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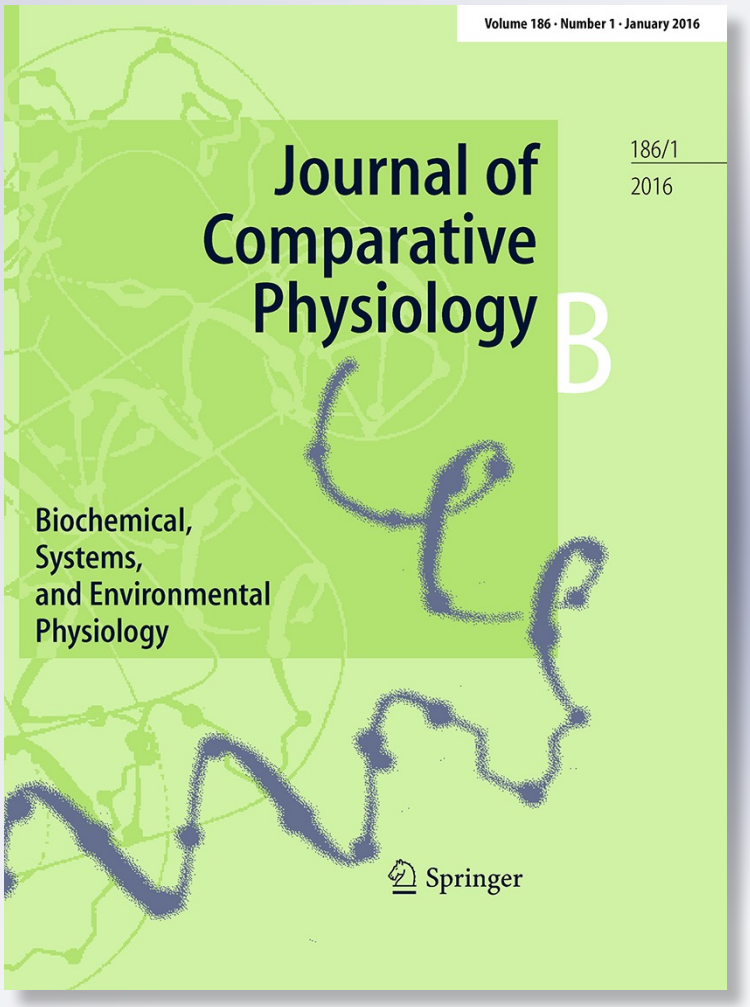


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ORIGINAL PAPER

# Peroxiredoxin 6 from the Antarctic emerald rockcod: molecular characterization of its response to warming

**A. M. Tolomeo[1] · A. Carraro[1] · R. Bakiu[2] · S. Toppo[3] · S. P. Place[4] · D. Ferro[5] · G. Santovito[1]**

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**Abstract** In the present study, we describe the purification and molecular characterization of two peroxiredoxins (Prdxs), referred to as Prdx6A and Prdx6B, from *Trematomus bernacchii*, a teleost widely distributed in many areas of Antarctica, that plays a pivotal role in the Antarctic food chain. The two putative amino acid sequences were compared with Prdx6 orthologs from other fish, highlighting a high percentage of identity and similarity with the respective variant, in particular for the residues that are essential for the characteristic peroxidase and phospholipase activities of these enzymes. Phylogenetic analyses suggest the appearance of the two *prdx6* genes through a duplication event before the speciation that led to the differentiation of fish families and that the evolution of the two gene variants seems to proceed together with the evolution of fish orders and families. The temporal expression of Prdx6 mRNA in response to short-term thermal stress showed a general upregulation of *prdx6b* and inhibition of *prdx6a*, suggesting that the latter is the variant most affected by temperature increase. The variations of mRNA accumulation are more conspicuous in heart than the liver, probably related to behavioral changes of the specimens in response to elevated temperature. These data, together with the peculiar differences between the molecular structures of the two Prdx6s in *T. bernacchii* as well as in the tropical species *Stegastes partitus*, suggest an adaptation that allowed these poikilothermic aquatic vertebrates to colonize very different environments, characterized by different temperature ranges.

**Keywords** Antarctica · Fish · Gene expression · Molecular evolution · Peroxiredoxins

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A.M. Tolomeo and A. Carraro contributed equally.

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## Introduction

Temperature is a physical parameter that affects many biological processes, especially those related to energy. The main characteristic of Antarctic seawater is its low constant temperature of about −1.8 °C that determines a high concentration of dissolved gasses, $O_2$ in particular, in the environment as well as in animal body fluids (Hardy and Gunther 1935). This condition can increase the formation rate of reactive oxygen species (ROS), molecules that are normally produced in cells from the partial reduction of $O_2$ (Halliwell and Gutteridge 1999), also increasing the risk of oxidative stress (Acworth et al. 1997). In the Antarctic fish this stress condition is favored by the presence of body lipid accumulations, considered to be involved in buoyancy and aerobic metabolism of these organisms (Eastman and

De Vries 1982; Eastman 1993; Roberfroid and Buc Calderon 1995).

Both enzymes and non-enzymatic compounds of the antioxidant systems are present in cells in order to prevent the damages caused by ROS. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and peroxiredoxin (Prdx), act together limiting the effects of oxidant molecules on cells and tissues and constitute the first line of defense.

Peroxiredoxins (Prdxs, EC 1.11.1.15) are antioxidant enzymes that catalyze the reduction of peroxides using redox-active cysteines (Randall et al. 2013; König et al. 2013). Up to six different isoforms are known, grouped in three different families (typical 2-Cys, atypical 2-Cys and 1-Cys) that differ for their structural and mechanistic features (Wood et al. 2003b).

The typical 2-Cys Prdxs are the largest class of Prdxs (isoforms from 1 to 4) and are identified by the conservation of their two redox-active cysteines, the peroxidatic cysteine and the resolving cysteine (Ren et al. 2014). Typical 2-Cys Prdxs are obligate homodimers containing two identical active sites. The peroxidase reaction is composed of two steps centered around a redox-active cysteine, the peroxidatic cysteine. In the first step peroxidatic cysteine (Cys–SPH) attacks the peroxide substrate and is oxidized to a cysteine sulfenic acid (Cys–SOH). In the second step of the peroxidase reaction, the peroxidatic cysteine sulfenic acid (Cys–SPOH) from one subunit is attacked by the resolving cysteine (Cys–SRH) located in the C-terminus of the other subunit. This condensation reaction results in the formation of a stable intersubunit disulfide bond, which is then reduced by one of several cell-specific disulfide oxidoreductases (e.g., thioredoxin, AhpF, tryparedoxin or AhpD), completing the catalytic cycle (Hall et al. 2009).

The second class of Prdxs are the atypical 2-Cys Prdxs (Prdx5), which have the same mechanism as typical 2-Cys Prdxs but are functionally monomeric. In these Prdxs, both the peroxidatic cysteine and its corresponding resolving cysteine are contained within the same polypeptide, with the condensation reaction resulting in the formation of an intramolecular disulfide bond (Wood et al. 2003a; Knoops et al. 2011).

Our study focused on the last subfamily, 1-Cys Prdxs, which are represented by peroxiredoxin 6 (Prdx6). It is a bifunctional protein, possessing the activities of both glutathione peroxidase and phospholipase $A_2$ ($PLA_2$; Liu et al. 2015; Rahaman et al. 2012). In particular, this enzyme is characterized by a single peroxidatic cysteine that after oxidation is reduced by glutathione S-transferase-bound GSH to complete the catalytic cycle (Manevich et al. 2004). The peroxidatic cysteine ($Cys^{47}$ in mammals) is placed in the catalytic center characterized by the amino acid sequence PVCTTE, analogous to the surrounding sequence in typical 2-Cys Prdxs, FVCPTE (Fisher 2011). In mammals, the peroxidase activity is due to a catalytic triad, represented by $Thr^{43}$ and $Arg^{130}$ residues together with the peroxidatic Cys introduced above. The $PLA_2$ activity of Prdx6 is blocked by serine protease inhibitors, providing evidence for a serine as the active center. The primary sequence of Prdx6 at amino acids 30–34 indicates a lipase motif (GXSXG), which occurs in essentially all serine-dependent lipases. This motif is not present in other peroxiredoxins. Furthermore, examination of the crystal structure of human Prdx6 suggests that $Ser^{32}$ is part of a surface-localized catalytic triad ($His^{26}$, $Ser^{32}$, $Asp^{140}$) that is common for serine-based $PLA_2$. Site-directed mutation of $Ser^{32}$ resulted in the loss of $PLA_2$ activity, which was interpreted to confirm a role for this amino acid moiety in catalysis (Manevich et al. 2007).

Prdx6s have received considerable attention in recent years as important cellular antioxidant proteins that help control intracellular peroxide levels. In addition, these proteins may also function, through peroxide-mediated inactivation, in hydrogen peroxide signaling in eukaryotes (Hall et al. 2009). Various gene sequences of Prdx6s from aquatic animals have been reported (Nikapitiya et al. 2009; Park et al. 2008; Smeets et al. 2008; David et al. 2007), although only few Prdx6 cDNAs have been sequenced from fish (Zheng et al. 2010; De Zoysa et al. 2012) and none from Antarctic species.

In the present study, we report on the molecular characterization of two Prdx6s from the emerald rockcod, *Trematomus bernacchii*, a teleost widely distributed in many areas of Antarctica. With the aim of evaluating differences at molecular level in relation to their evolutionary history, we also compared the sequence of Prdx6s from *T. bernacchii* with those from other vertebrate species. In this respect, the evolution of Prdxs is particularly interesting, since they are considered ancient proteins, evolved by duplication events during the past 2.5 billion years together with ROS and metabolic pathways. In particular, in eukaryotes, the higher number of genes coding for family members is only partly explained by the compartmentalization and the existence of mitochondrial, nuclear, peroxisomal and chloroplast isoforms (Knoops et al. 2007). Furthermore, we employed molecular modeling technique to perform a structural reconstruction of the two cold-adapted Prdx6s. In particular, using different in silico approaches, we investigated Antarctic fish Prdx6s (i.e., compared with other, non-cold-adapted, cytosolic Prdx6s) for the presence of molecular characteristics for cold adaptation.

Lastly, to gain some insight into the potential physiological role of the Pdrx6 isoforms in *T. bernacchii*, we characterized basal mRNA levels for both Prdx6A and Prdx6B in various organs and tissues. We also analyzed changes in transcript levels for both Prdx6 genes after exposer to short-term thermal stress to gain a better understanding

of their potential role in the thermal stress response of *T. bernacchii* to increased sea surface temperatures related to global climate change.

## Materials and methods

### Ethical procedures

The sample collection and animal research conducted in this study comply with Italy's Ministry of Education, University and Research regulations concerning activities and environmental protection in Antarctica and with the Protocol on Environmental Protection to the Antarctic Treaty, Annex II, Art. 3.

### Experimental animals

Adult samples of *T. bernacchii* were collected in the proximity of Mario Zucchelli Station in Terra Nova Bay, Antarctica (74°42′S, 167°7′E) and kept in aquaria supplied with aerated seawater at approximately 0 °C. Five specimens were immediately euthanized (tricainemethanesulfonate, MS-222; 0.2 g $l^{-1}$) and samples of liver, heart, spleen and skeletal muscle tissues were quickly excised, placed into cryotubes and snap-frozen in liquid nitrogen and later stored at −80 °C. To measure changes in mRNA abundance after a short thermal stress, we acclimated 25 specimens to a gradual increase in seawater temperature from 0 to 5 °C. The water temperature in the aquariums was increased at a rate of 0.05 °C/h until it reached the next degree centigrade. At 1, 2, 3, 4 and 5 °C, the water temperature was held constant for 4 days, after which five specimens were killed, tissue samples quickly removed, frozen in liquid nitrogen, and stored at −80 °C until analyzed. Twenty-five untreated specimens were maintained at a control temperature of 0 °C and $n = 5$ fish were killed at the same times as the warm-acclimated specimens.

### Primers design, RNA extraction, DNA synthesis, cloning and sequencing of peroxiredoxin 6A (Prdx6A)

For the primer design, amino acid and nucleotide sequences of Prdx6s from fish were obtained from NCBI database and aligned by Larkin et al. (2007). The considered species include Prdx6 of *Oplegnathus fasciatus* (barred knifejaw), *Sparus aurata* (Gilt-head seabream), *Psetta maxima* (turbot), *Ictalurus punctatus* (Channel catfish), *Danio rerio* (Zebrafish), *Oncorhynchus mykiss* (Rainbow trout), *Miichthys miiuy* (Miiuy croaker), *Oryzias melastigma* (Indian medaka), *Pundamilia nyererei* (Crimson Tide Flameback), *Oreochromis niloticus* (Nile tilapia), *Maylandia zebra* (Zebra Malawi Cichlid), *Oryzias latipes* (Japanese ricefish), *Takifugu rubripes* (Japanese pufferfish), *Salmo salar* (Atlantic salmon), *Anoplopoma fimbria* (Sablefish) and *Pseudopleuronectes americanus* (Winter flounder). The primers were designed in the conserved domains and the primer sequences were analyzed with IDT Oligo analyzer (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/). Primer sets are shown in Table S1.

Total RNA was purified from various tissues of *T. bernacchii* using TRIzol® reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was further purified with 8 M LiCl in order to remove glucidic contaminants (Ferro et al. 2015) and the quantification was performed using the ND-1000 spectrophotometer (Nanodrop, Wilmington, DE); RNA integrity was assessed by capillary electrophoresis using the Agilent Bioanalyzer 2100, with the RNA 6000 Nano (Agilent Technologies, Palo Alto, CA). The first strand of cDNA was reverse-transcribed at 42 °C for 1 h from 1 μg of total RNA in a 20 μL reaction mixture, containing 1 μL of ImProm-II™ Reverse Transcriptase (Promega) and 0.5 μg oligodT Anchor primer. PCR reactions were performed with 50 ng of cDNA. The PCR program was the following: 95 °C for 2 min and 35 × (95 °C for 30 s, $T_m$ for 30 s, 72 °C for 1 min); final elongation 72 °C for 10 min. $T_{ms}$ were indicated in Table S1.

All the amplicons were gel-purified with the NucleoSpin Extract 2 in 1 (Macherey–Nagel), ligated into the pGEM®-T Easy Vector (Promega) and cloned in XL1-Blue *E. coli* cells (Invitrogen). Positively screened clones were sequenced at the BMR Genomics (University of Padova) on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems).

### Semiquantitative RT-PCR (sqRT-PCR) analysis

In order to evaluate Prdx6 mRNA expression, semiquantitative RT-PCR (sqRT-PCR) analysis has been performed. cDNAs for Prdx6 A and Prdx6 B were amplified with the specific primers reported in Table S1. To control for variation in efficiency of cDNA synthesis and PCR amplification reactions, *T. bernacchii* β-actin (GenBank accession number: ADF45299.1) was used as housekeeping gene and amplified with species-specific primers (Table S1). PCR amplifications were carried out with the following program: 95 °C for 2 min, then a variable number of cycles of 95 °C for 30 s, 30 s at specific melting temperature (Table S1), 72 °C for 1 min. For all the genes, the number of amplification cycles was optimized (42 for Prdx6, 40 for β-actin) to ensure that PCR products were quantified during the exponential phase of the amplification.

The amplification products were separated by electrophoresis on 1.5 % agarose GelRed-stained gel (Biotium) and the relative intensities were quantified with the software Quantity-one through a quantitative ladder (Gene Ruler™, Fermentas). Transcript levels are reported as the

intensity (in arbitrary units, a.u.) of the gene of interest relative to the expression level of β-actin in the same sample.

### Phylogenetic analyses

Phylogenetic studies were based on both amino acid and nucleotide sequences of Prdx6s from *T. bernacchii* and other species available in GenBank database (Table S2).

The T-Coffee multiple sequence alignment package has been used to obtain multiple sequence alignment of Prdx6s sequences (Notredame et al. 2000). Even though this method is based on the popular progressive approach to multiple alignment, we decided to use it because it is characterized by a dramatic improvement in accuracy with a modest sacrifice in speed, as compared to the most commonly used alternatives (Notredame et al. 2000).

The jModelTest 2 (Darriba et al. 2012) was used to carry out statistical selection of best-fit models of nucleotide substitution. Analyses were performed using 88 candidate models and three types of information criterion (Akaike Information Criterion—AIC, Corrected Akaike Information Criterion—cAIC and Bayesian Information Criterion—BIC). To select the best-fit model of analyzed protein evolution ProtTest 3 was used (Darriba et al. 2011). One hundred and twenty-two candidate models and the three previously mentioned criteria were used in these statistical analyses.

Phylogenetic trees were built using the Bayesian inference (BI) method implemented in Mr. Bayes 3.2 (Ronquist et al. 2012). Four independent runs, each one with four simultaneous Markov Chain Monte Carlo (MCMC) chains, were performed for 1,000,000 generations sampled every 1000 generations. Furthermore, we also used the maximum likelihood (ML) method implemented in PhyML 3.0 (Guindon et al. 2010). Bootstrap analyses were performed on 100,000 trees using both kinds of tree topology improvement: nearest neighbor interchange (NNI) and subtree pruning and regrafting (SPR). FigTree v1.3 software was used to display the annotated phylogenetic trees.

Finally, we employed the mechanistic empirical model (MEC, Doron-Faigenboim and Pupko 2007) that accounts for the different amino acid replacement probabilities based on the WAG empirical substitution matrix, while estimating the codon rate matrix, thus allowing for positions undergoing radical amino acid exchanges to acquire higher dN rates than those with less radical exchanges. The codon-wise × estimates were mapped onto human protein tertiary structure (PDB: 1prx) using the Selecton-3D web server (http://selecton.tau.ac.il).

### Molecular modeling

Structural models have been obtained for the two Prdx6 isoforms of *T. bernacchii* and *Stegastes partitus*. Human peroxiredoxin-6 shares over 70 % of sequence identity with both isoforms and was used as structural template (pdb: 1PRX). Final models were built with SWISS-MODEL (Biasini et al. 2014) and further energy minimized with GROMACS 5.04 (http://www.gromacs.org/) software package using Amber ff99SB-ILDN (Lindorff-Larsen et al. 2010) force field. The systems were subjected to a steepest descent energy minimization until reaching a tolerance force of no greater than 1000 kJ $mol^{-1}$ $nm^{-1}$. Electrostatic surface potential was calculated for the energy minimized models using the programs PDB2PQR (Dolinsky et al. 2007) and APBS (Baker et al. 2001), with the nonlinear Poisson-Boltzmann equation and contoured at ±2 kT/e.

### Statistical analysis

All data were expressed as the average of five analyzed specimens ± standard deviation (SD). Statistical analyses were performed with the PRIMER statistical program. One-way ANOVA was followed by the Student–Newman–Keuls test to assess significant differences ($p < 0.05$).

## Results

### Organization of *T. bernacchii* Prdx6 genes

Cloning and sequencing from *T. bernacchii* liver yielded a partial cDNA sequence of Prdx6. This sequence has been compared with the *T. bernacchii* transcriptome database, recently published (Huth and Place 2013), that allowed us to complete this cDNA sequence and also to identify the presence of a second Prdx6. We named the respective genes *prdx6a* (GenBank accession number KP969452) and *prdx6b* (GenBank accession number KP941423).

The Prdx6A transcript is 986 nt long. The 5′- and 3′-UTR regions consist of 108 and 212 nt, respectively. The open reading frame (ORF) includes 666 nt and encodes a putative protein of 221 aa, with a deduced molecular weight of 24.28 kDa (Fig. S1). The 3′-UTR region includes a putative polyadenylation signal (AATAAA) at 853–858 nt.

The Prdx6B transcript is 2973 nt long. The 5′- and 3′-UTR regions consist of 1696 and 602 nt, respectively. The ORF includes 675 nt and encodes a putative protein of 224 aa, with a deduced molecular weight of 24.71 kDa (Fig. S2). The 3′-UTR region includes a putative polyadenylation signal (AATAAA) at 820–825 nt and two ATTTA sequences, at nt 709–713 and 934–938, which signal rapid degradation in certain mammalian mRNAs (Shaw and Kamen 1986).

The amino acid sequences of Prdx6A and Prdx6B are quite different from each other, as demonstrated by the identity and similarity percentages (72.2 and 90.1 %,

respectively). The comparison of these peroxiredoxins (Table S3) with the Prdx6 of the other fish highlights a high percentage of identity and similarity with the respective variant. In particular, Prdx6A of *T. bernacchii* shows the highest identity (89.6 %) and similarity (97.2 %) with the only known Prdx6 of *M. miiuy* (order Perciformes). Similarly, Prdx6B of *P. nyererei* (order Cichliformes) is the sequence with the highest identity (89.3 %) and similarity (96.4 %) with *B* variant of *T. bernacchii*.

## Phylogenetic analyses and molecular modeling

The jModelTest 2.1.3 software determined the GTR + G model as being the best-fit model of Prdx6 cDNA sequence evolution with a gamma shape value (four rate categories) of 0.72 using AIC, cAIC and BIC statistical criterion (−lnL = 16365.65). Phylogenetic relationships of Prdx6 cDNA sequences were determined using the most powerful statistical method of BI. BI and maximum likelihood (ML) methods generate phylogenies with the same topology, depicted in the cladogram of Fig. 1. *T. bernacchii* Prdx6s emerge in two clearly separated clusters, which include all known fish Prdx6s. In particular, *T. bernacchii* Prdx6A is more closely related to Prdx6s from *M. miiuy* and *S. aurata* (posterior probability 99 %; bootstrap values 58 %). *T. bernacchii* Prdx6B is most closely related to a Prdx6 from another Antarctic fish, *Notothenia coriiceps* (posterior probability 100 %; bootstrap values 100 %). These two clusters are clearly separated from Prdx6s from other vertebrates (posterior probability 96 %; bootstrap values 89 %).

ProtTest3 software determined WAG + G model as being the best-fit model of Prdx6 amino acid sequence

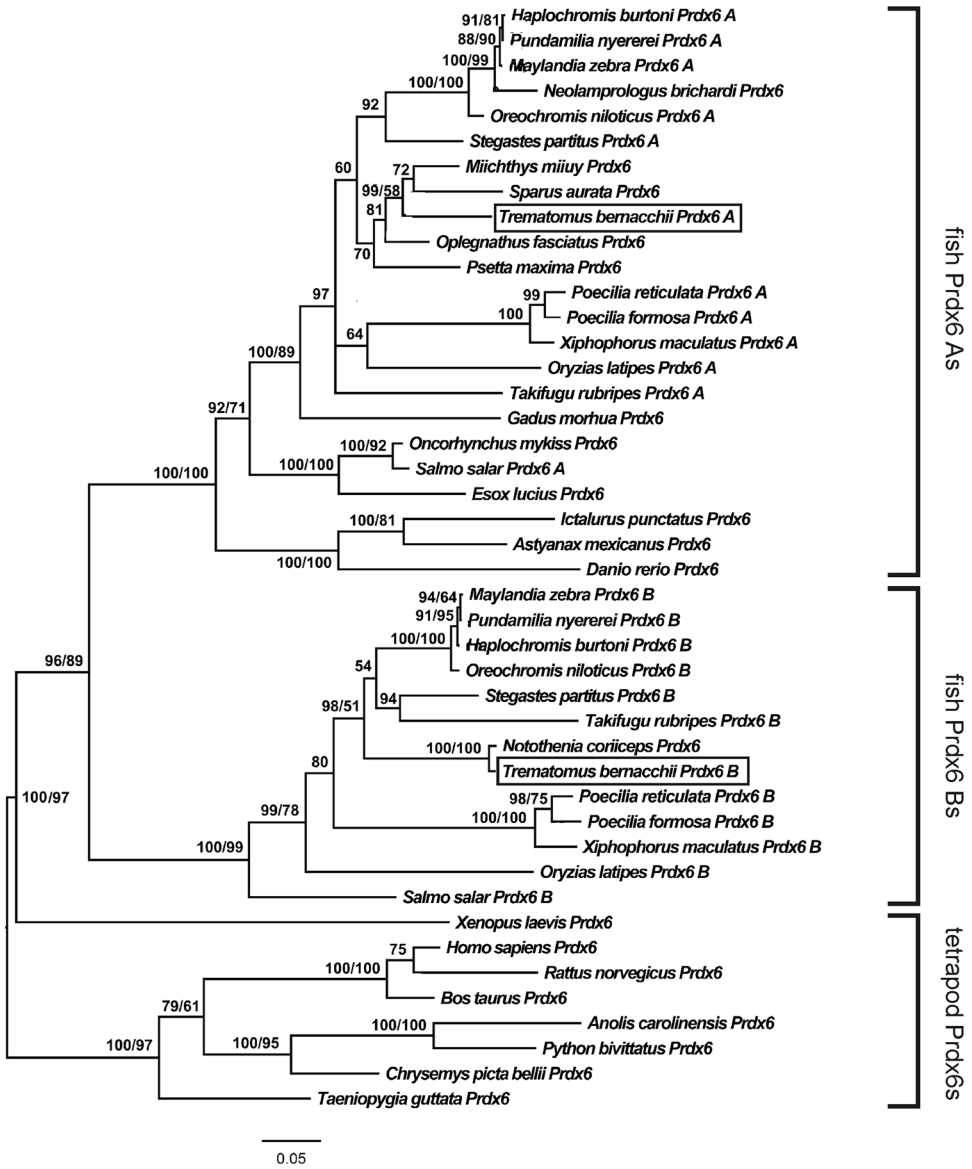


**Fig. 1** Phylogenetic relationships among different organisms Prdx6 reconstructed on the basis of cDNA coding region sequences and using both methods BI (arithmetic mean −11,513.54; harmonic mean −11,549.48) and ML (arithmetic mean −11,353.1). Bayesian posterior probability (first number) and bootstrap values higher than 50 % are indicated on each node, respectively. The *scale* for branch length (0.05 substitution/site) is shown below the tree. *T. bernacchii* Prdx6 are *boxed*

evolution with a gamma shape value (four rate categories) of 1.0 using all statistical criterion: AIC, cAIC and BIC ($-\ln L = -7241.91$). BI and ML methods generate phylogenies with the same topology and are depicted in the cladogram shown in Fig. S3. Again, *T. bernacchii* Prdx6s emerge in two clearly separated clusters, which include all known fish Prdx6s. Furthermore, the phylogenetic relationships among the various Prdx6 sequences are confirmed, even if with a lower degree of resolution.

MEC analysis indicated a strong prevalence of negative selection in the evolution of Prdx6s, but there are also amino acid residues that demonstrated the effect of positive selection on them (Fig. S4). In fact, these analysis highlighted Glu58, Arg79, Lys92, Cys94 and Cys95 (as referred to in zebrafish Prdx6) to be positively selected.

In Fig. 2, the deduced amino acid sequences of both *T. bernacchii* Prdx6A and Prdx6B were aligned with Prdx6s from other fish in addition to *Homo sapiens*. Amino acids that are essential for both peroxidase and $PLA_2$ activities are highly conserved in *T. bernacchii* Prdx6s as well as in the other species. In particular, the peroxidatic cysteine ($Cys^{46}$ in *T. bernacchii*), which is present in the functional pattern PVCTTE and forms a catalytic triad with $Thr^{43}$ and an Arg, at the position 130 in Prdx6A and 132 in Prdx6B, shows a high degree of conservation across all fish species. The one exception being the only known Prdx6 isoform in *O. mykiss*, where $Glu^{49}$ is replaced by Gly in the functional pattern PVCTTE. Similar to the peroxidatic cysteine, the amino acids responsible for the $PLA_2$ activity, represented by $His^{25}$, $Ser^{31}$ and $Asp^{138}$ ($Asp^{140}$ in Prdx6B), are also well conserved (Fig. 2). Here, the only notable exception is found within the functional pattern that includes $Ser^{31}$ (GXSXG) of *S. aurata* Prdx6, where $Gly^{43}$ is replaced by Arg.

In Prdx6A of *T. bernacchii* there are two specific substitutions involving amino acids that are not included in the catalytic group: $Ile^{34}$ is substituted by Val (also in Prdx6B) and $Lys^{124}$ is substituted by Ser. In Prdx6B of *T. bernacchii* there are five of these specific substitutions, namely: Cys replacing various amino acids in position 74; $Glu^{92}$ that is substituted by Asp; Leu that replaces Met, Ile or Val in position 127; $Leu^{169}$ that is substituted by Ala; $Ala^{176}$ that is substituted by Ser; $Pro^{191}$ that is substituted by Ala.

Though isoform A shows a greater negative isosurface compared to that of isoform B (Fig. 3a, b, respectively), the other side of the dimer (Fig. 3c, d, respectively) is in favor of a negative surface for isoform B. Isoform B is almost uniformly negative whereas isoform A has a highly negative side (Fig. 3a) and a weak positive side (Fig. 3c). The latter is also found in *S. partitus* isoforms that have a higher gap of total negative charge between isoform A and B (see Table S4). Template structure, human peroxiredoxin-6, does not have a prevailing negative surface and has a lower total negative charge compared to other species (see Table S4) and positive and negative potentials are equally distributed on its surface (data not shown).

### Analysis of gene expression in natural condition and after increased environmental temperature

Basal mRNA expression levels were determined in the in heart, liver, spleen and skeletal muscle of non-stressed specimens of *T. bernacchii* to characterize the tissue specific expression patterns of the two Prdx6 variants. Figure 4a shows that the gene *prdx6a* is expressed to a similar extent in all organs and tissues considered. A different result was obtained with the analysis of *prdx6b* transcript levels (Fig. 4b). In fact, liver and heart tissue displayed the highest level of mRNA expression, approximately double the levels observed in spleen and skeletal muscle ($p < 0.05$). No statistical difference between liver and heart mRNA levels was measured.

These results led us to focus our attention on liver and heart tissues for the subsequent analyses of mRNA accumulation in specimens exposed to environmental temperature changes. In liver tissues, *prdx6a* transcription showed a significant decrease in response to temperature increase. In particular, the control specimens had mRNA levels eight-fold higher ($p < 0.001$) than those measured in thermally stressed fish for all considered temperatures (Fig. 5a). In control fish, the mRNA levels remained constant during the entire experiment. In addition, no statistically significant differences were found between treatment fish held under various stress regimes. For *prdx6b*, the mRNA levels in specimens exposed to elevated temperatures were slightly higher than controls, but the differences were always statistically significant ($p < 0.05$; Fig. 5b). As seen with *prdx6a*, mRNA levels in the control fish remained constant during the entire experiment and there were no statistically significant differences among fish within the various stress treatments.

In heart tissues of control specimens, the mRNA level of both *prdx6a* and *prdx6b* remained constant during the entire experiment (Fig. 6). On the contrary, the specimens exposed to short-term thermal stress showed temperature-dependent variations. In particular, the *prdx6a* mRNA levels decreased at +2 °C (day 10, $p < 0.05$) and continued to decrease up to +3 °C (day 15, $p < 0.05$; Fig. 6a). In both these cases the differences are statistically significant with respect to controls ($p < 0.05$ and $p < 0.001$, respectively). At +4 °C (day 20) the *prdx6a* mRNA expression levels were similar to controls and we found no significant differences between these two groups. However, at +5 °C (day 25) *prdx6a* mRNA levels decreased again ($p < 0.05$), and were similar to those measured at +2 °C (day 10). The *prdx6b* mRNA levels remained more or less constant (and

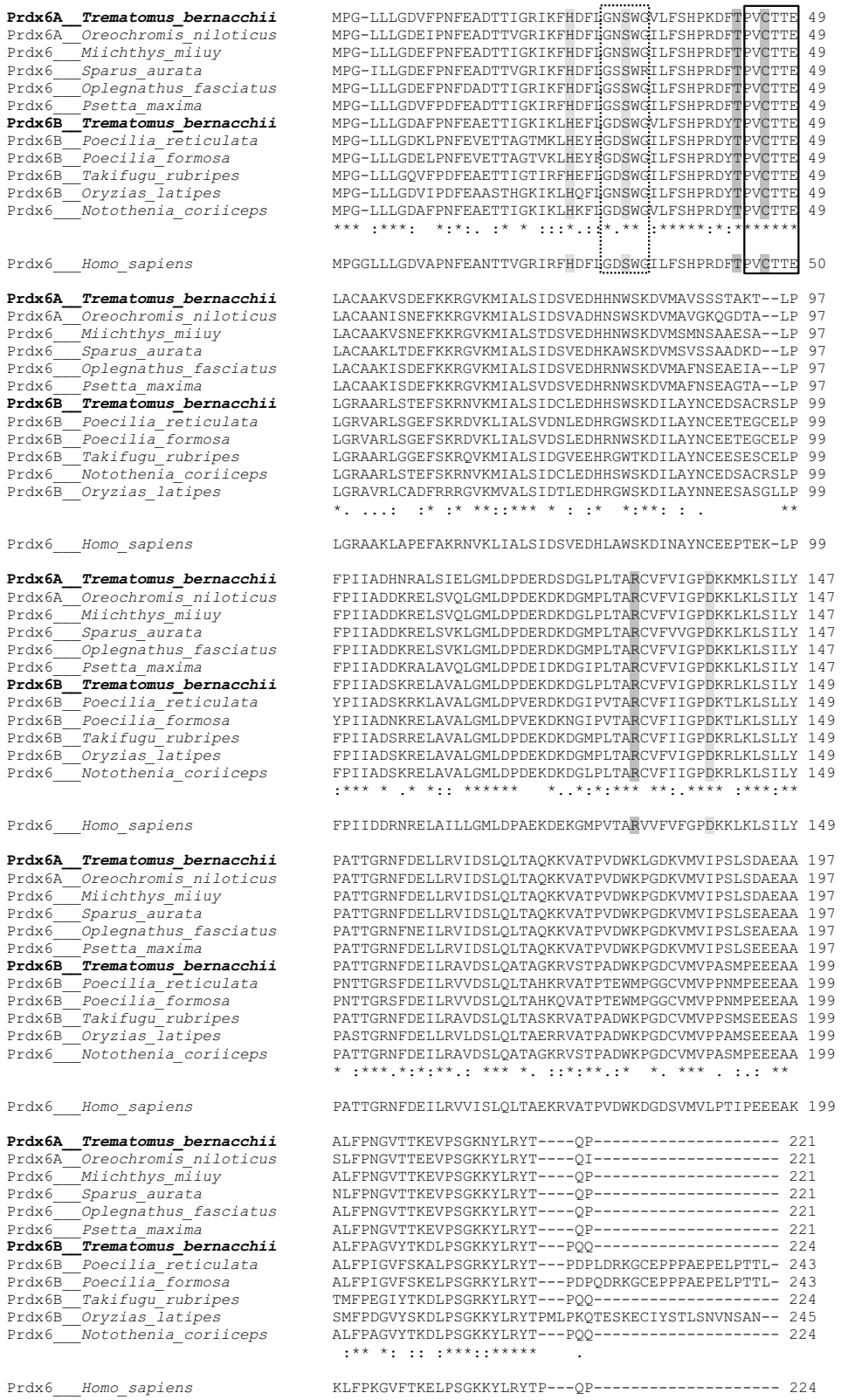


**Fig. 2** Multiple alignment of the amino acid sequences of 13 Prdx6s from Teleost species as well as from *H. sapiens*. The catalytic centers for peroxidase and phospholipase activities are *boxed* in *continuous* and *dotted lines*, respectively. The amino acid residues highlighted in *dark grey* represent the peroxidase triad. The amino acids of catalytic triad involved in phospholipase activity is highlighted in *light grey*. The *symbols* at the bottom of the teleost sequences correspond to the definitions of the CLUSTAL program: *asterisk* fully conserved, *colon* highly conserved, *dot* conserved substitution

significantly higher than controls) during the first phases of the stress treatment, but displayed a significant decrease at +4 °C (day 20, $p < 0.05$), with values 3-fold lower than controls ($p < 0.001$). However, at +5 °C (day 25) the level of *prdx6b* mRNA returned to initial levels and was higher than control values ($p < 0.01$; Fig. 6b).

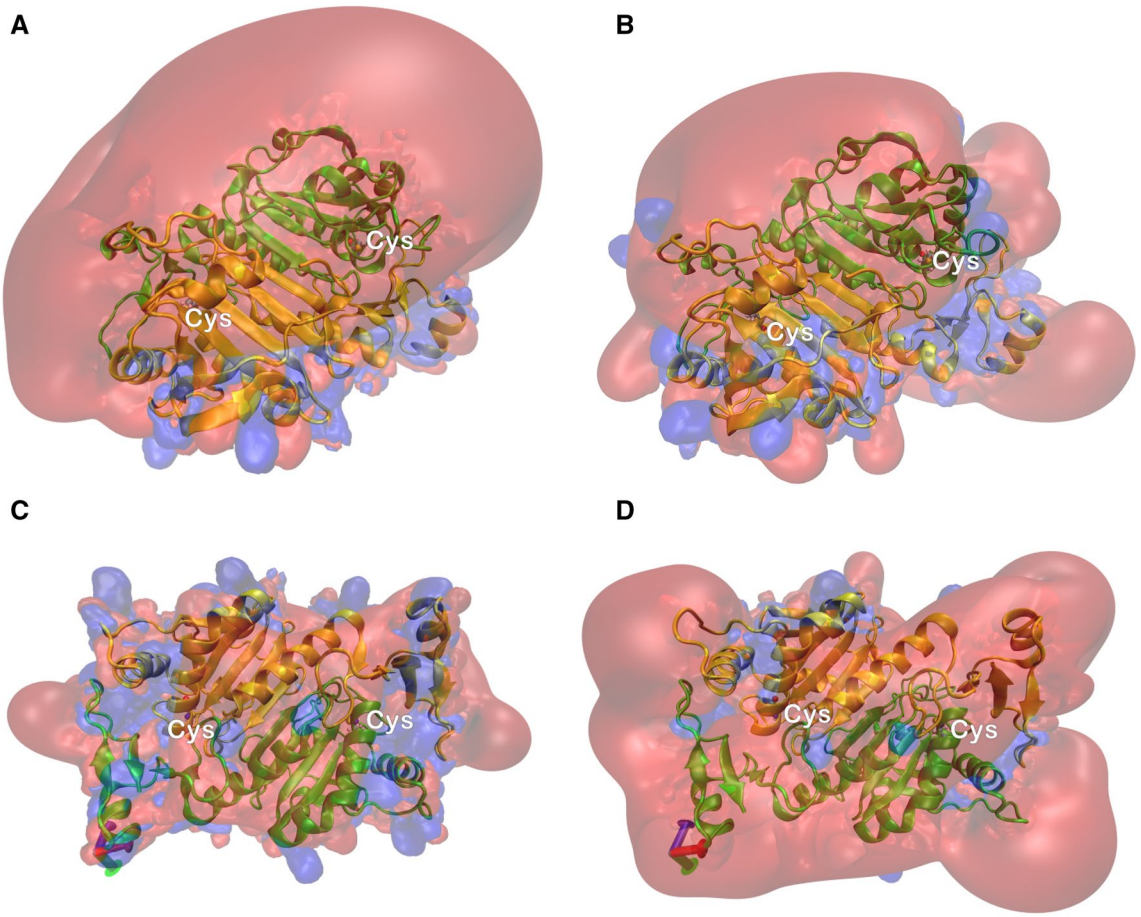


**Fig. 3** Potential isosurfaces are shown at +2 kT/e in *blue* and −2 kT/e in *red* for isoform A (**a**, **c**) and isoform B (**b**, **d**). Peroxidatic cysteines are reported in *ball* and *sticks*

## Discussion

In this paper, we report the characterization of Prdx6s in the Antarctic fish *T. bernacchii*. The presence of two Prdx6 variants in this species seems to be a common feature of fish, unlike most other vertebrate species that appear to encode a single Prdx6 isoform. Although the possibility exists that a second variant has simply not yet been identified in tetrapods, given the growing number of genome sequences available for these vertebrates, this seems unlikely. Despite the fact that only one Prdx6 isoform is described for most fish species represented in the NCBI database, several more recent studies have annotated two variants in several different teleost species. This suggests the appearance of the two *prdx6* genes through a duplication event likely occurred sometime before the speciation that led to the differentiation of fish families (beginning around 490 million years ago).

Furthermore, the evolution of the two variants seems to proceed together with the evolution of fish orders and families. In fact, in the phylogenetic analysis of coding sequences, the topology of the Prdx6A branch is very similar to that of Prdx6B, at least for Cichliformes and Cyprinodontiformes, in which sequences are grouped in well-supported clusters. Furthermore, Prdx6 sequences of species belonging to Perciformes, including *T. bernacchii*, fit this evolutionary pattern, emerging as a sister group of Cichliformes Prdx6s for both Prdx6A and Prdx6B. One peculiar exception to these trends are the positioning of *T. rubripes* (order Tetraodontiformes) Prdx6s. While variant *B* emerges with the ortholog from the Perciformes *S. partitus*, variant *A* emerges with the sequences of other Neoteleostei, yet it shares little correlation with both Ovalentaria and Eupercaria Prdx6s.

An unexpected result came from the comparison between the analyses of nucleotide and amino acid sequences of the Prdx6 isoforms. For Prdx6B, the two topologies were very similar; however, for Prdx6A we noted some important differences. In the Ovalentaria taxon the differences are limited, involving only the Prdx6s of *S. partitus*, while in the Eupercaria taxon, we found the

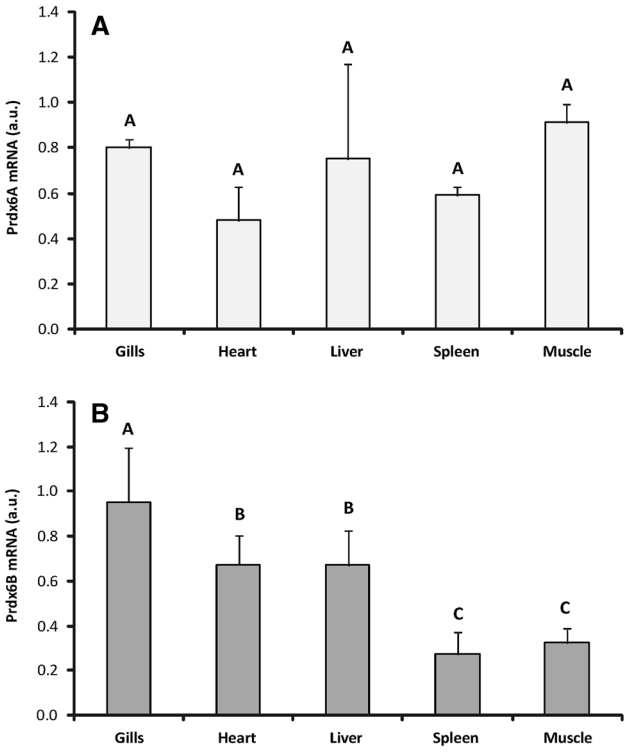


**Fig. 4** mRNA levels in heart, liver, spleen and skeletal muscle of *T. bernacchii* for *prdx6a* (**a**) and *prdx6b* (**b**). *Letters* Student–Newman–Keuls *t* test with respect to tissues ($p < 0.05$)

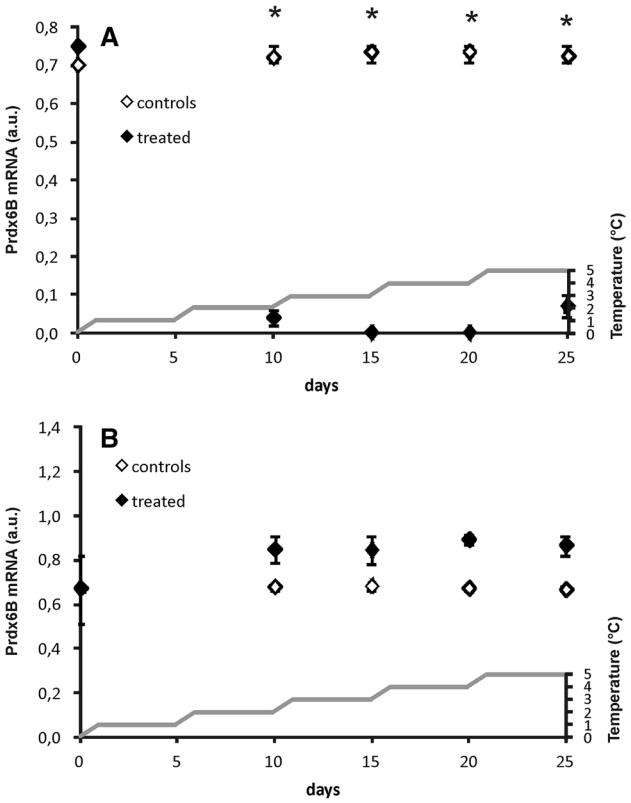


**Fig. 5** Accumulation of *prdx6a* (**a**) and *prdx6b* (**b**) mRNAs in liver of *T. bernacchii* in response to environmental temperature increase. *Asterisks* significant differences with respect to controls (***$p < 0.001$, **$p < 0.005$, *$p < 0.05$)

nucleotide sequences grouped together, but not the amino acid sequences. For instance, *Gadus morua* Prdx6 coding sequence clearly clusters as a sister group of Ovalentaria and Eupercaria Prdx6s, yet the amino acid analysis revealed weak relationships. Further analysis suggests that although substitutions at the nucleotide level are limited, a number of them are non-synonymous, leading to the higher variability observed at amino acid level. This could be an indication of a purifying selection effect. However, some residues could be under a strong positive selection, responsible for the higher degree of diversity among the vertebrate Prdx6 amino acid sequences. In particular, the last three residues of the positively selected amino acid sites ($Lys^{92}$, $Cys^{94}$ and $Cys^{95}$) are located within the motif FNQDKACC also subject to positive selection. This is a feature that is rarely seen investigating the presence of positive selection, and probably it could easily have contributed to the functional diversification of the two Prdx6 variants in fish.

Despite choosing a phylogenetically coherent group of organisms, and a protein that is highly conserved, it was difficult to identify amino acid changes in *T. bernacchii* that were possibly linked to thermal adaptation. From the data available so far, it is not possible to safely discuss whether the diversification of fish Prdx6s could also be correlated with specific physiological and behavioral characteristics of different families and species. Antarctic fish evolved in this environment, isolated for 10–12 million years, exposed to a very low and constant temperature as well as a high oxygen concentration. It has been proposed that such conditions may have affected their metabolic adaptive strategies during the evolution of this group of teleosts (Peck 2005). Indeed, several unique metabolic adaptations have been observed in these endemic fish, including increased mitochondrial density and the utilization of lipids as a primary energy source (Lin et al. 1974; Clarke et al. 1984; Johnston et al. 1998; Pörtner et al. 2005). It is thought these adaptations potentially result in an increased susceptibility to reactive oxygen species and oxidative stress (Roberfroid and Buc Calderon 1995; Abele and Puntarulo 2004), and the related evolution of efficient antioxidant systems (Albergoni et al. 2000; Santovito et al. 2000, 2006, 2012a, b). The high levels of Prdx6b mRNA measured in heart and liver of *T. bernacchii*, together with the relatively high antioxidant enzyme activities measured in the same organs of various Antarctic fish (Ansaldo et al. 2000; Santovito et al. 2012b; Enzor and Place 2014), where unsaturated fatty acids are predominantly accumulated, could be an example of this physiological adaptation in notothenioids.

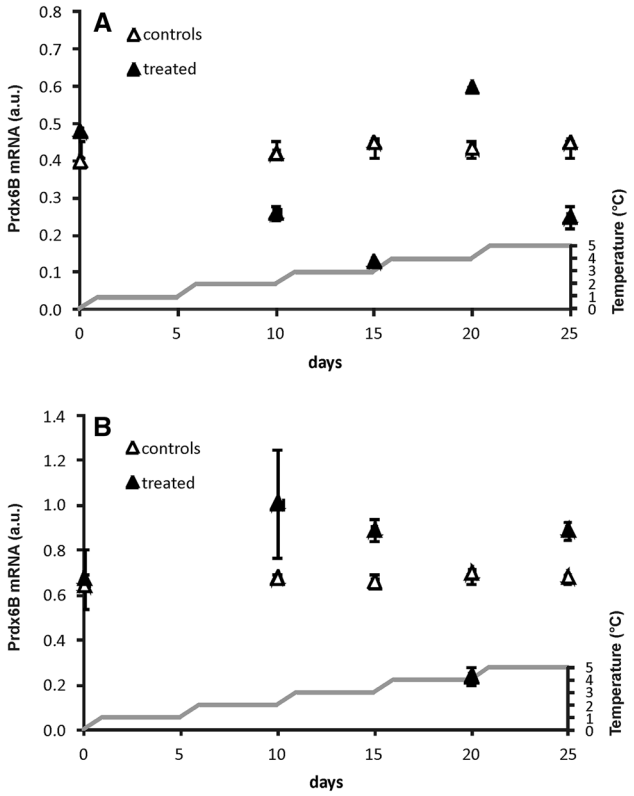


**Fig. 6** Accumulation of *prdx6a* (**a**) and *prdx6b* (**b**) mRNAs in heart of *T. bernacchii* in response to environmental temperature increase. *Asterisks* significant differences with respect to controls (***$p < 0.001$, **$p < 0.005$, *$p < 0.05$)

Further support for the perturbing effects of extreme cold temperatures on levels of oxidative damage have recently been published by Place and colleagues, who report a significantly higher level of oxidative damage occurs in multiple tissues of *T. bernacchii* when acclimated to −1.5 °C compared to specimens acclimated to +4 °C (Enzor and Place 2014). In this perspective, it might be interesting to note that Prxd6A of *T. bernacchii* has a unique structure with a particular surface charge distribution not seen in tetrapod orthologues. Variant *A* has a highly negative side and a weak positive opposite side whereas variant *B* is almost uniformly negative (similar to the surface charge of human Prdx). This situation is also observed in Prdx6 orthologues of *S. partitus*, suggesting this may be a common feature of all fish. Therefore, the presence of two variants of Prdx6s both in the Antarctic fish as well as in those of temperate zones could constitute an early adaptation that subsequently allowed these poikilothermic aquatic vertebrates to colonize very different environments, characterized by different temperature ranges. In this perspective, the possible thermal specialization of the two Prdx6 variants may have evolved prior the isolation of the Antarctic species, rather than constituting an adaptation to low temperatures that would have occurred in relatively recent times.

Although we cannot identify specific amino acid substitutions that are directly linked to cold adaptation in the Prdx6 variants, a number of amino acid substitutions unique to *T. bernacchii* suggest Prdx6A may display greater degree of flexibility at cold temperatures. Among these, $Ile^{34}$ is substituted by Val (this substitution is also present in the Prdx6B of *T. bernacchii* and *N. coriiceps*): both these amino acids are hydrophobic, but Val may confer more flexibility than Ile (Huang and Nau 2003). Given the proximity of this substitution to the functional motif that stabilizes $Ser^{32}$ (part of the phospholipase catalytic triad) this could potentially affect the phospholipase activity of Prdx6A. The same argument can be generated for the substitution of $Lys^{124}$ by Ser, which replaces a charged and rigid one amino acid with a polar, more flexible one (Huang and Nau 2003). This substitution occurs in a loop region close to $Arg^{132}$ (which is part of the peroxidase catalytic triad) and $Asp^{140}$ (which is part of the phospholipase catalytic triad) and could therefore affect both enzymatic activities of the protein. Finally, in *T. bernacchii* Prdx6A, $Pro^{183}$, whose ring structure provides greater rigidity, is replaced by Leu, which is also a hydrophobic amino acid but confers greater chain flexibility in another important loop region. Given that single point mutations in loop regions can confer dramatic changes in enzyme kinetics (Gekko et al. 2000), cumulatively, these amino acid substitutions may be related to low temperature adaptation (Römisch and Matheson 2003; Siddiqui and Cavicchioli 2006).

In addition to biochemical properties that suggest the Prdx6A orthologue in *T. bernacchii* may display temperature specialization, we generated gene expression data that also indicated Prdx6A seems to be the variant most affected by temperature. When acclimated to temperatures as little as 3 °C above ambient, we observed a significant decrease in the transcript levels for Prdx6A in both liver and heart tissues. Meanwhile, Prdx6B mRNA levels displayed significant increases in these same tissues. While the down-regulation of Prdx6A could potentially be viewed as an energetic trade-off, this would be counter-intuitive to previous studies that have shown *T. bernacchii* experiences increased levels of oxidative damage during short-term thermal stress, likely as a result of elevated metabolic rates (Enzor et al. 2013; Enzor and Place 2014). In fact, we have evidence that transcript levels for cytoplasmic superoxide dismutase (Cu, ZnSOD) increase in these same fish (in review). Increased Cu, ZnSOD activity, which converts superoxide ions into the less reactive species, $H_2O_2$, would result in elevated levels of substrate for enzymes such as Prdx6 and selenium glutathione peroxidase (GPx-1), which further reduce $H_2O_2$. We also have recent data that demonstrate mRNA levels of GPx-1 also increase in these fish (in review) that when combined with the increases in *prdx6b* and *Cu*, *ZnSOD* transcripts, suggest *T. bernacchii*

actively defends against ROS production at a time when Prdx6A is down-regulated. A more plausible hypothesis is that Prdx6B shares functional overlap with Prdx6A, and, if its structural stability is less temperature sensitive as suggested by the sequence and biochemical analyses, may compensate for the down-regulation of Prdx6A at elevated temperatures.

Ideally, it would be best to further test the hypothesis that Prdx6A is a "cold-adapted" enzyme by measuring the thermal stability of the active protein for the two variants of Prdx6s. Unfortunately, at the moment these data are not easy to obtain, since there is currently not an optimized methodology to test the activity of the 1-Cys Prdxs (unlike the 2-Cys Prdxs, for which a commercial kit also exists).

In conclusion, the data presented here are the first on the molecular and functional characterization of the genes encoding the Prdx6s of Antarctic fish, and constitute the starting point to study the expression of these enzymes in specific ecological contexts such as Antarctica. In particular, our expression analyses may be important for predicting climate change responses in this organism. In fact, the obtained results revealed rapid and specific responses of the Antarctic rockcode to temperature. This first demonstrated the effectiveness of using Prdx6s gene expression as biomarkers of stress in environmental field studies related to global warming. Furthermore, the presence of a Prdx (Prdx6B), whose expression is activated by increased temperatures, may be a condition that limits the stenothermy of *T. bernacchii*, rendering this species less vulnerable to moderate environmental temperature changes and other environmental perturbations associated with global climate change.

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