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ORIGINAL PAPER

Postnatal development of muscle biochemistry in nursing harbor seal (*Phoca vitulina*) pups: limitations to diving behavior?

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Abstract Adult marine mammal muscles rely upon a suite of adaptations for sustained aerobic metabolism in the absence of freely available oxygen (O_2). Although the importance of these adaptations for supporting aerobic diving patterns of adults is well understood, little is known about postnatal muscle development in young marine mammals. However, the typical pattern of vertebrate muscle development, and reduced tissue O_2 stores and diving ability of young marine mammals suggest that the physiological properties of harbor seal (*Phoca vitulina*) pup muscle will differ from those of adults. We examined myoglobin (Mb) concentration, and the activities of citrate synthase (CS), β-hydroxyacyl coA dehydrogenase (HOAD), and lactate dehydrogenase (LDH) in muscle biopsies from harbor seal pups throughout the nursing period, and compared these biochemical parameters to those of adults. Pups had reduced O_2 carrying capacity ([Mb] 28–41% lower than adults) and reduced metabolically scaled catabolic enzyme activities (LDH/RMR 20–58% and CS/RMR 29–89% lower than adults), indicating that harbor seal pup muscles are biochemically immature at birth and weaning. This suggests that pup muscles do not have the ability to support either the aerobic or anaerobic performance of adult seals. This immaturity may contribute to the lower diving capacity and behavior in younger pups. In addition, the trends in myoglobin concentration and enzyme activity seen in this study appear to be developmental and/or exercise-driven responses that together work to produce the hypoxic endurance phenotype seen in adults, rather than allometric effects due to body size.

Keywords Muscle · Harbor seal · Pups · Enzyme · Postnatal development · Diving physiology

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Introduction

In terrestrial mammals, exercise-related increases in metabolic rate are accompanied by increases in ventilation, heart rate and muscle perfusion, all of which promote continual delivery of both fuels and O_2 to the working muscles (Bangsbo and Hellsten 1998; Krustrup et al. 2004; Brooks et al. 2005). In contrast, diving marine mammals rely upon a suite of adaptations that reduce O_2 consumption rates while diving: these include bradycardia, vasoconstriction, decreases in body temperature, and low-cost swimming strategies (Davis et al. 1985; Kooyman 1988; Kooyman 1989; Castellini 1991; Butler and Jones 1997; Kooyman and Ponganis 1998). While these diving adaptations allow for sustained aerobic metabolism in the absence of freely available O_2, at the level of the working muscle, they result in reduced blood flow and, therefore, limit O_2 and fuel

(glucose and/or fatty acids) delivery (Blix et al. 1983; Cherepanova et al. 1993; Davis and Kanatous 1999; Kanatous et al. 1999).

Despite this, and in contrast to initial expectations that marine mammal muscles would be adapted for anaerobic metabolism (Hochachka and Storey 1975; Elsner and Gooden 1983), multiple studies have demonstrated that diving is a highly efficient process that relies mainly on aerobic, lipid-based metabolism (Kooyman et al. 1980, 1983; Kooyman 1989; Ponganis et al. 1997a, b; Davis and Kanatous 1999). At the level of the muscle, adaptations that support muscle functioning when fuels and O_2 are not being replenished by vascular supply include increased endogenous fuel and O_2 stores, and increases in biochemical and histological properties that promote efficient use of said reserves (Kanatous et al. 1999; Dearolf et al. 2000; Watson et al. 2003, 2007; Polasek et al. 2006). For example, seal muscles have myoglobin concentrations ([Mb]) that are far above those in terrestrial endurance and high-altitude acclimated athletes (Reed et al. 1994; Polasek and Davis 2001; Noren et al. 2002; Polasek et al. 2006). Seals also have high densities of intramuscular lipid droplets, elevated concentrations of aerobic enzymes such as citrate synthase (CS) and β-hydroxyacyl coA dehydrogenase (HOAD), and high densities of interfibrillar mitochondria (Reed et al. 1994; Kanatous et al. 1999, 2002; Polasek et al. 2006; Watson et al. 2007). In combination with high proportions of slow oxidative (type 1) and fast oxidative-glycolytic fibers (type IIA and IID/X; Reed et al. 1994; Kanatous et al. 1999; Watson et al. 2003, 2007), these adaptations produce locomotory muscles that are specifically adapted for efficient, aerobically poised metabolism while diving (Saltin and Gollnick 1983; Kanatous et al. 1999). However, while adult seal muscles may lack purely glycolytic (type IIB) fibers (Watson et al. 2003), they do have relatively high anaerobic enzyme activity, suggesting that when necessary, diving activity can be sustained by anaerobic metabolic processes (Polasek et al. 2006).

Although the importance of these adaptations for supporting the aerobic diving patterns of adults is well understood, little is known about muscle development in young marine mammals. However, several lines of evidence suggest that the physiological properties of pup muscle will differ from those of adults. First, differences are likely because neonatal vertebrates typically have muscles with lower enzyme activities, smaller fiber diameters, and different myosin heavy chain isoforms than those of the adults (Condon et al. 1990; Dietz and Ricklefs 1997; Dearolf et al. 2000; Shea et al. 2007). As animals grow, there is very little fiber hyperplasia; rather, most muscle growth is primarily due to fiber hypertrophy (Goldspink 1970; Garry et al. 1996). In addition, fiber-type profiles and enzyme activities shift toward the adult phenotype as juveniles develop, with neonatal and slow fibers developing before faster isoforms (Condon et al. 1990; Powers et al. 1991; Schiaffino and Reggiani 1994; Garry et al. 1996; Olson 2001). However, the pattern and rate of development differ with the function of the muscle and the animals' life history patterns, with the locomotory muscles of species in which the neonate is active early in life maturing more rapidly than in more altricial animals (Grand 1992; Choi et al. 1993; Dearolf et al. 2000; Shea et al. 2007).

The muscles of young marine mammals are also likely to have different metabolic characteristics than those of adults since pups do not possess all of the physiological adaptations to support underwater diving of the adults. For example, pups have significantly lower Mb and hemoglobin (Hb) stores (Burns and Castellini 1996; Burns et al. 2005; Noren et al. 2005; Richmond et al. 2006; Clark et al. 2007), giving them reduced O_2 storage and facilitated diffusion capabilities (Davis and Kanatous 1999). In addition, young pups have also reduced cardiovascular control and higher diving and apneic heart rates than adults (Castellini 1994; Greaves et al. 2005), which, in combination, may increase muscle perfusion while diving. Enhanced perfusion would increase O_2 and fuel delivery to the muscle, but is also likely associated with higher diving metabolic rates, which would limit the duration of the dive. In addition, pups have higher mass-specific metabolic rates than adults (Miller and Irving 1975; Miller et al. 1976; Worthy and Lavigne 1987; Rea and Costa 1992), which dictate higher flux rates through metabolic pathways to sustain resting levels of ATP production. Since aerobic enzyme activity levels are positively correlated with resting metabolic rates (RMRs) (Hochachka and Somero 2002), the muscles of young pups may be required to have higher enzyme activity levels than older animals, just to achieve equivalent functionality. In addition, the relatively high-fat and low-protein milk diet of nursing pups (Oftedal 2002) suggests that pup muscles will have high activities of the enzymes involved with aerobic lipid oxidation.

The objectives of this study were to examine the pattern of muscle development in harbor seal (*Phoca vitulina*) pups across the nursing period, and to compare the muscle biochemical parameters of pups to those of adults. Harbor seal pups are fairly unique among phocids in that they are often born in the water, and pups routinely begin diving within hours to days of birth (Lapierre et al. 2004; Greaves et al. 2005). While young pups do not dive to the depths and durations of the adults (Frost et al. 2001, 2006; Greaves et al. 2005), this early activity requires that their muscles be able to support underwater activity at a very early age despite immature [Mb], total body oxygen stores (TBO_2), and cardiovascular control (Greaves et al. 2005; Clark et al. 2007). In addition, because harbor seal pups have a fairly short period of dependency prior to initiating independent foraging activity (~28 days; (Dubé et al. 2003) muscle

development must either occur prenatally, or develop rapidly in the days to weeks following birth.

To determine the temporal pattern of muscle development, we focused on age-related changes in [Mb], and the activities of three enzymes: lactate dehydrogenase (LDH), CS, and HOAD. LDH supports anaerobic generation of ATP via glycolysis by regenerating NAD+ through the reduction of pyruvate to lactate (Hochachka and Somero 2002; Fluck 2006), and as such is often used as an indicator of the extent to which muscles can rely solely on glycolysis. CS is the first enzyme in the citric acid (TCA) cycle, catalyzes the conversion of oxaloacetate to citrate, and is often used as an indicator of aerobic metabolism (Emmett and Hochachka 1981; Hochachka and Somero 2002). Similarly, HOAD, an enzyme involved in the β-oxidation of lipids, is often used to indicate the relative contribution of fatty acids as fuels for aerobic respiration (Hochachka and Somero 2002). We hypothesize that as pups grow and their dives become longer and deeper (Jorgensen et al. 2001; Greaves et al. 2005; Frost et al. 2006), there will be increases in both aerobic and anaerobic enzyme activities in the muscles to support efficient underwater exercise.

Methods

Sample collection and preparation

Harbor seal pups and adult females were captured in the St. Lawrence River Estuary, Canada, throughout the 4-week nursing period from 2001 to 2003 (Clark et al. 2007). At initial capture, pup age in days (A_d) was estimated based on mass and appearance (Dubé et al. 2003; Clark et al. 2007). For subsequent captures, pup age was incremented based on time since initial capture. Animals were categorized as neonates (0–4 days), early nursing (5–16 days), late nursing (17–27 days), and weaned ($\geq$28 days). While some individuals were captured multiple times during the nursing period, only one sample from each animal was retained for analysis in this study to avoid pseudoreplication. At each handling, seals were weighed, sedated with an IV injection of Diazepam (0.3–0.8 mg kg^{-1}, Sabex, Inc., Canada), and a muscle biopsy was collected from the main swimming muscle (*longissmus dorsi*) using a disposable sterile 6 mm biopsy punch or cannula. Samples were flash frozen in liquid nitrogen in the field, then transported to the University of Alaska Anchorage and stored at −80°C until analysis, 3–4 years later.

Biochemistry

Frozen muscle samples were weighed, sonicated at 0°C in 15× dilution homogenization buffer (50 mM imidazole, 1 mM EDTA, 2 mM $MgCl_2$; (Polasek et al. 2006), and centrifuged at 10,000*g* for 5 min at 4°C. The supernatant was then partitioned and diluted as described below for total protein (TP), [Mb] and enzyme activity assays. TP content (mg protein g^{-1} wet tissue mass) of the supernatant was determined using Pierce Coomassie Blue "The Better Bradford" Total Protein Assay (Pierce Chemicals, Rockford, IL). For these assays, 10 µl of the initial supernatant was diluted to the concentration range of the assay in pH 7.0 imidazole buffer, 10 µl of the resulting 300× dilution was pipetted into three wells of a 96-well microplate, and 300 µl of the pre-diluted dye was added to each well. The plate was then incubated at room temperature for 10 min, and read at λ = 595 nm (Spectra Max 340PC, Sunnyvale, CA). TP was calculated from standard curves derived from bovine serum albumin (BSA) standards. Harbor seal tissue of known TP (as determined by "The Better Bradford" assay) was used as a tissue control.

Myoglobin concentration was determined for 24 pups (neonate, n = 1; early, n = 8; late, n = 10; weaned, n = 5). These values were combined with Mb values from an additional 100 harbor seals captured during the same period that were previously reported in Clark et al. (2007) prior to statistical analyses. The protocols of Reynafarje (1963) were modified as follows: 30 µl of the initial 15× supernatant was diluted further to a total volume of 120 µl in 0.04 M phosphate buffer, pH 6.6, a grain of sodium dithionite was added and the sample was vortexed until the sodium dithionite dissolved. Then, samples were transferred into individual wells of a 384-well microplate, the plate was placed into an airtight, CO-filled chamber for 20 min to reduce the myoglobin. This method of CO reduction was validated using harbor seal tissue of known myoglobin concentration reported in Burns et al. (2007). All assays were run in triplicate, and each run included tissue controls from an adult harbor seal of known [Mb] (Burns et al. 2007) and lyophilized horse Mb standards (Sigma–Aldrich, Allentown, PA).

LDH, CS, and HOAD activities were determined following the methods of Polasek et al. (2006) under substrate saturating conditions, with the following assay formulae—LDH (EC 1.1.1.27): 0.3 mM NADH, 1 mM pyruvate, 50 mM imidazole buffer, pH 7.0 at 37°C, ΔA_{340}, millimolar extinction coefficient ε_{340} = 6.22; CS (EC 4.1.3.7): 0.25 mM DTNB, 0.4 mM acetyl coA, 0.5 mM oxaloacetate, 50 mM imidazole buffer, pH 7.5 at 37°C, ΔA_{412}, millimolar extinction coefficient ε_{412} = 13.6; and HOAD (EC 1.1.1.35): 0.3 mM NADH, 1 mM EDTA, 0.2 mM acetoacetyl coA, 50 mM imidazole buffer, pH 7.0 at 37°C, ΔA_{340}, millimolar extinction coefficient ε_{340} = 6.22. Absolute activities (IU g^{-1} wet tissue mass) were calculated from the change in absorbance at the maximal linear slope of the assay. Harbor seal muscle tissue of known enzyme activities (previously verified against rat tissue, Burns, unpublished

data) was used as a tissue control. Each sample was assayed in four wells, and the assay was accepted if the tissue control was within its determined enzyme activity range, had a CV of less than 10%, and if the sample CV was also less than 10%. Precision of the assays was determined from all tissue control assays and is as follows: LDH CV = 8.3%; CS CV = 16.2%; HOAD CV = 6.0%.

CS/HOAD ratios were calculated to determine if aerobic muscle metabolism was fueled primarily by lipid oxidation, or by carbohydrates and/or glycogenic amino acids in proteins, with ratios of less than 1 indicating that muscle metabolism was primarily fueled by lipid oxidation (Winder et al. 1974; Reed et al. 1994; Kanatous et al. 1999; Polasek et al. 2006). In addition, LDH/CS ratios were used as an indicator of the anaerobic scope of the muscle, with higher values indicating that the muscle had a greater ability to maintain ATP production anaerobically once oxidative pathways were saturated or O_2 availability was low (Reed et al. 1994).

Statistical analysis

Differences in [Mb], enzyme activities (both absolute and metabolically scaled), and enzyme ratios between all age classes were tested using one-way ANOVA or ANCOVAs with Bonferroni post hoc tests. Mass was only included as a covariate in the examination of age effects on [Mb] because the relationship between mass and enzyme activity differed by age class (Fig. 1), thus violating the assumption of homogeneity of regression (Zar 1984). Because there was no correlation between enzyme activity and muscle protein content (LDH: $r = 0.059$, $p = 0.784$; CS: $r = -0.131$, $p = 0.541$; HOAD: $r = -0.032$, $p = 0.883$), enzyme activities were not scaled to TP prior to analysis of age effects. To account for the effect of age-specific differences in mass-specific metabolic rates on enzyme activity, absolute enzyme activities were also scaled to mass-specific RMRs (ml O_2 kg^{-1} min^{-1}), as reported in the literature (Table 1). These values are termed metabolically scaled enzyme activities (e.g. LDH_{met}). It was not possible to scale enzyme activities to muscle-specific metabolic rates, since such data do not exist for harbor seal pups. Statistical significance for all tests was set at $p < 0.05$ and all statistical analyses were performed using SPSS v.14.0 (Chicago, IL). Values are reported as mean ± SE unless otherwise noted.

Results

Myoglobin concentrations were determined for 124 animals (Table 1). Myoglobin concentration did not differ among the neonate, early, and late nursing pups; however, weaned pups had significantly higher myoglobin concentration than all other pups (Table 1; $F_{4,123} = 17.067$, $p < 0.001$). Adult myoglobin concentrations were significantly higher than all pup age categories (Table 1; $F_{4,123} = 17.067$, $p < 0.001$).

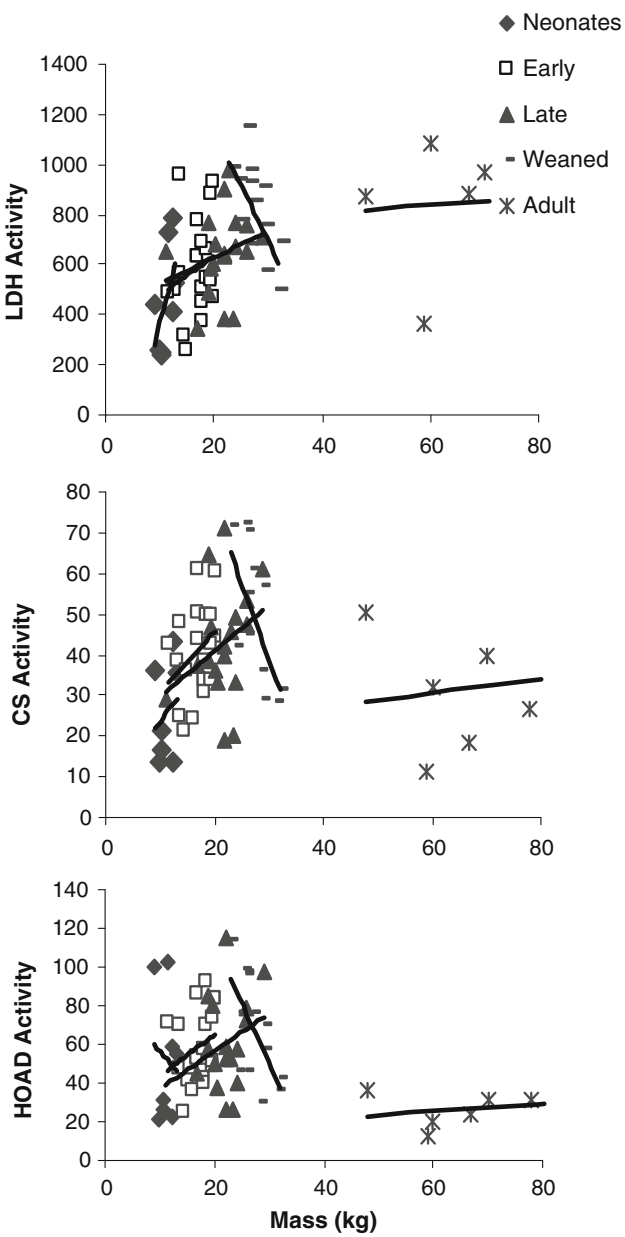

Fig. 1 Effects of mass on **a** LDH, **b** CS and **c** HOAD enzyme activity (IU g^{-1}). *Trend lines* are the best-fit regression lines depicting the different relationship between mass and enzyme activity for weaned pups and adults. All other age classes were non-significant

Enzyme activities were determined for 67 animals (Table 1). There were significant age-related differences in all enzyme activities on both an absolute and metabolically scaled basis. Absolute and metabolically scaled LDH (LDH_{met}) activity increased across the nursing period, with weaned pup and adult activity significantly higher than in

Table 1 Mean ± SE mass (kg), reported mass-specific resting metabolic rates (RMRs; ml−O_2 kg^{-1} min^{-1}) and absolute enzyme activities (LDH, CS and HOAD; IU g^{-1} wet tissue) for each age category

Age category	*N* (enzymes, Mb)	Mass (kg)	Reported RMR (ml O_2 kg^{-1} min^{-1})	Mb (mg g^{-1} wet tissue)	LDH (IU g^{-1} wet tissue)	CS (IU g^{-1} wet tissue)	HOAD (IU g^{-1} wet tissue)
Neonates	8, 18	11.2 ± 1.4	13.3[a]	16.6 ± 1.8*	456.1 ± 69.0*	25.7 ± 12.3*	52.3 ± 33.3*,†
Early nursing	20, 35	16.9 ± 2.5	12.3[b]	17.2 ± 0.6*	586.5 ± 43.6*,‡	41.0 ± 11.0*	58.8 ± 19.0†
Late nursing	18, 29	22.2 ± 3.0	10.2[c]	18.3 ± 0.7*	645.7 ± 45.9*,†,‡	42.6 ± 14.1*	60.7 ± 24.6†
Weaned pups	13, 26	27.1 ± 2.8	8.3[a]	24.3 ± 1.2†	825.7 ± 54.1†	49.7 ± 16.2†	68.3 ± 27.4†
Adults	8, 16	70.0 ± 17.3	4.6[d]	58.8 ± 1.7‡	798.3 ± 68.9†,‡	29.4 ± 15.6*	24.9 ± 10.4*

Different symbols indicate statistical difference within a column between age classes at $p < 0.05$

[a] Miller and Irving (1975)

[b] Miller et al. (1976)

[c] Clark et al. (2007)

[d] Davis et al. (1985)

the younger pups (absolute activity: $F_{4,62} = 6.28$, $p < 0.001$, Table 1; LDH_{met}: $F_{4,62} = 46.104$, $p < 0.001$, Fig. 2a). While weaned pups had similar LDH activity to adults when judged on absolute basis, LDH_{met} values were significantly lower than in adults. Similarly, there were increases in CS activity with pup age on both an absolute and metabolically scaled (CS_{met}) basis. However, in contrast to LDH, weaned pups had higher absolute CS activities as compared to adults, although levels were similar to adults when scaled to metabolic rates (absolute activity: $F_{4,62} = 4.963$, $p = 0.002$, Table 1; CS_{met}: $F_{4,62} = 11.314$, $p < 0.001$, Fig. 2b). In contrast, pups had significantly higher, and more variable, absolute HOAD activity than adults, but there was no difference among the different pup age classes ($F_{4,61} = 4.628$, $p = 0.002$, Table 1). However, metabolically scaled HOAD ($HOAD_{met}$) values increased with pup age with the highest levels of all age classes seen in weaned pups ($F_{4,61} = 5.523$, $p = 0.001$, Fig. 2c). Adults had significantly higher CS/HOAD ($F_{4,60} = 14.2$, $p < 0.001$, Fig. 3a) and LDH/CS ($F_{4,60} = 12.8$, $p < 0.001$, Fig. 3b) ratios than all pup age classes, and there were no age-related trends among pup age classes.

Discussion

The results of this study indicate that harbor seal pup muscles are not biochemically mature at birth or at weaning, despite the precocial appearance and early swimming activity (Jorgensen et al. 2001; Greaves et al. 2005). This immaturity can be seen in both the low O_2 carrying capacity (myoglobin concentration) and reduced catabolic enzyme (LDH and CS) activities. In combination, these findings suggest that pup muscle does not have the ability to support either aerobic or anaerobic performance to the same extent as adult seal muscle, and this limitation may contribute to the lower diving capacity and behavior in younger pups. In addition, the divergent effects of mass and age on enzyme activities suggest that the age-related differences in muscle biochemistry seen in this study are developmental and/or exercise-driven responses that together work to produce the hypoxic endurance phenotype seen in adults, rather than allometric effects due to body size.

As has previously been seen in penguins, cetaceans, and pinnipeds (Ponganis et al. 1997a, b; Dearolf et al. 2000; Burns et al. 2005; Noren et al. 2005; Richmond et al. 2006; Burns et al. 2007; Clark et al. 2007), neonatal harbor seals had much lower [Mb] in their muscles compared to older animals, and while there was a slight increase with age, pup [Mb] did not reach adult levels by the time pups were weaned. Although [Mb] are typically low in both terrestrial and marine mammal neonates (Whipple 1926; Longo et al. 1973; Tipler et al. 1978; Weller et al. 1986; Garry et al. 1996), in marine mammals, low [Mb] carries unique consequences because the diving response involves reduction of blood flow and substrate delivery to muscles (Kanatous et al. 1999). Thus, for young divers, lower [Mb] limits the amount of time that pups can remain submerged with aerobically working muscles, thereby reducing the ability of pups to sustain diving activities. Low endogenous Mb reserves in pup muscles may also increase reliance on anaerobic metabolism when foraging success requires dives longer than the aerobic dive limit (Burns and Castellini 1996; Burns 1999) or during periods of extreme vasoconstriction.

In muscle tissue, there is generally a close match between structural and biochemical properties, with fibers that rely more heavily on oxidative metabolism typically containing higher [Mb], and glycolytic fibers containing less Mb and having higher LDH activity (Saltin and Gollnick 1983; Garry et al. 1996; Hoppeler and Fluck 2002; Fluck 2006). This pattern appears to hold for adult muscles

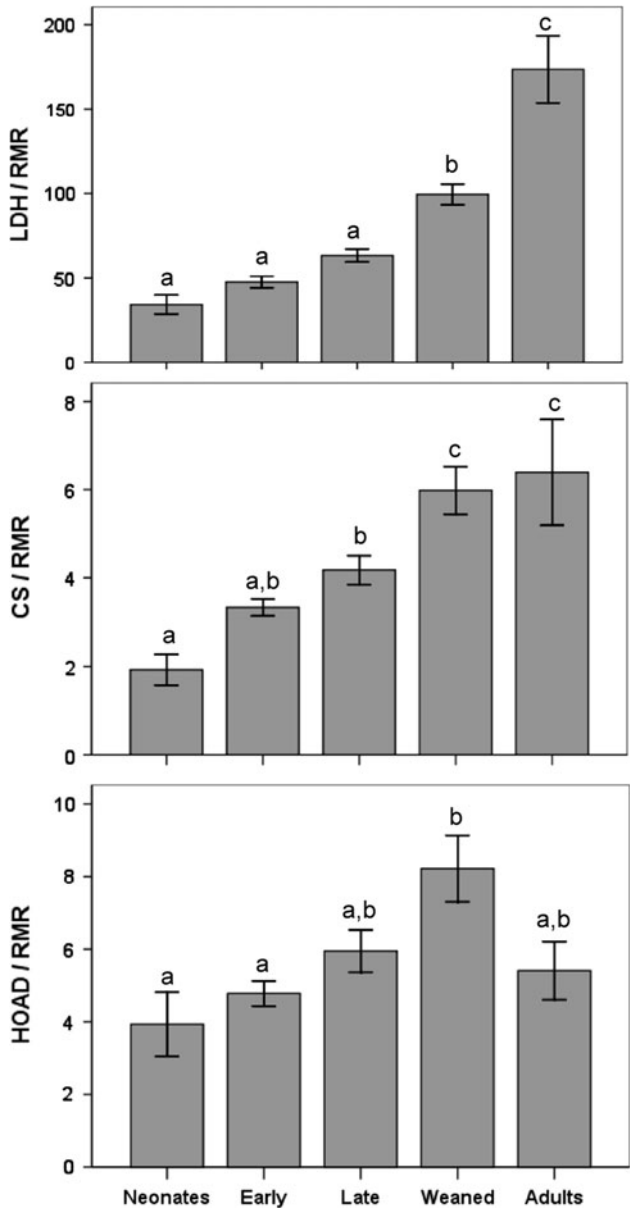

Fig. 2 Mean ± SE metabolically scaled (enzyme activity/ml O_2 kg min^{-1}) LDH, CS and HOAD. *Different letters* above *bars* indicate statistically significant differences between age categories within each enzyme. Sample size provided in Table 1

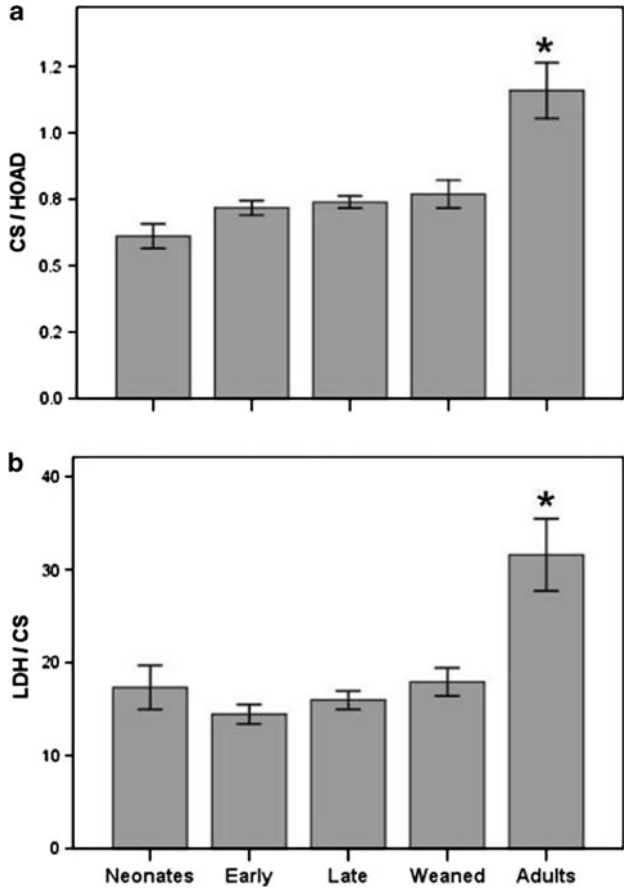

Fig. 3 Mean (±SE) CS/HOAD and LDH/CS for each age category. *Different letters* above *bars* indicate statistically significant differences between age categories within an enzyme ratio. Sample sizes as in Fig. 2

where the high [Mb] in the *longissimus dorsi* is associated with a large proportion of oxidative fibers (type 1 and IIA, Watson et al. 2003) as expected based on the need to support aerobic underwater activities. However, the LDH activity measured in adult harbor seal muscles in this study is intermediate between those of terrestrial endurance athletes such as the dog, and sprinters such as the cheetah (Williams et al. 1997; Polasek et al. 2006), suggesting that adults retain significant ability to produce ATP anaerobically, as would be necessary when dives exceed their aerobic capacity (Polasek et al. 2006). The LDH values measured in this study were within the range of those reported for other phocid seals (Castellini and Somero 1981; Hochachka and Foreman 1993; Reed et al. 1994; Polasek et al. 2006) suggesting that not only our methods and results are comparable to previous studies, but also the anaerobic metabolic potential of the main locomotory muscle in phocids is fairly uniform, reflecting similar use patterns.

In contrast to LDH activity, pup muscles have much lower [Mb] in their muscles than adults. One possible explanation is that their muscles contain more glycolytic fibers than adults, perhaps due to an increased proportion of anaerobic dives (Burns 1999). However, the low LDH activity in pup muscles does not support this idea, and indeed it runs counter to the prevailing pattern of muscle fiber development, where fast glycolytic fibers typically mature after the more oxidative forms (Condon et al. 1990; Powers et al. 1991; Schiaffino and Reggiani 1994; Garry et al. 1996; Olson 2001). Taken together, the lower absolute LDH and LDH_{met} activity indicates that pup muscles have a decreased ability both to convert lactic acid to

pyruvate, and to maintain the redox balance necessary to sustain glycolytic pathways when O_2 is limited. Similarly, the significantly lower LDH/CS ratio suggests that pup muscles have reduced ability to switch to anaerobic metabolism when aerobic stores are exhausted (Reed et al. 1994; Polasek et al. 2006). Thus, pup muscles do not appear to be as adapted for anaerobic underwater activities as adults.

Instead, the combination of low [Mb] and LDH activities in pup muscles may reflect both developmental constraints and use patterns. Postnatal development in many vertebrates is characterized by an increase in LDH activity (Blaise Smith 1980; Bishop et al. 1995; Agüera et al. 2001; Gondret et al. 2004), perhaps in association with increases in the proportion and/or size of type II fibers, and the increase in LDH activity with age (and mass) observed here suggests that harbor seal muscle development follows a broadly similar pattern. In addition, behavioral patterns suggest that LDH levels would likely not need to be high because the short and shallow dives made by pups are rarely long enough to exhaust circulating O_2 stores (Burns et al. 2005; Clark et al. 2007). Similarly, since harbor seal pups are unable to sustain low heart rates while diving (Greaves et al. 2005), pup muscles may be better perfused during dives than adult seal muscles. Since Mb can only serve as an endogenous O_2 reserve if tissue pO_2 levels drop significantly due to vasoconstriction, under higher perfusion rates myoglobin would serve primarily to facilitate O_2 delivery to mitochondria, and muscle pO_2 levels would remain high as long as did vascular levels (Davis and Kanatous 1999). This would favor oxidative metabolism and likely reduce lactate production.

The low [Mb] in the pups are also helpful in interpreting the aerobic capacities of their muscles. For example, given the high absolute enzyme activities one might expect that pup muscles are capable of supporting sustained aerobic activities, and consist of largely oxidative fibers with high mitochondrial densities, as seen in adults (Kanatous et al. 1999; Watson et al. 2003, 2007). However, since pups have relatively poor cardiovascular control (Greaves et al. 2005), low blood and muscle O_2 stores (Burns et al. 2005; Clark et al. 2007), and high mass-specific metabolic rates, it is unlikely that they can function long in the absence of freely available O_2. Instead, the relatively high absolute enzyme activities likely result from a need to match enzyme activity to metabolic rate (Hochachka and Somero 2002), as elevated CS and HOAD levels have been documented in a variety of precocial mammals and birds, but are less common in altricial species (Blaise Smith 1980; Glatz and Veerkamp 1982; Agüera et al. 2001; Krijgsveld et al. 2001; Shea et al. 2007; Kanatous et al. 2008). Despite these elevated levels, in harbor seals, when the high absolute enzyme activities are scaled to the mass-specific RMR of each age class, it becomes clear that younger pups have less ability to support aerobic metabolism than older animals.

That this is an age- and not mass-driven effect is evident by the fact that pups have much higher aerobic enzyme levels than expected based on their body mass and that the correlation between enzyme activity and body mass is negative for weaned pups (see Fig. 1), where older animals are lighter than younger ones (Muelbert and Bowen 1993). Indeed, the increases in enzyme activities observed during the nursing period run counter to the pattern predicted based on mass alone. Among adult animals, larger body size is associated with lower aerobic enzyme activities (Emmett and Hochachka 1981), likely due to the decline in mass-specific metabolic rates. In this study, the CS and HOAD activities measured in adult muscles were similar to those previously reported for harbor seals (Reed et al. 1994; Polasek et al. 2006), but higher than those reported for the larger Weddell and gray seals (Kanatous et al. 2002; Reed et al. 1994). One exception was the enzyme activities of fasting/lactating females, which had higher HOAD levels than would be expected based on body size alone (Burns et al. 2010; Kanatous et al. 2008).

In pups, the age-related increases in enzyme activity are consistent with the developmental patterns seen in other vertebrates, where hypertrophy is frequently accompanied by increases in catabolic enzyme activity and thermoregulatory abilities (Dearolf et al. 2000; Olson 2001; Shea et al. 2007). However, increases in Mb and enzyme activity during the nursing period may also be due to increases in the depth, duration, and frequency of dives (Jorgensen et al. 2001; Baechler et al. 2002; Greaves et al. 2005), as endurance exercise training and intermittent tissue hypoxia both increase the oxidative potential of muscle tissue by increasing mitochondrial densities and aerobic enzyme activities (Kayar et al. 1986; Hoppeler and Fluck 2002; Fluck 2006). Therefore, increased diving activity, in combination with exercise-induced hypoxia (due to low muscle Mb), likely augments developmental mechanisms to increase the endurance capabilities of pup muscles.

Age-related differences in enzyme activities also likely reflect dietary changes. In adult animals, diets high in fat are associated with increases in aerobic capacities of muscle due to elevated aerobic enzyme activities, and mitochondrial and lipid droplet densities (Miller et al. 1984; Reynolds et al. 2005; Garcia-Roves et al. 2007). For adult harbor seals, the high reliance on lipid-based aerobic respiration to fuel efficient underwater diving activities is reflected in CS/HOAD ratios much lower than in most terrestrial animals (Hochachka et al. 1983; Reed et al. 1994; Polasek et al. 2006) Yet, even these values are higher than observed in pups. However, during the nursing period, harbor seal pups subsist on milk that is much higher in fat (40–50%), and lower in both protein (9%) and carbohydrates

(Oftedal 2002; Lang et al. 2005) than the adult diet of fish and invertebrates (Bowen and Harrison 1996). The increased reliance on milk lipid as a fuel source is reflected in the much lower CS/HOAD ratio for nursing pups as compared to adults, while the low CS/HOAD ratio in weaned pups likely reflects their heavy reliance on the mobilization of blubber lipids to support metabolism as they learn to forage successfully (Muelbert and Bowen 1993; Lesage et al. 1999; Burns et al. 2005). Thus, provided that the vascular supply of O_2 is sufficient to compensate for their lower [Mb] content, pup muscles appear to be as metabolically primed for lipid-based aerobic respiration as adults.

The rate and extent of muscle development may depend on muscle function (Choi et al. 1993; Bishop et al. 1995; Agbulut et al. 2003), in which case the patterns observed here for the major locomotory muscle in harbor seals may not apply to all species and muscle. However, several lines of evidence suggest that the overall pattern is broadly applicable across marine mammal species. Age-related increases in enzyme activities in both locomotory and non-locomotory skeletal muscles, and in cardiac muscles, have been documented in harbor seals (this study), harp and hooded seals (Burns et al. 2010; Lestyk, unpublished data), Weddell seals (Kanatous et al. 2008), and Steller sea lions (Richmond, unpublished data), just as have been seen in many terrestrial mammals and birds (Gondret et al. 2004; Griffiths et al. 1994; Olson 2001; Shea et al. 2007). However, in both terrestrial and marine species, there are differences in the rate and magnitude of the developmental increases based on muscle use (Grand 1992), so behavior and morphology should be considered when making comparisons across species. In addition, when comparing enzyme activities across studies, it may also be necessary to consider the impact of season, diet, and animal condition, as all these factors are known to influence muscle metabolic activity in terrestrial species (Hoppeler and Fluck 2002; Kelsen et al. 1985).

In summary, in the context of a young, swimming and diving seal, neonatal harbor seals have low O_2 stores and decreased capacities to produce ATP both aerobically and anaerobically as compared to the adults, particularly when examined relative to metabolic rates. These immature parameters may not have much functional significance early in the nursing period when diving activity is limited, but become more important once pups are weaned and must forage on their own. By weaning, pup muscles have developed slightly, but are likely still constrained in their ability to sustain underwater aerobic metabolism by lower Mb content and LDH activity. In combination with lower TBO_2 stores, higher mass-specific RMRs, and poor cardiovascular control (Burns et al. 2005; Greaves et al. 2005; Clark et al. 2007), this study suggests that the diving behavior of weaned pups may be further limited by their underdeveloped muscle metabolic capacities.

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