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The ontogeny of aerobic and diving capacity in the skeletal muscles of Weddell seals

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SUMMARY

Our objective was to determine the ontogenetic changes in the skeletal muscles of Weddell seals that transform a non-diving pup into an elite diving adult. Muscle biopsies were collected from pups, juveniles and adults and analyzed for changes in fiber type, mitochondrial density, myoglobin concentrations and aerobic, lipolytic and anaerobic enzyme activities. The fiber type results demonstrated a decrease in slow-twitch oxidative (Type I) fibers and a significant increase in fast-twitch oxidative (Type IIA) fibers as the animals mature. In addition, the volume density of mitochondria and the activity of lipolytic enzymes significantly decreased as the seals matured. To our knowledge, this is the first quantitative account describing a decrease in aerobic fibers shifting towards an increase in fast-twitch oxidative fibers with a significant decrease in mitochondrial density as animals mature. These differences in the muscle physiology of Weddell seals are potentially due to their three very distinct stages of life history: non-diving pup, novice diving juvenile, and elite deep diving adult. During the first few weeks of life, pups are a non-diving terrestrial mammal that must rely on lanugo (natal fur) for thermoregulation in the harsh conditions of Antarctica. The increased aerobic capacity of pups, associated with increased mitochondrial volumes, acts to provide additional thermogenesis. As these future elite divers mature, their skeletal muscles transform to a more sedentary state in order to maintain the low levels of aerobic metabolism associated with long-duration diving.

Key words: diving, hypoxia, ontogeny, seals, skeletal muscle.

INTRODUCTION

Air-breathing diving vertebrates, especially species that make deep and long dives, exhibit physiological adaptations in their muscles (and other tissues) that sustain an aerobic, lipid-based metabolism under conditions of hypoxia and ischemia. These adaptations increase an animal's aerobic dive limit (ADL), which is the longest dive that an animal can make while relying primarily on oxygen stored in the blood and muscle to sustain aerobic metabolism. Our previous studies of adult Weddell seals (*Leptonychotes weddellii*), harbor seals (*Phoca vitulina*) and Steller sea lions (*Eumatopias jubatus*) have revealed that their muscle adaptations include: (1) an increased aerobic capacity (or one that is matched to routine levels of exertion), (2) a reliance on fatty acid catabolism for aerobic ATP production, (3) enhanced oxygen storage and diffusion capacity and (4) a reduced dependency on blood-borne oxygen and metabolites (e.g. decreased capillary density) compared with terrestrial mammals (Davis and Kanatous, 1999; Davis et al., 1991; Kanatous et al., 1999; Kanatous et al., 2001; Kanatous et al., 2002; Polasek et al., 2006).

The ontogeny of these skeletal muscle adaptations has been poorly described in diving mammals. By contrast, numerous studies involving terrestrial mammals have described changes in fiber type and metabolic profile of skeletal muscles as a function of exercise and ontogeny. For example, detailed transcriptional analyses have been undertaken to define upstream activation motifs including a CCAC box, A/T element, nuclear factor of activated T cells (NFAT) response element and E boxes that are necessary for muscle-specific transcription of the oxidative fiber program that includes the transcription of myoglobin (Chin, 2004; Chin, 2005; Chin et al., 1997; Chin et al., 1998; Chin et al., 2003). Following differentiation, myoglobin and oxidative fiber expression are coordinately regulated by neural and muscular activities that stimulate calcium signaling within the cell; specifically through calcium-induced calcium release through the interaction between cell surface l-type calcium channels and the ryanodine receptors of the sarcoplasmic reticulum. Stimuli that enhance intracellular calcium levels increase calcineurin (a Ca^{2+}/calmodulin-dependent serine phosphatase) activity and gene expression. Upon activation, calcineurin dephosphorylates the transcription factor NFAT, which translocates to the nucleus and combinatorially interacts with other transcription factors to regulate myoglobin and oxidative fiber gene expression during ontogeny of exercise in terrestrial mammals. Since myoglobin concentrations and oxidative capacities are important adaptations in the skeletal muscles of divers, the present study hypothesizes that the calcium calcineurin pathway will play an important role in the ontogeny of the skeletal muscles in Weddell seals.

Smaller, juvenile animals are less capable divers than adults, partly because of their higher mass-specific metabolic rate and

proportionately smaller blood and muscle oxygen stores (Burns, 1996; Burns et al., 1999; Kooyman et al., 1983). In addition, the muscle adaptations (as described above) that enhance diving performance may not completely develop until a young animal is several years old. As a result, they can neither dive as long nor as deep as adults. Young Weddell seals are therefore at a disadvantage in their ability to forage on deep-living prey such as Antarctic silverfish (*Pleuragramma antarcticum*), and this appears to influence survival during the initial years of life (Testa, 1987). Therefore, the ontogeny of muscle aerobic capacity, lipid metabolism and oxygen stores in the skeletal muscles is important for diving ability, yet we have only recently begun to describe the development of these physiological variables. In the present study, we investigate the ontogeny of skeletal muscle adaptations that ultimately determine the diving capabilities of Weddell seals and hypothesize a shift towards increased aerobic fibers and enzymes as Weddell seals develop.

MATERIALS AND METHODS

Animals

Twenty-four newborn Weddell seal pups (*Leptonychotes weddellii* L.) (age 3–5 weeks, mean mass 75±3 kg) were captured over three field seasons (October to December of 2002, 2005 and 2006) using mild physical restraint or a purse string net near the pupping colonies in McMurdo Sound, Antarctica. Likewise, 18 juvenile (age 1–2 years, mean mass 120±5 kg) and 26 adult Weddell seals (age ≥7 years, mean mass 385±13 kg) were captured with a purse string net along natural tidal cracks in McMurdo Sound. The ages of all the seals were determined from flipper tags using the data provided by personal communication with R. Garrott, J. Rotella and D. Siniff. The seals were sedated with ketamine [1.5 mg kg^{-1} (Davis et al., 1983; Davis et al., 1999)], weighed with a hanging digital scale (accuracy ±0.5 kg), and muscle biopsies were taken under local anesthesia. In order to standardize our sampling, all biopsies were taken from the mid-belly of the muscle and at the same location in all age classes (one-third of the body length from the tail). Pups were returned to their mothers within 30 min of recovery from mild sedation. Juveniles and adults were detained for less than one hour post-biopsy and were released near the site of capture once the animals had regained full voluntary locomotion.

Muscle biopsies

Three muscle samples of approximately 50 mg each were collected with a 6-mm biopsy cannula (Depuy, Warsaw, IN, USA) from the swimming (m. longissimus dorsi) muscle. Control samples were collected from the m. soleus, a predominantly slow oxidative muscle of laboratory rats (Sprague Dawley) and mice (C57/Bl/6) euthanized by cervical dislocation after 2–3 min of carbon dioxide anesthesia. Muscle samples were placed either into 2% glutaraldehyde fixative or frozen in liquid nitrogen immediately upon collection for western immunoblot analysis, measurement of enzyme activities and myoglobin concentration. Due to the extremely cold environmental temperatures at the time of sampling (–10 to –40°C), the muscle samples for fiber typing were split to determine the best cryo-preservation technique under these conditions. One half were mounted on foil in gum tragacanth and tissue freezing compound and frozen in liquid nitrogen-cooled isopentane for 15–30 s. The other half were fixed in 4% paraformaldehyde overnight, incubated in a sucrose/glycerol-based cryoprotectant for 30 min to minimize freeze artifact, mounted on foil in gum tragacanth and tissue freezing compound and frozen in liquid nitrogen-cooled isopentane for 15–30 s. Frozen samples were stored at –80°C until analysis for western immunoblot analysis, fiber typing, enzyme activity and myoglobin concentration.

Measurement of enzyme activities and myoglobin concentrations

Muscle samples were thawed, weighed and homogenized at 0°C in buffer containing 79% phosphate-buffered saline (PBS), 20% glycerol, 1% TWEEN 20, 1 mmol l^{-1} DL-dithiothreitol and protease inhibitor cocktail. The homogenates were spun for 4–5 min at 10,000 ***g***, and the supernatant was divided into aliquots and stored at –80°C until used for the assays. The enzymes assayed were as follows: citrate synthase (CS), important in the citric acid cycle; cytochrome *c* oxidase (COX), as a measure of the flux though the electron transport chain; β-hydroxyacyl CoA dehydrogenase (HAD), an indicator for the β-oxidation of fatty acids; lactate dehydrogenase (LDH), needed for the conversion of pyruvate to lactate in anaerobic glycolysis; total intramuscular lipase, important for the uptake of fatty acids from circulating triglycerides (TAG) in lipoproteins (e.g. chylomicrons and VLDL). Activities of CS and COX were measured with a Beckman Coulter DU800 Series spectrophotometer (Fullerton, CA, USA). Temperature was maintained at 37°C using a Beckman Peltier Temperature Controller. Activities of LDH, HAD and lipase were measured with a BioTek Synergy HT Multi-Detection microplate reader (Winooski, VT, Canada) at 37°C. The assay conditions were as follows. LDH: 50 mmol l^{-1} imidazole; 0.15 mmol l^{-1} NADH, pH 7.0 at 37°C; 1 mmol l^{-1} pyruvate; ΔA_{340}, millimolar extinction coefficient ($\in_{340}$)=6.22. HAD: 50 mmol l^{-1} imidazole, 1 mmol l^{-1} EDTA, 0.1 mmol l^{-1} acetoacetyl CoA, and 0.15 mmol l^{-1} NADH, pH 7.0 at 37°C; ΔA_{340}, $\in_{340}$=6.22. CS: 50 mmol l^{-1} imidazole; 0.25 mmol l^{-1} 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB); 0.4 mmol l^{-1} acetyl CoA; 0.5 mmol l^{-1} oxaloacetate, pH 7.5 at 37°C; ΔA_{412}, $\in_{412}$=13.6. COX: 0.1 mmol l^{-1} DTT; 0.22 mmol l^{-1} ferrocytochrome-*c*, 10 mmol l^{-1} Tris-HCl pH 7.0 with 120 mmol l^{-1} KCl; A_{550} $\in_{550}$=21.84. Total lipase activity was determined using a long-wavelength fluorescent assay kit from MGT Incorporated (product M1214; Eugene, OR, USA). Specific enzyme activities (μmol min^{-1} g^{-1} wet mass muscle) were calculated from the rate of change of the assay absorbance at the maximal linear slope. Aliquots from the supernatant after centrifugation were used for myoglobin assays. Myoglobin aliquots were diluted with phosphate buffer (0.04 mol l^{-1}, pH 6.6) and the resulting mixture centrifuged for 50 min at 28,000 ***g*** at 4°C. As previously described (Kanatous et al., 1999) the method of Reynafarje (Reynafarje, 1963) was adapted and used to determine myoglobin concentration. In brief, the supernatant was bubbled with 99.9% carbon monoxide (CO) for 3 min to convert the myoglobin to carboxymyoglobin. The absorbance of the supernatant at 538 and 568 nm was measured using a Bio-Tek PowerWave 340x microplate reader. A myoglobin standard (horse myoglobin, Sigma-Aldrich, St Louis, MO, USA) was included with each set of samples. The myoglobin concentrations were calculated as described previously (Reynafarje, 1963) and expressed in mg g^{-1} fresh tissue.

Immunohistochemical fiber typing

Cross sections of each muscle sample were cut into serial thin sections (7–9 μm) with a Shandon cryotome (Waltham, MA, USA) maintained at –20°C. Sections were placed onto glass slides, four serial sections per slide. Transverse orientation was verified using a standard light microscope. Fiber type distribution was determined and verified utilizing two methods.

In the first method, sections were stained using a metachromatic ATPase staining protocol modified from (Ogilvie and Feeback, 1990). Briefly, the procedure was as follows: (1) ATPase pre-incubation for 8 min (pH 4.5) at room temperature, (2) three 2-min rinses in Tris buffer (pH 7.8), (3) incubation with ATP for 25 min (pH 9.4), (4) three calcium chloride rinses, (5) counterstaining in 0.1% Toluidine Blue for 1 min, (6) dehydration in ethanol and (7) clearing in xylene. The proportion of slow oxidative (Type I), fast-twitch oxidative (Type IIA) and fast-twitch glycolytic (Type IIB) fibers was determined by standard point counting procedure and is presented as a percentage relative to the total number of fibers.

In the second method, slides were fixed in ice-cold alcohol–formalin acetic acid fixative in a Coplin jar, washed with PBS, and a proteinaceous blocking agent was applied to each section to minimize non-specific antibody binding. A series of monoclonal antibodies specific to myosin heavy chain isoforms Type I, Type IIA and Type IIB was applied to one section on each of the slides and incubated overnight in a humidity chamber at 4°C. Serial amplification of the primary antibody was accomplished using an incubation of biotinylated secondary antibody for 20 min, followed by a series of PBS washes, followed by a 20-min incubation with alkaline-phosphotase streptavidin conjugate. After washing with PBS, Fast Red substrate or DAB (3,3′-diaminobenzidine tetrahydrochloride) chromagen was applied to the slides. When adequate color development was seen, the slides were washed in water or a peroxidase to stop the reaction. The slides were counterstained with Mayer's hematoxylin, washed in water, and a coverslip was mounted onto the slide with Dako glycergel. A sample of approximately 200–400 artifact-free fibers showing good staining was counted from each section using a camera-mounted microscope attached to a PC loaded with BIOQUANT software (Bioquant, Nashville, TN, USA). Fibers showing a reaction to a specific antibody were considered to have that myosin heavy chain isoform. Percentages of Type I, Type IIA, and Type IIB fibers were generated for each sample (Kanatous et al., 2002). Since there were no differences in the results from the metachromatic or immunohistochemical staining techniques, the results for the metachromatic stain were reported.

Western blot analysis to define the changes in the expression of calcium regulatory and responsive proteins

Western blot analysis was performed according to a previously published protocol (Garry et al., 1998; Wu et al., 2000) to determine changes in the expression of proteins. Rabbit anti-myoglobin serum (1:3000; DAKO, Carpenteria, CA, USA), mouse anti-calsequestrin (1:1000; Affinity Bioreagents, Golden, CA, USA), mouse anti-calcineurin B (1:250; Affinity Bioreagents), mouse anti-InsP_3 (1:2000; Affinity Bioreagents) and mouse anti-SERCA2 ATPase (1:2500; Affinity Bioreagents) were used as the primary antiserum, which was detected using a horseradish peroxidase (HRP)-conjugated secondary antiserum. Protein bands were visualized using a chemiluminescent reagent (Pierce Supersignal reagent, Rockford, IL, USA) and intensity was quantified using a computerized digital analysis program (Scion Image 1.62c; Scion Corp., Frederick, MO, USA).

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) with Tukey *post-hoc* tests ($P \leq 0.05$, Sigmastat 2.0). Results are presented as means ± s.e.m.

RESULTS

Age-related changes in fiber type population and mitochondrial volume densities in swimming muscles

The results for the adults corroborate the studies we have previously reported (Kanatous et al., 2002). The swimming muscles of adult Weddell seals are composed of a mixed fiber type population consisting primarily of slow-twitch oxidative fibers (Type I), fast-twitch oxidative fibers (Type IIA) and a near absence of fast-twitch glycolytic fibers (Type IIB) (Fig. 1A). The total volume density of mitochondria was also similar to previously reported values (Kanatous et al., 2002) and comparable to sedentary terrestrial mammals of similar body mass (Fig. 2).

The fiber type results across the age classes showed a decrease in Type I fibers with a significant increase in Type IIA fibers as the animals matured (Fig. 1A). These results are further corroborated by the volume density of mitochondria analysis, which showed a significant decrease as the seals matured (Fig. 1B; 9.3±0.5%, 6.7±0.4% and 3.0±0.2% for pup, juvenile and adult, respectively, ANOVA $P \leq 0.05$). These results indicate that the aerobic potential of pups and juveniles is significantly greater than that of adults and similar to that of comparably sized athletic terrestrial mammals and short-duration divers (Fig. 2). To our knowledge, Weddell seals are the first reported species where there is a shift from aerobic Type

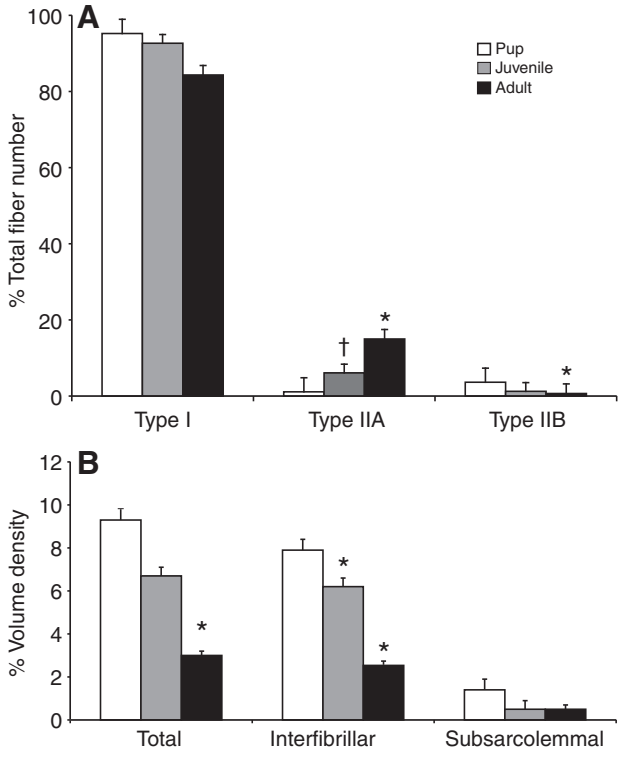

Fig. 1. (A) The percentage of fast-oxidative fibers (Type IIA) increased significantly in the swimming muscle as seals matured and increased their dive durations. Histogram showing the change in fiber type composition (% of total fiber number), in the swimming (longissimus dorsi) muscles of Weddell seals as they mature. (B) The mitochondrial volume densities in the swimming muscles decreased as the seals matured. Histogram showing the decrease in the volume density of mitochondria in the swimming muscle of Weddell seals as they mature. Values are means ± s.e.m. (N=6). * denotes significantly different from the pups; † denotes significantly different from adults (P<0.05, ANOVA).

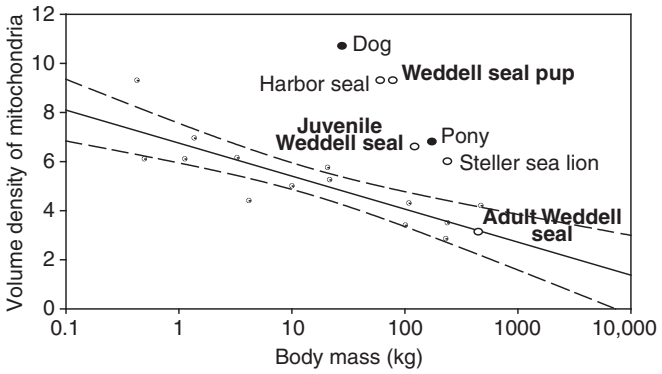

Fig. 2. Plot of muscle mitochondrial volume density against body mass in athletic (filled circles) and sedentary terrestrial mammals (small dots) and pinnipeds (open circles). The linear relationship [y=6.75–1.34(log x), r^2=0.70] was generated from the volume densities of the vastus medialis, a primary locomotory muscle, from various terrestrial mammals ranging in size from the dwarf mongoose to the steer (Kanatous et al., 1999) [terrestrial data from Hoppeler (Hoppeler et al., 1987)]. In contrast to the adult Weddell seals, whose mitochondrial volume densities are similar to that predicted for a sedentary terrestrial mammal of comparable size, juveniles and pups have mitochondrial volume densities similar to those of athletic terrestrial mammals and shorter-duration divers of similar size.

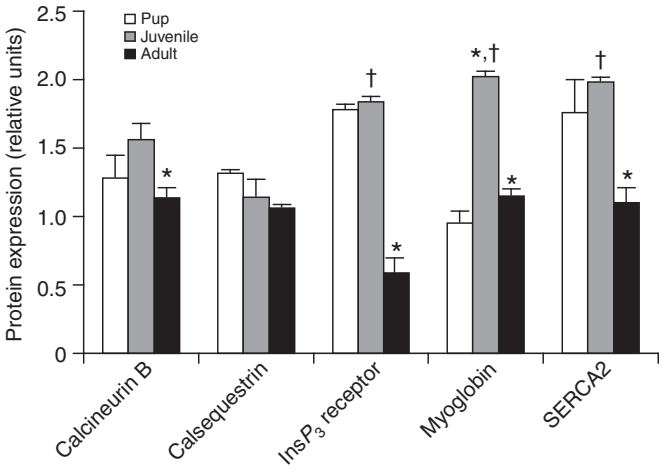

Fig. 4. Histogram displaying the changes in the expression of calcium regulatory and responsive proteins that may influence the expression of myoglobin. Adult seals had the lowest relative protein expression and were significantly different from the juvenile in all but one (calsequestrin) of the proteins measured. Protein concentrations were similar in pups and juveniles in all proteins measured except myoglobin, which was significantly greater in the juvenile than either the pup or adult. Values are means ± s.e.m. (N=6). * denotes significantly different from pups; † denotes significantly greater than adults (P<0.05, ANOVA).

I fibers to an increase in Type IIA associated with a significant decrease in mitochondrial density as the animals mature.

Age-related changes in myoglobin concentrations and enzyme activities

The changes in the concentration of myoglobin revealed some unexpected results. Myoglobin assays and western immunoblot analysis (Figs 3, 4) revealed that juveniles had a significantly greater concentration of myoglobin in their swimming muscles as compared with both adults and pups (72.4±7 *vs* 55.9±2.5 and 35.5±3 mg g^{-1} wet mass muscle, respectively). In addition, adult myoglobin values were significantly greater than those in pups (Fig. 3, 55.9±2.5 *vs* 35.5±3 mg g^{-1} wet mass muscle; ANOVA, P≤0.05).

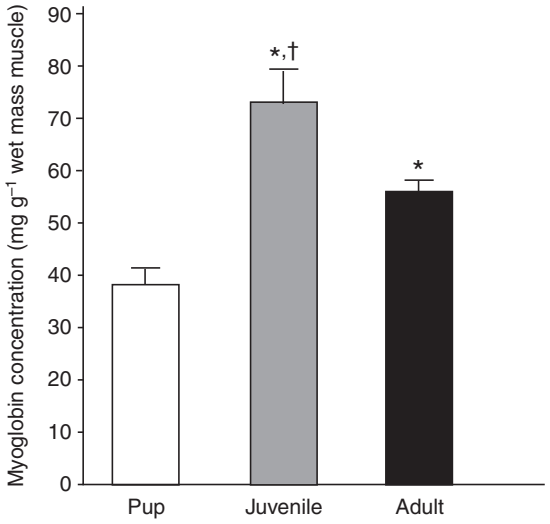

Fig. 3. The concentration of myoglobin in the swimming muscles of different age classes of Weddell seals. The concentration of myoglobin was significantly higher in the swimming muscles of juveniles compared with either pups or adults. In addition, myoglobin concentration was also significantly higher in adults compared with pups. Values are means ± s.e.m. (N=6). * denotes significantly greater than pups; † denotes significantly greater than adults (P<0.05, ANOVA).

There were no significant differences in enzyme activities between the pup, juvenile and adult for either of the aerobic enzymes CS or COX (Table 1). Although not statistically significant, LDH, a marker of anaerobic capacity, tended to be higher in pups than in either juveniles or adults. The pups had significantly higher HAD activity (2.5×) (Table 1) and a greater lipase activity (1.5×) (Table 1) than either the juveniles or adults. There was no statistical difference in any enzyme activity between the adult and juvenile age classes. The CS:HAD ratio, an index of the contribution of fatty acid metabolism to overall aerobic metabolism, was 0.3 in the juveniles and adults and 0.1 in pups. This indicates a greater reliance on fatty acid metabolism for the maintenance of aerobic metabolism in pups than in either juvenile or adult seals.

Ontogenetic changes in the expression of calcium regulatory and responsive proteins

Western blot gel electrophoresis showed that juvenile Weddell seals had the highest levels of relative protein expression for all the proteins. As seen previously with the myoglobin assay, the juveniles had a significantly greater expression of myoglobin compared with either the pups or adults (P<0.05). In addition, calcineurin, InsP_3 receptor and SERCA2 ATPase were significantly greater (P<0.05) in the juveniles and pups as compared with the adults (Fig. 4).

DISCUSSION

The main finding of this study is that Weddell seal pups have higher aerobic potential, as indicated by their significantly greater volume density of mitochondria and percentage of Type I muscle fibers, than either the relatively more active juvenile or adult seals. However, this enhanced aerobic potential was not witnessed in the

Table 1. Enzyme activities (i.u. g^{-1} wet mass muscle) in longissimus dorsi muscles of different age classes of Weddell seals

Age class	Citrate synthase	Cytochrome *c* oxidase	Lactate dehydrogenase	β-Hydroxyacyl CoA dehydrogenase	Lipase
Pup (*N*=8)	23.8±6.3	3.8±0.9	1221.2±290	187.9±22.7	7.5±0.8
Juvenile (*N*=9)	25.3±5.9	2.4±0.4	899.2±256.5	74.4±10.8*	7.0±1.4
Adult (*N*=9)	25.3±2.8	2.0±0.3	894.5±312.1	74.0±10.5*	4.7±1.1*

Values are means ± s.e.m.
*Significantly different from pups (P<0.05).

activity of key aerobic enzymes (citrate synthase and cytochrome *c* oxidase) between the three age classes. By contrast, muscle myoglobin concentrations were significantly lower in pups but unexpectedly rose to significantly greater concentrations in the juvenile seals than in any other age class. Another surprising result was that, even though the concentration of myoglobin increased, there was a decrease in Type I oxidative fibers and mitochondrial volume densities along with a concomitant increase in Type IIA oxidative fibers. To our knowledge, Weddell seals are the first reported example where there is a shift from aerobic Type I fibers towards an increase in Type IIA oxidative fibers with a significant decrease in mitochondrial density as the animals mature. By contrast, the locomotor muscles of precocial terrestrial mammals are similar in mass (as a percentage of total body mass) and fiber type composition across age classes (Cobb et al., 1994a; Cobb et al., 1994b; Dearolf et al., 2000; Schutt et al., 1994).

The difference in Weddell seal skeletal muscle physiology observed during this study may arise from the three very distinct stages of their life history. During the first few weeks of life, Weddell seal pups are a non-diving terrestrial mammal that must rely on lanugo (natal fur) for thermoregulation in the extremely harsh environmental conditions of Antarctica. Together with the thermoregulatory benefits of lanugo, an increased aerobic capacity may provide additional thermogenesis during lactation and weaning. Juveniles possess low whole-body oxygen stores and hence relatively poor diving capacity compared with adults (Burns et al., 2005; Burns et al., 2007; Clark et al., 2006; Clark et al., 2007; Fowler et al., 2006; Weise and Costa, 2007). This is reflected in both skeletal muscle physiology and mean dive durations (Burns, 1996) that are consistent with those of short-duration, shallower divers such as the Steller sea lion (Fig. 2). As juveniles continue to mature into elite deep divers, their skeletal muscles are transformed to a more sedentary state in order to maintain low levels of aerobic metabolism under the hypoxic conditions associated with long-duration diving. These results are in contrast to our original hypothesis, which expected a shift towards more aerobic fibers as Weddell seals matured and adapted to the hypoxic conditions associated with prolonged diving.

An important and novel finding in our present study is that the concentration of myoglobin is significantly higher in juveniles than adults. Previous studies investigating body oxygen stores in diving mammals have reported neonates with significantly lower total body oxygen stores compared with adults. This suggests that increases in oxygen stores are triggered by foraging (Burns et al., 2005; Clark et al., 2006; Clark et al., 2007; Fowler et al., 2006; Weise and Costa, 2007). Our findings that the pups had significantly lower concentrations of myoglobin and hence lower intramuscular stores of oxygen compared with either the adults or juveniles support these previous studies. The result that juveniles had increased myoglobin concentrations when compared with adults has not been shown in aquatic or terrestrial mammals. Although it has been reported that juvenile seals have significantly shorter dive durations, they are considerably more active swimmers than adults (Burns et al., 1999; Call et al., 2007). Recent work in our laboratory has found that hypoxia as a lone stimulus was not sufficient to induce the expression of myoglobin in either cell culture or whole-animal mouse studies. However, we found that hypoxia in combination with exercise became a powerful stimulus for the induction of myoglobin in skeletal and cardiac muscle (S.B.K., unpublished data). This suggests that the elevated energetic activity underwater in combination with breath-hold diving in juveniles may be the stimulus for their greater myoglobin expression when compared with adults. As the animals mature they employ energy-conserving modes of locomotion and lower their energetic output during diving (Davis et al., 1999; Kanatous et al., 2002) as myoglobin levels simultaneously decrease. In other words, the expression of myoglobin under hypoxic conditions is directly correlated to activity level in both terrestrial and diving mammals.

In contrast to total body oxygen stores, which significantly increase as these seals mature, the aerobic potential of the skeletal muscle is significantly greater in the muscles of pups than in either juveniles or adults. This would appear to be a paradox in that the pups do not dive and are quite sedentary compared with the diving, and therefore relatively more active, juveniles and adults. However, we hypothesized that, as homeotherms, Weddell seal pups would be under thermoregulatory stress in order to maintain a core temperature of 37°C in an environment with temperatures ranging between –10 and –40°C. Noren et al. recently observed that, although pups had the greatest proportion of blubber among the three age classes, their greater surface area to volume ratio and limited ability to minimize body-to-environment temperature gradients lead to the greatest calculated mass-specific heat loss (Noren et al., 2008). This implies that immature seals rely on elevated metabolic heat production to counter heat loss. This metabolic heat production is also substantiated by fatty acid analysis among age classes of Weddell seals, where levels of triglyceride-based fatty acids in the skeletal muscle were greatest in pups (S.J.T., unpublished data). Interestingly, it has been shown that acute exercise in humans is accompanied by an increase in muscle triglyceride breakdown, which increases whole-body fatty acid oxidation for up to 16 h, adding to overall heat production (Schenk and Horowitz, 2007). Therefore, we believe that this partitioning of more fatty acids toward triglyceride synthesis within locomotor muscles especially in pups, can provide a foundation in which thermoregulatory demands can be met. Moreover, the lack of enhanced aerobic enzyme activities associated with the significantly greater mitochondrial volume density and enhanced lipolytic enzyme capacities in the pups suggests a metabolic uncoupling in favor of heat production through non-shivering thermogenesis (Duchamp and Barre, 1993; Dulloo et al., 2002; Solinas et al., 2004). We suspect that elevated mitochondrial volume densities in the skeletal muscles of pups potentially have a greater role in uncoupled non-shivering thermogenesis (Blix and Steen, 1979; Blix et al., 1979; Grav and Blix, 1979). As the animals mature, their heat loss to the environment

decreases, reducing the need for additional metabolic heat production leading to an overall decrease in mitochondrial volume densities.

In an effort to unravel some of the underlying mechanisms regulating the ontogenetic changes in Weddell seal skeletal muscle physiology, we undertook western immunoblot analysis of selected calcium regulatory proteins. It has been well established in terrestrial animals that calcium signaling, as well as its downstream targets of calcineurin and NFAT, plays an important role in determining fiber type distribution, aerobic capacity and myoglobin concentrations in skeletal muscles (Chin et al., 1998; Schiaffino et al., 2007; Spangenburg and Booth, 2003). While numerous calcium regulatory and sensitive proteins were tested, due to the unique nature of our animal model our analysis was limited to those antibodies that gave reliable and reproducible results (calcineurin B, calsequestrin, InsP_3 receptor and SERCA2). As observed in terrestrial mammals, the protein expression pattern of calcineurin B was similar to the expression pattern of myoglobin (Chin et al., 1998). More specifically, these patterns were significantly higher in the juveniles compared with the adults. However, in contrast to the expression of myoglobin, calcineurin B was not significantly different in the pup but did show a trend toward being higher in the juvenile. These changes in calcineurin may aid in the fiber type conversion that is observed between the three age classes, but is not likely to be the sole determining factor.

The expression of InsP_3 receptor and SERCA2 in skeletal muscle also shows an age-related pattern where pups and juveniles have consistently higher expression levels than adults. With respect to calsequestrin, we found no differences between the age classes in the expression levels using western immunoblot analysis. These findings are in contrast to that observed in terrestrial mammals where an increase in InsP_3 receptor, SERCA, ryanodine and calsequestrin expression is observed between neonates and juveniles (de Jonge et al., 2006; Eizema et al., 2007). While the significance of these findings is limited on their own, the changes in these calcium regulatory proteins provide some preliminary insight into the regulation of calcium during the developmental changes within Weddell seal skeletal muscle.

It has been shown that the regulation of fiber type in terrestrial mammals is a combination of neural and molecular regulation and is independent of oxidative capacity (Chin, 2005). While our results support the hypothesis that there is a shift in fiber type population due to an increase in aerobic capacity and mitochondrial volume density as the animals become more active, our aquatically linked model differs from the terrestrial system in that the fiber type shift was associated with a significant decrease in mitochondrial density and no change in aerobic enzyme capacity. This would serve to decrease cellular and, in turn, overall metabolism necessary for increased diving capacity in deep and long-duration divers while maintaining the metabolic flux through the citric acid cycle in response to chronically hypoxic episodes associated with long-duration diving.

In addition to the results revealed here, we have successfully isolated RNA from all of the age classes and transformed it into cDNA for subtractive hybridization analysis. Our initial subtractions between adults and pups have yielded over 20 transcripts that are upregulated in the adult compared with the pup. In addition, the transcripts identified from the subtraction correlate with our physiological results. We have identified transcripts for myoglobin, myosin heavy chain IIa, calcineurin, cytochrome *c* oxidase and NADH dehydrogenase. The subtractive hybridization analysis further corroborated our physiological analysis indicating that the adults had a significantly greater percentage of fast-oxidative fibers as well as myoglobin concentration. These initial findings also suggest that some of the changes in physiology are regulated at the transcript level, further supporting the role of the calcium/calcineurin pathway in regulating the changes in mammalian skeletal muscle even in diving mammals. While this analysis is far from complete, we believe it is an important first step to be able to find transcripts that are representative of the changes in mammalian physiology.

In summary, newborn Weddell seal pups have an extremely high aerobic potential, with mitochondrial volume densities similar to those found in terrestrial animal athletes and short-duration divers. However, this enhanced aerobic capacity is not an adaptation towards diving but is due to their high fat diet and the need to offset thermoregulatory costs associated with the harsh environment of Antarctica. As the young seals begin to dive and mature into juveniles, their skeletal muscles begin to transform; as juveniles, they initiate the development of fast-oxidative fibers and

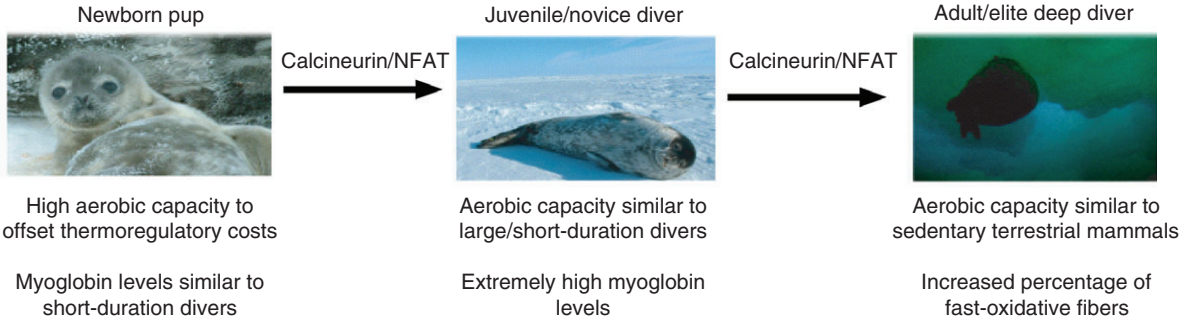

Fig. 5. Summary reveals the ontogeny of skeletal muscle adaptations that enable long deep dives in Weddell seals. As newborn pups, Weddell seals have an extremely high aerobic capacity, similar to that found in terrestrial animal athletes and short-duration divers. However, this enhanced aerobic capacity is not an adaptation towards diving but is due to their high fat diet and the need to offset thermoregulatory costs associated with using their lanugo (natal fur) for insulation in the extremely harsh environment of Antarctica. As the pups begin to dive and mature into juveniles, their skeletal muscles begin to transform. As juveniles, they initiate the development of fast-oxidative fibers and significantly increase their intramuscular stores of oxygen in the form of oxymyoglobin. As they continue to mature and increase their diving capacity, Weddell seals increase their percentage of Type IIA fast-oxidative fibers in their skeletal muscles. In addition, their skeletal muscles transform to a more sedentary state in order to maintain low levels of aerobic metabolism under the hypoxic conditions associated with long-duration diving. Similar to what has been found in terrestrial mammals; the results of our subtractive hybridization analysis indicate that these changes in skeletal muscle metabolic potential are regulated by calcium signaling and its downstream mediator, calcineurin.

significantly increase their intramuscular stores of oxygen in the form of oxymyoglobin. As they continue to mature and increase their diving capacity, Weddell seals increase their anaerobic capacity by significantly increasing their percentage of Type IIA fast-oxidative fibers in their skeletal muscles. In addition, their skeletal muscles transform to a relatively more sedentary state in order to maintain low levels of aerobic metabolism under the hypoxic conditions associated with long-duration diving (Fig. 5). The results of this study also indicate that these changes in skeletal muscle physiology are associated with changes in calcineurin expression. In conclusion, the skeletal muscles of Weddell seals undergo a unique transformation from a state of high aerobic potential, to offset the thermoregulatory costs as pups, to lower aerobic potential as adults in order to maintain the low levels of aerobic metabolism associated with long-duration diving.

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REFERENCES

Blix, A. S. and Steen, J. B. (1979). Temperature regulation in newborn polar homeotherms. *Physiol. Rev.* **59**, 285-304.
Blix, A. S., Grav, H. J. and Ronald, K. (1979). Some aspects of temperature regulation in newborn harp seal pups. *Am. J. Physiol.* **236**, R188-R197.
Burns, J. (1996). The development of diving behavior in juvenile Weddell seals: pushing physiological limits in order to survive. *Can. J. Zool.* **77**, 737-747.
Burns, J. M., Trumble, S. J., Castellini, M. A. and Test, J. W. (1998). The diet of Weddell seals in McMurdo Sound, Antarctica, as determined from scat collections and stable isotope analyses. *Polar Biol.* **19**, 272-282.
Burns, J. M., Castillini, M. A. and Testa, J. W. (1999). Movements and diving behavior of weaned Weddell seal (*Leptonychotes weddellii*) pups. *Polar Biol.* **21**, 23-26.
Burns, J. M., Costa, D. P., Frost, K. and Harvey, J. T. (2005). Development of body oxygen stores in harbor seals: effects of age, mass, and body composition. *Physiol. Biochem. Zool.* **78**, 1057-1068.
Burns, J. M., Lestyk, K. C., Folkow, L. P., Hammill, M. O. and Blix, A. S. (2007). Size and distribution of oxygen stores in harp and hooded seals from birth to maturity. *J. Comp. Physiol. B* **177**, 687-700.
Call, K. A., Fadely, B. S., Greig, A. and Rehberg, M. J. (2007). At-sea and on-shore cycles of juvenile Steller sea lions (*Eumetopias jubatus*) derived from satellite dive recorders: a comparison between declining and increasing populations. *Deep Sea Res. II* **54**, 298-310.
Chin, E. R. (2004). The role of calcium and calcium/calmodulin-dependent kinases in skeletal muscle plasticity and mitochondrial biogenesis. *Proc. Nutr. Soc.* **63**, 279-286.
Chin, E. R. (2005). Role of Ca^{2+}/calmodulin-dependent kinases in skeletal muscle plasticity. *J. Appl. Physiol.* **99**, 414-423.
Chin, E. R., Balnave, C. D. and Allen, D. G. (1997). Role of intracellular calcium and metabolites in low-frequency fatigue of mouse skeletal muscle. *Am. J. Physiol.* **272**, C550-C559.
Chin, E. R., Olson, E. N., Richardson, J. A., Yang, Q., Humphries, C., Shelton, J. M., Wu, H., Zhu, W., Bassel-Duby, R. and Williams, R. S. (1998). A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev.* **12**, 2499-2509.
Chin, E. R., Grange, R. W., Viau, F., Simard, A. R., Humphries, C., Shelton, J., Bassel-Duby, R., Williams, R. S. and Michel, R. N. (2003). Alterations in slow-twitch muscle phenotype in transgenic mice overexpressing the Ca^{2+} buffering protein parvalbumin. *J. Physiol.* **547**, 649-663.
Clark, C. A., Burns, J. M., Schreer, J. F. and Hammill, M. O. (2006). Erythropoietin concentration in developing harbor seals (*Phoca vitulina*). *Gen. Comp. Endocrinol.* **147**, 262-267.
Clark, C. A., Burns, J. M., Schreer, J. F. and Hammill, M. O. (2007). A longitudinal and cross-sectional analysis of total body oxygen store development in nursing harbor seals (*Phoca vitulina*). *J. Comp. Physiol. B* **177**, 217-227.
Cobb, M. A., Schutt, W. A., Jr and Hermanson, J. W. (1994a). Morphological, histochemical, and myosin isoform analysis of the diaphragm of adult horses, *Equus caballus*. *Anat. Rec.* **238**, 317-325.
Cobb, M. A., Schutt, W. A., Jr, Petrie, J. L. and Hermanson, J. W. (1994b). Neonatal development of the diaphragm of the horse, *Equus caballus*. *Anat. Rec.* **238**, 311-316.
Davis, R. W. and Kanatous, S. B. (1999). Convective oxygen transport and tissue oxygen consumption in Weddell seals during aerobic dives. *J. Exp. Biol.* **202**, 1091-1113.
Davis, R. W., Castellini, M. A., Kooyman, G. L. and Maue, R. (1983). Renal glomerular filtration rate and hepatic blood flow during voluntary diving in Weddell seals. *Am. J. Physiol.* **245**, R743-R748.
Davis, R. W., Castellini, M. A., Williams, T. M. and Kooyman, G. L. (1991). Fuel homeostasis in the harbor seal during submerged swimming. *J. Comp. Physiol. B* **160**, 627-635.
Davis, R. W., Fuiman, L. A., Williams, T. M., Collier, S. O., Hagey, W. P., Kanatous, S. B., Kohin, S. and Horning, M. (1999). Hunting behavior of a marine mammal beneath the antarctic fast Ice. *Science* **283**, 993-996.
de Jonge, H. W., van der Wiel, C. W., Eizema, K., Weijs, W. A. and Everts, M. E. (2006). Presence of SERCA and calcineurin during fetal development of porcine skeletal muscle. *J. Histochem. Cytochem.* **54**, 641-648.
Dearolf, J. L., McLellan, W. A., Dillaman, R. M., Frierson, D., Jr and Pabst, D. A. (2000). Precocial development of axial locomotor muscle in bottlenose dolphins (*Tursiops truncatus*). *J. Morphol.* **244**, 203-215.
Duchamp, C. and Barre, H. (1993). Skeletal muscle as the major site of nonshivering thermogenesis in cold-acclimated ducklings. *Am. J. Physiol.* **265**, R1076-R1083.
Dulloo, A. G., Stock, M. J., Solinas, G., Boss, O., Montani, J. P. and Seydoux, J. (2002). Leptin directly stimulates thermogenesis in skeletal muscle. *FEBS Lett.* **515**, 109-113.
Eizema, K., van der Wal, D. E., van den Burg, M. M., de Jonge, H. W. and Everts, M. E. (2007). Differential expression of calcineurin and SR Ca^{2+} handling proteins in equine muscle fibers during early postnatal growth. *J. Histochem. Cytochem.* **55**, 247-254.
Fowler, S. L., Costa, D. P., Arnould, J. P., Gales, N. J. and Kuhn, C. E. (2006). Ontogeny of diving behaviour in the Australian sea lion: trials of adolescence in a late bloomer. *J. Anim. Ecol.* **75**, 358-367.
Garry, D. J., Ordway, G. A., Lorenz, J. N., Radford, N. B., Chin, E. R., Grange, R. W., Bassel-Duby, R. and Williams, R. S. (1998). Mice without myoglobin. *Nature* **395**, 905-908.
Grav, H. I. and Blix, A. S. (1979). A source of nonshivering thermogenesis in fur seal skeletal muscle. *Science* **204**, 87-89.
Kanatous, S. B., DiMichele, L. V., Cowan, D. F. and Davis, R. W. (1999). High aerobic capacities in the skeletal muscles of pinnipeds: adaptations to diving hypoxia. *J. Appl. Physiol.* **86**, 1247-1256.
Kanatous, S. B., Elsner, R. and Mathieu-Costello, O. (2001). Muscle capillary supply in harbor seals. *J. Appl. Physiol.* **90**, 1919-1926.
Kanatous, S. B., Davis, R. W., Watson, R., Polasek, L., Williams, T. M. and Mathieu-Costello, O. (2002). Aerobic capacities in the skeletal muscles of Weddell seals: key to longer dive durations? *J. Exp. Biol.* **205**, 3601-3608.
Kooyman, G. C. M. A., Davis, R. W., Maue, R. A. (1983). Aerobic diving limits of immature Weddell seals. *J. Comp. Physiol. B* **151**, 171-174.
Noren, S. R., Pearson, L., Davis, J. W., Trumble, S. J. and Kanatous S. B. (in press). The thermoregulatory characteristics of Weddell seals (*Leptonychotes weddellii*) during ontogeny. *Physiol. Biochem. Zool.* **81**.
Ogilvie, R. W. and Feeback, D. L. (1990). A metachromatic dye-ATPase method for the simultaneous identification of skeletal muscle fiber types I, IIA, IIB and IIC. *Stain Technol.* **65**, 231-241.
Polasek, L. K., Dickson, K. A. and Davis, R. W. (2006). Metabolic indicators in the skeletal muscles of harbor seals (*Phoca vitulina*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **290**, R1720-R1727.
Reed, J. Z., Butler, P. J. and Fedak, M. A. (1994). The metabolic characteristics of the locomotory muscles of grey seals (*Halichoerus grypus*), harbour seals (*Phoca vitulina*) and Antarctic fur seals (*Arctocephalus gazella*). *J. Exp. Biol.* **194**, 33-46.
Reynafarge, B. (1963). Simplified method for the determination of myoglobin. *J. Lab. Clin. Med.* **61**, 138-145.
Schenk, S. and Horowitz, J. F. (2007). Acute exercise increases triglyceride synthesis in skeletal muscle and prevents fatty acid-induced insulin resistance. *J. Clin. Invest.* **117**, 1690-1698.
Schiaffino, S., Sandri, M. and Murgia, M. (2007). Activity-dependent signaling pathways controlling muscle diversity and plasticity. *Physiology (Bethesda)* **22**, 269-278.
Schutt, W. A., Jr, Cobb, M. A., Petrie, J. L. and Hermanson, J. W. (1994). Ontogeny of the pectoralis muscle in the little brown bat, *Myotis lucifugus*. *J. Morphol.* **220**, 295-305.
Solinas, G., Summermatter, S., Mainieri, D., Gubler, M., Pirola, L., Wymann, M. P., Rusconi, S., Montani, J. P., Seydoux, J. and Dulloo, A. G. (2004). The direct effect of leptin on skeletal muscle thermogenesis is mediated by substrate cycling between *de novo* lipogenesis and lipid oxidation. *FEBS Lett.* **577**, 539-544.
Spangenburg, E. E. and Booth, F. W. (2003). Molecular regulation of individual skeletal muscle fibre types. *Acta Physiol. Scand.* **178**, 413-424.
Testa, J. (1987). Juvenile survival and recruitment in a population of Weddell seals (*Leptonychotes weddellii*) in McMurdo Sound, Antarctica. *Can. J. Zool.* **65**, 2993-2997.
Weise, M. J. and Costa, D. P. (2007). Total body oxygen stores and physiological diving capacity of California sea lions as a function of sex and age. *J. Exp. Biol.* **210**, 278-289.
Wu, H., Naya, F. J., McKinsey, T. A., Mercer, B., Shelton, J. M., Chin, E. R., Simard, A. R., Michel, R. N., Bassel-Duby, R., Olson, E. N. et al. (2000). MEF2 responds to multiple calcium-regulated signals in the control of skeletal muscle fiber type. *EMBO J.* **19**, 1963-1973.