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ORIGINAL PAPER

Scaling matters: incorporating body composition into Weddell seal seasonal oxygen store comparisons reveals maintenance of aerobic capacities

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Abstract Adult Weddell seals (*Leptonychotes weddellii*) haul-out on the ice in October/November (austral spring) for the breeding season and reduce foraging activities for ~4 months until their molt in the austral fall (January/February). After these periods, animals are at their leanest and resume actively foraging for the austral winter. In mammals, decreased exercise and hypoxia exposure typically lead to decreased production of O_2-carrying proteins and muscle wasting, while endurance training increases aerobic potential. To test whether similar effects were present in marine mammals, this study compared the physiology of 53 post-molt female Weddell seals in the austral fall to 47 pre-breeding females during the spring in McMurdo Sound, Antarctica. Once body mass and condition (lipid) were controlled for, there were no seasonal changes in total body oxygen (TBO_2) stores. Within each season, hematocrit and hemoglobin values were negatively correlated with animal size, and larger animals had lower mass-specific TBO_2 stores. But because larger seals had lower mass-specific metabolic rates, their calculated aerobic dive limit was similar to smaller seals. Indicators of muscular efficiency, myosin heavy chain composition, myoglobin concentrations, and aerobic enzyme activities (citrate synthase and β-hydroxyacyl CoA dehydrogenase) were likewise maintained across the year. The preservation of aerobic capacity is likely critical to foraging capabilities, so that following the molt Weddell seals can rapidly regain body mass at the start of winter foraging. In contrast, muscle lactate dehydrogenase activity, a marker of anaerobic metabolism, exhibited seasonal plasticity in this diving top predator and was lowest after the summer period of reduced activity.

Keywords Aerobic dive limit · Body composition · Diving physiology · Enzymes · Myosin heavy chain · Oxygen stores

Abbreviations

BV	Blood volume
(c)ADL	(Calculated) aerobic dive limit
CS	Citrate synthase (IU g wet tissue^{-1})
DMR	Diving metabolic rate
FOG	Fast-twitch oxidative glycolytic
Hb	Hemoglobin (g dL whole blood^{-1})
Hct	Hematocrit (% whole blood)
HOAD	β-Hydroxyacyl CoA dehydrogenase (IU g wet tissue^{-1})
LBM	Lean body mass (kg)
LD	*Longissimus dorsi* skeletal muscle
LDH	Lactate dehydrogenase (IU g wet tissue^{-1})
Mb	Myoglobin (mg g wet tissue^{-1})
MCHC	Mean corpuscular hemoglobin concentration (%)
MHC	Myosin heavy chain
PV	Plasma volume
RBC	Red blood cell (10^6 μL whole blood^{-1})
SO	Slow-twitch oxidative
TBM	Total body mass (kg)
TBO_2	Total body oxygen stores

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Introduction

The proficiency with which marine mammals can forage depends, in part, on their ability to carry large endogenous oxygen (O_2) stores (Scholander 1940; Hochachka and Storey 1975; Kooyman et al. 1980; Butler and Jones 1997; Kooyman and Ponganis 1998; Costa and Sinervo 2004; Burns et al. 2007). In deep-diving phocid seals, lung collapse at depth ultimately renders this store inaccessible (Falke et al. 1985; Kooyman and Ponganis 1998) so the majority (~80 %) of the O_2 stores are located in the blood and muscle tissues (Lenfant et al. 1970; Kooyman and Ponganis 1998). Moreover, pinnipeds are adapted to utilize O_2 stores efficiently, comprising the graded dive response. At the onset of a deep dive, seals exhibit bradycardia and employ peripheral vasoconstriction to apportion blood O_2 stores to specific tissues (Butler and Jones 1997; Guyton et al. 1995; Davis et al. 2004), such as the heart, lung, and brain (Zapol et al. 1979). Diverting blood O_2 stores primarily to anoxia intolerant organs means that skeletal muscles are often un-perfused (Zapol et al. 1979; Guyton et al. 1995; Davis et al. 2004; Williams et al. 2011). To withstand this reduction of vascular O_2 supply during periods of underwater activity, pinniped skeletal muscles have 10–20-fold the amount of myoglobin (Mb) compared to terrestrial mammals (Reed et al. 1994; Guyton et al. 1995; Kanatous et al. 1999; Polasek et al. 2006).

Large aerobic capacities and reduced O_2 consumption allow pinnipeds to delay the production of lactate, thereby extending the aerobic dive limit (ADL) (Kooyman et al. 1980, 1983). The ADL can be estimated by dividing endogenous O_2 stores by diving metabolic rates (DMR), which is an accurate estimator of the diving lactate threshold in Weddell seals (Kooyman et al. 1980, 1983; Ponganis et al. 1993; Burns and Castellini 1996; Costa et al. 2001). The diving lactate threshold, or the "true" ADL, will vary on a dive-by-dive basis depending on O_2 saturation at the start of a dive, the magnitude of the dive response, and metabolic rates (Castellini et al. 1992b; Guyton et al. 1995; Davis and Kanatous 1999). However, given the difficulty of measuring the diving lactate threshold in free-living animals, the calculated (c)ADL is used as a proxy (Costa et al. 2001, 2004; Costa and Sinervo 2004).

Knowing when lactate accumulates above background levels has ecological significance because in many species, if the (c)ADL threshold is exceeded, the post-dive recovery period increases exponentially as lactate is cleared from the blood before the next dive (Kooyman et al. 1980). Alternatively, animals can continue diving despite high circulating lactate levels, but subsequent dives will be relatively short and aerobic until lactate levels decline (Castellini et al. 1988; Thompson and Fedak 1993). This ultimately decreases the total amount of time that can be spent foraging (Castellini et al. 1992b; Costa et al. 2001), and therefore, free-ranging pinnipeds are thought to limit the frequency of dives that exceed their ADL (Kooyman et al. 1980; Thompson and Fedak 2001). Because TBO_2 stores generally exhibit a linear relationship with body mass ($Mass^{1.0}$), whereas O_2 is depleted as an allometric function of $Mass^{0.75}$ (Kleiber 1947, 1975; Kooyman 1989), larger animals tend to have disproportionately longer ADL times (Schreer and Kovacs 1997; Halsey et al. 2006a, b). Further, O_2 is only stored in the lean body compartment, and therefore, the relationship of physiological parameters with animal size is complicated by extreme seasonal fluctuations in lipid stores (Costa et al. 1986; Wheatley et al. 2006; McDonald et al. 2008; Crocker et al. 2014).

In addition to animal mass and body composition (lipid stores), activity patterns may also have an impact on the ADL. This is because the production of many O_2 storage proteins is facilitated by exercise and hypoxia exposure (Hochachka et al. 1998; Hoppeler and Vogt 2001; Halvorsen and Bechesteen 2002; Haddad et al. 2003), both of which stimulate the NFAT/MEF-2, hypoxia inducible factor (HIF)-1, and Sp1 pathways to increase transcription of Mb, glycolytic enzymes, erythropoietin (EPO), and vascular endothelial growth factor (VEGF)-1 (Hochachka and Somero 2002, Kanatous and Mammen 2010; De Miranda et al. 2012). Weddell seals (*Leptonychotes weddellii*) likely reduce their diving activity during their breeding and molting periods, as do other phocid species (Kooyman 1975; Castellini et al. 1992a; Schreer and Testa 1996; Forcada et al. 2012). If cellular pathways regulating the production of O_2-storage proteins are down-regulated during this period, it would likely negatively impact foraging success, as O_2 stores and the ADL may be reduced.

In addition to having large O_2 stores, pinniped muscles must be able to generate sufficient power while consuming minimal O_2 during dives (Davis et al. 2004). The generation of muscle force for propulsion is largely determined by muscle fiber and myosin heavy chain (MHC) isoform composition. Endurance training leads to an increase in slow-twitch oxidative (SO) fiber types with predominately MHC I protein, high mitochondrial densities, elevated Mb loads, and greater aerobic enzyme activities such as citrate synthase (CS; initiates the TCA cycle) and β-hydroxyacyl CoA dehydrogenase (HOAD; in the β-oxidation chain for fatty acids), and pinnipeds have muscular profiles reflecting oxidative and endurance potential (Kanatous et al. 2002; Luedeke et al. 2004). Yet, SO fibers are the most vulnerable to atrophy during times of disuse (Booth 1977; 1982; Hudson and Franklin 2002). Muscles that can generate high force with rapid contractions are primarily composed of fast-twitch oxidative-glycolytic (FOG) fibers, containing MHC IIA and MHC IID/X (Baldwin and Haddad 2001). Muscles with primarily FOG fibers express greater lactate

Table 1 Mean ± SE Weddell seal straight length, total body mass, lean mass, and lipid (as %TBM) among reproductive groups

Body condition parameter	Fall	Spring	
	Non-reproductive	Non-reproductive	Reproductive
Straight length (cm)	234.1 ± 2.40 (*53*)[a]	230.5 ± 3.37 (*28*)[a]	250.1 ± 2.81 (*16*)[b]
Mass (kg)	321.6 ± 10.10 (*53*)[a]	335.6 ± 14.30 (*28*)[b]	413.7 ± 13.31 (*16*)[b]
Lean mass (kg)	221.0 ± 6.73 (*52*)	214.9 ± 9.79 (*27*)	266.5 ± 8.85 (*15*)
Body composition (%lipid)	30.88 ± 0.49 (*52*)[a]	36.08 ± 0.99 (*27*)[b]	36.30 ± 0.84 (*15*)[b]

Sample sizes are shown in parentheses, and *different letters* indicate significant differences between reproductive groups. Note that animal straight length was included as a covariate for total body and lean mass comparisons, and that there were yearly variations in animal mass and lipid stores (see Shero et al. 2014, 2015)

dehydrogenase (LDH) activities, so that they can rapidly convert pyruvate to lactate under low O_2 and burst-speed conditions (Peter et al. 1972; Baldwin et al. 1973; Kanatous et al. 2002). Because muscle mass and function are energetically costly to maintain, mammalian skeletal muscles show large capacities for phenotypic plasticity (Baldwin and Haddad 2001; Hoppeler and Flück 2002; Luedeke et al. 2004; Flück 2006), and are particularly affected by hormones, hypoxia, and exercise regimens (Stockdale and Miller 1987; Hoppeler and Flück 2002; Luedeke et al. 2004; Flück 2006).

This study aimed to determine whether the aerobic capacity and muscular efficiency of adult female Weddell seals is reduced following the molting period when seals have limited their underwater activities. Therefore, TBO_2 stores, as well as muscle structural and biochemical properties were measured directly post-molt and compared to those at the start of the summer breeding period, after intense winter foraging. We also determined whether females that returned to give birth after the austral winter foraging period had longer cADL times or muscle profiles indicative of greater power and efficiency, than those animals that returned but failed to produce a pup.

Methods

Animal handling

Fifty-three adult female Weddell seals were captured on the ice along the McMurdo Sound region, Antarctica in Erebus Bay (~77°S, 165°E) and the Victoria Land coastline (~76°S, 162°E) following the molt in January/February (austral fall), and forty-seven females (30 non-reproductive females; 17 reproductive females handled 7 days post-partum) were handled in October/November (austral spring) 2010–2012, following the winter foraging period. These animals comprise the large cross-sectional portion of this study. Of the Weddell seals handled in spring (year $t + 1$), twenty were animals that had been handled the previous fall (year t), and these animals constitute the longitudinal portion of this study. Twelve of these recaptured females returned with a pup the following year ($t + 1$), and eight animals did not give birth after the winter foraging period. All the post-molt animals were assumed to be non-reproductive because none of the molted known-age individuals handled and <15 % of fully molted females surveyed in the population in fall had given birth in the pupping period immediately preceding sampling (Burns et al. 2013; Beltran and Burns *unpublished*).

Animals were sedated with an initial intramuscular dose of ~ 1.0 mg kg^{-1} tiletamine/zolazepam HCl. Following a 10–15 min induction period, animals were captured via hoop net and additional intravenous injections of ketamine and diazepam (~0.2 and 0.012 mg kg^{-1}) were administered approximately every 10 min, or as necessary, to keep animals sedated while remaining eupneic. A straight length was taken and total body mass (TBM) was measured using a sling with a hand winch and scale (MSI-7200-IT Dyna-Link digital dynamometer, capacity 1000 ± 1.0 kg), while animals were suspended from a tripod. Body composition (%lipid) and lean body mass (LBM) were determined for each animal using tritiated water dilution as described in Shero et al. (2014, 2015) (Table 1).

Hematology and Blood Volume

Blood samples were collected in EDTA and heparinized vacutainers™ from the extradural vein. Hematocrit (Hct; packed RBC volume) was determined by whole blood centrifugation, and hemoglobin (Hb) concentrations (g dL^{-1}) were determined for each animal using the cyanomethemoglobin assay with Drabkin's reagent (Sigma Kit 625A) and a UV/Vis Beckman series 530 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA) at $\lambda = 540$ nm and concentrations of samples calculated through a linear regression of hemoglobin standards (Pointe Specific, Inc.). Mean corpuscular

hemoglobin concentration (MCHC) was calculated as follows:

$$\mathrm{MCHC}(\%) = \frac{\mathrm{Hb\ g\ dL^{-1}}}{\mathrm{Hct}} \times 100.$$

Medix™ Ery-Tic RBC test kits were used to count red blood cells (RBCs) (Medix Corp., Newbury Park, CA, USA).

Plasma volume (in liters; PV_L) was determined using the Evan's Blue technique. One pre-injection blood sample was taken followed by administration of ~0.5–1.2 mg kg^{-1} Evan's Blue dye into the extradural vein. Syringes used to distribute the dye were pre-weighed and flushed with blood to accurately determine the amount of dye injected for each animal. Injection was followed by three consecutive blood draws approximately 10 min apart, and the exact time of sample collections recorded. Blood samples were centrifuged and plasma stored at −80 °C until analyses. The plasma background absorbance values were measured at $\lambda = 740$ nm and were subtracted from optical density at $\lambda = 624$ nm. Evan's Blue dye stock (40 mg mL^{-1}) was used to construct standard curves and determine sample concentrations. Dilution of the dye was used to calculate PV_L as described in Foldager and Blomqvist (1991) and El-Sayed et al. (1995) and blood volume (in liters; BV_L) calculated as follows:

$$\mathrm{BV_L} = \frac{\mathrm{PV_L}}{(100 - \mathrm{Hct}) / 100.}$$

Myoglobin Concentrations

Longissimus dorsi (*LD*) skeletal muscle samples were taken from each animal in the field with a 6 mm biopsy punch and immediately frozen in liquid nitrogen before being stored at −80 °C until analysis. Muscle Mb concentrations (mg g wet $tissue^{-1}$) were assayed following Reynafarje (1963) as modified by Prewitt et al. (2010). Samples were run in quadruplicate in a Molecular Devices SpectraMax 340 microplate reader (Molecular Devices, Inc., Sunnyvale, CA, USA) alongside a lyophilized myoglobin horse standard (Sigma, mean recovery: 100.6 ± 2.82 %) and previously assayed harbor seal (*Phoca vitulina*) and Weddell seal tissue (Inter-assay CVs: harbor seal 6.5 %; Weddell seal 4.6 %). Samples were read at both $\lambda = 538$ and 568 nm to account for any Hb contamination.

Total body oxygen stores and the cADL

Blood O_2 stores were determined from Hb, Hct, and BV in arterial and venous systems with the following assumptions: (1) hemoglobin has an O_2 carrying capacity of 1.34 mL O_2 g^{-1} [Hb], (2) arterial blood is 33 % of total blood volume, with the remaining 66 % blood in the venous system, (3) the maximum O_2 saturation possible in the arterial system is 95 %, with a minimum of 20 % after O_2 has been transported to other tissues, and (4) venous blood is presumed to have 5 % less volume than the starting arterial O_2 stores and can be extracted to zero (Kooyman et al. 1983; Davis and Kanatous 1999; Burns et al. 2007). Muscle O_2 stores were determined for each animal assuming that Mb also had an O_2 carrying capacity of 1.34 mL O_2 g^{-1} [Mb] and total muscle mass was estimated as 38 % of lean body mass (Burns et al. 2007). Lung O_2 stores were calculated as follows:

$$\mathrm{Lung\ O_2} = V_i \times 0.15\mathrm{FO_2}.$$

V_i is the estimated diving lung volume (in liters) calculated as $0.5 \times 0.10(\mathrm{TBM})^{0.96}$, which assumes that lung volume is at 50 % total capacity at the onset of diving. Further, 0.15 FO_2 is the partial pressure of O_2 in the lungs (Kooyman 1989). Blood, muscle, and lung O_2 stores were summed to give total body oxygen (TBO_2) stores of animals for which all measurements were possible (Lenfant et al. 1970; Kooyman et al. 1983; Burns et al. 2007). Each animal's diving metabolic rate (DMR) was estimated from 1.6 × Kleiber (Kleiber 1975; Williams et al. 2004). Calculated aerobic dive limits (cADLs) were determined by dividing TBO_2 stores by DMR, and TBO_2 stores were scaled to TBM and LBM for seasonal comparisons.

Enzyme kinetics

To evaluate aerobic and anaerobic ATP production potential, citrate synthase (CS), β-hydroxyacyl CoA dehydrogenase (HOAD), and lactate dehydrogenase (LDH) kinetic activities (IU g^{-1} wet mass muscle) were measured. Spectrophotometric assays were run according to the procedures described by Polasek et al. (2006) and Prewitt et al. (2010) under substrate saturating conditions held at 37 °C. A buffer blank and a previously assayed muscle sample of known activity were measured as controls along with all experimental samples (Inter-assay CVs: CS 13.1 %, HOAD 10.4 %, LDH 7.0 %). CS:HOAD ratios were evaluated to determine the relative amount of aerobic metabolism that utilizes β-oxidation of fatty acids. Values less than 1 indicate higher dependence on lipid stores. The LDH:CS ratios were calculated to compare potentials for aerobic versus anaerobic metabolism (Polasek et al. 2006).

Myosin heavy chain isoform composition

Myosin heavy chain isoforms were separated using the SDS-PAGE technique as described by Blough et al. (1996) and Reiser and Kline (1998). Weddell seal samples were run in triplicate against a rat (*Rattus norvegicus*) sample that contained MHC I, IIA, IIB, and IID/X (50:50 soleus:

Table 2 Mean ± SE blood chemistry parameters with sample sizes shown in parentheses

Hematology parameter	Mixed model relationships		Fall	Spring	
	TBM	Body composition	Non-reproductive	Non-reproductive	Reproductive
HCT (%)	neg.	≈	62.1 ± 0.63 (*53*)[a]	62.1 ± 0.94 (*31*)[a]	56.2 ± 1.61 (*17*)[b]
Hb (g dL^{-1})	neg.	≈	25.2 ± 0.38 (*53*)	24.6 ± 0.31 (*31*)	23.6 ± 0.74 (*17*)
MCHC (%)	neg.	≈	40.3 ± 0.32 (*52*)	39.8 ± 0.54 (*31*)	39.9 ± 0.66 (*15*)
RBC (10^6 μL^{-1})	≈	≈	3.88 ± 0.06 (*53*)[a]	3.82 ± 0.08 (*18*)[ab]	3.63 ± 0.17 (*12*)[b]
PV (%TBM)			6.40 ± 0.09 (*53*)[a]	5.71 ± 0.14 (*28*)[b]	6.72 ± 0.16 (*16*)[a]
PV (%LBM)			9.26 ± 0.13 (*52*)[a]	8.94 ± 0.17 (*27*)[a]	10.44 ± 0.19 (*15*)[b]
BV (%TBM)			17.0 ± 0.28 (*53*)[a]	15.4 ± 0.45 (*28*)[b]	15.3 ± 0.44 (*16*)[b]
BV (%LBM)			24.7 ± 0.42 (*52*)	24.0 ± 0.62 (*27*)	23.5 ± 0.55 (*15*)

The relationship of parameters with LMM factors (total body mass; TBM and body composition) is noted: neg., negative correlation; ≈, no significant effect. Different letters indicate significant differences among reproductive groups. Plasma and blood volumes are shown scaled to total and lean body mass (TBM and LBM), respectively, and so TBM and body composition were not included in LMM models

extensor digitorum longus) as a standard. Gels were silver-stained and developed as described by Blough et al. (1996) and bands were quantified using digitizing software (UN-SCAN IT gel v. 6.1). The identity of Weddell seal MHC I, IIA, and IID/X bands was determined by comparing their relative migration distances to those in the rat standard, and those of other pinnipeds that had previously been confirmed by proteomic analysis (Shero et al. 2012) at the Ohio State University Mass Spectrometry and Proteomics Facility. New proteins were identified using capillary-liquid chromatography tandem mass spectrometry (Cap-LC/MS/MS) using a Thermo Scientific LTQ mass spectrometer (Bruker Daltonics Billerica, MA) and LC system (UltimMateTM 3000 System, Thermo Scientific). Dynamic exclusion was enabled on the mass spectrometer with two repeats within 10 s, a mass list size of 200, exclusion duration of 350 s, and a low mass width of 0.5 and high mass width of 2.5. A Mascot Daemon (Matrix Science v. 2.3.2, Boston, MA, USA) search against the SwissProt Database was performed to identify significant protein matches. Two missed cleavages for the enzyme were permitted.

Statistical analyses

Data were assessed for normality prior to statistical analysis, and transformed as necessary. To determine whether physiology differed seasonally and for animals that were in better condition (more lipid) for their mass, linear mixed models (LMMs) with Bonferroni post hoc comparisons were used with reproductive category as a fixed factor (fall non-reproductive, spring non-reproductive, spring reproductive), and total body mass (TBM) and body composition as covariates in models. Repeated measures were used to account for the fact that some individuals were handled in both fall and spring. Model residuals were evaluated for normality and homoscedasticity, and standardized residuals were used to evaluate outliers. Because MHC profiles were not normally distributed even after transformation, a Kruskal–Wallis H test was used to test for differences in MHC composition by reproductive group (SPSS v. 22, Chicago, IL, USA). Regression analyses were used to assess relationships between MHC profiles with animal condition and muscle biochemical properties. Overwinter changes in physiological parameters of females handled in both fall (year t) and spring ($t + 1$) were analyzed using paired t tests. Significance was defined at the 95 % level ($P < 0.05$) throughout, and results are reported as mean ± SE.

Results

Hematology and blood volume

In the cross-sectional study, blood Hct, Hb, and MCHC were negatively correlated with TBM (Table 2; Hct: $F_{1,\ 75.4} = 11.8$, $P = 0.001$; Hb: $F_{1,\ 73.1} = 20.7$, $P < 0.001$; MCHC: $F_{1,\ 44.6} = 5.0$, $P = 0.031$) and body composition did not significantly improve model fit. While Hb (24.7 ± 0.3 g dL^{-1}) and MCHC (40.1 ± 0.3 %) did not vary by reproductive status, Hct was significantly lower in spring reproductive females than non-reproductive females in both fall and spring (Table 2; $F_{2,\ 87.7} = 6.8$, $P = 0.002$). RBC numbers did not correlate with animal size or body composition, but spring reproductive females had significantly lower RBC counts than non-reproductive females in the fall ($F_{2,\ 33.8} = 4.7$, $P = 0.016$). In the smaller longitudinal study, Hct and Hb declined slightly as animals gained mass overwinter, but these trends were not significant as in the larger dataset. However, MCHC decreased significantly overwinter in both non-parous and parous females in the longitudinal study (non-parous: $t_5 = 3.9$, $P = 0.011$, parous females: $t_{10} = 2.6$, $P = 0.024$).

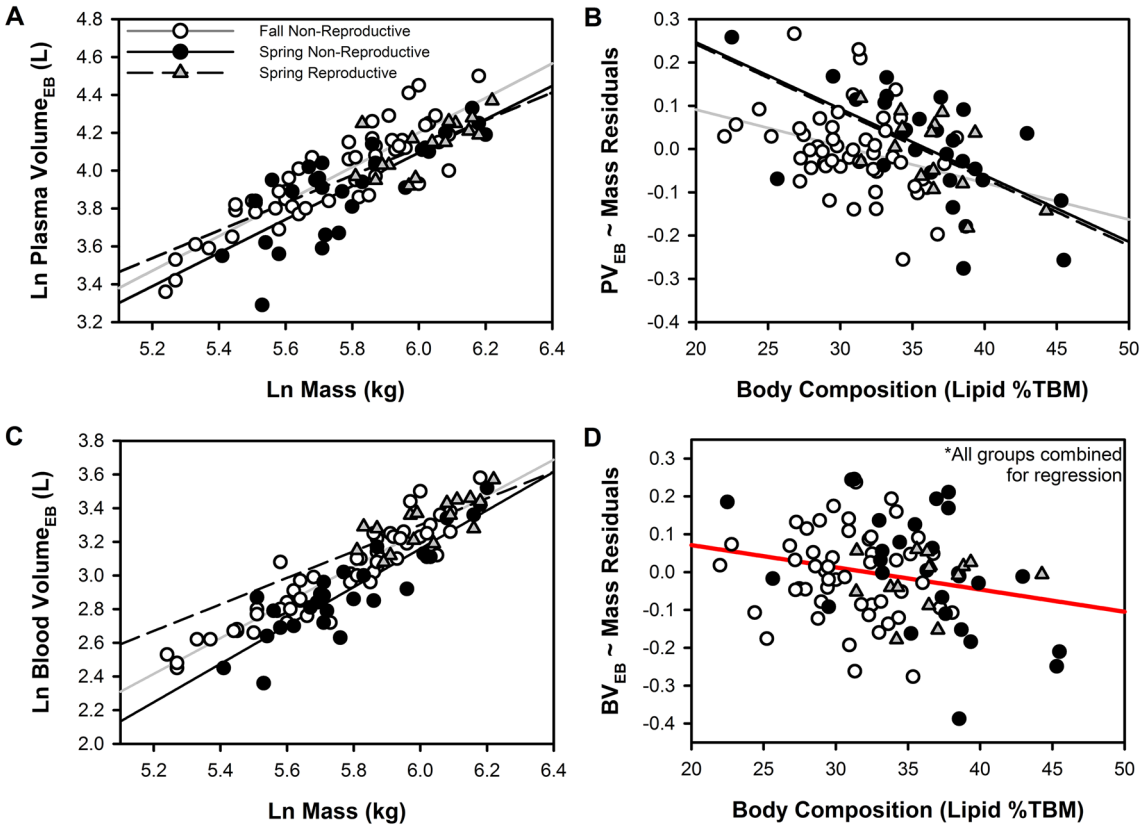

Fig. 1 Linear mixed models between Weddell seal plasma and blood volume, determined using Evan's blue dye, and total body mass (**a**, **c**). The LMM residuals from plasma or blood volume regressed against total body mass were significantly negatively correlated with body composition (**b**, **d**). Relationships differed by reproductive group in all LMM models (Fall Non-reproductive: *solid gray line*, Spring Non-reproductive: *solid black line*, Spring Reproductive: *dashed black line*), except for blood volume after body composition was accounted for (all reproductive groups combined: *single*, *thick solid red line*)

PV_L was significantly positively correlated with TBM and adding body composition significantly improved the model (Fig. 1a, b; Table 2; TBM: $F_{1,\ 80.4} = 623.4$, $P < 0.001$; Body Composition: $F_{1,\ 86.2} = 27.9$, $P < 0.001$). Non-reproductive females in spring had significantly higher plasma volume than reproductive females, but neither group differed from non-reproductive females in fall ($F_{2,\ 80.6} = 8.4$, $P < 0.001$). BV_L also exhibited a significant positive relationship with TBM (Fig. 1c, d; TBM: $F_{1,\ 81.3} = 254.4$, $P < 0.001$), and appeared to differ by reproductive group with fall non-reproductive females having the highest blood volume per unit body mass (Table 2). But this effect was an artifact of seasonal differences in body composition (spring > fall), as animals with lower than expected BV_L for their size were significantly fatter ($F_{1,\ 86.6} = 6.4$, $P = 0.013$). Similarly, blood O_2 stores scaled positively with TBM (Table 2; $F_{1,\ 84.9} = 56.6$, $P < 0.001$), yet models were not improved with the addition of body composition or reproductive group (14.3 ± 0.3 L O_2). In the longitudinal study, neither BV_L nor PV_L differed by season and mass-specific blood O_2 stores (scaled to TBM and LBM) did not change overwinter.

Muscle biochemistry and structure

In the large cross-sectional study, neither reproductive group, TBM, nor body composition accounted for significant variation in muscle Mb or aerobic enzyme activities (Table 3; overall averages Mb: 90.0 ± 1.7 mg g wet tissue^{-1}; HOAD: 23.2 ± 0.8 IU g wet tissue^{-1}; CS: 14.4 ± 0.4 IU g wet tissue^{-1}). The CS:HOAD ratio showed a significant negative relationship with body composition ($F_{1,\ 75.8} = 5.4$, $P = 0.023$), but did not differ by reproductive group or TBM. Among females that were captured in both fall (year t) and the subsequent spring ($t + 1$), Mb was maintained. Females that were reproductive in year $t + 1$ exhibited slight, but non-significant, decreases in CS and increases in HOAD, which together led to a significant decrease in the CS:HOAD ratio ($t_9 = 3.751$, $P = 0.005$). In the large cross-sectional study, muscle O_2 stores increased with TBM and the addition of body composition

Table 3 Mean ± SE muscle myoglobin (Mb), enzyme activities, and myosin heavy chain composition in the Weddell seal's primary locomotor muscle (*longissimus dorsi*) with sample sizes shown in parentheses

Muscle biochemistry parameter	Mixed model relationships		Fall	Spring	
	TBM	Body composition	Non-reproductive	Non-reproductive	Reproductive
Mb (mg g wet tissue^{-1})	≈	≈	90.4 ± 2.14 (*50*)	86.0 ± 3.52 (*26*)	95.8 ± 3.16 (*14*)
CS (IU g wet tissue^{-1})	≈	≈	14.9 ± 0.53 (*47*)	14.1 ± 0.68 (*26*)	13.3 ± 1.27 (*14*)
HOAD (IU g wet tissue^{-1})	≈	≈	23.3 ± 1.04 (*47*)	23.1 ± 1.33 (*26*)	22.9 ± 2.36 (*14*)
LDH (IU g wet tissue^{-1})	pos.	≈	505.8 ± 16.2 (*47*)^{a}	666.3 ± 24.8 (*26*)^{b}	592.8 ± 29.0 (*14*)^{ab}
CS:HOAD	≈	neg.	0.67 ± 0.03 (*47*)	0.64 ± 0.03 (*26*)	0.60 ± 0.04 (*14*)
LDH:CS	pos.	≈	36.7 ± 2.15 (*47*)^{a}	50.1 ± 2.98 (*26*)^{b}	49.9 ± 5.23 (*14*)^{ab}
MHC I (%)*	≈	≈	61.8 ± 1.9 (*51*)	62.9 ± 3.4 (*27*)	68.3 ± 3.8 (*14*)
MHC IIA (%)*	≈	≈	38.1 ± 1.9 (*51*)	37.0 ± 3.3 (*27*)	31.7 ± 3.8 (*14*)

The relationship of parameters with LMM factors (total body mass; TBM and body composition) is noted: pos., positive correlation, neg., negative correlation, ≈, no significant effect. *Different letters* indicate significant differences among reproductive groups. Asterisks indicate tests were run using Kruskal–Wallis tests

Table 4 Mean ± SE blood, muscle, total body oxygen stores, diving metabolic rate scaled to total and lean body mass (TBM and LBM), respectively, and the calculated aerobic dive limit, with sample sizes shown in parentheses

Oxygen store parameter	Mixed model relationships		Fall	Spring	
	TBM	Body composition	Non-reproductive	Non-reproductive	Reproductive
Blood O_2 (mL kg TBM^{-1})			45.4 ± 1.25 (*51*)^{a}	40.1 ± 1.55 (*28*)^{b}	38.1 ± 1.53 (*15*)^{b}
Blood O_2 (mL kg LBM^{-1})			65.7 ± 1.92 (*50*)	62.4 ± 2.32 (*27*)	58.4 ± 2.14 (*14*)
Muscle O_2 (mL kg TBM^{-1})			31.8 ± 0.85 (*49*)^{a}	27.7 ± 1.22 (*24*)^{b}	31.5 ± 1.23 (*12*)^{b}
Muscle O_2 (mL kg LBM^{-1})			45.9 ± 1.10 (*49*)	43.7 ± 1.89 (*24*)	49.4 ± 1.82 (*12*)
TBO_2 (mL kg TBM^{-1})			83.4 ± 1.55 (*49*)^{a}	72.5 ± 1.59 (*24*)^{b}	73.5 ± 1.37 (*12*)^{b}
TBO_2 (mL kg LBM^{-1})			120.8 ± 2.24 (*49*)	114.5 ± 2.23 (*24*)	115.5 ± 2.62 (*12*)
DMR (mL O_2 kg TBM^{-1} min^{-1})			4.30 ± 0.04 (*49*)^{a}	4.22 ± 0.05 (*24*)^{ab}	4.01 ± 0.04 (*12*)^{b}
DMR (mL O_2 kg LBM^{-1} min^{-1})			6.23 ± 0.06 (*49*)^{a}	6.69 ± 0.12 (*24*)^{b}	6.30 ± 0.12 (*12*)^{ab}
cADL (min)	≈	neg.	19.4 ± 0.33 (*49*)^{a}	17.2 ± 0.42 (*24*)^{b}	18.4 ± 0.30 (*12*)^{ab}

For all parameters scaled to TBM or LBM, mass and body composition were not included in LMM models. For the cADL, the relationship with LMM factors (total body mass; TBM and body composition) is noted: neg., negative correlation, ≈, no significant effect. Different letters indicate significant differences among reproductive groups

significantly improved model fit (Table 4; TBM: $F_{1,\ 74.3} = 113.0$, $P < 0.001$; Body composition: $F_{1,\ 78.9} = 11.2$, $P = 0.001$). Muscle O_2 stores did not differ by reproductive group (10.4 ± 0.3 L O_2). Similarly, muscle O_2 stores, whether scaled to TBM or LBM, in the smaller longitudinal study did not vary seasonally.

LDH activities and LDH:CS ratios were significantly positively correlated with TBM (Table 3; LDH: $F_{1,\ 75.5} = 10.5$, $P = 0.002$; LDH:CS ratio: $F_{1,\ 74.2} = 4.4$, $P = 0.040$), but the addition of body composition did not improve model fit. In contrast to indicators of aerobic metabolism, LDH activities and LDH:CS ratios differed by reproductive group (LDH: $F_{2,\ 57.3} = 13.5$, $P < 0.001$; LDH:CS ratio: $F_{2,\ 68.0} = 4.7$, $P = 0.012$) and non-reproductive females in spring had significantly higher LDH activities and LDH:CS ratios than post-molt seals in fall (LDH: $P < 0.001$; LDH:CS ratio: $P = 0.015$) but neither group differed from spring reproductive females. While females handled in both seasons showed a similar increase in LDH over winter as in the large cross-sectional dataset, the increase was not significant.

We identified four different MHC isoforms in Weddell seal *LD* muscle. MHC I and IIA were the two most abundant myosin isoforms, with MHC IID/X only present in six samples (Fig. 2). An additional MHC band was detected in six Weddell seals. Because this new MHC was very faint in Weddell seal muscle samples, not enough protein could be collected for proteomic analysis. However, this band migrated the same distance as one detected in greater concentration in hooded seal (*Cystophora cristata*) muscle. When this MHC band was sent out for proteomic analysis, a wide range of MHC isoforms were identified

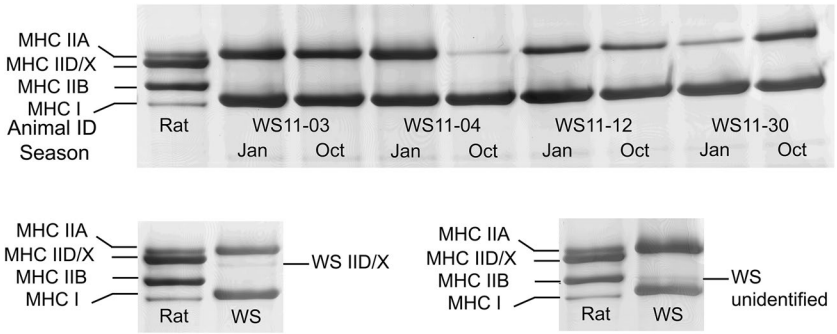

Fig. 2 Weddell seal (WS) myosin heavy chain (MHC) gels. (*Above*) Muscle MHC composition for females that were handled at two different times of year. MHC I and IIA were dominant. (*Below*) A few animals had the fast-twitch oxidative-glycolytic MHC IID/X isoform, or the unidentified isoform. All samples were run against a rat standard

as close matches in the MASCOT and SwissProt database, making it difficult to determine its functional role (Closest matches: (1) Accession number Q076A4, canine MYH8, slow-oxidative perinatal, (2) Accession number Q076A5, canine MYH4, fast-glycolytic MHC IIB, (3) Accession number Q8MJV0, horse MYH1, fast oxidative-glycolytic MHC IID/X, and (4) Accession number P49824, canine MYH7, slow-oxidative cardiac MHC beta). The number of unique peptide sequence matches ranged from 31 to 64, and Molecular Weight (MOWSE) scores to the unidentified band ranged from 531 to 1254 (scores >67 are significant; Pappin et al. 1993).

There were no differences in the relative proportion of either MHC I or IIA among reproductive groups (I: $\chi^2 = 2.9$, $P = 0.230$; IIA: $\chi^2 = 2.8$, $P = 0.244$) nor was MHC composition correlated with TBM or body composition. However, the MHC proportions were highly variable among individuals (range MHC I: 32.5–100 %; range MHC IIA: 0–66.0 %), and the variation was correlated with several measured muscle biochemistry parameters. For example, the relative proportion of MHC I was positively correlated with muscle Mb concentrations and HOAD activities (Fig. 3a–d; Mb: $F_{1, 88} = 28.9$, $P < 0.001$; HOAD: $F_{1, 83} = 12.0$, $P < 0.001$), while the relative proportion of MHC IIA was negatively correlated with the same parameters. In the longitudinal study, MHC composition remained constant in some recaptured animals while changing markedly in others, but there was no clear pattern with any measured variable.

Total body oxygen stores and the cADL

Total body O_2 stores were significantly positively correlated with TBM (Fig. 4a; $F_{1, 74.1} = 223.3$, $P < 0.001$) and when considered alone, the relationship differed by reproductive group. However, females with lower TBO_2 stores for their size had significantly greater lipid stores (Fig. 4b; $F_{1, 79.1} = 5.7$, $P = 0.020$), and once both size and body composition were accounted for, there were no differences in TBO_2 stores among reproductive groups (Table 4; 26.5 ± 0.6 L O_2). The slope of the relationship between TBO_2 and TBM was significantly lower than the overall mean mass-specific TBO_2 store value (slope: 65.9 mL O_2 kg^{-1}, 95 % CI 57.1–74.6 mL O_2 kg^{-1}; study mean: 78.9 ± 1.2 mL O_2 kg^{-1}), indicating that larger animals had significantly lower TBO_2 per unit body mass than smaller seals.

Once TBO_2 stores were divided by the estimated DMR, cADL times were not correlated with TBM (Table 4; Fig. 4c). When body composition was included in models, condition accounted for a significant amount of variation in cADL times (Fig. 4d; $F_{1, 78.9} = 6.9$, $P = 0.010$) and animals with shorter than expected cADLs were in better condition. cADL times differed by reproductive group ($F_{2, 78.5} = 3.2$, $P = 0.047$), and non-reproductive females in fall had significantly higher cADLs than non-reproductive females in spring ($P = 0.041$). There were no overwinter changes in the cADL of females handled in both fall (year t) and the subsequent spring ($t + 1$), regardless of whether they pupped or not.

Discussion

This study shows that despite the seasonal changes in Weddell seal activity budgets (Kooyman 1975; Castellini et al. 1992a; Schreer and Testa 1996; Forcada et al. 2012) and body condition (Wheatley et al. 2006; Shero et al. 2015), their ability to sustain aerobic metabolism while underwater as indicated by TBO_2 stores is conserved across the winter. Variation in O_2 stores was most strongly correlated with animal mass, and not season. Similarly, the aerobic nature of muscle structure and biochemistry was stable across seasons and reproductive status. Maintenance of aerobic capacities across the year would allow animals to forage effectively at the end of the annual molt. Rapid reacquisition of tissue stores following lactation and molt is likely critical for maintenance of early gestation and future reproductive success (Smith 1966; Atkinson 1997). In contrast, the summer period of reduced diving activity was associated with a decline in LDH activity, as post-molt seals had the lowest LDH values of animals handled in this

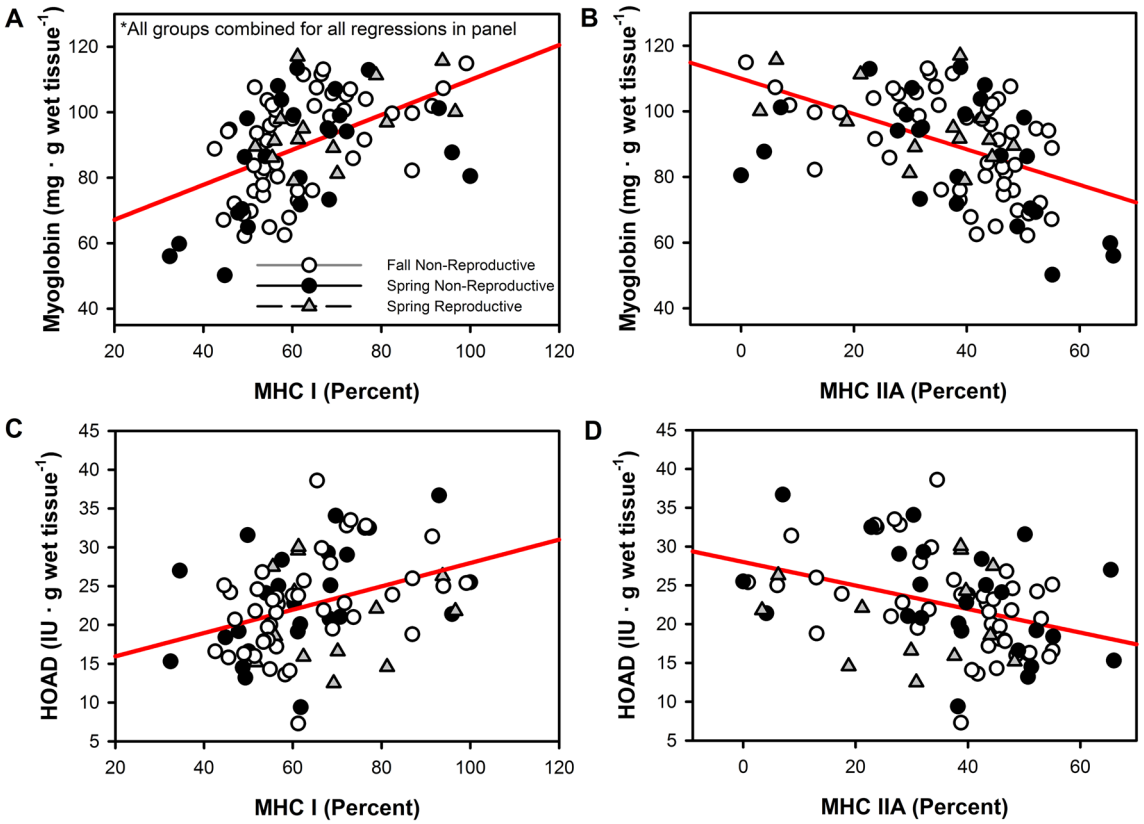

Fig. 3 Linear regressions showing the relationship between muscle biochemical and structural properties. Muscle (**a**, **b**) myoglobin and (**c**, **d**) *β*–hydroxyacyl CoA dehydrogenase (HOAD) activities were significantly correlated with MHC I and IIA composition in the *Longissimus dorsi* swimming muscle. *Single*, *thick solid red line* shows no effect of reproductive group in regression analysis, so trend shows all animals combined

study. Overwinter increases in LDH may allow animals to extend dive durations even further, just prior to the pupping period the subsequent year.

This study found no evidence for seasonal declines in O_2-storage capabilities in this diving predator. In both fall and spring, Weddell seal hematological parameters were similar to values seen in previous studies (Hindle et al. 2011; Mellish et al. 2011). Only blood Hct and RBCs were lower in reproductive Weddell seals, and Hb and BV did not change seasonally. Muscle O_2 stores, as indicated by Mb concentrations, were higher than previously reported in Weddell seals (Ponganis et al. 1993; Kanatous et al. 2002; 2008; Hindle et al. 2011), but were also maintained across the winter. As muscle Mb loads often correlate with dive durations (Noren and Williams 2000; Lestyk et al. 2009), higher Mb concentrations in deep-diving Weddell seals make their O_2-storage capacities more similar to those previously reported in other phocid species capable of extended breath hold diving (Burns et al. 2007; Lestyk et al. 2009; Hassrick et al. 2010). That Weddell seals expend energy to continue producing equivalent RBCs and iron-containing O_2-carrying proteins during all times of the year (i.e., reduced and intense foraging periods, and the energetically expensive molt period), and also through senescence (Hindle et al. 2009, 2011) demonstrates the importance of maintaining aerobic diving capacities. One possibility is that any summer diving activity and exercise would be sufficient to maintain protein production in Weddell seals. Pinnipeds also have sleep apneas, while hauled-out and this may provide additional "hypoxic exposure," that may serve to maintain aerobic capacity during periods when underwater activity is limited (Castellini et al. 1992b; Castellini 1994, 1996; Zenteno-Savin and Castellini 1998). Alternatively, because the turnover time for RBCs and muscle Mb is ~2–4 months (Hickson and Rosenkoetter 1981; Ben-David and Flaherty 2012), the breeding and molt periods may not provide sufficient time to observe a decrease in protein production, before animals would need to prepare for active foraging again. In combination, due to the preservation of blood and muscle O_2 storage proteins across the year, TBO_2 stores were also maintained.

An important outcome of this work is that both mass and body composition need to be considered when comparing TBO_2 stores and cADL among individuals and/or species.

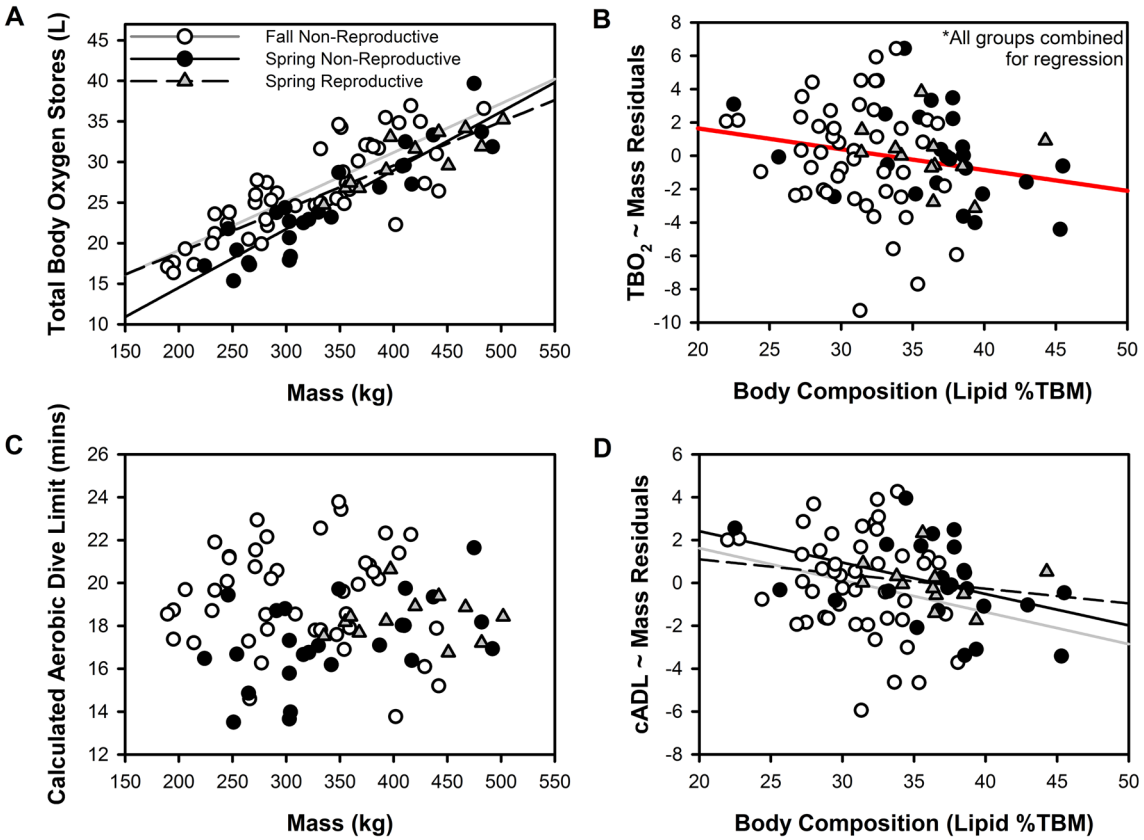

Fig. 4 Total body oxygen (TBO_2) stores were positively correlated with total body mass and the relationship differed by reproductive status (**a** fall non-reproductive: *solid gray line*, Spring Non-reproductive: *solid black line*, Spring Reproductive: *dashed black line*). Body composition accounted for significant variation in the model (**b** all reproductive groups combined*: Single*, *thick solid red line*). Conversely, the calculated aerobic dive limit (cADL) did not exhibit a significant relationship with body mass (**c**). Residuals were negatively correlated with body composition, with a reproductive effect (**d**)

Simply using mass as a scalar for O_2-stores ignores the dramatic variation in blubber and other lipid compartments that do not store O_2 but affect metabolism. When the relationship between TBO_2 and TBM was considered alone, it appeared as though animals had significantly lower TBO_2 stores, for their mass, in spring. However, when controlling for variation in lipid stores across individuals, TBO_2 stores did not differ by season. This comparison accounting for animal body composition is most physiologically relevant, as it tests whether there are differences in O_2-storage capacities in the lean body compartment. Weddell seals exhibited a relatively small over winter increase in body condition (lipid increased by 6 %TBM) as compared to other marine mammal species and at different times of the year (i.e., lipid may decrease by 15 %TBM across lactation; Costa et al. 1986; Beck et al. 2003; Wheatley et al. 2006; Crocker et al. 2014). Still, these small changes in lipid stores were enough to bias interpretations of seasonal differences in TBO_2 stores, before including body composition in statistical models. This further emphasizes the importance of incorporating body composition into TBO_2 store calculations. It should also be noted that because TBM was used to calculate lung O_2 stores, our estimate of TBO_2 stores is not entirely independent of TBM. However, lung O_2 stores only contribute ~7 % to Weddell seal TBO_2 stores, and so any remaining bias is likely small.

In addition to changes in O_2 stores, changes in DMR have the potential to greatly alter the ADL both on an individual dive basis (Castellini et al. 1992b; Williams et al. 2004; Fahlman et al. 2008), as well as across the year (Gerlinsky et al. 2014). For example, cetaceans tend to have lower TBO_2 stores than phocid seals, but their large size is associated with low DMRs, extending their aerobic capacities (Noren and Williams 2000; Croll et al. 2001). Diving mammals can, conversely, also greatly exceed their ADLs to reach rich or ephemeral prey patches (Costa et al. 2001, 2004). The relationship between animal size, O_2 stores, and dive durations also varies by taxonomic family (Schreer and Kovacs 1997; Halsey et al. 2006a, b). For example, otariids tend to have shorter dive durations than phocids of comparable size, as do diving seabirds, due to lower O_2 stores and higher mass-specific metabolic rates.

Within species, the ADL and dive durations increase during ontogeny (Kooyman et al. 1983; Burns and Castellini 1996; Richmond et al. 2006; Burns et al. 2007; Fowler et al. 2007; Weise and Costa 2007; Shero et al. 2012). However, among adults the relationship is less clear, and in this study larger Weddell seals had lower mass-specific TBO_2 stores than smaller seals. Lower estimated mass-specific metabolic rates (DMR $\propto$ $Mass^{0.75}$) in larger animals then resulted in equivalent cADLs across size classes. The cADLs in this study were comparable to the "true" ADL with increased lactate concentrations occurring at 20–25 min in Weddell seals (Kooyman et al. 1980; Williams et al. 2004).

Despite evidence that protein expression of muscle Mb, enzymes, and structural components is controlled by different regulatory pathways (Jansson et al. 1988; Terrados et al. 1990), all aerobic biochemical properties of Weddell seal primary locomotor muscles remained constant across both seasons in this study. Weddell seal locomotor muscles contained primarily MHC I and IIA (Kanatous et al. 2002, 2008), and other MHC isoforms were rare and/or not present in all individuals. In terrestrial mammals, such as humans, mice and rats, and sled and raccoon dogs, numerous aerobic aspects of muscles atrophy quickly during periods of inactivity and food deprivation (i.e., myofibers atrophy, capillary and mitochondrial densities drop, Mb and aerobic enzyme activities decrease, and MHC shifts from slow to fast-types; Lindboe et al. 1982; Baldwin and Haddad 2001; Flück 2006; Gerth et al. 2009; Kinnunen et al. 2015). Conversely, in Weddell seals, the great oxidative potential of muscles was reflected by stable CS and HOAD enzyme activities, in addition to high and relatively invariant Mb concentrations. These findings suggest that Weddell seals may be physiologically "programmed" to withstand periods of reduced activity while maintaining muscle integrity. Wild animals may need to forage effectively and escape predation after such periods of reduced activities, and indeed, similar patterns of atrophy resistance have been observed in hibernating bats and rodents, and winter lethargy in bears (Lohuis et al. 2007; Hershey et al. 2008; Lee et al. 2008; Nowell et al. 2011). Despite being composed of primarily slow-oxidative fibers that are particularly vulnerable to atrophy, Weddell seal muscles maintained aerobic MHC profiles. Muscle structure was strongly correlated with many biochemical features, and these relationships persisted throughout the year, further suggesting that preservation of muscular force and efficiency is critical to foraging success in this species.

While physiological indicators of aerobic capacity and muscle structure were largely conserved, anaerobic capacity, as judged from muscle LDH activity, exhibited seasonal plasticity. As would be expected based on controlled cell culture experiments and detraining protocols (Semenza et al. 1994; Mujika and Padilla 2001; De Miranda et al. 2012), reduced activity and hypoxic exposure during the Weddell seal's summer breeding period and molt were associated with ~25 % lower LDH activities, suggesting that glycolytic enzyme activities may atrophy faster than aerobic enzymes. Because LDH was also positively correlated with animal size, animals were larger in spring with higher LDH activities. Higher glycolytic enzyme activities may allow animals to extend their dives to longer durations, past the cADL, more frequently in the spring. Higher LDH activities would also clear lactate faster during surface recuperation periods by catalyzing its conversion to pyruvate (Thompson and Fedak 2001; Davis et al. 2004). The maintenance of aerobic capacity and increased anaerobic capacity across the winter may reflect a burst in foraging abilities prior to the pupping season the following year.

Studies conducted in other pinniped species have shown opposing results on seasonal changes in TBO_2 stores and the cADL. In multiple sea lion species, variation in blood and muscle O_2 stores emerged in response to changes in food availability and/or nutritional plane (Villegas-Amtmann and Costa 2010; Villegas-Amtmann et al. 2012; Gerlinsky et al. 2014). In contrast to otariids, the Phocidae family appears to follow a different pattern. In addition to TBO_2 and muscle biochemistry being conserved in Weddell seals, the northern elephant seal (*Mirounga angustirostris*) did not exhibit any changes in factors contributing to TBO_2 or the cADL across the post-molt or post-breeding foraging trips (Hassrick et al. 2010), and studies show that harbor seal (*Phoca vitulina*) muscle biochemistry does not change seasonally (Burns *unpublished*). Weddell seals and other phocids forage on unreliable prey resources spanning great distances in order to accumulate some or all of the reserves necessary for energetically costly periods of reproduction and the annual molt (Costa and Shaffer 2012). Loss of body condition during these periods likely makes the initiation of winter foraging and early gestation a critical recuperation period for female Weddell seals to prepare for the next year. Maintaining high O_2 stores throughout the year would be imperative to effective foraging, and also to buffer times when prey is scarce due to environmental perturbations or other anomalies.

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