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ORIGINAL PAPER

Development of the aerobic dive limit and muscular efficiency in northern fur seals (*Callorhinus ursinus*)

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Abstract Northern fur seal (*Callorhinus ursinus*; NFS) populations have been declining, perhaps due to limited foraging ability of pups. Because a marine mammal's proficiency at exploiting underwater prey resources is based on the ability to store large amounts of oxygen (O_2) and to utilize these reserves efficiently, this study was designed to determine if NFS pups had lower blood, muscle, and total body O_2 stores than adults. Pups (<1-month old) had a calculated aerobic dive limit only ~40% of adult females due to lower blood and, to a much greater extent, muscle O_2 stores. Development of the *Pectoralis* (*Pec*) and *Longissimus dorsi* (*LD*) skeletal muscles was further examined by determining their myosin heavy chain (MHC) composition and enzyme activities. In all animals, the slow MHC I and fast-twitch IIA proteins typical of oxidative fiber types were dominant, but adult muscles contained more (*Pec* ~50%; *LD* ~250% higher) fast-twitch MHC IID/X protein characteristic of glycolytic muscle fibers, than pup muscles. This suggests that adults have greater ability to generate muscle power rapidly and/or under anaerobic conditions. Pup muscles also had lower aerobic and anaerobic ATP production potential, as indicated by lower metabolically scaled citrate synthase, β-hydroxyacyl CoA dehydrogenase, and lactate dehydrogenase activities (all P values ≤ 0.001). In combination, these findings indicate that pups are biochemically and physiologically limited in their diving capabilities relative to adults. This may contribute to lower NFS first year survival.

Keywords Aerobic dive limit · Oxygen stores · Muscle development · Myosin heavy chain · Enzyme · Diving physiology · Northern fur seal

Abbreviations

ADL	Aerobic dive limit
BV	Blood volume (% body mass)
cADL	Calculated aerobic dive limit
CS	Citrate synthase (IU g^{-1} wet tissue)
DMR	Diving metabolic rate
Hb	Hemoglobin (g dL^{-1} whole blood)
Hct	Hematocrit (% whole blood)
HOAD	β-Hydroxyacyl CoA dehydrogenase (IU g^{-1} wet tissue)
LD	*Longissimus dorsi* skeletal muscle
LDH	Lactate dehydrogenase (IU g^{-1} wet tissue)
Mb	Myoglobin (mg g $tissue^{-1}$)
MCHC	Mean corpuscular hemoglobin concentration (%)
MHC	Myosin heavy chain
NFS(s)	Northern fur seal(s)
O_2	Oxygen
Pec	*Pectoralis* skeletal muscle
PV	Plasma volume (% body mass)
RMR	Resting metabolic rate
TBO_2	Total body oxygen stores
TP	Total protein (mg g $tissue^{-1}$)

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Introduction

Presently, fewer than 1.1 million northern fur seals (*Callorhinus ursinus*; NFS) reside in the North Pacific, with 55% of this population located on the Pribilof Islands off the coast of Alaska (NMFS 2007). This population has declined by approximately 5% per year since 1958 (Gentry 1998; Towell et al. 2006), and NFS are currently listed as a "depleted" species under the Marine Mammal Protection Act (Angliss and Lodge 2004). The reason behind the continued decline remains unknown, despite ongoing study (Towell et al. 2006; Lea et al. 2009, 2010). One potential factor could be reduced juvenile survival, particularly throughout the first winter when pups begin to forage independently (Lander 1979, 1981). Indeed, reduced juvenile foraging proficiency has been hypothesized as a cause of population decline for many marine mammal species (York 1994; Craig and Ragen 1999; Lea et al. 2009).

In foraging, pinnipeds exploit underwater prey resources, and one factor that affects diving capability is the amount of O_2 that can be stored in tissues. Not surprisingly, pinnipeds have larger total body oxygen (TBO_2) stores than terrestrial mammals due to a larger blood volume and greater packed red blood cell volume (hematocrit: Hct), hemoglobin (Hb) concentration, and muscle myoglobin (Mb) levels (Hochachka and Storey 1975; Butler and Jones 1997; Kooyman and Ponganis 1998; Burns et al. 2007). In otariids, the blood and muscle O_2 stores account for 60% of the TBO_2 (Lenfant et al. 1970; Richmond et al. 2006), but in many pinniped species, pups possess significantly smaller mass-specific TBO_2 stores than adults (Burns et al. 2005, 2007; Noren et al. 2005; Richmond et al. 2006; Spence-Bailey et al. 2007). Tissue O_2 stores, especially Mb loads, mature when pups begin to forage independently and perform more strenuous physical activity (Noren et al. 2005; Burns et al. 2007; Geiseler 2011). Once mature, Mb content in pinniped propulsive muscles is characteristically 10–20 times higher than in terrestrial mammals (Reed et al. 1994; Kanatous et al. 1999; Polasek et al. 2006), and Mb levels are positively correlated with dive duration in marine mammals (Kooyman and Ponganis 1998; Noren and Williams 2000; Lestyk et al. 2009).

Not only do pinnipeds have extensive O_2 stores, but seals are also able to control the rate at which they utilize these reserves. During dives, pinnipeds can lower their body temperature, exhibit bradycardia, and employ peripheral vasoconstriction to apportion which tissues receive the blood O_2 stores (Irving et al. 1942; Zapol et al. 1979; Hill et al. 1987). Vasoconstriction acts to prevent muscle Mb, with a higher O_2 affinity than Hb, from removing all the O_2 from the blood (Zapol et al. 1979). These adaptations lower the organism's diving metabolic rate and conserve O_2 stores for critical tissues to lengthen the animal's breath-hold capacity (Zapol et al. 1979; Castellini et al. 1992; Butler and Jones 1997; Kooyman and Ponganis 1998; Davis and Kanatous 1999; Davis et al. 2004).

Compared to the efficient dive response in adults; however, pinniped neonates have higher mass-specific metabolic rates due to their smaller size, faster growth rate, and relatively poor cardiovascular control (Miller and Irving 1975; Castellini et al. 1994; Donohue et al. 2000; Lapierre et al. 2004), which in combination with lower TBO_2 stores reduces the animal's breath-hold capacity, or aerobic dive limit (ADL). As O_2 stores are depleted, anaerobic respiration begins to predominate, and lactate accumulates in tissues and blood, requiring more extensive post dive recovery periods at the surface after dives (Kooyman et al. 1980). The ADL can be estimated by dividing endogenous O_2 stores by diving metabolic rates, and this calculated ADL (cADL) has been shown to be an accurate estimator of the onset of lactate accumulation (Kooyman et al. 1980, 1983; Ponganis et al. 1993). Because pups have both smaller mass-specific TBO_2 stores and faster metabolic rates, they have shorter cADLs, and therefore a reduced time window to forage compared with adults (Kooyman et al. 1983; Burns 1999; Spence-Bailey et al. 2007).

To lengthen the cADL and foraging times, marine mammals must conserve O_2 while at the same time meeting the demands for physical activity. This necessitates effective aerobic processes to maintain O_2 stores; however, pinnipeds also need efficient glycolytic processes for those times when local O_2 stores are depleted (Kooyman et al. 1980; Kanatous et al. 2002). At the level of the working muscle, Mb loads, fiber type and myosin heavy chain (MHC) profiles, and enzyme activity levels all influence endurance under aerobic and anaerobic conditions, and all these parameters are known to change in response to age and activity levels (Condon et al. 1990; Bishop et al. 1995; Hoppeler and Flück 2002; Shea et al. 2007; Kanatous et al. 2008; Prewitt et al. 2010). MHCs are classified as fast- or slow-twitch, referring to the speed at which chemical energy is converted into mechanical force via isoform-specific ATPase activities during cross-bridge cycling (Baldwin and Haddad 2001). In general, the percentage of fast-twitch MHC IID/X proteins, found in glycolytic fibers, and thus anaerobic capacities, increases as muscles develop (Baldwin and Haddad 2001). In neonates, muscles consist of mainly slow-twitch and oxidative fibers (Condon et al. 1990; Baldwin and Haddad 2001; Luedeke et al. 2004). Increased weight-bearing activity, degree of loading, contractile function, and hypoxia all increase the expression of fast-twitch and glycolytic fibers that primarily express MHC IIA and MHC IID/X isoforms (Baldwin and Haddad 2001, 2002; Hoppeler and Flück 2002; Putman et al. 2004; Flück 2006).

In addition to the MHC profiles associated with different muscle fiber types, each fiber type has a characteristic biochemical profile (Hoppeler and Flück 2002; Flück 2006) that is reflected in the relative activities of enzymes involved in aerobic and glycolytic metabolism. Muscles that primarily consist of fast-twitch and glycolytic fibers have higher proportions of MHC IIA and IID/X isoforms and typically express high lactate dehydrogenase (LDH) activity, indicating that pyruvate is regularly converted to lactate under low O_2 conditions (Peter et al. 1972; Baldwin et al. 1973; Kanatous et al. 2002) so that ATP production can continue even once local O_2 stores have been exhausted on a dive. Conversely, muscles with more slow-twitch and oxidative fibers typically have greater MHC I expression and aerobic capacities, with high citrate synthase (CS) and β-hydroxyacyl CoA dehydrogenase (HOAD) activities (Kanatous et al. 2002; Luedeke et al. 2004).

Citrate synthase activity has been used as an indicator of aerobic respiration capacities in many pinniped species (Emmett and Hochachka 1981; Reed et al. 1994; Kanatous et al. 1999, 2002; Polasek et al. 2006; Burns et al. 2010; Prewitt et al. 2010), as it marks the entrance to the citric acid cycle and is proportional to metabolic rate (Emmett and Hochachka 1981). While nursing, neonatal marine mammals have a largely lipid-based milk diet (Oftedal et al. 1987). Therefore, it is of interest to determine reliance on the oxidation of fatty acids via HOAD activities to fuel the citric acid cycle in NFS neonates and adults. In general, adult pinniped muscles have large aerobic capacity, which enables the animals to forage efficiently while diving under O_2-limiting conditions (Emmett and Hochachka 1981; Kanatous et al. 1999; Castellini and Castellini 2004).

Enzyme profiles in pinniped pups differ from those of adults, often in unexpected ways. For example, terrestrial neonate muscles typically exhibit lower enzyme activities than adult muscles and, corresponding with muscle fiber and MHC composition, particularly less LDH glycolytic activities (Baldwin et al. 1973; Bishop et al. 1995; Baldwin and Haddad 2001; Hoppeler and Flück 2002). However, the recent studies on early metabolic development show inconsistent patterns among pinniped species, with some neonates and weaned pups exhibiting higher enzyme activities than adults, and thus these trends differ from muscle maturation models of terrestrial vertebrates (Kanatous et al. 2008; Burns et al. 2010; Prewitt et al. 2010). Oxygen stores, enzyme activities, and muscle MHC profiles will all influence the ability of young pups to forage. The general correlations between muscle structure and biochemistry are indicative of mature muscle function and efficiency by utilizing complementary contractile properties and metabolic activities to meet changing energetic demands together (Flück 2006). While studies have shown age-related changes in O_2 loads and enzyme kinetics, as yet, there have been no studies directly linking MHCs and enzymes in pinnipeds.

As compared with other otariid species, NFS pups have a short dependent period of ~4–5 months before they must forage for themselves in the North Pacific (Baker and Donohue 2000; Lea et al. 2010). Because not having fully-developed physiological adaptations would limit diving ability, young NFS pup muscles might be more mature at birth than has been documented in other otariid species. To determine if more precocial NFS pups are physiologically unprepared to forage for prey relative to adults, this study assessed age-related differences in (1) endogenous O_2 storage capacities and the cADL, (2) MHC isoform composition, and (3) aerobic and anaerobic enzyme activities within these fibers. We hypothesized that NFS pups would have lower blood, muscle, and TBO_2 stores than adult females. Further, we expected that pup muscles would show more of the slow MHC I isoform and less fast-twitch IID/X protein. These differences would likely be reflected in lower enzyme levels than adults, particularly for LDH, which is associated with anaerobic potential. Thus, we predicted that pups would have smaller TBO_2 reserves and less potential for ATP production, corresponding with a shorter cADL and limited ability to sustain breath-hold diving activities. Together, these findings could elucidate the physiological diving constraints and potential influences on the foraging strategies and survival of NFS pups.

Methods

Animal handling

Blood and muscle samples were collected from nine adult and eight <1-month-old NFS on the Lovushki Island rookery in the Kuril Islands of far eastern Russia (48°33′14.4″N, 153°51′25.2″E) in 2008. Seals were captured by hoop net, transported to a research vessel, and anesthetized with isoflurane for blood and muscle sampling.

Hematology and blood volume

Hematocrit (%) was determined in the field by centrifugation of whole blood samples. Hemoglobin concentrations (g dL^{-1}) for each animal were determined in triplicate using the cyanomethemoglobin assay. Optical density of each sample was read using a UV/Vis Beckman series 530 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA) at $\lambda = 540$ nm and concentrations of samples were calculated through a linear regression of Hb standards (Pointe Scientific, Inc., Canton, MI, USA) based on obtained absorbance values. Mean corpuscular hemoglobin concentration (MCHC) was calculated as: MCHC (%) = (Hb g dL^{-1}/Hct) × 100.

Plasma volume (PV_{EB}) was determined using the Evan's Blue technique (Foldager and Blomqvist 1991; El-Sayed et al. 1995). In the field, approximately 0.5 mg kg^{-1} Evan's Blue dye was injected using individually calibrated syringes, and three consecutive blood draws were taken at 10 min intervals. Blood samples were centrifuged and plasma was collected and frozen for later analysis. The plasma sample background absorbance values at $\lambda = 740$ nm were subtracted from optical density at $\lambda = 624$ nm, and Evan's Blue dye stock standard curves were used to find sample concentrations (Foldager and Blomqvist 1991; El-Sayed et al. 1995). The change in dye concentration over time was modeled using regression analysis, and the *y*-intercept used to estimate PV_{EB}. If the R^2 value was less than 0.5, the average value of the three data points was used to determine PV_{EB}. Blood volume (BV_{EB}) was then calculated as: $BV_{EB} = PV_{EB}/[(100 - Hct)/100]$.

Muscle enzyme activity, myoglobin, and total protein assays

Pectoralis (*Pec*, a major locomotor muscle when on land and at sea) and *longissimus dorsi* (*LD*, used primarily in posture, and to a lesser extent when swimming) muscle samples (<150 mg) were taken from each animal in the field with a 4–6 mm biopsy punch. The *Pec* and *LD* muscles were chosen to compare the development in muscles used for different functions. All samples were stored at −80°C until laboratory analyses could be performed. In the laboratory, muscle samples were thawed, weighed, and sonicated on ice in homogenization buffer (50 mM imidazole, 1 mM EDTA, and 2 mM $MgCl_2$). Homogenates were then centrifuged 10,000*g* for 5 min at 4°C and the supernatant was diluted to experimentally determined values, and used to determine enzyme activity, Mb (mg g $tissue^{-1}$), and TP (mg g $tissue^{-1}$) content. Samples were assayed in quadruplicate in a Molecular Devices SpectraMax 340 microplate reader (Molecular Devices, Inc., Sunnyvale, CA, USA).

To evaluate aerobic and anaerobic ATP production potential, citrate synthase (CS), β-hydroxyacyl CoA dehydrogenase (HOAD), and lactate dehydrogenase (LDH) kinetic activities (IU g^{-1} wet mass muscle) were measured. Spectrophotometric assays were run according to the procedures described by Polasek et al. (2006) and Prewitt et al. (2010) under substrate saturating conditions held at 37°C. A buffer blank and a previously assayed muscle sample of known concentration were measured as controls along with all experimental samples. Assay conditions were as follows: CS (EC 4.1.3.7): 50 mM imidazole, 0.25 mM 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), 0.4 mM acetyl CoA, and 0.5 mM oxaloacetate at pH 7.5, and ΔA_{412}, $\varepsilon_{412} = 13.6$; LDH (EC 1.1.1.27): 50 mM imidazole, 0.3 mM NADH, and 1 mM pyruvate at pH 7.0, ΔA_{340}, $\varepsilon_{340} = 6.22$; and HOAD (EC 1.1.1.35): 50 mM imidazole, 1 mM EDTA, 0.3 mM NADH, and 0.2 mM acetoacetyl CoA at pH 7.0, ΔA_{340}, $\varepsilon_{340} = 6.22$. Enzyme activities are reported as both absolute and metabolically scaled (absolute enzyme activity/resting metabolic rate) values. Pups have much higher mass-specific metabolic rates than adults, so scaled enzyme values control for the disproportionate flux of metabolites through the metabolic pathways. Pup mass-specific resting metabolic rates (RMRs) were estimated from values reported in Donohue et al. (2000) and adult values were calculated as twice the values estimated from Kleiber's (1961) equation relating BMR to body mass (Williams et al. 2001) (Pup RMR: 15.3, adult RMR: 9.1 mL O_2 min^{-1} kg^{-1}). CS:HOAD ratios were evaluated to determine the relative amount of aerobic metabolism that utilizes β-oxidation of fatty acids. Values <1 indicated higher dependence on lipid stores. The LDH:CS ratios were calculated to compare potentials for aerobic versus anaerobic metabolism (Polasek et al. 2006).

The same supernatant (20× dilution in homogenization buffer) used for enzyme analysis was then assayed for Mb concentrations according to methods by Reynafarje (1963). For each sample, 60 µL supernatant and 50 µL of 0.4 M phosphate buffer (pH 6.6), was loaded into four microplate wells. Samples were reduced to their carbonyl derivative by placing the microplate in an air-tight chamber with $CO_{(g)}$ for 20 min, adding 10 µL of 10% sodium dithionate solution to wells, and then repeating $CO_{(g)}$ reduction for 5 more min. Samples were read at both $\lambda = 538$ and 568 nm to eliminate any Hb contamination. A lyophilized Mb horse standard (Sigma Aldrich, St. Louis, MO, USA) and previously assayed harbor seal (*Phoca vitulina*) tissue control samples were run with all unknown samples. Another aliquot of the supernatant was further diluted (300×) in pH 7.0 homogenization buffer for the TP assay to assess whether muscle protein concentrations or the proportion of TP consisting of Mb protein changed with age. In each microplate well, 10 µL of 300× diluted sample and 300 µL of Pierce Coomassie Blue reagent ("The Better Bradford" Total Protein Assay, Pierce Chemicals, Rockford, IL, USA) were added. Plates incubated for 10 min at room temperature and were read at $\lambda = 595$ nm. Each microplate also contained a bovine serum albumin standard curve (0–1.5 mg/mL) and a known tissue control.

Total body oxygen stores and the cADL

Mass-specific blood O_2 stores were estimated from Hb, Hct, and BV in arterial and venous systems with the following assumptions: (1) Hb and Mb have an O_2 carrying capacity of 1.34 mL O_2 g^{-1} [Hb/Mb], (2) arterial blood is 33% of total blood volume, with the remaining 66% blood in the venous system, (3) the arterial O_2 stores are depleted from

95 to 20% saturation, and (4) venous blood was depleted from 5 vol% less than the starting arterial O_2 stores to zero (Kooyman et al. 1983; Ponganis et al. 1993; Burns et al. 2007). Average whole body Mb was estimated from the average of measured *Pec* and *LD* Mb concentrations, as suggested by Lestyk et al. (2009). Total muscle mass was estimated to be 30% total body mass (Kooyman et al. 1983). Lung O_2 stores were estimated from NFS values obtained from Lenfant et al. (1970), with the additional assumption that diving lung volume was at 50% total lung capacity (Kooyman 1989). Blood, muscle, and lung O_2 stores were summed to give TBO_2 stores of animals for which all measurements were possible (Lenfant et al. 1970; Kooyman et al. 1983). The cADL for each animal was determined by dividing TBO_2 stores by an estimated diving metabolic rate (DMR) of 1.7× resting (Williams et al. 2004; Burns et al. 2007), using the same RMR values determined previously for scaling kinetic enzyme activities.

Myosin heavy chain isoform composition

Myosin heavy chain isoforms were separated using the SDS-PAGE technique as described by Blough et al. (1996) and Reiser and Kline (1998). All muscle samples (7–12 mg) were homogenized (Tissuemiser, Fisher Scientific, Inc., Pittsburgh, PA, USA) in gel sample buffer (8 M urea, 2 M thiourea, 0.05 M Trizma base, 0.075 M dithiothreitol, 3% (w/v) SDS, at pH 6.8, and 0.004 (w/v) bromophenol blue) (Blough et al. 1996) with 60 μL buffer mg^{-1} wet muscle (a 2× dilution). The homogenate was heated for 2 min at 65–95°C, chilled on ice, and then centrifuged for 4 min at 100,000 RPM. The supernatant was collected and brought to a final 10× dilution in gel sample buffer. The upper running buffer was 100 mM Tris base, 150 mM glycine, 0.1% SDS, and 800 mM Al 2-mercaptoethanol, and the lower was 50 mM Tris base, 75 mM glycine, and 0.05% SDS. The separating gel was 7% acrylamide:bis-acrylamide (50:1), 30% glycerol, 200 mM Tris buffer (pH 8.8 at 18°C), 100 mM glycine, and 0.4% SDS (w/v). The stacking gel was composed of 4% acrylamide:bis-acrylamide (50:1), 30% glycerol, 70 mM Tris buffer (pH 6.8 at 18°C), 4 mM glycine, 4 mM EDTA (pH 6.8) and 0.4% SDS (w/v). For gel electrophoresis, 3 μL of prepared sample was loaded into each well. These 0.75 mm thick gels were run for 20 h on a Hoefer standard vertical electrophoresis unit (Hoefer, Inc., Holliston, MA, USA) with a PS300-B power supply set at constant 300 V and cooling system at 8°C. Following completion of the run, gels were silver-stained and developed as described by Blough et al. (1996). Gels were then scanned and imaged using digitizing software (UN-SCAN IT gel v 6.1). NFS muscle samples were run against a rat muscle standard consisting of (50:50) plantaris:diaphragm using SDS-PAGE, and the rat MHC isoforms were identified by comparison with previously published studies using identical analytical techniques. Relative migrations of rat and NFS bands were used for preliminary protein identification. Bands were excised from one Coomassie stained gel and sent to the Ohio State University Mass Spectrometry and Proteomics Facility, where in-gel protease digestion using trypsin was performed, followed by nano liquid-chromatography–mass spectrometry (LC–MS/MS) for analysis of peptides. A Mascot Daemon database (Matrix Science, Ltd. v. 2.2.1, Boston, MA, USA) search with the NFS MHC primary sequences was used in order to confirm protein identity. MOWSE scores greater than 67 indicate a significant match ($P < 0.05$) (Pappin et al. 1993). Pinniped proteins do not exist within the database; the closest evolutionary relative with sequenced proteins is the canine.

Statistical analyses

For enzyme, MHC, Mb, and Hb assays, samples were run in triplicate or quadruplicate, and results only accepted if the coefficient of variance was <10% and the standards and/or controls fell within an acceptable range (±2SD of the experimentally determined value). Data were assessed for outliers and normality prior to statistical analyses. Age related differences in all O_2 stores were determined using unpaired *t* tests assuming unequal variance. General linear models (SPSS v. 18.0, Chicago, IL, USA) were used to examine differences in muscle Mb, TP, enzyme activities (both absolute and scaled to RMR) and ratios, and MHC isoform composition between <1-month-old pup and adult age classes as well as by muscle type (*Pec* and *LD*). Because two-way (age class × muscle) interactions were frequently significant, data were split by age and muscle, and analyzed separately throughout. In these subsequent analyses, unpaired *t* tests with unequal variance were used to compare parameters between age classes within each muscle. Because all values were not available for all animals, unpaired *t* tests were used instead of paired tests to determine how muscle biochemistry and structure differed within individual age classes due to muscle (*Pec* vs. *LD*). Pearson correlation analyses were used to determine significant relationships between MHC composition and enzyme activities. Significance was assumed to be $P < 0.05$.

Results

Oxygen stores and cADL

There was a positive relationship between age and all blood O_2 storage parameters (Table 1), and adult NFS Hb, Hct, and MCHC values in this study were similar to those

Table 1 Mean ± SE O_2 storage capacity factors used to obtain the cADL for NFS pups and adult females

Measured parameter	*N* (Pup:Adult)	Age class	
		<1-month pup	Adult
Mass (kg)	(8:7)	7.6 ± 0.2	36.9 ± 2.5*
Hemoglobin (g dL^{-1} whole blood)	(8:7)	12.6 ± 0.6	15.6 ± 0.9*
Hematocrit (%)	(8:7)	41.4 ± 1.6	45.3 ± 2.3
MCHC (%)	(8:7)	30.3 ± 0.6	34.5 ± 1.2*
Plasma volume (% body mass)	(8:6)	5.5 ± 0.2*	4.8 ± 0.2
Blood volume (% body mass)	(8:6)	9.5 ± 0.4	9.1 ± 0.4
Total body average Mb (mg g $tissue^{-1}$)	(6:6)	11.6 ± 0.8	35.8 ± 0.8*
cADL (min)	(6:6)	1.0 ± 0.1	2.6 ± 0.1*

* Age class with a significantly higher O_2 storage parameter (*t* test, $P < 0.05$)

reported in Norberg et al. (2011), which included a large sample size and animals that were likewise captured on Lovushki Island. Pups in this study had slightly lower Hct values ($t_{11} = -1.4$, $P = 0.184$) than adults, and significantly lower Hb concentrations ($t_{11} = -2.8$, $P = 0.018$) and MCHC values ($t_9 = -3.0$, $P = 0.011$). However, NFS pups had significantly higher mass-specific PV_{EB} than adults ($t_{11} = 2.8$, $P = 0.017$), which in combination with their lower blood Hct, resulted in no difference in mass-specific BV_{EB} values ($t_{11} = 0.6$, $P = 0.548$) due to age.

Both pup muscles had significantly lower Mb concentrations (by ~65%) than adults (Fig. 1, *Pec* $t_{10} = -10.3$, $P < 0.001$; *LD* $t_9 = -18.1$, $P < 0.001$). As a result, pups had smaller total body average Mb loads than adults

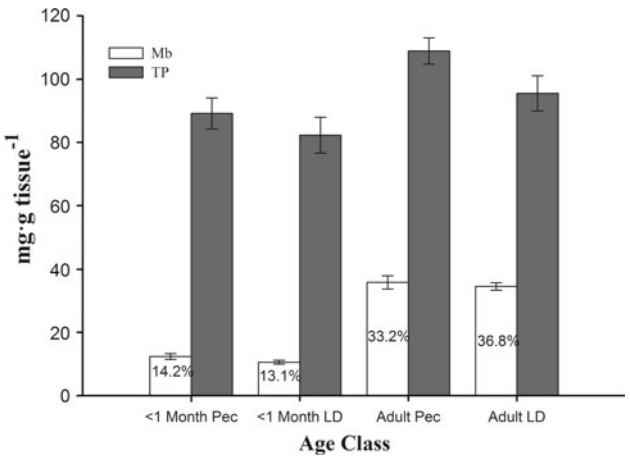

Fig. 1 Mean ± SE Mb and TP concentrations in *Pec* and *LD* muscles for <1-month-old pups and adult NFS. Percentage values indicate TP comprised of Mb. *N* = <1-month *Pec* 8, *LD* 6; adult *Pec* 8, *LD* 7

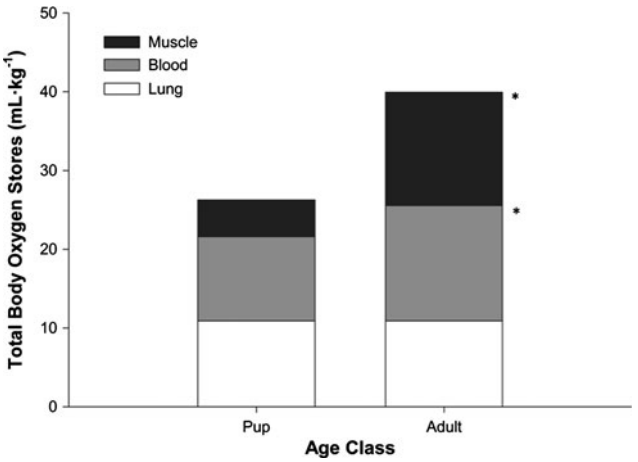

Fig. 2 Mean muscle, blood, and lung summed to TBO_2 stores for NFS pups and adults. *Asterisk* indicates significantly higher mass-specific O_2 storage variable, $N = 6$

(Table 1, $t_{10} = -22.0$, $P < 0.001$). Pup TP concentrations were also lower (by ~15%) than adults in both muscles; however, this difference between age classes was only significant in the *Pec* muscles (Fig. 1, *Pec* $t_{14} = -3.1$, $P = 0.009$; *LD* $t_{11} = -1.7$, $P = 0.124$). While Mb and TP concentrations were slightly higher in the *Pec* muscles for both age classes, differences between muscles were not significant (Mb pup: $t_{11} = 1.6$, $P = 0.127$; adult: $t_{11} = 0.5$, $P = 0.605$; TP pup: $t_{11} = 0.9$, $P = 0.379$; adult: $t_{12} = 1.9$, $P = 0.079$). In both muscles, Mb loads comprised a significantly larger component of muscle TP in adults relative to pups (*Pec* $t_{11} = -7.3$, $P < 0.001$; *LD* $t_8 = -9.6$, $P < 0.001$).

NFS pup mass-specific TBO_2 stores were only 65.7% adult values (Fig. 2, $t_9 = -8.7$, $P < 0.001$), with significantly lower mass-specific blood and muscle O_2 stores (blood: $t_{12} = -2.8$, $P = 0.016$; muscle: $t_{10} = -22.0$, $P < 0.001$). Additionally, the relative importance of blood, muscle, and lung O_2 stores to overall TBO_2 differed between age classes. The largest O_2 store in pups was the lungs (42.0% of TBO_2), closely followed by blood (40.2%), and lastly, muscle (17.8%). In adults, lung O_2 reserves were least prominent (27.4%), while blood and muscle O_2 contributed the most (36.6 and 36.0%, respectively) to TBO_2 stores. Similarly, pup cADL times were only ~40% those of adults (Table 1, $t_{10} = -20.7$, $P < 0.001$).

Muscle structure and biochemistry

MHC isoforms were confirmed via LC–MS/MS analysis, with all proteins most closely matched to the canine, the closest relative in the database to NFS. The three MHC isoforms expressed in NFS muscles were confirmed as follows: the slowest running band was identified as MHC IIA (77 unique peptides matched canine MHC IIA,

accession number Q076A7, SwissProt Database; MOWSE score = 5,695), the intermediate band was identified as MHC IID/X (75 peptides matched canine, accession number Q076A6, SwissProt Database; MOWSE score = 5,055), and the fastest running band identified as MHC I (67 peptides matched canine, accession number P49824 SwissProt Database; MOWSE score = 4,558) (Fig. 3). The migration order of NFS MHC proteins is similar to that of the rat, with only MHC IID/X migrating slightly further in the gel.

While pup and adult muscles contained the same three MHC isoforms, the relative proportion of these three isoforms differed due to muscle and age (Fig. 4). Comparing muscles, both the pup and adult *LD* contained higher proportions of MHC IIA (pup: $t_{14} = -2.3$, $P = 0.036$; adult: $t_{10} = -5.7$, $P < 0.001$) and MHC I (pup: $t_{13} = -4.9$, $P < 0.001$; adult: $t_{14} = -6.5$, $P < 0.001$) and a lower proportion of MHC IID/X (pup: $t_8 = 6.5$, $P < 0.001$; adult: $t_{12} = 9.5$, $P < 0.001$) than the *Pec*. When comparing muscle structure between age classes, both pup muscles had significantly greater proportions of slow MHC I protein than adults (MHC I *Pec* $t_{13} = 3.0$, $P = 0.011$; *LD* $t_{13} = 2.6$, $P = 0.023$) and less fast-twitch MHC IID/X protein. However, these differences in MHC IID/X were only significant in the *Pec* and not the *LD* (MHC IID/X *Pec* $t_{10} = -3.1$, $P = 0.011$; *LD* $t_9 = -1.8$, $P = 0.113$). There was no effect of age on the relative proportion of MHC IIA protein in either muscle (*Pec* $t_{11} = 1.7$, $P = 0.123$; *LD* $t_{14} = -1.0$, $P = 0.330$).

On both an absolute and metabolically scaled basis, the activity of all three enzymes assayed differed significantly between age classes and muscles (Table 2). CS_{abs} activities in pups were significantly lower than adults in the *Pec* ($t_{13} = -3.6$, $P = 0.003$), but not the *LD*. However, once scaled to metabolic rate, CS_{met} levels in pups were lower in both the *Pec* ($t_{10} = -9.4$, $P < 0.001$), and the *LD* ($t_{12} = -3.9$, $P = 0.002$). Only adults showed differentiation between the muscles, with adults having significantly higher CS_{abs} activities in the *Pec* muscles than the *LD* ($t_{13} = 6.6$, $P < 0.001$). In contrast, pups had significantly higher $HOAD_{abs}$ activity in both muscles (*Pec* $t_{13} = 3.4$, $P = 0.005$; *LD* $t_6 = 3.1$, $P = 0.019$), but when scaled by resting metabolic rate (RMR), the pup $HOAD_{met}$ values were lower than adults (*Pec* $t_{14} = -2.7$, $P = 0.017$; *LD* $t_9 = -2.9$, $P = 0.017$). Conversely, pup muscles had significantly lower LDH_{abs} and LDH_{met} activity than adult muscles (absolute: *Pec* $t_{13} = -5.1$, $P < 0.001$; *LD* $t_{12} = -3.3$, $P = 0.007$; scaled *Pec* $t_{10} = -9.4$, $P < 0.001$; *LD* $t_{11} = -6.9$, $P < 0.001$). In both pups and adults, HOAD and LDH activities were higher in *Pec* muscles than the *LD /Emphasis> (HOAD pup:* $t_{12} = 2.6$, $P = 0.022$; *adult:* $t_{10} = 3.4$, $P = 0.007$; *LDH pup:* $t_{11} = 2.3$, $P = 0.044$; *adult:* $t_{14} = 3.8$, $P = 0.002$).

There were significant differences in the CS:HOAD ratio due to both age and muscle (Table 2). The CS:HOAD ratio was similar in the *Pec* and *LD* muscles of pups, but it was much higher in the adult *Pec* than the *LD* ($t_{14} = 3.4$, $P = 0.005$). When comparing across ages, the CS:HOAD ratio in the *Pec* was significantly lower in pups than adults (*Pec* $t_{10} = -6.9$, $P < 0.001$), but there was no difference in the *LD*. Overall, pups had lower LDH:CS ratios than adults in both the *Pec* and the *LD* (*Pec* $t_{14} = -2.5$, $P = 0.024$; *LD* $t_{10} = -3.3$, $P = 0.008$), suggesting that adults could generate more ATP via anaerobic respiration if necessary than pups. The LDH:CS ratio did not differ between muscles in either pups or adults.

There were no correlations between the relative percent of any MHC isoform, Mb, CS, or HOAD levels in any pup or adult muscle. However, in adult NFS there was a significant positive correlation between the relative percent of MHC IID/X protein and LDH activity in both muscles ($y = 9.9x + 580.4$, $r = 0.867$, $P < 0.001$). There was also a significant negative correlation between the proportions of both MHC IIA and MHC I and LDH activity (MHC IIA: $y = -17.3x + 1{,}615.1$, $r = -0.784$, $P < 0.001$; MHC I:

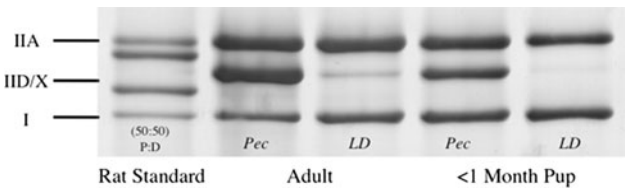

Fig. 3 MHC regions of separating gels showing three protein isoforms in *Pec* and *LD* muscles of NFS <1-month pup and adult run against a rat (50:50, *Plantaris:Diaphragm*) standard. The rat standard contained the MHC IIB isoform, but this was not present in NFS. Note that the rat and NFS MHC IID/X did not migrate the same distance in the gel

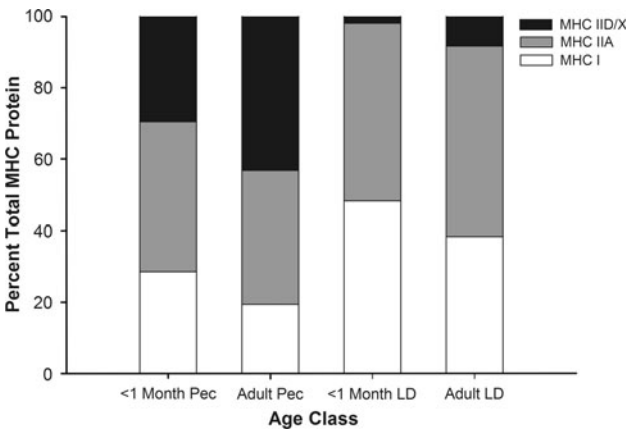

Fig. 4 Mean percent isoform composition for MHC IIA, IID/X, and I in <1-month-old pup and adult NFS *Pec* and *LD* muscles. The relative proportion of the MHC isoforms differed significantly between muscle types ($P < 0.05$), N = <1-month *Pec* 8, *LD* 8; adult *Pec* 9, *LD* 8

Table 2 Mean ± SE enzyme activity (IU g^{-1} wet tissue), from *Pectoralis* (*Pec*) and *Longissimus dorsi* (*LD*) muscle biopsies of pup and adult female NFS

Variables	Age class					
	<1-month pup			Adult female		
	Pec		*LD*	*Pec*		*LD*
N	8		6	8		8
CS_{abs}	31.8 ± 1.7	=	25.4 ± 2.7	**41.8 ± 2.3**	>	24.0 ± 1.7
CS_{met}	2.1 ± 0.1	=	1.7 ± 0.2	**4.6 ± 0.2**	>	**2.6 ± 0.2**
$HOAD_{abs}$	**56.6 ± 3.4**	>	**44.1 ± 3.3**	42.5 ± 2.8	>	33.2 ± 1.3
$HOAD_{met}$	3.7 ± 0.2	>	2.9 ± 0.2	**4.7 ± 0.3**	>	**3.6 ± 0.1**
LDH_{abs}	598.0 ± 45.2	>	442.6 ± 51.1	**988.7 ± 70.4**	>	**681.7 ± 59.8**
LDH_{met}	39.2 ± 3.0	>	29.0 ± 3.4	**108.1 ± 6.7**	>	**74.6 ± 5.7**
CS:HOAD	0.6 ± 0.1	=	0.6 ± 0.1	**1.0 ± 0.1**	>	0.7 ± 0.1
LDH:CS	18.9 ± 1.3	=	18.3 ± 2.5	**23.9 ± 1.5**	=	**28.7 ± 2.0**

Variables with subscript "*abs*" are absolute enzyme activities and subscript "*met*" indicate values have been scaled to RMR. Within each age class, symbols indicate whether the *Pec* had similar (=) or significantly greater (>) enzyme activities than the *LD*. Bolded font indicates the age class that had significantly higher enzyme activities within each muscle type (*t* test, $P < 0.05$)

$y = -16.1x + 1{,}303.9$, $r = -0.799$, $P < 0.001$). These patterns were not evident in pup muscles (Fig. 5).

Discussion

Results from this study show that 1-month-old NFS pups have significantly smaller mass-specific TBO_2 stores than adults, and therefore, a lower capacity to carry critical O_2 reserves to depth during dives. This difference is due to both lower mass-specific blood and muscle O_2 stores with the deficiency being much greater in muscle than blood, as found in other pinnipeds (Burns et al. 2005, 2007; Fowler et al. 2007; Spence-Bailey et al. 2007). The relative immaturity of the muscle is also reflected in a lower proportion of fast-twitch MHC proteins, and lower metabolically scaled aerobic and anaerobic enzymatic activity. In addition, MHC isoform composition and enzyme activities are not correlated in pups, and only correlated with LDH in adults. This suggests that structural components of muscle mature early during development in NFS, and are only later matched by increased Mb loads and enzyme activities. Further, these findings indicate that substantial physiological development must take place before pups begin actively diving.

NFS pups had lower mass-specific TBO_2 stores than adults due to both smaller blood O_2 and muscle O_2 reserves. As in other pinniped species, NFS mass-specific blood O_2 stores increased (~35%) between birth and maturity (Noren et al. 2005; Richmond et al. 2006; Fowler et al. 2007; Spence-Bailey et al. 2007). This increase was not due to a change in mass-specific blood volume, but rather an increase in Hct, Hb, and MCHC. That this increase in blood O_2 stores was driven solely by changes in the amount and phenotype of RBCs fits with developmental patterns in other terrestrial and marine mammals (Halvorsen and Halvorsen 1973; Horning and Trillmich 1997; Burns et al. 2004, 2005, 2007; Noren et al. 2005; Spence-Bailey et al. 2007). Similarly, muscle Mb concentrations were much less mature in young pups than adults, as Mb in both *Pec* and *LD* pup muscles were ~30% that of adult values. While NFS pup Mb loads were markedly reduced relative to adult values, they were fairly mature by comparison to similarly aged otariid pups in other species, which typically have Mb loads only ~10% adult levels (Richmond et al. 2006; Spence-Bailey et al. 2007). Instead, the relative maturity of NFS pup Mb loads are more similar to those of neonatal phocids, which have Mb concentrations ~30–40% of adult values (Burns et al. 2005, 2007; Noren et al. 2005; Lestyk et al. 2009; Prewitt et al. 2010).

As a result of their immaturity, the relative contribution of muscle O_2 to TBO_2 stores was much lower in pups than adults (17.8 vs. 36.0%). If NFS muscles, like phocid pup muscles, do not mature rapidly during the remaining 4-month nursing period, then naïve divers would need to rely primarily on blood O_2 stores to supply O_2 to working muscles (Burns et al. 2007). This may explain why blood O_2 stores mature more rapidly than those of the muscle (Davis et al. 2004; Burns et al. 2007). Typically, muscle Mb loads are similar among different muscles at birth, with Mb heterogeneity emerging during postnatal development as activity patterns change (Richmond et al. 2006; Lestyk et al. 2009; Geiseler 2011). Remarkably, Mb loads were similar between the *Pec* and *LD* not only in the pups, but also in the adults. This was unexpected, as primary locomotor muscles typically have higher Mb concentrations in marine mammals (Kanatous et al. 1999; Polasek et al.

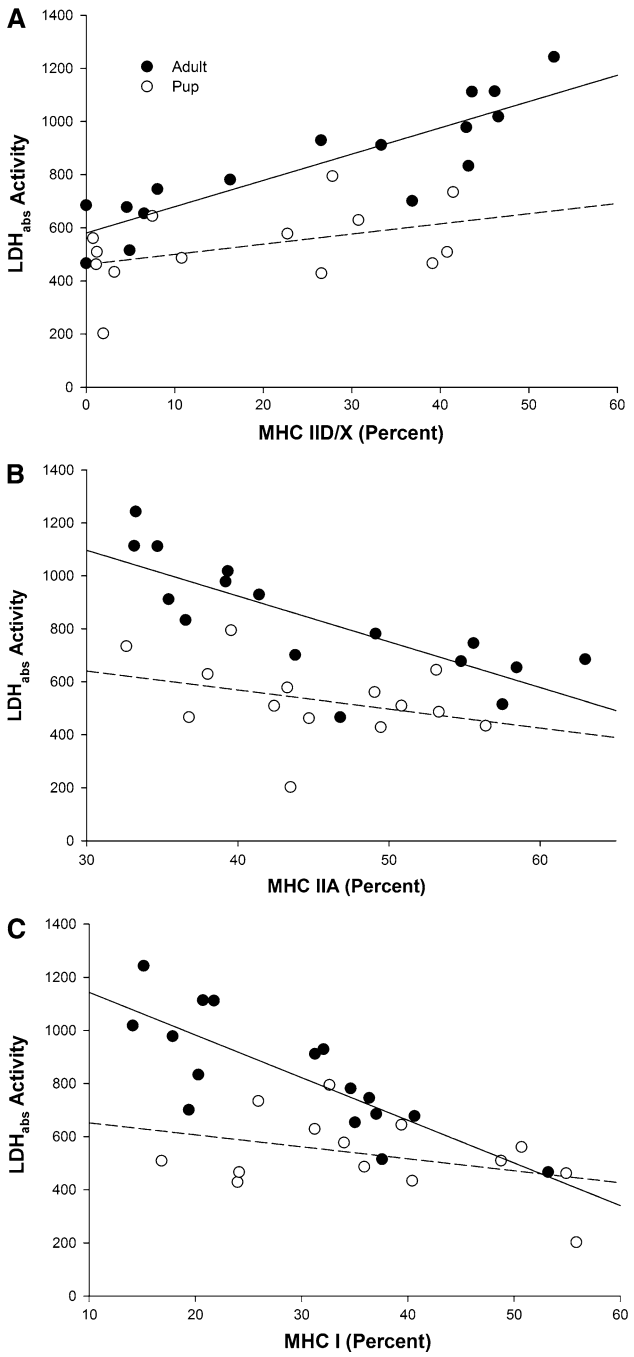

Fig. 5 Correlations between percent **a** MHC IID/X, **b** MHC IIA, and **c** MHC I isoforms and LDH activity (IU g^{-1} wet tissue) in both *Pec* and *LD* muscles combined. *Dark circles* represent adults ($N = 16$) and *open circles* represent <1-month-old pups ($N = 14$). *Solid lines* indicate significant Pearson correlations ($P < 0.001$) whereas *dashed lines* are not significant

2006; Richmond et al. 2006; Lestyk et al. 2009). Polasek et al. (2006) found a Mb gradient in harbor seal epaxial muscles, and regions of increased Mb loads were associated with areas that generate more propulsive power while swimming. Equivalent Mb levels in adult NFS *Pec* and *LD* muscles may reflect heavy use of the hindlimbs and *LD* for quadrupedal terrestrial locomotion (Beentjes 1990).

Similar Mb loads between muscles and/or the reduced Mb in pups might also be due to MHC isoform composition. The developmental shift in the relative proportion of different MHC proteins in both the *Pec* and *LD* fits with the gradual shift from slow (MHC I) isoforms toward faster isoforms (MHC IIA and IID/X) that occurs in most young vertebrates (Baldwin and Haddad 2001; Pette 2001). Similarly, the greater percentage of fast-twitch MHC proteins in the *Pec* of both pups and adults fits with the *Pec*'s use in underwater propulsion and burst-type activities (Baldwin and Haddad 2001). Thus, in both pups and adults, MHC isoform profiles appear to reflect use patterns. Muscle fibers that contain primarily MHC IID/X protein typically have much lower Mb loads, and CS and HOAD activities, than more oxidative fiber types such as MHC I or MHC IIA containing fibers, and therefore rely more heavily on glycolytic pathways to produce ATP (Peter et al. 1972; Baldwin et al. 1973). However, even though pups had a higher percentage of MHC I and IIA, typically found in oxidative fibers, and a lower percentage of MHC IID/X protein than adults, they had a lower Mb load, suggesting that both the structural and biochemical properties of pup muscles were immature.

In addition to having large endogenous Mb stores and more fast-twitch MHC proteins in major locomotory muscles when compared to pups, adult marine mammals typically have enzyme levels in those muscles that are consistent with sustained aerobic respiration and high anaerobic potential. This includes CS levels ~50–160%, HOAD levels ~170–450%, and LDH levels ~70–200% the enzyme levels in terrestrial mammal muscles (Reed et al. 1994; Kanatous et al. 1999; Polasek et al. 2006). Adult NFSs typically make fairly short (2.2 min) and shallow (68 m) dives (Gentry and Kooyman 1986), and their enzyme levels correspond with short aerobic dive and also burst-speed behaviors. In the NFS adults, enzyme activities were greater than those of terrestrial vertebrates and the shallow-diving Steller sea lion (*Eumetopias jubatus*; Kanatous et al. 1999), but less than some of the more deep-diving phocid seals (Burns et al. 2010). Similarly, all enzyme activities were greater in the *Pec* muscle relative to the *LD* in adults, as expected based on NFS's use of their foreflippers for propulsion underwater (Kanatous et al. 1999; Polasek et al. 2006).

In contrast to adults, pup muscles had a larger proportion of MHC I and IIA, but a lower aerobic capacity as indicated by CS_{abs} and CS_{met} activities. Pup muscles appeared to rely more heavily on lipid oxidation, as indicated by ~30% higher $HOAD_{abs}$ levels and ~40% lower CS:HOAD ratios, as befits their reliance on a high-lipid milk diet. In combination with the lower Mb levels, these

findings suggest that pup muscles have a reduced ability to generate ATP aerobically as compared to adults, despite the larger proportion of MHC I protein.

While the reduced Mb and aerobic enzyme activities might suggest that pups rely more heavily on anaerobic metabolism, their muscles contained a much lower proportion of the MHC IID/X protein that frequently predominates in glycolytic fibers. In addition, NFS pup muscles had lower LDH:CS ratios, and lower LDH_{abs} and LDH_{met} activities. In pups, there was no correlation between LDH activity and MHC composition in either muscle. In contrast, adult muscles with higher LDH activity also contained a larger proportion of MHC IID/X protein and a lower proportion of MHC I and IIA, as predicted based on the general correlation between structure and function in muscle fibers (Peter et al. 1972; Baldwin et al. 1973; Kanatous et al. 2002). Together these findings suggest that glycolytic pathways are also not as well developed in pups as in adults. Reduced ability to generate ATP both aerobically and anaerobically has been noted in young harp (*Pagophilus groenlandicus*) and hooded (*Crystophora cristata*) seals, suggesting that this is a common pattern among this order (Burns et al. 2010).

While there were developmental shifts in both structural and biochemical properties of fur seal muscle, pup muscles were much more similar to adult muscles in their structural components than their biochemical aspects. NFS muscles did not express embryonic or neonatal MHC isoforms, and only showed a shift from slow MHC I to fast MHC IID/X. In contrast, Mb values were 67% lower in pups and enzyme activities differed by as much as 30–40% on an absolute basis. In combination, these findings suggest that the structural fiber components of muscle tissues are laid down first in the development, to be followed by increasing enzyme activities, total protein, and Mb loads. Such a pattern could also indicate that there is a tradeoff between simply adding new MHC protein to existing muscle fibers to increase muscle mass during the development, versus synthesizing more mitochondria and increasing enzyme and Mb concentrations in the cytosolic matrix. Prioritizing structural over biochemical growth has been observed in rapidly developing bird species (Choi et al. 1993; Ricklefs et al. 1994; Shea et al. 2007). Similarly, differences in the rate at which enzymes and Mb levels matured suggest that different regulatory pathways control the expression of Mb versus oxidative and glycolytic enzyme activities (Winder et al. 1974; Jansson et al. 1988; Terrados et al. 1990).

In summary, this study shows that 1-month-old NFS pups have lower O_2 reserves than adults. Furthermore, it is unlikely that pups are able to utilize their already limited O_2 stores as efficiently as adults due to immature enzyme activities, and altered MHC profiles that do not correlate with metabolic profiles. Pups also may not be able to generate as much muscle power for burst-type behaviors, shown by lower LDH activities and fast-twitch MHC IID/X isoforms. These findings suggest that during diving activity, pups would exhaust their O_2 stores quickly, have decreased performance, and likely require increased recovery time at the surface. At the simplest level, this can be seen in the much lower cADL values of 1-month-old pups, as compared to adults. If, as in other pinniped pups, there is only limited development of O_2 stores during the nursing period (Burns et al. 2005, 2007; Noren et al. 2005; Richmond et al. 2006; Spence-Bailey et al. 2007; Prewitt et al. 2010), this physiological constraint likely persists through to weaning and contributes to the much shorter (0.7 ± 0.2 min) and shallower (6.6 ± 2.7 m) dives made by newly weaned pups during their first week at sea (Lea and Gelatt unpublished data; mean $\pm$ SD from 56 NFS pups across 1,176 6-h dive collection periods) than adults (2.2 min duration, 68 m depths; Gentry and Kooyman 1986). That this metric accurately reflects a real physiological limit is suggested by the fact that observed average dive times in both pups and adults are very close to the cADL. Shorter dives means that NFS pups may need to allocate their foraging time and efforts differently than adults. This may result in naïve NFS being unable to obtain the same quantity or quality of prey as compared to physiologically mature adults. While we do not yet know when NFS pup muscles and blood oxygen stores reach adult levels, dive depths and durations progressively increase until pups are $\sim$8–9 months of age (or $\sim$4 months after pups begin actively diving; Lea et al. 2010), suggesting that diving activity itself contributes to the final maturation of muscle biochemical and structural properties.

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