Seasonal Differences in Biochemical Adaptation to Fasting in Juvenile and Subadult Steller Sea Lions (*Eumetopias jubatus*)

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Accepted 12/3/2008; Electronically Published 3/26/2009

ABSTRACT

Nine Steller sea lions (*Eumetopias jubatus*) aged 1.75–6 yr were experimentally fasted for 7–14 d during the breeding and nonbreeding seasons to identify changes in plasma metabolites that are indicative of fasting and to determine whether the ability of sea lions to fast varies seasonally or with age. Although some animals approached the limit of their protein-sparing ability by the end of our fasting experiments, there was no sign of irreversible starvation biochemistry. Plasma blood urea nitrogen (BUN) concentrations decreased in all animals within the first week of fasting, reflecting a shift to a fasting-adapted state; however, significant increases in plasma BUN concentration at the end of the nonbreeding season fasts suggest that subadult Steller sea lions were not able to maintain a protein-sparing metabolism for a full 14 d during the nonbreeding season. In contrast, juveniles were able to enter protein sparing sooner during the nonbreeding season when they had slightly higher initial percent total body lipid stores than during the breeding season. Subadult and juvenile sea lions had low circulating ketone body concentrations compared with young sea lion pups, suggesting an age-related difference in how body reserves are utilized during fasting or how the resulting metabolites are circulated and catabolized. Our data suggest that metabolite concentrations from a single blood sample cannot be used to accurately predict the duration of fast; however, threshold metabolite concentrations may still be useful for assessing whether periods of fasting in the wild are unusually long compared with those normally experienced.

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Physiological and Biochemical Zoology 82(3):236–247. 2009. 1522-2152/2009/8203-8124$15.00
DOI: 10.1086/597528

Introduction

Homeotherms, such as bears, penguins, and phocid seals, limit protein degradation during predictable periods of long-term fasting by progressively increasing their reliance on catabolism of lipid stores (see review in Castellini and Rea 1992; Nørdoy et al. 1993; Rea 1995). Such changes in fuel catabolism are accompanied by predictable changes in plasma metabolite concentrations. Steller sea lions (*Eumetopias jubatus* [Schreber, 1776]) fast as a part of their natural life history while nursing pups on the rookery (adult females), defending breeding territories (males), and waiting for their mothers to return from feeding trips at sea (pups). The natural duration of these fasts are typically shorter in Steller sea lions than those experienced by phocid seals, penguins, or bears, but it is not known whether Steller sea lions utilize similar physiological mechanisms to ensure homeostasis and protein conservation during these shorter periods of restricted food intake.

In captivity, Steller sea lions have been shown to decrease their overall metabolic rate in response to complete fasting and specific types of reduced nutrition (Rosen and Trites 2002, 2005). These studies indicate that Steller sea lions can depress their metabolism to conserve energy when sufficient food is not available, as has been shown for species adapted to longer-duration fasting such as gray seal pups (Nordøy et al. 1990) and elephant seal pups (Rea and Costa 1992). They can also conserve mass during periods of food restriction (Rea et al. 2007), although it is unclear whether the noted decrease in the rate of mass loss during the early stages of fasting was due solely to metabolic depression or whether there were further modifications to metabolism that allowed the individuals to preferentially conserve vital lean tissue (i.e., muscle and internal organs) even during short-term fasting events. Rea et al. (2007) reported differences in the degree of reliance on lean versus lipid tissue reserves during fasting among individual sea lions, which suggested that at least some individuals employed protein-sparing strategies to conserve lean tissue during relatively short 7–14-d fasts. They also noted that initial body fat content of an animal before fasting appeared to influence the degree of reliance on lipid reserves for energy during fasting, although the success of these conservation tactics depended on the season of study and the age class of the sea lion.

Another means to further investigate the physiological adaptations responsible for energy and lean-mass conservation during fasting is to measure blood metabolite levels to identify changes in the types of body reserves that are being mobilized to meet energy requirements during food restriction. Changes in blood metabolite levels can be monitored to determine when or whether animals shift into a conservative protein-sparing metabolism (referred to as phase II fasting; Groscolas 1986; Le Maho et al. 1988; Nordøy et al. 1990; Rea 1995). Mammals tend to rely heavily on glucose and glycogen to fuel metabolism at the onset of fasting (phase I), and they supplement limited endogenous stores of glucose by gluconeogenesis from protein reserves.

Catabolism of protein during phase I results in significant concentrations of blood urea nitrogen (BUN) in circulation. As the fast progresses, lipid reserves are more heavily utilized for energy, with decreasing reliance on protein degradation, resulting in increases in the circulating levels of both nonesterified fatty acids (NEFA) and ketone bodies (β-HBA) and concomitant decreases in BUN concentration during phase II (Groscolas 1986; Castellini et al. 1987; Nordøy and Blix 1991; Rea 1995). When an animal has depleted lipid reserves to a threshold level, catabolism of protein reserves can no longer be limited and an increase in BUN concentration results during phase III of fasting (Le Maho et al. 1988). Phase III is considered to be a degradative stage of fasting during which homeostasis is often compromised.

Steller sea lions are a species that undergoes predictable periods of fasting in the wild as part of their life-history pattern, and they should therefore be able to maintain physiological homeostasis while fasting for short durations of up to 2 wk. They should maintain total protein concentrations within normal limits through homeostatic control, with no changes in hydration state. However, if physiological adaptation to fasting limits catabolism of protein (protein sparing), changes should occur in their blood metabolites. Circulating levels of protein metabolites (BUN) could be expected to decrease, while levels of lipid metabolites (NEFA and, secondarily, β-HBA) increase as lipid mobilization from body reserves and β-oxidation of lipids increase in phase II.

We experimentally fasted nine Steller sea lions in captivity for 7–14 d during the breeding season and the nonbreeding season to test the hypotheses that juvenile and subadult Steller sea lions exhibit changes in key plasma metabolites that are indicative of biochemical adaptation to fasting. In the wild, Steller sea lions fast during the summer breeding season as a part of their natural life-history pattern. This study was also designed to test whether the ability of juvenile and subadult animals to enter a fasting adapted state varies seasonally or whether they are equally prepared to undergo periods of fasting at all times of the year, independent of the life-history demands. Finally, we wanted to determine whether blood metabolite concentrations could be used as biochemical indicators of nutritional status in free-ranging juvenile and subadult Steller sea lions.

Material and Methods

The five subadult (aged 3–6 yr; 5.0 ± 1.2 yr) and four juvenile (aged 1.75–2.5 yr; 2.0 ± 0.2 yr) Steller sea lions that participated in our study were housed individually in research tanks filled with seawater at the Vancouver Aquarium (Vancouver, British Columbia, Canada) and the Alaska SeaLife Center (ASLC; Seward, Alaska). In the wild, juvenile sea lions of this age group can be observed to be closely associated with adult females and thus may presumably still be dependent on maternal partitioning (Pendleton et al. 2006); therefore, these individuals were considered separately in these analyses. The individual attention necessary to handle animals of the size of Steller sea lions meant that they had to be studied consecutively from June 1996 to November 1999. Although the mean age of subadult sea lions was slightly but not significantly older during the breeding season (t-test, $P = 0.527$), there was no difference in mean mass at the beginning of fasting (breeding season: 150.8 ± 13.2 kg; nonbreeding season: 154.3 ± 13.0 kg; t-test, $P = 0.839$; Rea et al. 2007). Our study animals experienced a complete fast for 7–14 d but were maintained on a normal training schedule, with ice cubes instead of fish as a reward for completing tasks (Christen et al. 1999). This study was conducted under Institutional Animal Care and Use Committee Protocols approved by the University of British Columbia, the ASLC (protocol 98-007), and the University of Central Florida (protocol 9807). Each study was terminated when the subadult animals had completed a fast of 14 d or the juvenile animals had completed 7 d of fasting, or if body mass loss exceeded 3% of an individual's body mass per day for 2 consecutive days or cumulatively exceeded a total mass loss of 15% of initial body mass. Similar fasting periods and mass losses are reported for wild otariids (Higgins et al. 1988; Baker et al. 1994) and were used as cutoff points to ensure that the health of our study animals was not compromised.

In addition to fasting trials, four subadult sea lions participated in food limitation experiments (Table 1). Over a 28-d period, these individuals were fed herring (*Clupea* spp.) at a level approximately one-half of their normal maintenance ration (ranging from 2.5 to 5 kg d^{-1}). Daily rations were adjusted to result in a slow and consistent mass loss throughout the duration of the study (0.45 ± 0.11 kg d^{-1}, $n = 4$) and a decrease in percent total body lipid (% TBL) reserves (decrease of 4.0% ± 1.4% over 28 d; range, 3%–6%; $n = 4$). This study design was intended to mimic a condition of suboptimal but constant food availability that could be found in the wild, at a level resulting in the inability to retain body mass and body condition.

Blood samples were collected at the onset of the study from each sea lion following an overnight fast. These data provided a control sample for each individual before prolonged fasting or food limitation was initiated. Body mass was measured daily on a platform scale (±0.1 kg) and blood samples were collected every 3–4 d during fasting trials and every 7 d during food limitation experiments. Because handling limitations required that blood samples be taken on different days for different

Table 1: Fasting and food limitation trials on Steller sea lions held at the Alaska SeaLife Center (ASLC) and the Vancouver Aquarium (VA)

Sea Lion, Animal ID, Fasting Season	Location	Date of Trial	Duration (d)	Age (yr)
M93WO:[a]				
M1:				
Breeding fast	ASLC	June 1999	14	6
Nonbreeding fast	VA	December 1996	14	3.5
Limitation	VA	April/May 1996	28	3
F93KI:[a]				
F2:				
Breeding fast	ASLC	June 1999	13	6
Nonbreeding fast	VA	October 1997	8	4.5
F93SU:[a]				
F1:				
Breeding fast	VA	June 1997	12	4
Nonbreeding fast	ASLC	December 1998	10	5.5
Limitation	VA	August/September 1996	28	3
M93AD:[a]				
M2:				
Breeding fast	VA	June 1997	14	4
Limitation	VA	April/May 1996	28	3
M93TA:[a]				
M3:				
Nonbreeding fast	VA	April 1997	9	4
Limitation	VA	November/December 1996	28	3.5
F97SI:[b]				
F3				
Breeding fast	VA	July 1999	7	2
Nonbreeding fast	VA	March 1999	7	1.75
M97KO:[b]				
M4:				
Breeding fast	VA	July 1999	7	2
Nonbreeding fast	VA	March 1999	7	1.75
F97HA:[b]				
F4:				
Breeding fast	VA	July 1999	7	2
Nonbreeding fast	VA	November 1999	7	2.5
M97TI:[b]				
M5:				
Breeding fast	VA	July 1999	7	2
Nonbreeding fast	VA	November 1999	7	2.5

Note. Animal ID also indicates sex of individual (M = male, F = female).

[a] 1993 cohort.

[b] 1997 cohort.

animals (± 1 d), the duration of fasting was divided into five intervals for statistical comparisons. Interval 1, or onset of fasting, sampled after an overnight fast; interval 2 ranged from 2 to 4 d of fasting; interval 3 ranged from day 5 to day 7; interval 4 ranged from day 8 to day 10; and the final interval, interval 5, ranged from day 11 to day 14 of fasting. Because of overall shorter fasting trials, data from juvenile sea lions are reported for intervals 1–3. Because of early termination of fasting in three subadult sea lions, comparisons between breeding season and nonbreeding season in interval 5 could not be completed for these individuals. Blood samples were drawn from the caudal gluteal vein directly into heparinized and nonheparinized Vacutainer blood collection tubes using a 2.5-inch, 18-gauge hypodermic needle and adapter system. Animals were held in a restraint cage during blood collection and were held under isoflurane anesthesia for all or part of this restraint period at the discretion of the attending veterinarian.

Hematocrit (Hct) was determined in duplicate using a

battery-operated field microhematocrit centrifuge (Compur M1100, Bayer, Germany; samples spun at 5,400 *g* for 3.5 min), and hemoglobin concentration (Hb) was measured spectrophotometrically using methanocyanide (Sigma Chemicals Kit 525-A) to provide veterinary monitoring of the health of the animal during the progress of the fasting trials (not reported). The remaining blood samples were centrifuged, and plasma was removed and frozen at −80°C for later analysis. Plasma metabolite concentrations were measured by spectrophotometric endpoint assay (BUN: Sigma Diagnostics 66-UV; NEFA: Wako Chemicals 990–75410; β-HBA: Sigma Diagnostics procedure 310-UV; and total protein: Sigma Diagnostics procedure 541). Plasma glucose concentrations were measured either using spectrophotometric endpoint assay (Sigma Diagnostics 16–20 Glucose HK) or using a YSI Model 2300 Stat Glucose/L-lactate autoanalyzer. Plasma concentrations of total ketone bodies (β-HBA + acetoacetate) were determined when possible ($n = 8$) by spectrophotometric assay (Wako Chemicals Autokit TKB). Because this analysis must be completed within days of blood collection, it was not possible to assay all samples collected at the Vancouver Aquarium after they had been stored frozen.

β-HBA was chosen over acetoacetate as the by-product of lipid catabolism to be monitored in Steller sea lions, given the high relative contribution of β-HBA to total circulating ketone body concentrations (see "Results") as well as the strong correlation between these two parameters and the greater stability of β-HBA in frozen plasma. Circulating creatinine concentrations were measured in serum using a clinical chemistry analyzer at the Central Laboratory for Veterinarians in Langley, British Columbia, and at the Veterinary Services Department of the Alaska SeaLife Center (Seward, AK). Plasma and whole-blood water content and specific gravity were measured using the methods previously described by Castellini et al. (1993).

All results are presented as means ± SD. One-way repeated-measures ANOVA (NCSS 6.0) was used to determine whether significant changes in blood metabolite concentrations or blood water concentrations occurred during intervals 1–5 of fasting among juvenile and subadult sea lions during the breeding season and the nonbreeding season, respectively, and to assess differences in concentrations observed between subadult and juvenile subjects during the first three intervals of fasting. Covariance matrix circularity was tested with each analysis, and when assumptions were violated, Geisser-Greenhouse adjustments were completed and either Geisser-Greenhouse epsilon probability levels (G.-G. *P* values) or Box epsilon probability levels were reported. When repeated-measures ANOVA indicated significant differences ($P < 0.05$), Bonferroni multiple-comparison post hoc analysis was used to indicate which intervals in the fast differed in metabolite concentration. Interval 1 and interval 2 blood metabolite concentrations were compared among juveniles and subadults fasting during the breeding season, along with previously reported data for 6-wk-old Steller sea lion pups (Rea et al. 2000), using one-way ANOVA (NCSS 6.0) and Tukey-Kramer multiple-comparison post hoc tests ($P < 0.05$). Seasonal differences in blood metabolite concentrations were assessed during three intervals of fasting in juvenile sea lions ($n = 4$) and four intervals of fasting for subadults ($n = 3$) using paired *t*-tests (NCSS 6.0; $P < 0.05$). Paired *t*-tests were also used to test the significance of changes in metabolite concentrations measured in initial samples (collected on the first day of food limitation) compared with concentrations measured in sea lions after 28 d of food limitation.

Results

The specific gravity of plasma did not change significantly with fasting (juvenile breeding season: $F_{2,11} = 3.12$, $P = 0.118$; juvenile nonbreeding season: $F_{2,11} = 3.10$, $P = 0.119$; subadult breeding season: $F_{4,19} = 1.59$, $P = 0.241$; subadult nonbreeding season: $F_{3,15} = 0.44$, $P = 0.732$); however, subadults had higher plasma specific gravity values than juveniles during the breeding season (breeding season: $F_{1,31} = 5.15$, $P = 0.031$; nonbreeding season: $F_{1,27} = 0.06$, $P = 0.808$; table 2). Plasma specific gravity was slightly elevated during the nonbreeding season (*t*-test, $P = 0.010$) compared with that of the matched interval of fasting in the breeding season. Plasma water content ranged from 90.6% to 93.4% and showed no significant change during fasting (juvenile breeding season: $F_{2,11} = 0.34$, $P = 0.725$; juvenile nonbreeding season: $F_{2,11} = 0.10$, $P = 0.904$; subadult breeding season: $F_{4,19} = 3.97$, G.-G. $P = 0.141$; subadult nonbreeding season: $F_{3,15} = 0.92$, $P = 0.470$). Slightly but significantly higher plasma water contents were measured in subadults than in juveniles during the breeding season (breeding season: $F_{1,31} = 12.06$, $P = 0.002$; nonbreeding season: $F_{1,27} = 0.27$, $P = 0.609$). Plasma water content was also slightly elevated in the nonbreeding season (*t*-test, $P = 0.006$) compared with that of the matched interval of fasting in the breeding season. In contrast, whole-blood specific gravity and water content values were similar in both age classes (breeding season: $F_{1,26} = 0.15$, $P = 0.699$ and $F_{1,26} = 3.15$, $P = 0.088$, respectively; nonbreeding season: $F_{1,19} = 0.94$, $P = 0.345$ and $F_{1,19} = 0.47$, $P = 0.502$, respectively). Whole-blood specific gravity was slightly elevated in the nonbreeding season (*t*-test, $P = 0.032$) compared with that of the matched interval of fasting. Whole-blood specific gravity (juvenile breeding season: $F_{2,11} = 2.40$, $P = 0.172$; juvenile nonbreeding season: $F_{2,11} = 2.27$, $P = 0.184$; subadult breeding season: $F_{4,14} = 4.17$, G.-G. $P = 0.178$; subadult nonbreeding season: $F_{3,7} = 1.86$, $P = 0.311$) and whole-blood water content (juvenile breeding season: $F_{2,11} = 3.35$, G.-G. $P = 0.165$; juvenile nonbreeding season: $F_{2,11} = 2.28$, $P = 0.184$; subadult breeding season: $F_{4,14} = 3.51$, $P = 0.141$; subadult nonbreeding season: $F_{3,7} = 1.42$, $P = 0.390$) remained stable throughout fasting and also did not change significantly with fasting duration ($P > 0.305$).

BUN concentrations decreased significantly, from 8.5 ± 2.8 to 5.0 ± 1.1 mM, between intervals 1 and 2 of the breeding season fast in subadult animals ($F_{4,19} = 8.14$, $P = 0.002$), and they remained at this low level throughout the study (Fig. 1). During the nonbreeding season fast, BUN concentrations decreased significantly, from 8.1 ± 2.0 to 5.2 ± 1.0 mM, between intervals 1 and 2 in subadult animals ($F_{3,15} = 18.32$, G.-G.

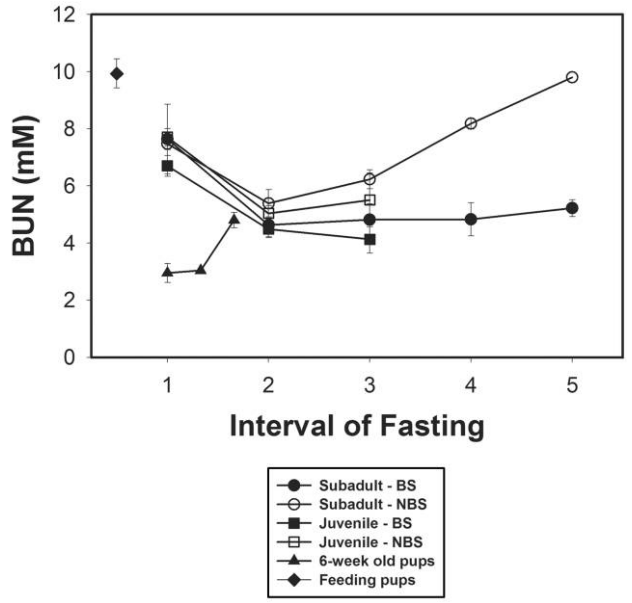

Figure 1. Changes in plasma concentrations of blood urea nitrogen (BUN; mM) for nine subadult and juvenile Steller sea lions fasting during the breeding season (BS) and the nonbreeding season (NBS). Comparative data for fasting 6-wk-old pups and feeding 9-mo-old Steller sea lion pups are provided (Rea et al. 2000).

$P = 0.023$), and they remained at this level through interval 3 (approximately the first 7 d of fasting). Significant increases in BUN concentration were evident between intervals 3 and 4 in subadult sea lions fasting during the nonbreeding season ($F_{3,15} = 18.32$, G.-G. $P = 0.023$). Juvenile sea lions fasting during the nonbreeding season significantly decreased plasma BUN concentrations, from 7.7 ± 0.6 mM at the beginning of the fast (interval 1) to 5.0 ± 0.5 mM in interval 2 of fasting ($F_{2,11} = 11.83$, $P = 0.008$). Similarly, during the breeding season, BUN concentration decreased significantly in juvenile sea lions, from 6.7 ± 0.7 mM at the beginning of the fast (interval 1) to 4.5 ± 0.6 mM in interval 2 of fasting ($F_{2,11} = 25.14$, $P = 0.001$). At the initiation of the fast in both seasons, plasma BUN concentrations in subadult and juvenile sea lions were significantly higher than values previously reported for 6-wk-old pups (breeding season: $P = 0.007$; nonbreeding season: $P < 0.001$; Rea et al. 2000). After 2–3 d of fasting (interval 2), there were no significant differences observed in BUN concentrations among these three groups ($P > 0.65$ in both seasons). Although there was a trend for mean BUN concentration to decrease during 4 wk of food limitation, from 8.8 ± 1.2 mM to 6.7 ± 1.7 mM, this trend was not significant (*t*-test, $P = 0.104$).

Total protein concentration in the plasma ranged from 6.58 to 8.74 mM during the study and did not change significantly with fasting (juvenile breeding season: $F_{2,11} = 0.13$, $P = 0.881$; juvenile nonbreeding season: $F_{2,11} = 1.24$, $P = 0.353$; subadult breeding season: $F_{3,11} = 1.72$, $P = 0.262$; subadult nonbreeding season: $F_{4,14} = 1.43$, $P = 0.306$), nor did it differ significantly between age classes (breeding season: $P = 0.318$; nonbreeding season: $P = 0.449$). Plasma concentrations of creatinine ranged from 0.083 to 0.163 mM and did not significantly change with fasting for subadults in the breeding season ($F_{4,19} = 2.59$, $P = 0.090$) or the nonbreeding season ($F_{3,15} = 1.65$, $P = 0.247$), nor did it significantly change for juveniles in the nonbreeding season ($F_{2,11} = 2.73$, $P = 0.144$). However, during the breeding season, juvenile sea lions showed a small but significant peak in creatinine concentrations during interval 2 ($F_{2,11} = 9.20$, $P = 0.015$). No consistent change in creatinine concentration was observed during 28 d of food limitation (*t*-test, $P = 0.202$). Subadults had higher plasma concentrations of creatinine during the breeding season than during the nonbreeding season (0.132 ± 0.018 mM and 0.111 ± 0.010 mM, respectively; $F_{1,31} = 23.92$, $P < 0.0002$), whereas juveniles displayed the opposite trend (breeding season: 0.109 ± 0.007 mM, and nonbreeding season: 0.120 ± 0.014 mM; $F_{1,24} = 5.02$, $P = 0.036$). Subadult levels of plasma creatinine were similar to those measured in juveniles both during the breeding season ($F_{1,23} = 3.14$, G.-G. $P = 0.126$) and during the nonbreeding season ($F_{1,23} = 1.32$, $P = 0.295$).

The ratio of plasma BUN concentration to creatinine concentration (UC ratio) significantly decreased between intervals 1 and 2 of fasting during the breeding season in subadult sea lions ($F_{4,19} = 6.43$, $P = 0.005$; Fig. 2). During the nonbreeding season, UC ratio significantly increased in subadults, from a minimum of 47.7 ± 8.7 during interval 2 to a maximum of 75.8 ± 8.3 during interval 4 of the fast ($F_{3,15} = 6.67$, Box epsilon $P = 0.042$). In fasting juveniles, a significant decrease in UC ratio was observed between intervals 1 and 2 during both the breeding season ($F_{2,11} = 27.78$, $P = 0.0009$) and the nonbreeding season ($F_{2,11} = 12.08$, G.-G. $P = 0.040$). There were

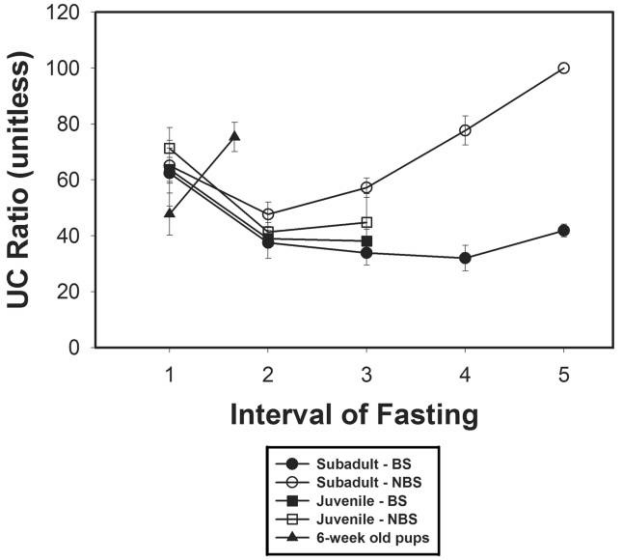

Figure 2. Changes in the ratio of plasma concentrations of blood urea nitrogen in mM to creatinine in mM (UC ratio; unitless) in nine subadult and juvenile Steller sea lions fasting during the breeding season (BS) and the nonbreeding season (NBS). Comparative data for fasting 6-wk-old Steller sea lion pups are provided (Rea et al. 2000).

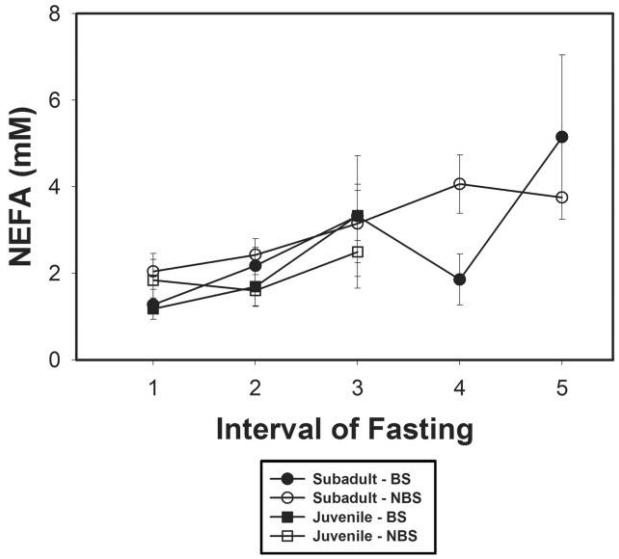

Figure 3. Changes in plasma concentrations of nonesterified fatty acids (NEFA; mM) for nine subadult and juvenile Steller sea lions fasting during the breeding season (BS) and the nonbreeding season (NBS).

no seasonal differences in UC ratios at the start of the fast in either subadults (*t*-test, $P = 0.597$) or juveniles (*t*-test, $P = 0.408$). During food limitation, the decrease in UC from 82.0 ± 12.9 at the start of the trial to 59.3 ± 14.9 after 28 d of limitation was not statistically significant (*t*-test, $P = 0.053$).

Plasma glucose concentrations did not differ significantly between age classes in the breeding season ($F_{1,23} = 0$, $P = 0.979$) or the nonbreeding season ($F_{1,23} = 1.50$, $P = 0.266$), nor did they differ significantly between seasons (juveniles: $F_{1,23} = 0$, $P = 0.998$; subadults: $F_{1,30} = 0.82$, $P = 0.373$; overall mean, 6.84 ± 0.96 mM). Glucose concentrations also did not change significantly during fasting (juvenile breeding season: $F_{2,11} = 5.50$, $P = 0.044$; however, no differences identified by Bonferroni post hoc evaluation; juvenile nonbreeding season: $F_{2,11} = 2.85$, $P = 0.135$; subadult breeding season: $F_{4,19} = 3.04$, $P = 0.060$; subadult nonbreeding season: $F_{3,15} = 1.29$, $P = 0.335$) or food limitation (*t*-test, $P = 0.050$). Although plasma NEFA tended to increase in concentration with the duration of fasting, concentrations did not change significantly in either age class or season (juvenile breeding season: $F_{2,11} = 4.44$, $P = 0.066$; juvenile nonbreeding season: $F_{2,11} = 1.12$, $P = 0.385$; subadult breeding season: $F_{4,19} = 2.15$, $P = 0.137$; subadult nonbreeding season: $F_{3,15} = 1.71$, $P = 0.235$; Fig. 3). There were no seasonal differences in NEFA concentrations, with the exception of lower NEFA values observed in subadults during interval 4 of fasting during the breeding season compared with nonbreeding season concentrations (*t*-test, $P = 0.029$).

Plasma concentrations of β-HBA were consistently higher than those of acetoacetate during fasting in sea lions, as illustrated by changes observed during the breeding fast of two subadult sea lions (Fig. 4). A strong correlation was evident between β-HBA and total ketone body concentrations during fasting (TKB = 1.17736[β-HBA] + 8.085 × 10^{-3}; $r^2 = 0.869$). Plasma concentrations of β-HBA were highly variable between individuals, and they remained below 0.34 mM in subadults and juveniles for the duration of these studies, significantly lower than values reported at the onset of fasting in another study of fasting 6-wk-old pups (interval 1: $F_{2,12} = 22.85$, $P = 0.0002$) and during interval 2 ($F_{2,12} = 9.61$, $P = 0.005$; Fig. 5; Rea et al. 2000). In subadults, β-HBA concentrations remained low throughout the breeding and nonbreeding seasons, with a maximum level of 0.21 ± 0.01 mM observed during the breeding season at interval 5 of fasting. Changes in juvenile β-HBA levels were not significant during the breeding season fast; however, significant increases in β-HBA levels were observed in juveniles between intervals 2 and 3 of fasting during the nonbreeding season ($F_{2,11} = 6.96$, $P = 0.027$). Plasma β-HBA concentrations increased significantly, from 0.05 ± 0.02 mM after an overnight fast to 0.12 ± 0.05 mM after 28 d of food limitation (*t*-test, $P = 0.044$).

The ratio of plasma β-HBA concentrations to BUN concentrations (HBA : BUN) increased gradually during the breeding season fast in subadults such that the value of this ratio reached a maximum at the end of the fast ($F_{4,19} = 2.72$, $P = 0.080$; Fig. 6). Also, no changes in this ratio were detected during the

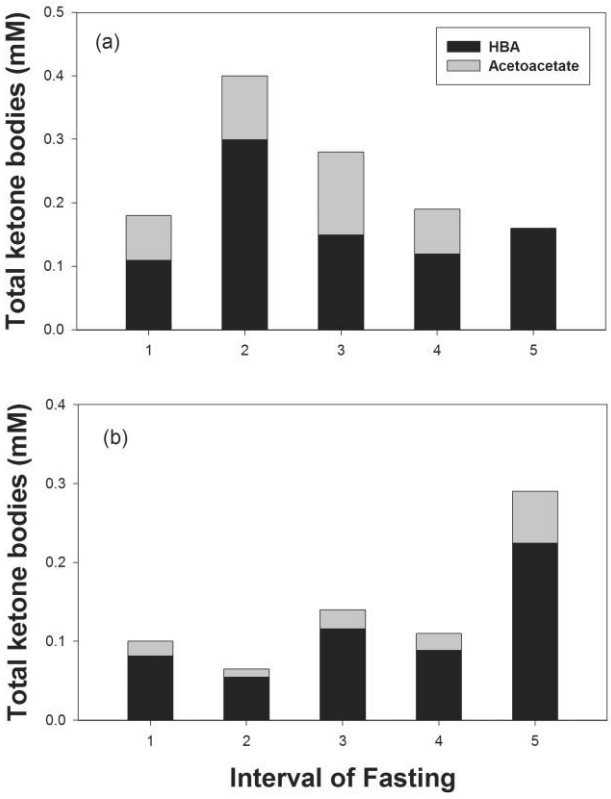

Figure 4. Changes in the plasma concentration of total ketone bodies (mM) measured in a subadult female sea lion (F93KI; *a*) and a subadult male sea lion (M93WO; *b*) fasting during the breeding season. Plasma levels of β-hydroxybutyrate (β-HBA; mM) are shown in black, and calculated estimates (total ketone bodies minus β-HBA) of acetoacetate concentration (mM) are shown in gray.

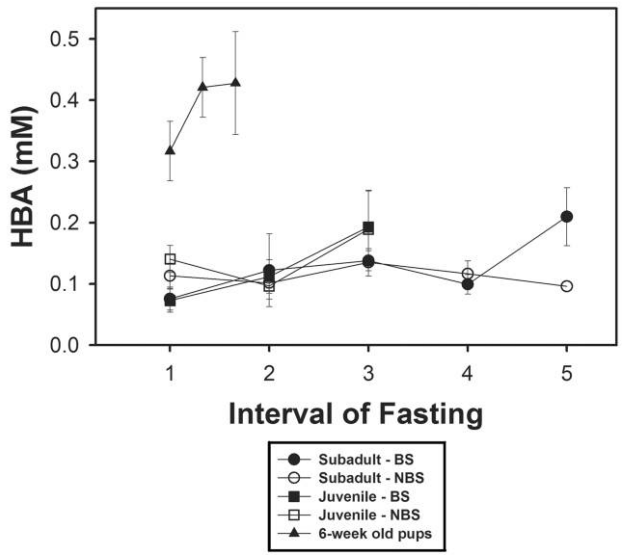

Figure 5. Changes in plasma concentrations of β-hydroxybutyrate (β-HBA; mM) for nine subadult and juvenile Steller sea lions fasting during the breeding season (BS) and the nonbreeding season (NBS). Comparative data for fasting 6-wk-old Steller sea lion pups are provided (Rea et al. 2000).

nonbreeding season fast in subadult sea lions. In contrast, juvenile sea lions exhibited an increase in HBA : BUN values between intervals 2 and 3 of fasting during the nonbreeding season ($F_{2,11} = 10.00$, Box epsilon $P = 0.032$). During the breeding season, HBA : BUN values previously reported for 6-wk-old pups were significantly higher than those measured in fasting subadults and juveniles at interval 1 ($F_{2,12} = 33.93$, $P < 0.001$) and at interval 2 ($F_{2,12} = 10.24$, $P = 0.004$). Apparent differences observed between subadults and juveniles after 1 wk of fasting were not significant (breeding season: $P = 0.160$; nonbreeding season: $P = 0.062$). A slight but statistically significant increase in HBA : BUN, from 0.006 ± 0.003 to 0.020 ± 0.009, was also detected over the duration of the 28-d limitation period ($P = 0.019$).

Discussion

Plasma total protein and glucose concentrations and measurements of plasma and whole-blood water contents remained stable during fasting among both age classes and between seasons. This illustrates the ability of Steller sea lions to maintain homeostasis during the short periods of fasting that are typical in the wild. Like many other fasting-adapted species, Steller sea lions showed no indication of dehydration during fasting, which suggests that any changes in plasma metabolite concentrations were a direct result of changes in metabolism that impact the mobilization or utilization of lipid, protein, and carbohydrate reserves.

Circulating levels of total protein (primarily albumin, globulins, enzymes, and hormones) serve an essential role in maintaining osmotic pressure of the circulatory system and thus should be defended during fasting. Fasting black bears (Nelson 1980), fasting northern elephant seal pups (Ortiz et al. 1978; Costa and Ortiz 1982), and food-restricted boars (Wolkers et al. 1993) have all shown the ability to maintain stable total protein concentrations during weeks to months of food limitation or complete fasting.

Although our animals received small amounts of daily freshwater intake in the form of ice cubes during training sessions, they should have had no difficulty maintaining water balance, even without access to freshwater, because of the metabolic water produced while metabolizing body fat reserves. Freshwater sources are not typically available to animals fasting in the wild. However, concern over dehydration may arise if fasting animals are required to primarily catabolize protein tissue with no access to freshwater, such as may happen if individuals are forced to begin a fast while they are in poor body condition (i.e., low initial % TBL content) or to fast for longer-than-normal periods. Catabolizing protein requires the excretion of water to expel nitrogenous waste products, and less metabolically derived water is available from catabolism of protein compared with that of lipids. Nelson et al. (1975) found several indicators of dehydration in bears that were fasted outside of the natural denning period, including a decrease in total body water.

Seasonal differences were measured in how Steller sea lions mobilized body stores during fasting. Within the first 3 d of fasting (by interval 2), all subadult animals showed biochemical adaptations to fasting that were indicative of protein sparing and independent of the season of the fasting trial. During the first 3 d of fasting, increased mobilization of body fat stores decreased the need to catabolize lean body tissue for energy

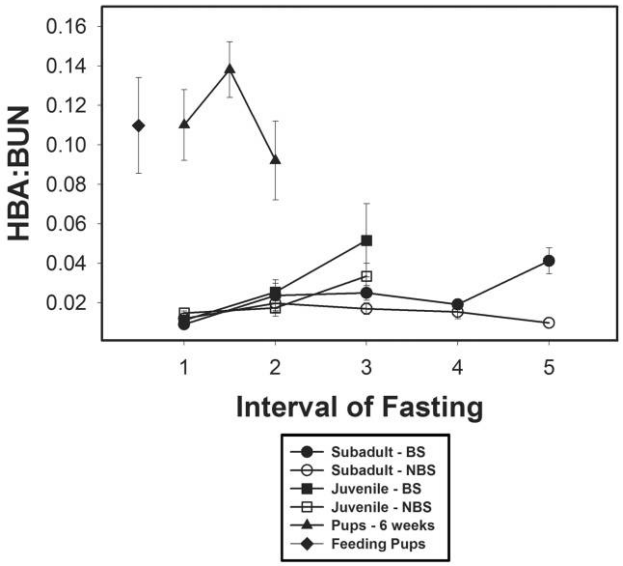

Figure 6. Changes in ratio of plasma concentrations of β-hydroxybutyrate (β-HBA; mM) to blood urea nitrogen (BUN; mM) in nine subadult and juvenile Steller sea lions fasting during the breeding season (BS) and the nonbreeding season (NBS). Comparative data for fasting 6-wk-old pups and feeding 9-mo-old Steller sea lion pups are provided (Rea et al. 2000).

during fasting, which in turn resulted in a decrease in circulating plasma concentrations of BUN. However, only those subadults who fasted during the breeding season were able to maintain this protein-sparing metabolism longer than interval 3 or the first week of fasting. These results directly mirror the ability of subadult Steller sea lions to depress rates of mass loss for the duration of the fast only during the breeding season (Rea et al. 2007), when they would be expected to fast in the wild. This suggests that conservation of body reserves during fasting is not only due to metabolic depression (Rosen and Trites 2002) but is also enhanced by preferential mobilization of lipid reserves to provide energy and conserve protein during fasting.

Significant increases in plasma BUN concentrations at the end of the nonbreeding season fasts coincided with a slight increase in the rate of mass loss during that period (Rea et al. 2007). This suggests that subadult Steller sea lions were not as able to adapt their metabolism to a depleted food environment during the nonbreeding season, and they experienced higher rates of catabolism of their protein energy reserves than was observed during the breeding season (Rea et al. 2007). This is similar to black bears, which have been shown to maintain a protein-sparing metabolism with low BUN concentrations for up to 2 mo during the natural denning period but which significantly increase BUN concentrations when fasted for only 2 wk outside of this period (Nelson et al. 1975).

Juveniles showed varying degrees of biochemical evidence of a protein-sparing metabolism during both seasons even though they were less successful at conserving lean tissue during fasting than subadults were (Rea et al. 2007). Contrary to patterns observed in subadults, juveniles were able to enter protein sparing sooner during the nonbreeding season, as evidenced by decreases in BUN concentration by interval 2 of the nonbreeding season fasts compared with interval 3 during the breeding season. This is consistent with the trend of juveniles to exhibit a higher rate of fat loss during the nonbreeding season (when they had slightly higher initial % TBL) than during the breeding season ($P = 0.09$; Rea et al. 2007), and it suggests that juveniles were better able to decrease their reliance on protein catabolism for energy during nonbreeding-season fasting.

The intensity of protein sparing and the duration of its effectiveness have been associated with the percentage of body fat stores at the induction of the fast (Robin et al. 1988; Cherel et al. 1992, 1993; Lindgård et al. 1992; Rea and Costa 1992; Atkinson et al. 1996; Arnould et al. 2001; Noren et al. 2003). In ptarmigan, lean subjects lost mass at a faster rate than subjects with higher initial fat contents because of an immediate reliance on lean-tissue catabolism for energy in the former (Lindgård et al. 1992). In both northern and southern elephant seals (Carlini et al. 2001; Noren et al. 2003), fatter pups lost proportionately more fat during the postweaning fast than did thinner conspecifics. Fasting rates of protein utilization have also been shown to be higher in lean lactating northern elephant seals compared with those with high body fat contents (Crocker et al. 1998). However, the full potential of protein-sparing metabolism may not yet be realized in immature animals because they do not need to fast like breeding adults during the breeding season.

Both subadult and juvenile sea lions showed higher initial and interval 1 BUN levels than did the 6-wk-old Steller sea lions that fasted overnight in another study (Rea et al. 2000). This likely reflects the higher protein content of the herring diet of older animals compared with the formula fed to young pups, as well as the rapid entrance of pups into a protein-sparing metabolism during fasting. The limited body energy reserves of pups, combined with faster rates of mass loss (a 5.1% $\pm$ 0.3% mass loss of pups over 2-d fast [Rea et al. 2000] vs. a 1.0%–2.0% mass loss per day in subadults and juveniles in our study and in Rea et al. [2007]), mean that a quick transition into a fasting-adapted metabolism would benefit young pups who are left on the rookery or haulout to fast for 1 or more days while their mothers forage at sea (Trites and Porter 2002; Milette and Trites 2003).

In general, Steller sea lions make the transition to a protein-sparing metabolism more swiftly than fasting penguins and phocid seals, which transition to phase II after approximately 6–10 d (king penguin chicks, Le Ninan et al. 1988; gray seal pups, Nordøy and Blix 1991; harp seal pups, Worthy and Lavigne 1987). A rapid transition to phase II fasting has also been documented after 2–3 d in Antarctic fur seal pups, another otariid species (Arnould et al. 2001).

Subadult and juvenile Steller sea lions did not show the progressive increase in β-HBA concentrations that has been documented in phocid seal pups and penguin chicks during prolonged fasting (northern elephant seal pups, Castellini and Costa 1990; gray seal pups, Nordøy and Blix 1991; harp seal pups, Nordøy et al. 1993; King penguin chicks, Le Ninan et al. 1988). A possible explanation for this unexpected finding is that the duration of the fast was shorter for our sea lions or there was a lower ketone body accumulation in the plasma of older animals. β-HBA concentrations were generally low and also highly variable between individuals, with higher levels only observed during fasting events that showed marked protein sparing, such as the end of the breeding season in subadults (interval 5) and in the middle of the nonbreeding season in juveniles (interval 2). With the exception of the smallest subadult female (after 12 d of fasting), β-HBA levels ranged from 0.03 to 0.17 mM, similar to concentrations seen in fasting adult bears (Ahlquist et al. 1984) and fasting adult female elephant seals (Castellini and Costa 1990; Williams 1995).

Low plasma levels of β-HBA may result from an age-related difference in how sea lions use body reserves during fasting or how the resulting metabolites are circulated. An earlier study on 6-wk-old Steller sea lion pups found elevated ketone body concentrations of up to 0.63 mM during a shorter fast of 2.5 d (Rea et al. 2000). In a subsequent study conducted when these same pups were 3 mo of age, β-HBA concentrations ranged from only 0.07 to 0.2 mM during a 2.5-d fast (L. D. Rea, D. A. S. Rosen, and A. W. Trites, unpublished data), which is similar to concentrations we found during this study on 1.75–6-yr-old animals. Although there are few data available for subadult or adult fasting pinnipeds, an age-related difference

Table 2: Blood hydration parameters for subadult and juvenile Steller sea lions during the breeding season (BS) and the nonbreeding season (NBS) fasting trials and for subadult sea lions during the 28-d food limitation trials

Age Group, Trial, Parameter	PL SG (g mL^{-1})	PL H_2O (%)	WB SG (g mL^{-1})	WB H_2O (%)
Subadults:				
BS fast:				
Mean ± SD	1.016 ± .007	92.2 ± .7	1.046 ± .008	81.5 ± 1.4
Minimum–maximum	1.007–1.025	90.8–93.4	1.034–1.056	79.1–84.1
Sample size	(19)	(19)	(19)	(19)
NBS fast:				
Mean ± SD	1.020 ± .005	92.4 ± .5	1.049 ± .004	82.1 ± 2.0
Minimum–maximum	1.009–1.025	91.4–92.9	1.043–1.054	79.0–85.2
Sample size	(17)	(17)	(9)	(9)
Limitation:				
Mean ± SD	1.024 ± .002	92.2± .3	1.050 ± .005	82.6 ± 1.0
Minimum–maximum	1.020–1.029	91.8–92.6	1.037–1.057	80.7–84.0
Sample size	(20)	(20)	(20)	(20)
Juveniles:				
BS fast:				
Mean ± SD	1.012 ± .005	91.5 ± .5	1.046 ± .006	81.2 ± .8
Minimum–maximum	1.002–1.020	90.6–92.2	1.037–1.053	80.2–82.3
Sample size	(12)	(12)	(12)	(12)
NBS fast:				
Mean ± SD	1.009 ± .004	91.5 ± .4	1.048 ± .004	81.3 ± .9
Minimum–maximum	1.005–1.014	91.1–91.8	1.041–1.055	80.3–83.3
Sample size	(5)	(5)	(12)	(12)

Note. PL SG = plasma specific gravity; PL H_2O = plasma water content; WB SG = whole-blood specific gravity; WB H_2O = whole-blood water content.

in plasma ketone body concentration has also been documented in king penguins (*Aptenodytes patagonica* Miller), with fasting adults developing a modest ketosis (1.5–2 mM) and chicks a more moderate response (4 mM; as reviewed by Groscolas 1990). Balasse and Féry (1989) also reported high β-HBA concentrations in 5-yr-old children who were fasted for 20–22 h (3.5 mM) compared with healthy adults who were fasted overnight (0.1–0.4 mM). It may be particularly important for young animals with high growth rates and high mass-specific metabolic rates to spare muscle and vital organ protein stores by maximizing reliance on lipid metabolism during fasting. In mammals, ketone bodies (β-HBA and acetoacetone) can be utilized heavily by heart and kidney tissues and less so by skeletal muscle, intestines, and the brain (Bruss 1989). High levels of circulating β-HBA can additionally spare protein by directly decreasing amino acid oxidation and increasing protein synthesis (Anonymous 1989; Bruss 1989). Thus, it would be beneficial for young growing animals to be able to shift readily to ketone body production during fasting.

Young Steller sea lions and harbor seals have been reported to have significantly higher β-hydroxyacyl coenzyme A activities in skeletal muscle compared with adults of the same species (Richmond et al. 2002; Prewitt et al. 2006). β-hydroxyacyl coenzyme A is a rate-limiting enzyme in β-oxidation that allows greater production of acetyl CoA from the catabolism of lipid reserves (predominantly long-chain fatty acids). If high β-hydroxyacyl coenzyme A activities also exist in the liver of young pinnipeds, high acetyl CoA concentrations would also result from hepatic β-oxidation. Tissues have a limited capacity to take up β-HBA; once that threshold is met, β-HBA concentrations can increase rapidly in circulating plasma (Balasse and Féry 1989). In a young, growing animal whose life history includes natural periods of fasting during or immediately following the period of dependence on a mother's milk, high β-hydroxyacyl coenzyme A activities would ensure high rates of oxidation of lipids to provide energy during fasting, resulting in higher circulating concentrations of ketone bodies, which allow protein to be spared for muscle and organ development.

In cases where significant concentrations of HBA do not accumulate in circulation as a clear indicator of duration of fasting, the ratio HBA : BUN can provide additional information about how these plasma metabolites change with the duration of fasting in relation to one another. Rea (1995) found HBA : BUN to increase significantly during fasting in postweaning northern elephant seal pups and during the short postweaning fast in Weddell seal pups. This ratio appears to be a very helpful tool to monitor biochemical transitions such as the shift toward protein catabolism when an animal has reached the physiological limit of protein sparing.

Decreases in UC ratio measured between intervals 1 and 2

in subadults fasting in the breeding season and in juveniles fasting during both seasons closely reflected similar trends for decreasing BUN concentrations during the first 3 d of fasting (between intervals 1 and 2). Similar trends of decreased BUN concentration and decreased UC ratios have been measured in northern elephant seal pups, while significant decreases in excretion rate (84%) facilitate protein sparing during the postweaning fast (Adams and Costa 1993). Neither plasma creatinine concentration nor UC ratios were reliable indicators during long-term undernutrition, when these animals did not enter into a protein-sparing metabolism.

Although NEFA concentrations were shown to increase during the transition to protein sparing during the breeding season in subadult sea lions, the high variability in the plasma concentrations of this metabolite (probably due to varying levels of activity before sampling resulting in recent muscle consumption of this circulating fuel) limits its use as a predictor of fasting duration. High variability in plasma concentrations of NEFA were similarly observed during fasting in elephant seal pups when these animals increased activity levels after 5 wk of fasting (Rea 1995).

One subadult male sea lion (M93WO) showed evidence of reaching its physiological limit of fasting during the nonbreeding season after experiencing a 21% decrease in mass over 14 d of fasting. Although this degree of mass loss is typical for adult male otariids that are fasting during the breeding season in the wild (Boyd and Duck 1991), this animal began the fast at a low body fat content (11% TBL), which decreased further to 8% TBL during fasting (Rea et al. 2007). This animal was the only subject to experience a decrease in hematocrit during fasting (dropping from 40% to 33%; L. D. Rea, D. A. S. Rosen, and A. W. Trites, unpublished data), and a low total serum protein concentration (6.6 mM) occurred when metabolite chemistry showed evidence of entrance into phase III fasting. After 14 d of fasting (interval 5), this male showed a maximum BUN of 9.8 mM, which was accompanied by a decreased β-HBA concentration (0.10 mM in interval 5 after a higher value of 0.12 mM in interval 3) and a decreased NEFA level (3.8 mM in interval 5 after a higher value of 4.9 mM in interval 3). A peak UC ratio of 100.0 was also measured after 14 d of fasting (interval 5). This is a clear indication of the impact of low lipid reserves on the ability of sea lions to maintain a protein-sparing fasting metabolism outside of their natural breeding season. This abrupt decrease in hematocrit prompted a change in study design such that all subsequent fasting trials were limited to a maximum of 15% total body mass loss to ensure the good health of the study subjects. Unfortunately, this also limited the ability of this study to further investigate the role of initial body condition in limiting the maintenance of metabolic adaptation during fasting in this species.

There was no evidence of animals entering a protein-sparing metabolism within 28 d of food limitation, during which time mass losses were maintained at approximately 0.5 kg per day. Although all animals were able to remain in physiological homeostasis, with stable glucose and blood water content levels, this long period of negative energy balance resulted in continued reliance on lean-tissue catabolism to provide energy. Although increasing β-HBA concentrations indicate that lipid reserves were being mobilized during the limitation studies, final concentrations after 28 d of undernutrition were lower than those observed during periods of fasting when protein sparing was particularly evident in subadults and juveniles. Although HBA : BUN was found to significantly increase during the food limitation experiments, peak levels were much lower than observed for this age class during fasting, again indicating that protein sparing is not significantly evoked while some food is available. In other food limitation studies of shorter (9-d) duration, Kumagai (2004) found that 64% of the trials resulted in slight decreases in BUN; however, final BUN concentrations (7.6 ± 1.7 mM) were not depressed to the same degree as in the animals that had entered a protein-sparing metabolism during complete fasting.

One of the goals of our study was to determine whether metabolite profiles of Steller sea lions could be used to assess the nutritional status of free-ranging juveniles, as has been done for young free-ranging pups (Rea et al. 1998). Previous studies have shown that ketone body (β-HBA) concentration provides the best indication of the relative duration of fasting in phocid seal pups, with BUN concentrations confirming a protein-sparing metabolism (Nordøy et al. 1990, 1993; Rea 1995). Unfortunately, the β-HBA concentrations in our subadult and juvenile animals did not gradually increase over the fasting period to the same degree as observed in phocid seal pups (Castellini and Costa 1990; Nordøy et al. 1990, 1993; Rea 1995), and elevated BUN concentrations were evident at the beginning and at the end of the fasting period in some animals.

On the basis of the results of our study, the task of evaluating the nutritional status of free-ranging subadult and juvenile sea lions from the metabolite profiles of a single blood sample seems problematic, with one of the key fasting indicators showing very little change even after 7–14 d of fasting compared with that of the pups. Our study suggests that β-HBA levels between 0.05 and 0.2 mM are typical for healthy juvenile and subadult sea lions undergoing a 2–14-d fast (β-HBA concentrations >0.2 mM were observed only after 12 d of fasting in one sea lion). Thus, conservatively, β-HBA concentrations above 0.2 mM would indicate a significant duration of fasting in free-ranging juvenile and subadult Steller sea lions, particularly when accompanied by BUN concentrations below 6.0 mM. Some β-HBA concentrations recently measured in free-ranging juvenile and subadult Steller sea lions have been shown to exceed levels measured during these experiments (Rivera et al. 2006), suggesting that this approach can be helpful in identifying free-ranging sea lions that were involved in fasts of long durations at the time of capture. However, because of the variability of concentrations exhibited between individuals in our controlled study, it would not be possible to accurately estimate the duration of these fasting periods.

In summary, seasonal differences in rates of mass loss reported previously for Steller sea lions (Rea et al. 2007) were consistent with seasonal differences in how animals mobilized lean and lipid tissue resources and were not simply due to

differences in overall energy expenditure. Subadult sea lions were able to enter and maintain a protein-sparing fasting metabolism during the breeding season, when they naturally undergo fasting in the wild, but they were more limited in their ability to conserve protein during the fast in the nonbreeding season. Although there was evidence that some animals had approached the limit of their protein-sparing ability by the end of our fasting experiments, there was no evidence of irreversible starvation biochemistry with this duration of fasting such that animals remained in glucose and water balance for the duration of the studies. Our data indicate that metabolite concentrations in a single blood sample cannot be used to predict the duration of fasting that an individual has undergone because of (1) the large individual variability in blood metabolite concentrations, (2) the generally low concentration of β-HBA accumulated during fasting in subadults and juveniles, and (3) the variable seasonal response in these age classes. However, single blood samples may still be informative to determine whether a sea lion exhibits threshold levels of these metabolites that would indicate that it has experienced an unusually long fast.

Acknowledgments

This study was authorized under the Marine Mammal Protection Act Permit (881-1443) held by the Alaska SeaLife Center (ASLC) and by Institutional Animal Care and Use Committee Protocols approved by the University of British Columbia (UBC), the ASLC (98-007), and the University of Central Florida (9807). This research was funded by the National Marine Mammal Laboratory Steller sea lion research program, through a grant to L.D.R. from the National Fish and Wildlife Foundation (98-244-008), and through grants from the National Oceanic and Atmospheric Administration and the North Pacific Marine Science Foundation to the North Pacific Universities Marine Mammal Research Consortium to support the UBC Steller sea lion program. We wish to thank D. Christen, C. Porter, T. Shannon, and G. Shephard, whose training skills and inventiveness made our study possible, as well as the staff at both the Vancouver Aquarium and the ASLC for their assistance with our study. We are also grateful to the late P. Hochachka for access to his analytical laboratory at UBC and wish to acknowledge the tireless laboratory assistance of M. Citron, T. Haase, K. Kimble, J. Richmond, and J. Smith. This manuscript was improved through the comments of two anonymous reviewers, and special thanks are extended to T. Loughlin for his support and review of this manuscript.

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