seal health, body condition and feeding ecology, we need data from seals on diets that vary in nutritional composition and basic information on the importance of fat in the energy metabolism of organs and tissues. Working with the Alaska SeaLife Center, we determined how fatty acid profiles in the blubber of captive harbor seals changed over time during controlled diets of herring and pollock. In addition, we assessed the aerobic capacity and lipid metabolism of skeletal muscle, heart, liver, alimentary organs and kidneys in wild harbor seals in Prince William Sound. This four year project was initiated in 1999 and continued in 2000 and 2001 as a contract between the State of Alaska, Dept. of Fish and Game (Division of Habitat and Restoration) and Texas A&M University. The project was completed in FY02 as Restoration Project 01441-BAA and resulted in the preparation of five manuscripts for publication in peer-reviewed journals.

Abstract: The purpose of this project was to examine how fatty acids in the blubber of captive harbor seals (*Phoca vitulina*) change over time during feeding of controlled diets of herring and pollock and to investigate the importance of fatty acids in energy metabolism. The fatty acid composition of seal blubber changed in response to changes in diet. 85% of the harbor seal blubber samples could be correctly classified according to the composition of their diet and changes in blubber lipid composition after four months on a herring or pollock diet. Results from muscle fiber typing showed that harbor seal swimming muscle was comprised of oxidative fibers. In addition, the volume density of mitochondria and aerobic enzyme activities (citrate synthase and b-hydroxyacyl CoA dehydrogenase) in the swimming muscles, heart, liver, kidneys, stomach and small intestine of harbor seals were elevated compared to terrestrial controls suggesting that these organs and tissues possess a heightened ability for aerobic, fat-based metabolism during the hypoxia associated with diving. Our results indicate that lipid is important in the energy metabolism of harbor seals and is probably derived from both the diet and blubber reserves. The fatty acid composition of blubber reflects that of the diet and can be used to detect dietary changes over a period of approximately four months.

Key Words: harbor seal, lipid, diet, metabolism, Prince William Sound

Project Data: Data were collected from live harbor seals held in captivity at the Alaska SeaLife Center and from wild harbor killed during a native subsistence hunt in Prince William Sound. Blubber and muscle were processed in different laboratories. All data were entered as Excel spreadsheets. Custodian - contact Randall Davis, Dept. Marine Biology, Texas A&M University, Galveston, Texas 77551.

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Adams, T.C. and Davis, R.W. (in prep.) The effect of diet on the fatty acid composition of blubber in harbor seals (*Phoca vitulina*). Marine Mammal Science.

Fuson, A.L., Cowan, D.F., Kanatous, S.B., Polasek, L.K., and Davis, R.W. (2003). Adaptations to diving hypoxia in the heart, kidneys and splanchnic organs of harbor seals (*Phoca vitulina*). Journal of Experimental Biology.

Polasek, L. K., Dickson, K. and Davis, R.W. (in press) Mapping aerobic and glycolytic enzyme activities and myoglobin concentration in the locomotory muscles of harbor seals (*Phoca vitulina)*. Journal of Experimental Biology.

Watson, R.R, Miller, T.A. and Davis, R.W. (2003). Immunohistochemical fiber typing of the locomotory muscles of harbor seals (*Phoca vitulina*). Journal of Experimental Biology.

Watson, R.R., Kanatous, S.B. and Davis, R.W. (submitted) Mapping mitochondrial volume density in the locomotory muscles of harbor seals (*Phoca vitulina*). Journal of Experimental Biology.

Adaptations to diving hypoxia in the heart, kidneys and splanchnic organs of harbor seals (*Phoca vitulina*).

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Summary

Pinnipeds (seals and sea lions) have an elevated mitochondrial volume density [Vv(mt)] and citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (HOAD) activities in their swimming muscles to maintain an aerobic, fat-based metabolism during diving (Kanatous et al., 1999). The goal of this study was to determine if the heart, kidneys and splanchnic organs have an elevated Vv(mt) and CS and HOAD activities as parallel adaptations for sustaining aerobic metabolism and normal function during hypoxia in harbor seals (*Phoca vitulina*). Samples of heart, liver, kidney, stomach and small intestine were taken from ten freshly dead harbor seals and fixed in glutaraldehyde for transmission electron microscopy or frozen in liquid nitrogen for enzymatic analysis. Samples from dogs and rats were used for comparison. Within the harbor seal, the liver and stomach had the highest Vv(mt). The liver also had the highest CS activity. The kidneys and heart had the highest HOAD activities, and the liver and heart had the highest LDH activities. Mitochondrial volume densities scaled to tissue specific resting metabolic rate [Vv(mt)/RMR] in the heart, liver, kidneys, stomach and small intestine of harbor seals were elevated (range 1.2 – 6.6x) when compared to the dog and/or rat. In addition HOAD activity scaled to tissue specific resting metabolic rate in the heart and liver of harbor seals were elevated compared to the dog and rat (3.2x and 6.2x in the heart and 8.5x and 5.5x in the liver, respectively). These data suggest that organs such as the liver, kidneys and stomach possess a heightened ability for aerobic, fat-based metabolism during hypoxia associated with routine diving. However, a heightened LDH activity in the heart and liver indicates an adaptation for the anaerobic production of ATP on dives that exceed the animal's aerobic dive limit. Hence, the heart, liver, kidneys and

gastrointestinal organs of harbor seals exhibit dual adaptations that promote an aerobic, fat-based metabolism under hypoxic conditions, but can provide ATP anaerobically if required.

Introduction

Based on forced submergence studies conducted from the 1940s through the early 1970s, it was believed that marine mammals relied primarily on anaerobic metabolism during diving (Elsner and Gooden, 1983; Butler and Jones, 1997). After a forced submergence, there was a net accumulation of lactic acid in the plasma, indicating that anaerobic glycolysis had provided ATP as the organs and tissues became hypoxic. Therefore, it was assumed that marine mammals might have enhanced enzyme activities for anaerobic glycolysis. However, Castellini et al. (1981) showed that marine mammals do not posses significantly elevated anaerobic enzyme activities when compared to terrestrial mammals. These results were difficult to reconcile with the apparent reliance of marine mammals on anaerobic metabolism during forced submergence. In the early 1980s, Kooyman et al. (1981) established the concept of the aerobic dive limit (ADL) by measuring the post-dive blood lactate concentration in Weddell seals (*Leptonycotes weddellii*) following voluntary dives from an isolated ice hole in Antarctica. The ADL is defined as the longest dive that a marine mammal can make while relying principally on oxygen stored in the lungs, blood and muscles to maintain aerobic metabolism. They found that dives shorter in duration than the ADL showed no post-dive increase in blood lactic acid, indicating that metabolism had remained aerobic. By attaching time-depth recorders to free-ranging Weddell seals, they showed that most voluntary dives were within the ADL. Similar results have been found in other species of marine mammals and birds (Butler and Jones, 1997; Ponganis et al., 1997).

A marine mammal's response to "exercise" during diving is somewhat counterintuitive in the context of our normal understanding of the mammalian exercise

response. When terrestrial mammals exercise, they increase ventilation and cardiac output, and peripheral vasodilation increases skeletal muscle perfusion and allows heat dissipation through the skin (Rowell, 1986; Wagner, 1991). In contrast, marine mammals undergo apnea (breath-holding), bradycardia (reduction in heart rate), and peripheral vasoconstriction, which are collectively known as the dive response. As cardiac output decreases, reflex peripheral vasoconstriction maintains central arterial blood pressure by reducing flow to all organs and tissues except the brain. Although the degree of bradycardia and peripheral vasoconstriction may vary with the dive duration or level of exertion, all organs and tissues, including the heart, kidneys, and splanchnic organs, experience a reduction in convective oxygen delivery resulting from both hypoxic hypoxia and ischemic hypoxia (Butler and Jones, 1997; Davis and Kanatous, 1999; Kanatous et al., 2001). By the end of aerobic dives, the blood Po_2 in Weddell seals is as low as 24 mm Hg (Qvist et al., 1986; Davis and Kanatous, 1999). This is equivalent to the degree of hypoxia experienced by human climbers on the top of Mt. Everest (approximately 8,850 m). At this altitude, the maximal oxygen consumption of climbers is reduced to 25% of that at sea level (West et al., 1983). Nevertheless, pinnipeds maintain aerobic metabolism during most free-ranging dives (Kooyman et al., 1983; Davis et al., 1991; Hochachka, 1992; Butler and Jones, 1997).

Previous research on adaptations that enable tissues to maintain normal function during diving have focused mostly on skeletal muscle. Kanatous et al. (1999) found that pinniped skeletal muscle has an increased Vv(mt) that is most pronounced in the muscles used for swimming. The increased Vv(mt) is thought to facilitate aerobic metabolism under hypoxic diving conditions by decreasing the diffusion distance between

mitochondria and intracellular oxygen stores in the form of oxy-myoglobin. The increased Vv(mt) results in an increased citrate synthase (CS) activity and maximum aerobic capacity, although this may not be important to marine mammals that make long dives because they use cost-efficient modes of locomotion to conserve oxygen stores and prolong dive duration (Williams et al., 2000; Davis et al., 2001). It was also found that the β-hydroxyacyl-CoA dehydrogenase (HOAD) activity in the skeletal muscle of pinnipeds was significantly greater than in the skeletal muscle of terrestrial mammals (Kanatous et al., 1999). HOAD activity is indicative of aerobic, fat-based metabolic potential. Since the skeletal muscles of pinnipeds show an increased Vv(mt) that facilitates aerobic metabolism under hypoxic conditions, the question arises: Do other organs and tissues show similar adaptations?

The goal of this study was to determine if harbor seals have an elevated Vv(mt) and enzymatic capacity for aerobic metabolism in their heart, kidneys and splanchnic organs. Our results show that harbor seal organs have an enhanced Vv(mt) when scaled to tissue specific resting metabolic rate that decreases the diffusion distance of oxygen between mitochondria and intracellular oxygen stores during hypoxia. When scaled to tissue specific resting metabolic rate, CS and HOAD activities in these organs are also elevated, indicating a reliance on aerobic, lipid-based metabolism. These adaptations enable harbor seals to maintain aerobic metabolism and physiological homeostasis under hypoxic conditions associated with voluntary dives.

Methods

Tissue Sampling. Tissue samples from 10 adult or sub-adult harbor seals were taken within seven hours of death as part of a native subsistence hunt in Alaska. The heart, kidneys, liver, stomach and intestines were removed in their entirety and weighed. Multiple samples (2-3 g) were taken from the left ventricle of the heart, a random reniculi of the right kidney, a random lobe of the liver, and the fundus of the stomach. Only the mucosal layer of the stomach was analyzed. Samples from the intestine were taken at half the length of the entire intestine, in the jejunum. For comparison, equivalent samples were obtained from three laboratory rats (Sprague-Dawley) from the Health and Kinesiology Department at Texas A&M University and three dogs sacrificed for research purposes at the Texas A&M College of Veterinary Medicine. Tissue samples were taken in accordance with guidelines for the humane treatment of animals at Texas A&M University.

Samples taken for electron microscopy were fixed in 2% glutaraldehyde and remained in the fixative for approximately 30 days before being minced and stored in 0.1M cacodylate buffer at pH 7.4 prior to embedding. Since samples were taken after death and were immersion fixed, some tissue autolysis may have occurred. However, serious autolysis was not apparent in the electron micrographs of any of the tissues. Samples for enzymatic analysis were immediately frozen in liquid nitrogen until they were returned to Texas A&M University, after which, they were stored at -70°C.

Mitochondrial Volume Density. Fixed samples were rinsed in 0.1M cacodylate buffer and postfixed for one hour in a 1% solution of osmium tetroxide. They were then rinsed with distilled water, stained en bloc with 2% uranyl acetate for 30 minutes at 60°C, dehydrated with increasing concentrations of ethanol (50-100%), and then passed through propylene oxide and increasing concentrations of epoxy (50-100%). They were finally embedded in epoxy and allowed to polymerize overnight at 60°C. Semi-thick sections (1 μm) were cut with a Leica Ultratome (Reichert Division of Leica Co., Vienna, Austria) and stained with toluidine blue. Ultrathin (50-70 nm) sections from four randomly selected blocks per sample were cut, placed on a copper grid (150 Mesh), and contrasted with lead citrate and/or uranyl acetate. Micrographs were taken with a Phillips 201 transmission electron microscope (FEI Company, Eindhoven, Netherlands). Final image magnification was approximately 18,150x. The number of micrographs taken for each block ranged from 10-20, yielding a total of 40-80 micrographs per sample. We calculated Vv(mt) from digitized micrographs using a standard point-counting technique (Hoppeler et al., 1981; Mathieu et al., 1981). Electron micrographs from the cardiac muscle were used only if the sections were transverse or oblique in orientation.

Mitochondrial Distribution. The intracellular distributions of mitochondria in the liver, kidney and stomach were semi-quantitatively characterized in ≥ 40 micrographs per species. Micrographs in which the mitochondria were more uniformly distributed were classified as “homogeneous,” and those with tightly packed mitochondria with areas of cytoplasm devoid of mitochondria were classified as “clustered” (Jones, 1984). The classification was conducted independently by two of the authors. The results were

compared, and those micrographs with differing classifications were discarded from the analysis. The percentages of micrographs with "homogeneous" and "clustered" mitochondria were calculated for the tissues of each species.

Enzyme Assays. Frozen tissue samples were thawed, blotted, weighed and immediately homogenized in a volume of buffer (1 mM EDTA, 2 mM $MgCl_2$, and 50 mM imidazole, pH 7.0 at 37°C) according to its mass and type (300x dilution for heart, 30x for liver, 10x for kidney and 5x for both the stomach and intestine) in a ground glass homogenizer (Reed et al., 1994). The homogenates were centrifuged at 2,900 g for 50 minutes at 4°C. Enzyme analyses were performed at 37°C on a $PowerWave_X$ 340 microplate reader (Bio-Tek Instruments, Inc., Winooski) according to the methods of Polasek et al. (in prep). The assay conditions for CS (EC 4.1.3.7) were: 0.5 mM oxaloacetate, 0.4 mM acetyl CoA, 5,5'-dithiobis(2-nitrobenzoic acid) and 50 mM imidazole; pH 7.5; DA_{412}, $\in_{412}$ = 13.6, where DA indicates absorbance wavelength and $\in$ is the extinction coefficient. Assay conditions for HOAD (EC 1.1.1.35) were: 0.1 mM acetoacetyl CoA, 0.15 mM NADH, 1 mM EDTA and 50 mM imidazole; pH 7.0; DA_{340}, $\in_{340}$ = 6.22. Assay conditions for LDH (EC 1.1.1.27) were: 1 mM pyruvate, , 0.15 mM NADH and 50 mM imidazole; pH 7.0; DA_{340}, $\in_{340}$ = 6.22. Enzyme activities in micromoles of substrate converted per minute (IU/g wet wt tissue) were calculated from the rate of change in absorbance at the maximum linear slope. Due to the extremely low CS activity in the small intestine, samples were combined to yield only one measurement for each species.

Statistical Analysis. Results are expressed as means ± SE. Vv(mt) was determined for six seals, three rats (exclusive of kidney) and three dogs. Enzyme activities were determined for 10 seals, three rats (exclusive of kidney), and three dogs. Inter-organ and inter-species comparisons of mean values of Vv(mt) and enzyme activities were analyzed using an ANOVA (Tukey HSD, p-value < 0.05). Rat kidney was not included in either analysis due to a sample size of only one. CS activity in the intestine was not analyzed statistically because only a single value could be obtained for each species. In addition to the above analyses, values for Vv(mt) and enzyme activities were scaled to each tissue's calculated specific resting metabolic rate (RMR) to adjust for differences in body mass between the seals and the contol species. Based on the work of Wang et al. (2001), the scaling exponent for the RMR of individual organs and tissues is more variable than for whole body RMR. Therefore, instead of scaling Vv(mt) and enzyme activities with the whole body RMR, estimated as 70 $M_b^{-0.25}$ (Schmidt-Nielsen and Duke, 1984), where M_b is the body mass of the animal (in kg), we used the estimated specific RMR for for each organ or tissue (Wang et al., 2001). The estimated tissue specific RMRs (KJ kg^{-1} d^{-1}) were as follows: liver RMR = 2861 $M_b^{-0.27}$, heart RMR = 3725 $M_b^{-0.12}$, kidney RMR = 2887 $M_b^{-0.08}$, stomach and intestine RMR = 125 $M_b^{-0.17}$. Statistical comparisons of scaled Vv(mt) and enzyme activities among species were made using an ANOVA (Tukey HSD p-value < 0.05). Statistical comparisons among species for CS/HOAD and LDH/CS ratios were also made using an ANOVA (Tukey HSD, p-value < 0.05). All statistical analyses were performed with SYSTAT version 10.

Results

Tissue Morphology. Mitochondria in cardiac muscle occurred mainly in columns or rows interspersed regularly among the myofibrils and were easily distinguishable. Cardiac mitochondria were generally round in the harbor seal and dog, but some rat mitochondria were irregularly shaped (Figure 1). Electron micrographs of hepatocytes were taken at a random orientation. Mitochondria in the liver were dispersed throughout hepatocytes with prominent organelles such as endoplasmic reticulum and nuclei. Hepatic mitochondria were easily distinguishable from other organelles due to their well-defined cristae and a thick, darkly staining double membrane (Figure 2). Electron micrographs were taken from random areas of the cortex of the kidney, and mitochondria were generally very round and highly distinguishable from other structures (Figure 3). There appeared to be two different populations of renal mitochondria in close proximity that exhibited differential staining; one stained lightly while the other took on a darker stain (Figure 4). Electron micrographs of the stomach were taken mostly at the mucosal surface where metabolism should be active due to the secretion of hydrochloric acid, potassium chloride, traces of other electrolytes, and a glycoprotein called "gastric intrinsic factor" which is essential for the absorption of vitamin B_{12} (Junqueira et al., 1998). The mitochondria in the stomach were generally very round with distinctive membranes and easily distinguishable from other structures (Figure 5). Micrographs of the small intestine were taken mostly near the microvilli, where nutrient absorption occurs. Mitochondria in the intestine were not as easily distinguishable due to areas of dense connective tissue and some cellular autolysis (Figure 6).

Harbor Seal Interorgan Comparisons. The mean Vv(mt) of the seal liver (26.4) was significantly greater than in the heart and kidney (18.6 and 21.4, respectively) (Table 1). Stomach Vv(mt) (24.5) was also significantly greater than the heart (18.6). Vv(mt) in the liver, heart, kidney and stomach were significantly greater than in the intestine (8.9). The mean CS activity in the heart (73.8) was significantly greater than in the liver (13.5), kidney (15.4) and stomach (11.4) (ANOVA, $p < 0.05$), but there were no significant differences among liver, kidney and stomach (Table 2). The mean HOAD activity in the kidney (2.4×10^2) was significantly greater than in the heart (1.0×10^2), liver (11.2), stomach (4.4) and intestine (3.4), but there were no significant differences between the liver and stomach. The mean LDH activity in the liver (1.1×10^3) was significantly greater than in the heart (6.9×10^2), kidney (1.9×10^2), stomach (1.6×10^2) and intestine (2.5×10^2), and the LDH activity in the heart was significantly greater than in the kidney, stomach, and intestine. There were no significant differences in LDH activity among the kidney, stomach and intestine. The CS/HOAD ratio ranged from 6.0×10^{-2} in the kidney to 2.6 in the stomach (Table 3). Except for the stomach, the CS/HOAD ratios were close to or less than unity, indicating that the β-oxidation of fatty acids could provide most of the acetyl-CoA for the citric acid cycle. The LDH/CS ratio ranged from 9.9 in the heart to 1583.1 in the intestine. Only one intestinal CS value was recorded for each species.

Mitochondrial Volume Density Among Species. Mean Vv(mt) in harbor seal liver was significantly greater than in the dog and rat (26.4, 17.3, and 13.2%, respectively) (Table 1, Figure 7). The Vv(mt) in harbor seal kidney was significantly greater than in the dog (21.4 and 16.6%, respectively). Stomach Vv(mt) in the harbor seal was significantly

greater than in the rat (24.5 and 13.3%, respectively). There were no significant differences in Vv(mt) among species in either the heart or the small intestine.

The Vv(mt)/RMR in the harbor seal heart, liver, stomach and intestine were significantly greater than in the rat. The Vv(mt)/RMR in the harbor seal liver, kidney, stomach, and intestine were greater than in the dog (Table 4, Figure 8). Dog heart, liver and stomach were also significantly greater than the rat.

Mitochondrial Distribution. Mitochondrial distribution in the liver of the harbor seal was very homogeneous (87.8% of the micrographs) (Figure 2), whereas only 8.7% and 4.8%, respectively, of dog and rat liver micrographs had homogeneously distributed mitochondria. For harbor seal and dog kidney, 49% and 50%, respectively, of the micrographs were classified as homogeneous (Figure 3). Again, rat kidney was not included in the analysis due to a sample size of only one. For harbor seal stomach, 91.3% of the micrographs were classified as homogeneous, whereas only 76% and 69%, respectively, of dog and rat stomach micrographs were classified as homogeneous (Figure 5).

Enzyme Activities. The mean CS activity in the rat heart was significantly greater than in the harbor seal or dog (124.7, 73.8, and 71. 8, respectively) (Table 2). The CS activities of harbor seal and rat liver (13.5 and 12.3, respectively) were not significantly different, but both were significantly greater than in the dog (8.2). CS activity in the dog kidney was significantly greater than in the harbor seal (21.3 and 15.4, respectively). Other organs exhibited no significant differences in CS activity among the three species. The CS/RMR activity of the harbor seal liver was significantly greater than that of the

dog and rat (1.3×10^{-2}, 5.2×10^{-3}, and 3.5×10^{-3}, respectively). No differences among species existed in the CS/RMR activity of the heart, kidney, or stomach. An analysis was not conducted for the intestine because to only one CS activity measurement was made for each species.

Mean HOAD activity in harbor seal heart was significantly greater (2.5x) than in the heart of dogs and rats (1.0×10^{2}, 38.3, and 27.5, respectively; Table 2). Harbor seal liver also had a significantly greater HOAD activity when compared to the dog and rat (11.2, 2.0, and 7.1, respectively). The HOAD activity in the liver of rats was significantly greater than in dogs. The HOAD activity in the small intestine of the rat was significantly greater than in the harbor seal and the dog (6.0, 3.4, and 2.4, respectively). HOAD activities in the kidney and stomach of the three species were not statistically different. Analysis of the HOAD/RMR showed that: 1) harbor seal heart was significantly greater than dog and rat heart (4.2×10^{-2}, 1.3×10^{-2}, and 6.8×10^{-3}, respectively), 2) harbor seal liver was significantly greater than dog or rat liver (1.1×10^{-2}, 1.3×10^{-3}, and 2.0×10^{-3}, respectively), and 3) the harbor seal intestine was significantly greater than dog intestine (5.1×10^{-2} and 2.8×10^{-2}, respectively; Table 5, Figure 10). There were no significant differences in the HOAD/RMR activities in the kidney or stomach among the three species.

Mean LDH activity in rat heart was significantly greater than in both the harbor seal and dog (1.3×10^{3}, 6.9×10^{2}, and 5.4×10^{2}, respectively), but seal heart LDH activity was significantly greater than in the dog (Table 2). The liver of harbor seals had a significantly higher LDH activity when compared to dogs and rats (1.1×10^{3}, 3.1×10^{2}, and 8.1×10^{2}, respectively), and LDH in the rat liver was also significantly greater than

in the dog. Dog kidney LDH activity was significantly greater than in seal kidney (2.19 x 10^2 and 1.9 x 10^2, respectively). The LDH activity of the small intestine of both the harbor seal and the rat were significantly greater than in the dog (2.5 x 10^2, 2.7 x 10^2, and 1.3 x 10^2, respectively). The stomach was statistically indistinguishable among species. The LDH/RMR ratio showed that: 1) harbor seal heart was significantly greater than the dog (2.9 x $10^{-1}$and 1.9 x 10^{-1}, respectively), 2) harbor seal liver was significantly greater than the dog and rat (1.1, 2.0 x 10^{-1}, and 2.3 x 10^{-1}, respectively), 3) harbor seal kidney was significantly greater than the dog (8.8 x 10^{-2} and 8.6 x 10^{-2}, respectively), 4) harbor seal stomach was significantly greater than the rat (2.4 and 1.2, respectively), and 5) the harbor seal intestine was significantly greater than the dog or rat (3.8, 1.6, and 1.9, respectively; Table 5, Figure 11).

The CS/HOAD ratio was calculated as an index of potential fatty acid oxidation versus the overall aerobic metabolism of the animal, with a ratio less than one indicating that fatty acids can provide most of the acetyl CoA for the Krebs cycle (Pette and Dölken, 1975; Simi et al., 1991). The CS/HOAD ratio ranged between 0.04 in the rat intestine to 5.2 in the dog stomach (Table 3). The most extreme difference in this ratio among species occurred in the heart. On the low-end of the spectrum, the harbor seal heart had a ratio of 0.7, whereas the rat heart had a ratio of 4.5, a 6-fold difference. The CS/HOAD ratio of the harbor seal heart was significantly less than the dog or rat, and the dog was significantly less than the rat. The CS/HOAD ratio in the liver was significantly different between the dog and harbor seal, with ratios of 4.1 and 1.2, respectively. The CS/HOAD in the harbor seal liver was significantly less than in the dog or rat, and the rat was less than the dog. The CS/HOAD ratio of the harbor seal kidney was significantly less than in

the dog (6.0×10^{-3} and 0.1, respectively). Ratios for the stomach were again significantly different between the dog and harbor seal (5.2 and 2.6, respectively). CS/HOAD ratios for intestine were similar among species (5.0×10^{-2}, 6.0×10^{-2}, and 4.0×10^{-2} for harbor seal, dog, and rat, respectively) but were not analyzed statistically due to the small sample size.

The LDH/CS ratio was calculated as an index of relative anaerobic *vs* aerobic metabolic capacities (Hochachka et al., 1982). The LDH/CS ratio ranged between 5.3 in the rat kidney to 1583.1 in the harbor seal intestine. The LDH/CS ratio of the harbor seal liver and kidney (80.5 and 12.2, respectively) were significantly greater than in the dog (38.3 and 9.8, respectively) (Table 3). The LDH/CS ratio in the rat liver was also significantly greater than in the dog (66.3). There were no differences among species in the LDH/CS ratio of the heart or stomach. The LDH/CS ratio of the intestine was not included in the statistical analysis.

Discussion

Studies of adaptations in mammals to hypoxia have fallen in to two major categories: 1) exposure to hypoxia and 2) altitude adapted mammals. For the first category of studies, research has shown that there is either no change or a decrease in aerobic indicators of the heart and liver (Kayar and Banchero, 1987; Costa et al., 1988; Lewis et al., 1999; Kennedy et al., 2001). For studies of the second type, CS (Hochachka et al., 1982) and LDH activities of the heart were increased (Vergnes, 1971; Penney, 1974; Barrie and Harris, 1976; Ohtsuka and Gilbert, 1995). Our studies of marine mammal organs suggest that they are more akin to altitude adapted mammals, than hypoxia exposed mammals The liver (Davis et al., 1983; Castellini et al., 1988), kidneys (Davis et al., 1983; Castellini et al., 1981), and gastrointestinal tract (Davis et al., 1983) all maintain aerobic metabolic function during natural diving. A model for convective oxygen transport and tissue oxygen consumption in Weddell during aerobic dives seals indicates that convective oxygen transport to the heart, liver, kidneys and gastrointestinal tract is sufficient to maintain aerobic metabolism despite a falling arterial Po_2 to as low as 22 mm Hg during an aerobic dive (Davis and Kanatous, 1999).

Harbor Seal Interorgan Comparisons. The function of the heart, liver, kidneys and digestive organs of mammals relies on the delivery of oxygen from the circulation. The estimated mass specific resting metabolic rate of harbor seal organs (taken from Weddell seal estimates, Davis and Kanatous, 1999) is, in descending order, heart (59.2 ml O_2 min^{-1} kg^{-1}), kidneys (38.4), liver (27.7), and the gastrointestinal tract (10.1). When blood flow is reduced, these organs increase their extraction of oxygen from the blood (Fisher, 1963; Jacobsen et al., 1969, Granger and Shepherd, 1973; Lutz et al., 1975; Nelson et al., 1988;

Fink, 2001) or, in the case of the kidneys, decrease their metabolic rate because glomerular filtration rate (GFR) is reduced (Brezis et al., 1984). If convective oxygen transport is insufficient to maintain aerobic metabolism, the tissue will become anoxic and cellular damage or death can result (Brezis et al., 1984). Although the seal heart has the highest resting metabolic rate among the organs, it did not have the highest Vv(mt). However, CS activity in the seal heart was 4.8-6.5x greater than the liver, kidney and stomach, indicating a higher density of citric acid cycle enzymes in heart mitochondria. This, combined with an elevated HOAD activity and a CS/HOAD ratio of 0.7, shows the high aerobic capacity of the heart and its ability to oxidize fatty acids.

Among the organs of the harbor seal, liver had the highest Vv(mt) followed by the stomach and kidneys. The HOAD activity of the seal kidney was significantly greater than all other organs, reinforcing the reliance on lipid metabolism. The intestine had the lowest Vv(mt), which may reflect the overall low resting metabolic rate of the gastrointestinal tract. The LDH activity of the liver was the highest among the seal's organs, reflecting its capacity to switch on anaerobic ATP production if necessary. However, the high LDH activity may also indicate an enhanced ability to convert lactate into pyruvate as the initial step in gluconeogenesis. Previous studies (Davis et al., 1983) have shown that most of the lactate produced when harbor seals are forcibly submerged or exercising is not oxidized but recycled, most likely back into glucose in the liver.

Interspecies Comparison of the Heart. We found that the unscaled Vv(mt) of harbor seal heart was not significantly different from that of the dog and rate, but the Vv(mt)/RMR was greater (1.4x) than in the rat. These results indicate a small increase in the Vv(mt) in

the seal heart relative to its metabolic requirements. We hypothesize that this increase in Vv(mt)/RMR aids in the maintenance of aerobic metabolism during diving by decreasing the diffusion distance between mitochondria and intracellular oxygen. We base this hypothesis on the rate of diffusion within a muscle fiber described by Fick's equation:

$$dQ/dt = -DA(du/dx)$$

where dQ/dt is the diffusive flux of substance Q over time dt, A is the area through which diffusion takes place, du/dx is the concentration (or partial pressure) gradient over distance dx, and D is the diffusion coefficient (Schmidt-Nielsen and Duke, 1979). As the diffusion distance (dx) decreases, the rate of oxygen flux (dQ/dt) increases. For the diffusion of intracellular oxygen, a decrease in diffusion distance is especially advantageous at low partial pressures (i.e., du is small) of oxygen experienced during diving.

The unscaled CS activity in the harbor seal heart was significantly less than in the rat heart. However, when CS activity was scaled to RMR, there were no significant differences among the three species, indicating that the high resting metabolic rate of the rat accounts for the high CS activity. As a result, overall aerobic capacity of harbor seal heart muscle is not elevated compared to the rat and dog when scaled to resting cardiac muscle metabolic rate. However, there is an increase in Vv(mt) that may enhance the diffusion of intracellular oxygen into mitochondria under hypoxic conditions.

Based on an average postabsorptive respiratory quotient (ratio of CO_2 production/O_2 consumption) of 0.74 in seals, previous studies (Kooyman et al., 1981; Davis et al., 1991) showed that seals rely heavily on lipid as a fuel for energy metabolism, especially during

exercise. Even without scaling for RMR, HOAD activity in the harbor seal heart was significantly greater (2.6x and 3.6x) than in the dog and rat, respectively. When scaled to RMR, the HOAD activity in the seal heart was 3.2x and 6.2x greater than in the dog and rat, respectively, indicating that seal heart relies heavily on lipid as a source of energy. This was further supported by a CS/HOAD ratio of 0.7 in the seal, while the ratio in the dog and rat were 1.9 and 4.5, respectively. This dependence on lipid as an energy source in seals results from a diet rich in fatty acids and protein but containing little carbohydrate (Roberts et al., 1943; Balazquez et al., 1971; Kettelhut et al., 1980; Davis et al., 1991). Studies of terrestrial mammals have shown that high-fat, low carbohydrate diets increase the rate of lipid oxidation (Roberts et al., 1996; Lee et al., 2001) and that this is accompanied by an increase in the concentration of enzymes required for fatty acid oxidation (Gollnick and Saltin, 1988; Roberts et al., 1996). A greater reliance on fatty acid oxidation also spares carbohydrate for red blood cells and the central nervous system, which are obligate glucose metabolizers.

Castellini et al. (1981) found that LDH activity in the hearts of marine and terrestrial mammals were not significantly different. In contrast, we observed that LDH activity in the seal heart was significantly greater (1.2x) than in the dog, and this difference was further enhanced (1.5x) when LDH activity was scaled to RMR. Since the heart is critical for survival, enhanced LDH activity may have survival advantage, even if the heart normally remains aerobic during dives. Ohtsuka and Gilbert (1995) studied the effects of high-altitude hypoxemia on cardiac enzyme activities in pregnant and non-pregnant sheep. The results showed that LDH activity increased by 24 and 27%, respectively, in the left ventricle of non-pregnant and pregnant adult sheep. Similar

results for animals exposed to high altitudes (hypoxia) have been reported by Vergnes (1971), Penney (1974), and Barrie and Harris (1976). Seal cardiac muscle shows adaptations for both aerobic and anaerobic metabolism under conditions of hypoxia. Oxygen will be used until a critical arterial PO_2 is reached during a dive. At that point, anaerobic metabolism will become increasingly important as a source of ATP. In Weddell seals, this critical PO_2 is less than 22 torr, and it appears that the seals rarely exceed this threshold during aerobic dives (Qvist et al., 1986; Davis and Kanatous, 1999). As a result, convective oxygen transport to the heart is normally sufficient to maintain aerobic metabolism during a dive. Nevertheless, enhanced anaerobic glycolytic enzyme activity is present if needed to protect the heart against hypoxia.

Interspecies Comparison of the Liver. Seal liver Vv(mt) was significantly greater (2x and 1.5x) than rat and dog liver, respectively. When scaled for RMR, seal liver Vv(mt) was still significantly greater (1.4x and 1.2x) than rat and dog liver, respectively. As with the seal heart, we hypothesize that this increase in Vv(mt) decreases the intracellular distance for oxygen diffusion and effectively increases diffusive conductance to maintain aerobic metabolism and organ function during periods of hypoxia while diving (Costa et al., 1988). Based on the hepatic clearance of indocyanine green (ICG) from the blood during voluntary dives, Davis et al. (1983) showed that hepatic function was maintained in subadult Weddell seals during voluntary dives, even though hepatic arterial and portal blood flow were reduced as a result of the dive response. The liver appears to compensate for a reduction in blood flow during a dive by increasing the extraction coefficient of ICG (Fisher, 1963; Jacobsen et al., 1969), thereby maintaining a predive

level of ICG clearance. Plevris et al. (1999) observed that reduced ICG clearance in laboratory animals is due mainly to impaired microcirculation in the liver and compromised hepatocyte function. Since ICG clearance in the seal is maintained during aerobic dives, there is no impairment of hepatic microcirculation or function. This conclusion is further supported by data that show little variation in the blood glucose concentration and blood urea nitrogen (BUN) during consecutive, aerobic dives (Castellini et al., 1988; Davis, unpubl. results), which would not be possible if liver function were disrupted.

Costa et al. (1988) showed that the livers of rats exposed to chronic, hypobaric hypoxia had a more homogeneous distribution of mitochondria than rats raised under normoxic conditions. In our analysis of hepatic mitochondrial distribution, 88% of micrographs from the seal were classified as homogeneous, whereas, only 9% of dog and 5% of rat liver micrographs were classified as homogeneous. Along with an elevated Vv(mt), we hypothesize that the homogeneous distribution of hepatic mitochondria decreases the intracellular diffusion distance for oxygen and helps maintain aerobic metabolism under hypoxic conditions.

CS activity in the harbor seal liver was significantly greater than that of the dog. Since CS is an enzyme found in the matrix of the mitochondria, it follows that an increase in Vv(mt) would result in an increase in CS activity. HOAD activity in the seal liver was 5.6x and 1.2x greater than in the dog and rat, respectively, and 3.2x and 6.2x greater, respectively, when scaled to RMR. The increased HOAD activity in the harbor seal probably results from the high-fat, low-carbohydrate diet discussed previously. These results are similar to those for skeletal muscle for harbor seals, Steller's sea lions, and

northern fur seals (Kanatous et al., 1999). Kennedy et al. (2001) found that HOAD activity in the liver of rats decreases after exposure to chronic hypoxia at high altitude, but HOAD activity and glycogen sparing have not been studied widely in the liver. Although the CS/HOAD ratio in the liver is not as low as in heart, kidneys and intestine, it indicates that lipid is an important source of fuel for energy metabolism in the liver.

Castellini et al. (1981) found that LDH activity (in the direction of pyruvate to lactate) in marine mammal liver was higher than in terrestrial mammals. Our data are in agreement, with harbor seals having a statistically greater LDH activity than either the dog or rat regardless scaling for RMR. However, our mean LDH activity was double that reported by Castellini et al. (1981) for marine mammals, with values of 1084.5 ± 66.5 and 538 ± 188 IU/g wet weight tissue, respectively. However, as noted by Castellini et al. (1981), some of the terrestrial mammals had high LDH activities and some marine mammals had low LDH activities. The LDH/CS ratio of the harbor seal liver is greater than the dog indicating a relatively high anaerobic capacity. Therefore, our results show a heightened ability for anaerobic metabolism in the liver of the harbor seal. As with the heart, the seal liver shows adaptations for both aerobic and anaerobic metabolism under conditions of hypoxia. Although Davis and Kanatous (1999) showed that the liver in Weddell seals receives sufficient oxygen to prevent anaerobic ATP production during dives within the ADL, but this source of energy may be important during longer dives.

Interspecies Comparison of the Kidney. Studies in which seals were forcibly submerged led researchers to believe that there was a pronounced decrease in blood flow to the kidneys during diving resulting from an extreme dive response (Blix et al., 1976).

Bradley and Bing (1942) and Murdaugh et al. (1961) came to the same conclusion when seals that were forcibly submerged experienced either a decrease or complete cessation in GFR. However, a study by Davis et al. (1983) of Weddell seals making voluntary dives came to a different conclusion. By injecting inulin into the blood of the seals, they were able to measure the seal's GFR during and after dives. They found that inulin clearance did not change from predive, resting levels and only decreased when the seals dived for longer than their ADL. They concluded that the kidneys functioned normally during dives shorter than the ADL due to a sustained renal blood flow and GFR.

Mammalian kidneys, regardless of species, require an abundance of mitochondria to provide ATP for active transport of electrolytes and metabolites across the renal tubules. Since mitochondria are the source of ATP production, they are present in the kidneys where the sodium pump enzymes reside (Abrahams et al., 1991). The harbor seal kidney had a Vv(mt), that was significantly greater (29%) than that of the dog. We hypothesize that this elevation in Vv(mt) is an adaptation to sustain aerobic metabolism and renal function during the hypoxia experienced during diving. When blood flow decreases to the kidneys of a terrestrial mammal, it concomitantly reduces its metabolic rate since the kidney's workload is directly proportional to the amount of plasma that must be filtered (Brezis et al., 1984). However, at very low renal blood flow, the metabolic rate of the kidneys is reduced to basal levels because there is little filtration and absorption (Lassen, 1964). The kidneys can suffer damage if there is a further decrease in blood flow (Brezis et al., 1984), although the seal kidney appears to recover from severe anoxia better than the dog kidney (Halasz et al., 1974). The elevated Vv(mt) in the harbor seal kidney may

aid in decreasing the intracellular diffusion distance of oxygen and thereby keeping renal metabolism aerobic and functioning normally.

Mitochondrial distribution in the harbor seal and dog kidneys were nearly identical. Unlike the liver, it appears that the harbor seal kidney needs no redistribution of mitochondria to facilitate the intracellular diffusion of oxygen. The increased volume density of the mitochondria may be enough to increase the effective oxygen diffusive conductance, or the intrinsic grouping of mitochondria in the mammalian kidney may be equally divided between a homogeneous to clustered distribution.

The CS activity in the dog kidney was significantly greater (1.2x) than in the seal, even though the Vv(mt) in the seal was greater (1.4x) than the dog. This result indicates a greater concentration (packing) of CS in the dog mitochondria. Kanatous et al. (1999) obtained similar results for an increase in CS activity in the mitochondria of pinnipeds skeletal muscle. However, when scaled for RMR, there were no statistical differences in the CS activity of harbor seal and dog kidneys. The enzymatic design of the mammalian kidney for oxidative metabolism may depend solely on body mass. HOAD activity in both the seal and dog kidneys were at least twice as great as any other organ examined (the rat showed a similar trend), but were not significantly different between the two species regardless of scaling. As a result, the CS/HOAD ratio for the harbor seal, dog and rat kidneys were very low (ca. 0.1), indicating that the mammalian kidney has an elevated enzymatic potential for aerobic lipid metabolism.

When LDH activity in the harbor seal kidney is scaled to RMR, it was significantly greater than in the dog. The LDH/CS ratio was also significantly greater than in the dog indicating a higher anaerobic capacity in the harbor seal kidney. The

enhanced LDH activity may confer a survival advantage, even though the kidneys remain aerobic during most voluntary dives. Again, when a dive exceeding an animal's ADL is required, there is additional LDH activity available for the glycolytic production of ATP. This ability was observed by Halasz et al. (1974), which explains the ability of the seal kidney to recover from severe bouts of hypoxia that would be rare in the wild.

Interspecies Comparison of the Gastrointestinal Tract. The Vv(mt) in the stomach of the harbor seal was significantly greater (1.8x) than in the rat and, when scaled for RMR, it was greater (3.9x and 1.5x, respectively) than in both the rat and dog. When scaled for RMR, the Vv(mt) of the harbor seal small intestine was also significantly greater (2.6x and 2x, respectively) than that of the rat and dog. We hypothesize that the increase in Vv(mt) in the stomach and small intestine of the harbor seal is an adaptation for maintaining aerobic metabolism and gastrointestinal function during hypoxia. This is supported by the observations of Davis et al. (1983), who found that the plasma of Weddell seals making foraging dives became very lipemic and opaque from the presence of chylomicrons. The lipemic plasma was an indication that the digestion and intestinal absorption of fat (Weddell seals usually feed on Antarctic silverfish [*Pleuragramma antarcticum*], which have a very high lipid content) was taking place during a forging bout.

Previous research has shown that the gastrointestinal organs are capable of compensating for alterations in blood flow by adjusting the amount of oxygen extracted from the blood (Granger and Shepherd, 1973). The oxygenation of the tissue is regulated by the balance between blood flow and oxygen extraction (Johnson, 1960;

Garg, 1979; Granger and Norris, 1980). Kvietys and Granger (1982) also found that at normal intestinal blood flows, the uptake of oxygen appears to be blood flow independent, whereas, at very low perfusion, oxygen uptake becomes flow dependent. An increased Vv(mt) would support the efficient extraction (by increasing the diffusive conductance) of oxygen needed to support aerobic metabolism and normal function during a dive.

Analysis of the distribution of mitochondria in the stomach revealed that approximately 91.3% of harbor seal micrographs and 76 and 69% of dog and rat micrographs, respectively were classified as homogenously distributed. As in the liver, the distribution of mitochondria in the mucosal surface of the stomach may aid in decreasing the effective diffusion distance of oxygen in the stomach lining.

The CS activities in the stomachs of the three species were not significantly different and, although intestinal CS activity was not included in the analyses, the single values obtained for each species were very similar. Mean HOAD activity in the stomach of the harbor seal was not significantly different from that of rat or dog. However, the HOAD/RMR of the seal intestine was significantly greater than that of the dog. The CS/HOAD ratio for the harbor seal intestine (and the rat and dog) was very low (< 0.1) indicating a high enzymatic potential for aerobic lipid metabolism. The CS/HOAD ratio of the harbor seal stomach (5.2) was higher than the intestine.

The LDH activity in the stomach of the harbor seal was not significantly different from that of the dog or rat. However, when scaled for RMR, LDH activity in the seal stomach was significantly greater than the rat. When scaled for RMR, LDH activity in the small intestine of the harbor seal was significantly greater than the dog and rat. The

higher LDH activity in the harbor seal digestive organs indicates a heightened ability to undergo anaerobic metabolism during a dive if necessary. However, given that most dives are within an animal's ADL and the LDH/CS ratio is not significantly different than dog or rat, we believe that the animal may only rely on this anaerobic production of ATP when undertaking a dive beyond its ADL.

Conclusions

The elevated Vv(mt)/RMR and CS/RMR, especially in the liver of the harbor seal, indicate adaptations to sustain aerobic metabolism during hypoxia by enhancing the diffusion of oxygen to mitochondria at low partial pressures. The high HOAD activity and low CS/HOAD ratio, along with a respiratory quotient less than 0.74, indicates that lipids are the primary substrate for aerobic metabolism. A heightened LDH activity indicates an adaptation for the anaerobic production of ATP on dives that exceed animal's ADL. Hence, the heart, liver, kidneys, and gastrointestinal organs of harbor seals exhibit dual adaptations that promote an aerobic, lipid-based metabolism under hypoxic conditions, but can provide ATP anaerobically if required.

Literature Cited

Abrahams, S., Greenwald, L. and Stetson, D.L. (1991). Contribution of renal medullary mitochondrial density to urinary concentrating ability in mammals. *Am. J. of Physiol*. 261, R719-R726.

Balazquez, E., Castro, M., Herrera, E. (1971). Effect of high-fat diet on pancreatic insulin release, glucose tolerance and hepatic gluconeogenesis in male rats. *Rev. Espan. Fisol*. 27, 297-304.

Barrie, S.E. and Harris, P. (1976). Effect of chronic hypoxia and dietary restriction on myocardial enzyme activities. *Am. J. Physiol*. 231, 1308-1313.

Blix, A.S., Kjekshus, J.K., Enge, I. and Bergan, A. (1976). Myocardial blood flow in the diving seal. *Acta Physiol. Scand*. 96, 277-280.

Bradley, S.E. and Bing, R.J. (1942). Renal function in the harbor seal (*Phoca vitulina L*.) during asphyxial ischemia and pyrogenic hyperemia. *J. Cell. Comp. Physiol*. 19, 229-239.

Brezis, M., Rosen, S., Silva, P., and Epstein, F.H. (1984). Renal ischemia: a new perspective. *Kidney Int*. 26, 375-383.

Butler, P.J., and Jones, D.R. (1997). Physiology of diving birds and mammals. *Physiol. Rev*. 77 (3), 837-899

Castellini, M.A., Davis, R.W. and Kooyman, G.L. (1988). Blood chemistry regulation during repetitive diving in Weddell seals. *Physiol. Zool*. 61(5), 379-386.

Castellini, M.A., Somero, G.N. and Kooyman, G.L. (1981). Glycolytic enzyme activities in tissues of marine and terrestrial mammals. *Physiol. Zool*. 54(2), 242-252.

Costa, L.E., Boveris, A., Koch, O.R. and Taquini, A.C. (1988). Liver and heart mitochondria in rats submitted to chronic hypobaric hypoxia. *Am. J. Physiol*. 255 (*Cell Physiol*. 24), C123-C129.

Davis, C.N., Davis, L.E. and Powers, T.E. (1975). Comparative body compositions of the dog and goat. *Am. J. Vet. Res*. 36, 309-311.

Davis, R.W. (1983). Lactate and glucose metabolism in the resting and diving harbor seal. *J. Comp. Physiol*. 153, 275-288.

Davis, R.W., Castellini, M.A., Kooyman, G.L. and Maue, R. (1983). Renal glomerular filtration rate and hepatic blood flow during voluntary diving in Weddell seals. *Am. J. Physiol*. 245, R743-R748.

Davis, R.W., Castellini, M.A., Williams, T.M. and Kooyman, G.L. (1991). Fuel homeostasis in the harbor seal during submerged swimming. *J. Comp. Physiol. B* 160, 627-635.

Davis, R.W., Fuiman, L.A., Williams, T.M., LeBoeuf, B.J. (2001). Three-dimensional movements and swimming activity of a northern elephant seal. *Comp. Biochem. Physiol. A Mol. Integr. Physiol*. 129, 759-770.

Davis, R.W. and Kanatous, S.B. (1999). Convective oxygen transport and tissue oxygen consumption in Weddell seals during aerobic dives. *J. Exp. Biol*. 202, 1091-1113.

Elsner, R. and Gooden, B. (1983). Diving and asphyxia. A comparative study of animals and man. *Monogr. Physiol. Soc*. 40, 1-168.

Fink, M.P. (2001). Cytopathic hypoxia: mitochondrial dysfunction as mechanism contributing to organ dysfunction in sepsis. *Crit. Care Clin*. 17, 219-237.

Fisher, A. (1963). Dynamics of the circulation in the liver. In *The Liver* (ed. C. Rouiller), vol.1, pp. 329-378. New York: Academic.

Garg, D.K. (1979). Cellular oxygenation and metabolism during ischemia in gastric mucosa. *Texas Med*. 75, 56-60.

Gollnick, P.D. and Saltin, B. (1988). Fuel for muscular exercise: role of fat. In *Exercise, Nutrition and Energy Metabolism* (ed. E.S. Horton and R.L. Terjung), pp. 72-87. New York: Macmillan.

Granger, H.J. and Norris, C.P. (1980). Intrinsic regulation of intestinal oxygenation in the anesthetized dog. *Am. J. Physiol*. 235, H836-H843.

Granger, H.J. and Shepherd, A.P. (1973). Intrinsic microvascular control of tissue oxygen delivery. *Microvasc. Res*. 5, 49-72.

Halasz, N.A., Elsner, E., Garvie, R.S. and Grotke, G.T. (1974). Renal recovery from ischemia: a comparative study of harbor seal and dog kidneys. *Am. J. Physiol*. 227, 1331-1335.

Hochachka, P.W. (1992). Metabolic biochemistry and the making of a mesopelagic mammal. *Experientia* 48, 570-575.

Hochachka, P.W., Stanley, C., Merkt, J. and Sumar-Kalinowski, J. (1982). Metabolic meaning of elevated levels of oxidative enzymes in high altitude adapted animals: an interpretive hypothesis. *Respir. Physiol*. 52, 303-313.

Hoppeler, H., Mathieu, O. Krauer, R, Claassen, H., Armstrong, R.B. and Weibel, E.R. (1981). Design of the mammalian respiratory system. VI. Distribution of mitochondria and capillaries in various muscles. *Respir. Physiol*. 44, 78-111.

International Life Sciences Institute Risk Science Institute. (1994). Physiological parameter values for PBPK models. Washington D.C.

Jacobsen, K.R., Ranek, L. and Tygstrup, N. (1969). Liver function and blood flow in normal man during infusion of vasopressin. *Scand. J. Clin. Lab. Invest*. 24, 279-284.

Johnson, P.C. (1960). Autoregulation of intestinal blood flow. *Am. J. Physiol*. 199, 311-318.

Jones, D.P. (1984). Effect of mitochondrial clustering on O_2 supply in hepatocytes. *Am. J. Physiol*. 247, C83-C89.

Junqueira, L.C., Carneiro, J and Kelley, R.O. (1998). Digestive Tract. In *Basic Histology* (ed. D.A. Barnes, A.M. Suver, J. Roche, M.B. Darrow and M. McKenney), pp. 285-287. Stamford, CT: Appleton & Lange.

Kanatous, S.B., DiMichele, L.V., Cowan, D.F. and Davis, R.W. (1999). High aerobic capacities in the skeletal muscles of pinnipeds: adaptations to diving hypoxia. *J. Appl. Physiol*. 86, 1247-1256.

Kanatous, S.B., Elsner, R. and Mathieu-Costello, O. (2001). Muscle capillary supply in harbor seals. *J. Appl. Physiol*. 90, 1919-1926.

Kayar, S.R. and Banchero, N. (1987). Volume density and distribution of mitochondria in myocardial growth and hypertrophy. *Respir. Physiol*. 70, 275-286.

Kennedy, S.L., Stanley, W.C., Panchal, A.R. and Mazzeo, R.S. (2001). Alterations in enzymes involved in fat metabolism after acute and chronic altitude exposure. *J. Appl. Physiol*. 90, 17-22.

Kettelhut, I.C., Foss, M.C. and Migliorini, R.H. (1980). Glucose homeostasis in a carnivorous animal (cat) and in rats fed a high-protein diet. *Am. J. Physiol*. 239, R437-R444.

Kooyman, G.L., Castellini, M.A. and Davis, R.W. (1981). Physiology of diving in marine mammals. *Annu. Rev. of Physiol*. 43, 343-356.

Kooyman, G.L., Castellini, M.A., Davis, R.W. and Maue, R.A. (1983). Aerobic diving limits of immature weddell seals. *J. Comp. Physiol. B* 151, 171-174.

Kvietys, P.R. and Granger, D.N. (1982). Relation between intestinal blood flow and oxygen uptake. *Am. J. Physiol*. 242, G202-G208.

Lassen, N.A. (1964). The metabolic demand of the kidney as related to variations in renal blood flow. *Circ. Res. Supplement* 1. XIV/XV, 183-184.

Lee, J.S., Bruce, C.R., Spriet, L.L. and Hawley, J.A. (2001). Interaction of diet and training on endurance performance in rats. *Exp. Physiol*. 86.4, 499-508.

Lewis, A.M., Mathieu-Costello, O., McMillan, P.J. and Gilbert, R.D. (1999). Quantitative electron microscopic study of the hypoxic fetal sheep heart. *Anat. Rec*. 256, 381-388.

Lutz, J., Henrich, H. and Bauereisen, E. (1975). Oxygen supply and uptake in the liver and intestine. *Pfluegers Arch*. 360, 7-15.

Mathieu, O., Krauer, R., Hoppeler, H., Gehr, P., Lindstedt, S.L., McNeill Alexander, R., Taylor, C.R. and Weibel, E.R. (1981). Design of the mammalian respiratory system. VII. Scaling mitochondrial volume in skeletal muscle to body mass. *Respir. Physiol*. 44, 113-128.

Murdaugh Jr., H.V., Schmidt-Nielsen, B., Wood, J.W. and Mitchell, W.L. (1961). Cessation of renal function during diving in the trained seal (*Phoca vitulina*). *J. Cell. Comp. Physiol*. 58, 261-265.

Nelson, D.P., Samsel, R.W., Wood, L.D.H. and Schumacker, P.T. (1988). Pathological supply dependence of systemic and intestinal O_2 uptake during endotoxemia. *J. Appl. Physiol*. 64, 2410-2419.

Ohtsuka, T. and Gilbert, R.D. (1995). Cardiac enzyme activities in fetal and adult pregnant and nonpregnant sheep exposed to high-altitude hypoxemia. *J. Appl. Physiol*. 79(4), 1286-1289.

Penney, D.G. (1974). Lactate dehydrogenase subunit and activity changes in hypertrophied heart of the hypoxically exposed rat. *Biochim. Biophys. Acta* 358, 21-24.

Pette, D. and Dölken, G. (1975). Some aspects of regulation of enzyme levels in muscle energy-supplying metabolism. *Adv. Enzyme Regul*. 13, 335-378.

Plevris, J.N., Jalan, R., Bzeizi, K.I., Dollinger, M.M., Lee, A., Garden, O.J. and Hayes, P.C. (1999). Indocyanin green clearance reflects reperfusion injury following liver transplantation and is an early predictor of graft function. *J. Hepatol*. 30, 142-148.

Ponganis, P.J., Kooyman, G.L., Baranov, E.A., Thorson, P.H. and Stewart, B.S. (1997). The aerobic submersion limit of Baikal seals, Phoca sibirica. *Can. J. Zool*. 75, 1323-1327.

Qvist, J., Hill, R.D., Schneider, R.C., Falke, K.J., Liggins, G.C., Guppy, M., Elliott, R.L., Hochachka, P.W. and Zapol, W.M. (1986). Hemoglobin concentrations and blood gas tensions of free-diving Weddell seals. *J. Appl. Physiol*. 61, 1560-1569.

Reed, J.Z., Butler, P.J. and Fedak, M.A. (1994). The metabolic characteristics of the locomotory muscles of grey seals (*Halichoerus grypus*), harbour seals (*Phoca vitulina*) and antarctic fur seals (*Arctocephalus gazella*). *J. Exp. Biol*. 194, 33-46.

Roberts, S., Samuels, L.T., Reinecke, R.M. (1943). Previous diet and the apparent utilization of fat in the absence of the liver. *Am. J. Physiol*. 140, 639-644.

Roberts, T.J., Weber, J., Hoppeler, H., Weibel, E.R. and Taylor, C.R. (1996). Design of the oxygen and substrate pathways. II. Defining the upper limits of carbohydrate and fat oxidation. *J. Exp. Biol*. 199, 1651-1658.

Rowell, L.B. (1986). *Human Circulation Regulation During Physical Stress*, p. 415. Oxford, New York: Oxford University Press.

Schmidt-Nielsen, K. and Duke, J.B. (1979). Appendix B: Diffusion. In *Animal Physiology: Adaptation and Environment*. pp. 533-535. New York: Cambridge University Press.

Schmidt-Nielsen, K. and Duke, J.B. (1984). Metabolic rate and body size. In *Scaling: Why Is Animal Size So Important*?, pp. 62-64. New York: Cambridge University Press.

Simi, B., Sempore, B., Mayet, M.H. and Favier, R.J. (1991). Additive effects of training and high-fat diet on energy metabolism during exercise. *J. Appl. Physiol*. 71, 197-203.

Vergnes, H. (1971). Modifications in the activity of enzymes in extracts from the myocardia of rats living at high altitudes. *Cardiology* 56, 222-223.

Wagner, P.D. (1991). Central and peripheral aspects of oxygen transport and adaptations with exercise. *Sports Med*. 11, 133-142.

Wang, Z., O'Connor, T.P., Heshka, S. and Heymsfield, S.B. (2001). The reconstruction of Kleiber's law at the organ and tissue level. *J. Nutr*. 131, 2967-2970.

West, J., Boyer, S. and Graber, D. (1983). Maximal exercise at extreme altitude on Mt. Everest. *J. Appl. Physiol*. 55, 688-698.

Williams, T.M., Davis, R.W., Fuiman, L.A., Francis, J., Le Boeuf, B.J., Horning, M., Calambodkidis, J. and Croll, D.A. (2000). Sink or swim: strategies for cost-efficient diving by marine mammals. *Science* 288, 133-136.

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Table 1. Mean body mass, percent organ mass and mitochondrial volume density [Vv(mt)] for heart, liver, kidney, stomach and intestine in the harbor seal, dog and rat.

Species	Mass, kg	*n*	Organ	% Body Mass	Volume Density, Vv(mt), %
Harbor seals	43.8 ± 6.3	6	Heart	0.7	18.6 ± 0.9
(*Phoca vitulina*)			Liver	2.7	26.4 ± 0.9†§
			Kidney	0.6	21.4 ± 0.9†
			Stomach	1.2	24.5 ± 1.5§
			Intestine	4.2	8.9 ± 1.0
Dogs	9.2 ± 0.5	3	Heart	0.8[+]	18.2 ± 0.8
(*Canis familiaris*)			Liver	2.3[+]	17.3 ± 1.2
			Kidney	0.6[+]	16.6 ± 1.0
			Stomach	0.8[+]	21.3 ± 3.4
			Intestine	2.7[+]	6.0 ± 0.5
Rats			Heart	0.3*	22.8 ± 4.7
(*Rattus norvegicus*)	0.48 ± 0.03	3	Liver	3.7*	13.2 ± 1.7
Strain: Sprague-Dawley		1 for kidney	Kidney	0.7*	18.7
			Stomach	0.5*	13.3 ± 3.8
			Intestine	2.2*	7.5 ± 1.8

Values are means ± SE. *n*, no. of animals. Vv(mt), volume density of total mitochondria, all quantities expressed per tissue volume. [+] Percent organ mass for dogs from Davis et al. (1975). * Percent organ mass for rats from International Life Sciences Institute Risk Science Institute (1994). † Significantly different from dog (ANOVA, $p<0.05$). § Significantly different from rat (ANOVA, $p<0.05$). ‡ Significantly different from harbor seal (ANOVA, $p<0.05$). Values with no SE were not included in the analysis and are presented for comparison.

Table 2. Enzyme activities of citrate synthase (CS), β-hydroxyacyl-CoA dehydrogenase (HOAD) and lactate dehydrogenase (LDH) in the heart, liver, kidney, stomach and intestine of harbor seals, dogs and rats.

Species	Mass, kg	*n*	Organ	CS, IU/g wet wt tissue	HOAD, IU/g wet wt tissue	LDH, IU/g wet wt tissue
Harbor Seals	46.1 ± 4.3	10	Heart	73.8 ± 5.1	$1.0 \times 10^2 \pm 3.8$†§	$6.9 \times 10^2 \pm 20.5$†
(*Phoca vitulina*)			Liver	13.5 ± 0.3†	11.2 ± 0.5†§	$1.1 \times 10^3 \pm 66.5$†§
			Kidney	15.4 ± 0.3	$2.4 \times 10^2 \pm 45.6$	$1.9 \times 10^2 \pm 6.1$
			Stomach	11.4 ± 2.0	4.4 ± 0.7	$1.6 \times 10^2 \pm 16.8$
			Intestine	0.16	3.4 ± 0.3	$2.5 \times 10^2 \pm 13.0$†
Dogs	9.2 ± 0.5	3	Heart	71.8 ± 2.1	38.3 ± 7.4	$5.4 \times 10^2 \pm 46.7$
(*Canis familiaris)*			Liver	8.2 ± 0.3	2.0 ± 0.1	$3.1 \times 10^2 \pm 18.9$
			Kidney	21.3 ± 0.7‡	$1.7 \times 10^2 \pm 66.0$	$2.1 \times 10^2 \pm 21.2$‡
			Stomach	21.8 ± 4.6	4.2 ± 0.9	$2.1 \times 10^2 \pm 18.9$
			Intestine	0.14	2.4 ± 0.2	$1.3 \times 10^2 \pm 3.2$
Rats	0.48 ± 0.03	3	Heart	$1.2 \times 10^2 \pm 7.7$*†‡	27.5 ± 1.1*	$1.3 \times 10^3 \pm 81.1$*†‡
(*Rattus norvegicus*)		1 for kidney	Liver	12.3 ± 0.8†	7.1 ± 0.4†	$8.1 \times 10^2 \pm 11.6$†
Strain: Sprague-Dawley			Kidney	18.7	143.8	1.0×10^2
			Stomach	16.8 ± 5.0	6.7 ± 1.5	$1.7 \times 10^2 \pm 12.2$
			Intestine	0.22	6.0 ± 0.7†‡	$2.7 \times 10^2 \pm 4.0$†

Values are expressed as means ± SE. *n*, no. of animals. CS, HOAD, and LDH activities are expressed as μmol product formed min^{-1} g wet wt $tissue^{-1}$. * Values from Polasek et al. (in prep). † Significantly different from dog (ANOVA, $p < 0.05$). § Significantly different from rat (ANOVA, $p < 0.05$). ‡ Significantly different from harbor seal (ANOVA, $p < 0.05$). Values with no SE were not included in the analysis but are presented for comparison.

Table 3. CS/HOAD and LDH/CS ratios for the heart, liver, kidneys, stomach and intestine of harbor seals, dogs and rats.

Species	*n*	Organ	CS/HOAD	LDH/CS
Harbor Seals	10	Heart	0.7 ± 0.05†§	9.9 ± 1.0
	8 for liver	Liver	1.2 ± 0.05†§	80.5 ± 5.4†
	9 for intestine	Kidney	0.06 ± 0.01†	12.2 ± 0.4†
		Stomach	2.6 ± 0.3†	15.7 ± 1.7
		Intestine	0.05	1583.1
Dogs	3	Heart	1.9 ± 0.4§	7.5 ± 0.6
		Liver	4.1 ± 0.2	38.3 ± 1.2
		Kidney	0.1 ± 0.04	9.8 ± 0.7
		Stomach	5.2 ± 0.1	10.2 ± 1.6
		Intestine	0.06	988.8
Rats	3	Heart	4.5 ± 0.4	10.9 ± 0.7
	1 for kidney	Liver	1.7 ± 0.2†	66.3 ± 4.5†
		Kidney	0.1	5.3
		Stomach	2.5 ± 0.3†	11.6 ± 2.3
		Intestine	0.04	1180.0

Values are expressed as means ± SE. *n*, no. of animals. † Significantly different from dog (ANOVA, $p < 0.05$). § Significantly different from rat (ANOVA, $p < 0.05$). ‡ Significantly different from harbor seal (ANOVA, $p < 0.05$). Values with no SE were not included in the analysis but are presented for comparison.

Table 4. Mitochondrial volume densities Vv(mt) for heart, liver, kidney, stomach and intestine scaled to tissue specific resting metabolic rate (RMR) for harbor seals, dogs and rats.

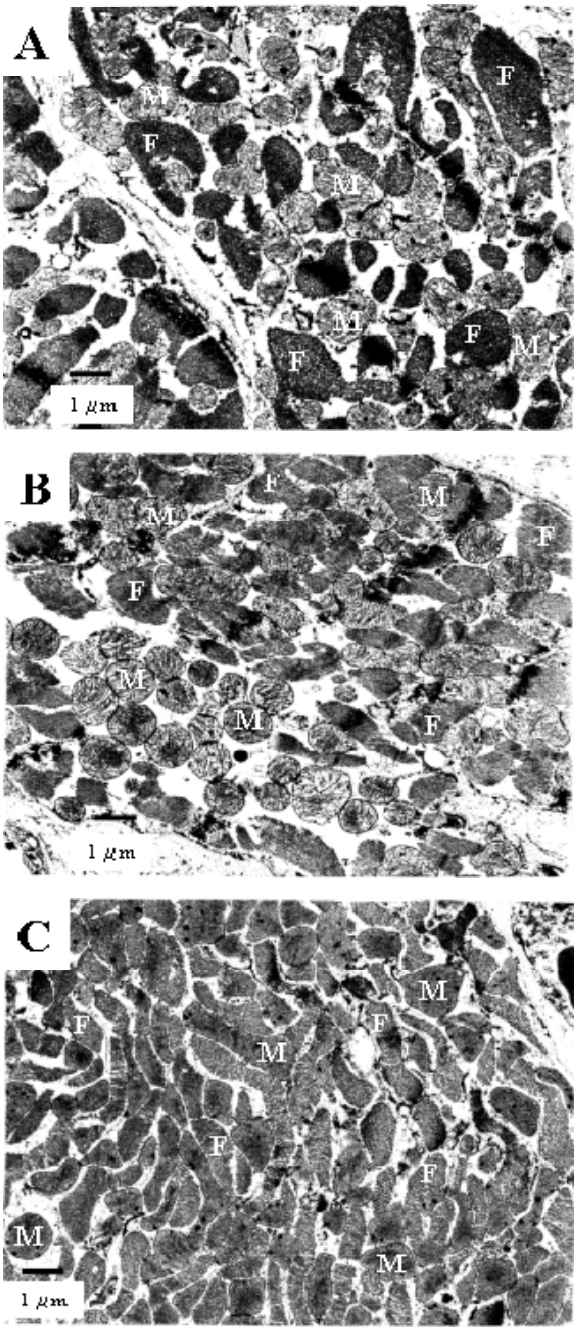
Species	Mass, kg	*n*	Organ	Tissue specific resting metabolic rate [kJ/(kg*d)]	Volume density ratio [Vv(mt)/RMR]
Harbor seals	43.8 ± 6.3	6	Heart	2.4×10^{3}	$7.8 \times 10^{-3} \pm 3.5 \times 10^{-4}$§
(*Phoca vitulina*)		5 for intestine	Liver	1.0×10^{3}	$2.5 \times 10^{-2} \pm 1.2 \times 10^{-3}$†§
			Kidney	2.1×10^{3}	$1.0 \times 10^{-2} \pm 5.2 \times 10^{-4}$†
			Stomach	6.6×10^{1}	$3.7 \times 10^{-1} \pm 2.6 \times 10^{-2}$†§
			Intestine	6.6×10^{1}	$1.4 \times 10^{-1} \pm 1.5 \times 10^{-2}$†§
Dogs	9.2 ± 0.5	3	Heart	2.9×10^{3}	$6.4 \times 10^{-3} \pm 2.6 \times 10^{-4}$
(Canis familiaris)			Liver	1.6×10^{3}	$1.1 \times 10^{-2} \pm 8.8 \times 10^{-4}$§
			Kidney	2.4×10^{3}	$6.9 \times 10^{-3} \pm 3.8 \times 10^{-4}$
			Stomach	8.6×10^{1}	$2.5 \times 10^{-1} \pm 3.8 \times 10^{-2}$§
			Intestine	8.6×10^{1}	$7.0 \times 10^{-2} \pm 5.2 \times 10^{-3}$
Rats		3	Heart	4.1×10^{3}	$5.6 \times 10^{-3} \pm 6.3 \times 10^{-4}$
(Rattus norvegicus)	0.48 ± 0.03	1 for kidney	Liver	3.5×10^{3}	$3.8 \times 10^{-3} \pm 4.7 \times 10^{-4}$
Strain: Sprague-Dawley			Kidney	3.1×10^{3}	6.2×10^{-3}
			Stomach	1.4×10^{2}	$9.4 \times 10^{-2} \pm 2.7 \times 10^{-2}$
			Intestine	1.4×10^{2}	$5.3 \times 10^{-2} \pm 1.3 \times 10^{-2}$

Values are means ± SE. n, no. of animals. Mb, body mass (in kg). Vv(mt), volume density of total mitochondria, all quantities expressed per tissue volume. † Significantly different from dog (ANOVA, $p < 0.05$). § Significantly different from rat (ANOVA, $p < 0.05$). ‡ Significantly different from harbor seal (ANOVA, $p < 0.05$). Values with no SE were not included in the analysis but are presented for comparison.

Table 5. Citrate synthase (CS/RMR), β-hydroxyacyl-CoA dehydrogenase (HOAD/RMR) and lactate dehydrogenase (LDH/RMR) activities scaled to tissue specific resting metabolic rate for harbor seals, dogs and rats.

Species	Mass, kg	*n*	Organ	CS	HOAD	LDH
				(mean enzyme activity/tissue specific resting metabolic rate)		
Harbor seals	46.1 ± 4.3	10	Heart	$3.1 \times 10^{-2} \pm 2.3 \times 10^{-3}$	$4.2 \times 10^{-2} \pm 1.7 \times 10^{-3}$†§	$2.9 \times 10^{-1} \pm 9.8 \times 10^{-3}$†
(*Phoca vitulina*)		8 for liver	Liver	$1.3 \times 10^{-2} \pm 4.9 \times 10^{-4}$†§	$1.1 \times 10^{-2} \pm 6.1 \times 10^{-4}$†§	$1.1 \pm 6.6 \times 10^{-2}$†§
		9 for intestine	Kidney	$7.2 \times 10^{-3} \pm 1.7 \times 10^{-4}$	$1.1 \times 10^{-1} \pm 2.1 \times 10^{-2}$	$8.8 \times 10^{-2} \pm 2.7 \times 10^{-3}$†
			Stomach	$1.7 \times 10^{-1} \pm 2.8 \times 10^{-2}$	$6.6 \times 10^{-2} \pm 1.0 \times 10^{-2}$	$2.4 \pm 2.3 \times 10^{-1}$§
			Intestine	2.4×10^{-3}	$5.1 \times 10^{-2} \pm 4.6 \times 10^{-3}$†	$3.8 \pm 1.9 \times 10^{-1}$†§
Dogs	9.2 ± 0.5	3	Heart	$2.5 \times 10^{-2} \pm 8.9 \times 10^{-4}$	$1.3 \times 10^{-2} \pm 2.6 \times 10^{-3}$	$1.9 \times 10^{-1} \pm 1.6 \times 10^{-2}$
(*Canis familiaris)*			Liver	$5.2 \times 10^{-3} \pm 2.4 \times 10^{-4}$	$1.3 \times 10^{-3} \pm 9.0 \times 10^{-5}$	$2.0 \times 10^{-1} \pm 1.2 \times 10^{-2}$
			Kidney	$8.8 \times 10^{-3} \pm 2.6 \times 10^{-4}$‡	$7.0 \times 10^{-2} \pm 2.7 \times 10^{-2}$	$8.6 \times 10^{-2} \pm 8.4 \times 10^{-3}$
			Stomach	$2.5 \times 10^{-1} \pm 5.1 \times 10^{-2}$	$4.8 \times 10^{-2} \pm 9.7 \times 10^{-3}$	$2.4 \pm 2.0 \times 10^{-1}$
			Intestine	1.6×10^{-3}	$2.8 \times 10^{-2} \pm 2.5 \times 10^{-3}$	$1.6 \pm 5.0 \times 10^{-3}$
Rats	0.48 ± 0.03	3	Heart	$3.1 \times 10^{-2} \pm 2.1 \times 10^{-3}$	$6.8 \times 10^{-3} \pm 2.8 \times 10^{-4}$	$3.3 \times 10^{-1} \pm 1.8 \times 10^{-2}$†
(*Rattus norvegicus*)		1 for kidney	Liver	$3.5 \times 10^{-3} \pm 1.7 \times 10^{-4}$	$2.0 \times 10^{-3} \pm 1.3 \times 10^{-4}$	$2.3 \times 10^{-1} \pm 5.9 \times 10^{-3}$
Strain: Sprague-Dawley			Kidney	6.2×10^{-3}	4.7×10^{-2}	3.3×10^{-2}
			Stomach	$1.2 \times 10^{-1} \pm 3.5 \times 10^{-2}$	$4.8 \times 10^{-2} \pm 1.0 \times 10^{-2}$	$1.2 \pm 7.4 \times 10^{-2}$
			Intestine	1.6×10^{-3}	$4.2 \times 10^{-2} \pm 5.2 \times 10^{-3}$	$1.9 \pm 2.1 \times 10^{-2}$

Values are expressed as means ± SE. *n*, no. of animals. † Significantly different from dog (ANOVA, $p < 0.05$). § Significantly different from rat (ANOVA, $p < 0.05$). ‡ Significantlydifferent from harbor seal (ANOVA, $p < 0.05$). Values with no SE were not included in the analysis but are presented for comparison. RMR for specific tissues were calculated as in Table 4.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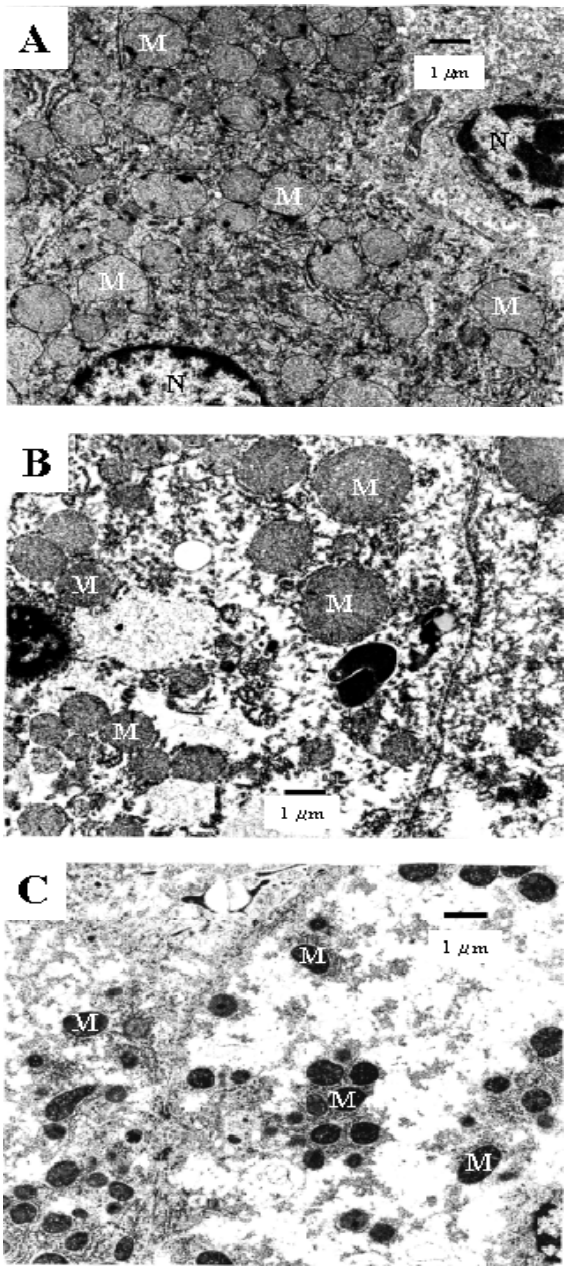
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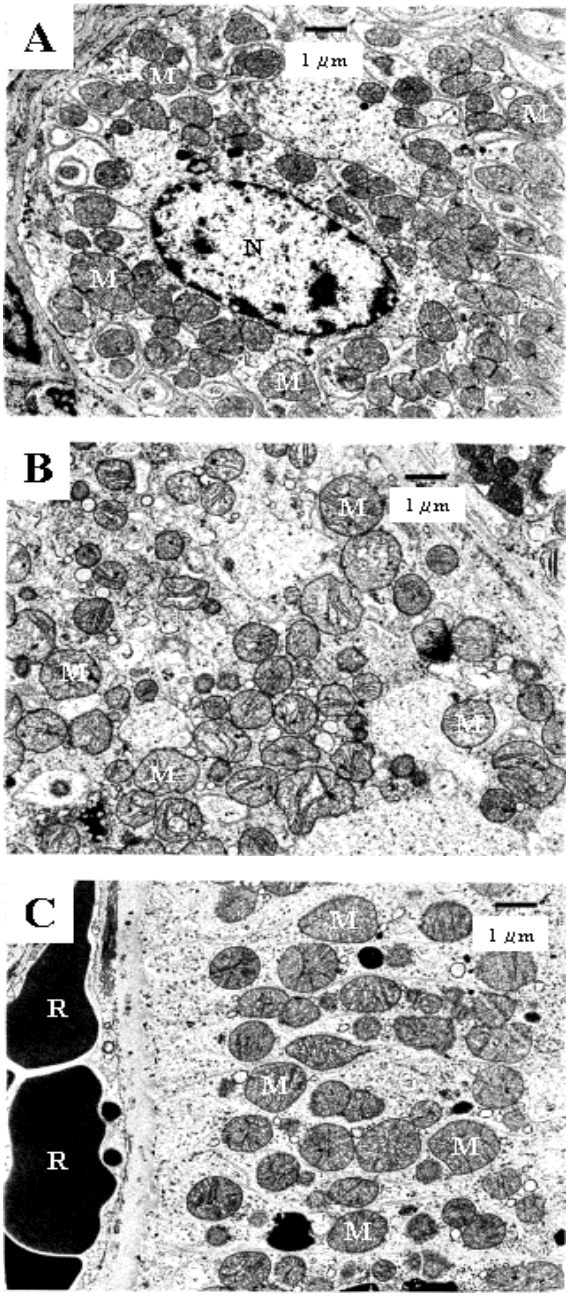
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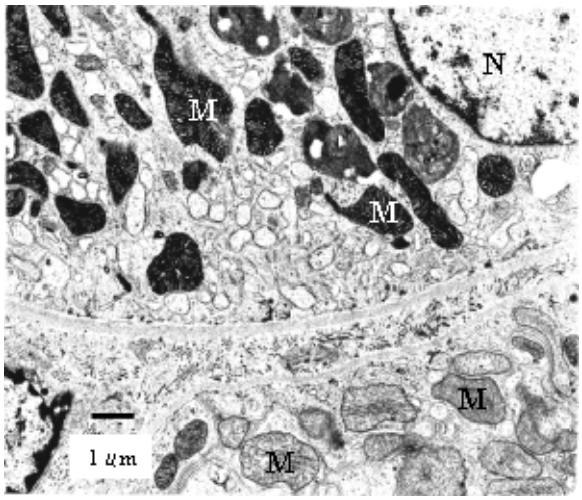
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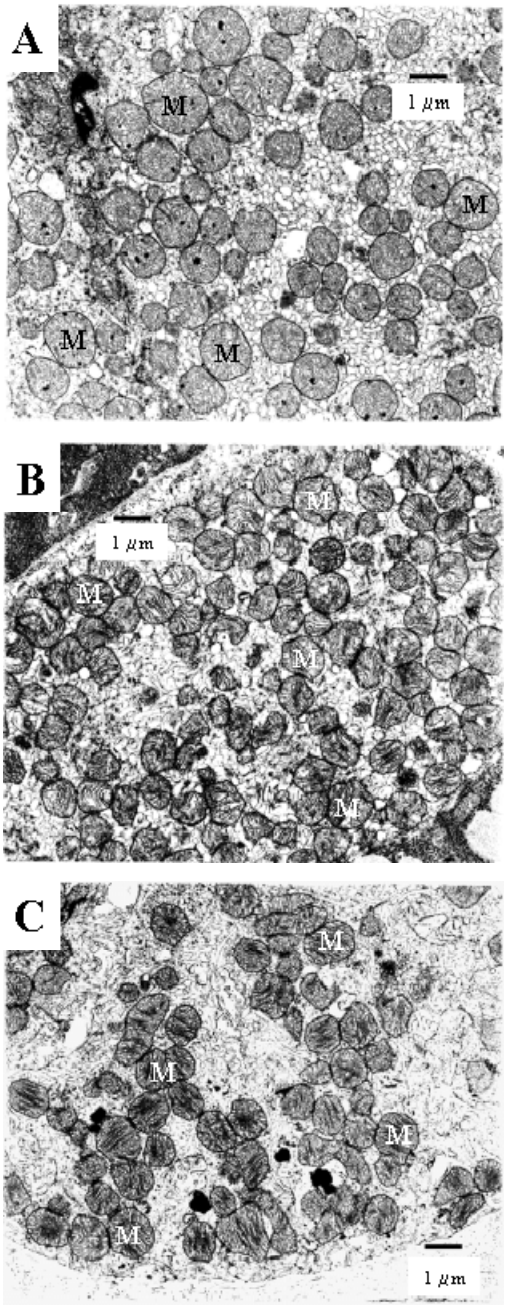
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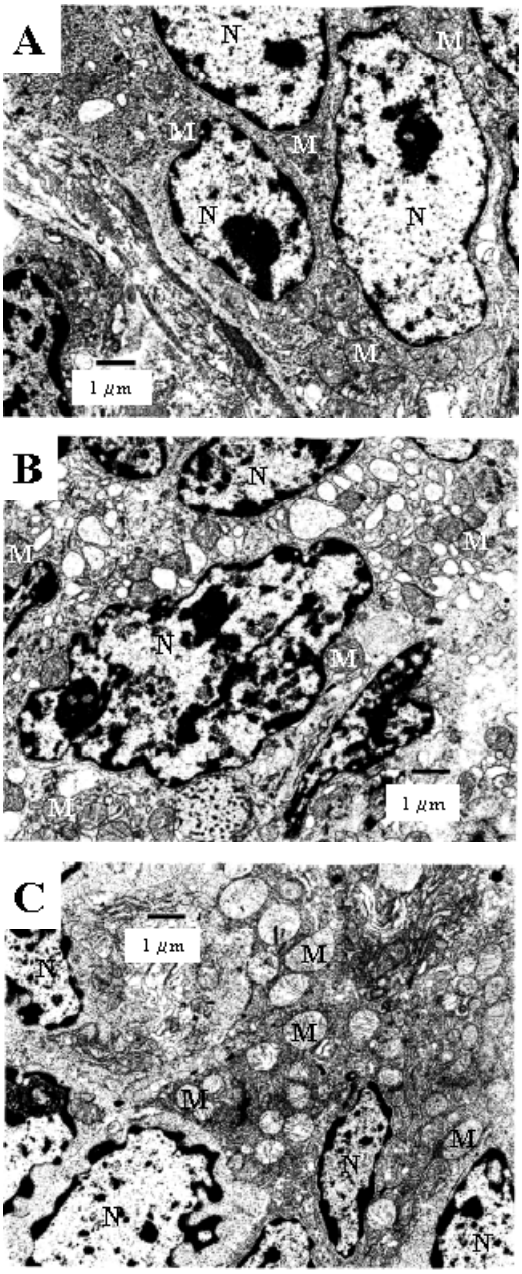
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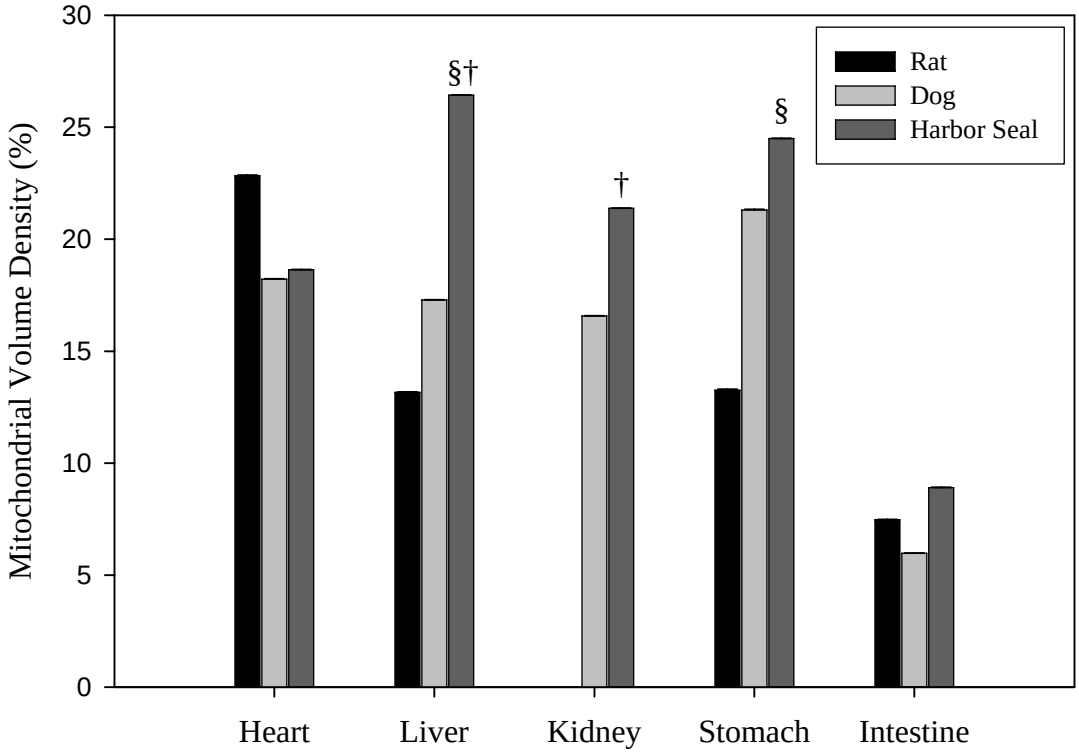
Mitochondrial Volume Density (%)
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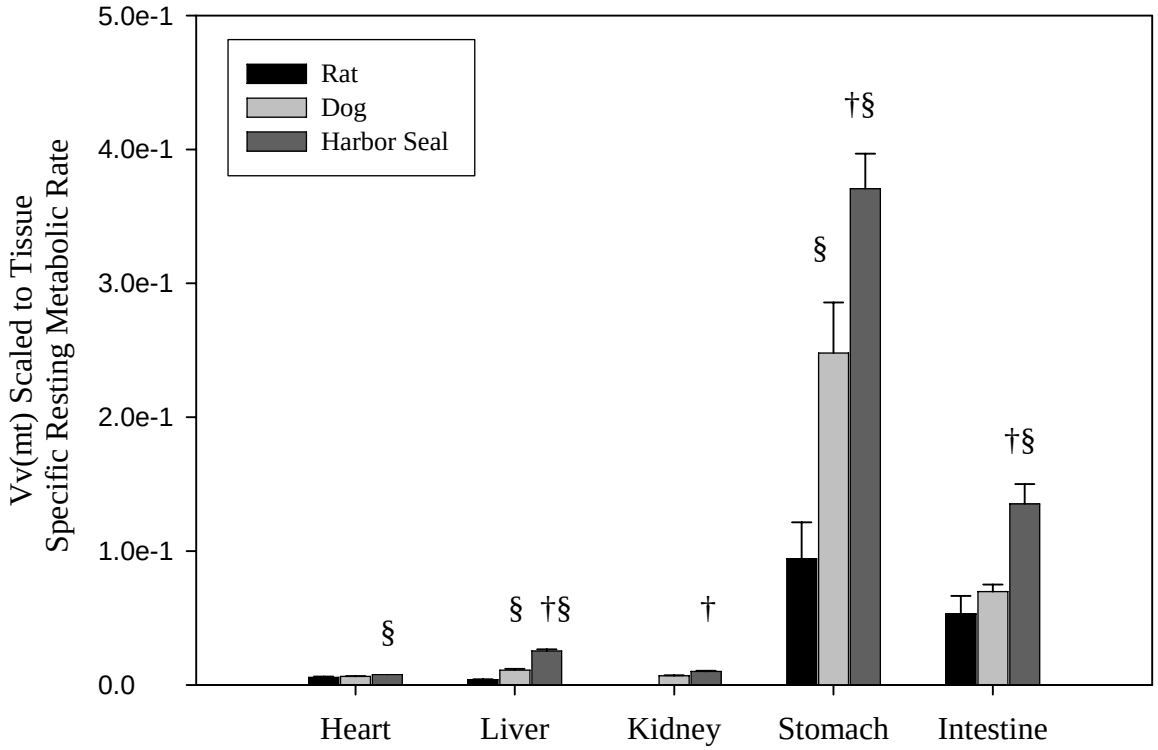
Kidney
Stomach
Intestine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Vv(mt) Scaled to Tissue
Specific Resting Metabolic Rate
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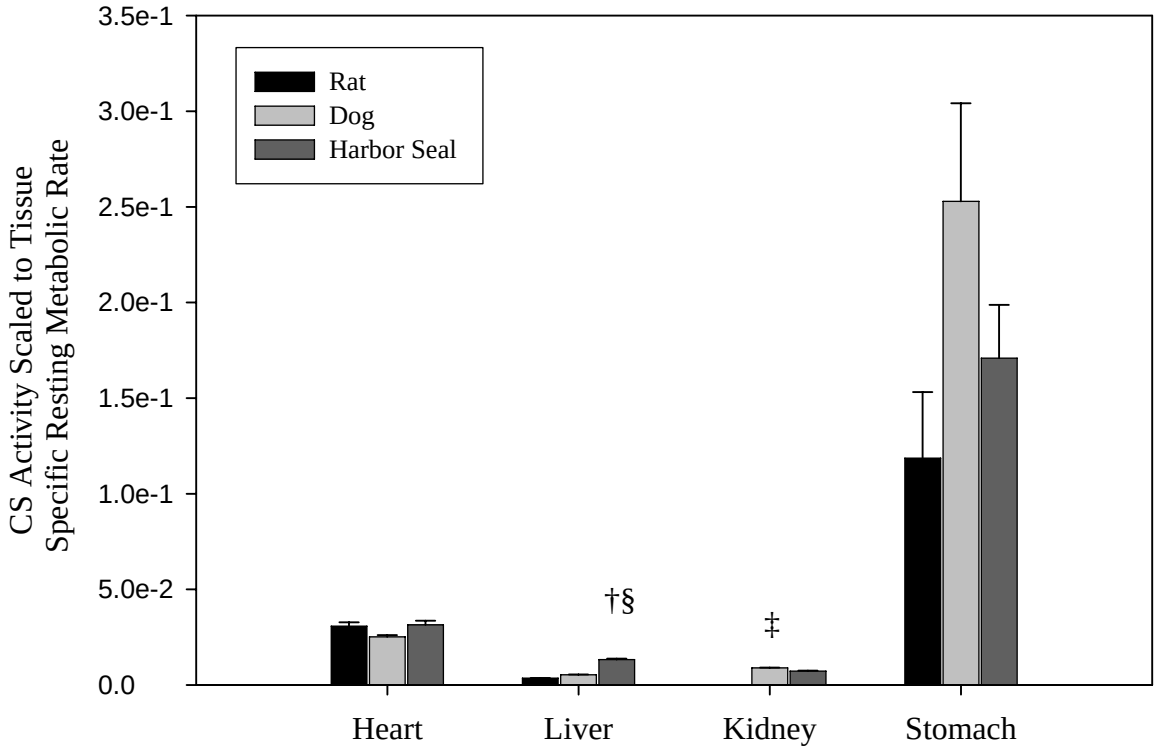
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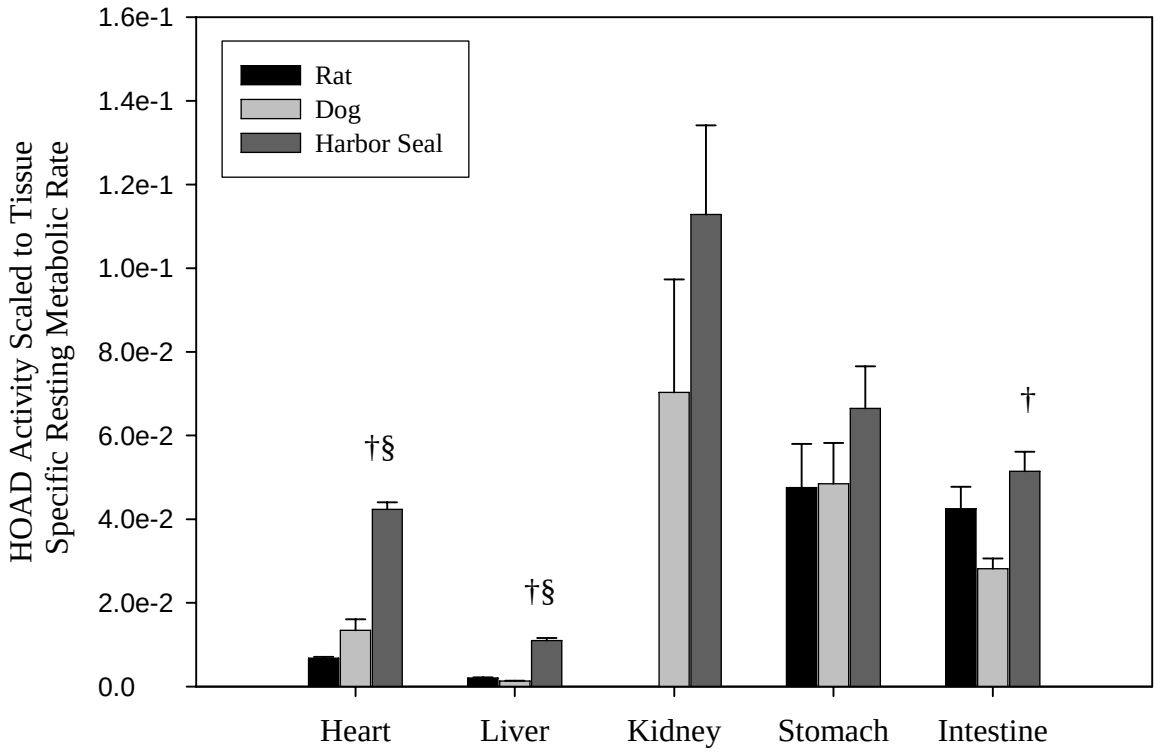
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HOAD Activity Scaled to Tissue
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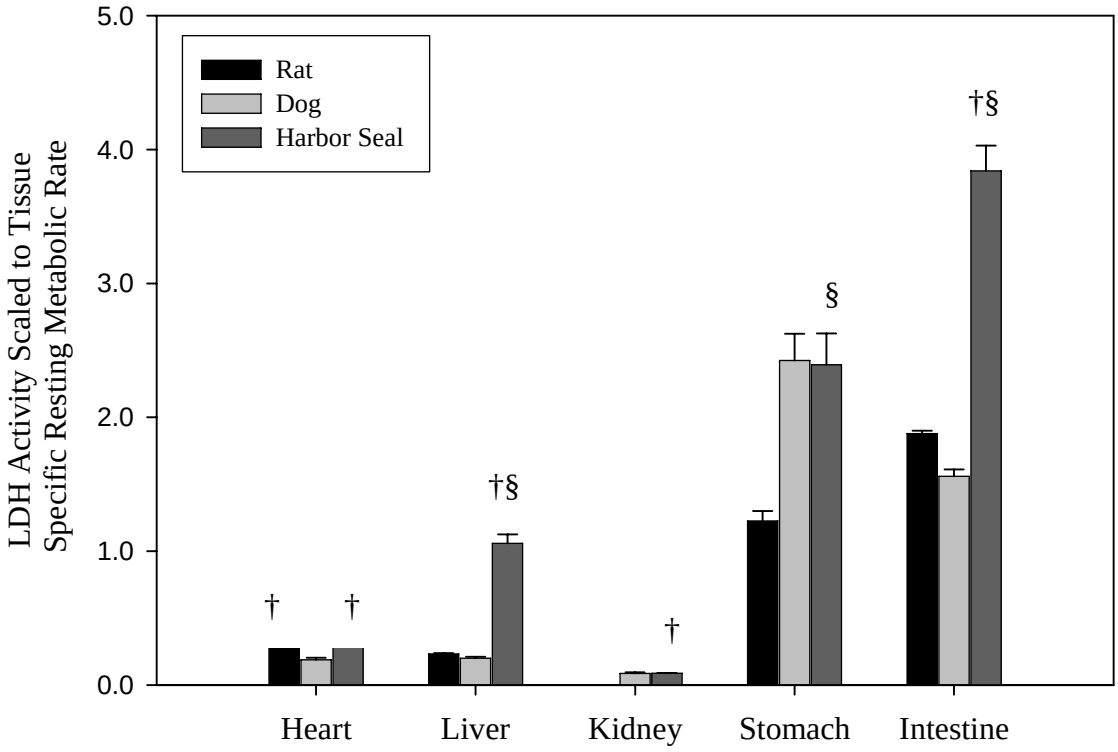
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2.0
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Rat
Dog
Harbor Seal
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†
†§
†
§
†§
Heart
Liver
Kidney
Stomach
Intestine

Mapping aerobic and glycolytic enzyme activities and myoglobin concentration in the locomotory muscles of harbor seals (*Phoca vitulina)*

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Summary

The goal of this study was to determine the distribution of citrate synthase (CS), β-hydroxyacyl CoA dehydrogenase (HOAD) and lactate dehydrogenase (LDH) activities and myoglobin (Mb) concentration in the locomotory muscles (epaxial muscles) of harbor seals (HS). In addition, single samples were taken from the hindlimb complex (locomotory muscles), *pectoralis* (non-locomotory muscle) and left ventricle of the heart. Ten HS were sampled as part of a native subsistence hunt in Prince William Sound, Alaska. The entire epaxial musculature was removed and weighed, and three transverse sections (cranial, middle, and caudal) were taken along the muscle bundle. Multiple samples (ca. 30 per transverse section) were taken along points on a circular grid using a 6-mm biopsy punch. A spectrophotometric technique was used to measure CS, HOAD and LDH activities and Mb concentration. Rats and dogs were used as controls. Mean values were calculated for four roughly equal quadrants (dorsal, ventral, right, left) in each transverse section. There were no significant differences among the quadrants within any of the transverse sections for the three enzymes or Mb. However, there were significant differences in the average transverse section enzyme activities and Mb concentrations along the length of the muscle. The middle and caudal sections had significantly higher mean CS, LDH, and Mb than the cranial section. The CS activity in the heart was 2.6-3.0x higher than in the epaxial muscles, hindlimb complex and *pectoralis*. HOAD activity in the hindlimb complex and *pectoralis* muscle was significantly less (ca. 31%) than in the epaxial muscles, but the heart and epaxial muscle HOAD activities did not differ significantly. LDH activity was significantly lower in the seal heart than in the epaxial muscles. There were no significant differences in LDH activities among the other tissues. The enzyme ratios CS:HOAD and

LDH:CS exhibited no variation within transverse sections or along the length of the epaxial muscles. No significant difference was seen in the LDH:CS ratios among the other muscles in the harbor seal. Relative to the rat and dog, the epaxial muscles and heart of the harbor seal had high HOAD levels and low CS:HOAD ratios which, taken together, indicate an increased capacity for aerobic lipid metabolism during diving.

Introduction

To maintain aerobic metabolism during diving, pinnipeds (seals and sea lions) generally exhibit a low metabolic rate and rely principally on oxygen stored in the blood and muscle (Kooyman et al., 1983). Reed et al. (1994) found that muscle myoglobin (Mb) concentrations in harbor seals are significantly greater (20–27x) than those of terrestrial mammals. Mb is a significant storage site for oxygen in the muscle and enhances the diffusion of intracellular oxygen, particularly under hypoxic conditions (Whittenberg and Whitttneberg 1989; Reed et al., 1994; Guyton et al., 1995). Recently, Kanatous et al. (1999) showed that the muscles of harbor seals have an increased mitochondrial volume density (Vv(mt)) and increased activities of citrate synthase (CS) and β-hydroxyacyl CoA dehydrogenase (HOAD) relative to terrestrial mammals. The increased Vv(mt) is thought to facilitate aerobic, metabolism under hypoxic diving conditions by decreasing the diffusion distance between mitochondria and both extra- and intra-cellular oxygen stores. Increased activities of CS and HOAD indicate greater aerobic capacity, with a significant contribution from fatty acid oxidation. These morphological and enzymatic adaptations maintain an aerobic, fat-based metabolism in muscles as oxygen partial pressure decreases during a dive and tissues become hypoxic (Davis and Kanatous, 1999).

Earlier studies of marine mammal muscle morphology and function have generally relied on single biopsies or spot samples from dead animals (Robinson, 1939; Ponganis and Pierce, 1978; Hochachka and Foreman, 1993; Reed et al., 1994; Butler and Jones, 1997; Kanatous et al., 1999). Mapping intramuscular enzyme activities and Mb concentration is a new approach for obtaining a better understanding of metabolic adaptations in the swimming muscles of marine mammals, but this approach requires a

high-density sampling regime in muscle cross-sections. Polasek and Davis (2001) used a mathematical mapping technique for contouring Mb concentration in the locomotory muscles of cetaceans. This method proved to be a successful technique for revealing heterogeneity within the muscle. In the present study, we used this approach to map the activities of CS, HOAD, and lactate dehydrogenase (LDH) and Mb concentration in transverse sections of the epaxial muscles (the primary locomotory muscles) of harbor seals. In addition, samples were analyzed from the hindlimb complex (locomotory muscle), the pectoralis (non-locomotory muscle) and the heart. For comparison, we analyzed comparable samples from the rat and dog. Our null hypothesis was that CS, HOAD, and LDH activities and Mb concentrations are homogenously distributed throughout the harbor seal muscles and that they do not differ significantly from terrestrial control species (dog and rat). Our alternative hypothesis was that regional heterogeneity reflects differences in the oxygen consumption and work performed by different areas of the muscle and that elevations in the aerobic muscle enzyme activities and Mb concentrations are adaptations that sustain aerobic, fat metabolism during dive-induced hypoxia.

Methods

Animals and Tissue Samples. Samples from the epaxial muscles, hindlimb complex, *pectoralis* muscle and heart were obtained from eight female and two male harbor seals during a native subsistence harvest in Prince William Sound, Alaska. Based on standard length (straight line distance from tip of nose to end of tail), the seals were sub-adults or adults (Pitcher and Calkins, 1979). All samples were taken within six hours of death. Two

locomotory muscles were sampled. The primary locomotory musculature lies along the vertebral column (Figure 1) and will be referred to as the epaxial muscles. The epaxial muscles on either side of the vertebral column alternately contract and relax to produce the lateral spinal flexions that generate thrust by the hind flippers during swimming. The entire epaxial musculature along one side of the spine was removed. After weighing the muscle, three transverse sections were taken in the cranial (CR), middle (MID) and caudal (CA) regions (Figure 1). The CR transverse section was taken at the 7th cervical vertebrae, MID was taken at the 14th thoracic vertebrae, and CA was from the lower lumbar region. Samples (ca. 0.5 g) were taken at points on a circular grid using a 6-mm stainless steel biopsy punch, averaging 20 samples per transverse section (Figure 2). A spot sample was taken from the hindlimb muscle complex, which also contributes to locomotion. By hindlimb muscle complex, we are referring to muscles on the dorsal surface of the femur that include, but are not exclusive of, the *gluteus maximus* and the *biceps femoris*. Single samples were taken from the *pectoralis* (a non-locomotory muscle) and the left ventricle of the heart. All samples were stored in liquid nitrogen until they were returned to Texas A&M University where they were stored at -70° C until analysis. Control samples were obtained from three male laboratory rats (Sprague-Dawley) and three female dogs sacrificed for research purposes at Texas A&M University. Samples were taken from the rat *soleus*, the dog *gastrocnemius*, and the rat and dog heart (left ventricle) and intercostal muscles. The rat *soleus* and dog *gastrocnemius* muscles contribute directly to locomotion, while the intercostal muscles contract during respiration. Tissue samples were taken in accordance with guidelines for the humane treatment of animals at Texas A&M University.

Enzyme Activities. Muscle samples were thawed, weighed and then homogenized cold in buffer containing 1 mmol·L^{-1} EDTA, 2 mmol·L^{-1} $MgCl_2$ and 50 mmol·L^{-1} imidazole at pH 7.6. In preliminary tests, we determined that sonication after homogenizing the sample was not necessary for complete enzyme extraction. Each homogenate was centrifuged for 4-5 min at 10,000 *g*, and the supernatant was used for the assays. Optimal in vitro activities were measured at 37°C under conditions of saturating substrate concentrations (determined prior to assays) for the following enzymes: Citrate synthase (CS), which catalyzes the first reaction in the citric acid cycle and is a measure of aerobic capacity; β-hydroxyacyl CoA dehydrogenase (HOAD), which catalyzes one reaction in the β-oxidation of fatty acids and is a measure of the capacity for fatty acid metabolism; lactate dehydrogenase (LDH), which catalyzes the conversion of pyruvate to lactate and is a measure of anaerobic glycolysis. All assays were performed using a SPECTRAmax® 340 microplate reader (Molecular Devices, Sunnyvale, CA). The assay conditions were as follows: Citrate synthase (EC 4.1.3.7): 0.5 mmol·L^{-1} oxaloacetate, 0.25 mmol·L^{-1} 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.4 mmol·L^{-1} acetyl CoA, and 50 mmol·L^{-1} imidazole, pH 7.5 at 37° C; ΔA_{412}, ϵ_{412}=13.6. β-hydroxyacyl CoA dehydrogenase (EC 1.1.1.35): 0.1 mmol·L^{-1} acetoacetyl CoA, 1 mmol·L^{-1} EDTA, 0.15 mmol·L^{-1} NADH, and 50 mmol·L^{-1} imidazole , pH 7.0 at 37° C; ΔA_{340}, ϵ_{340}=6.22. Lactate dehydrogenase (EC 1.1.1.27): 1 mmol·L^{-1} pyruvate, 0.3 mmol·L^{-1} NADH, and 50 mmol·L^{-1} imidazole, pH 7.0 at 37° C; ΔA_{340}, ϵ_{340}=6.22. Specific enzyme activities (μmol of substrate converted to product min^{-1} g^{-1} wet mass (IU)) were calculated from the rate of change of the assay absorbance on a linear slope for the duration of the reaction. Enzyme activity ratios (CS:HOAD, LDH:CS) were calculated to assess the relative importance of different

metabolic pathways in the muscle. The CS:HOAD ratio indicates the capacity of β-oxidation to generate acetyl-CoA relative to the capacity of the citric acid cycle to oxidize it, and the LDH:CS ratio indicates the relative glycolytic *vs.* aerobic metabolic capacity. Methods for enzyme analysis were adapted from Reed et al. (1994).

Myoglobin. Aliquots from the same supernatants used for enzyme assays were diluted with phosphate buffer (0.04 M, pH 6.6), and the resulting mixture was centrifuged for 50 min at 28,000 *g* at 4° C. The method of Reynafarje (1963) was used to determine myoglobin concentration. The supernatant was bubbled with 99.9% carbon monoxide (CO) for 5 min to convert the myoglobin to carboxymyoglobin (Polasek and Davis, 2001). After bubbling, the absorbance of the supernatant at 538 and 568 nm was measured using a Bio-Tek PowerWave 340x microplate reader. A myoglobin standard (horse myoglobin, Sigma-Aldrich, St. Louis, MO) was run with each set of samples. The myoglobin concentration was calculated as described by Reynafarje (1963) and expressed in mg g^{-1} of wet mass tissue.

Contours and Statistics. Statistical comparisons were made within each transverse section of the seal epaxial muscles, and among different muscles within the seals. Using the controls, interspecific comparisons were made between muscles with similar function (e.g., locomotory and non-locomotory). In addition, enzyme activities were scaled to each animal's calculated mass-specific resting metabolic rate (RMR), estimated as 70 $M_b^{-0.25}$ (Schmidt-Nielson, 1995), where M_b is the body mass of the animal. Scaling to mass-specific RMR was necessary to compensate for differences in the size of the seals and

control animals. Small animals, such as the rat, have a much higher mass-specific RMR than do larger animals such as the seal and dog. Analysis of variance was utilized for all statistical comparisons using InStat 3 (GraphPad Software, San Diego, CA) and SYSTAT 10 (SPSS Inc., Chicago, IL). Contour maps of enzyme activities and myoglobin concentration within the epaxial muscle transverse sections were made using Surfer (Golden Software, Inc., Golden, CO). Kriging was used to contour the data because it generates the best interpretation of medium to small data sets (Keckler, 1997). Values stated in the text are mean ± SD unless otherwise noted.

Results

Morphometrics. The harbor seals used in this study had a mean body mass of 46.1 ± 4.3 kg and a standard length of 126.2 ± 5.3 cm (Table 1). The epaxial muscles were 6.1% ± 0.4 of total body mass and 41.7% ± 0.9 of the standard length. The epaxial muscles are approximately circular in shape at their origin along the cervical vertebrae, but extend ventrally along the thoracic vertebrae to cover part of the rib cage before tapering back to a circular shape along the lumbar vertebrae (Figure 1). The muscle is dark red in color due to its high myoglobin content.

Enzyme Activities. We tested for the homogeneity of enzyme activities within each transverse section of the epaxial muscles using three comparisons. Due to variations in sizes of the seals and the epaxial muscles, tissue cores within each of the transverse sections were not taken at identical locations among animals. Clustering data was the best way to determine statistical variation with in the muscle. First, each section was divided

into roughly four equal quadrants (dorsal, ventral, right, and left) (Figure 3), and mean enzyme activities were calculated for each quadrant. There were no significant differences among the quadrants within any of the transverse sections for CS, HOAD or LDH activities (Figure 4). Second, each transverse section was divided into four equal quadrants along a vertical and horizontal plane (dorsal right, dorsal left, ventral right, and ventral left). Again, there were no significant differences among the quadrants within any of the transverse sections for the three enzymes. Finally, the transverse sections were divided into thirds along the horizontal axis, and no significant differences were found among the three regions. Although heterogeneity within the transverse sections of the epaxial muscles was visually apparent for the activities of CS, HOAD and LDH, no statistically significant pattern was detected because of inter-animal variation. As a result, mean enzyme activities were calculated for each transverse section.

There were significant differences in CS, HOAD and LDH activities along the length of the epaxial muscles. The mean CS and LDH activities in the middle (MID) (34.8 ± 0.8 and 1111.6 ± 34.4 IU, respectively) and caudal (CA) sections (34.8 ± 0.7 and 1027.3 ± 30.8 IU, respectively) were significantly greater ($p < 0.001$) than the CS and LDH activities (29.1 ± 1.1 IU and 868.8 ± 46.5 IU, respectively) in the cranial (CR) section (Table 2). HOAD activity showed no significant differences among sections (CR 26.4 ± 1.5, MID 26.4 ± 0.8, and CA 28.0 ± 0.8 IU). The CS:HOAD ratio (1.1 to 1.3) and LDH:CS ratio (29.5 to 31.9) exhibited no significant differences along the length of the epaxial muscles (Table 2).

Mean enzyme activities for the entire epaxial musculature were compared with activities in the hindlimb complex, the *pectoralis* and heart in the harbor seals (Table 3). For CS activity, the heart was the only tissue that was significantly different ($p<0.01$). The

heart had 2.6-3.0 times higher CS activity than did the epaxial muscles, hindlimb and *pectoralis*. HOAD activities in the hindlimb complex and *pectoralis* were significantly less (ca. 31%) than in the epaxial muscles ($p<0.001$), but the heart and epaxial muscle HOAD activities did not differ significantly. LDH activity was significantly lower in the seal heart than in the epaxial muscles ($p<0.01$). There were no significant differences in LDH activities among the other tissues. The CS:HOAD ratio in the epaxial muscles was significantly less than in the other muscles and heart in the harbor seal ($p<0.001$) (Table 3), indicating a high potential for oxidative fat metabolism. For the locomotory muscles, the harbor seal heart had a significantly lower LDH:CS ratio than was found in the epaxial muscles. No significant difference was seen in the LDH:CS ratios among the other muscles in the harbor seal.

Interspecific comparisons were made for muscles of similar function (Table 3). There was significantly higher CS activity in the seal epaxial muscles than in the rat *soleus* ($p< 0.01$), but not in the *gastrocnemius* of the dog. Both the seal and the dog non-locomotory muscles had higher CS activity than did the non-locomotory muscles in the rat ($p<0.01$). The rat heart had significantly higher CS activity than did the seal and dog heart ($p<0.01$). The seal had significantly higher HOAD activity in the epaxial muscles than the locomotory muscles in the dog and rat, and higher HOAD activity in the non-locomotory muscle than in the rat ($p<0.01$). However, HOAD activity in the locomotory hindlimb complex of the seal was not significantly different from the locomotory muscles of the dog and rat. All three species had similar HOAD activity in the heart. The seal had higher LDH activity than the dog and rat in both the locomotory and non-locomotory muscles

($p<0.01$). The seal and dog hearts had significantly lower LDH activities than did the rat heart ($p<0.01$).

The CS:HOAD ratio was significantly lower in the seal epaxial muscles than the locomotory muscles of the dog and rat ($p<0.01$) (Table 3). The CS:HOAD ratio was also lower in the hindlimb complex of the seal than the *gastrocnemius* in the dog ($p<0.01$), but did not differ significantly than the *soleus* in the rat (Table 3). The CS:HOAD ratio was significantly higher in the rat non-locomotory muscles than the seal pectoralis, but no difference was seen between the seal and dog non-locomotory muscles ($p<0.01$). No significant difference was seen in the CS:HOAD ratios for the heart of the three species. The seal locomotory and non-locomotory muscles had significantly higher LDH:CS ratios than did those of the dog ($p<0.01$) (Table 3). The seal and rat non-locomotory muscles had significantly higher LDH:CS ratios than the dog non-locomotory muscles. No significant difference was seen in the LDH:CS ratios for the heart of the three species.

To adjust for allometric differences due to body mass, we scaled the enzyme activities to the calculated RMR for each species (Table 4; Figures 5, 6, and 7). The seal and dog locomotory and non-locomotory muscles had significantly higher CS:RMR ratios (ca. 5.6x) than did those of the rat ($p<0.001$). There were no significant interspecific differences in the CS:RMR ratios for the heart. The seal had a significantly higher (ca. 5-25x) HOAD:RMR ratio than the rat in both locomotory muscles, but only the seal epaxial muscles had a higher ratio (ca. 10x) than the dog locomotory muscles ($p<0.001$). The seal non-locomotory muscles had a significantly higher HOAD:RMR ratio than did those of the rat ($p<0.001$). No significant interspecific differences were seen in the HOAD:RMR ratio

for the heart. The seal had significantly higher LDH:RMR ratios than did the dog and rat for all muscles (ca. 3-6x) and the heart (ca. 1.6x) ($p<0.001$).

Myoglobin Concentration. Similar to the enzyme activities, there was no statistically significant difference in the distribution of myoglobin concentrations within each of the transverse sections of the seal epaxial muscles (Figure 8), but there were significant differences along the length of the epaxial muscles. The mean myoglobin concentrations for the MID (38.0±0.9 mg g^{-1}) and CA (38.0±0.9 mg g^{-1}) sections were significantly greater ($p=0.001$) than that of the CR section (31.4±0.8 mg g^{-1}) (Table 3). The epaxial muscles had a significantly higher Mb concentration ($p<0.001$) than did the hindlimb complex and the *pectoralis* (37.0 ± 1.1, 30.5 ±1.4, and 26.2 ± 2.4 mg g^{-1}, respectively). Average myoglobin concentrations for all seal muscles were more than 20x those measured in the dog and rat muscles (Table 3). The seal heart myoglobin concentration (18.4±2.2 mg g^{-1}) was 10x greater than that in the dog (1.8±0.3 mg g^{-1}) and rat heart (1.7±0.1 mg g^{-1}).

Discussion

No consistent patterns of distribution were seen in the activities of CS, HOAD and LDH and the concentration of Mb within the cranial, middle and caudal transverse sections of the seal epaxial muscles. These results are consistent with the homogenous distribution of slow (Type I) and fast (Type IIa) twitch aerobic fibers within the respective transverse sections of the epaxial muscles (Watson, unpublished observations). In contrast, there were significant differences in enzyme activities and Mb concentration among the transverse sections of the epaxial muscles that appear to be correlated with power production during swimming. Seals generate thrust with their hind flippers by contralateral contraction of the epaxial muscles that cause lateral flexions in the posterior half of their body (Fish et al., 1988). Since the cranial section of the epaxial muscles is furthest from the hind flippers, the lower CS and LDH activities and myoglobin concentration in this region may reflect a smaller contribution to the generation of thrust during submerged swimming. In a study of four species of dolphins, Polasek and Davis (2001) found that the highest concentrations of Mb were located in the caudal region of the epaxial muscles where most of the thrust is generated during swimming (Fish, 1993; Pabst, 1993). However, the gradient of Mb concentration from head-to-tail was not as pronounced in the harbor seal as in the faster swimming dolphins. It appears that the capacity for aerobic metabolism, oxygen storage, and glycolytic metabolism in the epaxial muscles reflects their contribution to thrust generation during submerged swimming.

Comparisons were also made among the epaxial muscles, the hindlimb complex and *pectoralis* muscle of the harbor seals to test for differences between locomotor and non-locomotor muscles. Although there was no significant difference in CS and LDH

activities, HOAD activity and Mb concentration were significantly higher in the epaxial muscles than in the hindlimb complex and the pectoralis muscle. Kanatous et al. (1999) observed similar results for the epaxial and *pectoralis* muscles in the harbor seal. In addition, the harbor seal heart had higher CS activity and lower LDH activity than the epaxial muscles, reflecting a higher aerobic potential. It appears that the aerobic and anaerobic capacities (as measured by CS and LDH activities, respectively) of the epaxial and *pectoralis* muscles are not significantly different, reflecting cost efficient modes of locomotion and a similar degree of adaptation to hypoxia (Kanatous et al., 1999; Williams et al., 2000; Davis et al., 2001). However, there is a higher capacity to provide acetyl CoA from fatty acids and to store oxygen in the in the epaxial muscles than in the pectoralis and muscles of the hindlimb, which we hypothesize reflects their greater requirements for oxygen and fuel during swimming.

After scaling for RMR, we found no significant difference between the CS:RMR ratio in the seal epaxial muscles and the dog *gastrocnemius* muscle, but both were significantly greater than in the rat *soleus* muscle. An enhanced oxidative capacity has been observed in animal athletes such as dogs and in high altitude adapted and acclimated mammals (Reynafarje, 1962; Gollnick et al., 1972; Jansson and Kayser, 1977; Hochachka et al., 1982; León-Velarde, 1993; Brooks, 1998). Kanatous et al. (1999) showed an increased mitochondrial volume density and a more homogeneous distribution of mitochondria in the muscles of harbor seals, which reduces the distance for the intracellular diffusion of oxygen among mitochondria and enhances the diffusive flux of oxygen. This, along with the significant increase in Mb concentration, sustains aerobic metabolism in the muscle

during diving as the arterial partial pressure of oxygen declines to as low as 22 torr (Qvist et al., 1981; Davis and Kanatous, 1999).

The HOAD:RMR ratio in the seal epaxial muscles was 10x and 20x greater than in the dog and rat skeletal muscle, respectively, indicating that seal muscle has a much higher capacity to oxidize lipid as a source of energy. This is further supported by a CS:HOAD ratio of 1.2 in the seal epaxial muscles, while the ratio in the dog and rat were 7.2 and 4.2, respectively. Similar results have been found for harbor seals and other pinnipeds (Reed et al., 1994). Moreover, metabolic studies on seals have measured respiratory exchange ratios (CO_2 produced/O_2 consumed) of 0.75 at rest and 0.71 during exercise, which also indicates that fat is the main source of fuel (Davis et al., 1991). This dependence on lipid as an energy source in seals is most likely related to their diet, which is rich in fat and protein, but contains little carbohydrate (Kettelhut et al., 1980; Davis et al., 1991; Roberts et al., 1996). Studies of terrestrial mammals have shown that high-fat, low-carbohydrate diets increase the rate of lipid oxidation (Roberts et al., 1996) and that this is accompanied by an increase in the concentration of enzymes involved in fatty acid oxidation (Gollnick and Saltin, 1988; Roberts et al., 1996). A greater reliance on fatty acid oxidation within the locomotory muscles would also spare glucose for red blood cells and the central nervous system, which are obligate glucose metabolizers. Similar adaptations that spare glycogen and enhance lipid oxidation are seen in endurance trained terrestrial mammals (Brooks, 1998).

No significant difference was found in the LDH:CS ratios among the harbor seal skeletal muscles. Thus skeletal muscle appears to have similar anaerobic capabilities. The heart had a lower LDH:CS ratio than the skeletal muscle in the seal, reflecting the higher

aerobic potential of the heart. LDH activities were greater in the non-locomotory muscles of the seal in comparison to the dog. The elevated levels in the seal muscle indicate an adaptation to hypoxia. This is further supported by the higher LDH:CS ratio in all tissues of the seal compared to the dog. The harbor seal non-locomotory muscle enzyme activities, when scaled to RMR, were greater than that of the rat for all three enzymes. The only difference seen in the enzyme activity of heart for the seal, dog, and rat was in LDH levels. The heart of the seal had greater LDH activity than both the dog and rat when scaled to RMR. This elevated LDH activity suggests the capacity of the seal heart to undergo anaerobic glycolysis if necessary.

In conclusion, the elevated enzyme activities and Mb concentrations seen in the middle and caudal regions of the seal epaxial muscles reflect differences in the oxygen consumption and work performed by the locomotory muscles. Overall, enzyme activities and Mb concentrations are homogeneously distributed within the transverse sections. Similar to endurance trained athletic animals, harbor seals have high oxidative enzyme activities that are an indicator of enhanced aerobic capacity. The epaxial muscles and heart of the harbor seal have extremely high HOAD:RMR ratios and low CS:HOAD ratios which, taken together, indicate an increased capacity for aerobic lipid metabolism during diving. The elevated levels of LDH:RMR reflect an adaptation to hypoxia not formerly detected by non-scaled LDH activity.

Literature Cited

Brooks, G. A. (1998). Mammalian fuel utilization during sustained exercise. *Comp. Biochem. Physio. B.* 120, 89-170.

Butler, P. J. and Jones, D. R. (1997). Physiology of diving birds and mammals. *Physiol. Rev.* 77, 837-899.

Davis, R. W., Castellini, M. A., Williams, T. M., and Kooyman, G. L. (1991). Fuel homeostasis in the harbor seal during submerged swimming. *J. Comp. Physiol. B.* 160, 627-635.

Davis, R. W. and Kanatous, S. B. (1999). Convective oxygen transport and tissue oxygen consumption in Weddell seals during aerobic dives. *J. Exp. Biol.* 202, 1091-1113.

Davis, R. W., Fuiman, L. A., Williams, T. M., Le Boeuf, B. J. (2001). Three-dimensional movements and swimming activity of a northern elephant seal. *Comp. Biochem. and Physiol. Part A.* 129, 759-770.

Fish, F. E., Innes, S., and Ronald, K. (1988). Kinematics and estimated thrust production of swimming harp and ringed seals. *J. Exp. Bio*l. 137, 157-173.

Fish, F. E. (1993). Power output and propulsive efficiency of swimming bottlenose dolphins (*Tursiops truncates*). *J. Exp. Bio*l. 185, 179-193.

Gollnick, P. D., Armstrong, R. B., Saltin, B. (1972). Enzyme activity and fiber composition in skeletal muscle of untrained and trained men. *J. Appl. Physiol.* 33(3), 312-319.

Gollnick, P. D. and Saltin, B. (1988). Significance of skeletal muscle oxidative enzymes enhancement with endurance training. *Clin. Physiol*. 2, 1-12.

Guyton, G. P., Stanek, K. S., Schneider, R. C., Hochachka, P. W., Huford, W. E., Zapol, D. G., Liggins, G. C., and Zapol, W. M. (1995). Myoglobin saturation in free-diving Weddell seals. *J. Appl. Physiol.* 79(4), 1148-1155.

Hochachka, P. W., Stanley, C., Merkt, J., and Sumar-Kalinowski, J. (1982). Metabolic meaning of elevated levels of oxidative enzymes in high altitude adapted animals: an interpretive hypothesis. *Respir. Physiol.* 52, 303-313.

Hochachka, P. W. and Foreman III, R. A. (1993). Phocid and cetacean blueprints of muscle metabolism. *Can. J. Zool.* 71, 2089-2098.

Jansson, E. and Kaijser, L. (1977). Muscle adaptation to extreme endurance training in man. *Acta Physiol. Scand.* 100, 315-324.

Kanatous, S. B., DiMichele, L. V., Cowan, D. F. and Davis, R. W. (1999). High aerobic capacities in the skeletal muscles of pinnipeds: adaptations to diving hypoxia. *J. Appl. Physiol*. 86(4), 1247-1256.

Keckler, D. (1997). *Surfer for Windows*. Colorado: Golden Software, Inc. 483pp.

Kettelhut, I. C., Foss, M. C., and Migliorini, R. H. (1980). Glucose homeostasis in a carnivorous animal (cat) and in rats fed high-protein diet. *Am. J. Physio.* 239(5), R437-444.

Kooyman, G. L., Castellini, M. A., Davis, R. W., and Maue, R.A. (1983). Aerobic dive limits of immature Weddell seals. J. *Comp. Physiol. [B]* 151, 171-174.

León-Velarde, F., Sanchez, J., Bigard, A. X., Brunet, A., Lesty, C., and Monge-C, C. (1993). High altitude tissue adaptation in Andean coots: capillarity, fibre area, fibre type and enzymatic activities of skeletal muscle. *J. Comp. Physiol. B.* 163, 52-58.

Pabst, D. A. (1993). Intramuscular morphology and tendon geometry of the epaxial swimming muscles of dolphins. *J. Zoo., Lond.* 230, 159-176.

Pitcher, K. W. and Calkins, D. (1979). Biology of the harbor seal, *Phoca vitulina richardsi*, in the Gulf of Alaska. U.S. Dep. Commer. NOAA, OCSEAP Final Rep. 19:231-310.

Polasek, L. and Davis, R. W. (2001). Heterogeneity of myoglobin distribution in the locomotory muscles of five cetacean species. *J. Exp. Biol.* 204, 209-215.

Ponganis, P. J. and Pierce, R. W. (1978). Muscle metabolic profiles and fiber type composition in some marine mammals. Comp. Biochem. Physiol. 59B, 99-102.

Qvist, J., Weber, R. E., Zapol, W. M. (1981). Oxygen equilibrium properties of blood and hemoglobin of fetal and adult Weddell seals. *J. Appl. Physiol.* 50(5), 999-1005.

Reed, J. Z., Butler, P. J. and Fedak, M. A. (1994). The metabolic characteristics of the locomotory muscles of grey seals (*Halichoerus grypus*), harbour seals (*Phoca vitulina*) and Antarctic fur seals (*Arctocephalus gazella*). *J. Exp. Biol*. 194, 33-46.

Reynafarje, B. (1962). Myoglobin content and enzymatic activity of muscle and altitude adaptation. *J. Appl. Physiol.* 17(2), 301-305.

Reynafarje, B. (1963). Simplified method for the determination of myoglobin. *J. Lab. Clin. Med*. 61, 138-145.

Roberts, A. C., Butterfield, G. E., Cymerman, A. Reeves, J. T., Wolfel, E. E. (1996). Acclimatization to 4,300-m altitude decreases reliance on fat as a substrate. *J. Appl. Physiol.* 81(4), 1762-1771.

Robinson, D. (1939). The muscle hemoglobin of seals as an oxygen store in diving. *Science.* 90, 276-277.

Schmidt-Nielsen, K. (1995). *Animal Physiology: Adaptation and Environment.* Cambridge: Cambridge University Press. 602pp.

Whittenberg, B. A. and Whittenberg, J. B. (1989). Transport of oxygen in muscle. *Annu. Rev. Physiol.* 51, 857-878.

Williams, T. M., Davis, R. W., Fuiman, L., Francis, J., Le Boeuf, B. J., Horning, M., Calambokidis, J. and Croll, D. A. (2000). Sink or swim: Strategies for cost-efficient diving by marine mammals. *Science.* 288, 133-136.

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Figure 8. Contours of myoglobin concentration ($mg \cdot g^{-1}$) from one harbor seal). CR, MID and CA indicate cranial, middle and caudal transverse sections. The orientations of the contours are from the head looking towards the tail.

Table 1. Gender and morphometrics of the harbor seals used in this study.

Seal	Sex	Mass (kg)	Standard Length (cm)	Mass of Epaxial * (kg)	% of Body	Length of Epaxial (cm)	% Body Length of Epaxial
1	F	30.0	104.0	1.4	4.7%	48.0	46.2%
2	F	69.9	153.0	4.6	6.6%	61.0	39.9%
3	F	34.1	115.0	1.6	4.7%	45.8	39.8%
4	M	53.1	135.0	4.4	8.3%	60.0	44.4%
5	F	43.6	129.0	3.0	6.9%	53.5	41.5%
6	F	48.7	132.5	3.0	6.2%	56.5	42.6%
7	F	63.9	150.0	4.0	6.3%	57.0	38.0%
8	M	31.9	111.5	2.2	6.9%	50.7	45.5%
9	F	48.6	122.0	2.8	5.8%	48.0	39.3%
10	F	37.0	109.5	1.8	4.9%	44.0	40.2%
Mean		46.1 ± 4.3	126.15 ± 5.3	2.88 ± 0.4	6.1% ± 0.4	52.5 ± 1.9	41.7% ± 0.9

* The epaxial muscles were only removed from one side of the seal. Values were doubled to determine the mass of the entire epaxial complex. Mean values include ± SE.

Table 2. Mean enzyme activities (µmol substrate converted to product $min^{-1} \cdot g^{-1}$tissue wet weight), Mb concentration ($mg \cdot g^{-1}$) and enzyme ratios for the three transverse muscle sections from 10 harbor seals.

Epaxial Muscles	**CS**	**HOAD**	**LDH**	**Mb**	**CS:HOAD**	**LDH:CS**
Cranial (CR)	29.1 ± 1.1	26.4 ± 1.5	868.8 ± 46.5	31.4 ± 0.8	1.1 ± 0.01	29.9 ± 2.4
Middle (MID)	34.8 ± 0.8	26.4 ± 0.8	1111.6 ± 34.4	38.0 ± 0.9	1.3 ± 0.08	31.9 ± 1.7
Caudal (CA)	34.8 ± 0.7	28.0 ± 0.8	1027.3 ± 30.8	38.0 ± 0.5	1.2 ± 0.05	29.5 ± 2.0
	MID, CA>CR*		MID, CA>CR*	MID, CA>CR*		

Values are means± SE. * Significantly different ($p<0.01$).

Table 3. Mean (± SE) enzyme activities (µmol substrate converted to product $min^{-1} \cdot g^{1}$tissue wet weight), Mb concentration ($mg \cdot g^{-1}$), and enzyme ratios for the harbor seal, rat, and dog.

Species	***n***	**Muscle**	**CS**	**HOAD**	**LDH**	**Mb**	**CS: HOAD**	**LDH: CS**
Harbor seal	10	Locomotory (Epaxial)	32.9 ± 1.9 ‡	26.9 ± 0.5 † ‡	1002 ± 71 † ‡	37.0 ± 1.1 † ‡	1.2 ± 0.06 † ‡	30.5 ± 2.1 †
(Phoca vitulina)		Locomotory (Hindlimb)	28.5 ± 3.3	8.0 ± 1.3 §	986 ± 90 † ‡	30.5 ± 1.4 § † ‡	3.6 ± 0.5 § †	34.6 ± 4.0 †
		Non-Locomotory (Pectoralis)	30.1 ± 2.3 ‡	8.6 ± 1.2 § ‡	1075 ± 105 † ‡	26.2 ± 2.4 § † ‡	3.5 ± 0.8 § ‡	35.7 ± 1.4 †
		Heart	86.1 ± 11.7 § ‡	28.5 ± 5.6	737 ± 56 § ‡	18.4 ± 2.2 § † ‡	3.0 ± 0.8 §	8.6 ± 4.0 §
Dog	3	Locomotory (Gastrocnemius)	43.3 ± 8.0	6.0 ± 0.2 *	480 ± 27 *	1.5 ± 0.02 *	7.2 ± 2.3 *	11.1 ± 4.9 *
(Canis familiaris)		Non-Locomotory (Intercostals)	47.4 ± 5.4 ‡	6.5 ± 0.04	416 ± 9 *	1.6 ± 0.1 *	7.2 ± 1.7	9.5 ± 2.8 * ‡
		Heart	98.6 ± 9.1 ‡	24.9 ± 1.8	646 ± 92 ‡	1.8 ± 0.3 *	4.0 ± 1.1	6.6 ± 0.9
Rat	3	Locomotory (Soleus)	19.5 ± 2.0 *	4.4 ± 0.6 *	437 ± 47 *	1.5 ± 0.2 *	4.4 ± 0.9 *	22.4 ± 6.8
(Rattus norvegicus)		Non-Locomotory (Intercostal)	14.8 ± 2.1 * †	1.0 ± 0.2 *	655 ± 39 *	1.4 ± 0.02 *	14.4 ± 6.2 *	44.3 ± 20.2 †
Strain: Sprague-Dawley		Heart	193.3 ± 7.7 * †	27.7 ± 3.9	1347 ± 0.2 * †	1.7 ± 0.1 *	7.0 ± 1.3	7.0 ± 0.5

Values are means ± SE. *n*, no. of animals. § Significantly different from the harbor seal epaxial muscle ($p<0.001$). Statistical comparisons were made between similar muscle groups of the three species. * Significantly different from seal ($p<0.01$). For locomotory muscles * denotes significance from the epaxial muscles and denotes difference from the hindlimb. † Significantly different from dog ($p<0.01$). ‡ Significantly different from rat ($p<0.01$).

Table 4. Citrate synthase (CS:RMR), β-hydroxyacyl-CoA dehydrogenase (HOAD:RMR), and lactate dehydrogenase (LDH:RMR) activities scaled to mass-specific resting metabolic rate from muscle tissues of the harbor seal, dog, and rat

Species	*n*	Mass (kg)	Muscle	Estimated mass-specific metabolic rate (Watts·kg^{-1})	CS:RMR	HOA:RMR	LDH:RMR
Harbor seals	10	46.1 ± 4.3	Locomotory (epaxial)	27.2 ± 0.6	0.06 ± 0.004 ‡	0.05 ± 0.005 † ‡	1.8 ± 0.1 † ‡
(Phoca vitulina)			Locomotory (hindlimb)		0.05 ± 0.02 ‡	0.01 ± 0.002 ‡	1.7 ± 0.2 † ‡
			Non-Locomotory (pectoralis)		0.05 ± 0.02 ‡	0.01 ± 0.002 ‡	1.9 ± 0.1 † ‡
			Heart		0.15 ± 0.004	0.05 ± 0.009	1.3 ± 0.1 † ‡
Dogs	3	9.2 ± 0.5	Locomotory (gastrocnemius)	40.2 ± 0.6	0.05 ± 0.01 ‡	0.005 ± 0.001 *	0.6 ± 0.05 *
(Canis familiaris)			Non-Locomotory (intercostals)		0.05 ± 0.01 ‡	0.005 ± 0.001	0.5 ± 0.8 *
			Heart		0.11 ± 0.01	0.03 ± 0.005	0.8 ± 0.01 *
Rat	3	0.48 ± 0.03	Locomotory (soleus)	83.9 ± 1.3	0.009 ± 0.001 † *	0.002 ± 0.005 *	0.3 ± 0.04 *
(Rattus norvegicus)			Non-Locomotory (intercostal)		0.009 ± 0.001 † *	0.005 ± 0.009 *	0.4 ± 0.04 *
Strain: Sprague-Dawley			Heart		0.10 ± 0.009	0.015 ± 0.003	0.8 ± 0.05 *

Values are expressed as mean ± SE. *n*, no. of animals. Statistical comparisons were made between similar muscle groups of the three species. * Significantly different from seal ($p<0.001$). † Significantly different from dog ($p<0.001$). ‡ Significantly different from rat ($p<0.001$).

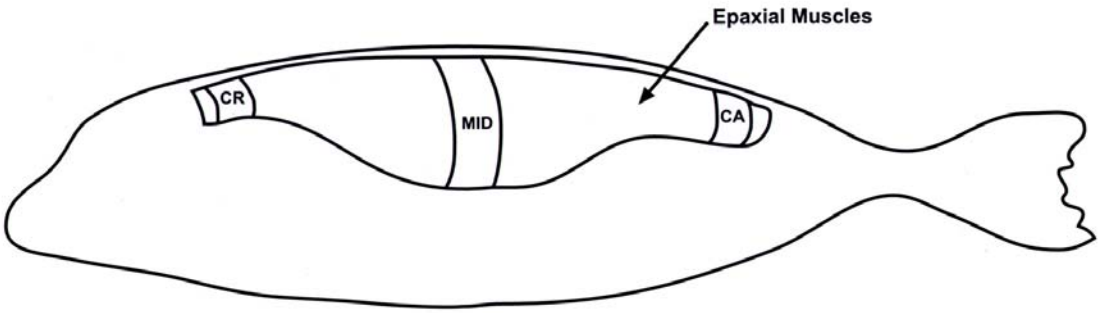


Figure 1. Schematic representation showing the location of the three transverse sections taken from the epaxial muscles. CR, MID and CA indicate the cranial, middle and caudal transverse sections, respectively.

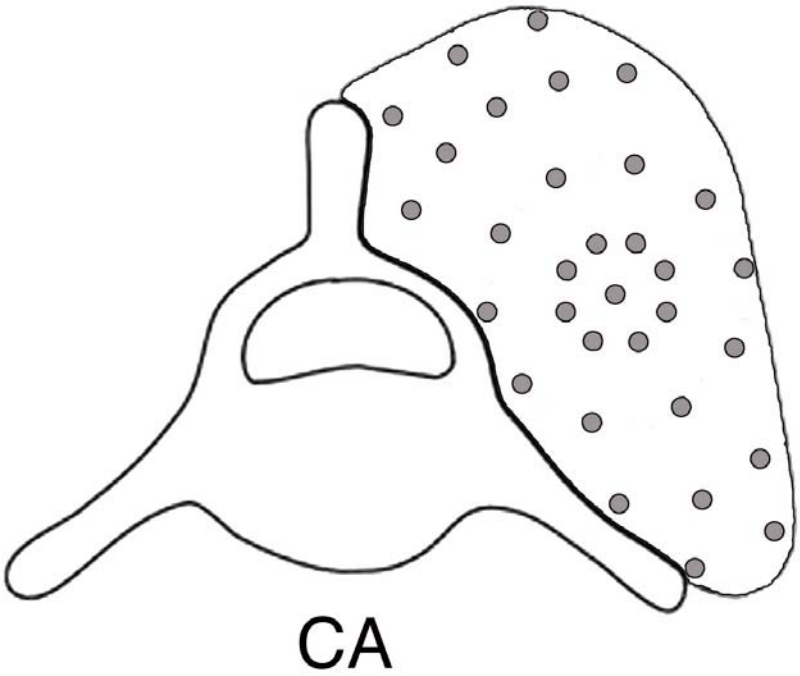
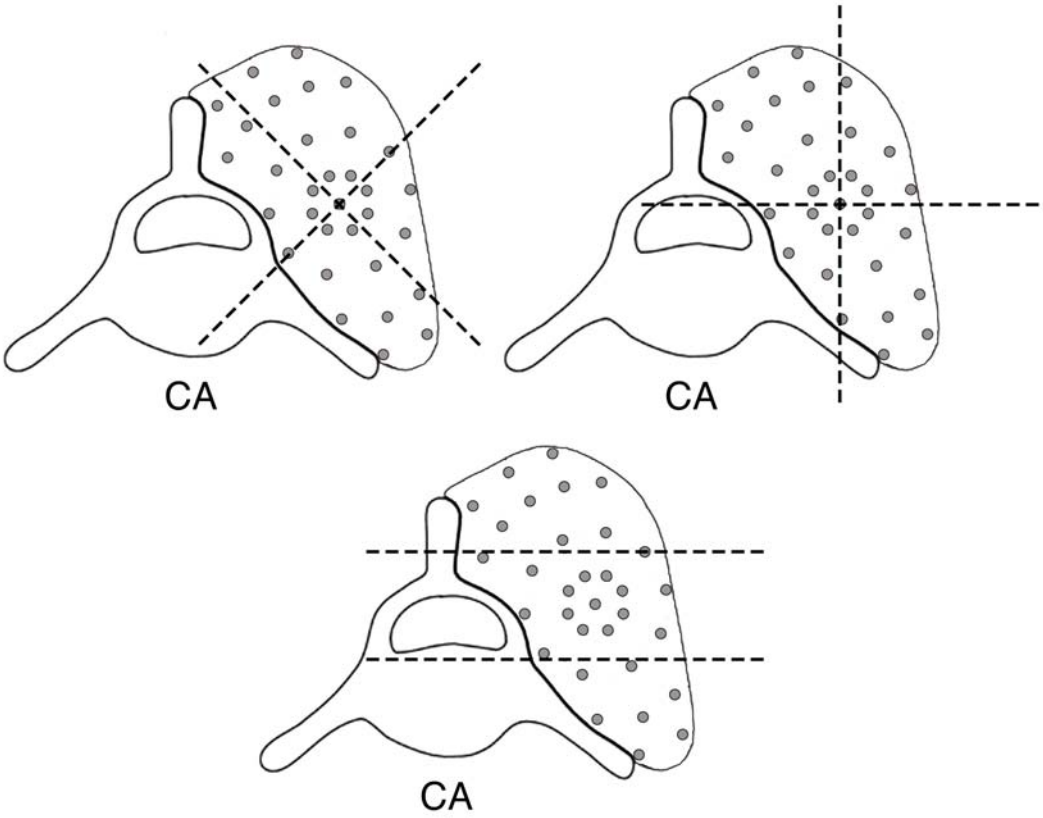


Figure 2. Diagrammatic representation of a transverse section through the vertebra and epaxial muscles showing the coring pattern for samples. CA indicates a caudal transverse section.



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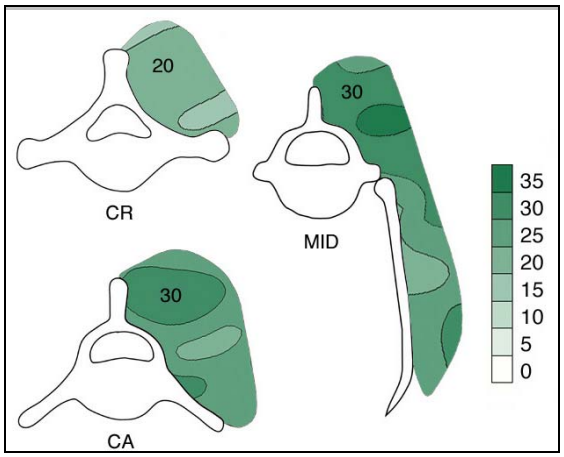

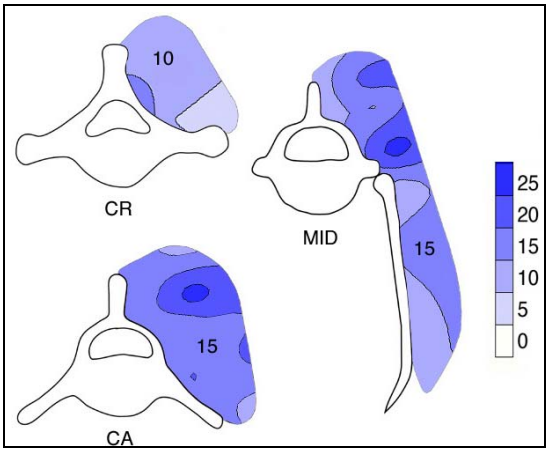

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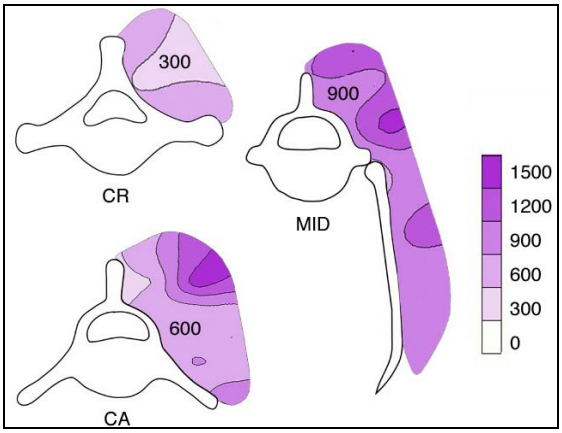

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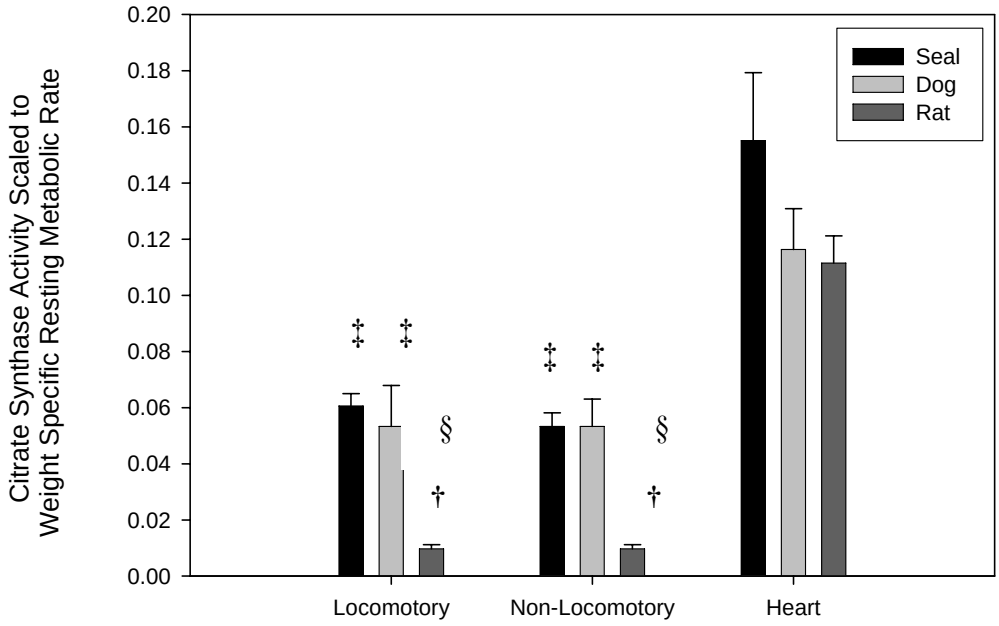
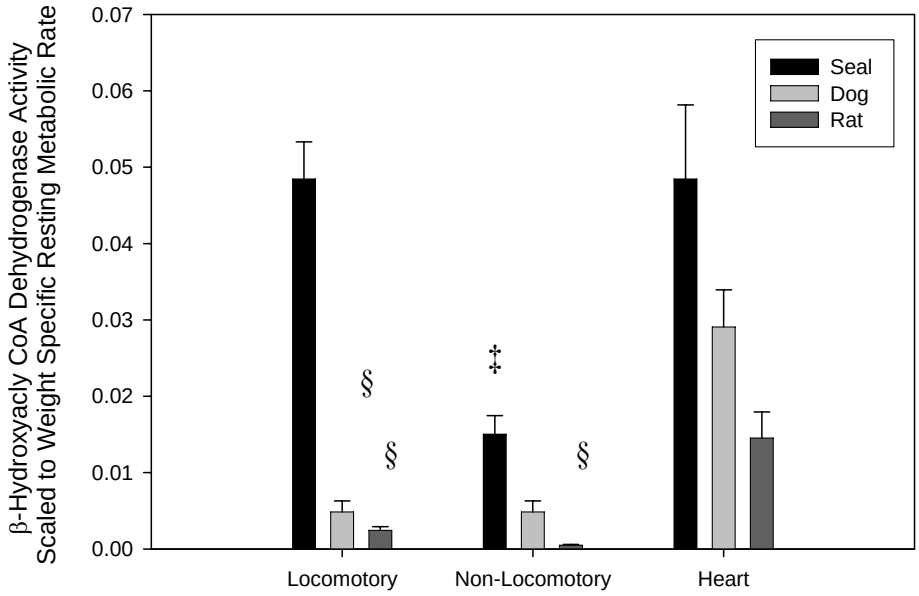


Figure 5: Citrate synthase activity scaled to mass-specific resting metabolic rate for harbor seals, dogs and rats. The epaxial, *gastrocnemius*, and *soleus* are the locomotory muscles for the seal, dog and rat, respectively. The non-locomotory muscle for the seal is the *pectoralis* and the intercostal for the dog and rat. § Significantly different from seal ($p<0.001$). † Significantly different from dog ($p<0.001$). ‡ Significantly different from rat ($p<0.001$).



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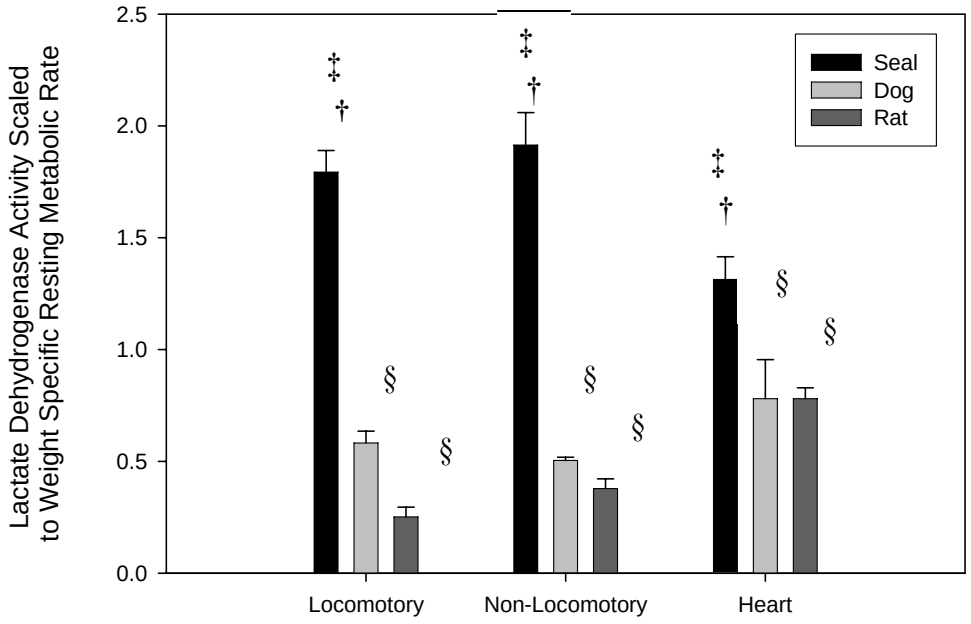

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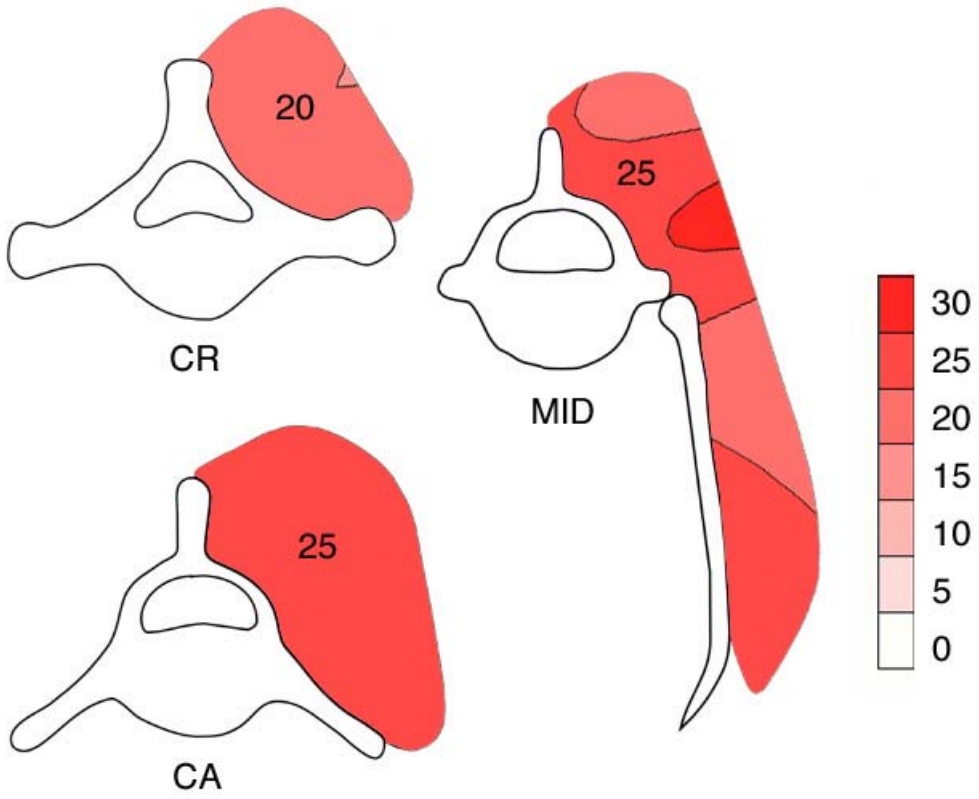

Figure 8. Contours of myoglobin concentration ($mg \cdot g^{-1}$) from one harbor seal). CR, MID and CA indicate cranial, middle and caudal transverse sections. The orientations of the contours are from the head looking towards the tail.

Immunohistochemical fiber typing of the locomotory muscles of harbor seals (*Phoca vitulina*)

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Summary

In seals, sea lions, fur seals (pinnipeds), the energy to power skeletal muscle contractions during subsurface swimming is derived primarily from aerobic metabolism. Initial studies in our laboratory using muscle biopsies showed that the swimming muscles in these pinnipeds are well adapted to localized hypoxia and ischemia experienced during diving. The objective of this study was to determine if the physiological indices of aerobic metabolism are consistent throughout the primary locomotory muscles of the harbor seal (*Phoca vitulina*). Results from fiber typing indicated that harbor seal swimming muscle (epaxial muscle) is comprised of 46.9% type I (slow twitch, oxidative) fibers and 53.0%, IIa (fast twitch, oxidative) fibers, which are homogeneously distributed throughout the muscle. No fast twitch, glycolytic (type IIb) fibers were detected in contrast to the published data on fiber typing of harbor seal epaxial muscle using traditional histochemical techniques. The extreme specificity inherent to the immunohistochemical fiber-typing procedure leads us to conclude that harbor seal swimming muscle is entirely comprised of oxidative fibers.

Introduction

The skeletal muscles of marine mammals appear to be well-adapted for maintaining aerobic metabolism under the hypoxic conditions that occur during diving. In previous studies, investigators reported high mitochondrial volume densities and elevated enzyme activities that support an aerobic, lipid-based metabolism in the skeletal muscles of some pinnipeds (Family Carnivora, Subfamily Pinnipedia: seals, sea lions and walrus) (Hochachka and Foreman, 1993; Reed et al., 1994; Kanatous et al., 1999). Myoglobin concentrations in the skeletal muscles of both cetaceans (Family Cetacea: whales and dolphins) and pinnipeds are elevated 10-20x compared to terrestrial mammals and provide an endogenous source of oxygen during dives (Cherepanova et al., 1993; Kanatous et al., 1999; Polasek and Davis, 2001). The high mitochondrial volume densities and myoglobin concentrations enhance the intracellular diffusion of oxygen into mitochondria under low oxygen partial pressure (Kanatous et al., 1999). In contrast, there appears to be little enhancement of glycolytic enzyme activities in pinniped skeletal muscle and other organs compared to terrestrial mammals (Castellini et al., 1981). These observations are in agreement with the well-accepted theory that marine mammals maintain aerobic metabolism during most voluntary dives (Kooyman et al., 1983; Davis, 1983; Thompson and Fedak, 1993; Butler and Jones, 1997).

There have been a few attempts to quantify fiber type composition in marine mammal skeletal muscles. Histochemical ATPase staining of the swimming muscles of seals has shown an average numerical composition of approximately 46% type I (slow twitch, oxidative fibers), 46% type IIb (fast twitch, glycolytic fibers), and the balance type IIa (fast twitch, oxidative fibers) (Hochachka and Foreman, 1993; Reed et al., 1994). These results conflict with the

suggestion that skeletal muscles of seals are adapted for aerobic metabolism, since type IIb fibers characteristically do not possess high concentrations of mitochondria or myoglobin. Although these two studies measured the oxidative capacity of the fibers by staining for NADH diaphorase and succinate dehydrogenase (SDH) activity, neither reported their results. Fiber typing of biopsies taken from the locomotory (epaxial) muscles of one Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) and the hypaxial and epaxial muscles of one live and one dead bottlenose dolphin (*Tursiops truncatus*) showed approximately 50% fast twitch, glycolytic fibers and 50% slow twitch, oxidative fibers (Ponganis and Pierce, 1978; Bello et al., 1983; Goforth, 1983). Of these, only Goforth (1983) performed SDH staining and verified that fast twitch, oxidative-glycolytic fibers were rare or absent due to the lack of staining overlap between SDH activity and fast twitch fibers.

The purpose of this study was to collect multiple samples from the swimming (*M. longissimus dorsi*) and non-swimming (*M. pectoralis*) musculature of the harbor seal (*Phoca vitulina*) and apply immunohistochemical (IHC) techniques to determine fiber types and their distribution. Based on previous studies of enzyme activities and mitochondrial volume density, we hypothesized that there would be a higher proportion of oxidative (type I slow twitch and type IIa FOG) fibers than glycolytic (type IIb, fast twitch FG) fibers. We also hypothesized that the fiber type distribution within the muscles would be heterogeneous (i.e., we expected to see fast twitch fibers located superficially and slow twitch fibers located deeper in the muscle). Instead, we found that all fibers in both of the muscles sampled were either type I or type IIa. The *pectoralis* muscle possessed significantly more type IIa fibers than the *longissimus dorsi*. In addition, fiber type distribution within the locomotory muscle was homogeneous.

Methods

Animals and sample collection. Samples from locomotory and non-locomotory muscles were collected within six hours of death from adult and subadult harbor seals (three males and seven females; average weight = 46.1 $\pm$ 13.5 kg) during a native subsistence hunt in eastern Prince Williams Sound, Alaska. The epaxial muscles lie along the vertebral column (Figure 1) and are alternately contracted and then stretched to produce the lateral spinal flexions that generate thrust by the hind flippers during swimming (Fish et al., 1988). The entire epaxial musculature along one side of the spine was removed, weighed, and three transverse sections were taken in the cranial (CR), middle (MID), and caudal (CA) regions (Figure 1). The CR transverse section was taken at the 7th cervical vertebrae, MID was taken at the 14th thoracic vertebrae, and CA was from the lower lumbar region. Seven samples (ca. 0.5 g) per transverse section were taken at points on a circular grid using a 6-mm stainless steel biopsy punch (Figure 2). Muscle samples were placed in a phosphate buffered saline (PBS) solution of 7% glycerol and 4% sucrose for 30 minutes prior to freezing in 3-methylbutane cooled with liquid nitrogen. After sample processing, a rectilinear grid was overlaid on the cross-section, and the location of each of sample was determined relative to a true dorso-ventral and medio-lateral orientation in the animal. Samples were transported back to Texas A&M University in liquid nitrogen and kept frozen at –70°C until analysis. For comparison with a non-swimming muscle, a single sample was taken from the center of the intact *M. pectoralis*.

Immunohistochemical Analysis of Muscle Fiber Types. Serial 7-μm cross-sections of frozen epaxial and *pectoralis* muscle samples were cut on a cryostat microtome and mounted on glass slides. Sections of muscle were fixed with cold AFA (50 ml of 37% zinc formalin + 370 ml 95% ethanol + 25 ml glacial acetic acid) for 5 min and then hydrated for 10 min in PBS prior to

blocking. PowerBlock (InnoGenex, San Rafael, CA) was added to the sections and incubated for 5 min at room temperature. Following removal of excess blocker, primary antibodies to the myosin heavy chains, type I (BA-D5), type IIa (SC-71), and type IIb (BF-F3) were added to the appropriate sections, and the slides were incubated at 4°C overnight in a humid chamber. Following incubation, slides underwent two 10-min washes in PBS with gentle rotation. After washing, a biotinylated goat anti-mouse Ig secondary antibody was added to the sections for 20 min at room temperature. After washing the slides as described above, streptavidin alkaline phosphatase conjugate was added, and the sections were incubated for 20 min at room temperature. The conjugate was removed by washing (as in prior steps), and a solution of naphthol phosphate buffer and Fast Red dye was added. The sections were then incubated until adequate color development was observed, counterstained with Mayer's Hematoxylin and mounted with Glycergel (Dako, Carpinteria, CA). Fibers containing the myosin heavy chains expressed a red color following exposure to the immunohistochemical staining procedure. Samples were analyzed using a Bioquant image analysis system (R&M Biometrics, Inc., Nashville, TN). This system consists of an Olympus BX-60 microscope with an attached Optronics DEI 470 camera interfaced with a personal computer. Serial muscle sections were also examined for IIx fibers (i.e., fibers that expressed no staining following exposure to any of the heavy chain antibodies). Fiber types were identified in each muscle cross section as described by Schiaffino et al. (1989) to determine their distribution and abundance.

Verification of antibody reactivity. We used a combination of mouse anti-rat primary antibody and goat anti-mouse secondary to differentiate between three myosin heavy chain isoforms. Western blot analysis and SDS-PAGE verified that the fiber types of seals matched the electrophoretic properties of rat fiber types (Figures 3 and 4). Metachromatic staining

performed in conjunction with IHC fiber typing on the epaxial muscles and *pectoralis* of Weddell seals showed similar results (Kanatous et al., 2002).

Data Analysis. Fiber type population differences among the three transverse sections were analyzed using a one-way ANOVA and a Tukey post-hoc test. For analyses performed within a transverse section of muscle, the grid used to identify the seven sample locations was divided into two sections delineated by the equation $y = 1x + 0$. Samples that fell on either side of the line were grouped into a category either proximal or distal to the vertebral column (Figure 5). Samples lying on the origin were discarded, and no samples fell on the line. Proximal and distal fiber type populations were averaged over all seals, and the two groups were compared using a Student's t-test. Comparisons of average fiber type abundance between the epaxial muscle and the *pectoralis* were analyzed using a Student's t-test. Fiber type populations were also analyzed with respect to seal sex and mass. All results are expressed as means ± 1 standard deviation and tested at a level of significance of $p<0.05$.

Results

The epaxial and *pectoralis* muscles were composed of type I and type IIa fibers, with type IIb fibers completely absent (Table 1). The average percentages of type I fibers for the CR, MID, and CA transverse sections of the epaxial muscle were 45.9%, 47.2%, and 48.2%, respectively. The average percentages of type IIa fibers for the three transverse sections were 53.6%, 52.8%, and 52.3%, respectively. The *pectoralis* muscle was comprised of 15.0% type I fibers and 84.5% type IIa fibers.

Fiber type composition was not significantly different among or within the transverse sections of the epaxial muscle, indicating that average fiber type distribution was homogeneous within the

swimming muscle (Table 1). However, we cannot rule out finer scale heterogeneity in fiber type distribution. Fiber type composition was also not significantly different between females (n = 7) and males (n = 3), nor was there a mass-specific relationship. However, there was a significant difference between fiber type populations of the epaxial muscle and the *pectoralis*. The *pectoralis* was composed of significantly less type I fibers and significantly more type IIa fibers when compared to the epaxial muscle (Table 1) ($p < 0.000$).

Discussion

Although published data based on traditional histochemical techniques agree with our results for type I fiber populations, there is a substantial difference in the results for the type IIa and type IIb fibers. Previous fiber typing of the harbor seal epaxial muscle showed a high percentage of type IIb fibers (approximately 45-47%) and few (<10%) type IIa fibers (Hochachka and Foreman, 1993; Reed et al., 1994). Our results show no type IIb fibers and a high percentage of type IIa fibers (approximately 53%) for the same muscle. Our results were also dissimilar to the fiber type compositions found in the epaxial and hypaxial muscles of dolphins, which were composed of approximately 50% Type I and 50% Type IIb fibers (Ponganis and Pierce, 1978; Bello et al., 1983; Goforth, 1983). This difference in fiber type composition may be attributed to analytical procedure. All of the fiber typing data cited above has been obtained using histochemical fiber typing procedures. The traditional histochemical staining procedure uses acidic and alkaline preincubations to selectively inhibit the ATPase of the different fiber types, allowing for differentiation (Brooke and Kaiser, 1970). This procedure is based on the correlation between velocity of muscle contraction and the concentration of actomyosin ATPase within each fiber type; fast twitch fibers contain more ATPase than slow twitch fibers. Although

the ATPase technique may produce reliable fiber typing results under stringently controlled conditions, the technique is subject to inaccuracies resulting from small changes in preincubation and incubation time, temperature, pH, preincubation buffer type, and the ionic composition of the preincubation medium (Matoba and Gollnick, 1984). This makes actomyosin ATPase fiber typing highly variable and sometimes irreproducible (Green et al., 1982). In addition, the ability of the histochemical fiber typing procedure to differentiate between myofibrillar ATPase activity and mitochondria ATPase activity remains questionable (Guth, 1973). These considerations raise questions about the interpretation of the results obtained from previous histochemical fiber typing of seal muscles (Ponganis and Pierce, 1978; Bello et al., 1983; Goforth, 1983; Hochachka and Foreman, 1993; Reed et al.; 1994). Due to the susceptibility of the histochemical fiber typing technique to small changes in the incubation protocol, we used IHC fiber typing to characterize the fiber composition of seal muscle. Since IHC fiber typing utilizes the specific antigenicity of myosin heavy chain (MHC) isoforms to differentiate between the fiber types, antibody binding capacity is binary and IHC staining eliminates the subjective determination of 'stain hue' to separate fiber type, making quantification more accurate. Although IHC fiber typing is not a new technique (Arndt and Pepe, 1975), it has not been applied to marine mammals. IHC fiber typing may be used on a wide range of mammalian species because the MHC sequence is well-conserved.

In general, we found that the fiber type population and the homogeneous nature of the fiber type distribution within the epaxial muscle matched the myoglobin and enzyme activity data of tissue samples collected from the same seals and from the same locations in the transverse muscle sections. Myoglobin concentrations (Mb), citrate synthase activity (CS; an indicator of aerobic metabolism), β-hydroxyacyl CoA dehydrogenase activity (HOAD; an indicator of fatty

acid metabolism), and lactate dehydrogenase activity (LDH; an indicator of anaerobic metabolism) were either comparable to or elevated in harbor seal muscle compared to rat and dog (Polasek et al., in prep). These results are consistent with the characteristics of type I and type IIa fibers, which are both oxidative. Moreover, mitochondria volume density in harbor seal swimming muscle was elevated compared to terrestrial mammals of comparable size, indicating that the type I and type IIa fibers found in the harbor seal may be highly aerobic (Kanatous et al., 1999).

Fiber type abundance in the locomotory muscle (epaxial muscle) of the seal was significantly different than the non-locomotory muscle (*pectoralis*). Whereas the epaxial muscle was composed of approximately 50% type I fibers and 50% type IIa fibers, the *pectoralis* possessed approximately 15% type I fibers and 85% type IIa fibers (Table 1). Seals swim using lateral undulations of their hind flippers to propel themselves through the water, and are characterized as thunniform swimmers (Fish et al., 1988). The epaxial muscles are therefore alternately contracted and stretched during underwater locomotion. This stretch-shortening cycle may allow for more efficient swimming by storing energy in the series elastic component during the stretch cycle, which is then released during the contraction phase (Moon, et al., 1991). Muscles involved in eccentric (active, lengthening) contractions generally have more type I fibers than muscles that perform concentric contractions (Armstrong and Phelps, 1984; Delp and Duan, 1996). Seal *pectoralis* may perform only concentric contractions and therefore contain fewer type I fibers than the epaxial muscles, which perform eccentric contractions during locomotion.

The physiological profile of the harbor seal skeletal muscle appears to be similar to that of terrestrial mammals adapted for sustained, aerobic exercise (e.g., horses and dogs). This

physiological profile includes an elevated mitochondrial volume density, increased enzymatic capacity to oxidize fats, elevated tricarboxylic acid cycle enzyme capacity, and a fiber type distribution of primarily type I and type IIa fibers in locomotory muscles. However, in seals, routine metabolic rate during diving is generally less than two times the resting, predive levels. (Castellini et al., 1992; Davis et al., 1985; Williams et al., in prep). Additionally, behavioral evidence indicates that seals are not active swimmers and may not maximize their aerobic capability *in vivo*. Rather, seals use energy saving locomotory strategies. Recent evidence shows that when seals dive, they often alternate between an active stroke phase and a passive glide phase to conserve energy and oxygen stores, a pattern that is demonstrated in a variety of diving mammals (Williams et al., 2000; Davis et al., 2001). Consequently, this behavioral information coupled with physiological data suggests that the elevated mitochondrial volume density found in seal skeletal muscle may have a primary function of decreasing the diffusion distance of oxygen stores in myoglobin to the site of oxidation at the mitochondria (Kanatous et al., 1999).

Our fiber typing results show that harbor seal skeletal muscle is made exclusively of slow twitch and fast twitch oxidative fibers. These results are consistent with the theory that diving in marine mammals is an aerobic activity. Fiber type distribution did not show pronounced spatial heterogeneity. Finally, differences in fiber type abundance in locomotory *vs*. non-locomotory muscle may be related to contraction velocity and ability to store elastic energy.

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Literature Cited

Armstrong, R. B. and R. O. Phelps. (1984). Muscle fibre composition of the rat hindlimb. *Am. J. Anat*. 171, 259-272.

Arndt, I. and F. A. Pepe. (1975). Antigenic specificity of red and white muscle myosin. *J. Histochem. Cytochem*. 23, 159-168.

Bello, M. A., R. R. Roy, I. Oxman, T. Martin, and V. R. Edgarton. (1983). Fiber type and fiber size distributions in the axial musculature of the dolphin (Tursiops truncates). *Physiologist* 26, A-26.

Brooke, M. H. and K. K. Kaiser. (1970). Three “myosin adenosine triphosphatase” systems: the nature of their pH lability and sulfhydryl dependence. *J. Histochem. Cytochem*. 18, 670-672.

Butler, P. J., and D. R. Jones. (1997). Physiology of diving of birds and mammals. *Physiol. Rev*. 77, 837-899.

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. *J. exp. Biol*. 165, 181-1994.

Castellini, M. A., G. N. Somero, and G. L. Kooyman. (1981). Glycolytic enzyme activities in tissues of marine and terrestrial mammals. *Physiol. Zool*. 54, 242-252.

Cherepanova, V., T. Neshumova, and R. Elsner. (1993). Muscle blood flow in diving mammals. *Comp. Biochem. Physiol*. 106A, 1-6.

Davis, R. W. (1983). Lactate and glucose metabolism in the resting and diving harbor seal. *J. Comp. Physiol.* 153, 275-288.

Davis, R. W., T. M. Williams, and G. L. Kooyman. (1985). Swimming metabolism of yearling and adult harbor seals *Phoca vitulina*. *Physiol. Zool*. 58, 590-596.

Davis, R. W., L. A. Fuiman, T. M. Williams, S. O. Collier, W. P. Hagey, S. B. Kanatous, S. Kohin, and M. Horning. (1999). Hunting behavior of a marine mammal beneath the Antarctic fast ice. *Science* 283, 993-996.

Davis, R. W., L. A. Fuiman, T. M. Williams, and B. J. LeBoeuf. (2001). Three-dimensional movements and swimming activity of a northern elephant seal. *Comp. Biochem. Physiol. Part A* 129, 759-770.

Delp, M. D. and C. Duan. (1996). Composition and size of type I, IIA, IID/X, and IIB fibers and citrate synthase activity of rat muscle. *J. Appl. Physiol*. 80, 261-270.

Fish, F. E., S. Innes, and K. Ronald. (1988). Kinematics and estimated thrust production of swimming harp and ringed seals. *J. exp. Biol*. 137, 157-173.

Goforth, H. W. (1984). Biochemical and anatomical characteristics of dolphin muscles. Naval Ocean Systems Center, San Diego, CA. Technical report No. 940.

Green, H. J., H. Reichmann, and D. Pette. (1982). A comparison of two ATPase based schemes for histochemical muscle fibre typing in various mammals. *Histochemistry* 77, 21-31.

Guth, L. (1973). Fact and artifact in the histochemical procedure for myofibrillar ATPase. *Exp. Neurology* 41, 440-450.

Hochachka, P. W. and Foreman III, R. A. (1993). Phocid and cetacean blueprints of muscle metabolism. *Can. J. Zool.* 71, 2089-2098.

Kanatous, S. B., L. V. DiMichele, D. F. Cowan, and R. W. Davis. (1999). High aerobic capacities in the skeletal muscles of pinnipeds: adaptations to diving hypoxia. *J. Appl. Physiol*. 86, 1247-1256.

Kanatous, S. B., R. W. Davis, R. Watson, L. Polasek, T. M. Williams, and O. Mathieu Costello. (2002). Aerobic capacities in the skeletal muscles of Weddell seals: key to longer dive durations? *J. exp. Biol*. 205: 3601-3608

Kooyman, G. L, M.A. Castellini, R. W. Davis, and R .A. Maue. (1983). Aerobic dive limits of immature Weddell seals. *J. Comp. Physiol*. 151, 171-174.

Matoba, H. and P. D. Gollnick. (1984). Influence of ionic composition, buffering agent, and pH on the histochemical demonstration of myofibrillar actomyosin ATPase. *Histochem*. 80, 609-614.

Moon, T.W., J.D. Altringham, and I.A. Johnston. (1991). Energetics and power output of isolated fish fast muscle fibres performing oscillatory work. J. Exp. Biol. 158:261-73.

Polasek, L. K. and R. W. Davis. (2001). Heterogeneity of myoglobin distribution in the locomotory muscles of five cetacean species. *J. Exp. Biol*. 204, 209-215.

Ponganis, P. J. and R. W. Pierce. (1978). Muscle metabolic profiles and fiber type composition in some marine mammals. *Comp. Biochem. Physiol*. 59B, 99-102.

Reed, J. Z., P. J. Butler, and M.A. Fedak. (1994). The metabolic characteristics of the locomotory muscles of grey seals (*Halichoerus grypus*), harbour seals (*Phoca vitulina*), and Antarctic fur seals (*Arctocephalus gazella*). *J. Exp. Biol*. 194, 33-46.

Schiaffino S., L. Gorza, S. Sartore, L. Saggin, S. Ausoni, M. Vianello, K. Gundersen, and T. Lømo. (1989). Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. *J. Muscle Res. Cell Motil*. 10, 197-205.

Thompson, D. and M. A. Fedak. (1993). Cardiac responses of grey seals during diving at sea. *J. exp. Biol.* 174, 139-164.

Williams, T. M., R. W. Davis, L. A. Fuiman, J. Francis, B. J. LeBoeuf, M. Horning, J. Calambokidis, and D. A. Croll. (2000). Sink or swim: Strategies for cost-efficient diving by marine mammals. *Science* 288, 133-136.

Figure Legends

Figure 1: Schematic diagram illustrating the location of the three transverse sections taken from the epaxial muscle. CR = cranial, MID = middle, and CA = caudal.

Figure 2: Representative transverse section showing the coring pattern of samples collected from the epaxial muscle. CA = caudal.

Figure 3. Western blot of rat *vastus medialis* muscle, seal epaxial muscle, and seal *pectoralis*. Lanes 1-3 are stained for all myosin heavy chains (type I, IIa, and IIb), lanes 4-6 are stained for type IIa only, and lanes 7-9 are stained for type I only. Type IIb band is absent in both seal muscles.

Figure 4. Silver stain (reverse western blot) of rat *vastus medialis* muscle, seal epaxial muscle and seal *pectoralis* muscle showing differentiation of myosin heavy chain fibers based on molecular weight. Type IIb band is absent in both seal muscles.

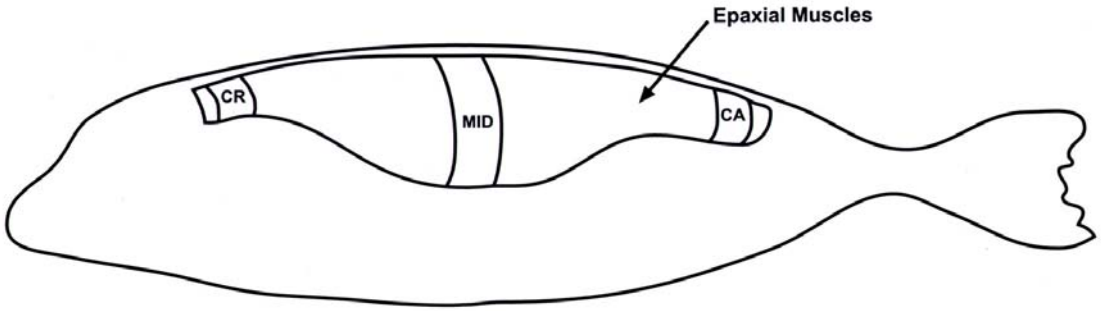
Figure 5: Representative transverse section showing the statistical division used for testing homogeneity within the epaxial muscle transverse sections. The dashed line (---) represents the natural vertical axis of the animal and the solid line (—) represents the line drawn at a 45 degree angle to the vertical axis to divide the epaxial muscle into proximal (P) and distal (D) sections. CA = caudal.

Figure 6. Representative serial cross sections from the epaxial muscle of a harbor seal stained for myosin heavy chain isoforms using a series of monoclonal antibodies. (A) is stained for type I (slow-twitch, oxidative) fibers, (B) is stained for type IIa (fast-twitch, oxidative) fibers, and (C) is stained for type IIb (fast-twitch, glycolytic) fibers. For comparison, the same fibers are

shown in each cross section. Lack of stain in (C) indicates an absence of type IIb fibers in this cross section of the muscle.

Table 1. Average population percentages of fiber types ± 1 standard deviation from three cross sections of the harbor seal epaxial muscle and *pectoralis*. The value in brackets is the number of animals averaged for each cross section. An asterisk (*) indicates a significant difference from the epaxial muscle at $p < 0.01$.

	Type I	**Type IIa**	**Type IIb**
Epaxial muscle			
Cranial (10)	45.9 ± 8.1	53.6 ± 8.2	0.0 ± 0.0
Middle (9)	47.2 ± 6.7	52.8 ± 6.8	0.0 ± 0.0
Caudal (9)	48.2 ± 9.1	52.3 ± 9.2	0.0 ± 0.0
Pectoralis (9)	15.0 ± 5.3*	84.5 ± 5.4*	0.0 ± 0.0



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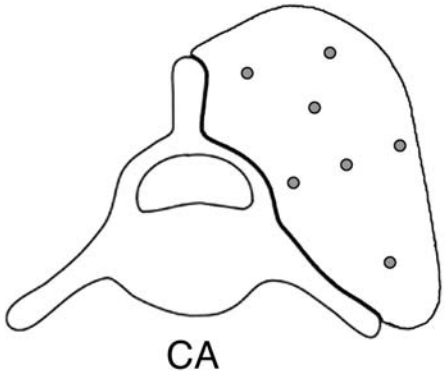
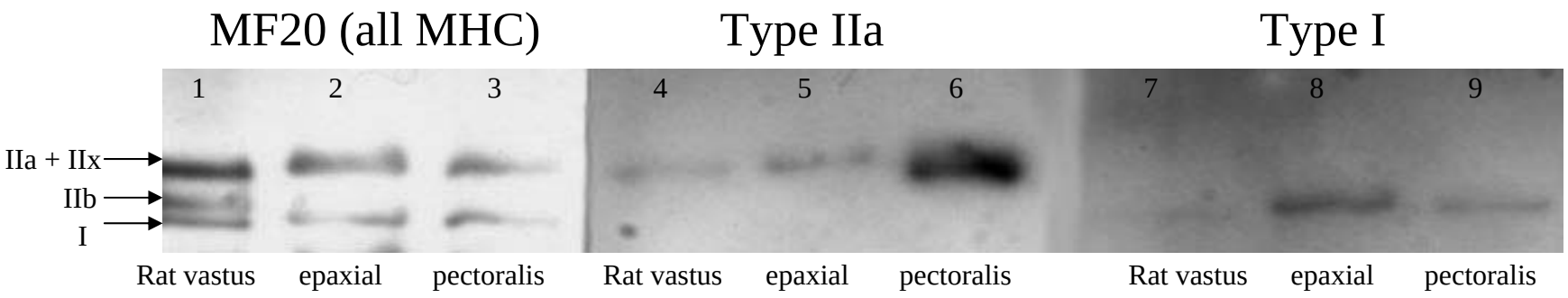


Figure 2: Representative transverse section showing the coring pattern of samples collected from the epaxial muscle. CA is the caudal transverse section of the epaxial muscle.



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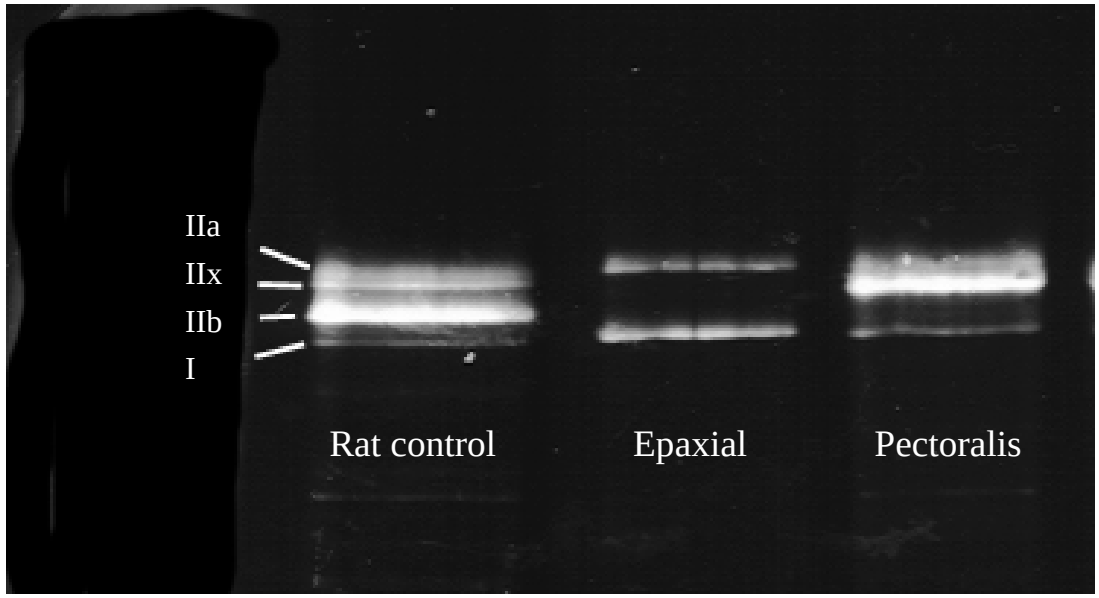

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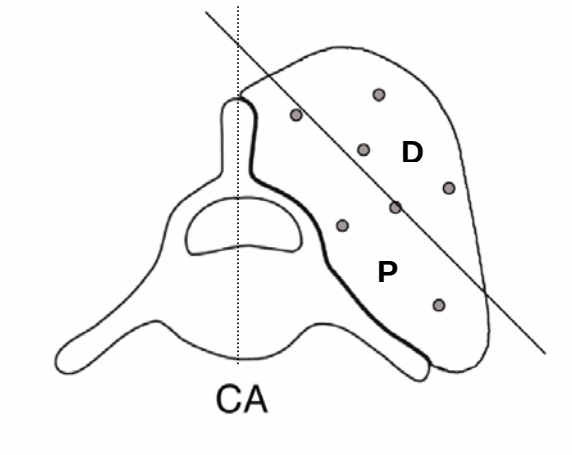

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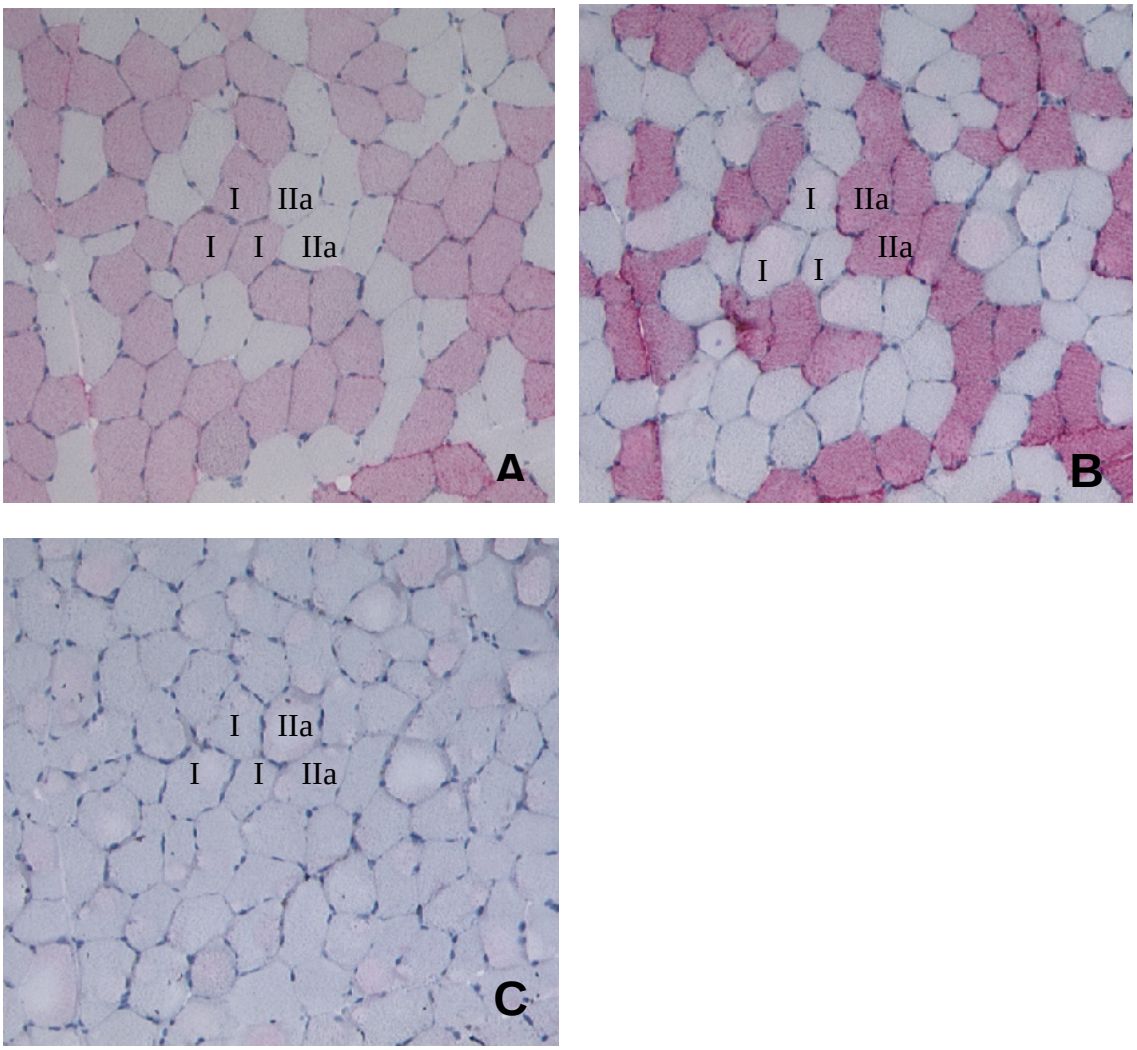

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Mapping mitochondrial volume density in the locomotory muscles of harbor seals (*Phoca vitulina)*

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Summary

Seals maintain aerobic metabolism during the majority of dives, and their skeletal muscle shows adaptations for aerobic energy production during hypoxia. Spot sampling in harbor seal locomotory (epaxial) muscle has shown high mitochondrial volume densities, elevated aerobic capacity, and an increased reliance on lipid metabolism. To determine the variability of aerobic indices in seal muscles, we analyzed entire transverse sections of the epaxial muscles of the harbor seal for mitochondrial volume density using electron microscopy. Mean volume density of mitochondria (Vv(mt,f)) in the muscle fibers was significantly higher than in the rat *soleus* muscle. These results are consistent with enzymatic data and fiber typing performed on the same muscle samples. In addition, regions of the epaxial muscles that were located proximal to the vertebra showed a significantly higher Vv(mt,f) relative to those regions distal to the vertebra. Thus, we found spatial heterogeneity of Vv(mt,f) within the epaxial muscles that may reflect regional differences in force generation during swimming.

Introduction

Seals maintain aerobic metabolism during the majority of their dives, and their skeletal muscle shows adaptations for aerobic energy production during hypoxia. Seal locomotory muscle (*Longissimus dorsi*) and non-locomotory muscle (*pectoralis*) have a high mitochondrial volume density and elevated aerobic capacity compared to terrestrial controls (Kanatous et al., 1999; Polasek and Davis 2001, Polasek et al. in prep). Because peripheral vasoconstriction occurs as part of the dive response, oxygen for aerobic metabolism is stored as oxy-myoglobin in the muscle. Myoglobin concentrations in the skeletal muscles of seals are elevated 10-20x compared to terrestrial mammals and provide an endogenous source of oxygen during dives (Cherepanova et al., 1993; Kanatous et al., 1999; Polasek and Davis, 2001). The high mitochondrial volume densities and myoglobin concentrations enhance the intracellular diffusion of oxygen into mitochondria under low oxygen partial pressure (Kanatous et al., 1999).

The purpose of this study was to determine the level of variability of mitochondrial distribution within the epaxial muscle of the harbor seal using electron microscopy. Our null hypothesis was that mitochondrial volume density was uniform throughout the epaxial muscle and did not differ significantly from terrestrial control species (dog and rat). Our alternative hypothesis was that regional heterogeneity in mitochondrial volume density reflects differences in the oxygen consumption and work performed by different areas of the muscle and that elevations in mitochondrial volume density is an adaptation to sustain aerobic metabolism during dive-induced hypoxia.

Materials and Methods

Animals and sample collection. Samples from locomotory and non-locomotory muscles were collected within six hours of death from adult and sub-adult harbor seals (1 male and 4 females; average weight = 51.6 $\pm$ 3.1 kg) during a native subsistence hunt in eastern Prince Williams Sound, Alaska. The epaxial muscles lie along the vertebral column (Figure 1) and alternately contract and relax to produce the lateral spinal flexions that generate thrust by the hind flippers during swimming (Fish et al., 1988). The entire epaxial musculature along one side of the spine was removed, weighed, and a caudal (CA) transverse section was taken from the lower lumbar region (Figure 1). For electron microscopy, seven samples (ca.0.5 g) from the transverse section were taken at points on a circular grid using a 6-mm stainless steel biopsy punch (Figure 2). Muscle samples were placed in a 6.25% gluteraldehyde solution in cacodylate buffer (pH 7.4). After sample collection, a rectilinear grid was overlaid on the cross-section, and the location of each of sample was determined relative to a true dorso-ventral and medio-lateral orientation in the animal. Samples were transported back to Texas A&M University and kept refrigerated until analysis. Control samples were collected from the *soleus* muscle of three Sprague-Dawley rats. Eight-month-old rats were anesthetized with pentobarbital sodium (60 mg/kg IP), decapitated, and the entire *soleus* muscle was excised and fixed according to the above protocol. Tissue samples were taken in accordance with guidelines for the humane treatment of animals at Texas A&M University.

Electron microscopy preparations. Tissue samples were cut into small blocks using a dissecting microscope and stored in 0.1 M cacodylate buffer (pH 7.4) at 4°C. The samples were rinsed in fresh cacodylate buffer, post-fixed for 1 hour in 1% osmium

tetroxide, and block-stained with 2% uranyl acetate for 30 minutes at 60°C. The samples were then passed through stepwise dehydration in increasing concentrations of ethanol (50-100%) and rinsed twice with 100% propylene oxide. After subjecting the samples to increasing concentrations of Poly/Bed 812 (Polysciences, Inc., Warrington, PA), they were embedded in flat molds with fresh Poly/Bed 812 and allowed to polymerize for 24 hr at 60°C.

Morphometry. Four blocks were randomly chosen from each sample for stereology. The samples were thick sectioned (1 μm) with a Sorvall MT6000 microtome to verify that the orientation of the sections was transverse or slightly oblique to the muscle fiber axis. Ultrathin sections were cut with a Reichert/Leica Ultracut S ultramicrotome, placed on 150-mesh copper grids, and stained with Reynold's (0.4%) lead citrate to improve contrast. Micrographs were made at a total magnification of 6,600x with a Phillips CM-100 transmission electron microscope at 60 kV. Ten representative micrographs were taken per block by photographing the section of tissue in consecutive corners of the copper grid. In this manner, a random sampling of the tissue cross-section was achieved (Weibel 1979). Micrographs were converted to digital images by scanning the negatives into Adobe Photoshop at high (1200 dpi) resolution with a Microtek Scanmaker 8700 flatbed scanner. Point counting was performed with a B-36 (144 test points) grid superimposed on the digital image. Relative standard errors among the blocks were maintained at <10%.

Data Analysis. Mitochondrial volume densities were compared between species using a Student's t-test. For analyses performed within the transverse section of muscle, the grid used to identify the seven sample locations was divided into two sections delineated by the equation $y = 1x + 0$. Samples that fell on either side of the line were grouped into a category either proximal or distal to the vertebral column (Figure 3). Samples lying on

the origin were discarded, and no samples fell on the line. Proximal and distal mitochondrial volume densities were averaged over all seals, and the two groups were compared using a Student's t-test. All results are expressed as means ± SE and tested at a level of significance of $p<0.05$.

Results

Total mitochondrial volume density (Vv(mt,f)) was elevated 56% in the seal epaxial muscles compared to the rat *soleus* muscle and was significantly different ($p = 0.00$) (Table 1). The elevation in total mitochondria volume density resulted from an increase in both subsarcolemmal (Vv(ms, f)) and interfibrillar (Vv(mi,f)) mitochondria (Table 1). There was evidence of heterogeneity within the harbor seal transverse sections. For all seals, the region proximal to the vertebrae showed a significantly higher (25%) Vv(mt, f) than the distal region ($p = 0.04$) (Table 1).

Discussion

Oxidative capacity of locomotory skeletal muscles in terrestrial mammals generally follows a trend related to the muscular anatomy of the limbs. "Deep" muscles (closer to the bone) and anti-gravitational muscles tend to have a higher population of slow twitch, oxidative (type I) fibers than superficial muscles or muscles that do not resist gravity (Peter et al., 1972; Gunn, 1978; Armstrong et al., 1982). The primarily slow twitch, oxidative muscles located proximal to the leg bones of a quadrupedal mammal are recruited along with fast twitch, oxidative muscles when an animal begins locomotion or engages in low-exertion exercise (Armstrong et al., 1982). As exertion increases in intensity, the fast-fatiguing, glycolytic muscles are recruited. Within muscles, fiber types show a similar pattern of recruitment (Armstrong and Laughlin, 1985). Locomotory fibers, especially fast-twitch glycolytic fibers, tend to occur on the periphery of the muscle, are less perfused with blood vessels, and are characteristically less well

innervated with motor units, being activated only during very energetic activities. Fibers located closer to the bone may be constantly tonic (as in quiet standing) and tend to be oxidative. Thus fiber type distribution and in the majority of terrestrial mammal locomotory muscles is heterogeneous.

The absolute Vv(mt,f) for the harbor seal epaxial muscles measured in this study was 5.26% and the Vv(mt,f) seal:rat ratio was 1.6:1. Harbor seal epaxial muscle Vv(mt,f) has been previously measured to be approximately 8.9%, yielding a seal:rat ratio of 2:1. (Kanatous et al., 1999; Kanatous et al., 2001). Thus the absolute values of Vv(mt,f) in both rats and seals were slightly lower in this study and probably resulted from variations in electron micrograph image analysis (Table 1). We hypothesize that this increase in Vv(mt) aids in the maintenance of aerobic metabolism during diving by decreasing the diffusion distance between mitochondria and intracellular oxygen. We base this hypothesis on the rate of diffusion within a muscle fiber described by Fick's equation:

$$dQ/dt = -DA(du/dx)$$

where dQ/dt is the diffusive flux of substance Q over time dt, A is the area through which diffusion takes place, du/dx is the concentration (or partial pressure) gradient over distance dx, and D is the diffusion coefficient (Schmidt-Nielsen, 1979). As the diffusion distance (dx) decreases, the rate of oxygen flux (dQ/dt) increases. For the diffusion of intracellular oxygen, a decrease in diffusion distance is especially advantageous at low partial pressures (i.e., du is small) of oxygen experienced during diving.

Immunohistochemical fiber typing of harbor seal epaxial muscle showed only oxidative fibers (type I and type IIa) and an absence of glycolytic fibers (IIb) (Watson et

al., in prep). These results agree with our mitochondrial volume density data. Although there was no significant heterogeneity of muscle fiber type distribution in the harbor seal epaxial muscle (Watson et al, in prep.), mitochondrial volume density was significantly higher in regions proximal to the vertebrae compared to the muscle periphery, indicating heterogeneity (Table 1). Thus the oxidative capacity of the epaxial muscle appears to be distributed in a heterogeneous fashion, whereas the distribution of the components of force generation (the myosin isoforms) appears homogeneous.

Oxidative capacity in harbor seal skeletal muscle has been assessed using enzyme assays. In general, the mitochondrial volume density data within the epaxial muscle matched the aerobic and anaerobic enzyme activities and myoglobin concentration of tissue samples collected from the same seals and from the same locations in the transverse muscle sections (Polasek et al., in prep). Citrate synthase activity (CS; an indicator of aerobic metabolism), β-hydroxyacyl CoA dehydrogenase activity (HOAD; an indicator of fatty acid metabolism), lactate dehydrogenase activity (LDH; an indicator of anaerobic metabolism) and myoglobin concentrations were either comparable to or elevated in harbor seal muscle compared to rat and dog. These data indicate a reliance on lipid-based, aerobic metabolism in seal swimming muscle. Elevated LDH activity in seals indicates an increased anaerobic metabolic capacity and does not necessarily preclude the maintenance of a high aerobic capacity. Although we found a trend towards heterogeneity within the epaxial muscles with our mitochondrial volume density analysis, a matching trend was not apparent in the enzyme activities or myoglobin concentrations. In this study, we analyzed only the caudal portion of the epaxial muscles, which generate most of the thrust involved with swimming. While many locomotory muscles of terrestrial mammals contain a mixed population of fiber types, some are homogeneous. For example, the *soleus* muscle in many digitigrade animals (e.g., rabbit, cat, and guinea pig) may be almost entirely composed of slow, oxidative fibers (Peter et al., 1972; Ariano

et al., 1973; Delp and Duan, 1996). However, in terrestrial mammals, these muscles primarily serve to resist gravity and maintain posture, which is not the case with the epaxial muscle of a seal.

In conclusion, an elevated mitochondrial volume density in harbor seal epaxial muscle indicates an adaptation to sustain aerobic metabolism during hypoxia by enhancing the diffusion of oxygen to mitochondria at low partial pressures. The elevated Vv(mt) enhances aerobic capacity, although this may be of minor significance to animals that routinely exhibit a low metabolic rate to extend dive duration. The higher Vv(mt) proximal to the vertebra may reflect greater force generation and energy metabolism in this region of the muscle during swimming. Parallel studies on the same harbor seals used in this study have shown a preponderance of fast-twitch oxidative and slow-twitch oxidative muscle fibers and enhanced aerobic enzyme activities in the epaxial muscles. Taken together, these data indicate an increased capacity for aerobic, lipid-based metabolism in the locomotory muscles of harbor seals during diving.

Literature Cited

Ariano, M.A., R.B. Armstrong, V.R. Edgerton. (1973). Hindlimb muscle fiber populations of five mammals. J. Histochem. Cytochem. 21(1):51-55.

Armstrong, R. B., C. W. Saubert, H. J. Seeherman, and C. R. Taylor. 1982. Distribution of fiber types in locomotory muscles of dogs. Am. J. Anat. 163:87-98.

Armstrong, R.B. and M.H. Laughlin. (1985). Rat muscle blood flows during high-speed locomotion. J. Appl. Physiol. 59(4):1322-1328.

Cherepanova, V., T. Neshumova, R. Elsner. (1993). Muscle blood flow in diving mammals. Comp. Biochem. Physiol. Comp. Physiol. 106(1):1-6

Delp, M.D., and C. Duan. 1996. Composition and size of type I, IIA, IID/X and IIB fibers and citrate synthase activity of rat muscle. J Appl Physiol 80:261-270.

Fish, F.E., S. Innes, K. Ronald. (1988). Kinematics and estimated thrust production of swimming harp and ringed seals. J. Exp. Biol. 137:157-73.

Gunn, H. M. 1978. Differences in the histochemical properties of skeletal muscles of different breeds of horses and dogs. J. Anat. 127:615-634.

Kanatous, S. B., L. V. DiMichele, D. F. Cowan, and R. W. Davis. 1999. High aerobic capacities in the skeletal muscles of pinnipeds: adaptations to diving hypoxia. Journal of Applied Physiology 86:1247-1256.

Kanatous, S.B., R. Elsner, O. Mathieu-Costello. (2001). Muscle capillary supply in harbor seals. J. Appl. Physiol. 90:1919-1926.

Peter, J. B., R .J. Barnard, V. R. Edgerton, C. A. Gillespie, and K. E. Stempel. 1972. Metabolic profiles of three fiber types of skeletal muscles in guinea pigs and rabbits. Biochem. 11:2627-2633.

Polasek, L. K. and R. W. Davis. 2001. Heterogeneity of myoglobin distribution in the locomotory muscles of five cetacean species. J. Exp. Biol. 204:209-215.

Schmidt-Nielsen, K. (1979). Animal Physiology. Cambridge University Press, Cambridge, U.K. 611pp.

Weibel, E. R. 1979. Stereological methods. Vol 1. Practical methods for biological morphometry. Academic Press, New York, NY. 415 pp.

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Table 1. Summary data for the electron microscopy of epaxial muscles of the harbor seals and rat soleus muscle. Values are average percents ± SE; body mass is in kg (seals) and g (rats). Vv(mt,f) is the volume density of total mitochondria, Vv(mi,f) is the volume density of interfibrillar mitochondria, and Vv(ms,f) is the volume density of subsarcolemmal mitochondria. *Significantly different from rat control ($p < 0.05$). **Significantly different from distal region ($p < 0.05$).

	Body mass	Vv(mt,f)	Vv(mi,f)	Vv(ms,f)
Harbor seal	51.6 ± 3.1 kg	5.26 ± 0.47*	4.23 ± 0.36	1.02 ± 0.26
Proximal region		5.80 ± 0.44**		
Distal region		4.66 ± 0.21		
Rat (Sprague-Dawley)	487.3 ±3 1.4 g	3.38 ± 0.37	3.08 ± 0.31	0.30 ± 0.11

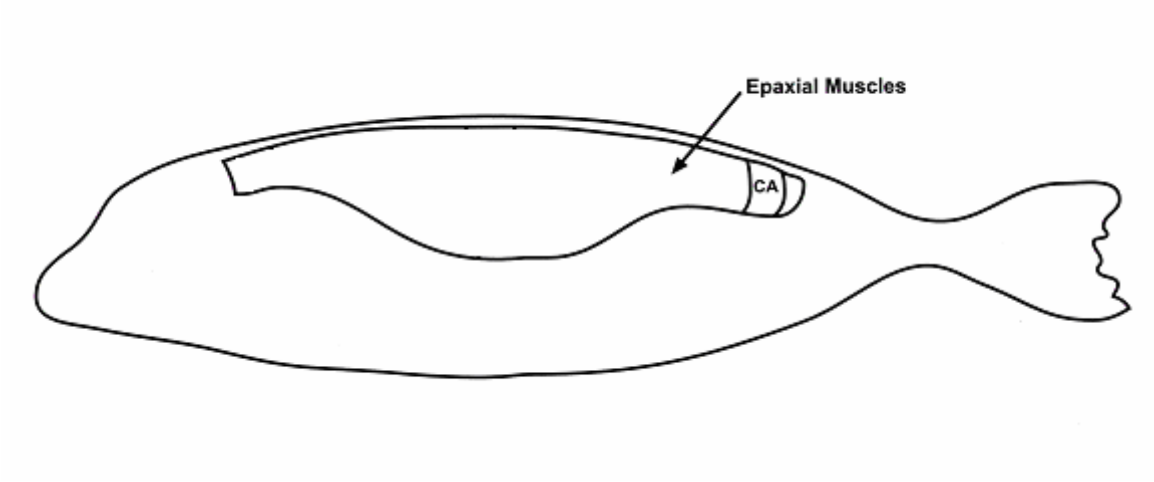
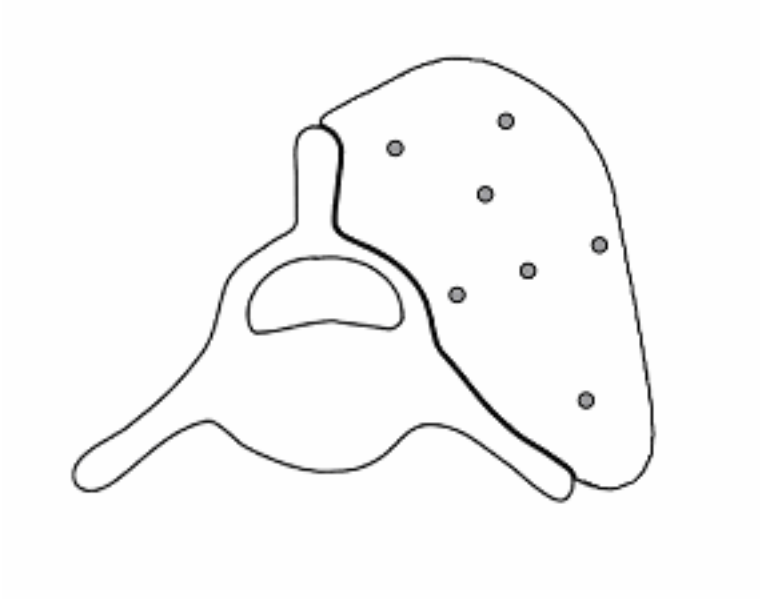


Figure 1: Schematic diagram illustrating the location of the three transverse sections taken from the epaxial muscle. CA indicates the caudal transverse section.



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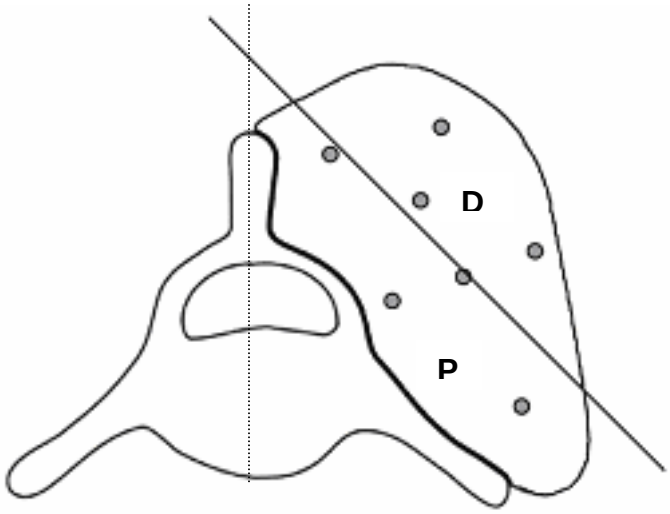

Figure 3. Diagram of a transverse section showing the statistical division used for testing homogeneity within the epaxial muscle. The dashed line (---) represents the vertical axis of the animal. The solid line (—) drawn at a 45° angle to the vertical axis divides the epaxial muscle into proximal (P) and distal (D) sections.

The effect of diet on the fatty acid composition of blubber in harbor seals (*Phoca vitulina*)

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Summary

Harbor seals in Alaska have experienced population declines similar to those observed for other marine mammals and piscivorous seabirds in the Bering Sea/Gulf of Alaska ecosystem. The decline is most likely the result of food limitation due to changes in the availability of prey resulting from the effects of both natural variability and fishing activities. As part of a larger program designed to examine how differing diets, seasons, and health status affect the body condition of harbor seals, this project examined how fatty acids in the blubber of captive harbor seals changed over time during feeding of controlled diets of herring and pollock. Despite small sample sizes, variation in the ages of seals, their reproductive status, prey consumption, changes in body mass and total body lipid, as well as the variation in the fatty acid profiles of the lots of prey species used for feeding, the fatty acid composition of the seal blubber was observed to change in response to changes in diet. When all of the blubber samples were combined, or only the end-point samples were used, 67% to 85%, respectively, of the harbor seal blubber samples could be correctly classified according to the composition of their diet using Classification and Regression Trees. This classification rate was substantially lower (59%) when using only the mid-point samples, suggesting that, while the fatty acid composition of the blubber changed with diet, these changes were more pronounced after four months, compared to two months, on a given diet. Using either inner or outer blubber layers, 75% of the samples were correctly classified by the seal's diet. Thus, while the fatty acid composition of these two blubber layers differed from each other, they were both influenced by changes in diet.

Introduction

In the Pacific, harbor seals (*Phoca vitulina*) range from Baja California, Mexico northward along the west coast of the United States, extending along the Aleutian and Kuril Islands and into northeastern Hokkaido, Japan (Ridgeway and Harrison, 1981). As with northern fur seals, Steller sea lions and some piscivorous sea birds, population censuses conducted since the1970s indicate significant declines in the abundance of harbor seals in Alaska (Wooster, 1992). While harbor seals are known to be affected by a variety of human activities, including incidental take in fisheries, disturbance from oil and gas resource development, pollution, disease and subsistence harvest, available data indicate that the decline is most likely the result of food limitation.

There are a number of ways in which food can be limiting, including a decline in overall prey abundance, a change in prey species composition, changes in size and/or age class composition of important prey species, and changes in the spatial/temporal distribution of prey species. There is insufficient data to determine which of these conditions might be affecting harbor seal food availability, but the abundance and distribution of fish stocks are a function of both natural variability and fishing activities. There is some evidence for an oceanographic regime shift that may have altered the distribution, abundance, or species composition of the region, thus affecting prey availability for the seals (Kerr, 1992; Francis and Hare, 1994; Trenberth and Harell, 1995). However, it is likely that normal fluctuations in oceanographic and climatic variables, which regularly affect prey species abundance and diversity, are now exacerbated or amplified by fishing activities such that food becomes locally limiting or prey availability is reduced for some populations or age classes of seals.

Harbor seals are generally non-migratory and exhibit a high degree of haulout site fidelity (Frost et al., 1996; Härkönen and Harding, 2001). Their localized movements are primarily associated with reproduction and changes in parameters such as tides and weather that affect prey distribution. Harbor seals are primarily found in nearshore waters where they feed on a variety of fish and invertebrates. The diet of newly weaned pups is composed almost exclusively of benthic crustaceans, including shrimp (Reeves et al., 1992). The principal prey species of adult harbor seals are small to medium-sized (up to ~30 cm in length) fishes and cephalopods (Frost and Lowry, 1986). Based on analysis of stomach contents, the predominant prey items in diets of harbor seals in the Gulf of Alaska were pollock (*Theragra chalcogramma*), octopus, capelin (*Mallotus villosus*), eulachon (*Thaleichthys pacificus*), and herring (*Clupea pallasii*) (Pitcher, 1980a). Within the Gulf of Alaska, harbor seal diets have been observed to vary regionally and seasonally (Pitcher and Calkins, 1979; Pitcher, 1980a; Pitcher, 1980b; Iverson and Frost, 1996). Although there is less data available for the Bering Sea and Aleutian Islands, the diet of harbor seals in these regions appears to be generally similar to that in the Gulf of Alaska (Lowry et al., 1982). However, the relative importance of a given species varies by region. For example, pollock and octopus were found to be the dominant prey items in the diets of harbor seals from the Gulf of Alaska and the Bering Sea and Aleutian Islands, whereas shrimp and capelin ranked above pollock in the diets of southeast Alaska harbor seals (Hoover, 1988). Fatty acid signature analysis also indicated variation in seal prey by region and season as well as differences in diet between seals in southeast Alaska, Kodiak Island, and Prince William Sound (Frost et al., 1996). As further evidence of their strong site fidelity, fatty acid signature analysis also found differences in seal diets within Prince William Sound, which correlated with average daily locations based on satellite tracking studies (Frost et al., 1996).

This study examined how fatty acids in the blubber of captive harbor seals changed over time during controlled diets of herring and pollock. The study had three main objectives within a larger program designed to better understand how differing diets, seasons, and health status affect the body condition of harbor seals. The objectives of this study were to examine whether the fatty acid signature of harbor seal blubber changes with a change in diet, whether these changes reflect the fatty acid signature of prey consumed, and whether these changes are different within the inner and outer layers of the blubber.

Methods

As described in Castellini et al. (2001), three groups of harbor seals at the Alaska SeaLife Center, Seward, Alaska were used to study the effects of diet on a variety of morphometric and physiological parameters. Over the course of two years, these seals were fed controlled diets of herring and pollock, and samples of blood and blubber collected at regular intervals. Group A consisted of two juvenile males and one reproductive-aged female seal. Group B consisted of two adult male seals not yet of reproductive age, and one reproductive-aged female seal. Group C was composed of two reproductive-aged female seals. The seals in group A were less than 10 years old: both males were two and the female was eight. In group B, the female was 23 years old and the males were 15 and 23. One female in Group C was seven years old, and the other was 14.

The seals were fed individually to a level of satiation as determined by the handlers so that the actual mass of food could vary throughout the experiment. Group C seals were fed a 50% herring:50% pollock diet throughout the study. In a repeated crossover study design (Table 1), the Group A and B seals received alternating diets of exclusively pollock or herring for periods

of four months. The feeding matrix allowed both groups of seals to experience a different diet at each of three metabolically defined times of the year: breeding, molting, and spring.

To ensure constant total protein, total lipid, water and caloric content, herring and pollock were purchased in large commercial batches and stored at -30°C until thawed in smaller batches for feeding. Storage time did not appear to affect gross lipid content or energy density, and the proximate composition of the diets remained constant throughout the experiment (Castellini et al., 2001). The herring used in these experiments had a substantially higher lipid content and energy density than the pollock. When analyzed over a broad range of lengths, there was a positive correlation between standard length and lipid content in small (11-14 cm) versus large (15-27 cm) herring (Castellini et al., 2001).

Morphometric measurements (standard length, girth, and mass) were made at least once every two weeks, including at the beginning, mid-point, and end of each diet trial. Total body water was also measured using deuterium dilution methods to assess body condition (body fat) at the time of diet switching. The results of these measurements are reported in Castellini et al. (2001) and will be discussed further as they relate to the results of the fatty acid study.

At the middle (two months) and end (four months) of each dietary period, a blubber sample was collected from each seal for the full depth of the blubber. Blubber samples were obtained from the ventral chest region, over the pectoralis muscle, with a 6-mm disposable punch biopsy inserted through a small incision in the skin. A local anesthetic (2% Lidocaine) was injected beneath the skin prior to making the incision. Each blubber sample was divided in half along its length and each piece immediately transferred to a separate cryovial for freezing in liquid nitrogen. The inner and outer blubber samples were stored at –70°C until analysis and analyzed separately.

Total lipids were extracted from seal blubber and homogenized fish samples using the Folch method (Folch et al., 1957) as modified by Iverson (1993). Fatty acid methyl esters (FAME) were prepared from the purified lipid extracts using the Hilditch reagent (0.5 N H_2SO_4 in methanol). The FAME were analyzed by temperature-programmed gas-liquid chromatography on a Perkin Elmer Autosystem XL Capillary FID chromatograph fitted with a coiled 30 m X 0.25 mm i.d. column coated with 50% cyanopropoyl polysiloxane (0.25 m film thickness: J & W DB-23; Folsom, CA) using helium as a carrier gas. Samples were analyzed in duplicate. Individual peaks of FAME were identified based on retention times of known standard mixtures (Nu-Check Prep, Elysian, MN) and also after isolation of fatty acid classes in selected samples using silver nitrate chromatography according to Iverson et al. (1992). The area under individual peaks was quantified by a computerized integration system (Turbochrom 6.1.1 software, PE Nelson). Individual fatty acids were expressed as weight percent of the total fatty acids and designated by shorthand IUPAC nomenclature of carbon chain length:number of double bonds and location (n-x) of the double bond nearest the terminal methyl group.

A small subset of "indicator" fatty acids were chosen and compared among groups, blubber layers, and diet trial stage using ANOVA (StatVeiw 4.5, Abacus Concepts, Inc., Berkley, CA 94704). Because the probability of Type 1 error increases as the number of comparisons relative to sample size increases for univariate analyses, a subset of fatty acid variables was chosen for t-test and ANOVA analyses. This subset included all of the dominant fatty acids (i.e., those that accounted for ≥ 1% of the total fatty acids by mass in the seal blubber samples) (Table 2), plus 20:4w3 and 22:1w9. Many of these fatty acids, in addition to being abundant in the samples, are important dietary indicators, originating only or mostly from prey. The ratios of the n-9 and n-11 isomers of 20:1 (r20:1) and 22:1 (r22:1) were also included because they have been shown to be

useful diagnostic indices in other feeding preference studies (Iverson et al., 1997). Paired t-test comparisons between diets (i.e., herring *vs*. pollock) for this same subset of "indicator" fatty acids were performed for Groups A and B, by blubber layer (inner and outer), and diet mid- and end-point using StatView. ANOVA was also used to compare these "indicator" fatty acids between the fish species. All results with $p \leq 0.05$ were reported as significant.

The fatty acid data were also analyzed using classification and regression trees (CART) in Systat (Version 10.2, SYSTAT Software Inc., Richmond, CA), a non-parametric multivariate technique for classifying data. CART uses a series of algorithms to split data into groups (nodes) that are as different as possible, based on measures of deviance; the splitting continues in a tree-like form until a classification is made at a terminal node. Each terminal node was classified based on the majority of observations in it, and an associated misclassification rate was based on the number of observations in the node that were not correctly classified.

Results

A total of 58 fatty acids and isomers were isolated and identified by gas chromatography in the seal blubber and fish samples. The 14 dominant fatty acids in the seal blubber samples (i.e., those that each accounted for ≥ 1% of the total fatty acids by mass in the sample) were 14:0, 16:0, 16:1w7, 18:0, 18:1w11, 18:1w9, 18:1w7, 18:2w6, 20:1w11, 20:1w9, 20:5w3, 22:1w11, 22:5w3, and 22:6w3, for all diets and both inner and outer blubber layers (Table 2). The 12 dominant fatty acids found in the herring samples were 14:0, 16:0, 16:1w7, 18:0, 18:1w9, 18:1w7, 18:4w3, 20:1w11, 20:1w9, 20:5w3, 22:1w11and 22:6w3. The pollock samples had these same 12 dominant fatty acids plus 17:0, 18:1w11, and 22:1w9.

Where ANOVA revealed significant differences, the subset of indicator fatty acids are presented in the form of bar graphs for visual comparison of the relative proportions by feeding group and diet for the seals, and by species and lot number for the prey. The three groups of seals could be distinguished by the fatty acids 14:0, 16:0, 16:1w7, 18:0, 18:1w9 and 18:2w6 in their outer blubber layer samples for all feeding trials (Figure 1). Group A had a greater percentage of 14:0, 16:0, 16:1w7 and 18:0 than both Groups B and C, which did not differ significantly in their proportions of these fatty acids. The proportion of 18:1w9 was significantly greater in the outer blubber layers of the Group C seals (fed the mixed diet throughout) than in the outer blubber of the Group A and B seals, which did not differ significantly from each other in the proportion of this fatty acid in their outer blubber. The proportion of 18:2w6 was greater in the outer blubber of the Group B seals than in the Group C seals, and the proportion of this fatty acid was greater in the Group C seals, than in the Group A seals.

When comparing the outer blubber samples among all three groups of seals, the only significant difference between herring and pollock diet trials was with the fatty acid 18:3w3 (Figure 2). The proportion of 18:3w3 was greater in the outer blubber layer on a herring diet than on a pollock diet. However, there were significant differences among numerous fatty acids between herring and the mixed diet (16:0, 18:1w9, 18:3w3, 18:4w3, 20:1w9, r20:1, 20:4w6, 20:5w3, r22:1, 22:6w3), where the proportion of all of these fatty acids in the outer blubber layer was higher for the herring diet than mixed diet, except 18:1w9. The proportion of 18:1w9 in the outer blubber layer was greater for the mixed diet than the pollock diet, but the proportions of 16:1w7, 20:1w9, r20:1, 20:4w6, 20:5w3, and 22:6w3 were higher for the pollock diet compared to the mixed diet.

When comparing the indicator fatty acids in the inner blubber layer by diet (Figure 3), there were fewer significantly different fatty acids overall among groups of seals, but more differences between herring and pollock than in the outer blubber layer. The proportions of the fatty acids 14:0, 18:3w3, 18:4w3, and r22:1 were significantly higher on the herring diet compared to the pollock diet. The inner blubber layer was lower in the weight percent of 20:1w9 and higher in 20:4w6 between the herring and mixed diets. The weight percent of 14:0 and 18:3w3 in the inner blubber layer was greater on the mixed diet compared to the pollock diet, but the proportion of 18:0 and 20:4w6 was lower on the mixed diet compared to the pollock diet.

Using the combined weight percents for mid- and end-points for the outer blubber layer, the fatty acids 14:0, 18:3w3, 18:4w3, and r22:1 were significantly different between herring and pollock feeding trials for the Group A seals (Figure 4). Both the inner and outer blubber layers were higher in the proportions of 18:3w3, 18:4w3, and r22:1 on the herring diet compared to the pollock diet. The inner blubber layer was higher in the proportion of these four fatty acids compared to the outer blubber layer for both herring and pollock diets. No significant differences were found for the Group B seals. When only the end-point blubber samples were compared, it was not possible to distinguish between herring and pollock diets for either Group A or B seals. Similarly, there were no significant differences between herring and pollock diets in any fatty acid for the Group B seals for the mid-point outer blubber layer. However, significant differences in 18:4w3 and r22:1 between herring and pollock diets were seen for the mid-point outer blubber layer for Group A seals. There were significant differences between herring and pollock diet trials for the inner blubber layer of Group B seals for 14:0, 16:1n-7, and 20:1n-11 (Figure 5).

Herring and pollock differed significantly in the amounts of 14:0, 16:0, 18:0, 18:1w9, 20:1w9, r20:1, 20:4w6, 20:5w3, r22:1, and 22:6w3 in their total lipids (Figure 6). With the exception of 20:4w6, these represent nine of the 18 "indicator" fatty acids described above. Even though they did not vary significantly in proximate composition, the pollock samples showed significant variation among lots in the weight percents of 12 of the 18 "indicator" fatty acids (14:0, 16:0, 16:1w7, 18:0, 18:1w9, 18:2w6, 20:1w11, 20:1w9, 20:5w3, 22:1w11, 22:1w9, and 22:6w3) in the total lipids, as well as three others (18:3w3, 18:4w3, and 20:4w6) (Figure 7). In comparing the fatty acids in the lipids among lots of herring (Figure 8), there were significant differences in 16:1w7, 18:1w9, 18:2w6, 18:3w3, 20:1w11, 20:1w9, r20:1, 20:5w3, 22:1w11, 22:1w9, and r22:1, despite a lack of significant difference among these lots in proximate composition (i.e., total lipid, total protein, water content and calorimetry) (Castellini et al., 2001). With the exception of 18:3w3, these were all "indicator" fatty acids.

Using CART, all of the herring and pollock samples could be distinguished from each other by their proportion of 14:0 (Figure 9). All the herring sampled from the two different lots used could be distinguished from each other by their proportion of 20:1w9 (Figure 10). Approximately 78% of the pollock used were correctly identified by lot using the proportion of first 16:1w7 and then 14:0 (Figure 11).

When all of the harbor seal blubbers were analyzed by CART, the "best fit" tree had five terminal nodes and a total error rate (samples not correctly placed in a terminal node according to diet) of 52 out of 158 observations (Figure 12). Nine of the 60 blubber samples from seals fed herring were placed incorrectly, as were 15 of the 40 samples from seals fed the mixed herring/pollock diet, and 28 of the 58 blubber samples from seals fed pollock. Thus, 67% of all blubber samples were correctly classified by diet using combined fatty acid profiles of inner and

outer blubber layers for both mid- and end-point samples. The fatty acid 18:1w7 used at the root node of this tree separated the majority of the herring and mixed samples (left side of tree) from more than half of the pollock samples (right side of tree). Of the samples on the right side, the same fatty acid (14:0) used to separate herring and pollock samples (Figure 9) was chosen by the algorithm to separate blubber samples from seals fed these two diets, although at a slightly lower proportion. The remaining blubber samples from seals fed pollock on the left side of the tree were not distinguished from the blubber samples from seals on the herring and mixed diets. However, most of the blubber samples from the seals fed herring and mixed diet on the left side of the tree were separated from each other, first using the proportion of 17:0 and finally 20:4w3.

Using only the endpoint samples, 85% (68/80) of the blubber samples were correctly classified by diet using the combined fatty acid profiles of inner and outer blubber samples (Figure 13). This tree had seven terminal nodes. The root node, the terminal node on the right, and the first node on the left used the same fatty acids (18:1w7, 14:0, and 17:0, respectively) in nearly the same proportions as the tree constructed using all blubber samples (Figure 12). The fatty acid 18:1w7 was selected again at the second node on the left. The final two terminal nodes on the left side were accomplished using 22:5w3 to separate blubber samples from seals fed herring, pollock and mixed diets.

Blubber samples from the mid-point of the feeding trials were not clearly distinguishable by diet (Figure 14). In this example, which had four terminal nodes, slightly more than half of the samples (46/72) were correctly classified by diet, and CART could not differentiate blubber samples from seals on the pollock diet from those consuming the herring and mixed diets. This tree used some of the same fatty acids, 17:0 at the root node, followed by 18:1w7 and then 22:5w3, as the previous two trees to discriminate between blubber samples from seals consuming

the three different diets. However, only 59% of these samples could be correctly identified by diet.

When only outer blubber layer samples were used, the error rate was improved compared to using inner blubber layer or all blubber samples combined, with 75% (56/75) of the observations being correctly classified by diet, regardless of whether blubber samples were from the mid- or end-point of a feeding trial (Figure 15). This tree began using 17:0 at the root node, which resulted in a terminal node on the left composed of blubber samples from the seals on the mixed diet. On the right side, 18:4w3 was used to spilt most of the remaining blubber samples from the seals fed mixed diets (on right branch) from blubber samples of seals fed herring (on left intermediate branch). A terminal split using 20:4w3 at the left intermediate branch point separated most of the blubber samples from seals fed pollock from those fed herring. On the right branch, 18:1w7 and then 14: were chosen by the alogorithm to distinguish blubber samples of seals fed pollock from those consuming a mixed diet of herring and pollock.

The same percentage of observations were correctly classified by diet when the analysis was performed using only the inner blubber samples (Figure 16). Once again, 75% (49/65) of the observations were placed in the appropriate terminal node according to the species composition of the diet. In contrast to the tree for the outer blubber layer, this tree began with 18:1w7 at the root node instead of 17:0, but 17:0 was then selected by the algorithm at a subsequent node. In the first split, 15/16 blubber samples from seals fed the mixed diet were separated into a terminal node, compared to 10/20 for the tree using outer blubber samples. Of the observations on the other side of the tree, using 17:0 separated 16 out of 25 of the blubber samples from seals fed herring into a terminal node which also contained five samples from seals fed pollock. Note that five of the blubber samples from seals fed herring were already split at the root node, so the

actual proportion of samples in the first terminal node on the left is 16/20. For the remaining observations on the left side of the tree, all but two out of 20 blubber samples were correctly classified by diet using 16:0.

Discussion

Nine of the 58 fatty acids identified in the seal blubber and their prey were chosen by the CART algorithm to distinguish blubber samples from seals fed different diets, as well as prey species, and lots of fish within a species. Of these fatty acids, 20:4w3, and 22:5w3 were used in two trees, and 14:0, 17:0, 18:1w7 were used in five trees. All of these are indicator fatty acids that have been shown to be useful in determining the diet of carnivores. In addition, these correspond to some of the same fatty acids that could be used to classify harbor seals by location within Prince William Sound, Alaska, and to separate their prey by species and age/size class (Iverson and Frost, 1996).

The proportion of individual fatty acids in the blubber is not only a function of the fatty acid composition of the diet, but can also be influenced by *de novo* fatty acids synthesis, as well as selective deposition or mobilization of individual fatty acids as animals gain or lose mass. Lipids are synthesized, deposited, or mobilized according to complex feedback mechanisms that are under dietary and hormonal control. In a simplified example, a seal that was consuming a surplus of calories would deposit more fatty acids in its blubber than a seal that was consuming only enough calories for its maintenance requirements. The lipids deposited by the seal would derive both from direct deposition of intact dietary lipids and *de novo* synthesis of lipids from excess dietary carbohydrates and protein. The proportions of fatty acids in the tissue of the seal at maintenance caloric intake would also be influenced by diet to some degree, but there would

be turnover (mobilization) of lipids to be used for energy as well as production of hormones, cell membranes, etc., which could alter the relative proportions of individual fatty acids in the blubber over time. However, it is likely that, the longer the seal remained at maintenance levels on a given diet, the more its tissue fatty acid profile would reflect that diet.

The fatty acid composition of Atlantic cod changed significantly in the direction of the patterns found in its prey within three weeks, despite being on low fat diets and even when the total body fat of the cod did not change (Kirsch et al., 1998). The results from our study indicate that fatty acid composition changes more slowly in harbor seals, perhaps due to a slower turnover rate of fatty acids in the blubber. When the mid- and end-point samples were combined, significant differences between diets were evident, but these changes were not consistently found when examining only the mid- or end-point samples, or using only the inner or only the outer blubber layers.

The period of time needed for the lipids in an animal's blubber to resemble those in its diet is a function of the caloric composition of the diet relative to the energy requirements of the animal. The mass of food consumed by seals during the various diet trials was not constant. It varied because they were fed to a level of satiation as determined by handlers. The seals altered their food intake and digestive physiology to match seasonal and dietary changes experienced in this study (Castellini et al., 2001). Thus, some seals may have consumed proportionately more or less of a given diet (i.e., herring or pollock) compared to the other seals. The body mass of the seals did vary during the course of the study. The older seals gained mass and body fat on both pollock and herring diets in the fall (molting season), whereas younger seals either lost or showed no change in body fat regardless of diet, but gained body mass on herring (Castellini et al., 2001). In the winter, all seals lost mass on pollock but gained mass on herring. During this

season, younger seals lost body fat on herring and gained body fat on pollock, whereas older seals lost body fat regardless of diet. Finally, during the summer, older seals lost mass and body fat on pollock, but gained it on herring, while younger seals increased mass regardless of diet, but only gained body fat on pollock. Some of these changes in body mass and lipid were likely related to the age of the seals, or more specifically, to the energetic requirements of young animals that were still growing.

Castellini et al. (2001) found that dry matter digestibility was greater for herring than for pollock. The seals may have needed to consume more (on a dry weight basis) on the pollock diet than the herring diet to obtain sufficient calories and/or nutrients because of the lower digestibility of pollock. Studies with other mammalian species demonstrated that animals tend to consume more when feed quality is low in some nutrient (Church and Pond, 1982), which may result in mass gain due to excess calories. Pollock have a lower total lipid content than herring, and, for the fish used in this study, were lower in their proportions of certain indicator fatty acids (14:0, 16:0, 18:1w9, r20:1 and r22:1), but higher in the proportions of others (18:0, 20:1w9, 20:4w6, and 22:6w3). As with other species studied, differences in the fatty acid profiles of herring and pollock are a function of their diet (Cowey and Sargent, 1972), which can change with age and size.

It is difficult to determine whether some of the more equivocal findings of this study are related to environmental factors (seasons), individual variation in food consumption and body condition, or to the variation in the fatty acid profiles of the lots of herring and pollock. However, Iverson et al. (1997) found that while the fatty acid signature of a fish species could vary with the age/size class of the fish, individual species of fish were distinguishable from other species regardless of the age/size class analyzed. Therefore, it is likely that the variation seen in

the blubber of the harbor seals was related to differing energetic requirements of younger versus older animals and/or to seasonal variation in lipid metabolism.

A study on the effect of ambient temperature on preference for dietary unsaturated fatty acids in the Djungarian hamster (*Phodopus sungorus*) concluded that thermal environment affects dietary fatty acid choice (Hiebert et al., 2000). The change in diet could be accomplished without any choice on the part of the animal. Exposure to cold induces an increase in the polyunsaturated content of plants in winter. Thus, by eating whatever plant foods were available in the environment, the proportion of unsaturated fats in the diet would increase. Seasonal shifts in dietary preference could also be driven by high reproductive requirements for protein, rather than by fatty acid saturation. The authors speculated that the regional heterothermy that develops in endotherms during cold exposure, in which skin appendage temperatures fall below core temperature, affects membrane function and/or lipid mobilization sufficiently to stimulate a change in dietary preference." The effect of environment (i.e., seasonal differences) on the lipid composition of seal blubber cannot be dismissed and merits further study. However, using the repeated crossover design ensured that all groups of seals experienced both herring and pollock diets for all seasons.

Returning to the issue of prey quality, excess dietary carbohydrates and proteins can be converted to lipids and deposited in blubber. Due to the limitations of the enzymes that regulate *de novo* lipid synthesis in mammals (i.e., where double bonds can be inserted), the fatty acid composition of seals consuming an excess of pollock to obtain sufficient amounts of some essential nutrient or lipid might have greater proportions of such fatty acids as 16:0, 18:0, and 18:1w9 compared to their diet, because these can be synthesized from non-lipid precursors. Growing animals may preferentially use or mobilize certain fatty acids needed for energy, the

production of hormones, etc. The variation in the ages, reproductive status, and prey consumption, coupled with the relatively small sample sizes within groups of seals, may have been confounding variables in determining how the fatty acid composition of seal blubber changes with diet. Nevertheless, it is apparent that, as with numerous studies on other carnivore species, the fatty acid composition of harbor seals is related to the fatty acid composition of their prey. This study suggests that the fatty acid composition of both the inner and outer portions of harbor seal blubber changes in response to the composition of the diet. The changes were somewhat evident after two months on a particular diet, but much more predictable after four months.

Adams (2000) found that Steller sea lions could be classified by geographic location based on the fatty acid composition of either their blubber or milk, which could also be distinguished from each other. This suggests that, even when physiological requirements may be influencing lipid metabolism (as with selective mobilization of lipids for lactation), the fatty acid composition of pinniped blubber reflects the composition of their diet. Iverson et al. (1997) have already demonstrated that harbor seal diets vary geographical on a fine scale. If the diet of harbor seals in the wild changes with season as well, either due to changes in the species composition of the prey available, or due to preferential selection of individual prey items, these changes should be evident in the blubber of the seals provided they maintain this diet for at least two months or longer. With adequate sampling of the potential prey available to harbor seals over time and space, it should be possible to determine both the spatial and temporal foraging patterns of harbor seals in Alaska based on analysis of the fatty acid composition of their blubber.

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Literature Cited

Adams, T.C. 2000. Foraging differences and early maternal investment in adult female Alaskan Steller sea lions (*Eumetopias jubatus*). Ph.D. Dissertation, Texas A&M University, College Station, TX.

Castellini, M.A., Castellini, J.M., and Trumble, S.J. 2001. Harbor seal Recovery. Phase II: Controlled Studies of Health and Diet. Restoration Project 01341 Final Report.

Church, D.C. and Pond, W.G. 1982. Basic Animal Nutrition and Feeding. 3rd Edition. John Wiley and Sons, Inc., New York.

Cowey, C.B. and Sargent, J.R. 1972. Fish nutrition. *In* Advances in marine biology. Vol. 10. *Edited by* Sir F. Yonge and Sir M. Yonge. Academic Press, London, U.K. pp. 383-492.

Folch, J., Lees, M., and Stanley, G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**: 497-509.

Francis, R.C., and Hare, S.R. 1994. Decadal-scale regime shifts in the large marine ecosystems of the North-east Pacific: a case of historical science. Fish. Oceanogr. **3**:279-291.

Frost, K.J. and Lowry, L.F. 1986. Sizes of walleye pollock, *Theragra chalcogramma*, consumed by marine mammals in the Bering Sea. Fish. Bull. **84**:192-197.

Frost, K.J., Lowry, L.F., Small, R.J., and Iverson, S.J. 1996. Monitoring, Habitat Use, and Trophic Interactions of Harbor Seals in Prince William Sound, Alaska. *Exxon Valdez* Oil Spill Restoration Project Annual Report (Restoration Project 95064), Alaska Department of Fish and Game, Division of Wildlife Conservation, Fairbanks, Alaska.

Härkönen, T. and Harding, K.C. 2001. Spatial structure of harbour seal populations and the implications thereof. Can. J. Zool. **79**: 2115-2127.

Hiebert, S.M., Fulkerson, E.K., Lindermayer, K.T., and McClure, S.D. 2000. Effect of temperature on preference for dietary unsaturated fatty acids in the Djungarian hamster (*Phodopus sungorus*). Can. J. Zool. **78**: 1361-1368.

Hoover, A.A. 1988. Harbor seal. Pp 125-157 in J.W. Lentfer (ed) Selected Marine Mammals of Alaska: Species Accounts with Research and Management Recommendations. Marine Mammal Commission, Washington, D.C.

Iverson, S.J. 1993. Milk secretion in marine mammals in relation to foraging: can milk fatty acids predict diet? *In* Marine Mammals: Advances in Behavioral and Population Biology: the Proceedings of a Symposium held at the Zoological Society of London on 9th and 10th April 1992, Symposium Zoological Society London No. 66. Edited by I.L. Boyd. Clarendon Press, Oxford. pp. 264-291.

Iverson, S.J., Sampugna, J., and Oftedal, O.T. 1992. Positional specificity of gastric hydrolysis of long-chain n-3 polyunsaturated fatty acids of seal milk triglycerides. Lipids, **27**: 870-878.

Iverson, S.J. and Frost, K.J. 1996. Appendix A: The use of fatty acid signatures to investigate foraging ecology and food webs in Prince William Sound, Alaska: harbor seals and their prey. In Monitoring, habitat use, and trophic interactions of harbor seals in Prince William Sound, Alaska. *Exxon Valdez* Oil Spill Restoration Science Study Number 95064, 1996 Annual Report. Alaska Dept. Fish and Game, Fairbanks, AK.

Iverson, S.J., Frost, K.J., and Lowry, L.F. 1997. Fatty acid signatures reveal fine scale structure of foraging distribution of harbor seals and their prey in Prince William Sound, Alaska. Mar. Ecol. Prog. Ser. **151**: 255-271.

Kerr, R.A. 1992. Unmasking a shifty climate system. Science **255**:1508-1.

Kirsch, P.E., Iverson, S.J., Bowen, W.D., Kerr, S.R., and Ackman, R. G. 1998. Dietary effects on the fatty acid signature of whole Atlantic cod (Gadus morhua). Can. J. Fish. Aquat. Sci. **55**: 1378-1386.

Lowry, L.F., Frost, K.J., Calkins, D.G., Swartzman, G.L., and Hills, S. 1982. Feeding habits, food requirements, and status of Bering Sea marine mammals. Document 19. North Pacific Fishery Management Council, Anchorage, AK. Unpublished.

Pitcher, K.W. 1980a. Food of the harbor seal, *Phoca vitulina richardsi*, in the Gulf of Alaska. Fish Bull. **79**:544-549.

Pitcher, K.W. 1980b. Stomach contents and feces as indicators of harbor seal, *Phoca vitulina*, foods in the Gulf of Alaska. U.S. Natl. Mar. Fish. Serv. Fish Bull. **78**(3):797-798

Pitcher, K.W. and Calkins, D. 1979. Biology of the harbor seal (*Phoca vitulina richardsi*), in the Gulf of Alaska. U.S. Dept. Commer., NOAA, OCSEAP Final Rep. 19(1983):231-310; Pitcher, K.W. 1980. Food of the harbor seal, *Phoca vitulina richardsi*, in the Gulf of Alaska. U.S. Natl. Mar. Fish. Serv. Fish Bull. **78**(2):544-549.

Reeves, R.R., Stewart, B.S., and Leatherwood S. 1992. The Sierra Club Handbook of Seals and Sirenians. San Francisco, CA.

Ridgeway, S.H and Harrison, R.H. (eds.). 1981. Handbook of Marine Mammals. Volume 2: Seals. Academic Press, London, England. 359pp.

Trenberth, K.E. and Harrell, J.W.. 1995. Decadal coupling atmospheric-ocean variations in the North Pacific Ocean. p. 15-24 in R.J. Beamish (ed) Climate change and northern fish populations. Can. Spec. Publ. Fish. Aquat. Sci. 121.

Wooster, W.S. 1992. Is it food? An Overview. p1-3 *in* Is It Food? Addressing Marine Mammal and Seabird Declines. Alaska Sea Grant Report 93-01.

Figure Legends

Figure 1. Comparison of selected fatty acids from the outer blubber layer of harbor seals by group. There were significant differences among the three groups ($p < 0.05$, ANOVA) in all fatty acids except 20:1n-9, 22:1n-11, and 22:1n-9. There were no significant differences between Groups B and C, except with 18:1n-9, 18:2n-6, 18:3n-3, 20:4n-6, and 22:6n-3. Groups A and B differed significantly in the proportion of 18:1n-9. Bars and lines indicate one standard deviation.

Figure 2. Comparison of selected fatty acids from the outer blubber layer of seals by diet. There were significant differences ($p < 0.05$, ANOVA) among diets in 16:0 (H > M; M < P), 16:1n-7 (M < P), 18:1n-9 (H < M; M > P), 18:3n-3 (H > M; H > P), 18:4n-3 (H > M), 20:1n-9 (H > M; M < P), r20:1 (H > M; M < P), 20:4n-6 (H > M; M < P), 20:5n-3 (H > M; M < P), r22:1 (H > M), and 22:6n-3 (H > M; M < P) where H is herring, P is pollock and M is a mixed diet of herring and pollock. Bars and lines indicate one standard deviation.

Figure 3. Comparison of selected fatty acids from the inner blubber layer of seals by diet. There were significant differences ($p < 0.05$, ANOVA) among diets in 14:0 (H > P; M > P); 18:0 (M < P); 18:3n-3 (H > P; M > P); 18:4n-3 (H > P); 20:1n-9 (H < M); 20:4n-6 (H > M; M < P); and r22:1 (H > P) where H is herring, P is pollock and M is a mixed diet of herring and pollock. Bars and lines indicate one standard deviation..

Figure 4. Comparison of selected fatty acids from the blubber of Group A seals by blubber layer and diet. There were significant differences between diets in both the inner and outer blubber for 14:0, 18:3n-3, 18:4n-3, and r22:1. Bars and lines indicate one standard deviation.

Figure 5. Comparison of selected fatty acids from Group B seals by blubber layer and diet. The fatty acids 14:0, 16:1n-7, and 20:1n-11 were significantly different ($p < 0.05$, ANOVA) between layers according to diet. Bars and lines indicate one standard deviation.

Figure 6. Comparison of selected fatty acids in food by species. There were significant differences between species ($p < 0.05$, ANOVA) in the amounts of 14:0, 16:0, 18:0, 18:1w9, 20:1w9, r20:1, 20:4w6, and 22:6w3. Bars and lines indicate one standard deviation.

Figure 7. Comparison of selected fatty acids in pollock samples by lot. There were significant differences in the amounts of all of the indicator fatty acids expect the r20:1 and r22:1. Bars and lines indicate one standard deviation.

Figure 8. Comparison of selected fatty acids in herring samples by lot. There were significant differences in the amounts of all of the indicator fatty acids expect 14:0, 16:0, 18:0, 18:4w3, 20:4w6, and 22:6w3. Bars and lines indicate one standard deviation.

Figure 9. Classification and Regression Tree (CART) for all fish samples. This CART, with two terminal nodes, correctly classified 100% of the fish by species using their proportions of 14:0. Each intermediate and terminal node is labeled according to the grouping with the largest

number of observations with that label in that node. The fatty acid listed at each branching point is the variable chosen by the algorithm to create the split: > and < values indicate the optimal splitting level (weight %) of the fatty acid. Fractions under each node indicate the number of misclassifications over the total number of observations in that node. Box plots within each node illustrate the distribution of the data

Figure 10. CART for all herring samples. Using the proportions of 20:1w9, 100% of the herring samples were correctly classified by the lot from which they derived. Details of the tree are as described in Figure 9.

Figure 11. CART for all pollock samples. This CART, with three terminal nodes, had a total error rate of seven out of 32 observations. Thus, 78% of the pollock samples were correctly classified according to the lot from which they were derived. Details of the tree are as in Figure 9.

Figure 12. (CART) for all harbor seal blubber samples. This CART, with five terminal nodes, had a total error rate of 52 out of 158 observations. Thus, 67% of all seals were correctly classified according to diet. Details of the tree are as described in Figure 9.

Figure 13. CART for harbor seal blubber samples taken at the end-point of each feeding trial in Table 1. This CART had seven terminal nodes and an error rate of 12 out of 80 observations. Thus, 85% of the seals were correctly classified according to diet. Details of the tree are as described in Figure 9.

Figure 14. CART for harbor seal blubber samples taken at the mid-point of each feeding trial in Table 1. This CART had four terminal nodes and a total error rate of 32 out of 78 observations. Only 59% of the seals could be correctly classified according to diet after approximately two months. Details of the tree are as described in Figure 9.

Figure 15. CART for the outer portion of the harbor seal blubber layer. This CART had six terminal nodes and a total error rate of 19 out of 75 observations. Thus, 75% of the seals could be correctly classified according to diet based on the fatty acid composition of the outer portion of their blubber. Details of the tree are as described in Figure 9.

Figure 16. CART for the inner portion of harbor seal blubber samples. This CART had four terminal nodes and a total error rate of 16 out of 65 observations. Thus, 75% of the seals could be correctly classified according to diet based on the fatty acid composition of the inner portion of their blubber. Details of the tree are as described in Figure 9.

Table 1. Feeding trial study design for seals in groups A and B.

Period	100% Herring Diet	100% Pollock Diet	Metabolic Season
Sep. 1998 - Jan. 1999	Group A	Group B	Molting
Jan. – May 1999	Group B	Group A	Spring
May – Sep. 1999	Group A	Group B	Breeding
Sep. 1999 – Jan. 2000	Group B	Group A	Molting
Jan. – May 2000	Group A	Group B	Spring
May – Sep. 2000	Group B	Group A	Breeding

Table 2. Dominant and indicator fatty acids identified in the blubber of harbor seals and their prey. Values are means ± SD.

Fatty acid	Weight (% of Total Fatty Acids)							
	Group A Seals		Group B Seals		Group C Seals		Prey	
	Inner Blubber (n = 29)	Outer Blubber (n = 29)	Inner Blubber (n = 30)	Outer Blubber (n = 30)	Inner Blubber (n = 20)	Outer Blubber (n = 20)	Herring (n = 30)	Pollock (n = 34)
C14:0	4.48 ± 0.94	3.86 ± 0.47	4.49 ± 1.19	3.09 ± 0.88	4.80 ± 0.94	3.23 ± 0.61	8.25 ± 1.13	4.02 ± 0.95
C16:0	7.79 ± 1.46	6.54 ± 1.03	8.34 ± 1.87	4.62 ± 1.85	7.93 ± 1.82	4.46 ± 1.42	15.78 ± 2.38	11.91 ± 2.78
C16:1n-7	11.88 ± 3.74	16.23 ± 3.70	9.39 ± 1.48	12.37 ± 1.20	9.83 ± 1.46	13.03 ± 1.72	5.75 ± 0.88	5.15 ± 1.94
C17:0	0.28 ± 0.08	0.25 ± 0.09	0.27 ± 0.07	0.14 ± 0.05	0.25 ± 0.09	0.29 ± 0.18	0.39 ± 0.15	1.08 ± 0.62
C18:0	0.94 ± 0.28	0.65 ± 0.18	1.02 ± 0.25	0.47 ± 0.20	0.84 ± 0.22	0.44 ± 0.21	1.51 ± 0.32	2.41 ± 0.89
C18:1n-11	4.44 ± 1.83	4.86 ± 2.10	5.00 ± 2.49	9.28 ± 3.72	5.23 ± 2.61	6.68 ± 3.25	0.42. ± 0.20	1.49 ± 0.65
C18:1n-9	20.62 ± 4.00	22.68 ± 1.78	19.24 ± 2.78	22.68 ± 3.53	19.21 ± 2.44	27.28 ± 6.44	13.66 ± 3.68	9.80 ± 4.49
C18:1n-7	3.42 ± 0.42	3.66 ± 0.28	3.41 ± 0.43	3.03 ± 0.28	2.90 ± 0.16	3.04 ± 0.31	2.47 ± 0.73	4.03 ± 1.93
C18:2n-6	1.08 ± 0.11	1.13 ± 0.12	1.10 ± 0.14	1.42 ± 0.19	1.12 ± 0.13	1.28 ± 0.15	0.82 ± 0.15	0.86 ± 0.19
C18:3n-3	0.65 ±0.12	0.61 ±0.08	0.64 ±0.12	0.61 ±0.05	0.68 ±0.10	0.58 ±0.06	0.68 ±0.24	0.62 ±0.24
C18:4w3	1.12 ± 0.25	1.01 ± 0.15	1.13 ± 0.27	0.87 ± 0.21	1.15 ± 0.24	0.86 ± 0.10	1.63 ± 0.40	1.87 ± 0.77
C20:1n-11	10.06 ± 3.81	6.54 ± 3.15	9.69 ± 2.60	5.79 ± 1.81	10.35 ± 2.80	5.77 ± 2.49	9.38 ± 4.83	8.71 ± 3.64
C20:1n-9	3.99 ± 1.68	3.77 ± 1.74	5.17 ± 2.30	6.56 ± 1.34	5.91 ± 2.08	6.66 ± 1.14	2.70 ± 0.88	3.44 ± 1.57
R20:1	2.81 ± 1.32	1.90 ± 0.88	2.36 ± 1.29	0.98 ± 0.59	2.13 ± 1.22	0.92 ± 0.58	4.02 ± 2.27	2.77 ± 1.02
C20:4n-6	0.37 ±0.08	0.43 ±0.09	0.35 ±0.05	0.35 ±0.02	0.31 ±0.03	0.33 ±0.02	0.30 ±0.09	0.58 ±0.23
C20:4n-3	0.47 ± 0.10	0.45 ± 0.06	0.51 ± 0.12	0.48 ± 0.08	0.49 ± 0.11	0.43 ± 0.08	0.45 ± 0.10	0.53 ± 0.22
C20:5n-3	4.86 ± 1.25	5.29 ± 1.16	4.58 ± 1.18	3.97 ± 0.75	4.39 ± 0.90	3.78 ± 0.28	6.43 ± 1.87	9.86 ± 2.42
C22:1n-11	3.70 ± 1.58	1.94 ± 1.16	3.52 ± 1.05	1.53 ± 1.00	3.56 ± 1.28	1.44 ± 1.06	11.57 ± 4.28	10.79 ± 6.07
C22:1w9	0.36 ± 0.12	0.20 ± 0.09	0.31 ± 0.11	0.18 ± 0.09	0.33 ± 0.12	0.16 ± 0.05	0.76 ± 0.53	1.00 ± 0.58
R22:1	11.26 ± 6.09	11.75 ± 11.23	12.63 ± 5.14	11.15 ± 10.29	12.20 ± 6.84	9.61 ± 7.59	21.66 ± 11.77	10.78 ± 3.14
C22:5n-3	3.42 ± 0.79	2.98 ± 0.46	4.44 ± 0.64	3.83 ± 0.43	3.98 ± 0.46	3.40 ± 0.69	0.591 ± 0.12	1.08 ± 0.41
C22:6n-3	6.71 ± 1.00	6.62 ± 0.63	7.26 ± 0.90	7.08 ± 0.37	6.65 ± 0.67	6.36 ± 0.61	5.92 ± 1.41	11.14 ± 2.78

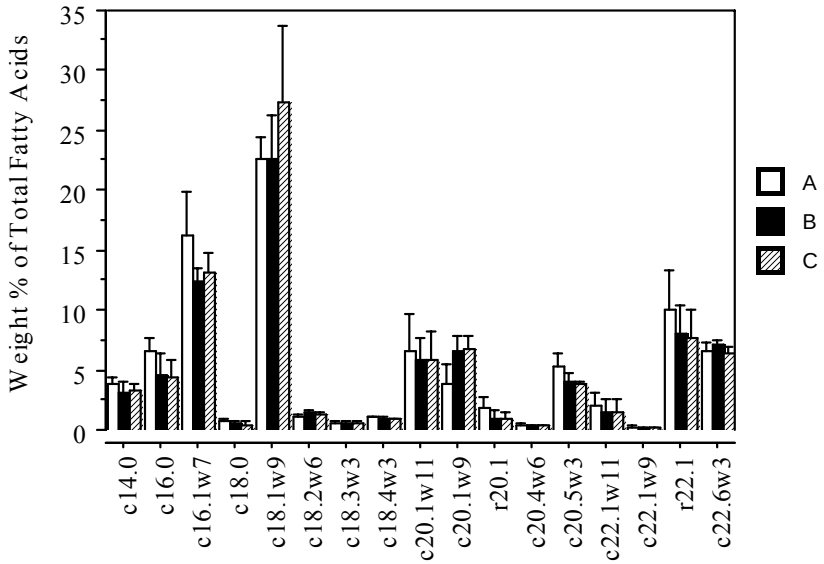
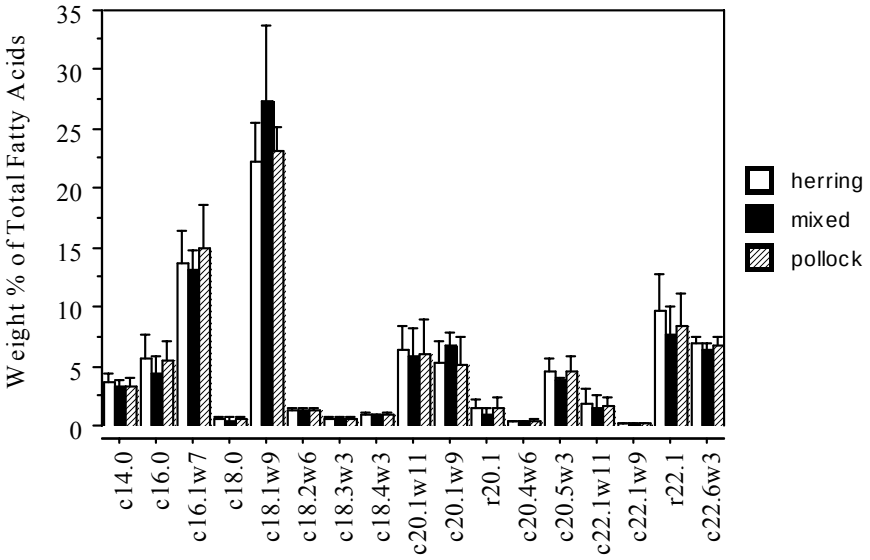


Figure 1. Comparison of selected fatty acids from the outer blubber layer of harbor seals by group. There were significant differences among the three groups ($p < 0.05$, ANOVA) in all fatty acids except 20:1n-9, 22:1n-11, and 22:1n-9. There were no significant differences between Groups B and C, except with 18:1n-9, 18:2n-6, 18:3n-3, 20:4n-6, and 22:6n-3. Groups A and B differed significantly in the proportion of 18:1n-9. Bars and lines indicate one standard deviation.



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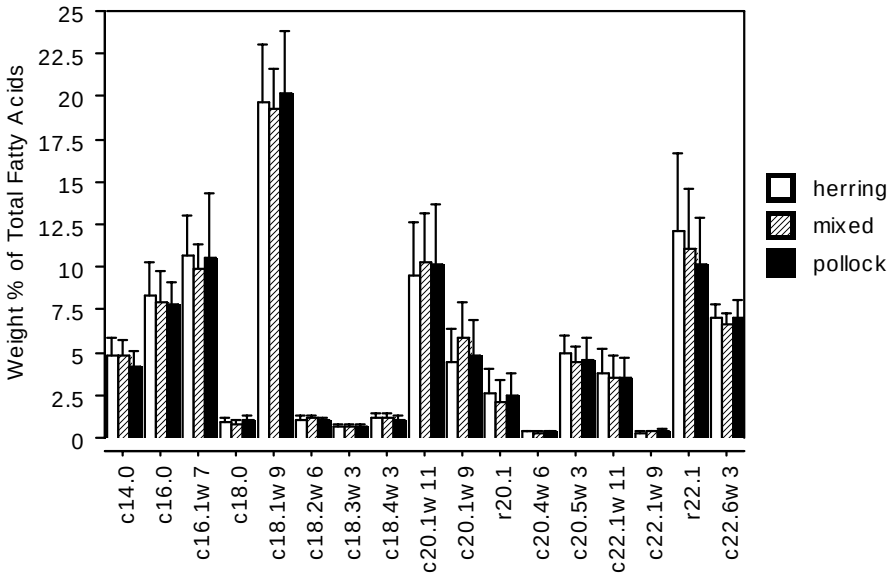

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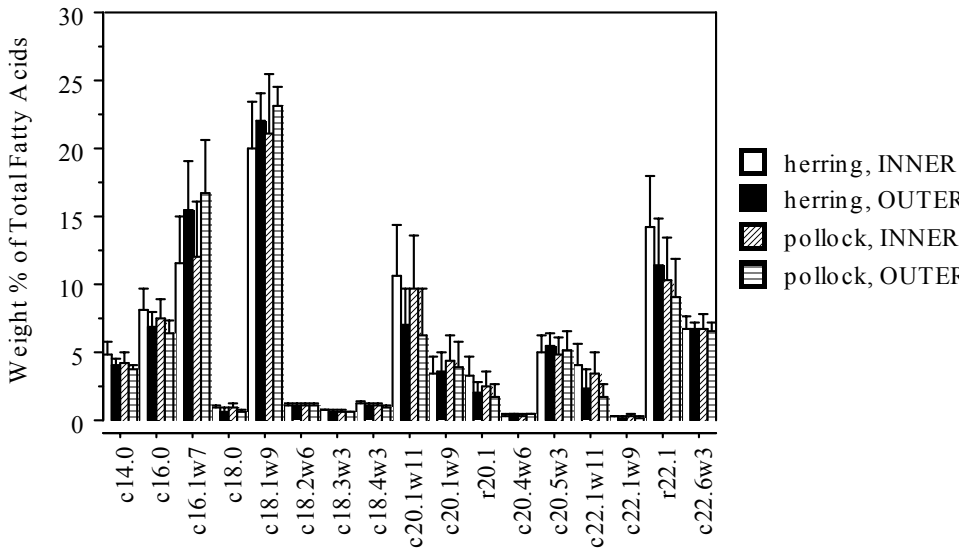

Figure 4. Comparison of selected fatty acids from the blubber of Group A seals by blubber layer and diet. There were significant differences between diets in both the inner and outer blubber for 14:0, 18:3n-3, 18:4n-3, and r22:1. Bars and lines indicate one standard deviation.

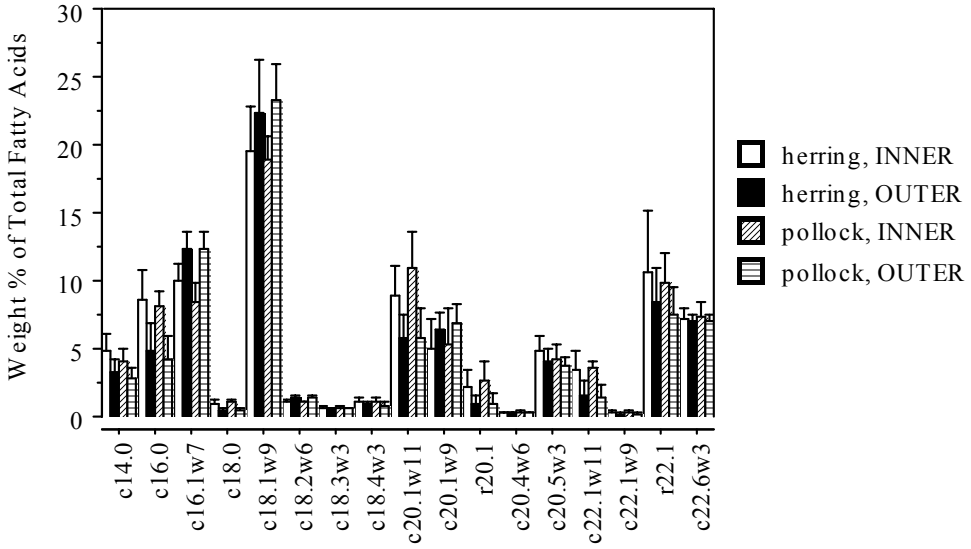

Figure 5. Comparison of selected fatty acids from Group B seals by blubber layer and diet. The fatty acids 14:0, 16:1n-7, and 20:1n-11 were significantly different ($p < 0.05$, ANOVA) between layers according to diet. Bars and lines indicate one standard deviation.

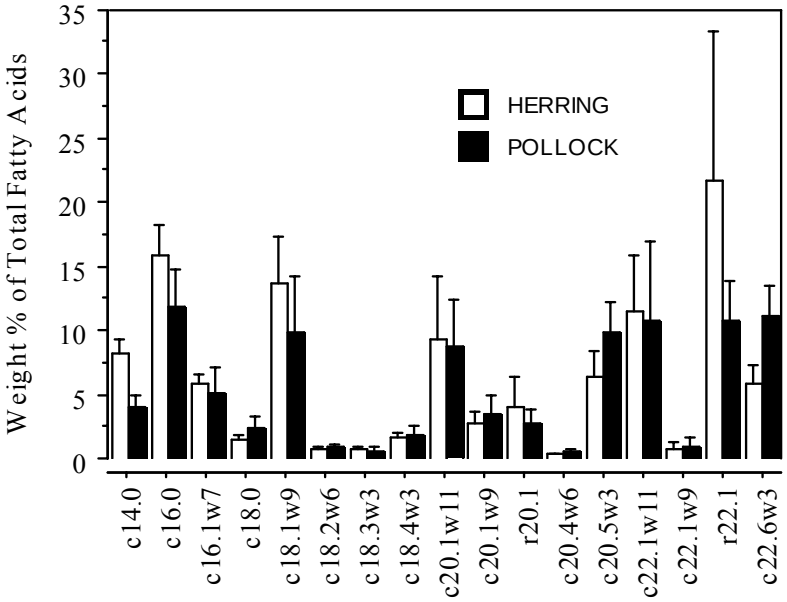

Figure 6. Comparison of selected fatty acids in food by species. There were significant differences between species ($p < 0.05$, ANOVA) in the amounts of 14:0, 16:0, 18:0, 18:1w9, 20:1w9, r20:1, 20:4w6, and 22:6w3. Bars and lines indicate one standard deviation.

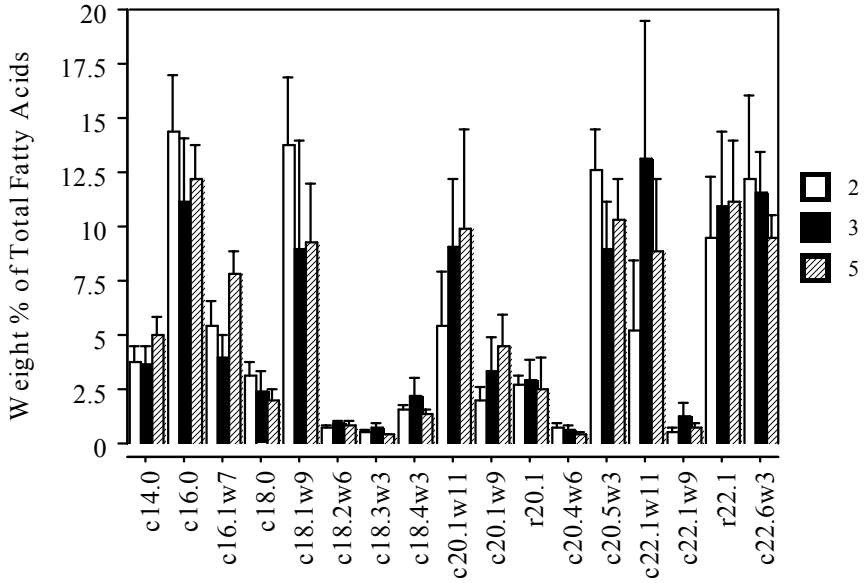

Figure 7. Comparison of selected fatty acids in pollock samples by lot. There were significant differences in the amounts of all of the indicator fatty acids expect the r20:1 and r22:1. Bars and lines indicate one standard deviation.

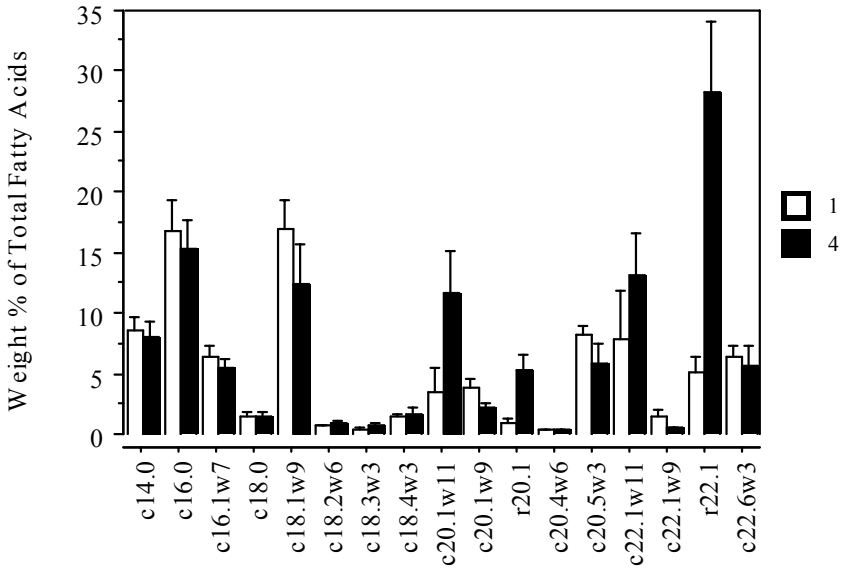

Figure 8. Comparison of selected fatty acids in herring samples by lot. There were significant differences in the amounts of all of the indicator fatty acids expect 14:0, 16:0, 18:0, 18:4w3, 20:4w6, and 22:6w3. Bars and lines indicate one standard deviation.

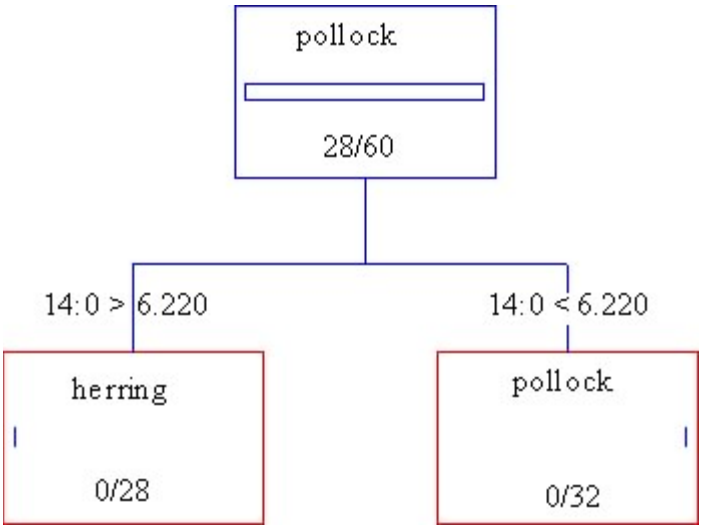

Figure 9. Classification and Regression Tree (CART) for all fish samples. This CART, with two terminal nodes, correctly classified 100% of the fish by species using their proportions of 14:0. Each intermediate and terminal node is labeled according to the grouping with the largest number of observations with that label in that node. The fatty acid listed at each branching point is the variable chosen by the algorithm to create the split: > and < values indicate the optimal splitting level (weight %) of the fatty acid. Fractions under each node indicate the number of misclassifications over the total number of observations in that node. Box plots within each node illustrate the distribution of the data

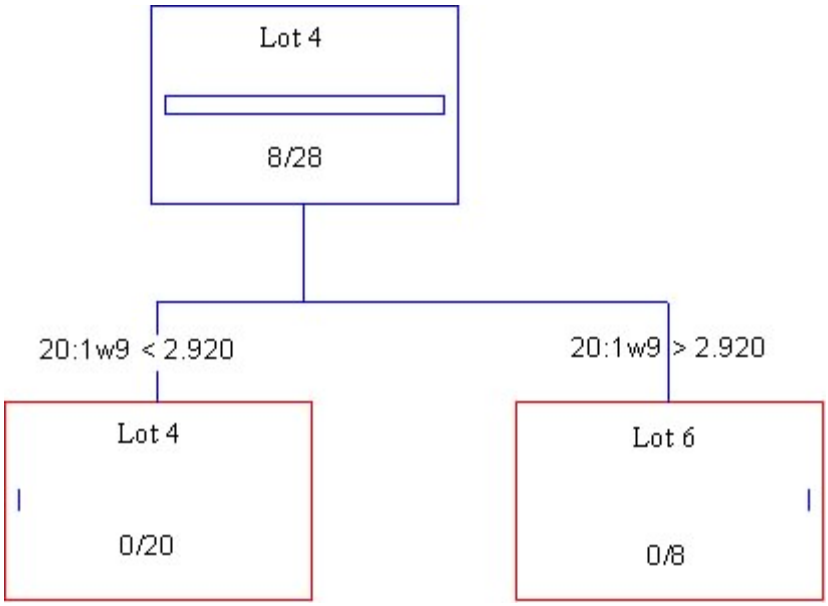

Figure 10. CART for all herring samples. Using the proportions of 20:1w9, 100% of the herring samples were correctly classified by the lot from which they derived. Details of the tree are as described in Figure 9.

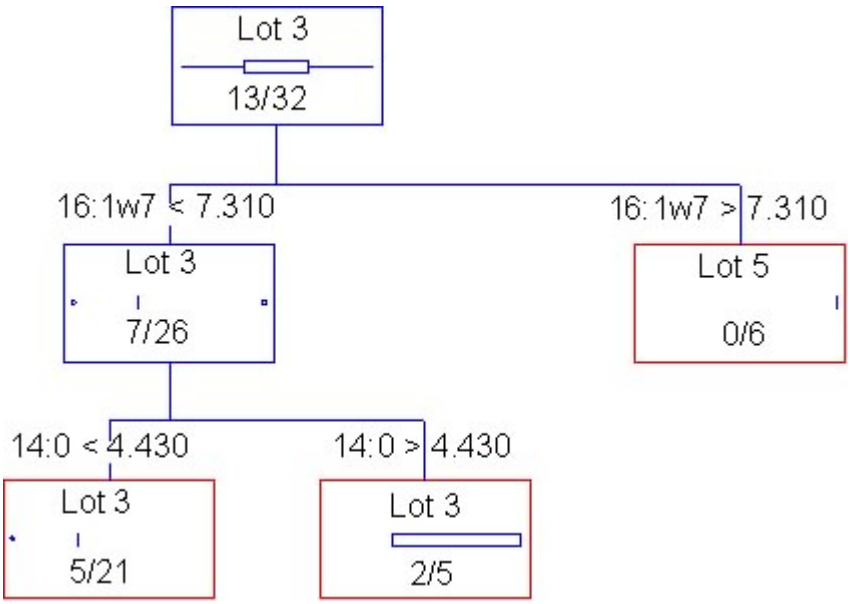

Figure 11. CART for all pollock samples. This CART, with three terminal nodes, had a total error rate of seven out of 32 observations. Thus, 78% of the pollock samples were correctly classified according to the lot from which they were derived. Details of the tree are as in Figure 9.

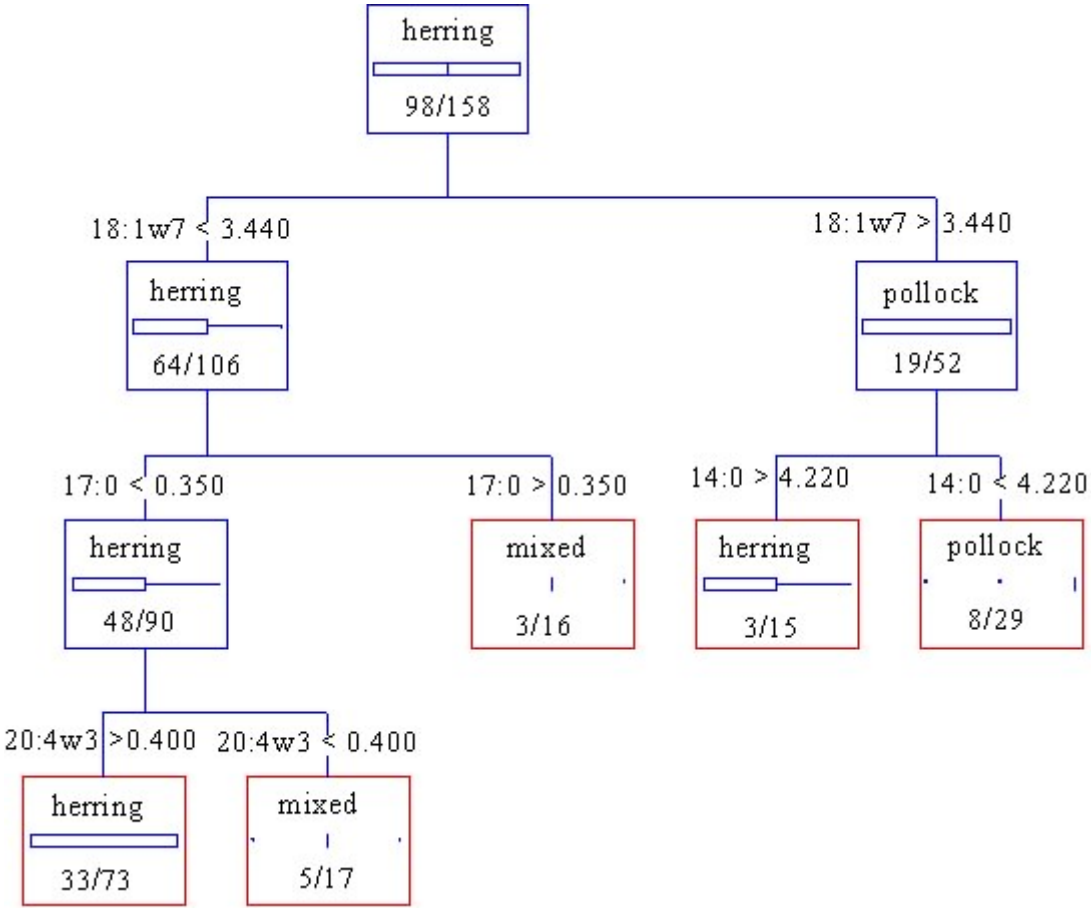

Figure 12. (CART) for all harbor seal blubber samples. This CART, with five terminal nodes, had a total error rate of 52 out of 158 observations. Thus, 67% of all seals were correctly classified according to diet. Details of the tree are as described in Figure 9.

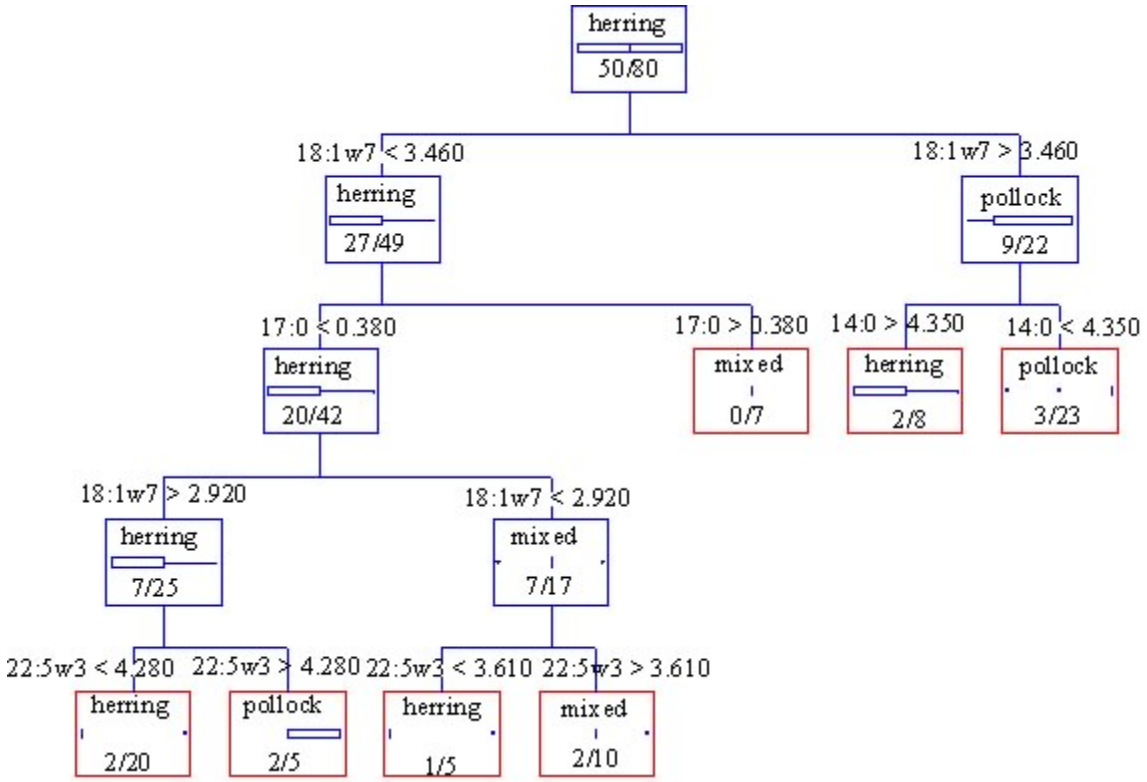

Figure 13. CART for harbor seal blubber samples taken at the end-point of each feeding trial in Table 1. This CART had seven terminal nodes and an error rate of 12 out of 80 observations. Thus, 85% of the seals were correctly classified according to diet. Details of the tree are as described in Figure 9.

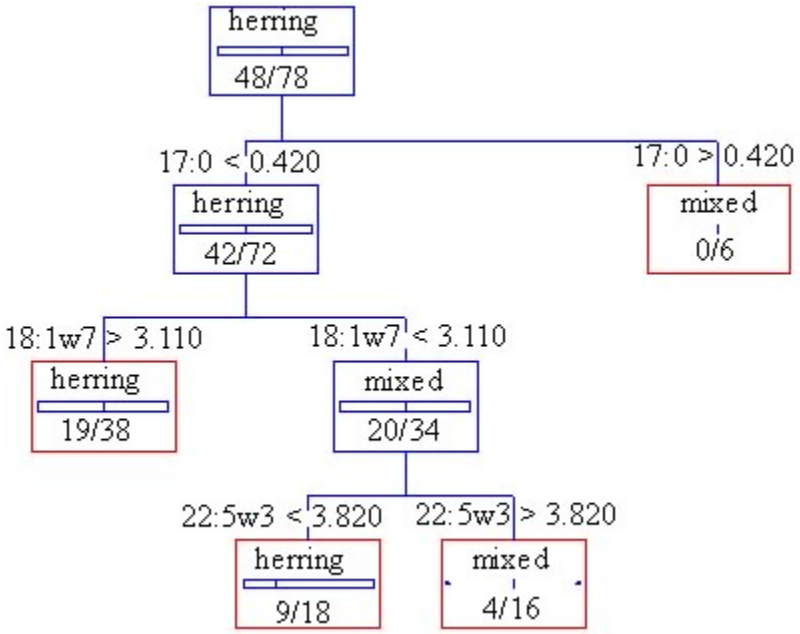

Figure 14. CART for harbor seal blubber samples taken at the mid-point of each feeding trial in Table 1. This CART had four terminal nodes and a total error rate of 32 out of 78 observations. Only 59% of the seals could be correctly classified according to diet after approximately two months. Details of the tree are as described in Figure 9.

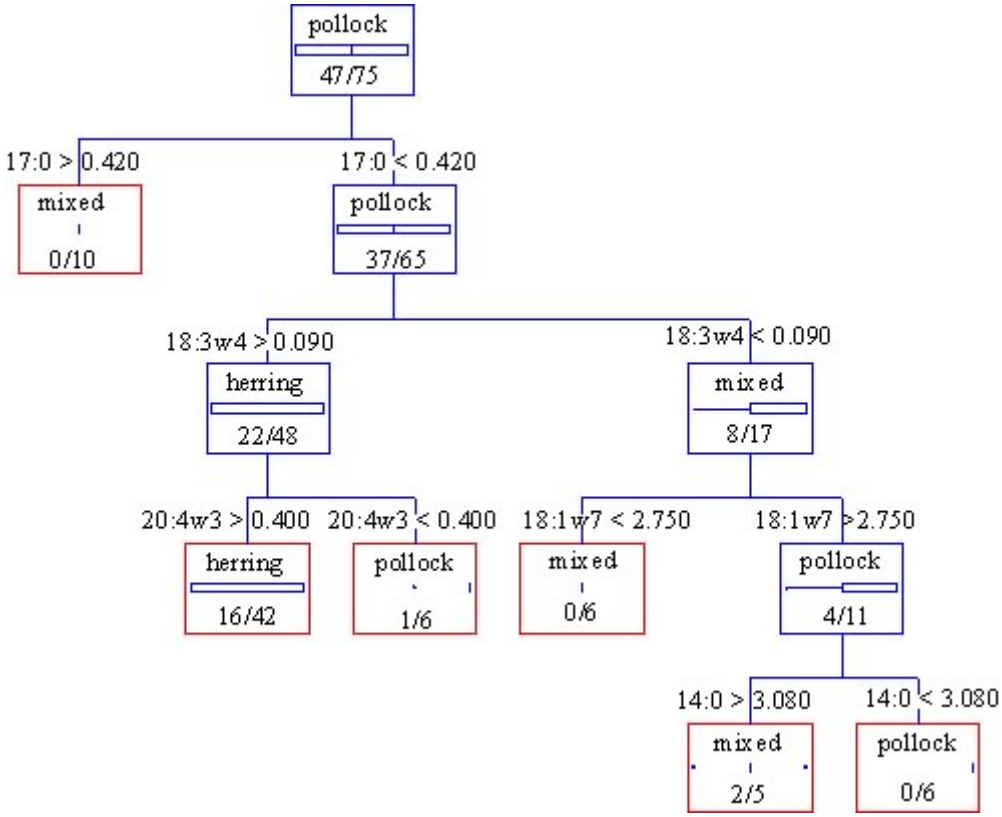

Figure 15. CART for the outer portion of the harbor seal blubber layer. This CART had six terminal nodes and a total error rate of 19 out of 75 observations. Thus, 75% of the seals could be correctly classified according to diet based on the fatty acid composition of the outer portion of their blubber. Details of the tree are as described in Figure 9.

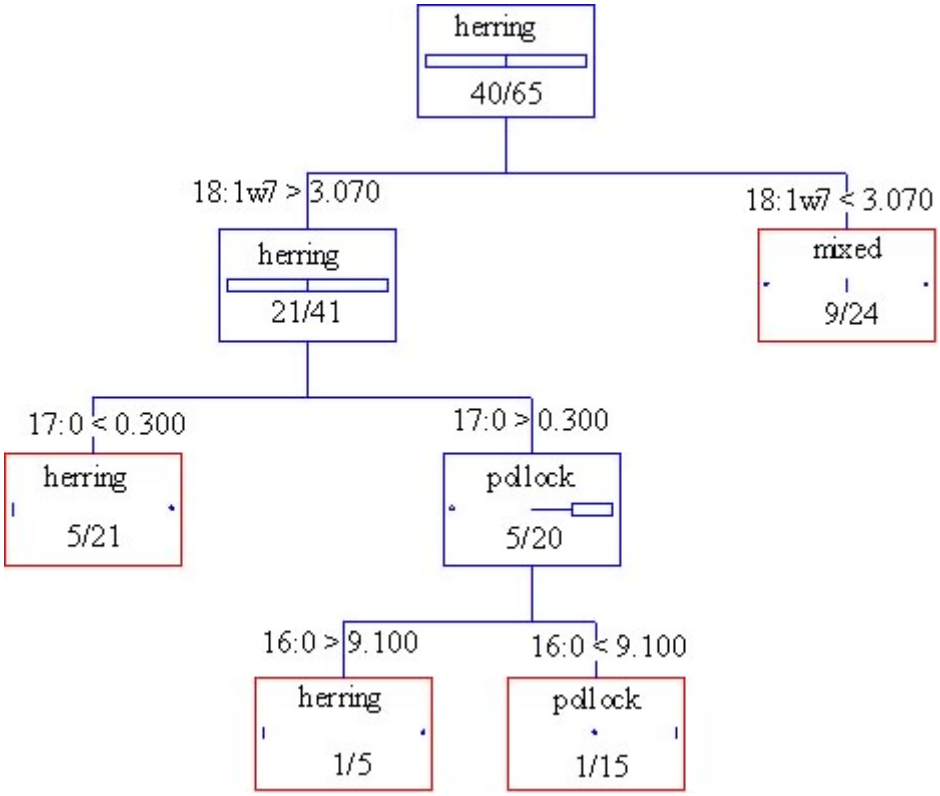

Figure 16. CART for the inner portion of harbor seal blubber samples. This CART had four terminal nodes and a total error rate of 16 out of 65 observations. Thus, 75% of the seals could be correctly classified according to diet based on the fatty acid composition of the inner portion of their blubber. Details of the tree are as described in Figure 9.