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Erythropoietin concentration in developing harbor seals (*Phoca vitulina*)

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Abstract

Tissue hypoxia elicits the production of erythropoietin (Epo), a hormone that stimulates red blood cell production. In young diving mammals, oxygen is stored primarily in the blood, and blood oxygen stores increase significantly during the first weeks of life. In an effort to establish the role of Epo during this period of blood development, this study measured Epo concentration in plasma of 134 harbor seal (*Phoca vitulina*) pups and adults. Concurrent measurements of hematocrit (Hct), hemoglobin concentration [Hb], and red blood cell (RBC) counts allowed the evaluation of the effect of Epo on blood oxygen store capacity. Erythropoietin and most blood parameters varied with age. At birth, neonatal [Hb], Hct, and RBC were elevated, possibly due to the rapid expansion of plasma volume associated with growth rates of 0.5 kg/day. In contrast, Epo concentration increased from 6.64 ± 0.83 mU/ml in newborns to 9.53 ± 0.86 mU/ml in early nursing pups. Erythropoietin concentration remained elevated above newborn and adult concentration (5.71 ± 0.79 mU/ml) through weaning, suggesting that Epo was responding to tissue hypoxia brought on by early anemia. Since similar changes in erythropoietin have been documented in terrestrial mammals, it appears that Epo plays a similar role in the blood development of harbor seals.

Keywords: Erythropoietin; Harbor seals; Development; Radioimmunoassay; Marine mammal

1. Introduction

Erythropoietin (Epo) is a glycoprotein hormone that stimulates red blood cell (RBC) production in response to changes in oxygen tension, oxygen storage capacity, and oxygen affinity of the blood (Jelkmann, 1992; Moritz et al., 1997). Prior to parturition, Epo is produced in the fetal liver. After birth, Epo is produced primarily in the kidneys (Jelkmann, 1992; Palis and Segel, 1998), with smaller amounts produced in the adult liver (Moritz et al., 1997), testes (Tan et al., 1992), and brain (Digicaylioglu et al., 1995; Masuda et al., 1994). As part of a negative feedback control mechanism, Epo production increases in response to hypoxia (low oxygen) and decreases in response to hyperoxia (high oxygen) (Krantz, 1991; Porter and Goldberg, 1994). Erythropoietin is highly specific with no known effect other than to enhance the production of red blood cells, or erythropoiesis (Jelkmann, 1992), particularly during anemic conditions.

Anemia, a condition of reduced hemoglobin concentration [Hb], hematocrit (Hct), and/or RBC count, has been associated with postnatal development in a variety of terrestrial mammals, such as mice, rabbits, rats, piglets, calves, and humans (Halvorsen and Bechensteen, 2002; Heikinheimo and Siimes, 1992; Sanengen et al., 1987; Sjaastad et al., 1996). Typically, this period of reduced blood oxygen stores in developing mammals is known as early anemia. Early anemia results from the rapid expansion of plasma volume and decreased RBC and/or [Hb] per unit blood volume (Feldman et al., 2000). This 'physiological anemia of infancy' typically occurs throughout

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the nursing period (Halvorsen and Bechensteen, 2002) and is not pathological in nature. The decreased oxygen storage capacity during the nursing period induces tissue hypoxia due to the reduced availability of oxygen for tissue use (Jelkmann, 1992). In response, Epo concentrations are elevated and stimulate red blood cell production to increase the oxygen carrying capacity of blood. Early anemia, therefore, can be detected by measuring blood parameters of rapidly developing young mammals.

With a relatively short postnatal developmental period (~28 days), young harbor seals (*Phoca vitulina*) enter the water soon after birth and follow their mothers throughout lactation (Bigg, 1969; Boulva and McLaren, 1979; Bowen et al., 1999; Knudtson, 1977). Harbor seal pups must quickly develop the physiological mechanisms necessary for diving and ultimately independent foraging. This includes rapid development of blood oxygen stores (Clark, 2004; Jørgensen et al., 2001), and the ability to reduce metabolic rate, regulate diving heart rate, and control peripheral vasoconstriction (Greaves et al., 2004, 2005; Kooyman, 1989; Lapierre et al., 2004). Since diving mammals are limited in their breathhold ability by the amount of oxygen available prior to a dive (Butler and Jones, 1997; Kooyman, 1989), and since harbor seals store more than 60% of their total tissue stores in blood (Clark, 2004), the development of blood oxygen stores is crucial to maximize breathhold ability. However, there have been no studies on the factors that stimulate or regulate the increase in blood oxygen stores in any phocid. Therefore, this study investigates Epo concentration changes in harbor seals from birth to weaning, to assess whether postnatal changes in Epo are coupled with simultaneous changes in [Hb], Hct, RBC counts, and blood oxygen stores. From this work, we will determine if the mechanism that regulates neonatal blood development in diving and terrestrial mammals are similar.

2. Materials and methods

2.1. Animal capture, handling, and sample collection

Harbor seal pups and adult females were captured during May–July of 2000, 2001, and 2002 near two haul-out sites, "Bic Island" (48°24′N, 68°51′W) and "Métis" (48°41′N, 68°01′W), along the south shore of the St. Lawrence River estuary in Quebec, Canada (Fig. 1). Seals were captured using a 5 m inflatable boat and a modified dip net (Dubé et al., 2003), and once captured basic morphometric measurements (e.g., sex, mass ± 0.5 kg) were taken. After weighing, pups were outfitted with uniquely numbered flipper tags (Jumbo Rototag, Dalton, England) and head tags (Hall et al., 2000). Pups were recaptured at approximately 1-week intervals throughout the 4-week lactation period. All research was approved by the Animal Care Committees of the University of Alaska and the University of Waterloo.

At initial capture, seals were aged by appearance and mass. Pups were classified as newborn if they had umbilical remnants and uncoordinated swimming ability (Dubé et al., 2003). Pups were considered weaned if they were difficult to capture (requiring many attempts to capture) and were never seen again with an adult female. In cases where pups could not to be classified as newborn or weaned, age was estimated by mass following Dubé et al. (2003). Using this method, four age categories were identified: newborn (0–4 days), early nursing (5–16 days), late nursing (17–27 days), and weaned pups (⩾28 day). Age was not estimated for adult females.

2.2. Blood collection and analysis

Blood samples, up to 120 ml, were taken from the extradural intravertebral vein (Geraci and Smith, 1975) using a 1.5″ 20G or a 3.5″ 18G spinal needle into vacutainer tubes (Becton–Dickinson Vacutainer® Brand) and were immediately stored on ice. The volume of blood collected was no more than 10% of the total blood volume (McGuill and Rowan, 1989). Within 8 h of collection, samples were centrifuged, and plasma was collected and stored at −20 °C before being transferred to an ultra-cold −80 °C freezer.

Hematocrit was measured from whole blood collected in K_3-EDTA vacutainers by direct centrifugation. Hemoglobin concentration was measured from the same sample using the cyano-methemoglobin photometric method (Sigma 525-A Kit). Red blood cell counts were determined by direct counting using a hemacytometer and Unopettes (Becton–Dickinson Vacutainer Systems). Manual counts were performed immediately using a compound microscope or the prepared hemacytometer was digitally photographed (Leitz Diaplan compound microscope and Leica Image analy-

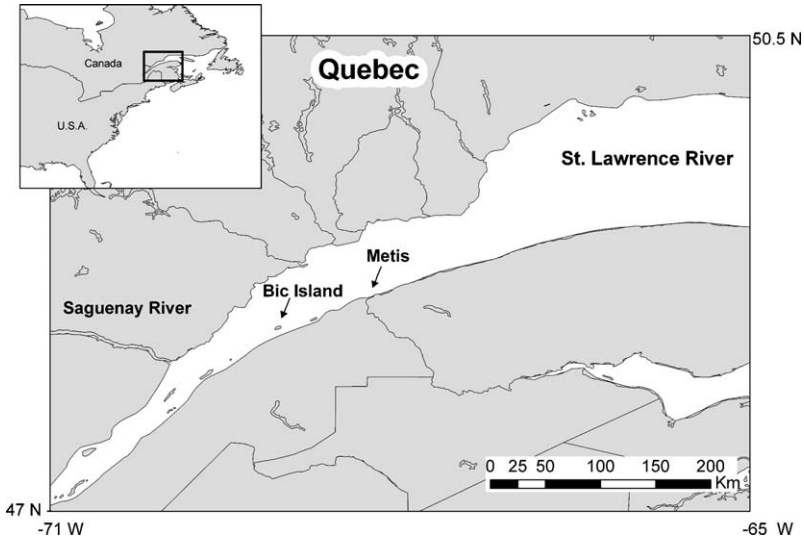

Fig. 1. The St. Lawrence River estuary with study sites Métis and Bic Island.

sis system) so images could be counted later. There was no significant difference in RBC counts between the two methods (paired t test, $P < 0.05$, $n = 20$), so results from the two techniques were combined for all subsequent analyses.

2.3. Radioimmunoassay

Harbor seal plasma Epo concentrations were determined in duplicate by radioimmunoassay (Diagnostic Systems Laboratories, DSL-1100, Webster, TX) following manufacturer instructions with the following modifications. The first incubation with rabbit anti-human Epo serum was extended from 4 to 8 h and was followed by an 18-h incubation with the I^{125} Epo label. Following the addition of the precipitating reagent, samples were further incubated at room temperature for 30 min, centrifuged at 0 °C at 1500*g* for 30 min, the supernatant decanted, and radioactivity of the pellet determined using a gamma counter (Diagnostic Products Corporation, Gamma-C12).

Validation assays were performed in duplicate on three groups of pooled samples; male pups (mp), female pups (fp), and adult females (af). Parallelism, how an assay responds to the standard curve and how accurately the results predict the standard (R^2 values) was analyzed. Serial dilutions of pooled samples showed a parallel relationship to the standard curve over the range of 1.25–200 mU/ml. Accuracy was high with a strong linear relationship between mass added (MA) and mass recovered (MR) in all pooled groups: $MR_{mp} = 2.68 + 0.89(MA)$, $R^2 = 0.982$; $MR_{mf} = 0.92 + 0.91(MA)$, $R^2 = 0.993$; and $MR_{af} = 1.42 + 1.05(MA)$, $R^2 = 0.970$. Percent recovery tests ranged from 82 to 100%. Intra-assay and inter-assay coefficient of variation were less than 14 and 7%, respectively. Based on these values, we believe the assay produced valid results.

2.4. Statistical analysis

Based on their capture frequency, pups were divided into two different treatment groups: cross-sectional (animals sampled only once) and longitudinal (animals sampled multiple times in different age categories).

Age-related changes in Hct, [Hb], RBC counts, and plasma Epo concentration were tested using one-way ANOVAs for the cross-sectional group, and linear mixed model effect in the longitudinal treatment group. Bonferroni post hoc comparisons ($P < 0.05$) were used to identify significant differences among age categories. To determine if there were differences due to handling, the mean values from the cross-sectional group were compared to the longitudinal group using Two-sample t test in which P values were adjusted using a Bonferroni correction factor to control for multiple comparisons and significant difference assumed only if $P < 0.013$.

Linear regressions were performed to determine whether Epo responded to changes in Hct, [Hb], and/or RBC. To establish whether Epo had an effect on overall blood development during the nursing period, mass-specific blood oxygen stores determined for these same pups (Clark, 2004) were compared with Epo concentration using linear regression. All statistical analyses were performed using SPSS® v 11.5.0, SPSS Inc. and statistical significance was assumed at $P < 0.05$.

3. Results

Over the 3 years of this study, 114 pups and 20 adults were sampled. Of these, 78 pups and 20 adult females were sampled only once, while 36 pups were sampled two or more times. There were significant age-related changes in plasma Epo concentration in the cross-sectional group (one-way ANOVA, $F_{4,93} = 3.398$, $P = 0.012$, Fig. 2). Comparisons between age categories revealed that newborns had low plasma Epo concentration that increased in early nursing (6.64 ± 0.83 to 9.53 ± 0.86 mU/ml, Fig. 2) and remained high in late nursing and weaned pups (7.79 ± 0.96 and 9.32 ± 1.15 mU/ml, respectively, Fig. 2). Erythropoietin concentrations in early nursing pups were also significantly higher than adult concentration (5.71 ± 0.79 mU/ml). There were no differences in Epo concentration due to pup sex or capture location.

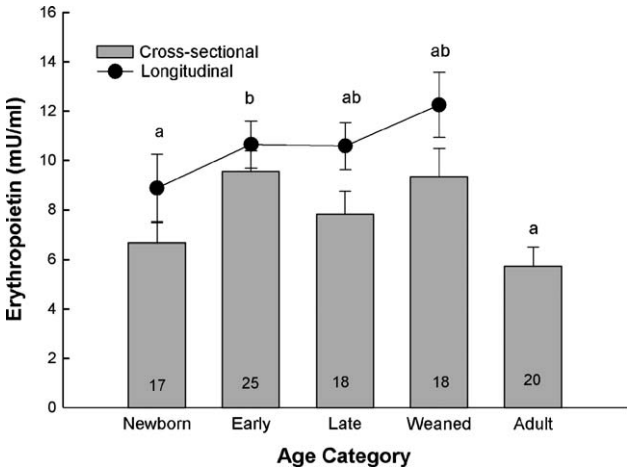

Fig. 2. Age-related changes in plasma Epo concentration (mean ± SE) in harbor seal pups and adult females. Different letters indicate statistically significant differences among cross-sectional age categories (Bonferroni, $P < 0.05$). The longitudinal group ($n = 36$) had no statistical differences in Epo concentration (linear mixed model $F_{3,83} = 1.049$, $P = 0.376$). Sample size for each age category is given in the corresponding bar.

Concurrent measurements of [Hb] revealed significant age-related differences in the cross-sectional (one-way ANOVA, $F_{4,91} = 5.283$, $P < 0.001$, Table 1) and longitudinal samples (linear mixed model $F_{3,84} = 11.089$, $P < 0.001$, Table 1). Newborns had the highest [Hb] (cross-sectional, 21.3 ± 0.6 g/dl, longitudinal, 21.1 ± 0.5 g/dl) when Epo concentrations were low (Fig. 3). Conversely, in early nursing pups when Epo concentrations were highest, [Hb] were lowest (cross-sectional, [Hb] = 18.7 ± 0.4 g/dl, longitudinal, [Hb] = 18.0 ± 0.3 g/dl, Fig. 3). For all age classes, while [Hb], Hct, and RBC had negative correlations with Epo concentration, only [Hb] was significantly correlated with Epo (EPO = 18.45–0.524[Hb], $R^2 = 0.069$, $P = 0.01$, EPO = 12.3–0.85Hct, $R^2 = 0.008$, $P = 0.379$, EPO = 10.5–0.005RBC, $R^2 = 0.006$, $P = 0.630$).

There were significant age-related changes in cross-sectional Hct and RBC counts (one-way ANOVA, $F_{4,93} = 10.616$, $P < 0.001$ and $F_{4,36} = 12.843$, $P < 0.001$, respectively, Table 1). Bonferroni post hoc tests revealed that Hct and RBC counts in newborn pups were elevated followed by a decrease in early nursing pups (Table 1). Both Hct and RBC values gradually increased during lactation and were higher in weaned pups compared with adult females (Table 1). Overall, hematology variables in the longitudinal group did not differ from the cross-sectional data (Table 1). There was a significant negative correlation between blood oxygen stores (BOS) and erythropoietin (EPO = 17.76–0.305BOS, $R^2 = 0.238$, $P = 0.047$). Overall, mass-specific blood oxygen stores decreased from birth to late nursing (Clark, 2004), while Epo concentration increased in early, then decreased in

Table 1
Mean values (± SE) for estimated age, body mass, [Hb], Hct, and RBC counts in harbor seal pups and adult females

Cross-sectional						Longitudinal				
Age category	Estimated age (days)	Mass (kg)	Hb (g/dl)	Hct (%)	RBC (10^6 µl)	Estimated age (days)	Mass (kg)	Hb (g/dl)	Hct (%)	RBC (10^6 µl)
Newborn	1.1 ± 0.4^{a}	11.3 ± 0.3^{a}	21.3 ± 0.6^{b}	54.9 ± 1.1^{b}	6.07 ± 0.15^{c}	2.1 ± 0.50	12.0 ± 0.3	21.1 ± 0.5	55.5 ± 0.8	6.03 ± 0.15
n	17	17	16	17	13	14	14	14	14	9
Early nursing	10.6 ± 0.6^{b}	16.6 ± 0.4^{b}	18.7 ± 0.4^{a}	48.8 ± 0.7^{a}	5.15 ± 0.14^{ab}	10.7 ± 0.6	16.1 ± 0.5	18.0 ± 0.3	47.3 ± 0.5	5.02 ± 0.11
n	25	25	25	25	11	30	30	30	30	20
Late nursing	20.8 ± 0.8^{c}	21.9 ± 0.6^{c}	19.7 ± 0.5^{ab}	49.4 ± 1.0^{a}	5.08 ± 0.23^{ab}	21.3 ± 0.6	22.0 ± 0.6	18.8 ± 0.3	49.6 ± 0.6	5.18 ± 0.11
n	18	18	17	18	5	29	29	29	29	19
Weaned	37.3 ± 2.0^{d}	26.5 ± 0.6^{d}	20.7 ± 0.4^{b}	54.5 ± 0.7^{b}	5.37 ± 0.16^{bc}	31.0 ± 0.9	24.9 ± 1.0	19.5 ± 0.4	52.3 ± 0.8	5.21 ± 0.17
n	18	18	18	18	5	15	15	15	15	8
Adult female	—	65.8 ± 2.6^{e}	20.1 ± 0.4^{ab}	51.7 ± 0.8^{ab}	4.42 ± 0.23^{a}	—	—	—	—	—
n		20	20	20	7					

[abcde]Different letters indicate statistically significant differences among cross-sectional age categories (Bonferroni, $P < 0.05$).
There were no statistical differences between cross-sectional and longitudinal hematological parameters among age categories.

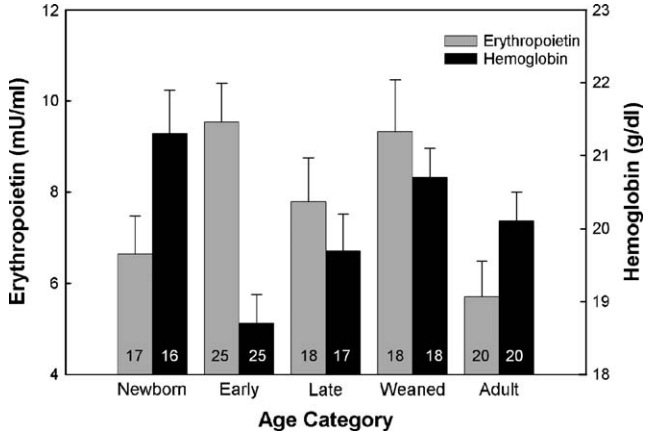

Fig. 3. Mean (± SE) plasma Epo and [Hb] determined for individual animals within each age category. Sample size for each age category is given in the corresponding bar.

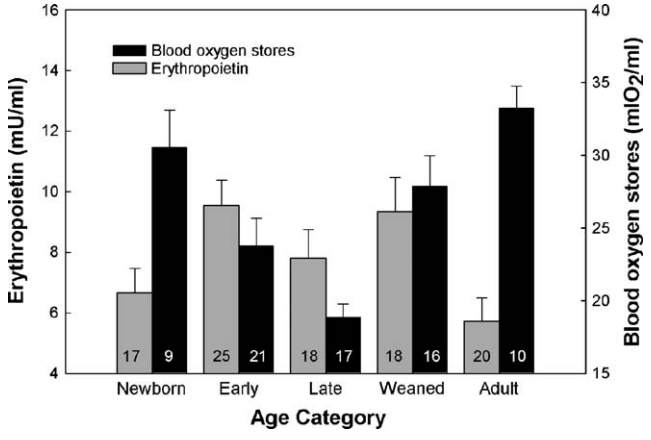

Fig. 4. Age-related changes in mean (± SE) plasma Epo concentration and mass-specific blood oxygen stores. Sample size for each age category is given in the corresponding bar.

late nursing (Fig. 4). In adults, Epo concentration was lower and blood oxygen stores higher when compared with pups (Fig. 4).

4. Discussion

This study provides the first measurements of plasma Epo concentration in developing harbor seals. As in terrestrial mammals, neonatal harbor seals had Epo concentrations that were higher than those of adults (Moritz et al., 1997; Sanengen et al., 1987) and Epo remained elevated throughout the lactation period. The rapid early increase in Epo concentration occurred within 4 days of birth when oxygen carrying capacity decreased due to the significant reduction in [Hb], Hct, and RBC counts. These hematological changes were associated with rapid growth (mass increase of 0.5 kg/day from birth to weaning) and a concurrent expansion of total plasma volume (Clark, 2004). In combination, increased plasma volume and reduced Hct resulted in lower mass-specific blood oxygen stores, which possibly provided the hypoxic stimuli necessary to induce Epo production (Jelkmann, 1992).

There has been extensive research on Epo and the role it plays in stimulating red cell production in sick adults (Das et al., 1975; Jelkmann, 1994; Sanengen et al., 1987) and healthy, developing juveniles (Halvorsen and Bechensteen, 2002; Kling et al., 1996; Palis and Segel, 1998). Studies on terrestrial neonates have shown that early anemia is associated with increased Epo concentration in humans (Kling et al., 1996), laboratory animals (e.g., mice, rabbits, and rats) and domesticated animals (e.g., pigs, calves, and lambs) (Halvorsen and Bechensteen, 2002). Thus, Epo is an important factor in the transition from an oxygen-deficit environment (intrauterine) to an oxygen-rich environment (air-breathing) in all terrestrial species studied to date. However, there is limited data on the role of Epo in the hematological development of marine mammals (Richmond et al., 2005). Marine mammals need to develop blood oxygen stores to sustain aerobic metabolism while diving, therefore one might expect that young pups should have a similar if not more pronounced response to Epo production as compared with terrestrial mammalian species (Halvorsen and Bechensteen, 2002; Sanengen et al., 1987). Indeed,

since harbor seal pups dive within a few hours of birth, which increases the demand for stored oxygen, it was not surprising to find that Epo was negatively correlated with Hct, [Hb], and RBC counts. As a result, elevated Epo concentration stimulated erythropoiesis thereby increasing Hct, [Hb], and RBC counts resulting in increased blood oxygen storage capacity. Similar findings in juvenile Steller sea lions (*Eumetopias jubatus*, Richmond et al., 2005) and the positive response of an anemic rough-toothed dolphin (*Steno bredanensis*, Manire and Rhinehart, 2000) to recombinant Epo suggest that marine mammals respond to hypoxic stress in a similar fashion as terrestrial species.

In young developing mammals, growth may contribute to early anemia. The rapid increase in body mass seen during the lactation period in harbor seals is associated with a simultaneous increase in absolute plasma volume (~29 ml/day), such that mass-specific plasma volume remains relatively constant across the lactation period (Clark, 2004). The challenge is for red blood cell production to keep pace with the expanding fluid associated with increasing body mass (Bechensteen and Halvorsen, 1996; Clark, 2004; Elsner and Gooden, 1983; Spensley et al., 1987). This study suggests that blood cells cannot be produced by young harbor seals as rapidly as plasma volume expands (Clark, 2004; Jørgensen et al., 2001), thus resulting in decreased Hct, [Hb], and RBC counts. This may also be compounded by the fact that neonatal RBC's have a shorter lifespan than adult cells (70 day vs. 120 day—Moritz et al., 1997), and are therefore destroyed more rapidly. Red cell production, therefore, may lag plasma volume for two reasons: constraints on the rate of red cell production due to cell death/destruction (7 days for mature red cell production, Harmening, 1997; Junqueira et al., 1998), and restrictions on new production imposed by micronutrient limitations (Burns et al., 2004; Halvorsen and Bechensteen, 2002; Sjaastad et al., 1996).

Regardless of the cause of early anemia, it results in reductions in mass-specific blood oxygen stores during the lactation period. As blood oxygen stores decline, Epo increases and acts to stimulate the production of new RBCs. While only [Hb] and Epo were significantly correlated, the significance was low, and this combined with no significance between Epo and Hct or RBC may be explained by a short lactation period, individual variation, and quick Epo response. In combination, these factors may make it difficult to detect correlations seen in other species with longer developmental periods (Halvorsen and Bechensteen, 2002; Jelkmann, 1994; Kling et al., 1996; Richmond et al., 2005). Regardless, the significant negative correlation between [Hb] and Epo supports the hypothesis that Epo is produced in response to decreased oxygen availability in order to increase blood oxygen stores and thus increase the amount of stored oxygen available for tissues. While young pups exhibit a slow and awkward diving ability, their tissue oxygen stores increase throughout the lactation period to prepare them for independent foraging. Further, the elevated Epo concentration throughout nursing and weaning, compared with adult concentrations, further supports the idea that Epo is closely tied with blood oxygen store development.

One interesting finding from this research was that the Epo concentration in harbor seals pups in the longitudinal treatment group increased from birth through weaning, while Epo concentration in the cross-sectional treatment group increased and decreased throughout lactation. This difference may be due to the fact that pups in the longitudinal sample had blood drawn repeatedly (up to 4 times) during the short lactation period (28 day), while cross-sectional pups were only subject to a single sampling event. While the amount of blood withdrawn each time was small (<10% of total blood volume), and well within animal handling guidelines (McGuill and Rowan, 1989), the need to replenish the total volume collected over the entire lactation period may have provided an additional stimulus that promoted the prolonged and increased Epo response in the longitudinal group as compared to the cross-sectional group.

In summary, results from this study indicate that harbor seal pups demonstrate the typical mammalian pattern of blood development, with Epo being produced in response to the hypoxic stress associated with early anemia. Following the increase in Epo concentration during nursing, [Hb], Hct, and RBC counts increase, such that by weaning pup hematological parameters are similar to those of adults. However, because erythropoietin concentrations for harbor seal pups were not elevated above those in terrestrial mammals, despite the additional hypoxic stress of diving, these findings suggest that the fundamental mechanisms driving erythropoiesis are similar in both terrestrial and marine mammals.

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