The Journal of Experimental Biology 212, 790-796
Published by The Company of Biologists 2009
doi:10.1242/jeb.025387

Diving into old age: muscular senescence in a large-bodied, long-lived mammal, the Weddell seal (*Leptonychotes weddellii*)

Allyson G. Hindle[1,2,*], Markus Horning[1,3], Jo-Ann E. Mellish[4] and John M. Lawler[5]

[1]Department of Marine Biology, Texas A&M University at Galveston, 5007 Avenue U, Galveston, TX 77551, USA, [2]Marine Mammal Research Unit, University of British Columbia, Room 247, 2202 Main Mall, Vancouver, BC, Canada V6T 1Z4, [3]Department of Fisheries and Wildlife, Marine Mammal Institute, Oregon State University, 2030 SE Marine Science Drive, Newport, OR 97365, USA, [4]School of Fisheries and Ocean Sciences, University of Alaska Fairbanks, Alaska SeaLife Center, 301 Railway Avenue, Seward, AK 99664, USA and [5]Department of Health and Kinesiology, Intercollegiate Faculty of Nutrition, Texas A&M University, College Station, TX 77843, USA

*Author for correspondence (e-mail: a.hindle@fisheries.ubc.ca)

Accepted 6 January 2009

SUMMARY

Classic aging theory postulates the absence of pronounced organismal senescence in wild animals since mortality probably occurs first. Large-bodied, long-lived mammals are a recognized exception to this tenet, yet organismal senescence has not been investigated to date in such mammals in the wild. Furthermore, oxidative stress theory of aging supports the suggestion that exercise hypoxia, as regularly incurred during apneustic foraging in diving mammals might lead to cellular dysfunction and accelerated aging. To determine if an aspect of organismal senescence occurs in wild marine mammals, we examined the pattern of skeletal muscle aging (contractile and connective tissue components of longissimus dorsi and pectoralis muscles) in free-ranging adult Weddell seals (9–26 years). The average myocyte cross-sectional area was 22% greater with age in the longissiums dorsi, but no significant increase occurred in the pectoralis. Cross-sectional area was not related to body mass. Changes in myocyte number per area were consistent with the 35–40% age-increase in extracellular space in both muscle groups. Also consistent with extracellular space remodeling, total and relative collagen contents were significantly elevated in older seals (115% in longissimus dorsi; 65% in pectoralis). The ratio of muscle myocyte to collagen declined with age (50–63%) at both sites. Additionally, a shift towards a higher ratio of type I to type III collagen occurred with advancing age in both muscle groups (79% increase in pectoralis; 49% in longissimus dorsi). We reject the classic tenet and null-hypothesis that Weddell seals do not survive to an age where muscular senescence becomes detectable.

Key words: muscle morphology, collagen, diving, aging.

INTRODUCTION

Mortality in wild populations is thought to typically occur prior to observable senescence (Kirkwood and Austad, 2000; Parsons, 2002), and aging theories suggest absence of selection for adaptations to aging (i.e. different phenotypes between old and young groups) as this would probably occur at the cost of the individual's inclusive fitness (Troen, 2003). However, large-bodied, long-lived mammals are an acknowledged exception (Gaillard et al., 1994; Hulbert et al., 2007), and oxidative stress theories of aging (Sohal and Weindruch, 1996; Hulbert et al., 2007) support the suggestion that breath-hold foragers such as diving, air breathing vertebrates may be particularly susceptible to oxidative stress and resultant cellular dysfunction and senescence. The life histories and physiological ecology of many diving mammals, particularly pinnipeds, are well described. Although ontogeny and early adulthood have received ample attention (Horning and Trillmich, 1997a; Horning and Trillmich, 1997b; Burns 1999; Burns et al., 1999; Noren et al., 2001; Kanatous et al., 2008), few studies have investigated processes, constraints and adaptations related to aging in older adult pinnipeds. There is limited, mixed evidence for reproductive senescence in pinnipeds (Boyd et al., 1985; Pistorius and Bester, 2002; Beauplet et al., 2006; Proffitt et al., 2007), and none for organismal senescence. The difficulty of conducting long-term ecological studies on land- or ice-breeding aquatic foragers contributes to the paucity of this information. Aging in wild populations is of ecological significance since recent life tables generated for several threatened or declining marine mammal species suggest 'aging' populations (Knowlton et al., 1994; Hanni et al., 1997; Holmes and York, 2003), with older animals constituting a greater proportion of adults, and increasing the significance of their reproductive output.

It is unknown whether aging processes, which are well-documented in humans and domesticated or laboratory-raised mammals, are detectable in long-lived, wild marine mammals. Because of the complex physiological adaptations and adjustments necessary for the daily activities of these breath-hold hunters, such aging processes are likely to impact overall performance (A. G. Hindle and M. Horning, in preparation). Many life history traits including foraging, migration, territory defense and predator avoidance are all critically dependent on skeletal muscle function. Proximate changes in skeletal muscle can be expected to play out at the whole animal level, thereby bridging the conceptual gap between cellular aging and population level senescence.

Skeletal muscle aging is typically characterized by the loss of both force-generating capacity and muscle endurance (Evans, 1995). In addition to the age-related loss of performance caused by declines in muscle mass and cross-sectional area, a loss of force generation per unit cross-sectional area (i.e. quality) has also been

documented in rodents, primates, domestic animals and humans (Brooks and Faulkner, 1994; Thompson, 1999). This multi-faceted decline of muscle mass, strength and quality with advancing age is termed 'sarcopenia'.

In humans and laboratory-raised animals, aging is equally detectable in the contractile and connective tissue components of skeletal muscle. Collagenous connective tissue makes up the extracellular matrix (ECM), which provides support and defines muscle framework. The mechanical properties of this ECM affect those of muscle as well, because the force generated by contractile tissue must overcome internal work created by connective tissue components in order to generate movement (Kjaer, 2004). Importantly, collagen content and collagen cross-linking increase with advancing age in rodents and humans (Mays et al., 1988; Kovanen and Suominen, 1989; Gosselin et al., 1998).

Though many collagen isoforms occur in skeletal muscle, the stiffer type I and more compliant type III are key in defining ECM mechanical properties (Kovanen, 2002; Kjaer, 2004). Despite functional differences amongst collagen isoforms, higher relative and overall collagen levels correlate with increased muscle stiffness, or length–passive tension (Alnaqeeb et al., 1984; Gosselin et al., 1994), which is expected to impair muscle contraction or relaxation events (Alnaqeeb et al., 1984). As with total collagen content, an adjustment in the ratio of the two key isoforms can influence contractile properties. Exercise training, injury, disease and aging are all associated with some degree of ECM remodeling (Mohan and Radha, 1980; Marshall et al., 1989; Williams et al., 1999; Miller et al., 2001; Mackey et al., 2004). Detectable signs of normal aging in skeletal muscle include increased deposition of total collagen (Mays et al., 1988; Kovanen and Suominen, 1989; Gosselin et al., 1998) resulting from increased resistance to collagen degradation and turnover (Mohan and Radha, 1980), as well as a relatively greater contribution of type I, at the expense of type III, to the collagen pool (Mays et al., 1988; Kovanen and Suominen, 1989). Both developments are expected to compromise the internal work capability of skeletal muscle with age.

Based on aging theory, we tested the null hypothesis that free-ranging Weddell seals do not survive to a time at which age-associated remodeling of myofibers and the ECM becomes detectable. Specifically, the histology of emerging senescence was quantified in contractile and connective tissue components of propulsive (longissimus dorsi) and maneuvering (pectoralis) muscles in individual, known-age seals.

MATERIALS AND METHODS

Capture, animal care and sampling

A group of 47 adult Weddell seals (*Leptonychotes weddellii* Lesson 1826) (ages 9–26 yrs; *N*=24 male, *N*=23 female) were sampled in Erebus Bay, McMurdo Sound, Antarctica (77 deg.51′S, 166 deg.40′E) during two reproductive seasons (October–December 2006, 2007). Animals were visually sexed and aged prior to capture with pre-existing, unique-numbered flipper tags (Cameron and Sinniff, 2004). Seals were restrained initially with a head-bag (Stirling, 1966) and then were chemically sedated with an intramuscular injection of ketamine hydrochloride combined with diazepam (2 mg kg^{-1} ketamine: 0.01 mg kg^{-1} diazepam) or midazolam hydrochloride [2 mg kg^{-1} ketamine: 0.1 mg kg^{-1} (J. E. Mellish et al., in preparation)]. Animals were weighed from a suspended force transduction scale (San Diego Scale Company, San Diego, CA, USA; accurate to 0.5 kg). Only males and non-pregnant, non-lactating females were sampled. Absence of late stage fetus and amniotic sac was confirmed by trans-abdominal ultrasonographic imaging (Sonosite 180 Vet; Bothwell, WA, USA) prior to sampling, and lactation status was derived from an available database of females noted to have produced a pup earlier in the season. Animal selection for the study was random, and independent from apparent health at time of capture, although one additional female was not sampled because of ethical considerations based on health complications that became apparent during initial sedation. Muscle biopsies were collected upon initial capture, or in the case of 19 females, only during the last of multiple sequential captures (10±1.0 days, 2–16 days after initial capture), to avoid muscle biopsies potentially affecting simultaneously recorded behavioral telemetry data. All work was conducted under permits through the Antarctic Conservation Act (#2007–007) and Marine Mammal Protection Act (#1034–1854), as well as local animal use committees (Texas A&M University Animal Use Protocol #2006–160; Alaska SeaLife Center Animal Use Protocol #05–004; and Oregon State University Animal Use Protocol #3454).

Biopsies were collected under sedation and application of a local anesthetic from longissimus dorsi and pectoralis muscles. Biopsy sites (5 cm×5 cm) were clipped and shaved, cleaned with povidone iodine scrub and 70% isopropyl alcohol, and blocked with 1 ml lidocaine (2%). After 10 min, an incision was made with a #10 scalpel, and the sterile biopsy needle [0.635 cm (1/4″) o.d. × 20.32 cm (8″) UCH Needle, Popper and Sons, NY, USA] was inserted. A maximum of six muscle biopsies were collected per site. Following sample collection the wound was flushed with cephazolin sodium (Webster Veterinary, Sterling, MA, USA; 0.5 g in 5 ml distilled water).

Muscle biopsies for histological analyses were placed immediately in ice-cold cryoprotectant (7% glycerol, 4% sucrose in PBS) for approximately 1 h. Samples were then removed, blotted dry, mounted in tissue freezing medium (TissueTek OCT, Sakura, Torrence, CA, USA) and frozen in liquid nitrogen-cooled isopentane. Muscle samples were stored at –80°C for up to 3 weeks prior to analyses.

Muscle morphology

Cryopreserved muscle biopsies were sectioned for all subsequent analyses at –20°C on a cryostat (TissueTek). Serial 7–9 μm sections were examined with a light microscope to confirm transverse orientation. Slides were air dried for up to 30 min, then stored at –80°C in airtight slide boxes for up to 3 weeks prior to processing. To visualize muscle morphological features such as fiber cross-sectional area and myocyte density, frozen slides were warmed at room temperature (RT), rinsed in PBS and stained with Hematoxylin (1 min). Extracellular space (ECS) was calculated for each slide from the measured fiber cross-sectional area and density (myocyte density × average cross-sectional area), and expressed as a volume percentage.

Collagen

Total collagen was visualized with Picrosirius Red histochemical staining (Sweat et al., 1964). The original method was modified by the addition of phosphomolybdic acid treatment, which has been reported to prevent uptake of the Picrosirius Red stain into the cytoplasm (Dolber and Spach, 1987). Sections were fixed in Bouin's solution for 30 min at room temperature. Slides were rinsed (1 min) in distilled water and placed in 0.2% phosphomolybdic acid for 5 min, prior to a 90-min immersion in Picrosirius Red solution (0.1% F3B Sirius Red in saturated aqueous picric acid). Slides were rinsed for 10 s each in acidified H_2O (0.5% glacial acetic acid) and picric alcohol (20% ethanol, 70% dH_2O, 10%

picric acid), then dehydrated (70%, 95%, 2×100% ethanol), cleared in xylene, and mounted.

Collagen types I and III were analyzed in muscle cross sections following the method described by Mackey et al. (Mackey et al., 2004). Briefly, frozen sections were fixed in acetone at –20°C, then blocked with 5% goat serum in TBS (50 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl, pH 7.5) for 60 min at room temperature. Sections were washed (0.5% Tween 20 in TBS) and then incubated with rabbit primary antibody (Rockland Immunochemicals, Gilbertsville, PA, USA) for 40 min at room temperature. Primary antibodies were diluted in 1% BSA-TBS in the ratios of 1:75 for type I collagen, and 1:100 for type III collagen. Sections were washed again and incubated for 30 min at room temperature in peroxidase-labeled goat anti-rabbit secondary antibody (Rockland Immunochemicals, 1:1000 diluted in 1% BSA-TBS). After a final wash, sections were stained with diaminobenzidine substrate-chromogen (5-min exposure; Dako, Carpinteria, CA, USA) and the amounts of the collagen isoforms determined. Slides were rinsed in dH_2O and then dehydrated in 95% and 100% ethanol, cleared with xylene and mounted.

Image analysis

All images were viewed with a Nikon E400 microscope, and collected using a Spot Pursuit Slider CCD camera. Images were calibrated (to the nearest 1 μm) using a stage micrometer. Muscle morphology, such as myocyte densities and cross-sectional areas, as well as areas occupied by type I, type III, and total collagen were quantified using ImageJ image analysis software (version 1.37s, National Institutes of Health, Bethesda, MD, USA). A minimum of 50 myocytes were included in the analyses for each animal. Cells directly adjacent to the edge of the section were excluded. Total collagen was identified by positive extracellular staining with Picrosirius Red. The ImageJ thresholding feature was used to isolate and quantify these stained intercellular regions. Regions identified as type I or type III collagen based on immunohistochemistry were similarly isolated and quantified.

Statistics

Adult seals were classified as either 'young' (ages 9–16 years) or 'old' (17+ years), per the reproductive population data available for this species (Proffitt et al., 2007). Histological variables were considered with respect to seal age (i.e. 'young' or 'old' cohort), sex and biopsy location (longissimus or pectoralis muscles) using three-way ANOVA procedures. LSD *post-hoc* tests for significant differences in sample means were performed when global *F*-tests were significant. When *F*-tests did not reveal any significant differences between males and females, data from both sexes were pooled for *post-hoc* comparisons. Data were tested for normality using the Shapiro–Wilkes statistic and homogeneity of variance was confirmed using a modified Levene test. Natural log transformations were employed when necessary to meet assumptions for parametric tests. Significance was set at the 5% level and means are presented ± 1 s.e.m. Statistical analyses were performed using SPSS software (version 11.5.1; Chicago, IL, USA).

RESULTS

Animals

Biopsies were collected from all animals listed here, but variations in quantity and quality of sampled tissue resulted in smaller sample sizes for specific measurements, as noted individually for each analysis and biopsy location (Tables 1 and 2). All available results are presented, and none were excluded. Of the 47 adult Weddell seals sampled (ages 9–26 years), N=26 belonged to the 'young' adult cohort and N=21 to the 'old' cohort. Mass was 397.2±10.9 kg (range: 292.3–508.2 kg) in the 'young' cohort, and 427.0±12.7 kg (range: 317.3–553.1 kg) for the 'old' adults. Myocyte cross sectional area did not correlate significantly with body mass in either muscle group examined (r^2=0.037, P=0.270 for longissimus; r^2=0.033, P=0.312 for pectoralis).

Myocyte morphology

In only one instance (myocyte density, see below) were differences detected between male and female (non-lactating, non-pregnant) seals in any of the histological variables measured. For all other variables in which no significant difference was seen between males and females, data from both sexes were pooled for *post-hoc* comparisons. Myocyte cross-sectional area (CSA) was significantly lower in pectoralis (4046±156 μm^2) compared with longissimus dorsi (5057±185 μm^2; $F_{1,64}$=22.593, P<0.001; Fig. 1; Table 1) muscle. CSA increased by 22% with age in the longissimus dorsi (P=0.003), but a similar increase did not occur in the pectoralis (P=0.565).

As CSA increased in the longissimus dorsi of older seals, myocyte density decreased (P=0.012; Table 1). A parallel decrease in myocyte density (16%) also occurred in the pectoralis (P=0.030; Table 1). Despite the similar degree of myocyte density decline in both muscle groups, a significant site effect was observed, with density 18.5% higher overall in the pectoralis ($F_{1,54}$=10.920, P=0.002; Table 1). Aging was associated with a 35–40% increase of ECS in both the longissimus and pectoralis ($F_{1,54}$=7.571, P=0.008), and was ~30% higher in the pectoralis overall ($F_{1,54}$=4.628; P=0.036; Table 1).

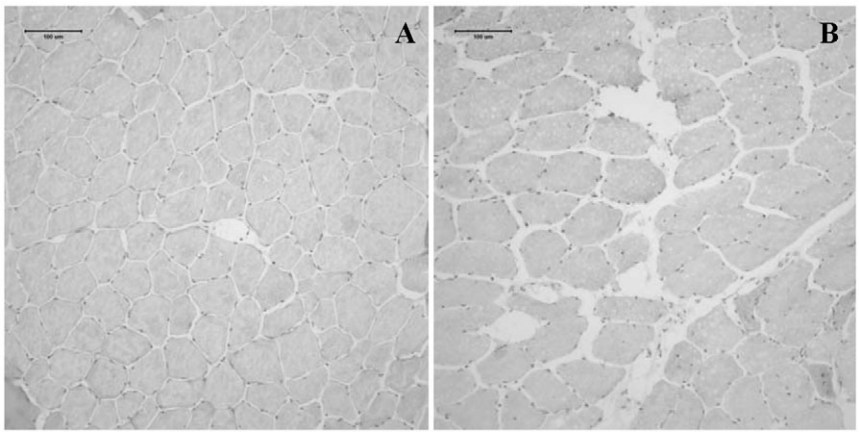

Fig. 1. Weddell seal longissimus dorsi muscle biopsies (7–9 μm transverse sections) from (A) young female; (B) old female. Sections were stained with Hematoxylin for contrast (200× magnification).

Table 1. Morphological characteristics of skeletal muscles in adult Weddell seals

	Longissimus dorsi		Pectoralis	
	Young (*N*)	Old (*N*)	Young (*N*)	Old (*N*)
Cross-sectional area (μm^2)	4632±210 (21)	5652±273* (15)	3958±219** (20)	4146±218** (17)
Myocyte density (cells mm^{-2})	170.3±8.3 (17)	134.6±7.1* (15)	197.7±14.1** (15)	166.3±9.0*,** (14)
Extracellular space (%)	18.1±2.6 (17)	24.5±1.8 (15)	22.7±3.6 (15)	31.6±2.9*,** (14)

Young: 9–16 years; old: 17–26 years. Data for both sexes were pooled (see Materials and methods). *Significant differences (α=0.05) between age classes; **significant differences between sampling locations. Values are means ± s.e.m.

Myocyte density was the only histological variable measured for which a significant sex difference was noted ($F_{1,54}$=4.098; P=0.048), with females showing 12% higher densities than males overall. This difference occurred alongside significant interactions between age and sex ($F_{1,54}$=10.920; P=0.002), as well as age, sex and muscle type ($F_{1,54}$=5.997; P=0.018).

Collagen

Total collagen content (%) was significantly elevated with age in both muscles (ln-transformed; $F_{1,72}$=44.674, P<0.001; Table 2) and was not significantly different between muscles ($F_{1,72}$=1.581, P=0.213). Total collagen increased 115% in the longissimus (from 5.9% in 'young'; P<0.001), and 65% in the pectoralis (from 7.6% in 'young'; P<0.001).

The combined result of age-related changes in myocyte morphology and collagen content is the ratio of muscle to collagen (volume ratio within analyzed biopsy area). This ratio is depressed in old adults (ln-transformed; $F_{1,53}$=41.897, P<0.001; Table 2) in the longissimus dorsi (63%; P<0.001) and in the pectoralis (49%; P<0.001; Table 2). The unique degrees of decline with age in the two muscles results in a significant location difference ($F_{1,53}$=5.821, P=0.019; Table 2). When compared for the 'young' cohort, muscle: collagen is ~30% lower in the pectoralis (P=0.029) and this ratio declines with age to levels that are not significantly different from each other (P=0.310; Table 2).

Collagen types were documented for only the 2006 data set because of a labeling failure with the 2007 antibody batch. Despite the limited data set, an age effect was seen in the ratio of collagen type I to III (ln-transformed; $F_{1,26}$=15.049, P=0.001; Fig. 2, Table 2). This ratio increased with age in both muscles (79% in the pectoralis, P=0.009; 49% in the longissimus dorsi, P=0.035), resulting in no significant effect of biopsy location (P=0.917; Table 2).

DISCUSSION

This is the first study to document both contractile and connective tissue remodeling with age in individuals of a large-bodied, long-lived, free-ranging mammal, the Weddell seal. Most notably, significant increases in collagen deposition at the expense of contractile tissue, and a shift towards a higher ratio of type I to type III collagen occurred with advancing age in both locomotor and maneuvering muscle locations. Myocyte density decreased, and ECS increased with advancing age. This effect was consistent with age-related myofiber loss at both sampling sites, and was coupled with elevation of average myocyte CSA in older individuals.

Muscle morphology

In free-ranging Weddell seals (aged 9–26 years) average myocyte CSA was higher in longissimus dorsi (5057 μm^2) than in pectoralis (4046 μm^2) muscle. The elevated CSA for the longissimus dorsi can in part be explained by a significant (22%) increase in this variable with age, compared with a mere 4% non-significant increase in the pectoralis. Even in young adults, myocyte CSA of the longissimus exceeds that of the pectoralis by 17% (Table 1). This difference could reflect muscle power output, in that the longissimus muscle serves primarily a propulsive function in phocid seals, whereas pectoralis is used for maneuvering, and controls pitch and directionality.

The age-related elevation of CSA in Weddell seal myocytes is at odds with expectations for mammalian muscle aging, which is generally associated with declining myocyte size (Evans, 1995). Although increasing mass with age is expected for Weddell seals (Fujise et al., 1985), this trend diminishes beyond prime reproductive age (J. E. Mellish et al., in preparation). The observed age-related elevation in myocyte CSA, therefore, cannot be explained by increased body mass. Our finding is also contrary to the classic expectations for muscle structure adjustments based on constraints associated with apneustic or hypoxic exercise. Hypoxic tissue environments, similar to the chronic hypoxia of mountaineering, are generally associated with CSA declines to improve tissue oxygen handling (Hoppeler et al., 1990). The regular apneustic exercise in diving, air breathing vertebrates should result in chronic submergence hypoxia (Guyton et al., 1985; Qvist et al., 1986; Davis and Kanatous, 1999; Ponganis et al., 2007). However, adaptations for apneustic exercise and tolerance to tissue hypoxia might reduce selective forces leading to CSA declines with age.

The observed age-related elevation in myocyte CSA could be explained by increased loading or use. Because body mass did not significantly increase along with myocyte CSA, it is unlikely that these increases are in response to loading outside the muscle. Instead this suggests that CSA increases are an adaptive response to

Table 2. Summary of collagen distribution within skeletal muscles in adult Weddell seals

	Longissimus dorsi		Pectoralis	
	Young (*N*)	Old (*N*)	Young (*N*)	Old (*N*)
Total collagen (% area)	5.9±0.6 (22)	12.8±1.2* (20)	7.6±0.8 (19)	12.6±1.2* (19)
Muscle: collagen ratio	19.3±2.5 (16)	7.2±0.6* (15)	13.0±1.5** (15)	6.6±1.0* (15)
Type I: type III collagen ratio	2.1±0.4 (10)	3.1±0.3* (6)	1.9±0.4 (9)	3.4±0.5* (9)

Young: 9–16 years; old: 17–26 years. Data for both sexes were pooled (see Materials and methods). *Significant differences (α=0.05) between age classes; **significant differences between sampling locations. Values are means ± s.e.m.

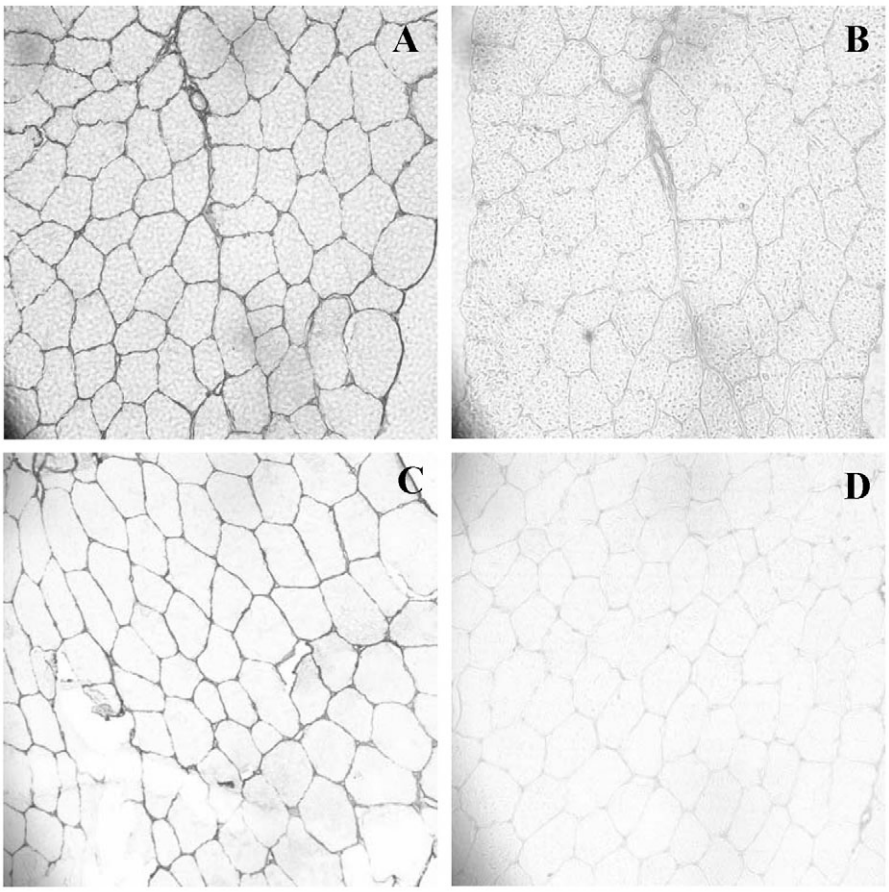

Fig. 2. Immunohistochemical staining for collagen subtypes in pectoralis muscle biopsies from female Weddell seals (7–9 μm transverse sections; 200X magnification). (A,C) Collagen type I and (B,D) collagen type III from young (A,B) and old (C,D) individuals.

increased internal work demands related to fibrosis and elevated muscle stiffness.

The large myocyte CSAs of Weddell seals correspond to elevated diffusion distance, and indeed low indices of capillarity are documented for this species (Kanatous et al., 2002). In compensation, Weddell seal muscles are characterized by a predominantly interfibrillar mitochondrial distribution, elevated volume density of interfiber lipid droplets, and high skeletal muscle myoglobin content (Kanatous et al., 2002). There is a correlation between the elevated myocyte CSA in the longissimus dorsi compared with the pectoralis, and the myoglobin contents of these muscles ($45.9\pm3.3\,mg\,g^{-1}$ wet weight *versus* $31.5\pm4.6\,mg\,g^{-1}$ wet weight) (Kanatous et al., 2002). The age-related elevation of CSA in the longissimus dorsi in this study is not, however, accompanied by a significant myoglobin increase (A. G. Hindle et al., in preparation). If elevated myoglobin content is not driving the increased myocyte CSA either, again we speculate that this occurs as a result of elevated internal resistance related to increases in the ECM and in relative collagen content. In any case, the lack of a concomitant myoglobin elevation alongside myocyte CSA could imply that aged longissimus dorsi muscles are more susceptible to the development of a hypoxic core during underwater or terrestrial activity, potentially affecting aerobic dive limit, or leading to fatigue and injury.

Myocyte density decreased with advancing age in both muscle groups examined (Table 1). This could be the result of the observed increase in myocyte CSA with age, or rather, the change in CSA may be a compensatory, exercise-induced hypertrophy of remaining fibers. The higher myocyte densities found in the pectoralis, over the longissimus, in both age groups is a function of its consistently lower fiber CSA (Table 1). Amongst significant interaction effects, myocyte density was the only variable for which a significant sex difference was noted. As muscle stress and injury contribute to apoptosis-driven myofiber loss (Yashuhara et al., 2000; Phaneuf and Leeuwenburgh, 2001; Pollack et al., 2002), perhaps the generally reduced fiber densities observed for males compared to females is the result of elevated muscle stress related to territory defense during the breeding season, when our sampling occurred.

Extracellular space

A significant increase of 35–40% in ECS occurred with age in free-ranging Weddell seals (Table 1). The elevation of ECS may diminish specific force of muscle (force per muscle CSA, and per muscle mass), given that a smaller proportion of total muscle volume is contractile tissue. Age-related motor unit loss via apoptosis or denervation (Dirks and Leeuwenburgh, 2002; Brooks and Faulkner, 1994) provides a mechanism for expanding ECS, as remodeling subsequent to motor unit removal generally compensates incompletely for fiber loss (Edström and Larsson, 1987; Brooks and Faulkner, 1994). Adjustments in collagen turnover with age (see below) probably contribute to ECS increases. Such contractile power loss may have important fitness considerations (A. G. Hindle and M. Horning, in preparation). Furthermore, declines in muscle performance may be of particular significance for males during the breeding season, in which aggressive territoriality by underwater defense of breathing holes is paramount to reproductive success.

Collagen

A striking increase in extracellular collagen within the endomysium was noted in mature seals in both muscle types (115% in the

longissimus, 65% in the pectoralis; Table 2). Muscle stiffening due to heightened collagen content or stability has been widely reported in terrestrial mammals (Mohan and Radha, 1980; Kovanen and Suominen, 1989; Gosselin et al., 1998). Such collagen remodeling in aged muscle (mediated by transforming growth factor-beta) has also been documented subsequent to mechanical, cytokine or oxidative tissue stress (Border and Noble, 1994; Cannon and St Pierre, 1998). As expected, increased total collagen content was accompanied by a decreased cross-sectional area ratio of muscle to collagen, generally accompanied by tissue stiffness (Kovanen et al., 1984; Gosselin et al., 1994; Gosselin et al., 1998). These age-related developments in the swimming muscles of Weddell seals could impede contractile force production and efficiency, and elevate energy requirements for locomotion and foraging.

Type I collagen is the 'stiffer' isoform, lending structural rigidity and storing elastic energy to increase fatigue resistance (Kovanen, 2002). Type III collagen, by contrast, confers compliance to muscle, more easily permitting structural changes and faster contractions (Kovanen, 2002). Type I was the dominant form of collagen in all samples analyzed (Fig. 2). The ratio of collagen type I to type III was similar in both muscles, increasing with age by 1.5× in the longissimus dorsi and 1.8× in the pectoralis (Table 2). These age-related changes were comparable to those in laboratory-raised rats (ratio of 2.1 at 1 month; 4.0 at 2 years) (Kovanen and Suominen, 1989), although on average this ratio did not exceed 3.5 in seals (Table 2).

Increased muscle stiffness with age, as a result of elevated total and relative collagen content and an increased type I:III collagen ratio, benefits the stability and fatigue resistance of slow-twitch fibers (Kovanen et al., 1984), the dominant muscle type in Weddell seals (Kanatous et al., 2002). This stability may, however, compromise sprint capacity and increase the likelihood of muscle injury and fatigue following burst-force generation (Kovanen et al., 1984). It may also compromise contractile efficiency, as a greater force output is necessary to overcome this heightened elastic component for a contraction of a given size. For seals exploiting the same prey resources throughout a lifetime, older individuals would be at an energetic disadvantage. Energetic simulations for aging Weddell seals reveal that even a small decline in the contractile ability of swimming muscle can produce a significant negative impact on energetic efficiency of foraging (A. G. Hindle and M. Horning, in preparation).

Conclusions

We reject the null-hypothesis that Weddell seals do not survive to an age where remodeling of myofibers and ECM become detectable. Age-specific reproductive rates in female Weddell seals remain elevated until the age of 20–22 and above (Cameron, 2001), and females have been recorded to pup to the age of 28 (Proffitt et al., 2007). This suggests that, contrary to predictions from classic theory of aging, muscular senescence does occur in these wild mammals well within their reproductive lifespan.

Our findings also suggest a probable effect of aging on sprint capacity and contractile efficiency. Modeling the effects of a possible reduction in contractile efficiency (A. G. Hindle and M. Horning, in preparation) in turn suggests a probable effect on foraging efficiency with age, as a result of such muscular senescence. Data and model combined suggests two potential outcomes for aging marine mammals in the wild: (1) tissue aging (in this case, skeletal muscle) is the basis for performance declines, leading to individual organismal senescence, altered energy balance, compromised individual condition and health, and ultimately reproductive senescence; (2) behavioral plasticity might compensate for the deleterious effects of aging on performance, allowing individuals to maintain positive energy balance and reproductive output, through adjustments of foraging behavior.

LIST OF ABBREVIATIONS

CSA	cross-sectional area
ECM	extracellular matrix
ECS	extracellular space

We sincerely thank M. Gray, R. Hill, S. Hill, J. Nienaber, J. Thompton, P. Tuomi, the staff of the Albert P. Crary Science & Engineering Center and Raytheon Polar Services Co. support personnel for their assistance. We thank R. Garrott and K. Proffitt for providing data on age and lactation status for tagged seals. Microscopy and imaging support provided by C. Marshall was invaluable. This work was supported by the National Science Foundation Office of Polar Programs under collaborative awards #0649609 (M.H.) and #0440715 (J.E.M.). Pilot data for this project was collected in 2005 with the kind assistance of S. Kanatous and his field team (supported by NSF Office of Polar Programs).

REFERENCES

Alnaqeeb, M. A., Al Zaid, N. S. and Goldspink, G. (1984). Connective tissue changes and physical properties of developing and ageing skeletal muscle. *J. Anat.* **139**, 677-689.

Beauplet, G., Barbraud, C., Dabin, W., Küssener, C. and Guinet, C. (2006). Age-specific survival and reproductive performances in fur seals: evidence of senescence and individual quality. *Oikos* **112**, 430-441.

Border, W. A. and Noble, N. A. (1994). Transforming growth factor beta in tissue fibrosis. *N. Engl. J. Med.* **331**, 1286-1292.

Boyd, I. L. (1985). Pregnancy and ovulation rates in grey seals (*Halichoerus grypus*) on the British coast. *J. Zool. A* **205**, 265-272.

Brooks, G. A. and Faulkner, J. A. (1994). Skeletal-muscle weakness in old-age: underlying mechanisms. *Med. Sci. Sports Exerc.* **74**, 71-81.

Burns, J. M. (1999). 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., Castellini, M. A. and Testa, J. W. (1999). Movements and diving behavior of weaned Weddell seal (Leptonychotes weddellii) pups. *Polar Biol.* **21**, 23-36.

Cameron, M. F. (2001). *The Dynamics of a Weddell Seal (*Leptonychotes weddellii*) Population in McMurdo Sound, Antarctica*. PhD Thesis, University of Minnesota, St Paul, MN, USA.

Cameron, M. F. and Sinniff, D. B. (2004). Age-specific survival, abundance, and immigration rates of a Weddell seal (*Leptonychotes weddellii*) population in McMurdo Sound, Antarctica. *Can. J. Zool.* **82**, 601-615.

Cannon, J. G. and St Pierre, B. A. (1998). Cytokines in exertion-induced skeletal muscle injury. *Mol. Cell. Biochem.* **179**, 159-167.

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.

Dirks, A. and Leeuwenburgh, C. (2002). Apoptosis in skeletal muscle with aging. *Am. J. Physiol.* **282**, R519-R527.

Dolber, P. C. and Spach, M. S. (1987). Picrosirius red staining of cardiac muscle following phosphomolybic acid treatment. *Stain Technol.* **62**, 23-26.

Edström, L. and Larsson, L. (1987). Effects of age on contractile and enzyme-histochemical properties of fast- and slow-twitch single motor units in the rat. *J. Physiol.* **392**, 129-145.

Evans, W. J. (1995). What is sarcopenia? *J. Gerontol. A* **50 Spec No**, 5-8.

Fujise, Y., Hidaka, H., Tatsukawa, R. and Miyazaki, N. (1985). External measurements and organ weights of five Weddell seals (*Leptonychotes weddellii*) caught near Syowa Station. *Antarct. Rec.* **85**, 96-99.

Gaillard, J.-M., Allaine, D., Pontier, D., Yoccoz, N. G. and Promislow, D. E. L. (1994). Senescence in natural populations of mammals: a reanalysis. *Evolution* **48**, 509-516.

Gosselin, L. E., Martinez, D. A., Vailas, A. C. and Sieck, G. C. (1994). Passive length-force properties of senescent diaphragm: relationship with collagen characteristics. *J. Appl. Physiol.* **76**, 2680-2685.

Gosselin, L. E., Adams, A., Cotter, T. A., McCormick, R. J. and Thomas, D. P. (1998). Effect of exercise training on passive stiffness in locomotor skeletal muscle: role of extracellular matrix. *J. Appl. Physiol.* **85**, 1011-1016.

Guyton, G. P., Stanek, K. S., Schneider, R. C., Hochachka, P. W., Hurford, W. E., Zapol, D. G., Liggins, G. C. and Zapol, W. C. (1985). Myoglobin saturation in free-diving Weddell seals. *J. Appl. Physiol.* **79**, 1148-1155.

Hanni, K. D., Long, D. J., Jones, R. E., Pyle, P. and Morgan, L. E. (1997). Sightings and strandings of guadalupe fur seals in central and northern California, 1988-1995. *J. Mammal.* **78**, 684-690.

Holmes, E. E. and York, A. E. (2003). Using age structure to detect impacts on threatened population: a case study with Steller sea lions. *Conserv. Biol.* **17**, 1794-1806.

Hoppeler, H., Kleinert, E., Schlegel, C., Claasen, H., Howald, H., Kayay, S. R. and Cerretelli, P. (1990). Morphological adatpions of human skeletal muscle to chronic hypoxia. *Int. J. Sports Med.* **Suppl. 1**, S3-S9.

Horning, M. and Trillmich, F. (1997a). Ontogeny of diving behaviour in the Galápagos fur seal. *Behaviour* **134**, 1211-1257.

Horning, M. and Trillmich, F. (1997b). Development of hemoglobin, hematocrit, and erythrocyte values in Galápagos fur seals. *Mar. Mamm. Sci.* **13**, 100-113.

Hulbert, A. J., Pamplona, R., Buffenstein, R. and Buttemer, W. A. (2007). Life and death: metabolic rate, membrane composition, and life span of animals. *Physiol. Rev.* **87**, 1175-1213.
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**, 3061-3068.
Kanatous, S. B., Hawke, T. J., Trumble, S. J., Pearson, L. E., Watson, R. R., Garry, D. J. and Davis, R. W. (2008). The ontogeny of aerobic and diving capacity in the skeletal muscles of Weddell seals. *J. Exp. Biol.* **211**, 2559-2565.
Kirkwood, T. B. L. and Austad, S. N. (2000). Why do we age? *Nature* **409**, 233-238.
Kjaer, M. (2004). Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol. Rev.* **84**, 649-698.
Knowlton, A. R., Kraus, S. D. and Kenney, R. D. (1994). Reproduction in North-Atlantic right whales (*Eubalaena glacialis*). *Can. J. Zool.* **72**, 1297-1305.
Kovanen, V. (2002). Intramuscular extracellular matrix: complex environment of muscle cells. *Exerc. Sport Sci. Rev.* **30**, 20-25.
Kovanen, V. and Suominen, H. (1989). Age- and training-related changes in the collagen metabolism of rat skeletal muscle. *Eur. J. Appl. Physiol. Occup. Physiol.* **58**, 765-772.
Kovanen, V., Suominen, H. and Heikkinen, E. (1984). Mechanical properties of fast and slow skeletal muscle with special reference to collagen and endurance training. *J. Biomech.* **17**, 725-735.
Mackey, A. L., Donnelly, A. E., Turpeenniemi-Hujanen, T. and Roper, H. P. (2004). Skeletal muscle collagen content in humans after high-force eccentric contractions. *J. Appl. Physiol.* **97**, 197-203.
Marshall, P., Williams, P. E. and Goldspink, G. (1989). Accumulation of collagen and altered fiber-type ratios as indicators of abnormal muscle gene expression in the mdx dystrophic mouse. *Muscle Nerve* **12**, 528-537.
Mays, P. K., Bishop, J. E. and Laurent, G. J. (1988). Age-related changes in the proportion of type I and III collagen. *Mech. Ageing Dev.* **45**, 203-212.
Miller, T. A., Lesniewski, L. A., Muller-Delp, J. M., Majors, A. K., Scalise, D. and Delp, M. D. (2001). Hindlimb unloading induces a collagen isoform shift in the soleus muscle of the rat. *Am. J. Physiol.* **281**, R1710-R1717.
Mohan, S. and Radha, E. (1980). Age-related changes in rat muscle collagen. *Gerontology* **26**, 61-67.
Noren, S. R., Williams, T. M., Pabst, D. A., McLellan, W. A. and Dearolf, J. L. (2001). The development of diving in marine endotherms: preparing the skeletal muscles of dolphins, penguins, and seals for activity during submergence. *J. Comp. Physiol. B* **171**, 127-134.
Parsons, P. A. (2002). Life span: does the limit to survival depend upon metabolic efficiency under stress? *Biogerontology* **3**, 233-241.
Phaneuf, S. and Leeuwenburgh, C. (2001). Apoptosis and exercise. *Med. Sci. Sports Exerc.* **33**, 393-396.
Pistorius, P. A. and Bester, M. N. (2002). A longitudinal study of senescence in a pinniped. *Can. J. Zool.* **80**, 395-401.
Pollack, M., Phaneuf, S., Dirks, A. and Leeuwenburgh, C. (2002). The role of apoptosis in the normal aging brain, skeletal muscle, and heart. *Ann. NY Acad. Sci.* **959**, 93-107.
Ponganis, P. J., Stockard, T. K., Meir, J. U., Williams, C. L., Ponganis, K. V., van Dam, R. P. and Howard, R. (2007). Returning on empty: extreme blood O_2 depletion underlies dive capacity of emperor penguins. *J. Exp. Biol.* **210**, 4279-4285.
Proffitt, K. M., Garrott, R. A., Rotella, J. J. and Wheatley, K. E. (2007). Environmental and senescent related variations in Weddell seal body mass: implications for age-specific reproductive performance. *Oikos* **116**, 1683-1690.
Qvist, J., Hill, R. D., Schneider, R. C., Falke, K. F., Liggins, G. C., Guppy, M., Elliot, R. L., Hochachka, P. W. and Zapol, W. C. (1986). Hemoglobin concentrations and blood gas tensions of free-diving Weddell seals. *J. Appl. Physiol.* **61**, 1560-1569.
Sohal, R. S. and Weindruch, R. (1996). Oxidative stress, caloric restriction, and aging. *Science* **273**, 59-63.
Sterling, I. (1966). A technique for handling live seals. *J. Mammal.* **47**, 543-544.
Sweat, F., Puchtler, H. and Rosenthal, S. I. (1964). Sirius Red F3BA as a stain for connective tissue. *Arch. Pathol.* **78**, 69-72.
Thompson, L. V. (1999). Contractile properties and protein isoforms of single skeletal muscle fibers from 12-and 30-month-old Fisher 344 Brown Norway F1 hybrid rats. *Aging Clin. Exp. Res.* **11**, 109-118.
Troen, B. R. (2003). The biology of aging. *Mt. Sinai J. Med.* **70**, 3-22.
Williams, P., Simpson, H., Kyberd, P., Kenwright, J. and Goldspink, G. (1999). Effect of rate of distraction on loss of range of joint movement, muscle stiffness, and intramuscular connective tissue content during surgical limb-lengthening: a study in rabbit. *Anat. Rec.* **255**, 78-83.
Yasuhara, S., Perez, M. E., Kanakubo, E., Yasuhara, Y., Shin, Y. S., Kaneki, M., Fujita, T. and Martyn, J. A. J. (2000). Skeletal muscle apoptosis after burns is associated with activation of proapoptotic signals. *Am. J. Physiol.* **279**, E1114-E1121.