Ready to dive? Early constraints help juvenile southern elephant seals (Mirounga leonina) acclimatize to aquatic life

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Abstract

Breath-holding foraging implies different adaptations to limit oxygen (O_2) depletion and maximize foraging time. Physiological adjustments can be mediated through O_2 consumption, driven by muscle mitochondria, which can also produce reactive oxygen species during reoxygenation. Southern elephant seals spend months foraging at sea, diving for up to one hour. Pups transition abruptly to aquatic life after a post-weaning period, during which they fast and progressively increase their activity, making this period critical for the development of an adaptive response to oxygen restriction and oxidative stress. We compared the functional capacity of a swimming muscle in 5 recently weaned and 6 adult female southern elephant seals. High-resolution respirometry was employed to examine muscle mitochondrial respiratory capacity and differences in protein and gene expressions of the main regulatory pathways were determined using LC-MS/MS and RT-PCR, respectively. Oxidative damages were measured in the plasma. Juveniles have higher mitochondrial coupling efficiency compared to adults probably as a response to growth and important physicial activity reported during the post-weaning. There were no differences in oxidative damage, but the adults had a higher level of antioxidant defenses. Both hypoxia and oxidative response pathways appeared less activated in the juveniles. This study highlights the differences in muscle metabolism and the likely adaptive response to hypoxia and oxidative stress between juveniles and adult south elephant seals. It also suggests that early constraints such as fasting, physical activity, and short-term low O_2 partial pressure exposure could contribute to immediate and long-term responses and to acclimatize juveniles to aquatic life.

Keywords : diving – hypoxia – oxidative stress – acclimatization – post-weaning

Summary statement

This study suggests that the post-weaning period contributes to acclimatizing juvenile southern elephant seals to aquatic life and the associated physiological constraints (limited O_2 supply and oxidative stress).

INTRODUCTION

Diving mammals are regularly exposed to oxygen (O_2) restriction, performing prolonged foraging dives in apneic conditions (Elsner and Gooden, 1983; Kooyman, 2012; Meir et al., 2009; Qvist et al., 1986; Ridgway et al., 1975). Breath-holding foraging implies increased physical activity despite the decline in O_2 availability, which requires physiological adjustments for optimal O_2 consumption, such as peripheral vasoconstriction, blood flow redistribution, or changes in mitochondrial efficiency, the cellular organelle responsible for oxidative phosphorylation (van den Berg et al., 2011). Prolonged diving is also associated with the generation of reactive oxygen species (ROS) and oxidative stress. During ischemia, superoxide radical are generated through the activation of pro-oxidant enzymes such as NADPH oxidase (Brookes et al., 2004; Granger, 1988; Granger and Kvietys, 2015; McCord, 1985). In addition, ROS are produced via reverse electron transport at mitochondrial complex I during reperfusion (Chouchani et al., 2016). ROS production and the associated biochemical damage increase with age. In parallel, a loss of function of the antioxidant enzymes is noted. This raises the question of how diving mammals cope with the alternation of low O_2 availability during apneas and reoxygenation after apneas and whether these adaptations are influenced by age.

Several studies have focused on physiological adaptations to low oxygen availability and / or oxidative stress in diving mammals. Previous studies on seals reported reduced cardiac output and blood flow redistribution toward obligatory oxygen-dependent tissues during the dives (Elsner, 1999; Kooyman and Ponganis, 1998; Lavigne et al., 1986). Massive oxygen stores, with 28% being in the muscles, have also been noted (94 ml O_2 kg^{-1}, i.e., four times more than in humans), along with physiological and behavioral adaptations to minimize oxygen depletion (Blix, 2018; Davis, 2014). Also, seals have mechanisms that limit oxidative damage in plasma, tissues and red blood cells. For instance, they are known to possess higher glutathione (GSH) levels than terrestrial mammals, and a rapid upregulation of genes involved in GSH metabolism in their endothelial cells was noticed during hypoxia exposure (Allen et al., 2024). Consequently, they exhibit lower levels of oxidative damage in their tissues, including muscles (Murphy and Hochachka, 1981; Vázquez-Medina et al., 2006; Vázquez-Medina et al., 2007; Wilhelm Filho et al., 2002; Wright et al., 2020; Zenteno-Savín et al., 2002). Muscle mitochondrial adaptations were also investigated in extreme divers such as the northern elephant seal (NES, *Mirounga angustirostris*). An acclimatization to the acute and repeated exposures of long breath-hold exercises was shown in adults, characterized by an increased efficiency in coupling phosphorylation (Chicco et al., 2014). The molecular pathways responsible for adaptations to low oxygen availability and oxidative stress are also well-identified. Low partial O_2 pressure triggers the hypoxia adaptive response through HIF-1α, a transcription factor involved in angiogenesis, erythropoiesis, and myoglobin production, helping in optimizing oxygen stores, redistribution, and consumption. On the other hand,

the transcription factor erythroid 2–related factor 2 (Nrf2) contributes to the activation of several antioxidant enzymes. Both HIF-1α and Nrf2 have been implicated in the seal's protection against apnea-induced hypoxemia and ischemia/reperfusion (Johnson et al., 2004; Johnson et al., 2005; Vázquez-Medina et al., 2011c). For instance, HIF-1α and Nrf2 levels increased after a voluntary submersion in NES (Vázquez-Medina et al., 2011c) or weddell seals (Murphy and Hochachka, 1981).

The ontogenesis of physiological adaptations to aquatic life in air-breathing mammals remains unclear. Juveniles of many phocid species undergo a post-weaning period, fasting for several weeks, before an abrupt transition to aquatic life. During this period, juveniles progressively increase their physical activity and at-sea movements, making the post-weaning critical in the development of diving abilities and adaptive response to low O_2 availability and oxidative stress (Noren et al., 2005; Prewitt et al., 2010; Somo et al., 2015; Thorson and Le Boeuf, 2023; Vázquez-Medina et al., 2011b). An increase in myoglobin abundance and maturation of the antioxydant system was already reported in juvenile weddell seals (*Leptonychotes weddellii*), hooded seals (*Cystophora cristata*), and northern elephant seals (NES, *Mirounga angustirostris*) (Geiseler et al., 2013; Moore et al., 2014; Vázquez-Medina et al., 2011a; Vázquez-Medina et al., 2011b). However, underlying mechanisms and molecular pathways of the ontogenetic development of diving abilities during this period remain unclear.

Southern elephant seals (SES, *Mirounga leonina*) spend months in the sea to feed, diving for up to one hour, reaching depths of up to 1,000 m (Hindell et al., 1991; Slip et al., 1994). General physiological adjustments are already described in NES, (Davis, 2014; Meir et al., 2009), but little is known about SES which remain in a polar environment. Moreover, few studies have investigated both mitochondrial performance and adaptive muscular and systemic responses to oxidative stress. As many other phocid species, pups abruptly transition to aquatic life after a 3 to 4 weeks post-weaning fast on land before leaving for their first trip to sea. This period is particularly costly for the juveniles as they progressively increase their physical activity and at-sea movements (Piot et al., 2024). The combination of costly events during the post-weaning period, the abrupt transition, and the ability of juvenile SES to perform extreme dives make them good model species for studying this post-weaning period as preconditioning, and the ontogenesis of physiological adaptations to O_2 restriction and oxidative stress.

The aim of this study was to compare the metabolic capacity of the swimming muscle in pups and adult SES to help understanding the ontogenesis of the adaptations to the hypoxic living conditions. High-resolution respirometry was employed to examine differences in muscle respiratory capacity between the two groups. We assessed protein and gene expression differences on the same tissue using liquid chromatography–mass spectrometry (LC-MS/MS) and gene expression measurements, respectively. Indices of oxidative stress were measured in their plasma by quantifying lipid peroxidation (isoprostanes, 8-isoPGF2a) and protein oxidation (protein carbonyls, PC). We hypothesized that adults would display greater mitochondrial efficiency than juveniles that had not yet been regularly exposed to

O_2 restriction and oxidative stress. We further assumed that the adaptive response to hypoxia and oxidative stress would increase in adults, resulting in no difference in oxidative damage. As juveniles remain physically active while fasting during this period, we thought they would show an upregulation of mitochondrial biogenesis and metabolic pathways to meet the energetic demand they face on land, which could contribute to increasing their adaptive response to physiological constraints they will face later in life.

MATERIALS AND METHODS

Data collection and analysis

Animal Sampling

All scientific procedures were approved by the Ethics Committee of the French Polar Institute and the French Ethics Committee (Cometh #32407-2021071212163407 v2) and followed the ARRIVE guidelines (Sert et al., 2020). The study was conducted in 2022, from late October to mid-November, in Port-Aux-Français (49°34'S, 70°21'E) in the Kerguelen Archipelago. Lactating adult females at the end of the weaning period to avoid disturbance (N = 6) and juvenile females of approximately 5–6 weeks old during the post-weaning period (N = 5) were sampled. All individuals remained on land fasting for 2 (juveniles) to 3 weeks (adults). Females were lactating and strictly remained on land. Juveniles were on the beach with the opportunity to play and swim in the sea. Animals were anesthetized with an initial dose of tiletamine and zolazepam (Zoletil® 100) via intramuscular injection. They were measured and weighted, and skeletal muscle biopsy and blood samples were collected, as described below.

Muscle biopsy and blood sampling

We performed a surgical biopsy of a swimming muscle (*longissimus dorsi*). During the surgical procedure, anesthesia was maintained by successive injections of tiletamine and zolazepam (Zoletil® 100) into the extradural vein every five to ten minutes. The biopsy area was sterilized using iodinated povidone 7.5% surgical scrub and iodinated povidone 10% solution (Vétédine®). Subsequent subcutaneous administration of local anesthesia (1% lurocaïne®) was made before making a small incision. Non-traumatic Mayo scissors were used to break adipose tissue until it reached the muscle layer. An 8-mm biopsy cannula was introduced, and two muscle samples of approximately 30 mg each were taken. One was placed into ice-cold BIOPS buffer (pH 7.1) containing 10 mM Ca2+-EGTA, 0.1 µM free calcium, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCL2, 5.77 mM ATP, and 15 mM phosphocreatine until processing for mitochondrial respiration experiments within the following hour; the other was placed into RNAlater® and frozen at -20°C for later analysis. A simple interrupted cutaneous suture with PDS® II D3T monofilament absorbable (2-0, 3/8, 24 mm; Ethicon) was made to

stitch the incision. The wound was cleaned with iodinated povidone 10% solution (Vétédine®) and protected with an aluminum healing spray (Alumisol®). Postoperatively, the animals received an intramuscular (IM) injection of both antibiotics (Penicillin, Streptomycin, Shotapen®, 13 mg/kg, and 16 mg/kg, respectively, without exceeding 12 mL per injection) and a non-steroidal anti-inflammatory drug (Meloxicam, Metacam®, 0.5 mg/kg). The whole capture and surgical procedure lasted less than one hour. After the biopsy, animals were monitored until complete voluntary locomotion was regained.
Blood samples were collected directly in the extradural vein in EDTA tubes after intramuscular anesthesia. The tubes were centrifuged for 5 min at 4,000 rpm, and the plasma was pipetted into an airtight vial and stored at -20°C until shipping and then at -80°C.

High-resolution respirometry

Tissue preparation

Muscle fiber bundles were selected from biopsies maintained in ice-cold BIOPS and mechanically separated in a petri dish on ice. Adipose and connective tissue were removed using fine forceps under a dissecting microscope. Teased fiber bundles (2–4 mg) were transferred to BIOPS containing 50 $\mu g.ml^{-1}$ saponin for 30 min of membrane permeabilization and washing of intracellular substrates (Gnaiger, 2009). Permeabilized muscle fibers were washed in ice-cold MiR05 respiration buffer containing 0.5 mM EGTA, 3 mM MgCl2, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose, and 1 g/l fatty acid free-BSA and gently rocked for 10 min to wash out residual saponin. Permeabilized fibers were blotted on Whatman filter paper to remove excess buffer, weighed, and immediately placed in the oxygraph chambers containing MiR05 at 37°C for stabilization before the respiration experiments described below.

Respirometry

High-resolution tissue respirometry was performed on permeabilized muscle fibers obtained from the *longissimus dorsi* using an Oxygraph-2K (O2K) respirometer (Oroboros Instruments, Innsbruck, Austria). All respirometry data were collected at 37°C less thant 2 hours after sampling. Before each protocol, the oxygen electrode for each chamber was calibrated using ambient air. Permeabilized muscle fibers were introduced into the chamber before partially sealing the chamber with a stopper. As respiration was low and a hyperoxygenated environment was not relevant in our model, we decided not to add oxygen before starting the measurements. Two protocols were performed for each sample (Table 1). Cytochrome C was used to assess mitochondrial membrane integrity. An increase in respiratory flux of more than 10% following ADP titration is usually used as a threshold to indicate that compromised outer mitochondrial membranes invalidated the respirometry measures for a given sample. One measurement had a ratio between the respiratory flux after adding cytochrome c and ADP of 14% and

another of 21%. Because all the experiments were run by the same person, under the same conditions, and the respiration values were good, we decided to keep both experiments in the final analysis.

Table 1. **High-resolution respirometry protocols (A and B) and associated respiratory states for muscle fiber mitochondrial respiration experiments.**

	Protocol components (in order of titration)	Respiratory state
A	Palmitoyl-carnitine (0.04 mM) + malate (0.1 M)	LEAK; uncoupled respiration in the presence of long and short chain fatty acid concentration
	Octanoyl-carnitine (0.2 mM)	
	ADP (5 mM)	OXPHOS FA; Fatty acid stimulated respiration
	Cytochrome c (0.01 mM)	CytC; Test of outer mitochondrial membrane integrity
	Malate (5 mM) + Pyruvate (5 mM) + Glutamate (10 mM) + Succinate (10 mM)	OXPHOS CI+CII ; Complex I+II coupled respiration
	Carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP, 0.001 mM)	MAX CI+CII; Maximal uncoupled Complex I and II capacity
	Rotenone (0.005 mM)	MAX CII; Maximum uncoupled Complex II capacity
	Malonate (5 mM) + Antimycine (0.0025 mM)	RESIDUAL; Residual oxygen concentration
B	Pyruvate (5mM) + Malate (5 mM)	LEAK; Complex I uncoupled respiration
	Glutamate (10 mM)	
	ADP (5 mM)	OXPHOS CI; Complex I coupled respiration
	Cytochrome c (0.01 mM)	CytC; Test of outer mitochondrial membrane integrity
	Succinate (10 mM)	OXPHOS CI+CII ; Complex I+II coupled respiration
	Carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP, 0.001 mM)	MAX CI+CII; Maximal uncoupled Complex I and II capacity
	Rotenone (0.005 mM)	MAX CII; Maximum uncoupled Complex II capacity
	Malonate (5 mM) + Antimycine (0.0025 mM)	RESIDUAL; Residual oxygen concentration

We assessed SES *longissimus dorsi* skeletal muscle oxygen consumption *ex vivo* in primary respiratory states, including measurement of LEAK respiration, OXPHOS, and MAX respiratory flux through respiratory complexes I and II (CI+CII). Uncoupled MAX respiration reflects the maximum mitochondrial

respiratory flux (Votion et al., 2012). OXPHOS respiratory capacity represents the portion of MAX capacity composed of both the respiratory flux coupled to mitochondrial oxidative phosphorylation through ATP synthase and non-coupled LEAK respiration. Steady-state respiratory values were corrected for baseline respiratory chamber oxygen flux determined at the beginning of each protocol. Oxygen flux was monitored in real-time following standardized instrumental and chemical background calibrations using Datlab software 7.4.0.4 (Oroboros Instruments).

Protein and RNA extraction

Muscle tissues were processed to extract protein and RNA simultaneously (Khudyakov et al., 2022). Muscle tissue (~30 mg) was isolated from RNA later while frozen and crushed. The tissue was processed using 1 ml of Trizol. RNA and proteins were separated through a chloroform-phase extraction. Proteins were precipitated from the organic phase using isopropanol, and pellets were washed with 0.3 mol/l guanidine hydrochloride in 95% ethanol and stored at -80°C until further processing. RNA was cleaned using RNeasy Mini Kit (Qiagen®). The amount and purity of RNA were measured with a Nanodrop and RNA stored at -80°C until further analysis.

Protein levels – quantitative mass spectrometry-based analysis

Muscle proteome analysis using DIA-based nanoLC-MS/MS

This method provides a robust method for large-scale, high-throughput protein analysis.

Detailed methods are provided in the Supplemental Materials (Supplementary Methods). From the protein extracts of elephant seal muscles, a reference sample comprising an equal protein quantity of all protein extracts was made for quality assessment of LC-MS/MS. Individual samples and the reference sample were electrophoresed using SDS-PAGE gels. A stacked protein band, as well as the part of the gel above this band, were excised from the gel, and proteins were in-gel reduced, alkylated and then digested at 37°C overnight with trypsin (Promega). A set of reference peptides (Indexed Retention Time [iRT] Kit, Biognosys AG) was added to the resulting peptides to allow the stability of instrument performance to be measured for QC purposes. Samples were analyzed on a nano–ultraperformance LC system (nanoElute 1) coupled to a hybrid trapped ion mobility spectrometry – quadrupole time of flight mass spectrometer (TimsTOF Pro 2) (Bruker Daltonics). The TimsTOF Pro 2 mass spectrometer was operated in data-dependent acquisition (DDA)-parallel accumulation-serial fragmentation (PASEF) mode (Meier et al., 2018) or repeated injections of the reference sample, allowing the stability of the system to be assessed throughout the experiment and to optimize the method used in data-independent acquisition PASEF (diaPASEF) mode (Meier et al., 2020) for differential analysis of the muscle proteome between adult and juvenile individuals. Mass spectrometry data acquired in DDA-PASEF mode were processed using Maxquant v2.4.7.03 (Tyanova et al., 2016), whereas those acquired in diaPASEF mode

were processed using directDIA implemented in Spectronaut v18 (Lou et al., 2023). The protein database that we used was obtained from the latest annotation of the *Mirounga leonina* (TaxID 9715) genome in Refseq (Refseq Assembly accession GCF_011800145.1; Assembly Name KU_Mleo_1.0). For DDA-PASEF data, the false discovery rate (FDR) was set to 1% for both peptide and protein spectrum matches. For diaPASEF data, identification was performed using a mutated method for decoy generation with a dynamic limit strategy, and the precursor Qvalue cutoff was set to 1% and the protein Qvalue cutoff to 1% (experiment and run).

Regarding the quantification of DDA-PASEF data, normalization and protein abundance estimation were performed using the label-free quantification (LFQ) option offered in MaxQuant (Cox et al., 2014). Quantification of diaPASEF data was performed using Spectronaut (Lou et al., 2023), using only protein group-specific peptides. The raw files from the analysis in DDA-PASEF mode of the repeated injections of the reference sample were specified to create a hybrid library and analyse accurately diaPASEF data, for which only the proteins quantified in at least four individuals per group were retained. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Perez-Riverol et al., 2022) with the dataset identifier PXD048683.

Data indicated a good reproducibility of quantitative measurements in DDA-PASEF, with a mean CV of 19% for all LFQ values determined from the repeated analysis of this reference sample. QC-related measurements also showed that HPLC performance remained good and stable throughout the whole experiment, with a median coefficient of variation of 0.67% concerning retention times of all iRT peptides when considering all injections.

Functional enrichment and pathway analysis

Hierarchical clustering of proteomics data was performed using Cluster V3.0 software (de Hoon et al., 2004), with the following parameters: median centering and normalization of genes for adjusting data and centroid linkage clustering for both genes and arrays. A dendrogram was generated and viewed using the Java Treeview V1.3.3 program (Page, 1996).

To enable automatic functional annotation of elephant seal proteins, we first identified their human homologs using BLAST searches (FASTA program v36; downloaded from http://fasta.bioch.virginia.edu/fasta_www2/fasta_down.shtml) against Swissprot-derived human protein sequences (20 427 entries, November 2023), combined with a careful examination of protein names and gene symbols. Only the best hits were retained, and inspection of the relevance of the matches one by one led us to reject only three entries out of the 1,708 quantified proteins. Functional annotation enrichment analysis was then performed using human protein identifiers from the list of differentially expressed proteins, including present/absent cases, using the desktop version of DAVID

(Ease V2.0) and an updated (November 2023) version of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases generated using the MSDA software (Carapito et al., 2014). All the Swissprot-derived human protein sequences used previously for BLAST searches were used here as the enrichment background. Enriched GO terms were filtered by only considering those with a Benjamini p-value lower than 0.05 and a fold enrichment higher than 1.5. Enriched GO terms were grouped together into broad functional categories, which were then considered as enriched broad functions. KEGG mapping tools (Kanehisa and Sato, 2019) were used for complementary biological interpretation of our proteomics dataset.

Muscle fiber typing

The proteome analysis allowed us to specify the fiber type profile of our samples through myosin isoform identification. Results are expressed as a percentage of the total myosin isoforms detected. Type I (slow oxidative fibers) were identified through myosin isoform Myh7 (Talmadge and Roy, 1993), and Type IIa (fast oxidative-glycolytic fibers) through myosin isoform Myh2 (Schiaffino and Reggiani, 1994). No Type IIb (fast glycolytic fibers, isoform Myh4) were found in our samples.

Gene expression – real-time reverse-transcription PCR

Five hundred nanograms of RNA were reverse transcribed using the Primescript RT Reagent kit (TAKARA BioEurope SAS) according to the manufacturer's instructions. cDNAs were used to perform real-time RT-PCRs. We investigated different genes involved in metabolic response to hypoxia, oxidative stress, and exercise. We selected a complex I subunit gene expression (NDUFS3), two genes involved in protein turnover (mTOR and Trim63), one related to muscular adaptive response to exercise and oxidative stress (PGC1α), one involved in lipogenesis (ACCb), and the two transcription factors HIF-1α and NRF2. Real-time PCRs were made using primers specially designed for the study (Table S1). Amplification (45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72°C for 45 s) was monitored using SYBR green (GoTag® qPCR kit, Promega, France). Three housekeeping genes were tested: actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and succinate dehydrogenase complex flavoprotein subunit A (SDHA). GeNorm software was used to choose the best internal control, and the combination of ACT/SDHA was kept (M = 0.029). Fold change was calculated by the Pfaffl method (Pfaffl, 2001). All results were obtained in duplicates (CV < 0.5%).

Plasma measurements

Plasma levels of 8-isoprostanes (8-isoPGF2α) were measured in duplicate using a competitive EIA assay kit (Cell BioLabs, San Diego, CA, USA). Plasma protein carbonyls (PC) were also measured in triplicate using commercially available ELISA kits (Cell BioLabs, San Diego, CA, USA,) (Vázquez-Medina et al., 2010).

Total protein content was measured using the BCA Protein Assay (ThermoFischer Scientific, Rockford, USA).

Statistical analysis

The normality of the data distribution and homoscedasticity were checked using the Shapiro-Wilk test ($P>0.01$) and Bartlett test ($P>0.01$), respectively, in the R software environment (v4.2.2) (R Core Team). For the LC-MS/MS analysis, protein abundances in juvenile and adult individuals were compared using Welch two-sample t-tests ($P<0.05$ required to conclude on differential expression levels). Of the differentially expressed proteins ($P<0.05$), subsequent analyses and interpretations took into account only those whose levels differed by at least 40% between the two groups of elephant seals. Results are expressed as mean ± standard error unless otherwise specified. Because of non-normality and heteroscedasticity, high-resolution respirometry, gene expression and oxidative damage results were compared using Wilcoxon tests.

RESULTS

The mean body mass of juvenile females SES (N = 5) was 103.5 ± 6.6 kg, with a mean body length of 140 ± 4.6cm. Adult females (N = 6) were sampled at the end of the lactation period with a mean body mass of 294 ± 19.8 kg, and a body length of 247.5 ± 8.8 cm.

High-resolution respirometry

Muscle mass-specific O_2 fluxes in juveniles and adults are illustrated in Figure 1, based on the sequential addition of each protocol component as described in Table 1. We found no differences in O_2 fluxes between juveniles and adult SES in the presence of either carbohydrate-related or fatty acid-related substrates, indicating a general similarity in mitochondrial functional capacity between the two stages of life. In both protocols, the addition of FCCP (MAX_{CI+CII}) did not increase O_2 fluxes, indicating that the muscle mitochondria of juvenile and adult SES were working at their maximal capacity. However, we observed a greater OXPHOS coupling control factor (OXPHOS-LEAK/OXPHOS) in the presence of fatty acids in the juveniles (0.576 ± 0.030) compared to the adults (0.377 ± 0.090, Wilcoxon test, $W = 3$, $p = 0.03$, Figures 1A and 1C). In adults, LEAK was higher in the presence of fatty acids than carbohydrate-like substrates (5.68 ± 0.75 vs 2.18 ± 0.75, Wilcoxon test, $W = 3$, $p = 0.02$). As our LEAK values were very low, we could not use residual oxygen consumption as a correction factor for all mitochondrial respiratory rates; we are likely at the limit of the sensitivity of the machine. We kept the raw values and reported the residuals (Figure 1).

LC-MS/MS

Southern elephant seals' muscle proteome changes between juveniles and adults

We identified 2,065 proteins in muscle, of which 1,708 could be accurately quantified, and we found significant age effects for the abundance of 270 muscle proteins, with 128 of them being increased and 142 decreased in juvenile SES compared with adults (Figure 2A, Table S2). Three proteins associated with protein turnover and small-molecule transport across the mitochondrial membrane were well detected in adults but remained below our detection limit for juveniles. The same applies to eight proteins related to cell integrity and mobility, cell matricial components, and the immune system, which were only detected in juvenile samples.

The most significant differences observed in muscle protein levels were in the mitochondrial pathways (Figure 2B) with up-regulation in juveniles (Figure 3). NADH dehydrogenase and cytochrome c oxidase of the respiratory electron chain were up-regulated, but there was no difference in citrate synthase. The expression of several proteins involved in the TCA cycle (pyruvate dehydrogenase kinase, fumarate hydratase, and isocitrate dehydrogenase) and fatty acid β-oxidation (ACADM, ECI1, and HCDH) was increased in juveniles. Mitochondrial transporters were also increased in juveniles (M2OM for malate/alpha-oxoglutarate, acylglycerol kinase, and AGK for protein import), as well as mitochondrial biogenesis and morphogenesis (identified by STML2, TMM11), and the energy cell sensor protein kinase AMPK γ1. No differences in glycolysis were, however, observed.

The expression of most of the proteins involved in oxidative defenses was lower in the muscle of the juveniles, including catalase, superoxide dismutase (SOD1), peroxidase (PRDX), glutathione peroxidase 3 (GPx3), one glutathione transferase (GST), thioreductase (TRXR1), and glutathione reductase (GR). Only two other GSTs (kappa and omega) were increased in juveniles. We found no difference in xanthine oxidase, GPx1 and 4, SOD2 and SOD3, or glutathione synthetase. As expected, because such molecules are generally expressed at low levels, Nrf2, the redox-sensitive transcription factor that controls the expression of antioxidant genes, was not detected with mass spectrometry.

Proteins of the HIF-1 signaling pathway involved in the adaptive response to hypoxia were detected. Although HIF1-α itself could not be detected, HIF1N, an HIF1-α inhibitor, was measured; no difference was observed between the juveniles and adults. However, the expression of EGLN1, a cellular oxygen sensor involved in hypoxia-influenced processes known to regulate the HIF1 signaling pathway, was even less pronounced in juveniles.

Finally, we specified muscle fiber types (Type I or IIa) through myosin isoform identification. Both juveniles and adults showed a similar high proportion of Type I fibers (60.9 ± 1.9 % and 67.6 ± 2.8 % , $p=0.09$). The proportion Type IIa were higher in juveniles but considerably low compared to Type I (myosin-2 isoform, 1.4 ± 0.3 % in juveniles and 0.29 ± 0.05 % in adults, $p=0.02$).

Gene expression

Transcript levels were expressed relative to ACT/SDHA and reported in Table 2. Individual values are available in Table S3. We found an increase in gene expression of mitochondrial respiratory chain complex I in juveniles (higher NDUFS3). An up-regulation of the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) was also noted in juveniles. Gene expression of both mTOR and Trim63, related to protein turnover, was increased in adults. No difference was observed between our groups for HIF-1 α, NRF2, and ACCb.

Table 2: Gene expression in juvenile (N=5) and adult (N=6) female southern elephant seals. Gene expression was evaluated by real-time RT-qPCR and normalized to the expression of the reference genes ACT/SDHA. Fold change was calculated by the Pfaffl method. All results were obtained in duplicates (CV < 0,5%). P values are indicated for the comparison of juvenile and adult southern elephant seal transcripts as determined by the Wilcoxon W-test. A positive fold change means an increased gene expression in juveniles compared to adults.

Gene	Fold change (juvenile / adults)	W	P value
NDUFS3	1.59	29	0.009
HIF-1α	-1.10	12	0.662
NRF2	-1.13	12	0.662
ACCb	-1.24	4	0.051
PGC1a	2.28	29	0.009
mTOR	-1.45	2	0.017
Trim63	-2.83	2	0.017

Oxidative damages

Plasma contents of 8-isoPGF2a and protein carbonyls were measured as proxies of oxidative stress (Fig 4). No difference was observed in either 8-isoPGF2a (juveniles: 171 ± 19 ng/ml, adults: 213 ± 10.9 ng/ml, Wilcoxon test, W = 7, p-value = 0.18) or protein carbonyls (juveniles: 6.0 ± 0.3 nmol/mg, adults: 6.0 ± 0.2 nmol/mg, Wilcoxon test, W = 15, p-value = 1.0).

DISCUSSION

SES maintain significant levels of physical activity during deep dives despite limited O_2 availability (Hindell et al., 1991). This ability is supported by a suite of complex behavioral and physiological adaptations (Davis, 2014). The present experimental design compares the metabolic capacity of the swimming muscle in pups and adult SES. Although the single sampling of early-fasting juveniles does not allow us to conclude on the development of the antioxidant defenses and metabolic adjustments needed to cope with breath-hold diving, this study is the first in the southern species to shed light on the ontogenesis of metabolic adaptations to hypoxic conditions

Our results show an important proportion of Type I muscle fibers and a low one of Type IIa in both groups, although more Type IIa were found in juveniles compared to adults. No Type IIb were found in both groups. This is in line with the ontogenetic changes in muscle fibers along the post-weaning already described in NES and weddell seals (Kanatous et al., 2003; Moore et al., 2014). This suggests juveniles' muscle structure was close to adults' when the sampling occurred.

In this study, we found a higher OXPHOS coupling control factor in juveniles in the presence of fatty acids, explained by a higher OXPHOS relative to LEAK capacity. This finding was supported by proteomic data showing an increase of proteins involved in several mitochondrial metabolic pathways in juveniles compared to adult females (beta-oxidation, citrate cycle, mitochondrial imports, and biogenesis). The opposite was found in juvenile and adult male NES, which indicates that a higher coupling control factor in adults was a mitochondrial adaptive response to diving (Chicco et al., 2014). However, the OXPHOS coupling control factor is notably lower for fatty acids in our adult female SES compared to the values reported by others in adult post-breeding male NES (0.3 *versus* 0.8) (Chicco et al., 2014). In our study, the females were lactating, a physiological state associated with a dramatic increase in energy demands for milk production. Consistent with this, studies in rats have reported a reduction in fatty oxidation in skeletal muscles as a potential mechanism to spare fatty acids for milk production (Gutgesell et al., 2009; Xiao et al., 2004). The reduced coupling efficiency observed in our lactating female SES may therefore reflect an adaptative metabolic response to their energy-intensive productive state.

Many lactating mammals increase their energy intake by 60–200% to meet the energy demand of milk production (Gittleman and Thompson, 1988). In contrast, female elephant seals fast during the 3-week nursing period, relying exclusively on fat stores (Crocker et al., 2001). In non-fasting and still-active lactating mammals muscle mitochondrial activity typically remain unchanged (Favorit et al., 2021). However, the limited energy availability in fasting female SES is associated with behavioral and physiological adaptations, such as reduced physical activity. This contrasts with males, that expend significant energy fighting during the breeding season, and growing juveniles that progressively increase activity during the post-weaning period (Lavigne et al., 1986; Modig et al., 1997; Piot et al., 2024). These differences could explain the higher mitochondrial coupling efficiency observed in our juvenile SES, comparable to values reported in recently weaned juvenile NES (Chicco et al., 2014). This is supported by previous studies showing increased PGC1α gene expression and activation of the AMPK pathway in skeletal muscle of juveniles – both being key drivers of the muscular adaptive response to exercise (Baar et al., 2003; Tadaishi et al., 2011). These findings align with our observed up-regulation of mitochondrial energetic pathways, consistent with earlier reports in juvenile NES (Piotrowski et al., 2021). Thus the variations in mitochondrial coupling efficiency across groups may result primarily from differences in

activity levels. Additional evidence comes from studies focusing on molting, another prolonged and energy-intensive on-land period. Molting female NES showed no changes in muscle respiratory capacity before and after molting, despite undergoing prolonged caloric restriction (Wright et al., 2020). However, emerging data suggest that SES females exhibit greater activity during molting than than during lactation (Charlanne et al., 2024), challenging the assumption they fast strictly and remain on land throughout the molting period. This highlights the importance of integrating behavioral monitoring with physiological data to better understand the drivers and implications of metabolic adaptations in this species.

Finally, the observed mitochondrial adaptations may also reflect the effect of fasting or hypoxia, as the AMPK pathway is known to be up-regulated in response to both low energy availability and low oxygen partial pressure (Mungai et al., 2011; Weir et al., 2017). Consequently, the changes in mitochondrial function and energy metabolism observed in juveniles likely result from the combined effects of these physiological constraints. Early exposure to energetically demanding conditions may activate pathways and adaptations that prepare SES juveniles to cope with the constraints of prolonged, deep dives in adulthood.

Our results also show a major contribution of CII to the electron transport chain. In both groups, either through the use of substrate-like fatty acids or carbohydrates, CII accounts for 60 to 80% of the respiratory ratio. The same finding was reported in juvenile and adult NES, but not in pups (Chicco et al., 2014). CII was recently suggested to play a role in the development of the reserve respiratory capacity (RRC), the difference between the maximum respiratory capacity and basal respiratory capacity, known to increase supply when energy demand exceeds reserves (Pfleger et al., 2015). Indeed, CII can mediate a synchronized increase of substrate entry into the citrate cycle and of the electron transport chain activity, known as a potential source of RCC (Bourgeron et al., 1995). In that study, the authors identified CII as a target of different metabolic sensors such as PDH and AMPK, maximizing the cellular RCC and enhancing cell survival after hypoxia (Pfleger et al., 2015). Consequently, both juvenile and adult SES could benefit from a high CII activation as they experience long fasting periods and/or hypoxia exposure. This could explain the observed lower contribution of CII in pups that have not experienced these challenges yet.

Taken together, these data highlight the critical role of mitochondrial respiratory plasticity in adapting to energy imbalances and physiological constraints experienced by diving mammals during their time on land.

Cellular and molecular responses involved in the seal's adaptations to low O_2 availability and protection against ischemia/reperfusion are well described. HIF-1 controls the adaptive response to hypoxia by stimulating many physiological responses, such as erythropoiesis, angiogenesis, or myoglobin level (Johnson et al., 2004; Johnson et al., 2005; Vázquez-Medina et al., 2011c). No difference was found

in HIF-1α gene expression between juveniles and adults, and HIF-1α factor was not detected at the protein level. However, the HIF pathway involves several molecular interactions likely responsible for the degradation of HIF-1α by intracellular proteases, highlighting the complex regulation of this adaptive response. In our proteomic analysis, several markers involved in HIF-1α pathway were down-regulated in juveniles (HIF-N, EGLN1). This is consistent with a progressive increase in the diving performance and aerobic capacity of adult muscles, as already reported in post-weaned seals (NES (Somo et al., 2015); weddell seals (Kanatous et al., 2008); Antarctic fur seals (LaRosa et al., 2012)). An increase of HIF-1α was reported in NES pups from the seventh week of the post-weaning period (Soñanez-Organis et al., 2013). Because our juveniles were sampled early during the post-weaning period, they may not have been yet sufficiently exposed to short apnea episodes to stimulate this pathway.

The adaptive response to oxidative stress consists of upregulating antioxidant genes in response to increased oxidant production, mediated through the Nrf2 transcription factor. An increase in antioxidant defenses and Nrf2 abundance was previously reported in NES after a voluntary submersion (Vázquez-Medina et al., 2011c). In our study, we could not detect Nrf2 at the protein level, and no difference in Nrf2 gene expression was found. In contrast, we found that antioxidant defenses, such as SOD, PRDX, Gpx, and catalase, were up-regulated in adults compared to juveniles. Similarly, markers of protein turnover, such as mRNA levels of mTOR and Trim63, were higher in adults, potentially reflecting mechanisms for the removal of oxidized soluble cell proteins (Rivett, 1985). This age-related increase in muscle expression of antioxidant-related genes is consistent with previous findings reported in NES (Crocker et al., 2016; Piotrowski et al., 2021) and likely reflects the more pronounced oxidative stress experienced by adults. Despite these differences, we found no difference in oxidative damage to lipids and proteins between groups (Fig 4) (Levine et al., 1994; Roberts and Morrow, 2000). These results align with previous studies suggesting that seals experience minimal oxidative damage despite repeated exposure to ischemia-reperfusion, likely thanks to robust antioxidant defenses (Murphy and Hochachka, 1981; Vázquez-Medina et al., 2006; Vázquez-Medina et al., 2011c; Wilhelm Filho et al., 2002; Wright et al., 2020; Zenteno-Savín et al., 2002).

Finally, oxidative stress is also a direct consequence of prolonged fasting, as it is associated with increased activity of xanthine oxidase, a pro-oxidant enzyme (Hille and Nishino, 1995). Moreover, oxidative stress has been identified as a potential physiological cost of reproductive effort (Monaghan et al., 2009; Speakman and Garratt, 2014). Supporting this, female NES exhibited elevated oxidative damage to proteins after 4 weeks of lactation, accompanied by increased plasma xanthine oxidase, catalase and GPX activity (Sharick et al., 2015). In our study, the females were 3 weeks into their lactation fast, while the juveniles had been fasting for approximately 2 weeks. Therefore, the oxidative damage and changes in antioxidant enzyme activity in this experimental design likely reflect not only the impact of hypoxia exposure but also the combined physiological demands of fasting and lactation.

In summary, this study highlights muscle metabolism and functional capacity according to age and constraints experienced by the animals. Muscle mitochondrial plasticity likely allows animals to meet the energetic demand on land. Hypoxia adaptive response and maturation of the antioxidant system are higher in adults, probably reflecting an adaptive response to regular constraints exposure. These results suggest the combination of fasting, physical activity, and short-term low O_2 partial pressure exposure could contribute to acclimatize juveniles to aquatic life. As young animals only perform short at-sea travels and apneas compared to those during their first and subsequent long offshore trips, a long post-weaning fast could be mostly required to activate these adaptations rather than intense physical training to perform extreme dives. Further studies investigating both physiological and behavioral adaptations during post-weaning, as well as diving behavior during their first year at sea, may reveal additional information on the development of diving abilities and the impact of first-year at-sea survival in this species.

LIST OF ABBREVIATIONS

EGLN1	Hypoxia-inducible factor prolyl hydroxylase 2
8-isoPGF2a	8-isoprostanes
GSH	Glutathione
GPx	Glutathione peroxidase
H_2O_2	hydrogen peroxide
HIF-1α	hypoxia inducible factor
Nox4	NADPH oxidase
Nrf2	NF-E2-related factor 2
PC	Protein carbonyls
PRDX	Peroxidase
SOD	Superoxide dismutase

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Competing interest

The authors declare no competing or financial interests.

Author contributions :

C. G., A. B., A. A., and L. C. conceived and designed the overall study. L. C. and L. H. collected the data. A. G. and F. B. made the LC-MS/MS analyses. S. Z. designed the primers and made the genomic analyses. L. C. and I. C. performed the molecular analyses on oxidative damages. L. C. performed the statistical analyses and drafted the manuscript. All authors reviewed and agreed on the content of the manuscript.

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Data and code availability:

Data and R-code are available on figshare at 10.6084/m9.figshare.26403949

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Figure legends

Figure 1. Mitochondrial respiratory flux in juvenile (N = 5) and adult (N = 6) southern elephant seals's muscle: A. Protocol A (fatty acids). B. Protocol B (carbohydrates) and respiratory ratios (C. Protocol A, D. Protocol B). Ratios of respiratory rates were calculated to assess the OXPHOS coupling control factor, calculated as ($OXPHOS_{CI}$-LEAK)/$OXPHOS_{CI}$, the contribution of Complex I+II coupled respiration to Maximal non-coupled Complex I+II respiration ($OXPHOS_{CI+CII}$/MAX_{CI+CII}), and the relative contribution of Maximal non-coupled Complex II respiration capacity to Maximal non-coupled Complex I+II respiration (MAX_{CII}/ MAX_{CI+CII}). Statistical significance (*p<0.05, Wilcoxon test). Results are expressed as mean ± standard error.

Figure 2. Overview of SES muscle proteomic between juvenile and adult SES. Changes in the proteome of the *longissimus dorsi* muscle between juveniles (N = 5) and adults (N = 6) are shown as heat maps of

differentially expressed proteins that were produced by hierarchical clustering (A). Signal values between groups were successfully discriminated (green, black, and red boxes represent downregulated, intermediate, and upregulated proteins, respectively). Functional annotation analysis from differential proteins revealed enriched Gene Ontology terms, which allowed the determination of broad functions significantly affected by age, i.e., regular exposition to stressful events, physical activity, fasting (B; filled circles represent the broad functions depicted by proteins that are discussed in this paper).

Figure 3. Regulation of key metabolic pathways in the *longissimus dorsi* muscle of juveniles (N = 5) and adult SES (N = 6). The relative abundance is shown using the following color code: significantly (Welch student and paired Student tests; $P < 0.05$) up-regulated proteins in juveniles are shown in dark orange boxes with white text. Down-regulated proteins in juveniles are in light orange boxes with black text. Blue boxes show proteins that did not change, and black boxes show undetected proteins. (A) Mitochondrial pathways. (B) Schematic representation of the proposed mechanisms that activate protective responses to oxidative stress, hypoxia, calorie restriction, and / or physical activity.

Figure 4: cellular oxidative damage in adult (N = 6) and juvenile (N = 5) female southern elephant seals. (A) plasma concentration of isoprostanes (8-isoPGF2α). (B) Plasma concentration of protein carbonyls (nmol per mg proteins). No difference was observed in either 8-isoPGF2a (Wilcoxon test, W = 7, p-value = 0.18) or protein carbonyls (Wilcoxon test, W = 15, p-value = 1.0). Results are expressed as mean ± standard error.

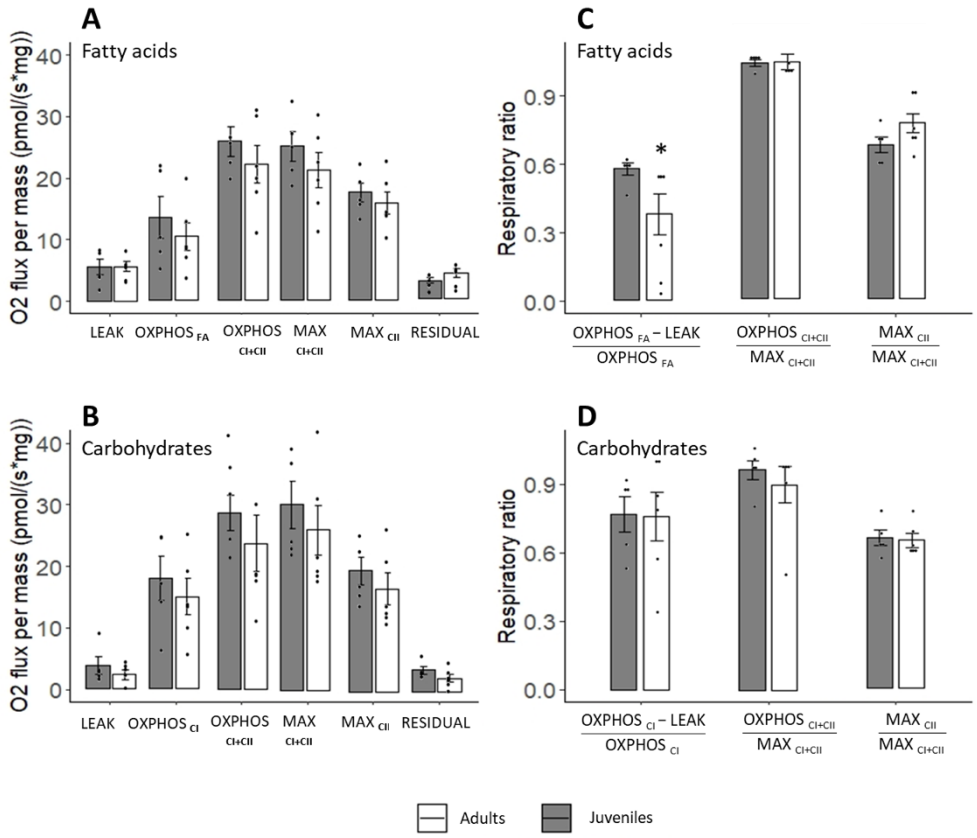

A
Fatty acids
O2 flux per mass (pmol/(s*mg))
LEAK
OXPHOS FA
OXPHOS CI+CII
MAX CI+CII
MAX CII
RESIDUAL
C
Fatty acids
Respiratory ratio
OXPHOS FA – LEAK / OXPHOS FA
OXPHOS CI+CII / MAX CI+CII
MAX CII / MAX CI+CII
B
Carbohydrates
O2 flux per mass (pmol/(s*mg))
LEAK
OXPHOS CI
OXPHOS CI+CII
MAX CI+CII
MAX CII
RESIDUAL
D
Carbohydrates
Respiratory ratio
OXPHOS CI – LEAK / OXPHOS CI
OXPHOS CI+CII / MAX CI+CII
MAX CII / MAX CI+CII
Adults
Juveniles

Figure 1

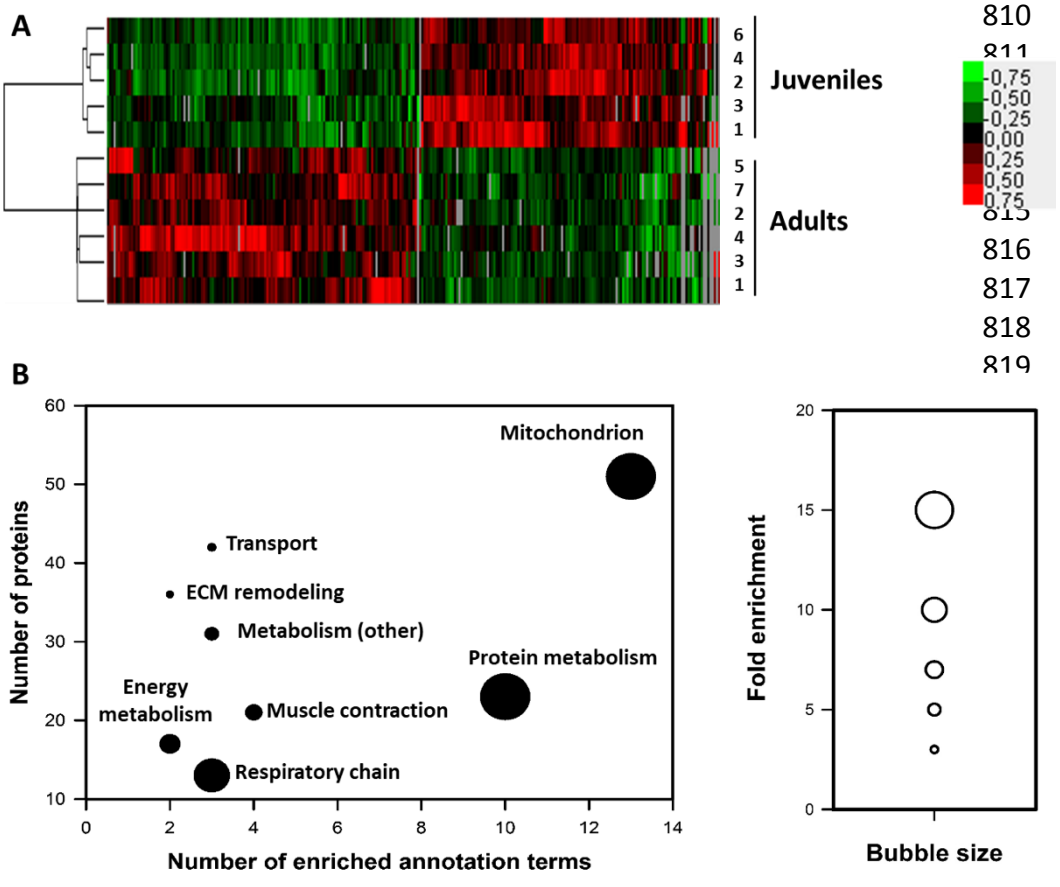
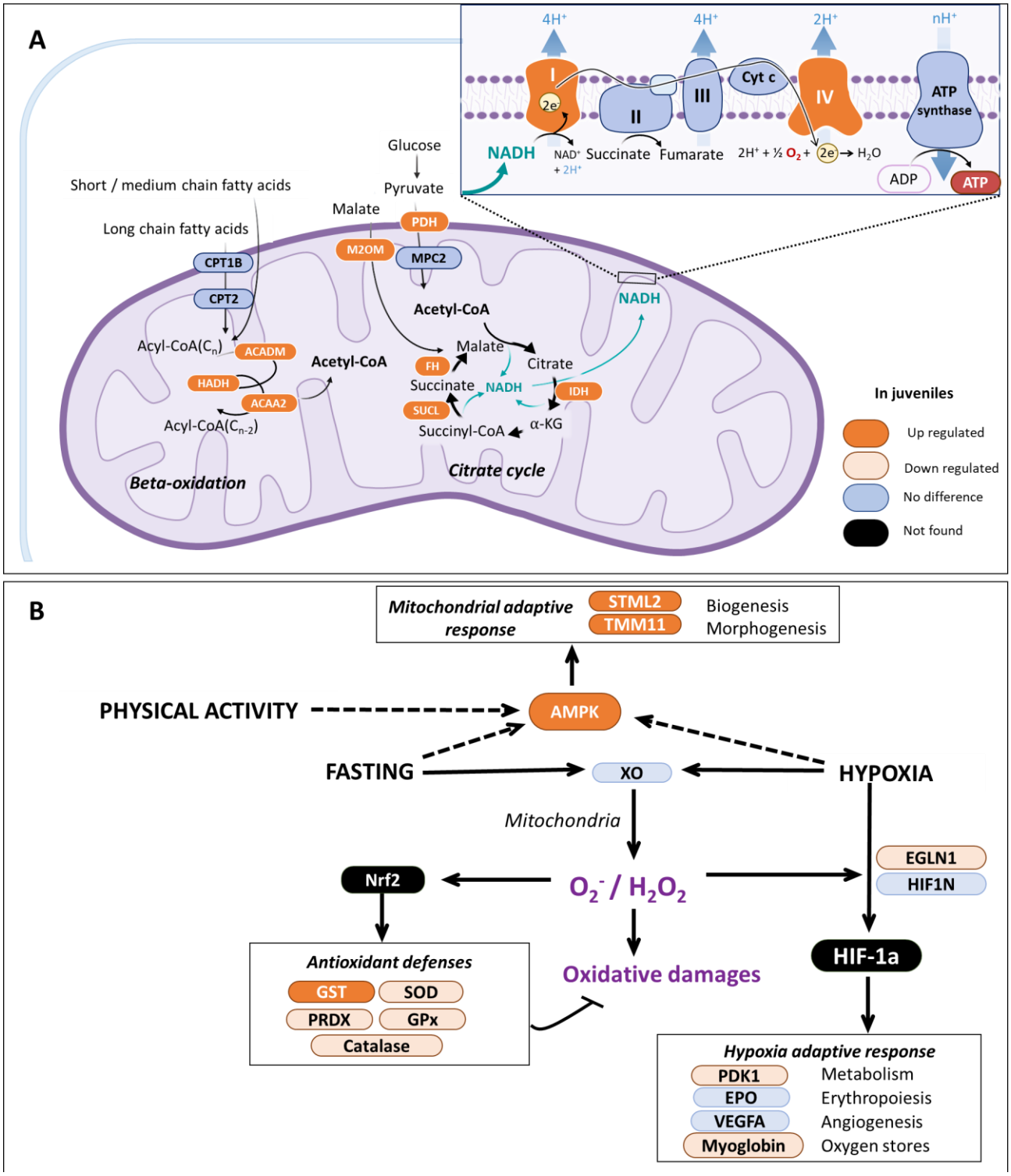

A
Juveniles
Adults
B
Mitochondrion
Transport
ECM remodeling
Metabolism (other)
Protein metabolism
Energy metabolism
Muscle contraction
Respiratory chain
Number of proteins
Number of enriched annotation terms
Fold enrichment
Bubble size

Figure 2

Figure 3


A
4H+
4H+
2H+
nH+
I
II
III
IV
Cyt c
ATP synthase
2e-
NADH
NAD+ + 2H+
Succinate
Fumarate
2H+ + ½ O2 + 2e- → H2O
ADP
ATP
Glucose
Pyruvate
Malate
PDH
M2OM
MPC2
Short / medium chain fatty acids
Long chain fatty acids
CPT1B
CPT2
Acyl-CoA(Cn)
ACADM
HADH
ACAA2
Acyl-CoA(Cn-2)
Acetyl-CoA
Acetyl-CoA
Malate
FH
Citrate
Succinate
NADH
IDH
SUCL
Succinyl-CoA
α-KG
NADH
Beta-oxidation
Citrate cycle
In juveniles
Up regulated
Down regulated
No difference
Not found
B
Mitochondrial adaptive response
STML2
TMM11
Biogenesis
Morphogenesis
PHYSICAL ACTIVITY
AMPK
FASTING
XO
HYPOXIA
Mitochondria
Nrf2
O2- / H2O2
EGLN1
HIF1N
HIF-1a
Antioxidant defenses
GST
SOD
PRDX
GPx
Catalase
Oxidative damages
Hypoxia adaptive response
PDK1
Metabolism
EPO
Erythropoiesis
VEGFA
Angiogenesis
Myoglobin
Oxygen stores

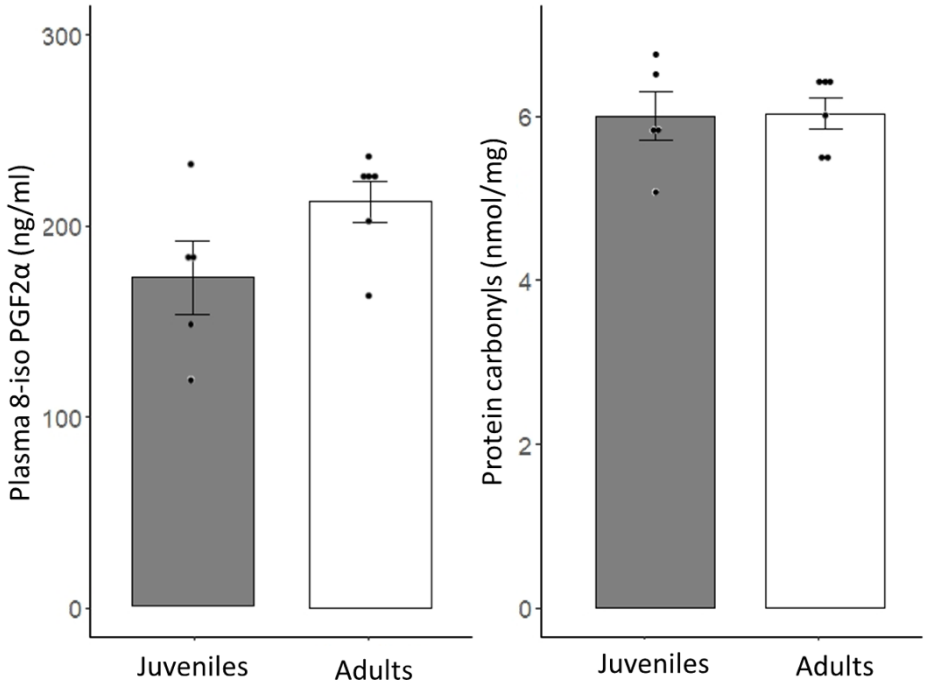

Plasma 8-iso PGF2α (ng/ml)
300
200
100
0
Juveniles
Adults
Protein carbonyls (nmol/mg)
6
4
2
0
Juveniles
Adults

Figure 4