

RESEARCH ARTICLE

Antioxidant capacity develops with maturation in the deep-diving hooded seal

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SUMMARY

Maturation in hooded seals is characterized by the rapid development of their physiological diving capacity and is accompanied by increases in oxidant production but not oxidative damage. To test the hypothesis that the antioxidant system of hooded seals develops as they transition from a terrestrial to an aquatic environment, we obtained the complete cDNA sequence that encodes the NF-E2-related factor 2 (Nrf2), a central regulator of the antioxidant response, and compared Nrf2 mRNA and protein expression levels in muscle samples from neonate, weaned pups and adult hooded seals, along with glutathione (GSH) levels and the activity/protein content of the antioxidant enzymes catalase, glutathione peroxidase (GPx), peroxiredoxin VI (PrxVI), thioredoxin 1 (Trx1), thioredoxin reductase (TrxR), glutaredoxin 1 (Glrx1), glutathione disulphide reductase, glutathione S-transferase and glutamate-cysteine ligase. The Nrf2 of the hooded seal is 1822 bp long and encodes a protein of 606 amino acids with a leucine zipper domain and Keap1-mediated proteosomal degradation residues, which are key for Nrf2 function and regulation. Although neither Nrf2 mRNA nor Nrf2 nuclear protein content are higher in adults than in pups, GSH levels along with GPx, PrxVI, Trx1, TrxR and Glrx1 activity/protein content increase with maturation, suggesting that the potential for peroxide removal increases with development in hooded seals, and that these enzymes contribute to the regulation of the intracellular redox state and the prevention of oxidative damage in these deep-diving mammals.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/214/17/2903/DC1>

Key words: antioxidant, ischemia, reperfusion, maturation, seal.

INTRODUCTION

While diving, seals experience bradycardia and peripheral vasoconstriction to maximize the use of their oxygen stores and are consequently exposed to prolonged ischemia and hypoxemia (Elsner, 1999; Kooyman and Ponganis, 1998; Meir et al., 2009). Ischemia/reperfusion and blood reoxygenation after hypoxemia increase oxidant production and oxidative stress because ATP degradation during ischemia and hypoxemia results in the accumulation of hypoxanthine (HX) and in the proteolytic conversion of xanthine dehydrogenase to xanthine oxidase (XO). Upon reperfusion/reoxygenation, XO is able to reduce HX, generating superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (Kuppusamy and Zweier, 1989; McCord, 1985; Thompson-Gorman and Zweier, 1990).

Although they are routinely exposed to diving-induced hypoxemia and ischemia/reperfusion, seals do not show higher basal levels of oxidative damage than terrestrial mammals (Vázquez-Medina et al., 2007; Wilhelm Filho et al., 2002; Zenteno-Savín et al., 2002). Interestingly, seal tissues do accumulate HX after *in vitro* exposure to ischemia (Elsner et al., 1998). Moreover, the basal capacity of tissue to produce $O_2^{\bullet-}$ is higher in seals than in non-diving mammals, and $O_2^{\bullet-}$ production, but not oxidative damage, is higher in seal than in terrestrial mammal tissues when exposed to an oxidant-generating system (Zenteno-Savín et al., 2002). The latter suggests that seals, as well as other diving vertebrates, are constantly exposed

to diving-induced oxidant production (Furtado-Filho et al., 2007; Valdivia et al., 2007; Zenteno-Savín et al., 2010). Seals, however, have higher activities of several antioxidant enzymes and higher glutathione (GSH) content than terrestrial mammals, which likely helps them to cope with the potentially damaging effects of diving-induced hypoxemia and ischemia/reperfusion (Elsner et al., 1998; Murphy and Hochachka, 1981; Vázquez-Medina et al., 2006; Vázquez-Medina et al., 2007; Wilhelm Filho et al., 2002).

In animal cells, enzymatic and non-enzymatic antioxidants scavenge $O_2^{\bullet-}$, H_2O_2 and lipid hydroperoxides, preventing oxidative damage. Superoxide dismutases (SODs) transform $O_2^{\bullet-}$ into H_2O_2 whereas catalase, glutathione peroxidases (GPxs), glutathione-S-transferases (GSTs) and peroxiredoxins (Prxs) remove H_2O_2 and lipid hydroperoxides. GPx, GST and Prx use GSH in their catalytic reactions, yielding glutathione disulphide (GSSG). GSH is a non-enzymatic antioxidant thiol synthesized in a two-step enzymatic process by glutamate-cysteine ligase (GCL) and glutathione synthase. GSSG is reduced to GSH by glutathione disulphide reductase (GR) but can also react with protein thiol groups causing protein S-glutathionylation, a process that is reversed by glutaredoxins (Glrxs). Thioredoxins (Trxs) can also help to reduce protein disulphide bridges by oxidizing themselves. Thioredoxin reductase (TrxR) then reduces oxidized Trx, maintaining intracellular balance (Halliwell and Gutteridge, 2007). The GSH, Glrx and Trx systems are thus key modulators of the intracellular

redox state, which controls the activation of the NF-E2-related factor 2 (Nrf2), a central regulator of the antioxidant response (Jaiswal, 2004).

Nrf2 induces the transcription of genes involved in antioxidant defense, such as GST, GCL, catalase and heme oxygenase (Immenschuh and Baumgart-Vogt, 2005; Jaiswal, 2004). Nrf2 is, under unaltered conditions, bound to its repressor protein Keap1, which targets its ubiquitin conjugation and, thus, its proteosomal degradation (Itoh et al., 1997). Oxidative/electrophilic stress causes the modification of the Cys273 and Cys288 residues in Keap1, inhibiting Nrf2 ubiquitination and promoting its translocation, nuclear accumulation and binding to the antioxidant response element (Bloom and Jaiswal, 2003; Kobayashi et al., 2004; Kobayashi et al., 2006; Zhang and Hannink, 2003).

It is well established that the diving capacity of hooded seals [*Cystophora cristata* (Erleben 1777)] develops with maturation (Burns et al., 2007; Burns et al., 2010; Folkow et al., 2010; Lestyk et al., 2009). It is not known, however, whether their antioxidant system develops as they transition from a terrestrial to an aquatic environment. We have previously shown that maturation increases $O_2^{\cdot-}$ production without increasing oxidative damage to lipids, proteins or DNA in hooded seals (Vázquez-Medina et al., 2011a). In the present study, we compared muscle GSH levels and antioxidant enzyme activities and/or protein content among three age groups of hooded seals to test the hypothesis that the post-natal development of the hooded seal is accompanied by increases in GSH and upregulation of antioxidant enzymes. We also cloned the full-length cDNA sequence of hooded seal Nrf2 and quantified its expression, at transcript and protein levels, along with Nrf2 nuclear content in muscle samples from newborn, weaned and adult hooded seals, in an effort to elucidate its potential role in the development of the seal's antioxidant system.

MATERIALS AND METHODS

Animal handling and sample collection

Animal capture and handling protocols were reviewed and approved by the Canadian Department of Fisheries and Oceans (DFO) and the University of Alaska Anchorage IACUC Committees. Samples were imported into the United States under Marine Mammal permits 782-1399 and 782-1694-02.

Fifteen healthy hooded seals were captured on the pack ice near the Magdalen Islands, Gulf of St Lawrence, Canada ($\sim 47^{\circ}23'N$, $61^{\circ}52'W$). Animals were categorized visually as adults (five reproductive females, mean body mass: 249.3 ± 25.2 kg) or pups. Pups were categorized as neonates (three males and two females, mean body mass: 21.6 ± 3.3 kg, age=1 day) or weaners (three females and two males, mean body mass: 48.1 ± 4.5 kg, age=5–14 days) following Bowen et al. (Bowen et al., 1987). Seals were physically restrained, drugged with diazepam (0.2 – 0.3 mg kg $^{-1}$) or tiletamine-zolazepam (0.7 – 1.0 mg kg $^{-1}$) and killed using methods approved for scientific collection by DFO. Tissue sample collection was performed in parallel with other studies to maximize the use of the samples obtained (Burns et al., 2007; Burns et al., 2010; Lestyk et al., 2009). Samples of longissimus dorsi muscle (ca. 2 g) were placed in cryogenic vials, fast-frozen in liquid nitrogen and stored at $-80^{\circ}C$ until analyzed.

Western blot

Frozen tissue samples were homogenized 1:10 (w/v) in 50 mmol l $^{-1}$ potassium phosphate buffer containing 1 mmol l $^{-1}$ EDTA, 1% Triton X-100, 1% phenylmethylsulfonyl fluoride (PMSF) and 1% protease inhibitor cocktail (Sigma, St Louis, MO, USA) (crude extracts), or

using the NE-PER nuclear protein extraction kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions (nuclear fractions). Total protein content in crude extracts and nuclear fractions was measured using the Bio-Rad Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). One independent crude extract and one nuclear fraction were prepared from each of the individual samples available and used for the western blot experiments ($N=5$ per group). Either 20 μ g of total or 10 μ g of nuclear protein were mixed with sodium dodecyl sulfate Laemmli sample buffer, boiled and resolved in either 4–15% or 10–20% (for Glrx1 and Trx1 analyses) Tris-HCl gradient gels under denaturing conditions. Electrophoresis was performed for 65 min at 100–120 V. Proteins were electroblotted for 25 min at 25 V using the Bio-Rad Trans Blot SD semi-dry cell onto 0.45 μ m nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) containing 0.05% of Tween 20 (PBS-T), and incubated overnight with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against mammalian GCLm (sc-22754), Glrx1 (sc-32943), Trx1 (sc-18215), PrxVI (sc-134478) or Nrf2 (H-300) (sc-13032) diluted 1:500–1:3000 in PBS-T with 1% BSA. Membranes were washed, incubated for 1 h at room temperature with IgG horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) diluted 1:5000, re-washed and developed using the Immun-Star Western C kit (Bio-Rad). Blots were visualized using a Chemi-Doc XRS system (Bio-Rad) and quantified using Bio-Rad's Quantity One software. Results were expressed as percent change from neonates. Actin and lamin were used as loading controls for crude extracts and Nrf2 nuclear protein content, respectively. An immunizing peptide (Santa Cruz Biotechnology, sc-18215P) was used to validate Trx1 measurements as multiple bands were detected in the proximity of the predicted molecular weight of the protein of interest.

Glutathione assay

Frozen tissue samples ($N=5$ per group) were independently homogenized (1:10 w/v) in 5% ice-cold sulfosalicylic acid previously bubbled with nitrogen gas for 10 min to prevent artifactual GSH oxidation during the extraction procedure. Tissue extracts were bubbled with nitrogen gas for 10 s and centrifuged at 19,000 g at $4^{\circ}C$ for 5 min. Supernatants were used immediately to measure total glutathione content (GSH-Eq=GSH+2GSSG) by following the enzymatic recycling method of Griffith (Griffith, 1980) with minor modifications as previously described (Vázquez-Medina et al., 2007). Results are expressed in nmol GSH-Eq g $^{-1}$ wet tissue.

Enzyme activities

Frozen tissue samples (50 mg) from each individual were independently homogenized (1:20 w/v) in 50 mmol l $^{-1}$ potassium phosphate buffer containing 1 mmol l $^{-1}$ EDTA, 1% Triton X-100,

Table 1. Primers used for cDNA cloning and qRT-PCR

Primer name	Sequence (5'–3')
Nrf2Fw1	CATGATGGACTTGGAGTTGCC
Nrf2Fw4	CTTAGGGCAAAAGCTCTCC
ccNrf2Fw1	GGAAGTGAACAAGATTTGG
ccNrf2Rv1	CACAAGGAATACATTGCCATC
NrfRv4	CACATCACAGTAGGAAGTTTATG
Nrf2Rv3	CTAGTTTTCTTTGTATCTGGC
GAPDHFW	CAGAACATCATCCCTGCCTC
GAPDHRV	CTGCTTCACCACCTTCTTGA

1% PMSF and 1% protease inhibitor cocktail (Sigma). Homogenates were centrifuged at 2000g for 20 min at 4°C. Supernatants were taken and used immediately for antioxidant enzyme activity analyses. Catalase, GPx, GST and GR activities were measured as described previously (Vázquez-Medina et al., 2006; Vázquez-Medina et al., 2007). TrxR activity was measured using a commercially available kit (Cayman Chemical, Ann Arbor, MI, USA). Results are expressed as mU mg⁻¹ protein. Total protein content in tissue homogenates was measured with the Bio-Rad Bradford protein assay.

cDNA cloning and mRNA expression of Nrf2

Total RNA was isolated from each frozen muscle sample available (*N*=5 per group) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA integrity was confirmed by measuring the ratio of absorbance at 260 nm/280 nm and by 1% agarose gel electrophoresis (Sambrook and Russell, 2001). First-strand cDNAs were reverse-transcribed from total DNA-free RNA (1 µg) using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA) and oligo-dT (0.5 µg). Annealing and extension steps were performed at 42°C for 30 min and 95°C for 3 min, respectively.

A full-length sequence that encodes for Nrf2 was obtained using primers designed based on mammalian Nrf2 nucleotide sequences. Initial PCR reactions (PCR1) were performed with Nrf2Fw1 + Nrf2Rv4 primers under the following conditions: 26 µl of Platinum PCR SuperMix (Invitrogen), 3 µl of cDNA and 1 µl (20 µmol l⁻¹) of each primer. The Nrf2 5'- and 3'-end extremes were obtained using Nrf2Fw1 + Nrf2Rv3 and Nrf2Fw4 + Nrf2Rv3 primers, respectively (Table 1), under the following conditions: 26 µl of Platinum PCR SuperMix (Invitrogen), 2.5 µl of PCR1 and 1 µl (20 µmol l⁻¹) of each primer. Amplification conditions consisted of one 3 min cycle at 94°C, and 40 cycles at 94°C for 40 s, 55°C for 60 s and 68°C for 120 s, and an overextension step at 68°C for 10 min. PCR fragments of ~900 bp (*Nrf25'*) and 1400 bp (*Nrf23'*) were obtained for each reaction and cloned using the pGEM-T Easy Vector System (Promega Corporation, Fitchburg, WI, USA). Sequences were identified as Nrf2 by comparing them with GenBank data using the Blast algorithm (Altschul et al., 1990). A full-length sequence of 1822 bp (*ccNrf2*) that encodes for Nrf2 was obtained by overlapping *Nrf25'* and *Nrf23'* sequences. Predicted amino acid sequences were obtained using a free-access translation program (<http://arbl.cvmbs.colostate.edu/molkit/translate/>) and aligned with other Nrf2 sequences using Clustal W (Thompson et al., 1994).

Nrf2 mRNA expression was quantified using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal standard (GeneBank accession number NM_002046). Nrf2 and GAPDH transcripts were measured by quantitative reverse transcription PCR (qRT-PCR) using *ccNrf2Fw1* + *ccNrf2Rv1* and GAPDHFw + GAPDHRv primers (Table 1). Two PCR reactions per sample corresponding to each available individual (*N*=5 per group) were run for qRT-PCR on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Final PCR reactions consisted of 10 µl SYBR Green PCR Master Mix (Applied Biosystems), 6 µl H₂O, 0.5 µl of each primer (20 µmol l⁻¹) and 3 µl cDNA (150 ng of total RNA). After an initial denaturing step at 95°C for 5 min, amplifications were performed for 40 cycles at 95°C for 30 s, 60°C for 35 s and a final step of 55 s at 72°C, with a single fluorescence measurement and a final melting curve program decreasing 0.3°C every 20 s from 95 to 60°C. Positive and negative controls were included. Standard curves of Nrf2 and GAPDH were run to determine amplification efficiency, which

was 99.2% for Nrf2 and 99.5% for GAPDH, using dilutions from 5 × 10⁻⁴ to 5 × 10⁻⁹ ng µl⁻¹ of PCR fragments.

Statistics

Differences among age groups were detected by one-way ANOVA with Bonferroni *post hoc* tests. Groups were considered statistically different at *P*<0.05. Statistical analyses were performed using the SYSTAT[®] 12.0 software (Cranes Software International Ltd, Richmond, CA, USA). Results are presented as means ± s.e.m.

RESULTS

Protein detection using antibodies

For hooded seal GCLM, a single band was observed at ~28 kDa. For PrxVI, a single band of ~25 kDa was detected (supplementary material Fig. S1). Using the Nrf2 (H-300) antibody, which maps near the N-terminus of human Nrf2, a single band was detected at ~68 kDa. In the nuclear extracts, additional weaker bands of approximately 37 and >100 kDa were observed (supplementary material Fig. S1). A single band at ~15 kDa was also detected with the Glrx1 (FL-106) antibody, whereas multiple bands were observed at ~75 kDa and above 25 and 15 kDa using the Trx1 (N-20) antibody. The use of an immunization peptide revealed that the band at ~12 kDa is Trx1 (supplementary material Fig. S2). All the observed bands indicate that the approximate molecular weight of each of the studied hooded seal proteins is very similar to that of human, bovine, rodent and porcine proteins (Swiss-Prot Protein knowledgebase).

Maturation increases GSH and peroxide-removing enzymes in the muscle of hooded seals

GSH-Eq, PrxVI expression, GPx and catalase activities were measured in the skeletal muscle of newborn, weaned and adult hooded seals to determine whether maturation increases GSH and peroxide-removing enzymes in these mammals during their transition from land to aquatic lifestyles. GSH content was higher (*P*<0.05) in adults (647±47 nmol g⁻¹ wet tissue) than in newborns (313±61 nmol g⁻¹ wet tissue) (Fig. 1A). PrxVI protein expression was twofold higher (*P*<0.01) in adults (238±38%) than in neonates (100±15%) or weaned pups (101±16%) (Fig. 1B) whereas GPx activity was more than fourfold higher (*P*<0.001) in adults (223±7 mU mg⁻¹ protein) than in neonates (47±3 mU mg⁻¹ protein) or weaned pups (51±3 mU mg⁻¹ protein) (Fig. 1C). Catalase activity was higher (*P*<0.05) in weaned pups (108±15 U mg⁻¹ protein) than in neonates (54±11 U mg⁻¹ protein) or adults (36±10 U mg⁻¹ protein) (Fig. 1D).

Trx, TrxR and Glrx increase with maturation in hooded seals

GR and TrxR activities, as well as Trx1 and Glrx1 protein expression, were measured in newborn, weaned and adult hooded seals to determine whether maturation increases these redox regulators. TrxR activity was higher (*P*=0.034) in adults (2.5±0.4 mU mg⁻¹ protein) than in neonates (1.0±0.2 mU mg⁻¹ protein) (Fig. 2A) whereas GR activity was higher (*P*<0.001) in weaned pups (7.4±0.3 mU mg⁻¹ protein) than in neonates (5.1±0.4 mU mg⁻¹ protein) or adults (4.9±0.3 mU mg⁻¹ protein) (Fig. 2B). Trx1 protein expression was 30–40% higher (*P*<0.05) in weaned pups and adults than in neonates (Fig. 2C) whereas Glrx1 protein content was 50% higher (*P*<0.05) in adults than in neonates (Fig. 2D). Taken together, these results suggest that the antioxidant capacity to remove H₂O₂ develops with maturation in hooded seals.

Nrf2 coding sequence of the hooded seal

The complete Nrf2 coding sequence from *C. cristata* (*ccNrf2*; GenBank accession no. JF429697) was obtained, cloned and

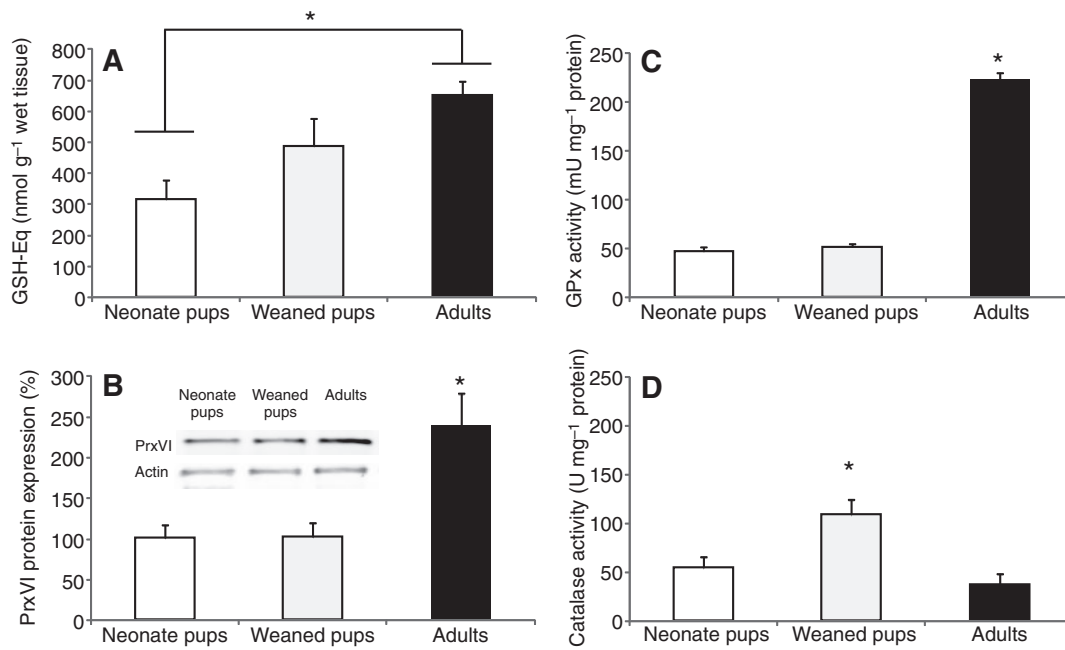


Fig. 1. Maturation increases glutathione content and peroxide-removing enzymes in skeletal muscle of hooded seals. (A) Total glutathione content (GSH-Eq), (B) 1-cys peroxiredoxin (PrxVI) protein expression, (C) glutathione peroxidase (GPx) and (D) catalase activities in neonates, weaned pups and adult hooded seals. One unit of GPx is defined as the amount of enzyme that oxidizes 1 μ mol NADPH min⁻¹ at 25°C. One unit of catalase is defined as the amount of enzyme needed to reduce 1 μ mol H₂O₂ min⁻¹ at 25°C. Data are means \pm s.e.m. *, $P < 0.01$.

sequenced from muscle cDNA. The *ccNrf2* sequence is 1822 bp long and encodes a polypeptide of 606 amino acids with a predicted molecular weight of 67.6 kDa, which is similar to other mammalian homologs (Fig. 3). The *ccNrf2* predicted amino acid sequence shows high identity to Nrf2 of the giant panda (*Ailuropoda melanoleuca*, 97%), the domestic dog (*Canis lupus familiaris*, 95%), the wild boar (*Sus scrofa*, 90%), humans (*Homo sapiens*, 90%) and the rat (*Rattus norvegicus*, 80%). The conserved leucine zipper domain is located in the N terminus (residues 520 to 606) and includes the key residues for nuclear export signal (Leu554, -558,

-560 and -562) and nuclear location signal (Lys597, -599 and -600; Ser598). Also, *ccNrf2* has the conserved N-terminal domain (Leu23 and -30; Gln26; Asp27, -77 and -29; Gly30 and -81; Glu57, -79 and -82; Thr80) essential for the Keap1-mediated degradation of Nrf2 by the proteosomal system (Fig. 3).

Nrf2 and its downstream genes during post-natal development in hooded seals

Nrf2 mRNA expression levels and protein content was measured in crude extracts and nuclear fractions to determine whether

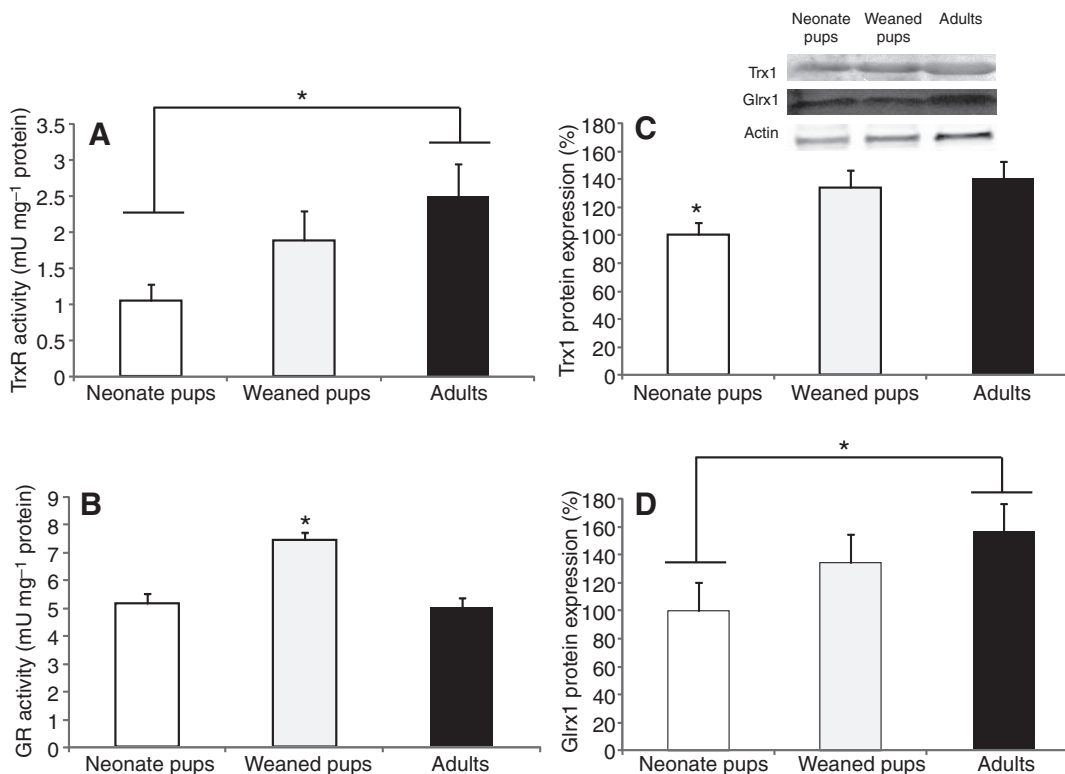


Fig. 2. Thioredoxin, thioredoxin reductase and glutaredoxin increase with maturation in hooded seals. (A) Thioredoxin reductase (TrxR) activity, (B) glutathione disulphide reductase (GR) activity, (C) thioredoxin (Trx) protein expression and (D) glutaredoxin 1 (Glxr1) protein expression in neonates, weaned pups and adult hooded seals. One unit of TrxR activity is defined as the NADPH-dependent production of 2 μ mol 2-nitro-5-thiobenzoate min⁻¹ at 22°C. One unit of GR is defined as the amount of enzyme needed to oxidize 1 nmol of NADPH to NADP⁺ per minute at 25°C. Data are means \pm s.e.m. *, $P < 0.05$.

		* * *		*		* * * *
Seal	MMDLELPPPLPSQQDMDLIDILWRQDI DLGVS REVFDSPQRKKEHELEKQKKLEKERQEQLQKEQKAFFAQLQLDEETGE FLPI QPAQHIPSETSGSANYFPGAHI PKPDALYFDDCM	120				
PandaV.....YSQV.....	120				
HumanY.....Q.....YSQV.....S.....	120				
RatQ.....DY.....QTD.....VSYSQV.....Q.....E.....	120				
Seal	QLLAETFPFVDNDNEVSSAAFSQLVPDIPSQIENPVFIAPNQAAQSPTLVVQSVIADLDNMQQDIEQVWEELLSIPELQCLNIQNDKLVETSTVPSPETKMTEIDNSYHFFSSMPSLEKEV	240				
PandaV...A.V..T.....V.....N.....Y.....	240				
HumanQ.....T.....GH..S.....E.S.A.VAPV..G.....E.....TM.....A.L.V.-..Y.I.M.....	239				
RatH-----E..AL...HV.SS..TT.D....LDSSLETAMT..SSI..M...Q..F.....TE.KQA..T.....ATL..M.SN...Y.I.....	232				
Seal	GNCSPHFLSAFEDSFSSILSTDSSQLTVNSLNSDATINTDFGDEFYSAFIAEPSSSNMPSSSATLSQSLSELNGP IDVDSL LSC KAFNQNH PEST- EFNDS DSGI SLNTS PGMAS PEH	359				
PandaPN.....V.....I.....P.....H.....T.....C.....	359				
HumanN.....SV.....	359				
Rat	DS.....HG.....D.A.--..D.NP.L.....L...GGG...AI.....G...EGC.....K.T.G.V.....SR.....	350				
Seal	SVESVSGSDTPLGFSDSEMEEIDSAPGSVQONGPKTPPVQSGAGTAQALS PSPGHSA PARDAQCENT PKKELVPSPGHRKTPFTKD KHSSRL E AHLTR DELRAKALHIPFVPEKIINLPV	479				
PandaY.....K.....Q.....SP.....	479				
HumanS.....L.....V.....L.....K.....H.S..MV.P...Q.Q.THVH.....E.....	478				
RatIY..P.P.....L.....K.....AQ.TH.S...V.P..AQ...AVHES.....T...V.....Q.V.....	470				
Seal	DDFNEMMSKEQFSEAQLALIRDIRRGKNKVAAQNCRK RKLENIVPLEQDLHLKDEKEKLLREKGENDSKSLHKKKQSTHYEV FVSMRLRDEDGKPY SPSEYSLQQ TRDGNVFLVP	599				
PandaN.....K.....	599				
Human	V.....N.....K.....	59				
RatN.....G.....R.....RN.....RK.....	590				
Seal	PDTKKN	606				
Panda	...T...	606				
Human	...V...	605				
Rat	...T...	597				

Fig. 3. Alignment of the predicted hooded seal Nrf2 amino acid sequence (*ccNrf2*) compared with its mammalian homologs. Seal: hooded seal *Cystophora cristata* (JF429697). Panda: giant panda *Ailuropoda melanoleuca*, 97%. Human: *Homo sapiens* (NP_006155). Rat: *Rattus norvegicus* (NP_113977). Leucine zipper domain (outlined), nuclear export signal (black shading), nuclear location signal (gray shading) and residues for the Keap1-mediated proteosomal degradation (*) are shown.

maturation increases this transcriptional regulator. Nrf2 transcript levels decreased fourfold ($P<0.01$) in adult seals compared with weaned pups (Fig. 4A). Nrf2 protein content in crude extracts was also higher ($P<0.01$) in weaned pups ($134\pm 12\%$) than in adults ($56\pm 7\%$) (Fig. 4B), whereas nuclear Nrf2 was higher ($P<0.05$) in neonates ($100\pm 4\%$) than in adults ($67\pm 7\%$) (Fig. 4C). GCLm protein expression and GST activity, both of which are known to be regulated by Nrf2, were also higher ($P<0.01$) in weaned pups ($107\pm 8\%$; 52 ± 4 mU mg⁻¹ protein) than in adults ($70\pm 7\%$; 21 ± 4 mU mg⁻¹ protein) (Fig. 4D,E), suggesting that the development of the antioxidant system in hooded seals is not related to increased Nrf2 expression.

DISCUSSION

Maturation in hooded seals is characterized by the development of their diving capacity (Burns et al., 2007; Burns et al., 2010; Folkow et al., 2010; Lestyk et al., 2009) and is associated with increases in $\text{O}_2^{\cdot -}$ production but not with increases in oxidative damage (Vázquez-Medina et al., 2011a). In the present study we found that the antioxidant system of the hooded seal develops with maturation, likely contributing to the prevention of the accumulation of oxidative damage.

Diving seals undergo a number of cardiovascular adjustments that allow them to maximize the use of their oxygen stores but at the same time result in ischemia and hypoxemia (Elsner, 1999; Kooyman and Ponganis, 1998; Meir et al., 2009). After a dive, perfusion of ischemic tissues presents seals with the potential for oxidant production and oxidative stress due to the accumulation of the ATP degradation product HX and the activation of XO during ischemia (Elsner et al., 1998; Kuppusamy and Zweier, 1989; McCord, 1985; Thompson-Gorman and Zweier, 1990). Comparative studies have shown that antioxidant protection is higher in seals than in non-diving mammals, which likely allows seals to cope with recurrent ischemia/reperfusion cycles associated with their diving

lifestyle (Elsner et al., 1998; Murphy and Hochachka, 1981; Vázquez-Medina et al., 2006; Vázquez-Medina et al., 2007; Wilhelm Filho et al., 2002; Zenteno-Savín et al., 2002). The present study shows that GSH content gradually increases with maturation in hooded seals, and that PrxVI expression and GPx activity are higher in adult hooded seals than in neonates or weaned pups. Both PrxVI and GPx use GSH as a cofactor to remove H_2O_2 . H_2O_2 is the main oxidant produced after ischemia/reperfusion due to the HX/XO pathway, which is activated after experimental ischemia in seal organs (Brown et al., 1988; Elsner et al., 1998). Our results suggest that the capacity to remove H_2O_2 increases as hooded seals transition from terrestrial to aquatic lifestyles. This is consistent with the previously reported higher $O_2^{\cdot -}$ production, MnSOD protein content and SOD activity in adult seals than in pups because SOD dismutates $O_2^{\cdot -}$ into H_2O_2 (Vázquez-Medina et al., 2011a). This also suggests that GPx and Prx, and not catalase, are the main enzymes responsible for H_2O_2 removal in seal muscle under basal conditions. Although GPx activity has been shown to be higher in muscle of ringed seals than in pigs under basal conditions, catalase activity is higher in pigs and no difference exists between terrestrial and marine mammal red blood cells (Vázquez-Medina et al., 2006; Wilhelm Filho et al., 2002).

The results also show that Trx1 and Glrx1 expression as well as TrxR activity increase with maturation in hooded seals, suggesting that these enzymes contribute to counteract the potential increases in peroxide production. Thioredoxins are the major cellular protein disulfide reductases; therefore, they serve as electron donors for enzymes such as Prx and GPx (Arnér and Holmgren, 2000; Björnstedt et al., 1994). Higher Trx1 and Glrx1 expression, as well as higher TrxR activity, are consistent with higher PrxVI protein content and GPx activity and suggest that the Trx and Glrx systems are key modulators of the intracellular redox balance in adult hooded seals.

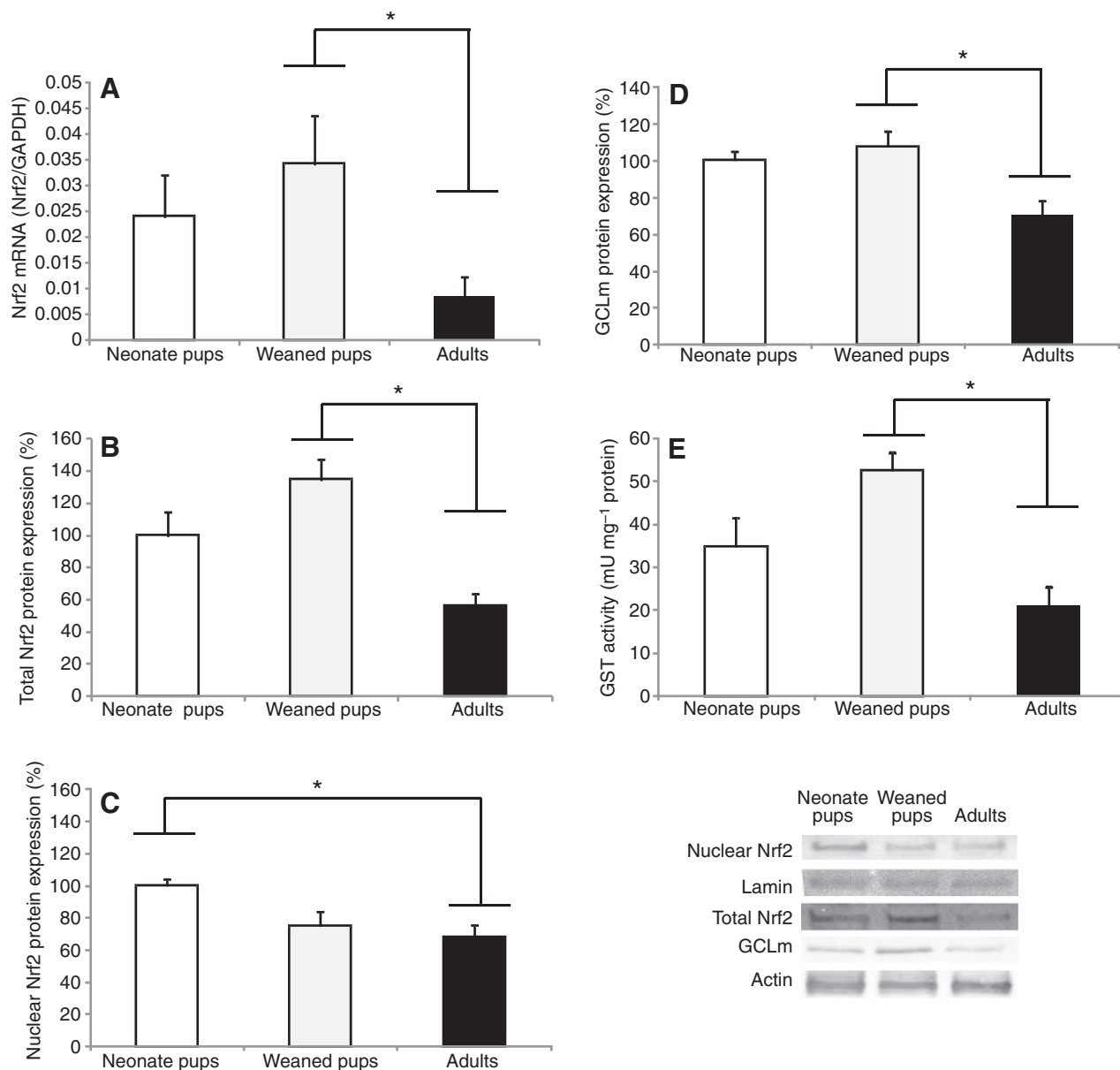


Fig. 4. Nrf2 and two of its downstream targets during the postnatal development of the hooded seal. (A) Nrf2 mRNA expression, (B) total Nrf2 (crude extract), (C) Nrf2 nuclear content, (D) glutamate-cysteine ligase modulatory subunit (GCLm) protein expression and (E) glutathione S-transferase (GST) activity in neonates, weaned pups and adult hooded seals. Nrf2 relative mRNA levels were calculated by quantitative real-time RT-PCR and compared with GAPDH levels. One unit of GST is defined as the amount of enzyme that synthesizes $1 \mu\text{mol product min}^{-1}$ at 25°C . Data are means \pm s.e.m. *, $P < 0.05$.

Although Nrf2 mRNA and protein were detected in crude extracts and nuclear fractions from the three age groups, neither transcript nor Nrf2 protein content increased with maturation, suggesting that Nrf2 activation is not stimulated by maturation-related increases in oxidant production in hooded seals (Immenschuh and Baumgart-Vogt, 2005; Jaiswal, 2004). The latter is consistent with the decreases observed in three downstream enzymes controlled by Nrf2 (GST, GCLm and catalase) in adult seals compared with weaned pups (Chan and Kan, 1999; Cho et al., 2002; Itoh et al., 1997; Yang et al., 2005). These results can be explained by the observed increases in PrxVI expression and GPx activity, as increased intracellular levels of H_2O_2 are among the main mechanisms responsible for the activation of Nrf2, and both GPx and PrxVI scavenge H_2O_2 . Lower Nrf2 and catalase in adults than

in weaned pups, as well as higher GPx and PrxVI in adults than in pups, also suggests that intracellular H_2O_2 production is kept at physiological levels during development in hooded seals because GPx and Prx are responsible for the removal of physiological concentrations of H_2O_2 whereas catalase activity and Nrf2 translocation increase in response to increased H_2O_2 production (Halliwell and Gutteridge, 2007; Prester and Talalay, 1995). Although PrxVI has been shown to be controlled by Nrf2 (Chowdhury et al., 2009), its transcription is also responsive to hormonal regulation and PrxVI levels increase with postnatal development in terrestrial mammals (Fisher, 2010; Kim et al., 2002).

An alternative explanation for the observed lower Nrf2 levels in adults than in weaned pups is that Nrf2 nuclear translocation in hooded seals responds to particular events, such as a prolonged

ischemic episode induced by a particularly extended dive. Although adult hooded seals are able to dive to depths in excess of 1000 m for nearly 1 h, and can remain submerged for approximately 80% of their time at sea, they perform routine dives of >20 min (Folkow and Blix, 1999). In addition, the higher Nrf2, catalase, GCLm and GST levels observed in weaned pups than in adults can be explained by the physiological condition of the weaned seals. We have shown that fasting increases catalase, GCLm and GST in postweaning elephant seals (Vázquez-Medina et al., 2010; Vázquez-Medina et al., 2011b) but apparently not in fasting adult female seals (Vázquez-Medina et al., 2011b).

In summary, our results show that the antioxidant system of hooded seals develops with maturation, likely preparing them to cope with the potential diving-induced oxidant production. Results also show that peroxide-removing enzymes such as GPx, PrxVI, Trx and Glrx1 increase with maturation, suggesting that they contribute to the regulation of intracellular redox state in hooded seal muscle. Lastly, Nrf2 of the hooded seal has functional and regulatory domains similar to those of most terrestrial mammals, although its nuclear translocation does not seem to be controlled by potential increases in maturation-related oxidant production.

LIST OF ABBREVIATIONS

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCLm	glutamate-cysteine ligase modulatory subunit
Glrx1	glutaredoxin 1
GPx	glutathione peroxidases
GR	glutathione disulphide reductase
GSH	glutathione
GSH-Eq	total glutathione content
GSSG	glutathione disulphide
GST	glutathione-S-transferases
HX	hypoxanthine
Nrf2	NF-E2-related factor 2
PrxVI	peroxyredoxin VI
SOD	superoxide dismutase
Trx1	thioredoxin 1
TrxR	thioredoxin reductase
XO	xanthine oxidase

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