

## Total body oxygen stores and physiological diving capacity of California sea lions as a function of sex and age

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### Summary

A defining physiological capability for air-breathing marine vertebrates is the amount of oxygen that can be stored in tissues and made available during dives. To evaluate the influence of oxygen storage capacity on aerobic diving capacity, we examined the extent to which blood and muscle oxygen stores varied as a function of age, body size and sex in the sexually dimorphic California sea lion, *Zalophus californianus*. We measured total body oxygen stores, including hematocrit, hemoglobin, MCHC, plasma volume, blood volume and muscle myoglobin in pups through adults of both sexes. Blood and muscle oxygen storage capacity was not fully developed by the end of the dependency period, with blood stores not fully developed until animals were larger juveniles (70 kg; 1.5–2.5 years) and muscle stores not until animals were sub-adult size (125 kg; 4–6 years). Differences in aerobic

diving capacity among size classes were reflective of these major milestones in the development of oxygen stores. Male sea lions had greater absolute blood volume than females and reflected the larger mass of males, which became apparent when animals were large juveniles. Adult female sea lions had greater muscle myoglobin concentrations compared to males, resulting in greater mass-specific muscle and total oxygen stores. Delayed development of oxygen stores is consistent with the shallow epi-mesopelagic foraging behavior in this species. We hypothesize that the greater mass-specific oxygen stores of female sea lions compared to males is related to differences in foraging behavior between the sexes.

Key words: development, blood, muscle, oxygen stores, aerobic dive limit, pinniped, *Zalophus californianus*.

### Introduction

For air-breathing vertebrates, an individual's ability to acquire food resources in the marine environment results from the interaction between an animal's behavior and its physiological capability, both of which may vary by age, body size and gender. Development of oxygen storage capacity is particularly important for marine mammals and birds that rely on onboard stores for diving and foraging activity during and following their transition to nutritional independence. Although blood typically accounts for more than 50% of the total oxygen reserves of adult pinnipeds (Kooyman, 1985), previous studies indicated that neonates and juveniles have significantly lower oxygen stores in their blood than adults (Thorson and LeBoeuf, 1994; Horning and Trillmich, 1997; Ponganis et al., 1999b; Costa et al., 1998; Burns, 1999; Noren et al., 2001; Noren et al., 2002; Burns et al., 2005; Fowler, 2005; Kuhn et al., 2006; Noren et al., 2005; Richmond et al., 2006). In addition to lower blood oxygen stores, myoglobin has been shown to have the longest development time of all oxygen storage compartments (Thorson, 1993; Noren et al., 2001; Burns et al., 2005; Fowler, 2005; Noren et al., 2005; Richmond et al., 2006).

Sexual dimorphism is evident among marine mammals, and physiological diving capacity, maximum diving depths and dive durations exhibit an allometric relationship with body size (Stonehouse, 1967; Lasiewski and Calder, 1971; Piatt and Nettleship, 1985; Gentry et al., 1986; Hudson and Jones, 1986; Prince and Harris, 1988; Burger, 1991; Wilson, 1991; Watanuki et al., 1996; Schreer and Kovacs, 1997; Halsey et al., 2006). Furthermore, the requirement for greater energy intake and increased diving capacity with size may lead to different foraging strategies and differences in ecology between the sexes. Although differences between male and female foraging behaviors of sexually dimorphic marine mammals have been examined [northern elephant seal *Mirounga angustirostris* (Le Boeuf et al., 2000); southern elephant seal *M. leonine* (Slip et al., 1994); grey seal *Halichoerus grypus* (Beck et al., 2003); New Zealand fur seal *Arctocephalus forsteri* (Page et al., 2005)], little is known about differences between sexes in the physiological diving capacity.

In pinnipeds, studies on neonates revealed that pups, like most young terrestrial mammals, had disproportionately greater metabolic rates, and limited control of heart rate and metabolic

processes (Elsner et al., 1977; Rea and Costa, 1992; Castellini et al., 1994). For many species the implications of these physiological limitations on the diving and foraging ability of young pinnipeds has not been considered. Recently, studies of the development of blood oxygen storage capacity indicated that the rate at which pups mature is closely tied to the length of the dependency period (Thorson and LeBoeuf, 1994; Horning and Trillmich, 1997; Merrick and Loughlin, 1997; Costa et al., 1998). In most true seals (Family Phocidae), nursing is a short period (4–50 days) and weaning is abrupt when the females abandon pups and return to the sea to forage (Costa, 1991; Costa, 1993). In contrast, for sea lions and fur seals (Family Otariidae), nursing lasts between 6 months and 3 years, with the females making short foraging trips lasting 3–14 days during that time (Costa, 1991; Costa, 1993).

In general, phocids store a greater proportion of their total oxygen stores in their blood while otariids store a greater proportion of their total oxygen in their muscle; therefore, the development of myoglobin stores could particularly constrain the diving behavior of young sea lions (Kooyman, 1989). Because otariid pups begin to dive well before weaning and the prolonged development of muscle oxygen stores, the full development of blood oxygen stores at weaning was expected. More recently, postnatal development of myoglobin oxygen stores was found to be dependent upon the initiation of independent foraging rather than the length of the dependency period in several marine mammal species [bottlenose dolphins *Tursiops truncatus*; northern elephant seals (Noren et al., 2001); Antarctic and sub-Antarctic fur seals, *Arctocephalus gazella*, *A. tropicalis* (Arnould et al., 2003); Steller sea lions, *Eumatopias jubatus* (Richmond et al., 2006)]. Further, myoglobin content increased significantly in phocids during the time between weaning and the onset of independent foraging. This time frame likely corresponds to increased activity levels, thermal demands, and time spent in apnea during swimming and diving, which have been hypothesized to explain changes in myoglobin content in immature animals (Noren et al., 2001; Arnould et al., 2003; Fowler, 2005) because of their effect demonstrated in adult animals (Morrison, 1966; Stephenson et al., 1989; MacArthur, 1990; Saunders and Fedde, 1991).

Few researchers have simultaneously measured the development of blood and muscle oxygen stores in otariids [Steller sea lion *Eumatopias jubatus* (Richmond et al., 2006); Australian sea lion *Neophoca cinerea* (Fowler, 2005)], and to date no research has included all age classes and both sexes through adulthood in the analysis. Limited data are available on the oxygen stores of California sea lions (Lenfant et al., 1970; Ponganis et al., 1997; Kuhn et al., 2006), and no data are available on differences between the sexes or the development of oxygen stores, and their affect on diving capacity in this species.

The California sea lion is sexually dimorphic, with adult males greater than four times the size of adult females. Female sea lions give birth at island rookeries in southern California from late May through late June each year and remain in the

area of breeding rookeries throughout lactation until weaning at 6–11 months (Melin, 1995). California sea lion pups are not precocial when born and do not enter the water for several weeks after birth, and may not begin foraging until 7 months of age at the earliest (Boness, 1991). Following the breeding season, most sub-adult and adult males disperse along the coast to central and northern California (Weise, 2006), and possibly as far as Oregon and Washington (Bartholomew, 1967), whereas females remain on the rookery with their pups. Adult male and female sea lions exhibit a shallow epi-mesopelagic foraging strategy with mean diving depth between 32 and 58 m and durations of 1.9 to 2.1 min, respectively (Feldkamp et al., 1989; Costa et al., 2004; Kuhn, 2006; Weise, 2006). Here we examine the effects of body size, age and sex on blood and muscle oxygen stores in sea lion pups through adults and evaluate how differences in oxygen stores may affect diving capacity and foraging behavior.

## Materials and methods

### Field sites and data collection

A total of 31 adult male, 37 adult females, 16 sub-adult males, 30 juveniles, 9 nine-month old pups and 11 five-month old pups were captured during this study. Adult and sub-adult male California sea lions (*Zalophus californianus* Lesson) were captured in the municipal harbor of Monterey, California, USA (36°36.5'N, 121°53.4'W) during September through December 2003 and 2004, using a modified aluminum enclosure (3 m×4 m×2 m) and squeeze cage (2.5 m×1 m×1.5 m). The enclosure was situated on a floating platform (4 m×8 m) and secured to the United States Coast Guard wharf near the base of the rock jetty where animals normally haul out on land to rest. Captures involved the voluntary entry of sea lions into the enclosure followed by the manual triggering of a sliding type door to enclose the animals inside. Animals were transferred to the squeeze cage where they were physically restrained and if necessary sedated with Midazolam intramuscularly at 0.20 mg kg<sup>-1</sup> (mixed with atropine at 0.02 mg kg<sup>-1</sup>) in conjunction with isoflurane gas (0.5–2.5%w/O<sub>2</sub>) or with isoflurane alone.

Adult females and juveniles were captured on Año Nuevo Island (37°6'N, 122°20'W) during September through October 2002, and adult females, juveniles and pups were captured on San Nicolas Island (32°16.0'N, 119°29.8'W) during late October 2003 and 2004, and March 2005. Animals were selected based on healthy appearance, accessibility with minimal disruption to the rookery, alertness and proximity to other alert individuals. Adult females, juveniles and pups were captured with a modified hoop net (Fuhrman Diversified, Seabrook, TX, USA). The nets were a soft mesh with a hole at the end for the animal's nose to facilitate breathing. Once in the net animals were transferred to a kennel for holding and, if necessary, given an intramuscular dose of Midazolam (0.20 mg kg<sup>-1</sup> mixed with atropine at 0.02 mg kg<sup>-1</sup>). Isoflurane gas was administered with oxygen *via* a portable vaporizer (Gales and Mattlin, 1998). Lengths and girths were measured

on all animals, including adult and sub-adult males, at six locations along the long axis of an animal and each animal was weighed (Dyna-Link MSI-7200; Measurement Systems International, Seattle, WA, USA).

Age of pups, juvenile, and sub-adult males was assessed using date of capture, body morphometrics and degree of canine tooth eruption. We were unable to verify the specific age in years for any size class, and young animals were broken into two categories based on size. Adult males were distinguished from sub-adult males by size and presence of a sagittal crest. Adult females were distinguished from juveniles by size and presence of lactation.

#### *Blood and muscle sample collection*

Blood samples were collected from the caudal gluteal vein, interdigital rear flipper vein, or jugular vein. Hematocrit (Hct) was measured in a subset of animals of each size class by collecting an initial blood sample prior to the induction of isoflurane gas, because of the decline of Hct as the spleen expands following isoflurane delivery (Zapol et al., 1989; Castellini et al., 1996; Costa et al., 1998). Following isoflurane induction and full sedation of animals a blood sample (time,  $T=0$ ) was taken and placed in a heparin Vacutainer (BD Vacutainer SST, Franklin Lakes, NJ, USA). Each animal received an intra-venous injection of Evan's Blue dye at a dose of  $0.6 \text{ mg kg}^{-1}$  and a concentration of  $10, 20$  or  $30 \text{ mg ml}^{-1}$  to determine blood volume (El-Sayed et al., 1995). Sequential 8–10 ml samples were taken as described above at approximately  $T=10, 20$  and  $30$  min post injection. Syringes were weighed with a portable field balance ( $\pm 0.001 \text{ g}$ ) before and after injections to accurately determine the mass of dye injected. Upon completion of injections, but before removal of the needle, blood was drawn into the syringes to flush the contents of the syringe into the vein, ensuring that all dye was injected. Blood samples were stored on ice until transported to the lab, centrifuged and stored at  $-20^\circ\text{C}$  until analysis.

Muscle biopsies of approximately  $50 \text{ mg}$  each were collected from live animals during anesthesia by making a 2–3 cm incision through the blubber layer then using a 6 mm canula or a biopsy needle. The biopsy site was cleaned with Betadine (Fisher Scientific, Pittsburgh, PA, USA) administered with sterile wipes before and after the procedure. Biopsies were collected from the dorsal triceps complex and supraspinatus located above the scapula, which are primary locomotor or swimming muscles in otariids. Muscle samples were stored on ice until transported to the lab and stored at  $-80^\circ\text{C}$  until analysis.

#### *Laboratory analysis*

Complete blood counts (CBCs) were determined for juvenile, sub-adult male, adult male and female sea lions collected in 2003 in the Monterey Harbor and Año Nuevo Island using an Animal Blood Counter (ABX Diagnostics, Montpellier, France), located at the Marine Mammal Center in Sausalito, California, USA. This Animal Blood Counter was calibrated for California sea lion blood analysis and programmed for the size and shape of their red blood cells. Red

blood cell (RBC), hemoglobin (Hb) concentration, hematocrit (Hct), and mean corpuscular hemoglobin concentration (MCHC) were determined for each animal. Automated Coulter counters not calibrated for marine mammals have resulted in values 4–15% greater than values obtained from microcentrifugation (Castellini et al., 1996). To ensure the accuracy of our method, we determined Hct for a subset of sea lions using standard clinical microhematocrit centrifugation and compared estimates based on Coulter counter methods on the same individuals. No difference in Hct ( $F_{1,24}=0.009$ ,  $P=0.926$ ) was found between the two methods.

Hematocrit using microcentrifugation and hemoglobin using the methanocyanide technique was measured on all animals sampled in the Monterey Harbor in 2004, and San Nicolas Island during 2003 and 2004. Upon returning from the field,  $10 \mu\text{l}$  aliquots of whole blood from  $T=0$  heparinized tubes were added to test tubes containing  $2.5 \text{ ml}$  of Drabkins Reagent (Ricca Chemical Co., Arlington, TX, USA). Samples were read at  $540 \text{ nm}$  on a split-beam spectrophotometer (Spectronic 1001, Bausch and Lomb, Rochester, NY, USA), and Hb was calculated using a linear regression based on absorbance values from a series of standards (Fisher Scientific, Pittsburgh, PA, USA).

Remaining blood samples collected at 0, 10, 20 and 30 min were centrifuged at  $3000 \text{ g}$  for 15 min, within 4 h of being collected, to separate blood cells from plasma. Supernatant containing blood plasma was frozen and stored at  $-20^\circ\text{C}$ . Maximum absorption of the Evan's Blue dye in sea lion plasma using a spectrophotometer was determined to be  $624 \text{ nm}$ . Photometric absorbance values were determined at  $624$  and  $740 \text{ nm}$  for all plasma samples. As  $740 \text{ nm}$  does not absorb blue, these values were used to calculate the blank optical density at  $624 \text{ nm}$  to account for possible hemolysis and precipitate (Foldager and Blomqvist, 1991). Serially collected Evan's Blue samples were logarithmically transformed and fit to a regression line, and the instantaneous dilution volume was determined from the  $y$ -intercept (El-Sayad et al., 1995). Blood volume ( $V_B$ ) was calculated from the hematocrit and plasma volume [ $V_P = \text{mg dye injected} / \text{dye concentration}$  (Swan and Nelson, 1971)] as follows:

$$V_B = V_P / [(100 - \text{Hct}) / 100]. \quad (1)$$

Myoglobin content ( $\text{g Mb } 100 \text{ g}^{-1}$  wet muscle) was determined for all animals using Reynafarje's procedure (Reynafarje, 1963).

For all sea lions sampled in the Monterey Harbor in 2004, and San Nicolas Island 2003 and 2004, we estimated the average MCHC using the following equation:

$$\text{MCHC} = (\text{Hb} / \text{Hct}) \times 100. \quad (2)$$

Total available oxygen stores were calculated for each sea lion using the following methods (Kooyman, 1989; Davis and Kanatous, 1999; Costa et al., 2001):

$$\text{CaO}_2 = V_B \times 0.33 B_{\text{BO}_2} \times 0.80, \quad (3)$$

$$\text{CV}_{\text{O}_2} = V_B \times 0.67 B_{\text{BO}_2} \times S\bar{v}_{\text{O}_2}, \quad (4)$$

Table 1. Morphometric measurements used to determine different age classes of California sea lions

Age class	N	Mass (kg)	Standard length (cm)	Curvilinear length (cm)	Anterior foreflipper (cm)	Canine* (mm)	Incisor* (mm)
5-month pups	11	20.9±1.4	100.2±1.4	105.2±1.4	31.0±0.6	NA	NA
9-month pups	9	23.2±1.2	100.4±1.7	105.4±1.7	29.6±0.7	3.7±0.5	6.5±0.3
Small juvenile	16	39.1±1.6	126.4±2.5	129.0±3.2	37.3±0.7	8.3±0.7	8.6±0.4
Large juvenile	12	70.1±2.7	156.4±2.0	163.9±2.6	47.4±0.8	NA	NA
Sub-adult male	16	125.4±3.8	187.5±2.0	197.1±1.9	54.8±0.5	NA	NA
Adult female	37	86.7±2.1	165.4±2.0	173.2±2.2	48.2±0.7	NA	NA
Adult male	31	175.0±7.8	205.6±2.3	216.2±2.3	58.8±0.6	NA	NA

Values are means ± s.e.m.; NA, not available.

\*Data from 9 month old pups ( $N=5$ ) and small juveniles ( $N=9$ ) captured on San Nicolas Island in March 2005.

where  $Ca_{O_2}$  is arterial blood oxygen (ml),  $Cv_{O_2}$  is venous blood oxygen (ml),  $V_B$  is blood volume (ml), percent arterial blood=0.33%, percent venous blood=0.66%,  $B_{BO_2}$  is capacitance coefficient of blood ( $g\ Hb\ l^{-1} \times 1.34\ ml\ O_2\ g^{-1}\ Hb$ ), assuming Hb oxygen-carrying capacity of  $1.34\ ml\ O_2\ g^{-1}$  (Dill et al., 1932), and  $S\bar{v}_{O_2}$  is oxygen saturation of mixed venous blood, which assumes an oxygen content that was 5% by volume less than the initial arterial blood oxygen. We assumed that the arterial blood was 100% saturated at the beginning of the dive as a result of pre-dive hyperventilation and 20% saturated at the end of the dive (Kooyman et al., 1980; Kooyman, 1989; Ponganis et al., 1993). Total muscle oxygen stores were calculated as follows:

$$C_{MO_2} = M_b \times 0.37 \times Mb \times 1.34, \quad (5)$$

Where  $C_{MO_2}$  is skeletal muscle oxygen concentration (ml),  $M_b$  is body mass (kg), 0.37 is the fraction of muscle mass in the body (Ponganis et al., 1997),  $Mb$  is myoglobin concentration, and  $1.34\ O_2\ g^{-1}$  is oxygen binding capacity of myoglobin and complete saturation at the beginning of the dive (Gayeski et al., 1987; Schenkman et al., 1997). Muscle oxygen content was based on the assumption that myoglobin was uniformly distributed throughout the musculature. Diving lung oxygen stores ( $C_{LO_2}$ ) were calculated using the following:

$$C_{LO_2} = (V_L \times M_b \times 0.15F_{O_2}), \quad (6)$$

where  $V_L$  is the measured diving lung volume ( $55\ ml\ O_2\ kg^{-1}$ ) of the closely related Steller sea lion (Lenfant et al., 1970),  $M_b$  is body mass, and  $0.15F_{O_2}$  is the oxygen extracted from the air in the lungs (Kooyman et al., 1971).

### Statistics

Size and age class of sea lions were defined using a hierarchical cluster analysis to detect discontinuous groupings or 'clumps' of data points. Euclidean distance and an average linkage function were used as this measure maximized the cophenetic correlation coefficient and thus best represented the raw data structure (Gauch, 1982; McGarigal et al., 2000) (Table 1). Change in oxygen stores with body size and during the development of California sea lions was examined using a one-way ANOVA followed by a Tukey pairwise comparison

test to compare inter-age differences in blood oxygen storage parameters (Hct, Hb, MCHC,  $V_P$ ,  $V_B$ ), muscle oxygen stores ( $C_{MO_2}$ ), absolute and mass-specific total oxygen stores (blood and muscle). Sex differences in the blood and muscle oxygen store parameters were analyzed using *t*-tests in size classes containing both sexes (5-month old pups, small juveniles and adults). ANCOVA was used to compare differences in the rate (slope) of development between sexes, based on least-squares regression, of myoglobin, and blood, muscle and total mass-specific oxygen stores. All variables were tested for normality and homogeneity of variances. Statistical analysis was completed using SYSTAT 11.0 software package. Values are reported as means ± standard error (s.e.m.). Values were considered significant if  $P \leq 0.05$ .

## Results

### Age and size related changes in blood and muscle oxygen stores

There were significant size and age related changes in blood oxygen stores of Hb ( $F_{6,127}=15.644$ ,  $P<0.001$ ), Hct ( $F_{6,68}=33.034$ ,  $P<0.001$ ) and MCHC ( $F_{6,116}=2.203$ ,  $P=0.048$ ; Fig. 1). The most pronounced changes in Hct, Hb and MCHC occurred in pups between 5 and 9 months of age; however, a slight decrease in MCHC from 5 to 9 month old pups occurred followed by an increase from juveniles (39–70 kg) to adults. While Hct increased during early development, the oxygen carrying capacity of individual red blood cells decreased initially prior to increasing to relatively stable levels.

Absolute  $V_P$  ( $F_{6,106}=190.604$ ,  $P<0.001$ ) and  $V_B$  ( $F_{6,106}=192.570$ ,  $P<0.001$ ; Fig. 2A), and mass-specific  $V_P$  ( $F_{6,106}=7.977$ ,  $P<0.001$ ) and  $V_B$  ( $F_{6,106}=12.507$ ,  $P<0.001$ ; Fig. 2A) increased linearly with greater body mass, although much less variation was explained in the relationship with mass-specific  $V_P$  and  $V_B$ . *Post-hoc* tests revealed that absolute  $V_P$  (5 and 9 months,  $P=1.000$ ; 9 months and small juvenile,  $P=0.498$ ; small and large juvenile,  $P<0.001$ ; sub-adult,  $P<0.001$ ; adult,  $P<0.001$ ) and  $V_B$  (5 and 9 months,  $P=1.000$ ; 9 months and small juvenile,  $P=0.313$ ; small and large juvenile,  $P<0.001$ ; sub-adult,  $P<0.001$ ; adult,  $P<0.001$ ) of larger juvenile through adult-size sea lions were significantly greater



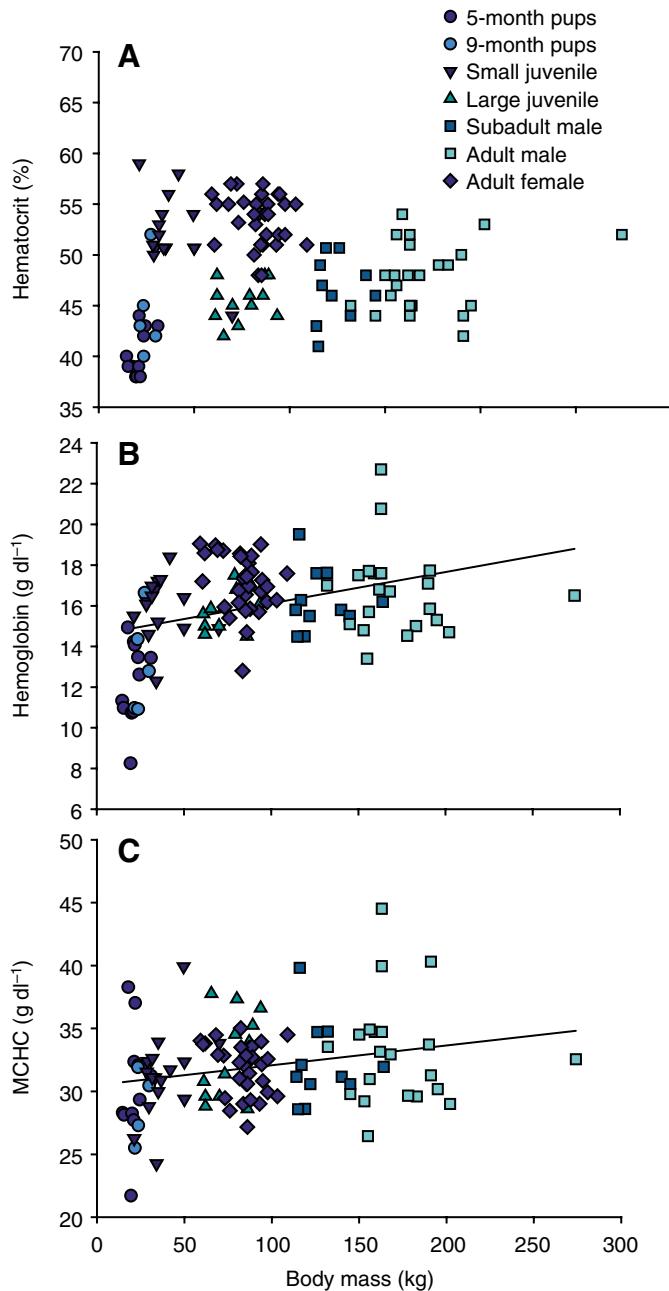


Fig. 1. Developmental changes in (A) hematocrit, (B) hemoglobin content and (C) mean corpuscular hemoglobin content as a function of body size in California sea lions from 5-month old pups to adults (5-month pups,  $N=13$ ; 9-month pups,  $N=7$ ; small juvenile,  $N=18$ ; large juvenile,  $N=12$ ; sub-adult male,  $N=15$ ; adult male,  $N=22$ ; 87 kg adult female,  $N=37$ ). For all animals combined the relationship between body size and Hct was not significant ( $r^2=0.01$ ,  $P=0.413$ ), whereas there was a significant linear relationship with Hb ( $r^2=0.14$ ,  $P<0.001$ ) and MCHC ( $r^2=0.06$ ,  $P=0.009$ ).

than younger animals. By contrast, mean mass-specific  $V_p$  of larger juvenile through adult-size sea lions were significantly greater than younger animals (5 and 9 months,  $P=0.474$ ; 9 months and small juvenile,  $P=0.136$ ; small and large juvenile,  $P=0.680$ ; sub-adult,  $P=0.098$ ; adult,  $P=0.001$ ), and smaller

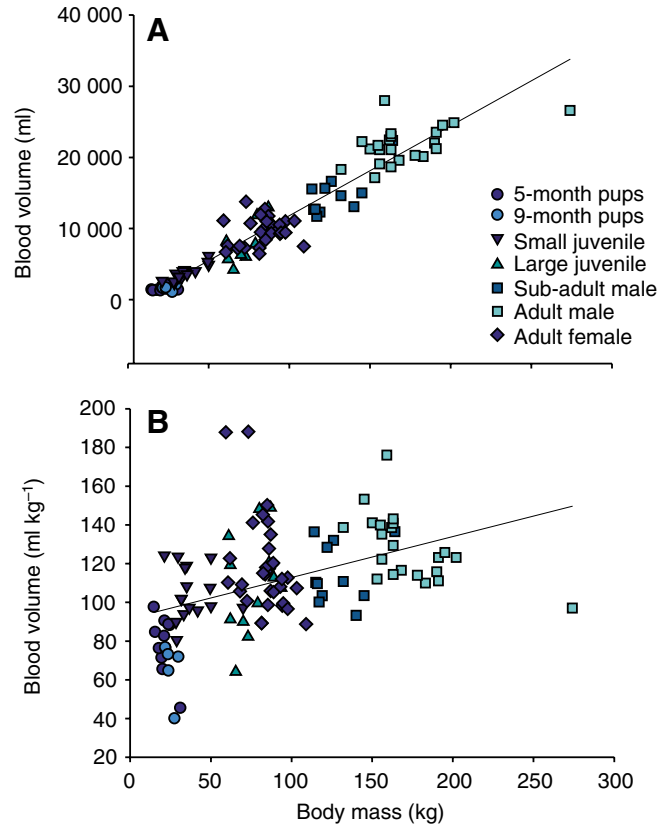


Fig. 2. Developmental changes in (A) absolute blood volume and (B) mass-specific blood volume with increasing body size in California sea lions from 5-month old pups to adults. There was a significant linear relationship during the development of absolute ( $r^2=0.92$ ,  $P<0.001$ ) and mass-specific blood volume ( $r^2=0.20$ ,  $P<0.001$ ).

juveniles through adults had greater mass-specific  $V_B$  than pups (5 and 9 months,  $P=0.887$ ; 9 months and small juvenile,  $P=0.005$ ; large juvenile,  $P=0.002$ ; sub-adult,  $P<0.001$ ; adult,  $P<0.001$ ). Mean mass-specific  $V_p$  and  $V_B$  slightly decreased from 5- to 9-month old pups before increasing significantly through large juveniles to adult size, and was likely the source of greater variation in the relationship with body mass. Muscle myoglobin concentration increased significantly ( $F_{6,91}=48.013$ ,  $P<0.001$ ) from pups (21–23 kg) to juvenile and sub-adult stages (39–125 kg), and from the sub-adult size to adults (Fig. 3).

As a result of ontogenetic changes in blood and muscle oxygen stores, total blood ( $F_{6,91}=124.127$ ,  $P<0.001$ ), muscle ( $F_{6,91}=157.481$ ,  $P<0.001$ ) and total oxygen storage capacity ( $F_{6,91}=213.616$ ,  $P<0.001$ ) increased significantly throughout development (Fig. 4). The proportion of oxygen stored in muscle was initially high in pups, resulting from a relatively low proportion of oxygen in immature blood stores. Subsequently, the proportion of oxygen stored in the muscle increased with greater body size, whereas the proportion of oxygen stored in matured blood stores stabilized beyond pups aged 5- and 9-months old. Mass-specific blood ( $F_{6,91}=20.036$ ,  $P<0.001$ ), muscle ( $F_{6,91}=48.026$ ,  $P<0.001$ ) and total oxygen

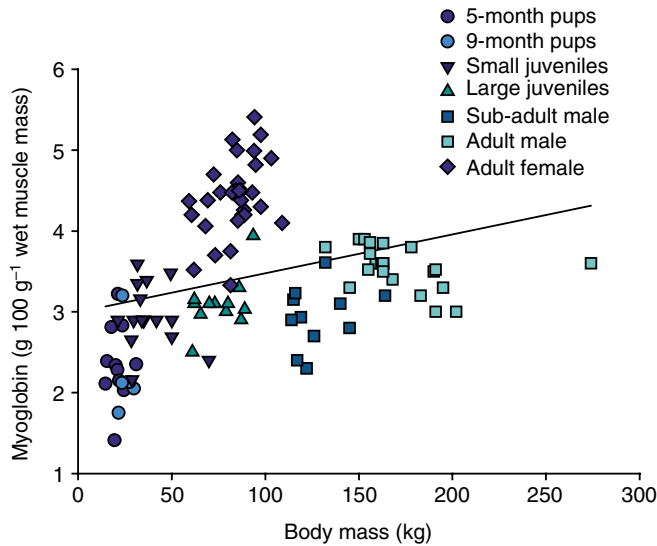


Fig. 3. Mean muscle myoglobin content in relation to body size for California sea lions from 5-month old pups to adults. Line represents least-squares regression ( $r^2=0.10$ ,  $P=0.001$ ).

stores ( $F_{6,91}=35.172$ ,  $P<0.001$ ) changed significantly with increasing size (Table 2).

#### Sex differences in oxygen stores

Differences in the development of some blood oxygen store parameters and absolute and mass-specific blood, muscle, and total oxygen stores among size classes resulted from differences between sexes. No significant differences in Hct and Hb were found between sexes for any age class; however, MCHC was significantly greater in adult males than adult females ( $F_{1,62}=4.565$ ,  $P=0.037$ ) indicating a greater capacity for red blood cells of males to carry oxygen than females. There were no differences in absolute  $V_p$  with sex in pups and juveniles, however  $V_p$  was significantly greater in adult males ( $F_{1,62}=4.565$ ,  $P=0.037$ ); by contrast, a significantly greater absolute  $V_B$  of males was first apparent in small juveniles (39 kg;  $F_{1,14}=4.790$ ,  $P=0.046$ ) and continuing through adult sizes ( $F_{1,52}=395.067$ ,  $P<0.001$ ). Muscle myoglobin was not significantly different between the sexes until adulthood ( $F_{1,41}=81.001$ ,  $P<0.001$ ); however, the rate (slope) of development of Mb stores was significantly greater in female sea lions ( $t=4.93$ ,  $P<0.001$ ; Fig. 5).

Total blood and muscle oxygen stores and total oxygen stores were significantly greater in males than females beginning with the small juvenile age class (blood:  $F_{1,14}=4.319$ ,  $P=0.047$ ; muscle:  $F_{1,14}=6.212$ ,  $P=0.026$ ; total:  $F_{1,14}=5.242$ ,  $P=0.038$ ; Fig. 5) continuing through adulthood (blood:  $F_{1,41}=142.122$ ,  $P<0.000$ ; muscle:  $F_{1,41}=75.419$ ,  $P<0.000$ ; total:  $F_{1,14}=202.189$ ,  $P<0.000$ ). Differences in absolute  $V_p$ ,  $V_B$  and blood, muscle and total oxygen stores between sexes were not surprising, given that adult males are significantly larger than adult females. Sex differences in oxygen stores as a function of age/size class became evident while animals were small

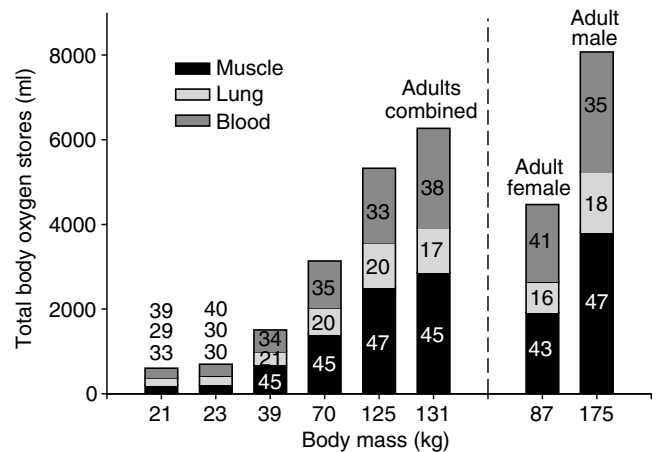


Fig. 4. Comparison of absolute total body oxygen stores in the blood, lung and muscle of California sea lions during their development from 5-month old pups to adults (131 kg; male and female combined). Adult female and male also compared to right of broken line. Percentage of oxygen stores in blood, lung and muscle are listed in text for each compartment.

juveniles, even though there were no differences in mass as a function of sex at this age.

As expected, adult males had a greater absolute amount of oxygen in their blood and muscle stores than adult females due to greater body mass; however, what was unexpected was that female mass-specific muscle oxygen stores were significantly greater than adult males ( $F_{1,41}=81.413$ ,  $P<0.001$ ; Table 2), although mass-specific blood oxygen stores were not different ( $F_{1,41}=0.521$ ,  $P=0.475$ ). Similarly, the rate of development of female mass-specific muscle oxygen stores was significantly greater than males ( $t=4.47$ ,  $P<0.001$ ; Fig. 6B), whereas, the rate (slope) of development of male mass-specific blood oxygen

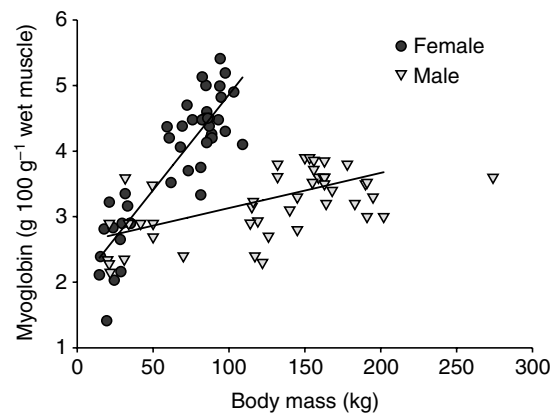


Fig. 5. Muscle myoglobin as a function of body size for male and female California sea lions from pup size through adulthood. The relationship between body size and myoglobin was significant for males ( $y=0.005x+2.56$ ;  $r^2=0.36$ ,  $P<0.001$ ) and females ( $y=0.03x+1.95$ ;  $r^2=0.78$ ,  $P<0.001$ ), and the rate of development was significantly different for the sexes.

Table 2. Mean body mass, mass-specific oxygen store parameters and calculated minimum and maximum aerobic dive limits based on diving and resting metabolic rates for different size classes of free-ranging California sea lions

Size class	N	Mass (kg)	Mass-specific oxygen stores (ml kg <sup>-1</sup> )			Calculated aerobic dive limit	
			Blood	Muscle	Total	Minimum <sup>a</sup>	Maximum <sup>b</sup>
5-month pup	11	20.9±1.4	9.4±0.8	11.1±0.7	28.7±1.4	1.4±0.1	3.7±0.2
9-month pup	9	23.2±1.5	8.3±0.7	11.3±1.9	27.8±1.6	1.4±0.1	3.7±0.2
Small juvenile	16	38.9±3.0	17.7±0.7	13.8±0.4	39.8±1.1	2.2±0.1	5.9±0.2
Large juvenile	12	75.7±3.4	18.2±1.4	14.5±0.4	41.0±1.4	2.7±0.1	7.3±0.2
Sub-adult male	16	125.2±4.2	19.5±1.1	13.8±0.5	41.5±1.2	3.1±0.1	6.4±0.2
Adult female	37	86.7±2.1	22.3±1.0	21.0±0.5	51.5±1.1	3.4±0.1	5.2±0.1
Adult male	31	174.6±6.2	22.5±1.0	16.5±0.2	47.3±1.1	3.8±0.1	7.9±0.2

Values are means ± s.e.m.

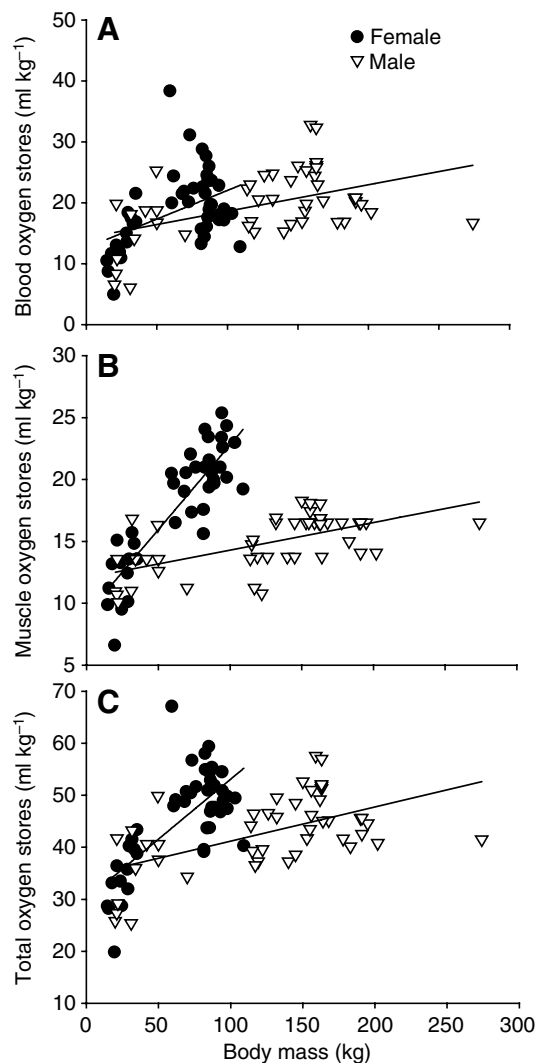
Maximum and minimum calculated aerobic dive limits (cADL) were based on diving and resting metabolic rate (MR), respectively.

<sup>a</sup>Min cADL=2.22×age (months or years)−2.5533;  $r^2=0.7006$ ,  $P=0.050$ .

<sup>b</sup>Max cADL=0.2357×age (months or years)+1.3571;  $r^2=0.7479$ ,  $P<0.000$ .

stores was no different than females ( $t=-0.37$ ,  $P=0.714$ ; Fig. 6A). Although mass-specific total oxygen stores of females were significantly greater than those of males

( $F_{1,41}=202.189$ ,  $P<0.001$ ), the rate of development in females was not significantly different than males ( $t=1.43$ ,  $P=0.155$ ; Fig. 6C).



Discussion

Our findings demonstrate pronounced differences in blood and muscle oxygen stores of California sea lions as a function of size, age and sex. The most prominent finding is the surprisingly long period of development required for blood (1.5–2.5 years) and muscle (4–6 years) oxygen stores to reach adult values. In fact, sea lions were foraging independently well before oxygen stores reach adult levels. Although a difference in myoglobin between sexes might be expected given the sexual dimorphism in this species, the greater Mb concentrations in the muscles of smaller adult females compared to larger adult males was surprising. These greater Mb levels gave adult females greater mass-specific muscle, and consequently, greater mass-specific total oxygen stores compared to adult males.

#### Effects of age and body size on oxygen stores

Blood oxygen stores were not fully developed at weaning and differences among size classes were related to age and

Fig. 6. Mass-specific (A) blood oxygen stores, (B) muscle oxygen stores and (C) total oxygen stores as a function of body size was significant for male and female California sea lions. The relationship between body size and mass-specific blood oxygen stores was significant for females ( $y=0.094x+12.66$ ;  $r^2=0.20$ ,  $P=0.002$ ) and males ( $y=0.03x+14.29$ ;  $r^2=0.99$ ,  $P=0.001$ ), and significant for muscle oxygen stores in females ( $y=0.14x+9.13$ ;  $r^2=0.78$ ,  $P<0.001$ ) and males ( $y=0.02x+12.06$ ;  $r^2=0.37$ ,  $P<0.001$ ), and total oxygen stores for females ( $y=0.23x+30.04$ ;  $r^2=0.50$ ,  $P<0.001$ ) and males ( $y=0.07x+35.60$ ;  $r^2=0.30$ ,  $P<0.001$ ). However, the rate of development in mass-specific muscle oxygen stores was greater in females than males, although no greater in blood and total oxygen stores.

body size. Among the blood parameters, only hematocrit had reached adult levels when pups were weaned at around 9 months. Differences in absolute plasma and blood volume and consequently blood and total oxygen stores were not surprising, given the differences in body size among age/size classes. While mass-specific  $V_P$  and  $V_B$  increased with greater body size, among age classes there was a lack of an increase in mass-specific blood volume until juveniles were larger (70 kg). This was likely related to a trade off between development of blood oxygen stores *versus* other components of growth, and differential growth and body composition between sexes and among individuals. In fact, no relationship between  $V_P$ ,  $V_B$  and age was found when scaled to lean body mass *versus* total body mass in harbor seals (Burns et al., 2005) and Steller sea lions (Richmond et al., 2006).

Similar to our results, Australian sea lion at the onset of independent foraging had blood oxygen stores lower than adult values (Fowler, 2005). This is in contrast to what was found in Steller sea lions (Richmond et al., 2006) and Galapagos fur seals, *Arctocephalus galapagoensis* (Horning and Trillmich, 1997), where juveniles had reached adult levels at weaning. Hct, Hb and RBC in yearling Galapagos fur seals were found to be similar to adult values (Horning and Trillmich, 1997); however, blood volume was not measured so a direct comparison of blood oxygen stores was not possible.

Although the development of blood oxygen stores was delayed past weaning in some otariids, it has been suggested that the development of Hct and Hb corresponds to lactation intervals (Richmond et al., 2006). For example, longer lactating species, such as Australian sea lion and Galapagos fur seal, do not reach adult levels of Hct and Hb until 18 months (Fowler, 2005) and 15 months of age (Trillmich, 1992), respectively. By contrast, species with shorter lactation intervals had adult levels of Hct and Hb at weaning, including Steller sea lions [Hct, 5 months; Hb, 9 months (Richmond et al., 2006)] and California sea lions in the southern Gulf of California [Hct and Hb, 9 months (Kuhn et al., 2006)]. In this study, however, California sea lions do not follow this pattern because they were weaned between 7 and 11 months of age and did not have adult levels of Hct and Hb until they were small juveniles with an estimated age of 1.5 to 2.5 years. Mean blood volume ( $96 \text{ ml kg}^{-1}$ ) for juvenile California sea lions reported by Ponganis et al. was within the range of values for small juvenile sea lions reported ( $81\text{--}124 \text{ ml kg}^{-1}$ ) in this study (Ponganis et al., 1997). Although young animals do not have the blood oxygen storage capacity of adults, the amount of oxygen they do have has been closely linked to the amount of time a young animal can spend underwater foraging (Costa, 1993).

Total body oxygen stores at the transition to independent foraging (9-month pups) were 54% of adult values, and male sea lions did not reach the equivalent of adult levels until the sub-adult stage (4–6 years). The magnitude of this difference between juveniles and adult females was consistent with California sea lions on the island of Los Islotes, in the southern Gulf of California that had 59% of adult stores (Kuhn et al., 2006), and similar to related species such as Steller sea lion

pups that had 80% of adult females (Richmond et al., 2006), Australian sea lion pups that had 50% of adult female values (Fowler, 2005), and juvenile New Zealand sea lions that had 87% of adult stores (Costa et al., 1998).

Our results support the prediction that myoglobin concentration would not be fully developed until adulthood, as seen in seabirds (Weber et al., 1974; Haggblom et al., 1988; Ponganis et al., 1999a), cetaceans (Dolar et al., 1998) and pinnipeds (Lenfant et al., 1970; Thorson and LeBoeuf, 1994; Burns et al., 2005; Richmond et al., 2006). Development of Mb was likely reflective of major developmental milestones in the ontogeny of total body oxygen stores and diving behavior. The first significant change in Mb during development was between 9-month old pups and small juveniles, which corresponded to animals' shift to foraging independently. Pups at weaning (9-months old) had approximately 60.7% of adult myoglobin content and small juveniles had 73.7% of adult values. Mean Mb levels (3.0 g%) and muscle oxygen stores ( $520 \text{ ml O}_2$ ) in small juveniles were consistent with previously published values for juvenile California sea lions [mass, 40 kg; Mb, 2.8 g%; muscle  $\text{O}_2$  stores,  $505\text{--}631 \text{ ml O}_2$  (Ponganis et al., 1997)].

Delayed development of myoglobin in juvenile sea lions may reflect reduced energetic intake of animals at the onset of independent foraging (Calkins and Pitcher, 1982; Merrick et al., 1988), which has been associated with high mortality and reduced growth in other sea lion species (Le Boeuf et al., 1994; Bowen et al., 2003; Richmond et al., 2006). Northern elephant seals lose mass during their first bout at sea foraging independently, and Mb during this time is lower than previously (Le Boeuf et al., 1994). The amount of oxygen stored in the blood was greater than in muscle throughout development, but the proportion of oxygen stored in the muscle increased with body mass and age, indicating a growing dependence on muscle oxygen stores.

Within pinnipeds, there appears to be a marked difference in the required period of postnatal development of Mb between phocids and otariids. Thorson and Kohin found that 300-day old elephant seals returned from their first trip to sea with 100% of adult Mb levels (Thorson, 1993; Kohin, 1998), and Burns et al. indicated that harbor seal yearlings in Alaska and California had similar Mb concentrations as adults (Burns et al., 2005). Sub-adult males in this study (estimated age 4–6 years), however, had Mb concentrations only 83% of adult values. Although total mass-specific oxygen stores of sub-adult males were not significantly different than adult males, their mass-specific muscle oxygen stores and consequently their calculated aerobic dive limits (cADL) were significantly less than adult males (Table 2). Determining the driving force in differences between phocids and otariids ontogeny of diving capacity is confounded by differences in lactation strategies. While otariids pups are provisioned by females for prolonged periods (months) of time resulting in a decreased necessity to forage independently early in life, phocids are weaned within weeks of birth and must rapidly learn to forage independently.



*Differences between sexes in diving capacity*

While differences in adult Mb levels between sexes have been observed in pinnipeds (Richmond et al., 2006), it was unexpected that adult female Mb concentrations, mass-specific muscle and total oxygen stores would be greater than adult males. Similar to Steller sea lions (Richmond et al., 2006), these differences were not apparent in younger age classes. Differentially greater Mb levels in adult females were consistent with other research that suggested that Mb and muscle oxygen stores are malleable (Morrison, 1966; Stephenson et al., 1989; Macarthur, 1990; Noren et al., 2001). Further, changes in only muscle oxygen stores and not blood oxygen stores in adult female sea lions indicated that blood stores may be at their developmental capacity and only muscle stores are malleable once sexual maturity is reached.

Different Mb levels between the sexes of sexually dimorphic species has been suggested to be related to different foraging or diving strategies, as might be expected with significant differences in body size (Le Boeuf et al., 2000; Richmond et al., 2006). Metabolic rate scales to  $M_b^{0.75}$ , whereas oxygen storage capacity scales to  $M_b^{1.0}$  (Kooyman, 1989). All things being equal, therefore, large adult male sea lions should be able to dive for greater duration and deeper than smaller adult females, based simply on body size. Greater exposure to increasing periods of apnea during diving resulting in increased Mb levels may explain how adult females extend their physiological diving capacity to compensate for the smaller body size. Conversely, less exposure to periods of apnea during shorter duration dives in adult males may explain lower Mb levels. Males may not be approaching their physiological capacity, and therefore, avoid incurring the energetic costs associated with developing muscle oxygen stores, yet were meeting their energetic demands using the advantage of greater body size. Further support for this hypothesis was found in sex differences in mass-specific total oxygen stores (blood and muscle combined), which were greater in adult female sea lions than in males corresponding to observed differences in patterns of diving in adult female and male California sea lions.

*Aerobic dive limit*

Previous studies of trained freely swimming sea lions showed that the metabolic rate (MR) of submerged California sea lions decreased as the period of submergence increased (Hurley and Costa, 2001). This suggests that while diving sea lions can vary the hypometabolic response as appropriate for the needs of the dive. A point estimate of cADL, therefore, does not take this into account and does not reflect the range of their aerobic capacity. To encompass this range we calculated an upper and lower limit of cADL from measurements of resting MR and estimates of diving MR derived from juvenile sea lions.

For pups and juveniles the lowest diving MR (DMR), which yields the highest cADL value, was assumed to be the resting metabolic rate of  $6.6 \text{ ml O}_2 \text{ min}^{-1} \text{ kg}^{-1}$  reported (Ponganis et al., 1997). The low DMR estimate for adult males was derived from measurements of submergence MR of

$6.43 \text{ ml O}_2 \text{ min}^{-1} \text{ kg}^{-1}$ ; mean mass 128 kg, and for adult females  $10.23 \text{ ml O}_2 \text{ min}^{-1} \text{ kg}^{-1}$ ; mean mass 66 kg (Hurley and Costa, 2001). To account for differences in mass of animals in this study submerged MRs were scaled to  $M_b^{0.75}$  for pups and juveniles ( $16.6 \text{ ml O}_2 \text{ kg}^{-0.75} \text{ min}^{-1}$ ), sub-adult and adult males ( $21.6 \text{ ml O}_2 \text{ kg}^{-0.75} \text{ min}^{-1}$ ) and adult females ( $29.2 \text{ ml O}_2 \text{ kg}^{-0.75} \text{ min}^{-1}$ ). The metabolic rates were the lowest rates recorded for these animals while they were sitting at the bottom of a pool. As such these rates do not include any costs associated with locomotion and thus should reflect the absolute lowest DMR possible.

The upper limit of DMR was derived from the only direct measurements of ADL in a California sea lion. Ponganis et al. directly measured the ADL of juvenile California sea lions otariid, and found that blood lactate levels increased during dives longer than 2.3 min (Ponganis et al., 1997). Given the oxygen stores measured in these juvenile sea lions, an ADL of 2.3 min would be equivalent to a DMR of  $17.8 \text{ ml O}_2 \text{ min}^{-1} \text{ kg}^{-1}$ . This DMR was used to calculate cADL for all groups of sea lions assuming that DMR varied as a function of  $M_b^{0.75}$  or  $44.8 \text{ ml O}_2 \text{ kg}^{-0.75} \text{ min}^{-1}$ . Admittedly, this extrapolation does not account for differences associated with sex and age, but at least it is derived from direct measurements of ADL and thus incorporates a consistent approach.

Significant differences among estimates of cADL for different age classes of sea lions were reflective of major milestones in the development of total body oxygen stores ( $F_{6,90}=68.48$ ,  $P<0.001$ ). The first major change in cADL occurred between pup and juvenile stage, when animals begin foraging independently, and corresponded with increased Hb and Hct. The second change among cADLs estimates occurred between juveniles and adults, which was consistent with the final stage of Mb development.

Aerobic diving capacity of juvenile California sea lions ranged from 48.5% to 56.7% of adult male diving capacity and 64.0% to 85.7% of adult female capacity at the transition to independent foraging (Table 2). During the next 3 to 5 years, at least in males, cADL increased to 69.7% to 85.1% of adult capacity, paralleling the development of total oxygen stores. This delay in cADL is not surprising given the delayed development of blood (2.5–3.5 years) and to a greater extent muscle oxygen stores (4–6 years), reflecting a transition from a greater reliance upon blood oxygen stores to muscle oxygen stores.

The minimum cADL determined for adult female and male sea lions in this study was consistent with the mean dive duration observed for free-ranging adult females [1.5–2.8 min (Feldkamp, 1987; Kuhn, 2006)] and adult males [1.9–2.3 min (Weise, 2006)], whereas maximum cADL was less than the maximum dive durations for individual males [range 4.4–11.1 min (Weise, 2006)] and less than those for adult females [range 6.0–9.9 min (Feldkamp et al., 1989)]. However, Hurley and Costa suggested the hypometabolic response during submergence was proportional to submergence duration (Hurley and Costa, 2001), and Butler suggested that aerobic metabolic rate during diving may be below resting level

(hypometabolism) for a portion of dives (Butler, 2006). Therefore, if we use the lowest recorded metabolic rate during submergence for an adult female (approximately  $6 \text{ ml O}_2 \text{ min}^{-1} \text{ kg}^{-1}$ ) and male sea lion [approximately  $3 \text{ ml O}_2 \text{ min}^{-1} \text{ kg}^{-1}$  (Hurley and Costa, 2001)], cADL increases to 8.6 min and 15.8 min, respectively, and maximum dive durations are close to or within maximum cADL.

Males dive well within their cADL, despite lower myoglobin levels and mass-specific muscle and total oxygen stores compared to adult females. For most of the year male sea lions forage in different geographic regions (central and northern California) than females on rookeries in southern California (Weise, 2006). Weise reported that 37% of male sea lions tagged in central California returned to rookeries in southern California outside the breeding season (Weise, 2006). While in southern California male diving behavior shifted towards the deeper female diving pattern with mean depth 64 m ( $\pm 94$  m) and durations of 3.3 min ( $\pm 2.3$  min). Dispersal of males northward of rookeries may be explained by the animals' ability to optimize their oxygen stores and meet their energetic needs more efficiently (shallow dives of less duration with less overall foraging effort) in central and northern California than in southern California.

#### Summary

This study confirms that blood and muscle storage parameters are not developed by the end of the dependency period, which is consistent with the ontogeny of oxygen stores found in a diverse array of cetaceans, phocids and otariids. Although our findings were consistent with other marine endotherms (pinnipeds, penguins and cetaceans) that require a period of postnatal development for Mb concentrations, sea lions were particularly delayed as sub-adult animals (4–5 years old) possessed Mb concentrations only 83% of adult values. There may be a fundamental difference in the physiological development of muscle in otariids compared with phocids. These limitations may help to explain the greater mortality of juvenile California sea lions during environmental perturbations and limited prey availability associated with El Niño events (DeLong et al., 1991). Sex differences in oxygen storage capacity, and consequently diving capacity, are likely based in differences in foraging strategies and effort between the sexes. The intrinsically greater dive capability of males due to their larger body size coupled with a less intense foraging effort observed in central and northern California apparently does not require elevated myoglobin concentration. By contrast, females apparently compensate for their smaller size and greater diving performance associated with pup rearing with greater myoglobin concentrations, in order to attain greater mass-specific total oxygen stores and associated increases in cADL.

#### List of abbreviations

cADL	calculated aerobic dive limit
CBC	complete blood count
DMR	diving MR

Hb	hemoglobin concentration
Hct	hematocrit
$M_b$	body mass
Mb	myoglobin
MCHC	mean corpuscular hemoglobin concentration
MR	metabolic rate
RBC	red blood cell concentration
$T$	time
$V_B$	blood volume
$V_L$	lung volume
$V_M$	skeletal muscle volume
$V_P$	plasma volume

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