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Postnatal ontogeny of erythropoietin and hematology in free-ranging Steller sea lions (*Eumetopias jubatus*)

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Abstract

The hormone erythropoietin (EPO) is responsible for the increased production of red blood cells (RBC) in response to tissue hypoxia. While the role of EPO in hematological development has been established in humans and terrestrial mammals, this relationship has never been examined in marine mammals that rely heavily on stored oxygen to maintain aerobic metabolism while diving. Since blood is the major oxygen storage site in marine mammals, it was hypothesized that EPO may have a significant influence on the development of hematology parameters associated with the expansion of blood oxygen stores during development. To explore this hypothesis, serum EPO concentrations were determined by radioimmunoassay in 235 free-ranging Steller sea lions (*Eumetopias jubatus*), throughout their Alaskan range. Hematocrit (Hct), hemoglobin (Hb), and red blood cell (RBC) counts were also measured, and mean corpuscular hemoglobin content (MCHC), mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCV) values determined. Erythropoietin and most hematological parameters varied with age. Hematocrit, Hb, RBC, and MCHC decreased after birth, reached their lowest values at two to three months of age, and then increased to values similar to those of adults by five months of age. Since changes in Hct and Hb account for the majority of the changes in blood oxygen stores and EPO was negatively correlated with both, it appears that EPO may play an important role in blood development of Steller sea lions, similar to previous studies on terrestrial mammals.

Keywords: Blood oxygen stores; Development; Erythropoietin; Hematology; Marine mammal; Population differences; Radioimmunoassay

1. Introduction

Erythropoietin (EPO) is a glycoprotein hormone that stimulates the production of red blood cells (RBC) in response to a reduction in oxygen availability caused by anemia, tissue hypoxia, or an increased hemoglobin affinity for oxygen (Jelkmann, 1992; Ratcliffe et al., 1997). Under conditions of low oxygen partial pressures, the hypoxia inducible factor (HIF-1a) binds to the enhancer element downstream to the EPO gene and stimulates transcription (Gassmann and Wenger, 1997). Once released into the blood stream, primarily from the kidney, circulating EPO then travels to the bone marrow where it increases the proliferation of new RBC, and prevents the programmed cell death of circulating RBC (Jelkmann, 1994; Palis and Segel, 1998). While an increase in EPO concentration can be observed within 1–2 h after hypoxic stimulus, the greatest concentrations of EPO in the blood occur 6–24 h after initial stimulus. However, reticulocytosis, or the increase in RBCs in circulation, does not occur until 3–4 days after initial EPO increase (Eckardt, 1994; Jelkmann, 1994). In addition, even moderately elevated concentrations of

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EPO can maintain an increased production of RBCs, or erythropoiesis (Eckardt, 1994).

Although research has primarily focused on the importance of EPO under conditions of physiological stress and disease, EPO may also play an important role in maintenance of oxygen homeostasis during neonatal development. Shortly after birth, most mammalian neonates experience a reduction in circulating hematocrit (Hct) and hemoglobin (Hb) concentrations due primarily to the relatively oxygen rich environment experienced by the neonate compared with that of the fetus (Palis and Segel, 1998). This normal 'physiological anemia of infancy' generally persists throughout the nursing period with Hct and Hb concentrations typically reaching their lowest point around weaning (Halvorsen and Bechensteen, 2002). Following the transition to independent foraging, Hct and Hb values slowly increase as animal growth stimulates tissue demand for more oxygen (Halvorsen and Bechensteen, 2002). Erythropoietin has been shown to play a role in these developmental changes (Kling et al., 1996). Typically, EPO concentrations are low at birth, become elevated when Hct and Hb reach their lowest point, and gradually decline towards normal adult values as Hct and Hb increase to adult values (Halvorsen and Bechensteen, 2002; Kling et al., 1996). A similar inverse relationship between EPO and Hct (and Hb) has been observed in adult mammals in response to hypoxia (Goldberg et al., 1993; Jelkmann, 1992).

Blood development is particularly important in species that rely heavily on increased oxygen storage capacity for diving and foraging activity. To sustain aerobic function during dives, adult marine mammals have a larger blood volume, and elevated hematocrit (Hct) and hemoglobin (Hb) values as compared with terrestrial species (Lenfant et al., 1970). While blood typically accounts for more than 50% of the total oxygen reserves in adult marine mammals (Kooyman, 1985), neonates have blood oxygen stores that are significantly lower than those of adults (Burns and Castellini, 1996; Clark, 2004; Horning and Trillmich, 1997), and these stores must increase in size before juveniles can dive and forage efficiently.

Steller sea lions (*Eumetopias jubatus*) provide a unique subject in which to study the role of EPO in the development of blood oxygen storage capacity. Young sea lions have a protracted nursing period that can last for up to 2 years (Calkins and Pitcher, 1982; Merrick et al., 1988). Pups do not enter the water for several weeks after birth, and do not begin to swim and dive routinely until 5–6 months of age (Loughlin et al., 2003; Raum-Suryan et al., 2002). Juveniles are not capable of movements and dive behavior similar to adults until at least 1 year of age (Loughlin et al., 2003; Pitcher et al., In review). Because pups begin to dive well before weaning, blood oxygen stores must increase prior to weaning. To assess whether this earlier development of blood oxygen stores is due to increased EPO production, as in terrestrial young, this study investigated developmental changes in hematology and in serum EPO concentrations in juvenile Steller sea lions.

2. Methods

2.1. Animal collection and age determination

Between 2000 and 2003, 455 Steller sea lions ranging in age from 1 month to greater than 3 years were captured throughout their Alaskan range by the Alaska Department of Fish and Game (ADFG), the National Marine Mammal Laboratory (NMML), and the Alaska SeaLife Center (ASLC). Archived serum samples were obtained from two adult males and four adult females captured in 1977, and five adult females captured in 1996. Individuals originated from three different populations: Southeast Alaska (SEA) ranging from the southern border of Alaska north to Cape Suckling, Prince William Sound (PWS) from Cape Suckling west to the Gulf of Alaska, and the Aleutian Islands (AI), including Kodiak west to the Alaskan end of the Aleutian chain (Fig. 1; Raum-Suryan et al., 2002). One-month-old sea lions were captured on their natal rookery using hoop nets, 2-month to 3-year-olds were captured using an underwater capture method developed by the ADFG (Raum-Suryan et al., 2004), and adult animals were captured following remote immobilization with telazol (Heath et al., 1996). Pups captured on rookeries that lacked an umbilicus were estimated to be 4 weeks of age (±2 weeks) based on the average pupping date of June 15 (Pitcher et al., 2001). Age determination for older animals was assessed using date, body size, and degree of canine tooth eruption, or canine growth annuli (King et al., 2003; Laws, 1962).

2.2. Blood collection and analysis

Approximately, 1–2 h postcapture sea lions were anesthetized with isoflurane gas based on methods outlined in Heath et al. (1997). Blood was collected from the interdigitial rear flipper vein or caudal gluteal vein as soon as anesthesia took full effect to standardize protocols and minimize the effect of isoflurane on Hct (Castellini et al., 1996). Two additional blood samples were collected at a minimum of 2 and 2.5 h after initial sampling on a subset of 29 individuals. Hematocrit was determined in duplicate using heparanized whole blood on a standard clinical microhematocrit centrifuge, and Hb were analyzed using the methanocyanide technique (525-A, Sigma–Aldrich, St. Louis, MO). Red blood cell counts were performed in duplicate using a hemocytometer on blood collected into EDTA-vacutainers. Mean

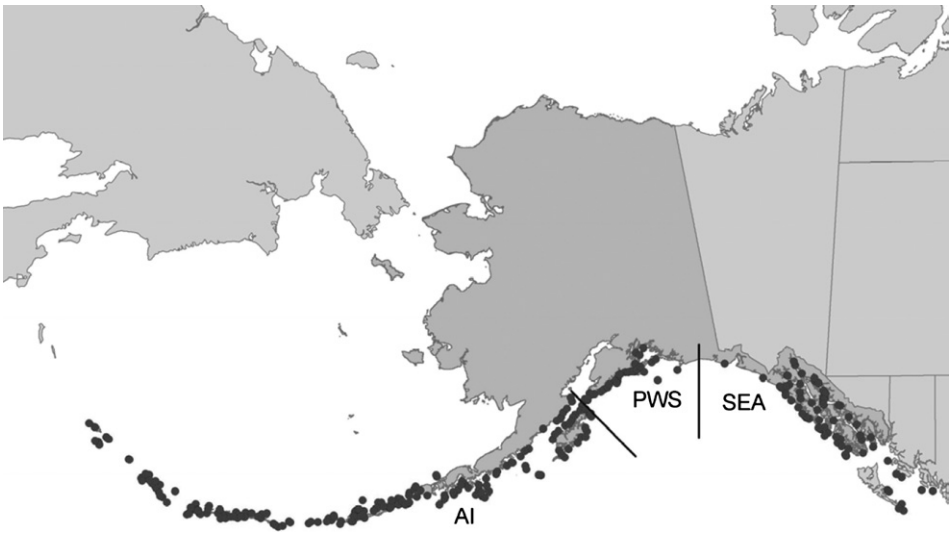

Fig. 1. Map of Alaska showing Steller sea lion haulouts with black circles, and divisions between Southeast Alaska (SEA), Prince William Sound (PWS), and Aleutian Islands (AI) sampling regions.

corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated in individuals from measured Hct, Hb, and RBC values. Blood collected into serum separator vacutainers was allowed to coagulate for 30–60 min, after which samples were centrifuged for 8 min, and the serum was decanted and frozen on dry ice prior to storage at −80 °C until analysis.

Serum EPO concentrations were determined in duplicate by radioimmunoassay (RIA 1100, Diagnostic Systems Laboratories, Webster, TX). Assay protocols were slightly modified for use with Steller sea lion serum as follows: sea lion serum was incubated with rabbit anti-human EPO serum for 8 h, ^{125}I EPO label was added and incubated for 18 h, samples were incubated for 30 min after the addition of the precipitating reagent, samples were then centrifuged at 0 °C at 1500*g* for 30 min, and the supernate was decanted. The radioactivity of the pellet was determined using a Diagnostic Products Corporation (Los Angeles, CA) C-12 Gamma counter. The standard curve ranged from 1.25 to 200 mU/ml. Four groups of pooled samples were used for assay validations: female pups, male pups, adult females, and adult males. Serial dilutions of the pooled samples showed a parallel relationship to the standard curve. Accuracy was high with a strong linear relationship between mass added (MA) and mass recovered (MR) in all pooled groups: male $\text{pup}_{\text{MR}} = 1.001(\text{MA}) - 1.396$, $R^2 = 0.996$; female $\text{pup}_{\text{MR}} = 0.989(\text{MA}) - 0.750$, $R^2 = 0.993$; male $\text{adult}_{\text{MR}} = 0.868(\text{MA}) - 0.210$, $R^2 = 0.996$; female $\text{adult}_{\text{MR}} = 0.9575(\text{MA}) - 1.0132$, $R^2 = 0.992$. Percent recovery ranged from 74 to 98%. Intra-assay and inter-assay coefficient of variation were less than 10 and 14%, respectively. Immunoblot analysis with horse sera showed that the linear parallelism demonstrated by serial dilution from the RIA for EPO did accurately reflect EPO concentrations and therefore no further validations were performed (Kearns et al., 2000).

2.3. Statistical analysis

To determine if EPO concentrations changed predictably over the holding period, serial samples collected from individuals were analyzed using a repeated measures ANOVA, for animals in three age classes: young of the year (<12 months), yearlings (12–24 months), and juveniles (>24 months). The effect of time of day was evaluated using a periodic regression analysis (Batschelet, 1981). To determine if sex, population, age, or their interactions significantly influenced EPO concentrations or any other blood parameters independently, animals were grouped into 1 month age categories, and three-way ANOVAs were run. When possible the initial blood sample was used in this analysis. Tukey post hoc tests were used to identify significant within factor differences. If region was identified as a significant factor, then each region was analyzed separately for age and sex effects. Due to small sample size, data from Aleutian Islands animals are shown for comparison purposes only. To establish whether EPO concentrations varied in response to Hct, Hb, or RBC, a linear regression analysis was performed for those animals in which both values were known ($n = 219$, $n = 154$, $n = 59$, respectively). Statistical analyses were completed using SPSS software package. Prior to all analyses, variables tested passed the Kolmogorov–Smirnov test for normality. Means are reported ±SE. Values were considered significant if $P \leqslant 0.05$.

3. Results

3.1. Erythropoietin

Erythropoietin concentrations were determined for 235 sea lions ranging in age from 1 month to 10 years. There were no systematic changes in EPO concentrations during handling ($F_{2,24} = 2.453$, $P = 0.107$; Fig. 2), and the average absolute individual change was less than the differences observed among age classes (mean 3.3 ± 2.8 mU, range 0–10.8 mU). Therefore, we conclude that the findings reported here, are not artifacts of handling but instead represent developmental changes. No circadian pattern was detected in EPO concentrations with respect to time of day ($F_{12,198} = 0.361$, $P = 0.698$, $R^2 = 0.004$).

EPO concentration varied significantly in response to animal age, population, and the interaction of age and population ($F_{1,144} = 6.056$, $P = 0.015$; $F_{18,144} = 5.082$, $P < 0.001$; $F_{8,144} = 2.144$, $P = 0.035$), respectively, while sex had no effect ($F_{1,144} = 0.878$, $P = 0.350$). Even though sea lions from SEA generally had greater EPO concentrations than those of the same age from PWS, the overall pattern of change with age was similar between populations (Fig. 3). In general, EPO concentrations were elevated in neonates (3.5–64.4 mU/ml, 20.2 ± 1.9), decreased in animals around 5 months of age (1.5–26.3 mU/ml, 9.1 ± 0.3), and then remained fairly constant with age, at concentrations similar to those of adults (4–18 mU/ml, 7.6 ± 0.5). One severely anemic 5-month-old pup with an Hct of 15% (normal for that age class was 39%) was not included in the analysis, but had an EPO concentration of 113.7 mU/ml, more than 12 times the average for that age class.

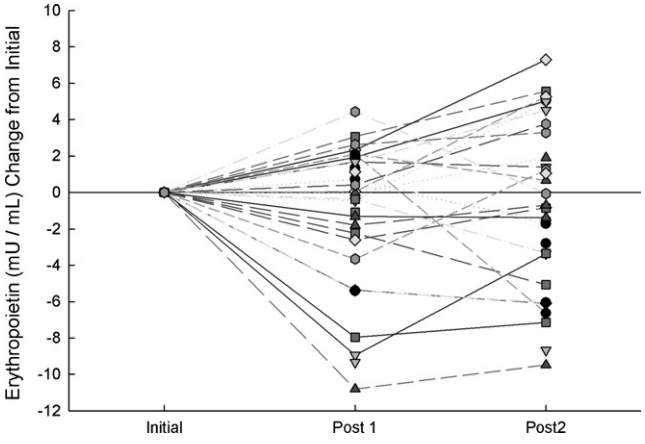

Fig. 2. The absolute change in erythropoietin (EPO) concentration during the holding time of 29 sea lions onboard research vessel. Values shown indicate difference from initial sample, so that negative post values represent a decrease in EPO concentration from the initial sample. Post 1 and 2 sample times are a minimum of 2 and 2.5 h from initial sample, respectively. Lines connect samples from individual sea lions.

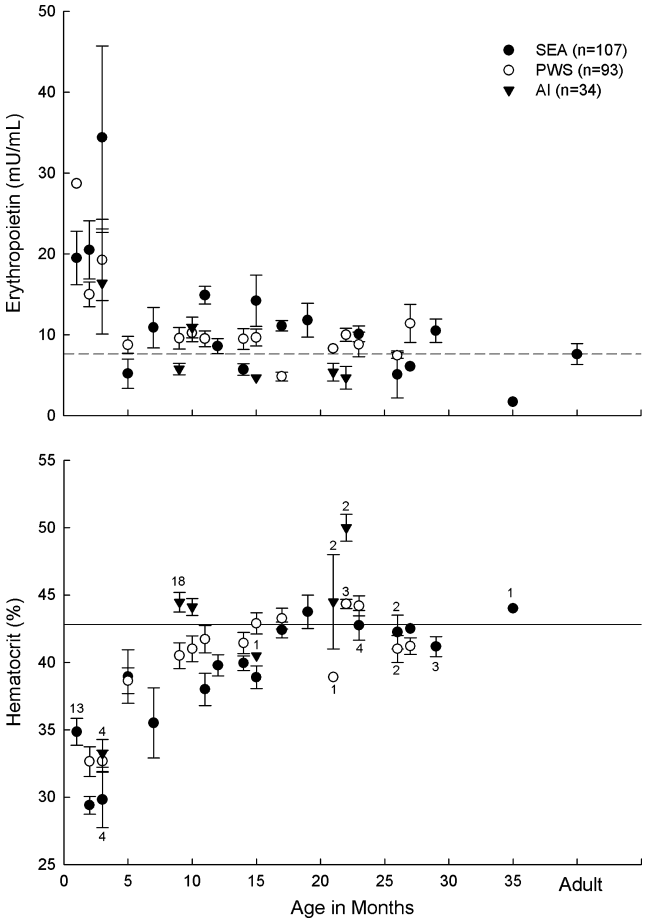

Fig. 3. Change in erythropoietin (EPO) and hematocrit (Hct) concentration with age for Steller sea lions captured in Southeast Alaska (SEA), Prince William Sound (PWS), and the Aleutian Islands (AI). Values from AI are shown for comparison only. Sample sizes for populations are given in legend. Age category sample sizes range from 5 to 10 individuals unless otherwise annotated in Hct graph. Values shown are mean ± SE. Average EPO concentrations above dotted line (mean adult value) represents moderate elevation. Adult female hematocrit represented by solid line shown for comparison (Castellini et al., 1996).

3.2. Hematology parameters

Hematocrit showed the opposite developmental pattern to EPO. Similar to EPO, Hct varied significantly with population and age ($F_{1,311} = 9.818$, $P = 0.002$; $F_{17,311} = 31.999$, $P < 0.001$), but not sex ($F_{1,311} = 0.708$, $P = 0.401$). Hematocrit values decreased after birth, reached the lowest point in animals of approximately 3 months of age, and then increased until values were similar to those of older age classes by 5 months of age (SEA 38.6 ± 0.8, PWS 39.2 ± 0.6). In general, sea lions from PWS tended to have greater Hct values than those from SEA, but the overall pattern of change with age was similar.

The developmental patterns seen in Hb, RBC, and MCHC were similar to that of Hct (Fig. 4). All blood

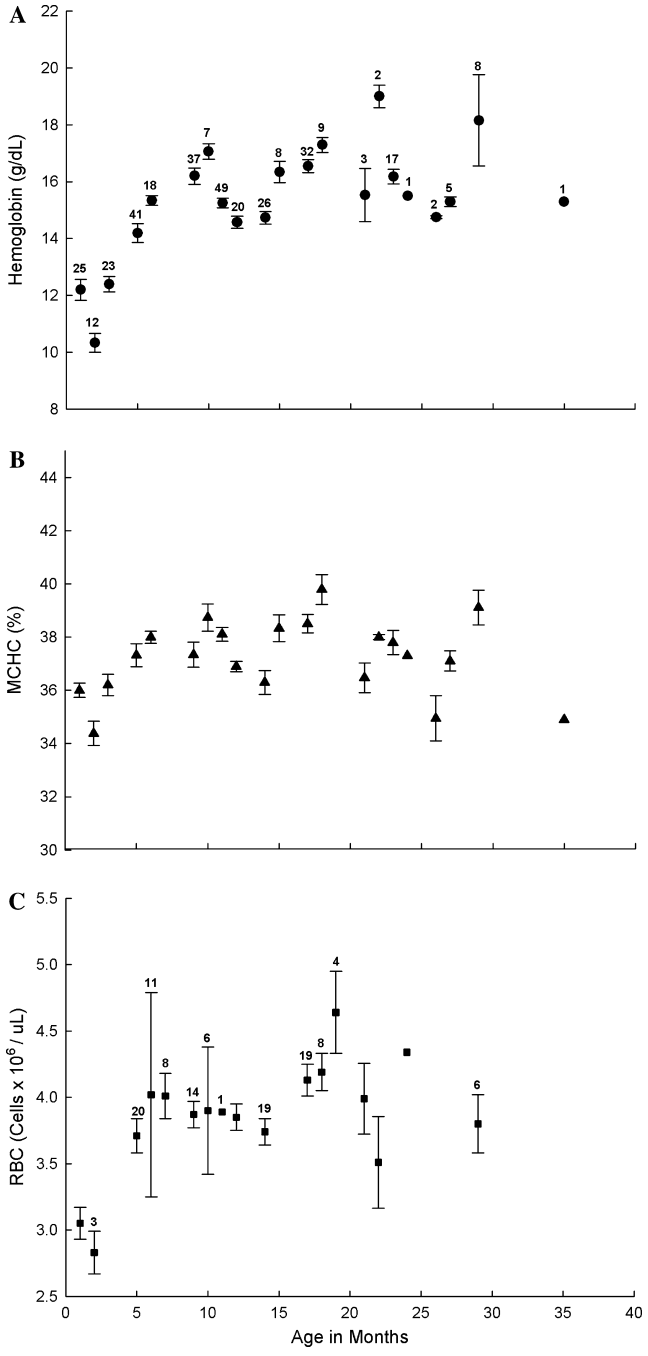

Fig. 4. Developmental trends in (A) hemoglobin ($n = 346$), (B) mean corpuscular hemoglobin content (MCHC, $n = 346$), and (C) red blood cell counts (RBC, $n = 170$) for Steller sea lions from all regions combined. Sample size for each age category given above data point in hemoglobin graph. Age categories sample sizes are identical among hematology parameters unless otherwise annotated.

parameters measured, except MCH, showed a significant relationship with age but not sex or population (age effects Hb: $F_{15,233} = 25.973$, $P < 0.001$; MCHC: $F_{15,233} = 6.888$, $P < 0.001$; RBC: $F_{11,116} = 7.657$, $P < 0.001$; MCV: $F_{11,116} = 2.984$, $P = 0.002$). Contrary to the developmental pattern seen in other blood parameters, MCV decreased for the first 7 months, and then increased to values similar to older individuals by 9 months of age (Table 1).

There were significant correlations between EPO concentrations and the hematological parameters upon which EPO acts. Over all age classes, EPO was negatively correlated with Hct ($EPO = -0.987Hct + 50.458$, $R^2 = 0.389$, $P < 0.001$; Fig. 5) and Hb ($EPO = -2.049Hb + 41.145$, $R^2 = 0.362$, $P < 0.001$). In addition, when animals younger than 3 months of age were analyzed separately from animals older than 3 months, Hct had a larger effect and explained more of the variance in concentrations of EPO in younger animals (Fig. 5).

Table 1
Hematology values for Steller sea lions throughout development, with all populations combined

Age in months	Sample size	MCH (pg)	MCV (fL)
1	25	40.6 ± 0.9	112.6 ± 2.3
2	3	35.7 ± 2.5	106.2 ± 5.9
5	18	40.7 ± 1.9	107.1 ± 5.2
6	10	38.1 ± 0.9	101.0 ± 2.0
7	8	ND	90.6 ± 4.2
9	12, 13	42.2 ± 0.7	113.8 ± 2.1
10[a]	5	48.4 ± 8.1	122.9 ± 19.2
12	20	38.3 ± 1.2	104.0 ± 3.2
14	18	39.5 ± 0.8	110.7 ± 2.7
17	19	40.3 ± 1.0	104.6 ± 2.7
18	8	37.6 ± 3.1	104.5 ± 7.7
19	4	ND	95.2 ± 5.5
21	2	37.6 ± 3.1	104.5 ± 7.7
22[a]	2	54.5 ± 4.2	143.3 ± 11.2
24	1	35.7	95.6
29	4	44.8 ± 1.4	111.4 ± 3.7

Mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) values shown are mean ± SE. ND indicates no data available.
[a] Samples from Aleutian Islands only.

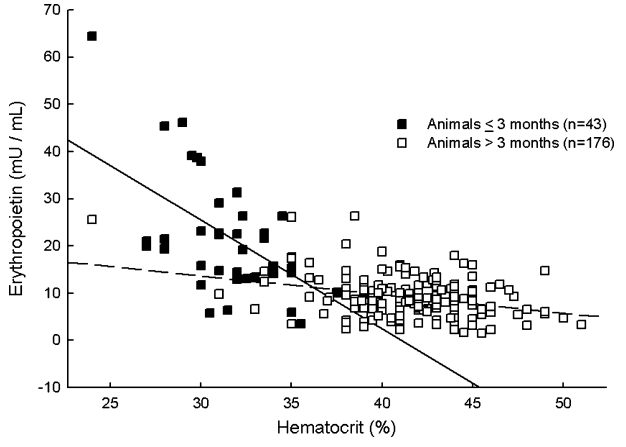

Fig. 5. Correlation between hematocrit and erythropoietin for individual sea lions for two different age groups. The best-fit least squares regression line for pups 1–3 months of age is shown with a solid line ($EPO_{1-3mo} = -2.306Hct_{1-3mo} + 94.653$, $R^2 = 0.416$, $P < 0.001$) and animals greater than 3 months are shown with broken line ($EPO_{>3mo} = -0.396Hct_{>3mo} + 25.533$, $R^2 = 0.104$, $P < 0.001$).

4. Discussion

This study successfully measured EPO concentrations in free-ranging Steller sea lions throughout development. Erythropoietin values determined for adult Steller sea lions (4–18 mU/ml) were similar to values reported for humans (6–32 mU/ml), cats (3–38 mU/ml), dogs (7–37 mU/ml), rough-toothed dolphins (7–29 mU/ml), and harbor seals (1–12 mU/ml) (Clark, 2004; Cook and Lothrop, 1994; Jelkmann, 1994; Manire and Rhinehart, 2000). As in terrestrial mammals, age significantly influenced concentrations of EPO in Steller sea lions. One-month-old pups had EPO values greater than adults which continued to increase and peak at 3 months of age, after which they declined to adult concentrations. Although this pattern of increase followed by decline has been seen in other species (Halvorsen and Bechensteen, 2002; Kling et al., 1996), Steller sea lions maintained elevated EPO concentrations for an extended period of time compared with terrestrial species. For example, in humans EPO concentrations are low after birth, increase twofold during the second month of life, but begin to decline towards adult values by 3 months of age (Halvorsen and Bechensteen, 2002; Jelkmann, 1992; Kling et al., 1996). Steller sea lion pups not only sustained elevated EPO concentrations throughout the first 3 months of life, but their EPO values also reached concentrations three times greater than those seen in adults. Since the half-life of EPO is less than 3 h (Jelkmann, 1992), these sustained concentrations represent a significant maintenance production, and suggest that EPO plays an important role in blood development in a young diving mammal.

The negative correlation between EPO and developmental changes in hematology was similar to that seen in terrestrial mammals. As with other species, increased EPO was associated with increases in Hct and Hb (Bechensteen et al., 1996; Kling et al., 1996; Pechereau et al., 1997). In Steller sea lions, this relationship was probably stronger in early development due to the substantial role that EPO plays in the regulation of erythropoiesis during the early anemia of infancy (Kling et al., 1996). Once Hct increased and anemia was alleviated, Steller sea lion EPO concentrations decreased to adult values, similar to the pattern seen in other species (Halvorsen and Bechensteen, 2002). For Steller sea lions, this occurred at 5 months of age, once young pups began to spend more time in the water and dive to increased depth (Pitcher et al., In review), suggesting that diving activity may be delayed until blood oxygen stores have matured. In addition, the decline in growth rates that occurs in animals around 5 months of age (ADFG, unpublished data) may reduce the burden of erythropoiesis associated with growth. Remarkably, increases in Hct and Hb were seen even after EPO activity diminished. This implies that either maintenance concentrations of EPO were enough to continue elevated levels of erythropoiesis, or suggests that other factors were involved in the continued increase in blood oxygen stores. Since previous work has shown that even moderately elevated values of EPO can maintain increased erythropoiesis activity (Eckardt, 1994), EPO could still influence later blood development in the Steller sea lion.

An unexpected result from this research was the difference in hematology and EPO values among sea lions from different populations. Previous research comparing hematology of neonate Steller sea lions in the Aleutian Islands with individuals in the Gulf of Alaska (comparable to our PWS region) indicated no difference between populations (Rea et al., 1998). However, we were unable to compare PWS and the Aleutian Islands due to small sample size in the Aleutians, and may have found similar results if comparisons between these two populations were possible. Variability in the magnitude of the early anemia can differ among populations in response to environmental factors, such as parasite load and protein intake (Davis et al., 1995; Halvorsen and Bechensteen, 2002; Roletto, 1993). Indeed, preliminary research suggests that greater than 50% of Steller sea lions between 2 and 3 months of age have hookworms, and hookworm-infected animals have lower Hct levels (Burek et al., 2004). While it is not yet possible to compare infection rates among populations, it may be that Steller sea lions in SEA sustain higher infestations than animals in PWS. Regional differences in diving and foraging behavior have been found in some studies (Loughlin et al., 2003; Raum-Suryan et al., 2004), but differences are not large (Pitcher et al., In review) and are therefore unlikely to strongly influence hematology in juveniles. Regardless of the reason for the difference in hematology, since Steller sea lions in SEA had both lower Hct and higher EPO concentrations than animals in PWS, the direction of the differences supports our hypothesis that Hct and EPO are correlated.

While the developmental pattern of Hct is similar in marine and terrestrial mammals, Hct in marine species generally begins to increase well before independent foraging occurs, whereas terrestrial species reach their Hct nadir around the time of weaning (Halvorsen and Bechensteen, 2002; Horning and Trillmich, 1997; Noren et al., 2002; Spensley et al., 1987). Concurrent with the hematology changes that facilitate increased oxygen storage, mass specific plasma volume and blood volume decreased slightly from 5 to 21 months of age (Richmond, 2004). Since increased blood volume is considered a primary means by which marine mammals increase circulating oxygen, this decline was unexpected (Butler and Jones, 1997; Kooyman, 1985; Lenfant et al., 1970). However, the effect on total blood oxygen stores were minimized in young Steller sea lions by the rapid increase in Hct and Hb. The extended period of elevated EPO concentration in Steller sea lions caused an

increase in Hct that enhanced their blood oxygen storage capacity to help support diving activity. Thus EPO, through its effect on Hct and Hb, appears to play an important role in the development of dive capacity.

When other hematology parameters were considered, a mechanism by which EPO could influence blood oxygen stores became apparent. When EPO was elevated, Hct, Hb, RBC, and MCHC values all increased while MCV decreased. This suggests that elevated EPO concentrations led to an increased RBC production resulting in a greater number of smaller immature cells in circulation (Jelkmann, 1992). Even though the newly released cells were smaller, the increase in MCHC indicates that these new cells had an elevated Hb content, as might be expected for young diving animals facing new environmental and behavioral challenges requiring increased breath-hold ability.

Hematology values reported in this study fell within previously reported values for Steller sea lions and other otariids (Steller sea lion: Castellini et al., 1993, 1996; Lenfant et al., 1970; Rea et al., 1998; Australian sea lion: Needham et al., 1980; Fur seals: Horning and Trillmich, 1997; Sepulveda et al., 1999), and the developmental pattern was similar to that observed in other marine and terrestrial species (Noren et al., 2002; Rawson et al., 1992; Roeder et al., 1990; Sepulveda et al., 1999; Thorson and LeBoeuf, 1994). Contrary to the findings here, some studies have not found age-related changes in MCHC (Horning and Trillmich, 1997; Matoth et al., 1971; Rietkerk et al., 1994). This discrepancy may be a result of sampling interval since the timing of physiological anemia is highly variable among species (Halvorsen and Bechensteen, 2002). In comparison to terrestrial mammals such as bears (Hellgren et al., 1993), Steller sea lions, like other diving mammals, tended to have fewer larger RBC with a greater Hb content.

In summary, marine mammals are limited in their aerobic dive capacity by the amount of oxygen they can store in blood, muscle, and lung (Butler and Jones, 1997; Kooyman, 1985). While adult Steller sea lions store more than half their oxygen reserves in their blood (Richmond, 2004), neonatal sea lions are born with reduced blood oxygen storage capacity due to low Hct and Hb. This study has demonstrated that age-related increases in Hct and Hb are correlated with elevated concentrations of EPO, suggesting that EPO plays a role in the development of blood oxygen stores in marine mammals similar to observations in terrestrial mammals. The variation in hematological parameters among populations was unexpected, particularly as animals with the lowest Hct values were from SEA where the population is slightly increasing, while animals from PWS where numbers are declining had greater Hct values (Calkins et al., 1999; Sease et al., 2001). Future research into factors that can influence Hct such as parasite load and nutrient intake, including protein, iron, copper, and folic acid (Burns et al., 2004; Davis et al., 1995; Halvorsen and Bechensteen, 2002; Roletto, 1993), may help elucidate why these population differences occur.

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