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Age-related differences in skeletal muscle lipid profiles of Weddell seals: clues to developmental changes

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SUMMARY

Our objective was to elucidate age-related changes in lipids associated with skeletal muscle of Weddell seals and to suggest possible physiological implications. Muscle biopsies were collected from pups, juveniles and adults in McMurdo Sound, Antarctica and analyzed for intramuscular lipid (IML) and triacylglyceride (IMTG) amounts, fatty acid groups, as well as individual fatty acid profiles. The results from this study suggest a switch from primarily saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) in the skeletal muscle of young pups to increases in polyunsaturated fatty acids (PUFAs) as the percentage of blubber increases, resulting in possible thermoregulatory benefits. As Weddell pups continue to develop into juveniles, fatty acids associated with the skeletal muscle changes such that MUFA levels are relatively higher, which may be in response to energy depletion associated with their restricted diving ability and rapid growth. As juveniles transform into adults, a reduction in n-3 PUFA levels in the muscle as the percentage of blubber increases may be indicative of a trigger to prepare for deep diving or could be a mechanism for oxygen conservation during long-duration dives. We speculate that the observed change in lipids associated with the skeletal muscle of Weddell seals is related to ontogenetic differences in thermoregulation and locomotion.

Key words: fatty acids, IMTG, lipids, skeletal muscle, Weddell seals.

INTRODUCTION

Diving phocids (true seals, Order Carnivora, Family Phocidae) must sequester adequate amounts of lipids to maintain sufficient blubber thickness to meet nutritional demands, buoyancy regulation and maintain thermoregulatory homeostasis (Burns et al., 2003; Castellini et al., 2009; Crocker et al., 1997; Gales and Burton, 1987; Kanatous et al., 2008; Noren et al., 2008; Rosen and Renouf, 1997; Ryg et al., 1988; Sato et al., 2003; Webb et al., 1998). Consequently, the selective pressures for maintaining blubber thickness are typically a combination of these demands, varying with age, diet and reproductive status (Castellini et al., 2009). For example, differences in blubber thickness among four species of high Antarctic seals (crabeater seal, *Lobodon carcinophagus*; Ross seal, *Ommatophoca rossii*; Weddell seal, *Leptonychotes weddellii*; and leopard seal, *Hydrurga leptonyx*) sampled during the same time period indicate species-specific nutritional and/or thermoregulatory requirements (Castellini et al., 2009). In addition, within-species comparisons for Weddell seals revealed that blubber thickness differences change with age in response to shifting thermoregulatory or energetic demands (Noren et al., 2008). These changes have also been shown to correspond with ontogenetic changes in the percentage of muscle fiber types and mitochondrial volume density found in the locomotor muscles of Weddell seals, which reflect age-class energetic requirements in their modes of locomotion, ranging from ice-platform-based non-diving pups to deep diving adults (Burns, 1999; Kanatous et al., 2008).

The energy supply for skeletal muscles during contraction/locomotion is tightly regulated to meet the increased energetic demands with ATP synthesis. Success in locomotion not only relies on the fiber type or mitochondrial density in the locomotor muscles but also on the nature of the oxidative fuels available. In humans, fatty acids (FAs) are the major fuel for low to moderate intensity exercises with muscular triacylglycerides (intramuscular triacylglycerides, IMTG) as well as total muscular lipids (intramuscular lipids, IML) increasing as the percentage of body fat increases (Hoeks et al., 2006; Hulver et al., 2003; van Loon et al., 2001). During long-distance flights, stored IMTG also serves as the primary fuel source for birds (McWilliams et al., 2004), with the selective mobilization of specific lipids from adipocytes an important step in this process (Pierce et al., 2005; Price et al., 2008). Specifically, utilizing n-3 polyunsaturated fatty acids (PUFAs) increased the aerobic capacity during long-distance migration in some bird species (Maillet and Weber, 2006; Pierce et al., 2005). It has been suggested that the total amount of stored unsaturated fats may play an important role because they are more easily transported from adipose stores to the muscle during exercise as well as having higher whole-organism oxidation rates (Leyton et al., 1987). While the mechanistic relationship between PUFAs and exercise remains unclear, *in vitro* studies involving fish, birds and mammals have shown that PUFAs stimulate cellular capacity for fat oxidation in hepatocytes, cardiomyocytes and adipocytes, which ultimately elicits a signal to prime locomotor muscles for exercise (Guo et al., 2005; Moya-Falcon, 2004; Yamazaki et al., 1987); thus, becoming a pathway for increased heat production.

Age-related differences in lipids associated with skeletal muscles in diving mammals have not been previously studied. However, it has been shown that with increasing age the Weddell seal skeletal muscle switches away from highly aerobic (pup) towards a more

sedentary state (adult) in order to maintain the low levels of aerobic metabolism associated with long-duration dives (Kanatous et al., 2008). Noren et al. also revealed that while Weddell seal pups had the greatest proportion of blubber, they also had the greatest calculated mass-specific heat loss and thus relied on additional heat production (Noren et al., 2008).

In this study, we investigated the relationship between blubber thickness and skeletal muscle lipids (saturated fatty acid, SFA; monounsaturated fatty acids, MUFA; PUFA) of pups (3–5 weeks old), juveniles (1–2 years old) and adults (>7 years old). We predict that the nature of the fuel utilized in the skeletal muscle lipids, along with FA composition, will correspond with age-specific metabolic demands. Specifically, we predict that PUFA amounts in the skeletal muscle of Weddell seals will change during development such that (1) pups will accumulate stores in the skeletal muscle for increased thermoregulatory benefits, and (2) a utilization of unsaturated FAs, for muscle oxygen conservation, in the muscle of adults will results in lower PUFA values.

MATERIALS AND METHODS

Animals

The vertebrate animal use committee at Colorado State University, Fort Collins, CO, USA, approved all protocols for this project. A total of 50 (*N*=50) Weddell seals (*Leptonychotes weddellii* Lesson 1826) were sampled during the field seasons of 2005 and 2006 near McMurdo Station, Antarctica; 20 pups (*N*=10 pups per year) observed to be without their mothers (age 3–5 weeks postpartum), 12 juveniles (age 1–2 years; 7 males and 5 females) and 18 sexually mature adult male (>7 years). Weddell seals were mildly restrained (head bag) near the pupping colony at Tent Island, McMurdo Sound, Antarctica, during October to December in 2005 and 2006. Animals were aged by verifying numbered tags as a part of a long-term study in McMurdo Sound on Weddell seal population demographics (J. Rotella, Montana State University). The seals were immobilized with an intravenous injection of telezol (intravertebral extradural vein, 0.1 mg kg^{-1}) (Wheatley et al., 2008), loaded onto a sling and subsequently weighed to the nearest 0.5 kg using an electronic scale (San Diego Scale Inc., San Diego, CA, USA) mounted on a cross-beam sling. To access the large dorsally located locomotor muscle, longissimus dorsi, a 2 cm^2 area was cleaned using alcohol and a betadine solution and shaved before each incision. Muscle biopsies were collected (30–50 mg taken under local anesthetic; 1 ml, Lidocaine®, Whitehouse Station, NJ, USA) using a 6 mm biopsy cannula (Depuy, Warsaw, IN, USA) several centimeters away from subcutaneous adipose tissue to ensure minimal contact with visible fat. However, if needed, samples were dissected free of any visible fat and connective tissue and then were frozen and stored in liquid nitrogen until later analysis. 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). All seals were detained for less than 60 min and were released when the animals had regained full locomotion.

Quantification of total muscle lipids and triacylglycerides

All muscle samples were weighed after thawing to the nearest 0.001 g (wet mass). Total IML from l. dorsi samples were determined gravimetrically in duplicate after extraction with chloroform–methanol 2:1 (Folch et al., 1957). Total IMTG was determined on additional samples in duplicate using a modification of the chloroform–methanol method by Frayn and Maycock (Frayn and Maycock, 1980). For IMTG analysis, extracts were dried, hydrolyzed in alcoholic KOH, neutralized with HCl, and triacylglyceride concentration was then determined by measuring the liberated glycerol spectrophotometrically using a commercially available enzymatic method (Sigma T2449, Sigma Chemical Co., St Louis, MO, USA). IML and IMTG are expressed in g 100 g^{-1} (wet mass) whereas FAs are expressed as a percentage of the total recovered.

FA methyl esters (FAME) were prepared as described in Iverson et al. (Iverson et al., 1997). Protocols for preparing and analyzing samples for FA using a Varian 3900 gas chromatograph–flame ionization detector (GC–FID) (Walnut Creek, CA, USA) followed Budge et al. (Budge et al., 2006) with the following modifications: CP-Select for FAME (CP7419, Varian) 100 m length × 0.25 mm inner diameter × 0.25 μm film thickness was our column of choice. The injector temperature was 250°C with a 1 μl injector split ratio 50:1. Column flow was 1.0 ml min^{-1} programmed at 210°C for 9.0 min and ramped at 15°C min^{-1} to 260°C for 7.7 min. Detector temperature was set at 300°C with a hydrogen flow of 30 ml min^{-1} and air flow of 300 ml min^{-1}. The internal standard was C19:0 (Fluka 72332, Milwaukee, WI, USA). Each FA recovered was calculated as a percentage of the cumulative of all FAs in the skeletal muscle. A subset of five muscle samples was sent to Lipid Analytical Laboratories Inc. (Guelph, Canada) for quality comparisons.

Blubber tissue sampling

While obtaining muscle samples was the focus of this study, opportunistic blubber samples were collected at the muscle biopsy site of adults (*N*=3). Blubber samples were stored in glass containers and immediately frozen in liquid nitrogen. FAME were prepared as described in Iverson et al. (Iverson et al., 1997). FA groups were determined to compare with muscle IML values.

Histological examination of skeletal muscle

Frozen samples used in histological assays were embedded in mounting medium and frozen in liquid-nitrogen-cooled isopentane before being sectioned using a cryostat (7–8 μm). Fiber type distribution was determined and verified using metachromatic ATPase staining (Kanatous et al., 2008).

Oil Red O (ORO) solution was used to stain lipids in serial (fiber type) cross-section slides of Weddell seal swimming muscles (Koopman et al., 2001). ORO was prepared by adding 0.5 g ORO powder to 100 ml 98% isopropanol. Prior to staining, 60 ml of this solution was diluted in 40 ml of water and left to stand for 24 h. The working solution was filtered to remove any crystals. Slides were air dried for 30 min and stained in ORO solution for >16 h in a humidifier. Slides were de-differentiated in 60% isopropanol (Sigma Chemical Co.) and washed in distilled water. The stained muscle samples were visualized, imaged and overlaid with fiber type sections using a Nikon Eclipse 90i upright microscope (×20) (Melville, NY, USA). Software (Simple PCI®, Sewickley, PA, USA) was used to analyze droplet size and number as a percentage of fiber area in approximately 20 fibers per cross-section in 10 serial sections (*N*=3 for each age class).

Statistical analysis

While 36 FAs were quantified, a subset of 15 FAs was used for all statistical analyses. These 15 FAs accounted for approximately 92% of the total recovered from lipids found in the skeletal muscles (Table 1). Lipid profile data were log-transformed and all proportion data (FA) were arcsine transformed to increase normality before statistical analysis. Levene's test was used to test for homogeneity of variance for all analyses. Values are presented as means ± s.e.m. Alpha level (α) was set at 0.05 for all statistical tests.

Table 1. Percentage of fatty acid composition of total intramuscular lipids (IML) and triacylglycerides (IMTG) found in Weddell seals collected during 2005 and 2006 near McMurdo Station, Antarctica

	IML			IMTG		
Fatty acids	Pup (*N*=20)	Juvenile (*N*=12)	Adult (*N*=18)	Pup (*N*=20)	Juvenile (*N*=12)	Adult (*N*=18)
Saturated						
14:0	8.39±0.04^{b}	8.35±0.13^{b}	11.69±0.06^{a}	10.26±0.08^{c}	11.89±0.24^{b}	13.95±0.04^{a}
16:0	15.23±0.14^{b}	17.67±0.15^{a}	15.43±0.24^{b}	14.26±0.14^{b}	15.15±0.16^{a}	13.61±0.17^{b}
18:0	3.84±0.02^{b}	4.97±0.02^{a}	3.09±0.03^{c}	2.24±0.01^{b}	3.06±0.05^{a}	1.75±0.02^{c}
Monounsaturated						
14:1n-5	0.77±0.09^{b}	0.77±0.02^{b}	0.94±0.04^{a}	0.96±0.08^{b}	0.85±0.03^{b}	1.16±0.02^{a}
16:1n-7	11.07±0.15^{a}	8.35±0.08^{b}	10.94±0.18^{a}	12.74±0.14^{a}	10.39±0.14^{b}	12.37±0.26^{a}
18:1n-11	3.31±0.20^{a}	2.50±0.14^{b}	2.78±0.12^{a,b}	3.20±0.18^{a}	2.40±0.24^{b}	2.58±0.15^{b}
18:1n-9	32.53±0.42^{b}	27.57±0.28^{b}	28.92±0.22^{b}	33.68±0.37^{a}	30.54±0.21^{b,c}	29.53±0.17^{c}
18:1n-7	4.91±0.14^{a}	4.49±0.14^{b}	5.04±0.15^{a}	4.30±0.13^{a}	3.78±0.10^{b}	3.93±0.24^{a,b}
20:1n-11	2.58±0.15^{b}	3.08±0.20^{a}	3.21±0.17^{a}	2.50±0.12^{c}	3.81±0.10^{a}	3.45±0.20^{b}
20:1n-9	2.05±0.21^{c}	3.01±0.15^{a}	2.54±0.14^{b}	2.04±0.16^{b}	3.20±0.24^{a}	2.15±0.18^{b}
22:1n-11	0.24±0.01^{c}	3.08±0.04^{a}	1.85±0.07^{b}	0.49±0.02^{c}	3.12±0.08^{a}	1.90±0.04^{b}
Polyunsaturated						
18:2n-6	2.35±0.05^{a}	1.93±0.03^{b}	1.74±0.03^{b}	1.74±0.01^{a}	1.56±0.04^{b}	1.53±0.05^{b}
20:5n-3	2.86±0.08^{c}	3.79±0.12^{a}	3.19±0.15^{b}	2.22±0.17^{b}	2.17±0.28^{b}	2.95±0.10^{a}
22:5n-3	1.23±0.03^{a}	1.10±0.03^{a}	0.78±0.02^{b}	1.23±0.02^{a}	1.25±0.03^{a}	0.77±0.05^{b}
22:6n-3	3.16±0.05^{b}	3.64±0.06^{a}	3.07±0.06^{b}	3.19±0.08^{b}	3.90±0.07^{a}	3.26±0.20^{b}
%IMTG/IML				73.56±0.05	58.81±0.08	67.91±0.13
ΣSaturated	28.03±5.83	31.47±7.10	30.75±6.42	27.15±5.91	31.78±6.81	30.92±6.07
ΣMonounsaturated	58.9±16.4	53.96±13.9	57.63±14.1	61.79±15.8	58.45±13.8	59.43±13.6
ΣPolyunsaturated	12.79±1.01	14.58±1.34	11.65±0.90	10.03±1.26	9.73±1.41	10.30±1.33
n-3/n-6	2.5±1.6	2.5±0.3	5.8±0.8	3.5±0.4	3.3±0.2	5.7±0.5

Of the 36 fatty acids quantified only values greater than 1% are listed with exception of n-3/n-6 ratio. Dissimilar superscript letters among age class for each fatty acid indicate statistical differences (P<0.05).

A general linear model [analysis of variance (ANOVA) SPSS® v.17, Chicago, IL, USA] was used to examine age-class differences in FA groups (SFA, MUFA, PUFA). ANOVA was also used to examine interannual differences between individual FAs in muscle samples collected from Weddell seals during 2005 and 2006. Only males were sampled in the adult age group (due to sampling protocol). Therefore, no sex-related statistical analyses were performed; however, sex-related differences were examined using ANOVA for pup and juvenile age groups. Differences among individual group means were analyzed using a Bonferroni correction.

Blubber mass was determined for a subset of the animals (2006 samples only) in a concurrent study (Noren et al., 2008) and was used to calculate blubber as a percentage of body mass. Surface area, body volume (excluding flippers) and blubber volume were determined by standard geometric equations for a series of truncated cones following the methods of Gales and Burton (Gales and Burton, 1987). Blubber mass was calculated by multiplying blubber volume by the density of pinniped blubber (0.95 g cm^{-3}) (Gales and Burton, 1987). Simple linear regression was used to determine the relationship between IML and the relative contribution of each FA group (SFA, MUFA, PUFA) as a function of the percentage of body fat for each age class. Analysis of covariance (mixed-model ANCOVA, SPSS®) was used to compare regression slopes. Along with ANCOVA, coincidence and parallelism of slopes were also verified using F-values from ANOVA tables (with n–2 degrees of freedom) (Garcia-Berthou, 2001). A simple linear regression was also used to test that an increase in the percentage of body fat would result in a concurrent increase in total muscle lipids and IMTG.

RESULTS

A total of 50 seals were sampled with 10 pups sampled each year (N=20, age 3–5 weeks postpartum, mean mass 75±3 kg), 5 juveniles were sampled in 2005, 7 juveniles in 2006 (N=12, age 1–2 years, mean mass 120±5 kg) and 10 adults in 2005 and 8 adults in 2006 (N=18, mean mass 385±13 kg). Mean values for FAs and IML across age classes are summarized in Tables 1 and 2, respectively. There were no year (2005 and 2006) or gender (pup and juvenile) differences between FA values ($F_{2,544}$=1.034, P>0.05); therefore, all data were pooled and analyzed accordingly. Levene's test indicated homogeneity of variance among samples ($F_{35,508}$=3.363, P>0.05). There were significant differences between age classes among FA groups ($F_{2,544}$=24.43, P<0.001) and individual FAs ($F_{11,544}$=11.807, P<0.001). Thirty-six FAs were identified from Weddell seal muscle samples during 2005–2006 Antarctic field seasons; however, only those with contributions greater than 1% by mass are noted (N=15, Table 1). Fifteen FAs (14:0, 16:0, 18:0, 14:1n-5, 16:1n-7, 18:1n-11, 18:1n-9, 18:1n-7, 20:1n-11, 20:1n-9, 22:1n-11, 18:2n-6, 20:5n-3, 22:5n-3, 22:6n-3) accounted for 89.4–92.3% of the total IMLs (Table 1). Three FAs (16:0, 16:1n-7, 18:1n-9) combined to account for 53.6–60.7% of the total contribution of all FAs (Table 1). IMTGs averaged 66.3±9.1% of total lipids for all animals sampled (Table 1). A subset of samples was sent to an external laboratory (Lipid Analytical Laboratories, Inc.) for quality control comparison; there were no significant differences in FA profile composition between laboratories (univariate *t-tests* with Bonferroni correction; P<0.05).

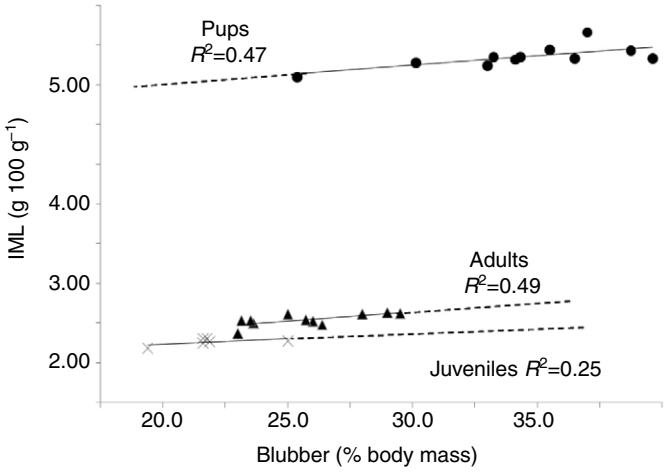

Fig. 1. Least linear regressions of the percentage of blubber and total intramuscular lipids (IML) in Weddell seal pups, IML=4.42+0.03 (% blubber) P<0.05; juveniles, IML=1.4+0.02 (% blubber), P<0.05; adults, IML=1.33+0.03 (% blubber), P<0.05.

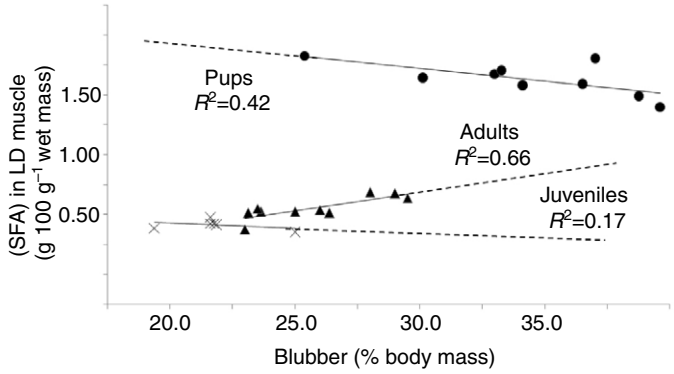

Fig. 2. Least linear regressions of saturated fatty acids (SFAs) in the skeletal muscle (longissimus dorsi; LD) against the percentage of subcutaneous blubber in Weddell seal pups, SFA=2.15–0.02 (% blubber), P<0.05; juveniles, SFA=0.73–0.01 (% blubber), P>0.05, adults, SFA=–0.11+0.03 (% blubber), P<0.05.

IML

As the percentage of blubber increased for animals within each age class, the total amount of IML increased (R^2=0.47, 0.25 and 0.49 for pups, juveniles and adults, respectively; P<0.05 for all regressions, Fig. 1). There was a 22-fold difference in the IML range among age classes for all lipid classes (Table 2); however, within FA groups the difference in age-class IML amounts varied between 3.3 (SFA) and 4.65 (PUFA, Table 2). Mean IML amounts were significantly increased in pups when compared with adults and juveniles (Table 2). However, juvenile and adult IML values were not different among FA groups (Table 2).

SFA

Mean SFA levels accounted for 29.9±1.7% in skeletal muscle lipids recovered whereas mean IMTG SFA levels were 30.1±1.1% (Table 1). Weddell seal pups had significantly greater mean SFA in the skeletal muscle than adults and juveniles ($F_{2,544}$=24.43, P<0.001, Tables 1 and 2). Juvenile seals had increased palmitic acid (16:0) and stearic acid (18:0) mean values whereas adults had greater myristic acid (14:0) levels ($F_{11,508}$=P<0.001, Table 1). There was a significant negative relationship between the percentage of blubber and SFAs (g 100 g^{-1} wet mass) in the skeletal muscle of pups (R^2=0.42, P<0.05, Fig. 2) whereas no significant relationship was observed in juveniles (R^2=0.17, P>0.05, Fig. 2). By contrast, there was a significant and positive relationship between SFA and blubber in adults (R^2=0.66, P<0.05, Fig. 2). There was no coincidence in slopes and the slopes were not parallel among any age class (ANCOVA; P<0.05).

MUFA

Mean MUFA levels were highest in pups, juveniles and adults when compared with SFA and PUFA values (Table 2). Similar to that observed in SFA levels, MUFA levels were highest in pups and lowest in juveniles, with no difference between juvenile and adult amounts (Table 2). MUFA levels accounted for 56.8±2.8% in skeletal muscle lipids recovered whereas mean IMTG MUFA levels were 59.9±3.1% (Table 1). The percentage of MUFAs found in skeletal muscle was statistically different among age classes ($F_{2,544}$=78.06, P<0.001, Table 1). There was a statistically lower contribution of palmitoleic (16:1n-7), vaccenic (18:1n-7, 18:1n-11) and oleic (18:1n-9) acids to the skeletal muscle lipids of juveniles when compared with adults and pups (Table 1). Weddell seal pups had lower levels of gadoleic acid (20:1n-11) and cetoleic acid (22:1n-11) compared with adults and juveniles in both IML and IMTG ($F_{8,50}$=P<0.05; Table 1). There was a significant, but weak, negative relationship between body fat and MUFAs in pups (R^2=0.19, P<0.05, Fig. 3), indicating decreased levels of MUFAs within their skeletal muscle as blubber mass increased. This was contrary to juveniles and adults which had significant and strongly positive

Table 2. Intramuscular lipid (g 100 g^{-1} wet mass) mean±s.e.m. amounts for age classes in Weddell seals collected during 2005 and 2006 near McMurdo Station, Antarctica

Groups	Mean 1	Mean 2	P-value (Bonferroni)
Saturated fatty acids			
Pups *vs* adults	1.50±0.11	0.64±0.07	P≤0.001
Pups *vs* juveniles	1.50±0.11	0.53±0.04	P≤0.05
Juveniles *vs* adults	0.53±0.04	0.64±0.07	P=0.05 (NS)
Monounsaturated fatty acids			
Pups *vs* adults	3.19±0.55	1.19±0.24	P≤0.001
Pups *vs* juveniles	3.19±0.55	1.04±0.14	P≤0.001
Juveniles *vs* adults	1.04±0.14	1.19±0.24	P≥0.05 (NS)
Polyunsaturated fatty acids			
Pups *vs* adults	0.69±0.10	0.19±0.02	P≤0.005
Pups *vs* juveniles	0.69±0.10	0.20±0.03	P≤0.001
Juveniles *vs* adults	0.20±0.03	0.19±0.02	P≥0.05 (NS)

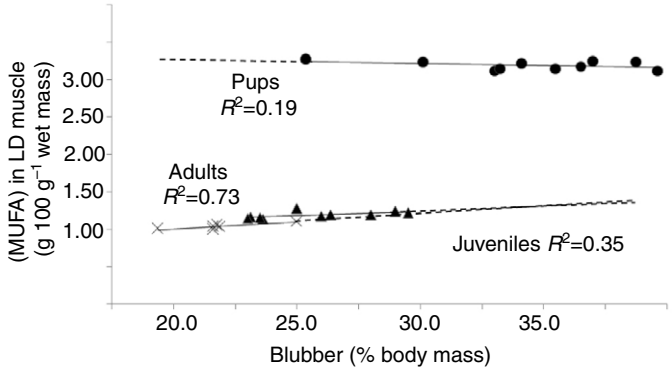

Fig. 3. Least linear regressions of monounsaturated fatty acids (MUFAs) in the skeletal muscle (longissimus dorsi; LD) against the percentage of subcutaneous blubber in Weddell seal pups, MUFA=3.4–0.01 (% blubber), P<0.05; juveniles, MUFA=0.87+0.01 (% blubber), P<0.05; adults, MUFA=0.54+0.02 (% blubber), P<0.05.

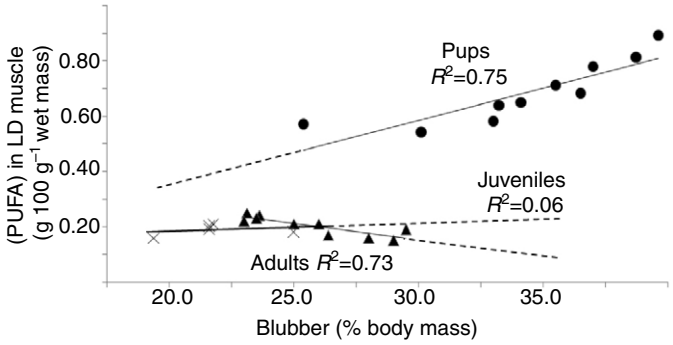

Fig. 4. Least linear regressions of polyunsaturated fatty acids (PUFAs) in the skeletal muscle (longissimus dorsi; LD) against the percentage of subcutaneous blubber in Weddell seal pups, PUFA=–0.23+0.03 (% blubber), P<0.05; juveniles, PUFA=0.57–0.01 (% blubber), P>0.05; adults, PUFA=0.12+0.01 (% blubber), P<0.05.

relationships between the percentage of body fat and MUFA (R^2=0.35 and 0.73 for juveniles and adults, respectively, P<0.05, Fig. 3), indicating blubber mass and MUFAs in the skeletal muscle increased concurrently. Regression slopes for juveniles and adults were parallel and not significantly different (ANCOVA; P>0.05). The regression slope for pups was significantly different from the juvenile and adult regression slopes (ANCOVA; P<0.05).

PUFA

Pups had a 5-fold higher mean PUFA level than juveniles and adults on a mass per muscle mass basis (Bonferroni; F=24.43, P<0.05, Table 2). The overall mean percent of PUFA contribution to the IML sampled from all age classes was 13.0±1.4% whereas the mean IMTG PUFA value was 10.0±1.6% (Table 1). Eicosapentaenoic acid (EPA, 20:5n-3) was highest in juveniles when compared among age classes (Bonferroni; F=17.46, P<0.05, Table 1). There was a significant and strongly positive relationship between percentage body fat and PUFAs in pups (R^2=0.75, P<0.05; Fig. 4) whereas no relationship for juveniles was determined (R^2=0.06, P>0.05; Fig. 4). There was a significant and strongly negative relationship in PUFAs and blubber in adults (R^2=0.73, P<0.05; Fig. 4). The regression slopes for PUFAs were significantly different among all age classes (ANCOVA; P<0.05). To determine the relative importance of PUFAs in the skeletal muscle, the ratio of (n-3)/(n-6) was calculated by adding the intake of all measured (n-3) PUFAs [α-linolenic acid (18:3n-3), stearidonic acid (18:4n-3), EPA (20:5n-3), docosapentaenoic acid (DPA, 22:5n-3) and docosahexaenoic acid (DHA, 22:6n-3)] and dividing by the intake of all measured (n-6) PUFAs (linoleic acid (18:2n-6) and arachidonic acid (20:4n-6). All age classes had IML and IMTG ratios favoring elevated n-3 PUFAs in the skeletal muscle with adult ratios ranging from 38% to 57% higher than in pups and juveniles (Table 1).

Muscle histology

While the percentage of type I muscle fibers was significantly greater when compared with type IIa fiber types among age classes (F=8.31, P<0.05), we found no significant difference in type I muscle fibers from the l. dorsi among age groups (Bonferroni, F=0.781, P>0.05, Fig. 5A). There was a significant difference in type IIa muscle fibers among age groups with adult animals having a greater percentage of type IIa (Bonferroni, F=3.18, P<0.05, Fig. 5A). Juveniles had a significantly lower percentage of lipid associated with both type I and type IIa fibers when compared with other age groups (Bonferroni, F=11.87, P<0.05, Fig. 5B, P<0.05). While having the lowest percentage of type IIa fibers among age classes, Weddell seal pups had a 4–10-fold increase in lipid in type IIa fibers (Fig. 5B).

DISCUSSION

Weddell seal skeletal muscle, specifically the l. dorsi, has varying levels of SFAs, MUFAs and PUFAs among age classes or stages of development. Specifically, we predicted PUFA levels in the skeletal muscle would provide developmental clues among age classes. Indeed, we observed a switch from primarily SFAs and MUFAs in the skeletal muscle of young pups to increased PUFAs as the pups accumulated blubber. This was contrary to what was observed in adults; SFA increased in the skeletal muscle as the percentage of blubber increased. Our results also revealed positive relationships between total IMLs and the percentage of body fat for all age classes. While the concept of selective use and mobilization of FAs has been recently introduced in lactating Weddell seals (Wheatley et al., 2008), this study is the first to describe changes in FA groups with age class within the skeletal muscle and their relationships to the percentage of body fat. While it has been previously reported that surviving in Antarctica presents selective pressures for maintaining blubber thickness to meet nutritional demands, buoyancy regulation and maintaining thermoregulatory homeostasis, it also appears that the FA composition of lipid stores in the locomotor muscle changes during development.

Lipids in muscle support thermoregulation of pups

Results from this study suggest that an increase in body fat in Weddell seal pups translates into an increase in stored IML levels, comprised mostly of PUFAs and MUFAs (Figs 1–3). Recent data have indicated preferential mobilization of MUFAs from female Weddell seal blubber to milk, resulting in increased storage of SFAs and MUFAs in the blubber of pups (Wheatley et al., 2008). Our results suggest that levels of SFAs and MUFAs in pup skeletal muscle decrease as blubber thickness increases whereas PUFA levels increase as pups accumulates blubber (Fig. 3). The change in milk-related SFAs and MUFAs transported to pup blubber from the mother (Wheatley et al., 2008), combined with the subsequent depletion in the muscle found during this study, may reflect specific SFA/MUFA use by the skeletal muscle in this age class. Wheatley

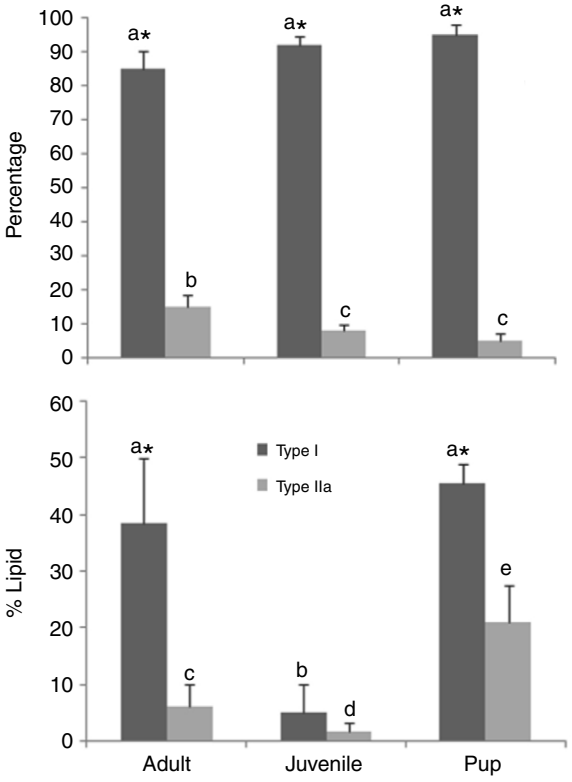

Fig. 5. Percentage of type I and IIa fibers in longissimus dorsi muscle (A) and percentage intramuscular lipid (IML) content (B) associated with fiber type in pup, juvenile and adult Weddell seals. Dissimilar letters indicate statistical difference among age classes whereas asterisks (*) indicates differences between fiber type (P<0.05).

et al. also demonstrated that as lactation progresses PUFAs in the pup adipose tissue decline (Wheatley et al., 2008). This may also suggest that pups preferentially maintain SFA and MUFA levels in their blubber while transporting PUFAs from the blubber to the intramuscular lipid droplets. It is worth noting that approximately 56% of the FAs received from milk were detected in the pup blubber, indicating that foraging by the adult female during lactation could account for the increase in skeletal muscle PUFAs in the pup (Wheatley et al., 2008). Either scenario of preferentially utilizing SFAs/MUFAs from the muscle or mobilizing PUFAs from the adipose tissue to the muscle would lead to an increase in PUFAs in the locomotor muscle of pups. This may have potential thermoregulatory benefits for pups inhabiting Antarctica. It has been reported that an increased intake of SFAs, as witnessed in Wheatley et al. (Wheatley et al., 2008), decreases sympathetic activity in brown adipose tissue, resulting in the promotion of body fat accumulation (Takeuchi et al., 1995). Moreover, an increase in PUFAs (specifically n-3) in the muscle may be selectively thermogenic (Oudart et al., 1997). During this study, two PUFAs, EPA and DHA, were recovered in greater amounts within the IML of pups and may result in increased non-shivering thermogenesis (NST) (Noren et al., 2008) (Table 1). Along with increased PUFAs, pup IML content also appeared to be related to muscle fiber type, such that type IIa muscle fibers had lipid values of 21.0% (±6.7%) whereas type I fibers had 45.6% (±3.5%) lipid content. This increase may be due to the higher oxidative capacity of type I muscle fibers, the spatial relationship between mitochondria and lipids or differences in lipolysis between the various fiber types. It has been reported that Weddell seal pups have increased mitochondrial volume density compared with juveniles or adults; thus, increasing the proximity of mitochondria to IMTGs and therefore potentially increasing metabolism (Watt et al., 2002; Kanatous et al., 2008). Therefore, pups may combine the fat accumulation benefit of metabolizing SFAs directly postpartum with storing PUFAs as the percentage of blubber increases. These factors, along with increased mitochondrial volume density to increase NST, may be used in concert to combat the high surface area to volume ratio (SA/V) of seals in the extremely low ambient temperatures of Antarctica.

Lipids in muscle as energy reserves in juveniles

Juvenile IML levels were approximately 3 times lower relative to pups and adults, and were mostly comprised of SFAs and MUFAs (Table 2). While we were unable to collect prey data during this study, we hypothesize that this muscle lipid composition may be a reflection of diet (Iverson et al., 1997), assuming a conserved role among age classes of FA elongases and desaturases in this species (Jakobsson et al., 2006). Weddell seals in McMurdo Sound forage on a fairly predictable diet of *Pleurogramma antarcticum* [range 70–100% of all scats (Burns et al., 1998; Williams et al., 2004)], and analysis of this notothenioid species has revealed high levels of MUFAs (54.3±3.4%) (Hagen et al., 2000). This reflects what was contained in the juvenile skeletal muscles during this study. It has also been reported that 22:1 isomers (22:1n-11, 22:1n-9) are notably higher in *P. antarcticum* (Hagen et al., 2000), which was consistent with increased IMTG levels detected in juvenile Weddell seals, and to a lesser extent adult seals, sampled during this study. The isomers of 22:1 were under-represented in the blubber of adults (present study, N=3, 1.62±0.05%) and from the blubber of lactating females (1.77%) (Wheatley et al., 2008), indicating this MUFA may be conserved in juvenile muscle. Cooper et al. reported similar findings in the gray seal (*Halichoerus grypus*) and suggested that the discrepancy between diet and blubber is a reflection of 22:1 efficient peroxisomal chain-shortening systems (22:1 to MUFAs) and *de novo* synthesis (22:1 to SFAs) (Cooper et al., 2006). While pups had significantly lower IMTG 22:1 values, these values correspond with published milk 22:1 values (Wheatley et al., 2008). As with pups, we detected consistently high DHA and EPA levels in juveniles (Table 1); however, the ratio of n-3/n-6 FAs did not support a switch to n-3 usage to offset a lower blubber thickness. A weak negative relationship between SFAs and the percentage of body fat may also suggest that juveniles are metabolizing SFAs at a slightly higher rate than MUFAs and PUFAs or mobilizing SFAs at a lower rate from blubber stores (Fig. 2). It would benefit juveniles to offset lower blubber thickness with increased metabolism of SFAs/MUFAs because it has been reported that these FAs may offer optimal characteristics by providing somewhat higher energy density than PUFAs (Maillet and Weber, 2006). Juvenile Weddell seals can have blubber reserves reduced by as much as 32% by one year postpartum as they learn to forage independently. Similar values have been reported for harbor seals (*Phoca vitulina*) (Muelbert et al., 2003). Thus, we suspect the IML levels found in juveniles are representative of a transition from balancing lipid stores for thermogenic homeostasis to utilizing lipids as an energy reserve. Similar findings were reported in a recent study by Castellini et al., who found that the ratio of blubber ring depth to core diameter in juvenile Weddell seals decreased as core diameter increased (Castellini et al., 2009). A lower blubber thickness in ice seals has been shown to increase heat loss and as a result increase energetic costs associated with thermogenesis (Kvadsheim and Folkow,

1997). While a pup or an adult may be able to offset this deficiency by skeletal muscle thermogenesis from blubber stores and consistent feeding, juveniles are at a distinct disadvantage being relatively poor divers with lower blubber reserves (Burns et al., 2005; Burns et al., 2007; Clark et al., 2006; Clark et al., 2007; Noren et al., 2005; Weise and Costa, 2007). This relatively poor diving ability is reflected in both skeletal muscle physiology and mean dive durations of juvenile Weddell seals (Burns and Castellini, 1996; Kanatous et al., 2008). Kanatous et al. reported that juvenile Weddell seals have higher concentrations of myoglobin than adults, which may suggest increased metabolic output of muscles through shivering (Kanatous et al., 2008). It has been reported that myloglobin levels in northern red-backed voles (*Myodes rutilus*) positively correlate with increased metabolic rates and shivering thermogenesis (Morrison et al., 1966). For inferior diving juvenile phocids, oxygen consumption rates 1.6 times predicted by Kleiber (Boily and Lavigne, 1996; Hansen and Lavigne, 1997; Noren, 2002) may indicate heat regulation when subcutaneous fat layers are minimal (Davydov and Makarova, 1964; Heath et al., 1977; Lavigne et al., 1986; Miller et al., 1973; Noren et al., 2008). It is worth noting that these metabolic levels have also been observed in non-diving adult Weddell seals (Castellini et al., 1992; Williams et al., 2004). Interestingly, the lowest metabolic rates observed during diving in adults (>14 min) were offset by feeding (assimilation costs), which increased metabolism by nearly 45% for several hours (Williams et al., 2004). Recent estimates of heat production for Weddell seal juveniles suggest that levels of heat production are nearly double that of adults and increased by 20% when heat is considered to be generated only by lean body mass (Noren et al., 2008).

Lipids minimize aerobic costs for prolonged diving in adults

Results from this study suggest that FA groups in the muscle changed during transition from juvenile to adult status in Weddell seals such that as the percentage of blubber increased, skeletal muscle PUFAs ($g\,100\,g^{-1}$) decreased by over 50% (Fig. 4). Assuming the diet consists mainly of *P. antarcticum* and because stored PUFAs are accumulated directly from the diet, we would predict similar PUFA values in the skeletal muscle of adults. Indeed, the mean percentage of PUFAs found in adult skeletal muscle is consistent with dietary sources (adult Weddell seal PUFA, 13.2±1.2%; *P. antarcticum* PUFA, 14.5±1.2%) (Hagen et al., 2000). Assuming no changes in diet and all FA proportions remaining consistent, a decrease in IML PUFA as the percentage of blubber increased (Fig. 4) would indicate increased mobilization of MUFAs and SFAs from adipose tissue (thereby decreasing the ratio of PUFAs in the muscle) or an increase in PUFA metabolism within the muscle. Since it has been established that mammalian cells do not produce *de novo* PUFAs and therefore reflect diet (Darios et al., 2007), we would also expect similar PUFA values between adipose tissue and prey. Interestingly, PUFA levels from adult adipose tissue were lower (present study, N=3, 9.8±1.3%), suggesting no preferential mobilization of MUFAs and SFAs from the blubber to the muscle but suggesting that PUFAs were preferentially mobilized by the muscle for energy. Several studies suggest that PUFAs may affect the performance of skeletal muscles and locomotion in a variety of vertebrates such as humans, salmon, birds and hares (Helge et al., 2001; Infante et al., 2001; McKenzie et al., 1998; Valencak et al., 2003). It has been reported that long-distance migratory birds (*Calidrius pusilla*) ingest a diet high in n-3 PUFAs prior to migration, which potentially then act as a molecular signal to prime flight muscles and increase aerobic efficiency (Maillet and Weber, 2006; Price et al., 2008). This would be invaluable to a deep diving mammal. We speculate PUFAs, especially n-3s, play an important role in the development of foraging from pups to adults and that this FA group may be functionally related to oxygen management, locomotor performance, life stage-specific energetic demands and diving ability. In a recent study involving rats switched from a SFA diet to fish oil (high n-3 PUFAs), myocardial oxygen consumption, coronary flow and the percentage of oxygen extraction were significantly reduced while the integrity of contractile function in the heart muscle was maintained (Pepe and McLennan, 2007). In many animal studies, an increase of n-3 PUFAs or a high n-3/n-6 ratio of FAs has resulted in increases in calcium transport and calcium absorption in PUFA-deficient rats (Kruger and Horrobin, 1997; Weiss et al., 2005). It has been established in terrestrial animals and diving mammals that calcium signaling, as well as the downstream targets of calcineurin and NFAT, play an important role in determining fiber type distribution, aerobic capacity and myoglobin concentrations in skeletal muscles (Chin et al., 1998; Kanatous et al., 2008; Schiaffino et al., 2007; Spangenburg and Booth, 2003).

Perspectives and significance

In every age class, as blubber increased (percentage of body mass) the relative amount of lipids in the skeletal muscle increased. Also, based on age-class differences in IML quantities, pups had 2.5 times the lipid amounts of adults and nearly 5 times those of juveniles. These findings are similar to the findings reported for the harp seal (*Phoca groenlandica*) where IMTG levels in the psoas muscle were 3 times that of adult levels (Worthy and Lavigne, 1983). Worthy and Lavigne speculated that increased lipid stores were used to sustain the seals during their post-weaning fast (Worthy and Lavigne, 1983). Our data support this finding such that the relatively weak correlation of blubber (percentage of body mass) and IML, as well as the relatively large variability associated with FA group in juveniles, may be indicative of this transition period and the subsequent utilization of all lipid stores during post-weaning hypophagia. The relatively robust and significant correlations in the pup and adult FA groups (SFA and PUFA) also provide evidence of a change in FA groups in the skeletal muscle throughout development. However, while the results of this study are novel and intriguing, it must be mentioned that no direct measurements of lipid utilization (e.g. FA labeling) were made during this study. Despite this limitation, we believe the variation or switch in FA group utilization may be explained by the expression of different metabolic pathways. For example, FA utilization can be altered by changes in oxidative or glycolytic enzymes often associated with muscle fiber type. Kanatous et al. (Kanatous et al., 2008) highlighted the developmental changes of enzymes in the muscle of these animals and, similar to this study, a significant decrease in type IIa fibers in pups when compared with adults. The difference in oxidative capacity between fiber types may provide some clue to age-related differences in FA groups found during this study. The variability observed in the juvenile FA group is likely to be a result of a small sample size for this age class. In conclusion, as Weddell seals continue to develop through the juvenile stage, lipid usage from the muscles appears to be in response to energy depletion potentially due to their limited diving/foraging ability and to support overall body maintenance. As juveniles mature to adults, the increased metabolism of n-3 PUFAs may be a molecular trigger to prepare for deep diving or could be a mechanism for oxygen conservation during long-duration dives.

LIST OF ABBREVIATIONS

DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
EPA	eicosapentaenoic acid
FAME	fatty acid methyl esters
FAs	fatty acids
IML	intramuscular lipids
IMTG	intramuscular triacylglycerides
MUFAs	monounsaturated fatty acids
NST	non-shivering thermogenesis
ORO	Oil Red O
PUFAs	polyunsaturated fatty acids
SFAs	saturated fatty acids

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