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Protection from Oxidative Stress in Immunocytes of the Colonial Ascidian *Botryllus schlosseri*: Transcript Characterization and Expression Studies

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Abstract. *Botryllus schlosseri* is a cosmopolitan colonial ascidian that undergoes cyclical generation changes, or take-overs, during which adult zooids are resorbed and replaced by their buds. At take-over, adult tissues undergo diffuse apoptosis and effete cells are massively ingested by circulating phagocytes, with a consequent increase in oxygen consumption and in production of reactive oxygen species (ROS). The latter are responsible for the death of phagocytes involved in the clearance of apoptotic cells and corpses by phagocytosis-induced apoptosis. However, the majority of phagocytes and hemocytes do not die, even if they experience oxidative stress. This fact suggests the presence of detoxification mechanisms assuring their protection. To test this assumption, we searched for transcripts of genes involved in detoxification in the transcriptome of *B. schlosseri*. We identified and characterized transcripts for Cu/Zn superoxide dismutase (SOD), γ-glutamyl-cysteine ligase modulatory subunit (GCLM), glutathione synthase (GS), and two glutathione peroxidases (*i.e.*, GPx3 and GPx5), all involved in protection from ROS. We also carried out a phylogenetic analysis of the putative amino acid sequences, confirming their similarity to their vertebrate counterparts, and studied the location of their mRNAs by *in situ* hybridization on hemocyte monolayers. We also analyzed gene transcription during the colonial blastogenetic cycle, which is the interval of time between one take-over and the next, by qRT-PCR. In addition, we investigated the effects of cadmium (Cd), an inducer of oxidative stress, on gene transcription. Our results indicated that i) antioxidant gene expression is modulated in the course of the blastogenetic cycle and upon exposure to Cd, and ii) hemocytes synthesize both enzymatic and nonenzymatic antioxidants, in line with the idea that they represent a major detoxification system for ascidians.

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Abbreviations: AG, adenine guanine (splicing consensus signal); ATG, start signal; CDS, coding sequences; Cu/Zn SOD, Cu-Zn superoxide dismutase; EST, expressed sequence tag; GCL, γ-glutamyl-cysteine ligase; GCLC, catalytic subunit of γ-glutamyl-cysteine ligase; GCLM, modulatory subunit of γ-glutamyl-cysteine ligase; GPx, glutathione peroxidase; GS, glutathione synthase; GSH, glutathione; GSSG, oxidized glutathione; GT, guanine timine (splicing consensus signal); ISH, *in situ* hybridization; MC, mid-cycle; ME, minimum evolution; ML, maximum likelihood; MP, maximum parsimony; NADPH, nicotinamide adenine dinucleotide phosphate; NJ, neighbor-joining; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PO, phenoloxidase; RACE, rapid amplification of the cDNA ends; ROS: reactive oxygen species; SEC, selenocysteine; SECIS, selenocysteine insertion sequence; SOD, superoxide dismutase; SODb, type B SOD; TAG, stop codon; TGA, thymine, guanine, and adenine nucleotides (stop codon); TO, take-over; UPGMA, unweighted pair group with arithmetic mean; UTR, untranslated region.

Introduction

Increasing evidence indicates that stressful conditions lead animals to increase the production of reactive oxygen species (ROS) by NADPH-, mitochondrial-, and microsomal-oxidase activity, which partially reduces molecular oxygen (Kaloyanni *et al.*, 2009; Tomanek, 2014; Canesi, 2015; Puppel *et al.*, 2015; Zeeshan *et al.*, 2016). Reactive oxygen species, including superoxide anions ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), peroxyl radicals ($\cdot RO_2$), and hydroxyl radicals ($\cdot OH$), exert microbicidal activity and prevent potentially pathogenic microorganisms from entering the weakened organisms. They can also activate signal transduction pathways mediating cell growth and apoptosis (De la Fuente and Victor, 2000; Lesser, 2006). Even in the immune system, phagocytes, once they are activated by the recognition of foreign molecules, increase their oxygen consumption in a process known as oxidative burst. This involves the activation of an inducible membrane oxidase and the consequent production of ROS.

When ROS levels exceed a threshold value, an imbalance occurs between the production of ROS and the ability of the cell and/or organism to readily detoxify the reactive intermediates or to repair the resulting damage. This condition, currently known as oxidative stress, is dangerous for cells and tissues because it can lead to the oxidation of lipids, proteins, and nucleic acids, producing irreversible structural and functional alterations. To prevent the negative effects of ROS, organisms evolved antioxidant defenses that can reestablish the cellular redox equilibrium, relying on both enzymatic and nonenzymatic mechanisms. Enzymes such as superoxide dismutase (SOD), catalase, glutathione reductase, and glutathione peroxidase (GPx) belong to the first category, whereas thiol-rich molecules, such as glutathione (GSH), metallothioneins, and phytochelatins, number among the nonenzymatic mechanisms.

Tunicates are invertebrate chordates and are considered the sister group of vertebrates (Delsuc *et al.*, 2006). For this reason, they are interesting organisms for evolutionary studies. Ascidians are the richest in species class of tunicates and thus are the most studied animal of this class.

Botryllus schlosseri is a colonial ascidian that performs cyclical (weekly, at 20 °C) generation changes, or take-overs, allowing recurrent rejuvenation of colonies (Manni *et al.*, 2007; Ballarin *et al.*, 2010). Colonies include three blastogenetic generations represented by mature, filter-feeding zooids, primary buds on zooids, and secondary buds (budlets) emerging from the primary buds (Manni *et al.*, 2007). During the generation change, lasting 24–36 h, tissues of adult zooids undergo diffuse apoptosis (Lauzon *et al.*, 1992, 1993; Cima and Ballarin, 2009; Ballarin *et al.*, 2010), and cells and corpses are rapidly ingested by phagocytes infiltrating the tissues after having left the circulation (Cima *et al.*, 2003; Manni *et al.*, 2007; Ballarin *et al.*, 2008a, b). In addition, a fraction of hemocytes, corresponding to 20%–30% of the total circulating cells, die by apoptosis at take-over and are replaced by new, undifferentiated hemocytes that enter the circulation from the hematopoietic sites (Ballarin *et al.*, 2008b). Among these are the phagocytes having ingested effete cells and corpses that tend to die by phagocytosis-induced apoptosis as a consequence of excessive respiratory burst (Cima *et al.*, 2010; Franchi *et al.*, 2016). Reactive oxygen species are also produced when cytotoxic morula cells sense the presence of nonself (Ballarin *et al.*, 2001) and release the enzyme phenoloxidase, which is stored in an inactive form inside their granules (Cima *et al.*, 2004; Franchi *et al.*, 2015). Phenoloxidase, acting on polyphenol substrata that are also released by morula cells, causes the production of ROS with microbicidal activity, for instance, during the nonfusion reaction between in-contact, genetically incompatible colonies (Ballarin *et al.*, 2002; Franchi *et al.*, 2015). Therefore, at take-over, when massive phagocytosis occurs, and during cytotoxic immune responses the majority of hemocytes need to protect themselves from the potential damages induced by ROS.

Until now, ascidian antioxidant strategies have been studied in the solitary species *Ciona intestinalis* (Franchi *et al.*, 2012, 2014; Ferro *et al.*, 2013) and *Halocyntia roretzi* (Abe *et al.*, 1999). Available data suggest that circulating hemocytes, in addition to their role in immune responses (Ballarin *et al.*, 2008b), are directly involved in the synthesis of ROS-scavenging molecules (Franchi *et al.*, 2012, 2014; Ferro *et al.*, 2013).

In the present study, we started a characterization of the ROS detoxification mechanisms in the hemocytes of *B. schlosseri*. New transcripts for *Botryllus* Cu/Zn superoxide dismutase (SOD), γ–glutamyl-cysteine ligase modulatory subunit (GCLM), glutathione synthase (GS), and two glutathione peroxidases (GPx3 and GPx5) are described, and their location in hemocytes is demonstrated through *in situ* hybridization (ISH). We also compared the level of mRNA transcription in colonies exposed to Cd—a known inducer of oxidative stress (Liu *et al.*, 2009) with respect to untreated colonies—by qRT-PCR. Our results indicated that immunocytes (both phagocytes and cytotoxic morula cells) are active in the transcription of genes involved in ROS detoxification, and their activity is modulated during the blastogenetic cycle and by the presence of Cd.

Materials and Methods

Animals

Colonies of *Botryllus schlosseri* (Tunicata, Ascidiacea) were collected near Chioggia, in the southern part of the Lagoon of Venice. They were reared according to the method of Gasparini *et al.* (2015), affixed to glass slides (5 × 5 cm), in aerated aquaria filled with 0.45-μm filtered seawater (FSW) that was changed every other day, held at a constant temperature of 19 °C, and fed with Liquifry marine (Liquifry Co., Dorking, UK). Under these conditions, colonies reproduce asexually by palleal budding and undergo take-over weekly. Within 24–36 h, old zooids are resorbed and replaced by their buds. A colonial blastogenetic cycle is defined as the period of time between one take-over and the next. Colonial developmental phases lasting more than one day from the preceding, or following, generation change are collectively known as mid-cycle (MC; Manni *et al.*, 2007).

Hemocyte collection

A colorless hemolymph containing various kinds of circulating hemocytes flows inside the lacunae and sinuses of the zooid open circulatory system and in the tunic vasculature that connects all the zooids and buds of the colony. Most of the circulating hemocytes are immunocytes, represented by phagocytes (both spreading and round) and cytotoxic morula cells (Ballarin and Cima, 2005).

Hemolymph was collected with a glass micropipette after puncture, using a fine tungsten needle, of the tunic marginal

vessels of the colonies. It was diluted 1:1 in 0.38% Na-citrate in FSW (as an anti-agglutinating agent) with pH 7.5, then centrifuged at 780 *g* for 10 min at room temperature. The resulting pellet was then resuspended in FSW to get a final concentration of 5×10^5 hemocytes/ml.

Exposure to cadmium

A storage solution was prepared by dissolving $CdCl_2$ in distilled water, whose concentration was determined by atomic absorption spectrometry, using a PerkinElmer 4000 spectrometer (PerkinElmer, Watham, MA), resulting in 45 mmol l^{-1}. It was subsequently diluted in FSW to obtain a working solution with a final concentration of 0.2 μmol l^{-1}. This concentration, although higher than those found in the environment, was effective in inducing oxidative stress in the hemocytes of *Botryllus schlosseri* (Franchi and Ballarin, 2013), and was within the concentration ranges used in toxicological experiments with other aquatic organisms (Jeppe *et al.*, 2014; Koutsogiannaki *et al.*, 2015; Mùgica *et al.*, 2015).

Nine colonies of comparable size (around 25 zooids each) were exposed to 0.2 μmol l^{-1} $CdCl_2$ in FSW, in 3 9-l aquaria (3 colonies per aquarium), at 16 °C. Three additional, unexposed colonies were used as controls. To avoid interference with the ROS production associated with the generation change (Cima *et al.*, 2010), exposed colonies were at the mid-cycle phase of the blastogenetic cycle; exposure time was limited to 2, 4, and 6 h. Previous results indicated that the effects of Cd exposure on hemocytes were already observable after a one-hour exposure (Franchi and Ballarin, 2013). After the exposure, colonies were collected, blotted dry, removed from the glass slides with a razor blade, frozen in liquid nitrogen, and stored at −80 °C until use.

Primer design, RNA extraction, cDNA synthesis, cloning, and sequencing

Our EST collection was aligned on the *Botryllus* genome already available online (Voskoboynik *et al.*, 2013). With this approach, many coding sequences (CDS) were recognized and recorded in our database (Campagna *et al.*, 2016). Comparison of our CDS collection (Campagna *et al.*, 2016) with the sequences of the vertebrate genes of interest allowed us to identify a series of nucleotide sequences and to design specific primers (Table 1) for PCR amplification. We focused our attention on the sequences of the predicted transcripts for GCLM, GS, Cu/Zn-SOD, GPx3, and GPx5, known as BsGCLM, BsGS, BsCu/Zn-SOD, BsGPx3, and BsGPx5, respectively. In all cases, the obtained EST sequences contained a 5′-terminal untranslated region (UTR) and the entire coding region. The 3′-rapid amplification of the cDNA ends (RACE) was performed using the 5′/3′ RACE Kit 2nd Generation (Roche Molecular Systems, Inc., Pleasanton, CA).

Table 1

PCR primers used and relative melting temperatures (Tm)

Primer	Tm (°C)	Sequence 5′–3′
BsGCLF	53	GTTGGAAAGAATCGGTAGGG
BsGCLFR	57.4	GCTTGGAATGACTTCTCAGGGAG
BsGCLF-RT	53.9	CGAAAGCGTTGAGTGTATGG
BsGCLM-RT	56.9	CAAAATCAGTCACGCCGATGTG
BsGSF	55.2	CGAAGCCAACATCATCCGA
BsGSR	55	CTCGGTTCGCTCTCATCTG
BsGSF-RT	60	CATGCGATCAGTCAAGATCC
BsGSR-RT	60	TTGCCATTGCAGTCTTCTTG
BsGPx5F	57.8	CATTGCTTGTTGCGAGTGCC
BsGPx5R	57	GCCACCAGAGTGTCCCAATA
BsGPx5F-RT	60	GGAAATGGATGGACGCCGCA
BsGPx5R-RT	60	CCTAACTCTTCGGTGTATGCGGGAC
BsGPx3F	58	CGTCGCTACAAGACAAGGTGG
BsGPx3R	55	ACATCTCCAACGCAAGTCC
BsGPx3R-RT	59.3	GGAAGCCACGACACCTTGC
BsSODF	58.4	CCACGGGTTTCACATTCACGAG
BsSODR	60.9	AATCCAATCACGCCACACGCC
BsSODF-RT	60	CTGTGCAAGGACTGACTCCA
BsSODR-RT	60	CCGGCATGATCAACCTTAGT
BsACTF-RT	60	ACTGGGACGACATGGAGAAG
BsACTR-RT	60	GCTTCTGTGAGGAGGACAGG
M13F	55	TTGTAAAACGACGGCCAGT
M13R	50	CAGGAAACAGCTATGACC
dT Anchor	57	ACCACGCGTATCGATGTCG (dT)16
Anchor	57	ACCACGCGTATCGATGTCG

PCR, polymerase chain reaction.

Total RNA was isolated from *B. schlosseri* colonies using the SV Total RNA Isolation System (Promega Corp., Madison, WI); its purity was determined spectrophotometrically by the A_{260}/A_{280} and A_{260}/A_{230} ratios. The integrity of RNA preparation was checked by visualizing the rRNA in ethidium bromide-stained 1.5% agarose gels. The first strand of cDNA was reverse-transcribed from 1 μg of total RNA according to the Improm II manual (Promega Corp.). cDNA amplification was performed with Go-Taq Polymerase (Promega; 5 U/μl), using the following cycling parameters: 94 °C for 2 min, 40 cycles of 94 °C for 30 s, melting temperature (Tm) for 30 s (Tms for the various primers are shown in Table 1), 72 °C for 1 min, and, a last step, at 72 °C for 10 min. Amplicons were subjected to electrophoresis and the corresponding bands were purified with ULTRAPrep Agarose Gel Extraction Mini Prep Kit (AHN Biotechnologie GmbH, Nordhausen, Germany), ligated in pGEM T-Easy Vector (Promega Corp.), and cloned in DH-5α *Escherichia coli* cells (Tang *et al.*, 1994). To confirm the sequences and their expression, positively screened clones were sequenced at BMR Genomics (University of Padova) on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Inc., Foster City, CA). Gene reconstructions were based on a *B. schlosseri* database using Spidey's algorithm (http://www.ncbi.nlm.nih.gov/spidey/).

Quantitative real-time PCR (qRT-PCR)

To estimate the total amount of mRNA for BsGCLM, BsGS, BsCu/Zn-SOD, BsGPx3, and BsGPx5, we used the qRT-PCR with the SYBR green method (FastStart Universal SYBR Green Master-Rox, Roche Molecular Systems, Inc.). In the first experimental series, mRNA was extracted from three colonies at take-over and three at mid-cycle (reference colonies) and maintained in FSW, to evaluate transcription changes under physiological conditions. In the second series, colonies at MC were exposed to 0.2 μmol l^{-1} $CdCl_2$ for 2, 4, and 6 h, and mRNA was extracted from three colonies for each exposure time. mRNA from three unexposed colonies (Cd concentration = 0) was used as reference control. Forward and reverse primers for BsGCLM (BsGCLF-RT and BsGCLR-RT), BsGS (BsGSF-RT and BsGSR-RT), BsCu/Zn-SOD (BsSODF-RT and BsSODR-RT), BsGPx3 (BsGPx3F and BsGPx3R-RT), BsGPx5 (BsGPx5F-RT and BsGPx5R-RT), and Bsβ-actin (BsACTF-RT and BsACTR-RT) transcripts—the last one (Bsβ-actin) used as a housekeeping gene—were synthesized by Sigma-Aldrich (St. Louis, MO) (Table 1). The stable expression of Bsβ-actin level (Campagna *et al.*, 2016) explains the choice of cytoplasmic actin as reference gene for quantitative PCR experiments. To exclude contamination by genomic DNA, all of the designed primers contained parts of contiguous exons; a qualitative PCR was also carried out before qRT-PCR. Furthermore, analysis of the dissociation curve of the qRT-PCR gave no indication of the presence of contaminating DNA.

qRT-PCR analyses were performed using Applied Biosystems 7900 HT Fast Real-Time PCR System, using the following cycling parameters: 95 °C for 10 min, then 40 cycles of 95 °C for 10 s and 60 °C for 1 min. cDNA synthesis was carried out as described above. Each set of samples was run three times and each plate contained cDNA from three different biological samples ($n = 3$) and negative controls. The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) was used to estimate the total amount of mRNA. The amounts of transcripts in different conditions were normalized to β-actin to compensate for variations in the amounts of cDNA.

Sequence alignment and phylogenetic analyses

Amino acid sequences of the proteins of interest were obtained by *in silico* translation. Sequence alignment and phylogenetic analyses were performed to compare the obtained sequences with those of the corresponding proteins from metazoans (Supplementary Table 1, view online). Alignments were carried out with Clustal W software (Larkin *et al.*, 2007) and assessed using the Molecular Evolutionary Genetics Analysis (MEGA) ver. 6 program (Tamura *et al.*, 2013) to infer evolutionary relationships among the various orthologous isoforms.

Phylogenetic reconstructions were performed according to unweighted pair group with arithmetic mean (UPGMA; Sneath and Sokal, 1973), minimum evolution (ME; Rzhetsky and Nei, 1992), neighbor-joining (NJ; Saitou and Nei, 1987), maximum parsimony (MP; Sourdis and Nei, 1988), and maximum likelihood (ML; Guindon and Gascuel, 2003) methods.

In situ *hybridization (ISH)*

For localization of mRNAs, sense and antisense probes for BsGCLM, BsGS, BsCu/Zn-SOD, BsGPx3, and BsGPx5 transcripts were obtained using T7 RNA- and SP6 RNA-polymerase. Probes were further purified with mini Quick Spin Columns (Roche Molecular Systems, Inc.). Whole colonies at MC (both Cd-treated and untreated) as well as hemocytes were used for ISH. Hemocytes, prepared as described above (see *Hemocyte collection* above in Materials and Methods), were left to adhere to Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA) for 30 min. Colonies and hemocytes were fixed in freshly prepared MOPS buffer (0.1 mol l^{-1} MOPS, 1 mmol l^{-1} $MgSO_4$, 2 mmol l^{-1} EGTA, and 0.5 mol l^{-1} NaCl) and 4% paraformaldehyde for 30 min and 2 h, respectively. After a prehybridization step in Hybridization Cocktail 50% Formamide (AMRESCO, Solon, OH) for 1 h at 58 °C, colonies and hemocytes were incubated with sense and antisense probes (2 μg/ml biotin-labeled riboprobe in Hybridization Cocktail) overnight at 58 °C. They were then incubated with the ABC Complex (Vector Laboratories, Inc., Burlingame, CA), and positivity was revealed by incubation in 0.025% DAB and 0.004% H_2O_2 in phosphate-buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 1.15 g/l Na_2HPO_4, pH 7.2) for 10 min. Colonies were then dehydrated, included in Paraplast Plus Xtra (Sigma-Aldrich), and 7-μm sections were obtained with a Jung micrometer. Hemocytes were mounted with Acquovitrex (Carlo Erba Reagents, Cornaredo, Italy). Finally, slides were observed under a light microscope at 1250× magnification.

Statistical analyses

Each experiment was replicated three times with three independent colonies ($n = 3$); data are expressed as means ± SD. Multiple comparisons were carried out with ANOVA; means were compared using Duncan's test (Snedecor and Cochran, 1980).

Results

Gene and transcript organization

The PCR amplification of BsGCLM produced an amplicon of 535 base pairs (bp). The coding sequence is 870 bp long, and is flanked by 5′-UTR and 3′-UTR regions of 54 and 626 bp, respectively (GenBank ID no. KT12002). The sequence includes one exon of 1550 bp (Fig. 1).

Amplification with BsGSF and BsGSR resulted in an amplicon of 304-bp sharing similarity with other deuterostome glutathione synthases (GSs) (GenBank accession no. KT120025).

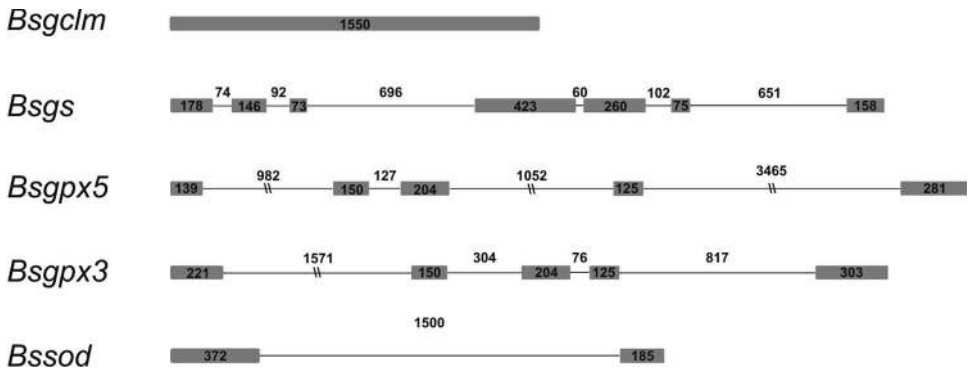

Figure 1. Gene organization of *Botryllus schlosseri* glutathione synthase (*bsgs*), *B. schlosseri* modulatory subunit of γ-glutamyl-cysteine ligase (*bsgclm*), *B. schlosseri* Cu-Zn superoxide dismutase (*bscu/znsod*), and *B. Schlosseri* glutathione peroxidase 3 (*bsgpx3*) and 5 (*bsgpx5*). Exons are denoted by gray rectangles, and introns by lines. Numerals refer to sequence length in base pairs (bps).

The coding sequence consists of 1278 bp and the gene includes 7 exons (Fig. 1) with the ATG start codon located in the first exon and the TAG stop codon in the last exon. All of the introns were provided with the canonical guanine timine (GT) and adenine guanine (AG) splicing signal consensus.

Amplification with BsGPx5F and BsGPx5R produced an amplicon of 493 bp that, after sequencing and BLAST comparison, resulted in vertebrate transcripts similar to those of *gpx3* and *gpx6*. This transcript presents a 675-bp coding sequence, with 5′-UTR and 3′-UTR regions of 58 and 184 bp, respectively (GenBank ID no. KT120026). The structure of the gene was analyzed by comparing the cDNA and the genomic sequences. It includes 5 exons (Fig. 1), with the ATG start codon located in the first exon and the TAG stop codon in the last exon. All of the introns were provided with the canonical GT and AG splicing signal consensus.

The PCR amplification with BsGPx3F and BsGPx3R gave an amplicon of 662 bp that, after sequencing and BLAST comparison, resulted in transcripts similar to those of *gpxb* and *gpxc* of *Ciona intestinalis*. The *bsgpx3* transcript has a coding sequence of 636 bp, with 5′ UTR and 3′ UTR regions of 128 bp and 239 bp, respectively (GenBank accession no. KT120027). The gene structure was analyzed by comparing the cDNA and the genomic sequences. It includes 5 exons (Fig. 1), with the ATG start codon located in the first exon and the TGA stop codon in the last exon. All of the introns were provided with the canonical GT and AG splicing signal consensus.

BsSODF and BsSODR amplified a sequence of 322 bp, similar to Cu/Zn SOD from other deuterostomes. The coding sequence of this transcript spans 447 bp in length and is flanked by 5′ UTR and 3′ UTR regions of 305 bp and 305 bp, respectively (GenBank accession no. KT120028). The structure of the gene was analyzed by comparing the cDNA and the genomic sequences. It includes two exons and one intron (Fig. 1) with canonical GT and AG splicing signal consensus.

Protein organization

In silico translation of the *bsgclm* transcript resulted in a putative protein of 289 amino acids with an Aldo/keto reductase superfamily domain extending from residues 85 to 208, required for antioxidant activity (Fig. 2A; Supplementary Fig. 1A, view online). BsGCLM, when aligned with the same protein of other deuterostomes, showed identities ranging from 34.3% (*C. intestinalis*) to 28.7% (*Xenopus laevis* and *Salmo salar*) (Supplementary Fig. 1A, view online).

In silico translation of the transcript of *bsgs* gave a putative protein of 425 amino acids with an eu-GS superfamily domain, typical of glutathione synthases (GSs) and necessary for the creation of the ATP-dependent bond between γ-glutamylcysteine and glycine, spanning from amino acid 10 to 400 (Fig. 2B; Supplementary Fig. 1B, view online). BsGS, when aligned with the same protein of other deuterostomes, showed identities ranging from 38.8% (*Branchiostoma floridae*) to 32.6% (*Danio rerio*). By comparing multiple alignments of the predicted amino acid sequence of BsGS with other deuterostome GSs, we recognized the amino acids of the active sites (Met^{129}, Ile^{143}, Lys^{309}, Asn^{368}, Tyr^{370}, the MEKI motif, Glu^{427}, Lys^{454}); the ATP-binding amino acids (Ile^{143}, Lys^{309}, Val^{365}, Lys^{367}, the MEKI motif, Glu^{427}, Lys^{454}); the magnesium-binding sites (Glu^{144}, Asn^{146}, Glu^{371}); and the GSH-binding sites (Arg^{123}, Ala^{148}, Ser^{150}, Glu^{215}, Asn^{217}, Gln^{221}, Arg^{452}, Val^{463}, Ala^{464}), all conserved in the *Botryllus schlosseri* sequence (Supplementary Fig. 1B, view online).

In silico translation of the transcript of *bsgpx5* resulted in a putative protein of 224 amino acids that included a conserved GSH-peroxidase domain (residues 30–145), necessary for hydroperoxide reduction by GSH, which acts as an electron donor (Fig. 2C; Supplementary Fig. 1C, view online). BsGPx5, when aligned with the same protein of other deuterostomes, showed identities ranging from 34.5% (*C. intestinalis* GPxc) to 24.8% (*B. floridae*). By comparing multiple alignments of

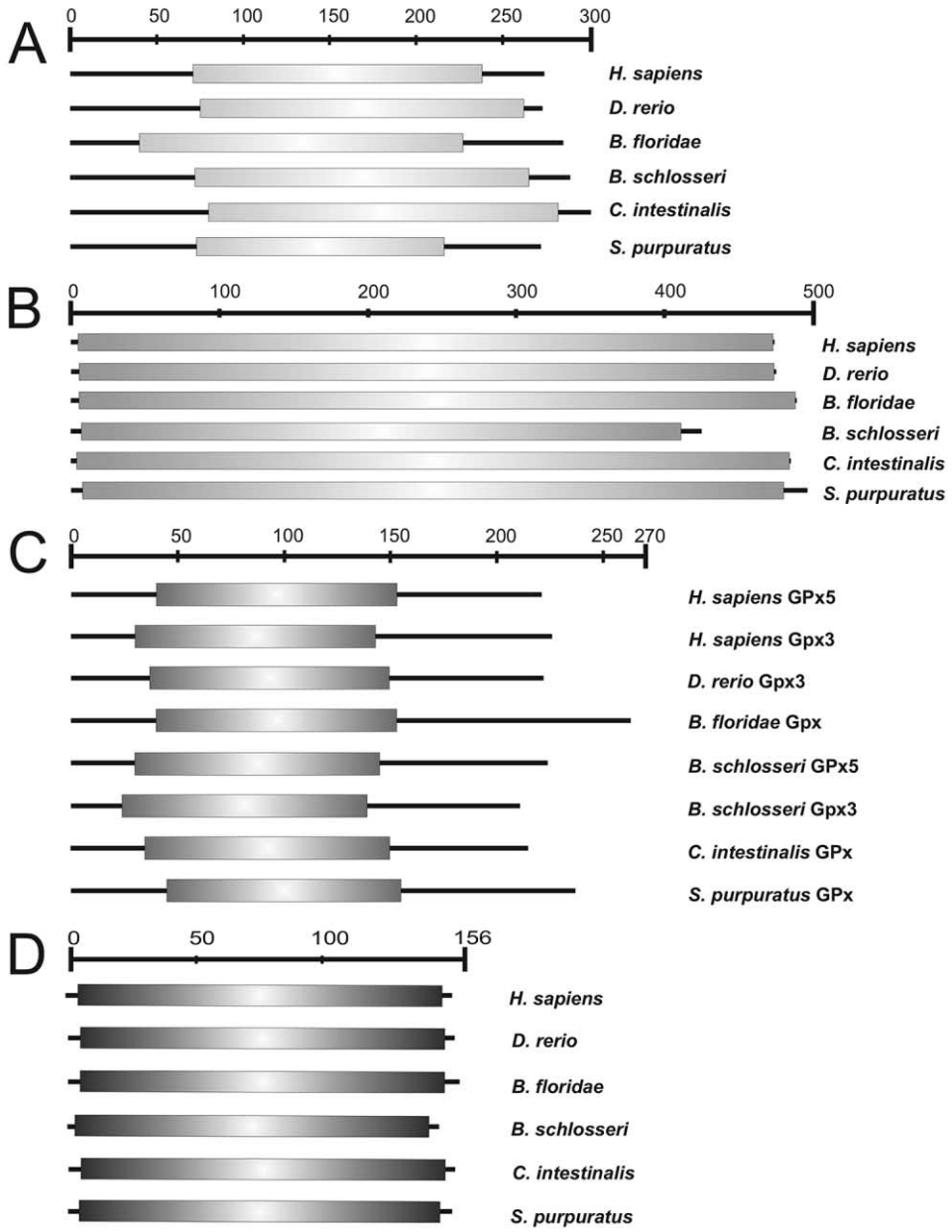

Figure 2. Schematic deuterostome domain organization of the modulatory subunit of γ-glutamyl-cysteine ligase (GCLM) (A); glutathione synthase (GS) (B); glutathione peroxidase 3 (GPx3) and 5 (GPx5) (C); and Cu-Zn superoxide dismutase (Cu/ZnSOD) (D) proteins. Numbers refer to the length of the amino acid sequences.

the predicted amino acid sequence of BsGPx5 with other deuterostome GPx, we identified two conserved amino acids of the active sites involved in catalytic activity in other deuterostomes (Gln^{95}, Trp^{173}). Residue 61, aligning with conserved U/C (cysteine with serine/cysteine) in vertebrates, is represented by a Ser, as in *B. floridae* (Supplementary Fig. 1C, view online).

In silico translation of the *bsgpx3* transcript resulted in a putative protein of 211 amino acids that included a conserved GSH-peroxidase domain, from residue 34 to 149 (Fig. 2C; Supplementary Fig. 1C, view online). BsGPx3, when aligned with the same protein of other deuterostomes, showed identities ranging from 38.2% (*C. intestinalis* GPxb) to 25% (*B. floridae*). By comparing multiple alignments of the predicted amino acid sequence of BsGPx3 with those of other deuterostome GPxs, we recognized the three amino acids of the active sites involved in catalytic activity (Sec/Cys^{161}, Gln^{95}, Trp^{173}) (Supplementary Fig. 1C, view online).

In silico translation of the transcript of *bscu/znsod* resulted in a protein of 148 amino acids, with the Cu-Zn superoxide

dismutase superfamily domain extending from residue 1 to 140 (Fig. 2D; Supplementary Fig. 1D, view online). BsCu/ZnSOD, when aligned with the same protein of other deuterostomes, showed identities that ranged from 57.9% (*Ovis aries*, *Bos taurus*, *Bos grunniens*) to 20.5% (*C. intestinalis*). By comparing multiple alignment of the predicted amino acid sequence of BsCu/ZnSOD with other deuterostome Cu/ZnSODs, we were able to recognize the amino acids of the active sites that bind cadmium (His^{41}, His^{43}, His^{115}), zinc (His^{66}, His^{75}), zinc and cadmium (His^{58}), as well as those involved in antioxidant reactions (Thr^{132}, Arg^{138}) (Supplementary Fig. 1D, view online).

Phylogenetic analyses

Phylogenetic trees were obtained from multiple alignments, using Clustal W on the predicted amino acid sequences of each considered transcript. All of the methods used gave similar results, but only trees that were obtained using maximum likelihood (ML) are presented. Trees of GCLM and GS showed that the tunicate cluster, represented by *Botryllus schlosseri* and *Ciona intestinalis*, is always positioned close to the cephalocordate + vertebrate clade (Figs. 3, 4).

As regards the phylogenetic reconstruction of deuterostome GPxs, BsGPx5 clusters together with *C. intestinalis* GPxc and *Xenopus laevis*, *Xenopus tropicalis*, and *Danio rerio* GPx3, as the sister group of vertebrate GPx1, GPx2, and GPx4 (Supplementary Fig. 2, view online), whereas BsGPx3 groups with *C. intestinalis* GPxc, *X. laevis*, and *X. tropicalis* GPx3, and *Branchiostoma floridae* GPx (Supplementary Fig. 3, view online). BsSOD clusters with *B. floridae* SOD; *C. intestinalis* SOD appears unrelated to the vertebrate group (Fig. 5).

qRT-PCR

When analyzed in the course of the blastogenetic cycle, the total amount of mRNAs for BsGCLM, BsCu/ZnSOD, and BsGPx5 significantly ($P < 0.001$) decreased during take-over with respect to MC. Conversely, BsGPx3, in the same conditions, significantly ($P < 0.001$) increased its mRNA level. The amount of mRNA for BsGS did not significantly change during TO phase with respect to mid-cycle (Fig. 6A).

Upon cadmium (Cd) exposure, the relative expression of the considered genes was deeply regulated. The quantity of mRNAs of *bscu/znsod*, *bsgpx3*, and *bsgs* that resulted were significantly ($P < 0.05$) increased, reaching the maximum amount of mRNAs, from 3- to 13-fold induction, after 2 h of treatment with Cd. The quantity then gradually decreased, with the lowest value seen at 6 h of treatment. Conversely, the level of mRNAs for BsGCLM and BsGPx5 decreased with respect to the control; BsGPx5 returned to the control value after 6 h (Fig. 6B).

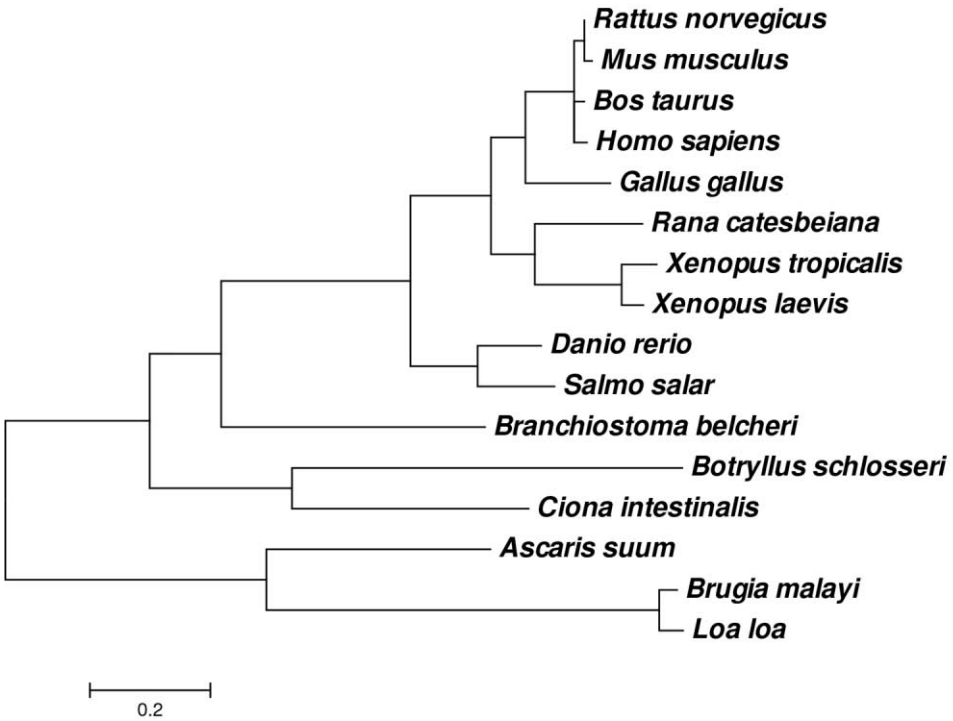

Figure 3. Evolutionary relationships (maximum likelihood; ML) among metazoan modulatory subunits of γ-glutamyl-cysteine ligases (GCLMs). Similar topologies were obtained with neighbor-joining (NJ), minimum evolution (ME), and unweighted pair groups with arithmetic mean (UPGMA). Branch length scale = 0.2 substitutions per site.

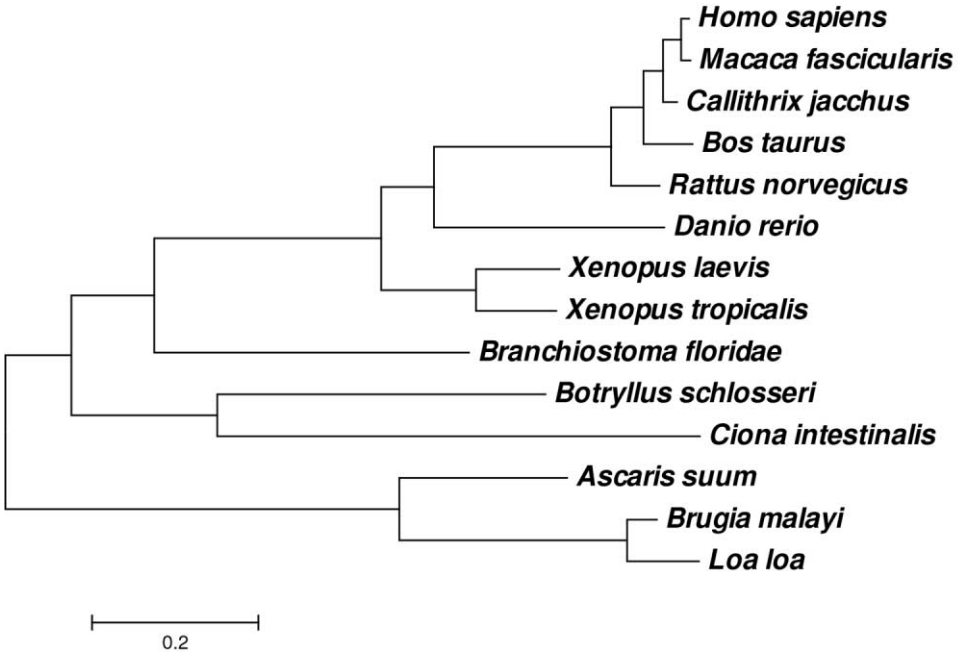

Figure 4. Evolutionary relationships (maximum likelihood; ML) among metazoan glutathione synthases (GSs). Similar topologies were obtained with neighbor-joining (NJ), minimum evolution (ME), and unweighted pair groups with arithmetic mean (UPGMA). Branch length scale = 0.2 substitutions per site.

In-situ *hybridization*

In colony sections, only hemocytes contained detectable levels of transcripts for BsGCLM, BsGS, BsCu/ZnSOD, BsGPx3, and BsGPx5 (data not shown). A more detailed analysis of hemocyte smears revealed that only immunocytes were labeled. In the presence of the specific riboprobes for BsGCLM and BsGPx3, cytotoxic morula cells and phagocytes were labeled, the former (BsGCLM) prevailing at take-over, and BsGPx3 prevailing at mid-cycle. Morula cells, at take-over, and phagocytes, at mid-cycle and take-over, were also recognized by antisense probes for BsGS and BsCu/ZnSOD, whereas only phagocytes were labeled by the probe for BsGPx5 (at mid-cycle and take-over). In addition, undifferentiated young cells, also called hemoblasts, appeared stained with the specific probes for GPx5 and Cu/ZnSOD in both mid-cycle and take-over. Incubation with the sense probes gave no labeling of the cells (Fig. 7).

Discussion

Despite the phylogenetic position of tunicates as the vertebrate sister group, their stress responses have been poorly investigated until now. A limited but increasing body of evidence indicates that ascidian hemocytes play important roles in stress responses by producing antioxidant molecules able to counteract the stress-related increase of ROS production (Franchi *et al.*, 2011, 2012, 2014; Ferro *et al.*, 2013).

In the compound ascidian *Botryllus schlosseri*, high quantities of ROS are produced both during the non-fusion reaction between genetically incompatible colonies, resulting in diffuse cytotoxicity along the contact region (Ballarin *et al.*, 2002) and at take-over, as a consequence of the increased respiratory burst in phagocytes that have ingested apoptotic cells and corpses deriving from the tissues of the old zooids (Cima *et al.*, 2010; Franchi *et al.*, 2016). In both processes, hemocytes are directly involved. Although some of them undergo ROS-induced cell death, most hemocytes do not die, suggesting their ability to overcome unfavorable conditions.

In the present work, we identified and characterized the transcripts for five *Botryllus schlosseri* enzymes (BsSOD, BsGCLM, BsGS, BsGPx3, and BsGPx5) involved in ROS detoxification mechanisms. To our knowledge, this is the first study of these genes in *Botryllus.* In addition, we demonstrated the modulation of the transcription of the above-reported genes during take-over and on exposure to cadmium. In both cases, increased production of ROS has been reported (Cima *et al.*, 2010; Franchi and Ballarin, 2013). This suggests that oxidative stress is the cause of the observed gene modulation and that cells face an increasing level of ROS by producing thiol-containing molecules, such as glutathione (GSH), or antioxidant enzymes. The location of the transcripts in immunocytes, as revealed by *in situ* hybridization, supports our previous observations in the solitary ascidian *Ciona intestinalis* (Franchi *et al.*, 2011, 2012, 2014; Ferro *et al.*, 2013), indicating that, in the absence of detoxifying or-

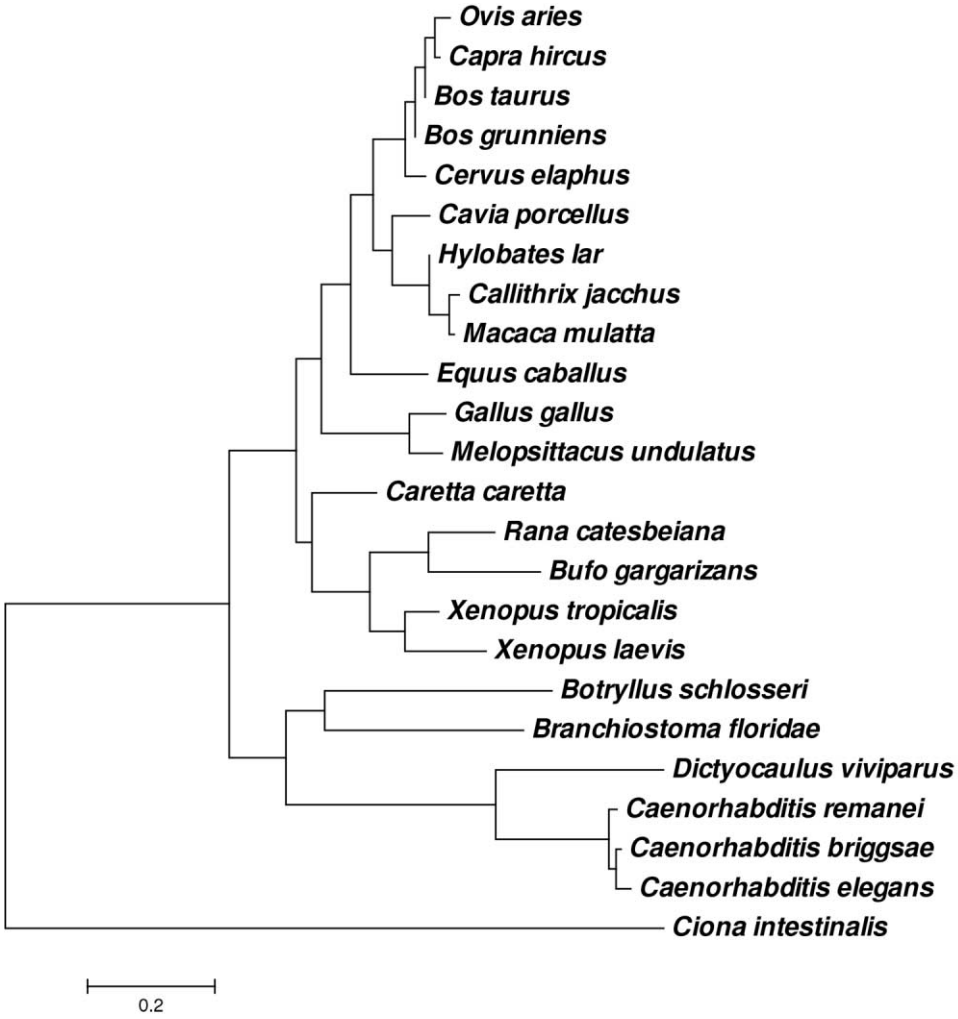

Figure 5. Evolutionary relationships (maximum likelihood; ML) among deuterostome glutathione peroxidase (GPxs). Similar topologies were obtained with neighbor-joining (NJ), minimum evolution (ME), and unweighted pair groups with arithmetic mean (UPGMA). Branch length scale = 0.2 substitutions per site.

gans, hemocytes represent the main detoxification system of tunicates. The reported presence of some transcript-related labeling in young hemocytes probably marks their first steps towards fully differentiated circulating cells.

The main intracellular antioxidant molecule is represented by GSH, a tripeptide (γ-glutamylcysteinylglycine) with a thiol group able to react with ROS, resulting in the formation of oxidized GSH (GSSG). In mammals, the synthesis of GSH involves two ATP-dependent reactions catalyzed by different enzymes. The first is γ-glutamyl-cysteine ligase (GCL), composed of a catalytic and a modulatory subunit (GCLC and GCLM, respectively) (Griffith, 1999; Dickinson and Forman, 2002). The second enzyme is glutathione synthase (GS), which catalyzes the binding of L-glycine to previously formed γ-glutamylcysteine. The transcripts for both genes are present in *Botryllus* immunocytes, either in phagocytes or morula cells. The amount of transcripts for BsGCLM decreases during take-over, as well as during Cd-treatment, suggesting a weak contribution of this subunit and, consequently, a probable major role of GCLC in the regulation of GSH synthesis in *B. schlosseri*. The increase in the amount of mRNA for BsGS in hemocytes from Cd-exposed colonies is probably due to Cd-induced oxidative stress, in accordance with the known induction of GSH synthesis by ROS (Franchi *et al.*, 2012; Jeppe *et al.*, 2014). In contrast, the absence of modulation in the level of transcript for BsGS, during the generation change, probably represents the equilibrium between the increase in gene expression, as a consequence of oxidative stress, and the decrease in the total number of aged cells as a consequence of apoptosis at take-over.

Among the detoxifying enzymes, superoxide dismutase (SOD) catalyzes a redox reaction, converting superoxide anions into molecular oxygen and hydrogen peroxide (Fridovich, 1986). GPxs, however, catalyze the reduction of peroxides

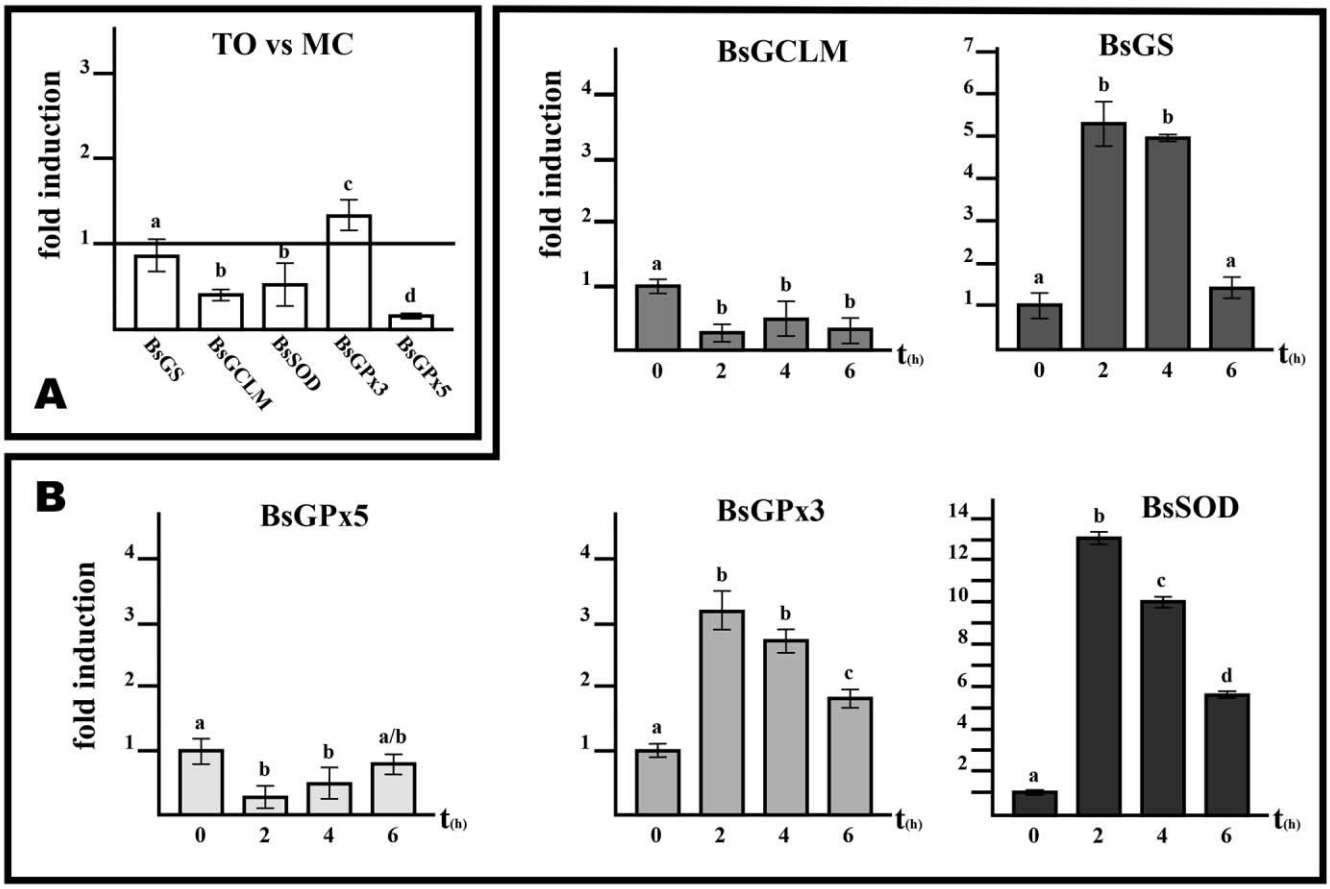

Figure 6. (A) Relative levels of mRNA in five *Botryllus schlosseri* enzymes: glutathione synthase (BsGS), *B. schlosseri* subunit of γ-glutamyl-cysteine ligase (BsGCLM), *B. schlosseri* Cu-Zn superoxide dismutase (BsCu/ZnSOD), and *B. schlosseri* glutathione peroxidase 3 (BsGPx3) and 5 (BsGPx5) at take-over with respect to mid-cycle (MC; reference control set to 1). (B) Relative levels of mRNA for BsGS, BsGCLM, BsCu/ZnSOD, BsGPx3, and BsGPx5 after treatment with 0.2 μmol l^{-1} $CdCl_2$ with respect to unexposed colonies (Cd concentration = 0; reference control set to 1). Normalization of expression was achieved using endogenous β-actin as housekeeping gene. Each histogram bar corresponds to the average of three independent experiments, each with a different colony ($n = 3$) ± SD. Different letters denote significant ($P < 0.05$) differences from the reference control. TO, take-over.

using GSH as substrate (Sunde and Hoekstra, 1980). Members of the GPx family can include a selenocysteine (SEC) residue in their *N*-terminal region. This residue is related to the presence of a SEC insertion sequence (SECIS) in the corresponding mRNA that allows the translation of the UGA codon of the catalytic site as SEC instead of as a STOP codon (Brigelius-Flohé, 1999; Brigelius-Flohé and Maiorino, 2013). In mammals, eight types of GPx have been identified so far (Brigelius-Flohé and Maiorino, 2013), expressed in various tissues (Ghyselinck *et al.*, 1993; Arthur, 2000; Toppo *et al.*, 2008; Brigelius-Flohé and Maiorino, 2013).

As stated earlier, our results indicated that the transcripts of these enzymes change during take-over and upon Cd exposure. For Cd, we observed an increase in the amount of mRNA for BsGS, BsGPx3, and BsCu/ZnSOD in exposed colonies. This finding is in agreement with the results obtained in solitary ascidians, which saw an increase in the transcription of genes for GCLC, GCLM, GS, metallothioneins, phytochelatin synthase, and SODb in *Ciona intestinalis* after treatment with 10 μmol l^{-1} $CdCl_2$ (Franchi *et al.*, 2011, 2012, 2014; Ferro *et al.*, 2013). The metal, cadmium, can deeply influence cysteine metabolism, acting at the level of the trans-sulfuration pathway, and cysteine is essential in detoxification processes because the amino acid is required for the synthesis of nonenzymatic, thiol-rich molecules, such as GSH, metallothioneins, and phytochelatins (Hughes *et al.*, 2009; Jeppe *et al.*, 2014).

The identified BsCu/ZnSOD lacks the signal peptide and can be considered an intracellular enzyme. According to the *in silico* hybridization results, its mRNA is located in morula cells and phagocytes. The lower amount of transcript at take-over is probably related to the fact that the transcripts are

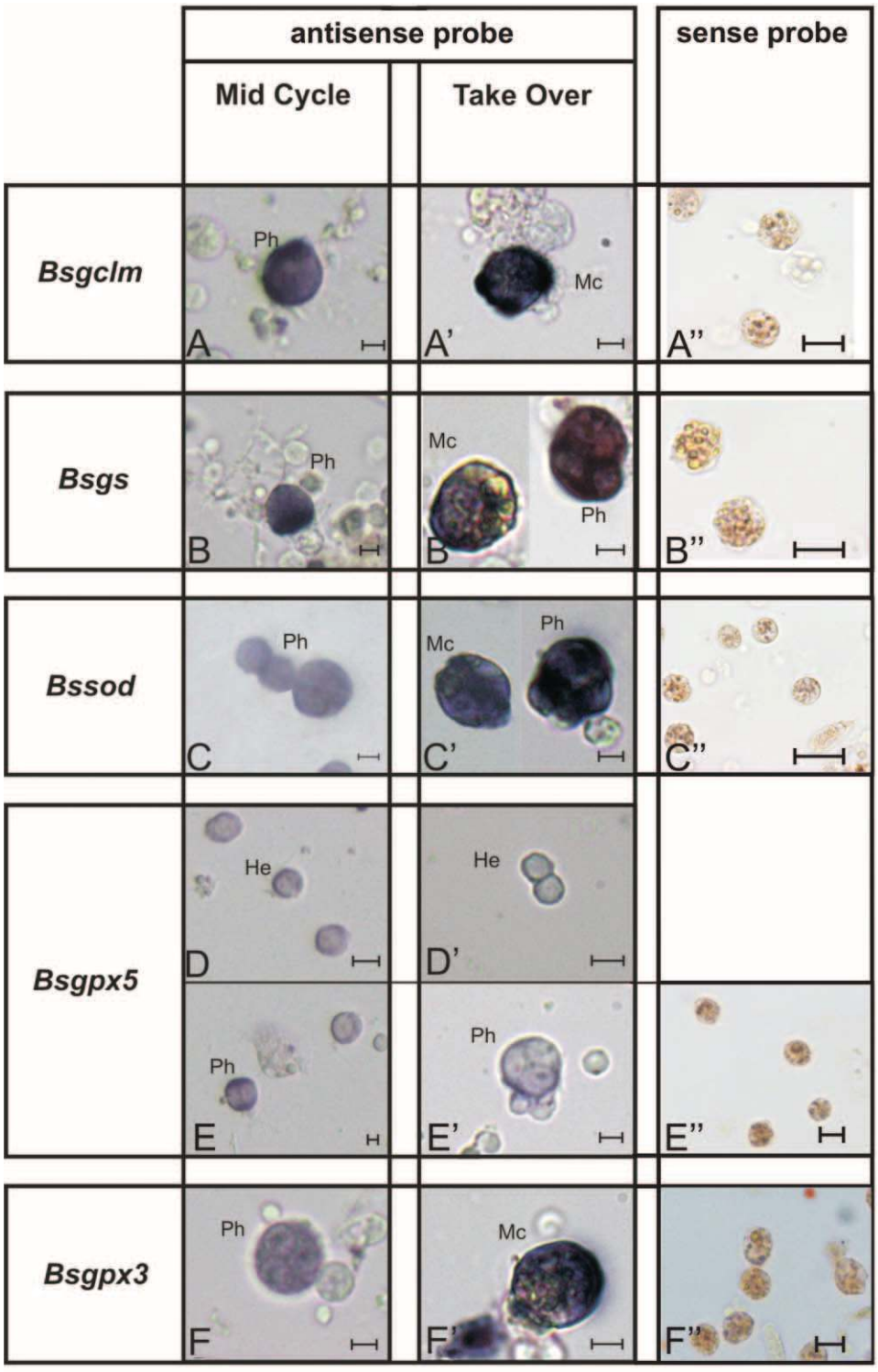

Figure 7. *In silico* hybridization of hemocytes stained with antisense riboprobes for transcripts of *bsgclm*, *bsgs*, *bscu/znsod*, *bsgpx3*, and *bsgpx5* in mid-cycle (A–F) and at take-over (A′–F′). A''—F'': sense probes (control). He, hemoblast; Mc, morula cell; Ph, phagocyte. Scale bar = 5 μm.

present in mature immunocytes, most of which, in this phase of the colonial blastogenetic cycle, undergo cell death by apoptosis (Cima *et al.*, 2010). However, the gene is activated by Cd exposure, resulting in an increase in the transcript level, in agreement with what was observed for the intracellular SOD of *C. intestinalis* (Ferro *et al.*, 2013).

GPxs include enzymes with or without the SECIS element, corresponding to a SEC or a Cys residue in the active site of the proteins, respectively. BsGPx3 shares with vertebrate GPx3 and GPx6 the presence of the SECIS. In addition, like vertebrate GPx3, BsGPx3 has the signal peptide that is absent in vertebrate GPx6. BsGPx5 lacks the SECIS and presents the signal peptide, as with vertebrate GPx5. What is unusual in BsGPx5 is the substitution of a Cys with a Ser residue, which has no reported antioxidant activity, in the catalytic site. The mRNA of both enzymes is mainly located in phagocytes, with BsGPx3 mRNA detectable only during the mid-cycle, when most of the phagocytes assume the spreading morphology and are not massively involved in phagocytosis (Ballarin *et al.*, 1994).

Different structure, pattern of expression, and response to $CdCl_2$ treatment strongly suggest different roles for the two BsGPx enzymes, with BsGPx3 more active in oxidative stress response and BsGPx5 probably involved in cellular homeostasis. This fits the observed increase in the amount of the mRNA for BsGPx3 at the generation change and in the presence of Cd, both the situations being marked by high ROS production. The decrease of the transcript level for BsGPx5, as detected by qRT PCR, at the take-over can be related to the decrease in the number of mature phagocytes in the colonial circulation.

Since changes in the amount of total mRNA do not necessarily correlate with changes in protein synthesis, these transcriptional data need to be augmented by quantification of the products (*e.g.*, through the measure of enzyme activity), the focus of future research.

Acknowledgments

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A: Amino acid alignment of gclm sequences obtained by CLUSTAL W

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Rattus norvegicus       ----MGTDSRAAGALLARASTLHLQTGNLLNWGRLRKKCPSTHSEELRDCIQKTLNEWSS 56
Mus musculus            ----MGTDSRAAGALLARASTLHLQTGNLLNWGRLRKKCPSTHSEELRDCIQKTLNEWSS 56
Bos taurus              ----MGTDSRAAGALLARASTLHLQTGNLLNWGRLRKKCPSTHSEELRDCIQKTLNEWSS 56
Homo sapiens            ----MGTDSRAAKALLARARTLHLQTGNLLNWGRLRKKCPSTHSEELHDCIQKTLNEWSS 56
Gallus gallus           ----MGTEG--ARALLERARTLNLQTGNLLSWGCGRKKCPVTPSEEVRDCIQKTLTEWSS 54
Xenopus tropicalis      ----MGTDSTAARTLLDKADKLVIQTGNLLNWGCLKKKCPSTPSEELQDCIRTTLNEWSQ 56
Xenopus laevis          ----MGTDSTAARTLLDKADTLILQTGNLLNWGCLKKKCPTTPSEELQDCIRTTLNEWNQ 56
Rana catesbeiana        ----MGTDT--ARSLLSAARTLILHTGNLMNWGRLKKKCPVTPGEELGDCIRITLSEWSS 54
Danio rerio             ----MEHHVN-AKRLFSHATTLKLHTGNLVNRSRLKKKCPSSPSEELQDCIQGTLSEWFT 55
Salmo salar             ----METHTTGAKVLLNHATTLNLHTGNLVNRSRLKKKCPSSPSEEIQDCVRATLSEWFA 56
Branchiostoma belcheri  --MSAVPVEEIARLMLSSTNNLTLHTGNIINWNHLKRKTSQNAGEEVLECLSATLNVWSS 58
Botryllus schlosseri    --------MDXAQMXVDQADTVVIXSGNIINSYSLKKRVGQKSSDELADSIEKTFTSWKE 52
Brugia malayi           MSTKVNHSSMEIISQVQKSSAVRLNTGNIQRHPELFRQMFRNSAEELVAALEHQL----D 56
Loa loa                 MSTKVSQSSVEIMSQVQKSSAVCLNTGNIQRHPELFRQLFKNSAEELVAALEHQL----D 56
Ascaris suum            MESKAYNK-----SELR---VFRLNTGNINSCGELKRRACKDSAEELVAALQIEL----N 48


Rattus norvegicus       QIS-PDLVREF-PDVLECTMSHAVEKINPDEREEMKVSAKLFIVGSNSSSSTRNAVDMAC 114
Mus musculus            QIS-PDLVREF-PDVLECTMSHAVEKINPDEREEMKVSAKLFIVGSNSSSSTRSAVDMAC 114
Bos taurus              QIS-PDLIREF-PDVLECTVSHAVEKINPDEREEMKVSAKLFIVGSNSSSSTRNAVDMAC 114
Homo sapiens            QIN-PDLVREF-PDVLECTVSHAVEKINPDEREEMKVSAKLFIVESNSSSSTRSAVDMAC 114
Gallus gallus           KVG-RDQNQEM-VEVLECTVAQAVEKMNPEERDELKVSAKLFIVGSNSSS-IRDAVDMAC 111
Xenopus tropicalis      KFS-PELVKEI-PQTLECTVPQAMETINLDEREEMKVSVKLFIVSPSHSS-VTQAIDMAC 113
Xenopus laevis          KIS-PELVKDI-SQTLECTVSQAMETINLDEREEMKVSVKLFIVSPSLSS-VTEAIDMAC 113
Rana catesbeiana        KAS-PEHIQDS-SQTLECAVNQALETINPEEREVMKVSAKLFVLDSGLSS-IGHAVDMAC 111
Danio rerio             AMP-SSIDSEL-PSVLDCSIPENTEAITPEEREELKVSVKLFLTEWDCSS-IRSAVDMAC 112
Salmo salar             TIPPPTSPTDL-PDTIDCSIPQATEAITPEEREELKVSVKLFICEADLSS-IKDAVDKAC 114
Branchiostoma belcheri  TVDPSWMNEES-NNALIITNPNQMERISEDERSKLKVSVKIFLLKWDPDL-VVEAVDQVC 116
Botryllus schlosseri    SVGPEYMNIPEPIHTLNVTNDALTWSHTDEIRSDLKISAKIFLSEVEPQL-VRNAVXNVC 111
Brugia malayi           GPNGPDLEMRP-DGTIMLTRDSENCRLNTIEKNDMKITLKVFMSSLDIKQ-VEESIDTAL 114
Loa loa                 GPNGPDLAMRP-DGTVMLARDSENCRLNTIEKADMKITLKVFMSSLDIKQ-VEESIDTAL 114
Ascaris suum            KRD--DLHFAN-DQTISLAAMET----NNYPRNDLKITLKVFMSSSDFSQ-VTDCLNATK 100
                                  .:  :        .   :  :*:: *:*.     .     .:: .

Rattus norvegicus       SVLGVAQLDSVIMASPPIEDGV---------NLSLEHLQPYWEELENLVQSKKIVAIGTS 165
Mus musculus            SVLGVAQLDSVIMASPPIEDGV---------NLSLEHLQPYWEELENLVQSKKIVAIGTS 165
Bos taurus              SVLGVAQLDSVIIASPPVEDGV---------NLSLEHLQPYWEELQNLVQSKKIVAIGTS 165
Homo sapiens            SVLGVAQLDSVIIASPPIEDGV---------NLSLEHLQPYWEELENLVQSKKIVAIGTS 165
Gallus gallus           SSLGVAQLDSVIIAPSAIEDET---------SLSLEYLQPYWQELETLVENKKIVAIGTS 162
Xenopus tropicalis      STLGVAQIDSMIIAPPPLEDGR---------SFSLEDLQPYWEELESLVRNGKVVSIGTS 164
Xenopus laevis          STLGVAQIDSVIIAPPPLEDGR---------SVSLENLQPYWEELERLVRDAKVVSIGTS 164
Rana catesbeiana        STLAVGQLDSVIIAPPPLEDGV---------ILTLEHLQPYWRELESLVQDNKVVAIGTS 162
Danio rerio             LSLGVSQLDSVFIAPPPLPEGE---------AQTLTHLQPLWQELESLVQSQKIAAIGTS 163
Salmo salar             QALAVSQLDSVIIAPPALPEEE---------SQTLANLQPAWRELEGLVQSHKIATIGTS 165
Branchiostoma belcheri  NELDIGVVDSVLLALPPLEAEMG-------EELTVNHILPMWEPMERLYDVERVSAIGTS 169
Botryllus schlosseri    KTLGVXYLDSAVVXFPXXXPEAY-------VEGSFSKALSVWREMEAMTKEGLVKHIGVT 164
Brugia malayi           HEINTKSISQLIIAFPPDDKLDIDPSVPTEVEEWLSHILPFWTQLETLVRTHKINTLGVA 174
Loa loa                 REINTKSISQLIIAFPPDDKLDIDLSVPSEVQRWLSHILPFWKQLETLVRTHKVNTLGVA 174
Ascaris suum            SLLGVESIEQLIMSFN-NFEPESEDSEDKELKNWVENVISVWEKIEALVKNGEISTVGVA 159
                          :    :.. .::                    .    . *  :: :     :  :*.:

Rattus norvegicus       DLDKTQLEQLYQW-AQVKPNSNQVNLASCCVMPPDLTAFAKQFDIQLLTHNDPKELLSEA 224
Mus musculus            DLDKTQLEQLYQW-AQVKPNSNQVNLASCCVMPPDLTAFAKQFDIQLLTHNDPKELLSEA 224
Bos taurus              DLDKTQLEQLYQW-AQVKPNSNQVNLASCCVMPPDLTAFAKQFDIQLLTHNDPKELLSEA 224
Homo sapiens            DLDKTQLEQLYQW-AQVKPNSNQVNLASCCVMPPDLTAFAKQFDIQLLTHNDPKELLSEA 224
Gallus gallus           DLDKTLLEQLYVW-AQVKPSSNQVNLASCCVMPPDLTAFAKQFDIQLLTHNDPKELLCEA 221
Xenopus tropicalis      DLDKALLEQLYLW-SQVKPASNQVNLASCCIMPPDLTEFAKQFDIQLLTHNDPKELLSEE 223
Xenopus laevis          DLDKALLEQLYLW-SQVKPASNQVNLASCCIMPPDLTEFAKQFDIQLLTHNDPKELLSEE 223
Rana catesbeiana        DLDKPLLEQLYLW-AQIKPSSNQVNLASCCIMPPDLTAFAKEFDIQLLTHSDSKEILSEE 221
Danio rerio             DLDKTLLEQLYNW-AQIKPSSNQVNLASCCVMPPDLTAFAKEFDIQLLTHSDPKELISAA 222
Salmo salar             DLDKELLEQLYNW-AQVKPSSNQVNLASCCVMPPDLTAFAKEFDIQLLTHNDPKELITAA 224
Branchiostoma belcheri  DLDKEMLEQTHGM-ARVKPTINQVNVVSCCVIPPELTAFAKENDIQLLTHSDPRDVLPTP 228
Botryllus schlosseri    DFDKTAMQNLYRC-ATVKPTIDQIKTGHDFNVPDDLIKLSEEYDIELLSHDDAADMLPEK 223
Brugia malayi           DLDYEQLKALYESTNDHRPMIDHYSTEHCCTVPPELREYAKQKDIQLLTHNDPNLHSINE 233
Loa loa                 DLDYEQLKALYESTDDHRPMIDHYSTEHCCTVPPELREYAKQKDIQLLTHNDPN-LYSIS 233
Ascaris suum            DFDLNHLKALYDG-AEIKPRIAHFNIAGCCSVPKDLQDYARENDIQLLTHNDPK-PFVTA 217
                        *:*   ::  :      :*   : .      :* :*   :.: **:**:*.*.

Rattus norvegicus       SFQEALQESIP-DIEAQEWVPLWLLRYSVIVKSRGIIKSKGYILQAKRKGS--------- 274
Mus musculus            SFQEALQESIP-DIEAQDWVPLWLLRYSVIVKSRGIIKSKGYILQAKRRGS--------- 274
Bos taurus              SFQEALQESIP-DIRAHEWVPLWLLRYSVIVKSRGIIKSKGYILQAKRKGS--------- 274
Homo sapiens            SFQEALQESIP-DIQAHEWVPLWLLRYSVIVKSRGIIKSKGYILQAKRRGS--------- 274
Gallus gallus           SFQEVLQESIQ-NVKAHEWIPLWILRYSVIVKSRGIIKSKGYIMQAKRNAS--------- 271
Xenopus tropicalis      AFQEALKESAQ-ECHSSAWSPIWILRYSVIVKTRGIIKLKGYILQAQRKGSL-------- 274
Xenopus laevis          DFQEALKESVP-ECHFNAWSPIWILKYSVIVKTRGIIKLKGYILQAKRKGSF-------- 274
Rana catesbeiana        SFQEVLRDSVE-GSDADEWSPVWVLRYSVIVKTRGIIKSKGYIVQAKRKAPH-------- 272
Danio rerio             GFQEAVQGSSQ-ELQVDDWRLEWVLRYSIIVKSRGIIKAKGYIVHAKKSNNH-------- 273
Salmo salar             SFQEAVQESTQ-DLKVADWRLEWVLRYSIIVKSRGIIKAKGYLVHAKRSSL--------- 274
Branchiostoma belcheri  TFQEILRGSSH-DDHVDEWQPFWVLRYTVMVKCRGVIKAKGYIVNGRRHATDMPLSVA-- 285
Botryllus schlosseri    SFQAIMEDNFPNSTSASEWLPSYAVRYSAIARCRGIVRSKGYIVEAGKRLSNGYYTVTTG 283
Brugia malayi           ERLDATT---RKLFGNEHFDLLFIARLTVWLRSRSIIVGKGYILKFMRKIS--------- 281
Loa loa                 ERLDATT---RKLFGNEHFELLFIARLTVWLRSRSIIVGKGYILKFLRKI---------- 280
Ascaris suum            DGLKDICNNEKYPLCDHDYKPSWASRYTVWVRGRSIIAAKGYMVQFERS---------- 266
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Rattus norvegicus	------
Mus musculus	------
Bos taurus	------
Homo sapiens	------
Gallus gallus	------
Xenopus tropicalis	------
Xenopus laevis	------
Rana catesbeiana	------
Danio rerio	------
Salmo salar	------
Branchiostoma belcheri	------
Botryllus schlosseri	GEGYID 289
Brugia malayi	------
Loa loa	------
Ascaris suum	------

B: Amino acid alignment of gs sequences obtained by CLUSTAL W. Green highlight active sites, pink highlight ATP binding sites, yellow highlight magnesium binding sites and red highlight GSH binding sites. Grey highlight amino acid with catalytic and ATP binding activity.

```
Homo sapiens              ----------------MATNWGS-LLQDKQQLEELARQAVDRALAEGVLLRTSQEPTSSE 43
Macaca fascicularis       ----------------MATNWGS-LLQNEQQLEELARQAVDRALAEGVLLRTSQEPTSSE 43
Callithrix  jacchus       ----------------MATNWGS-LLQDEQQLEELARQAVDRALAEGVLLRTSQEPTSSD 43
Bos taurus                ----------------MATGWGS-LLQDEQQLEELARQAVDRALAEGVLLRTSQAPSSSH 43
Rattus norvegicus         ----------------MATSWGS-ILQDEKQLEELAQQAIDRALAEGVLLRSAKNPSSSD 43
Xenopus laevis            ----------------MADLWDD-IYNDTKLLEELAPIAIDAALLQGVLMRTKESPNSSD 43
Xenopus tropicalis        ----------------MEALWED-IYSDTTLLEELAPIAIDSALLQGVLMRTKASPNSSD 43
Danio rerio               ----------------MSVKLED-TLKDENLIKRLEEIAKDTALLHGVLMRTKDTPNSPE 43
Branchiostoma floridae    ----------------MEPALPL-PLAPP-LLASAADSAKDWAILHGVAMRTEEQPNSSD 42
Botryllus schlosseri      ----------------MDAATELSKFLEQRSVGQLVLDAVDYAICNGIVMRTRSKPTSSD 44
Brugia malayi             MDEMPIRNNDDS----VLKYYPKLELIDGKLIKQLVEDTVDWAHAHGMVMRTAMTTDRSD 56
Loa loa                   MDETSVRSENDS----VLEYYPKLELIDRKLIKQLVEDTVDWAHAHGMVMKTETAADRGD 56
Ascaris suum              MNVDAERKQQQSDGSNIPNRYTFSLPLHELPLKDMLEDAVDWAHGCGMVMRTPQHKDRSD 60
                                          :              :      : * *   *: :::       .

Homo sapiens              VVSYAPFTLFPSLVPSALLEQAYAVQMDFNLLVDAVSQNAAFLEQTLSSTIKQDDFTARL 103
Macaca fascicularis       VVSYAPFTLFPSLVPSALLEQAYAVQMDFNLLVDAVNQNAAFLEQTLSSTIEQDDFTARL 103
Callithrix  jacchus       VVSYAPFTLFPSLVPSALLEQAYAVQMDFNMLVDAVSQNAAFLEQTLASTIKRDDFTARL 103
Bos taurus                VVSYAPFTLFPSPVPSALLEQAYAVQADFNLLVDAVSQNAVFLEQTLSSTIKRDSFTARL 103
Rattus norvegicus         VVTYAPFTLFPSPVPSTLLEQAYAVQMDFNILVDAVSQNSAFLEQTLSSTIKKDEYTARL 103
Xenopus laevis            VVSFAPFALLPSPVPKALFEQAKCVQEDFNTLVDRISQDTSFLEQVLSSTIKVDDFIRRL 103
Xenopus tropicalis        VVNFAPLTLLPSPVPKALFEQAKSVQEDFNLLVDRISQDTDFLEKALSSTAKVDDFIQRL 103
Danio rerio               VVSYAPFTLFPTSVPAALFHQALAVQTQFNRLVDRVSQNHSFLEDTLASTIKVDDFTARL 103
Branchiostoma floridae    VNFAPFLLLPSPVPRILFDQAKAVQRDFNLLVHRLSQDTNFLTSCLKSTIQVDDFTCRL 102
Botryllus schlosseri      VVQHAPFTLLPSPVPKNLYEQAISVQKDFNFLMHAISQDPEFMKSAFSTIIHSDDFTRRL 104
Brugia malayi             ICQTAPCTLFPSPFPYNLFQEAMDIQRTFSLLYFRISWDFDFLIKSHAEVVKTDDFTRHF 116
Loa loa                   ICQIAPCTLFPSPFPYSLFQEAMDIQQAFSLLYFRISWDFDFLIKSHAEVVKTDDFTKHF 116
Ascaris suum              ICQTAPFTLLPSPFPRHIFQQAVDVQQATNLLYFRMSWDYEFLIESHAEVVKTDDFTRHF 120
                              **  *:*: .*  : .:*  :*   . *   :. :  *: .      . *.:  ::

Homo sapiens              FDIHKQVLKEGIAQTVFLGLNRSDYMFQRSA-----DGSP----------ALKQIEINTI 148
Macaca fascicularis       FDIHKQVLKEGIAQTVFLGLNRSDYMFQRST-----DGSP----------ALKQIEINTI 148
Callithrix  jacchus       FDIHKQVLKEGIAQTVFLGLNRSDYMFQCSA-----DGSP----------VLKQIEINTI 148
Bos taurus                FDIHKQVLKEGIAQTVFLGLNRSDYMFQCNP-----DGSA----------ALKQIEINTV 148
Rattus norvegicus         FDIYKQVLKEGIAQTVFLGLNRSDYMFQCSA-----DGSK----------ALKQIEINTI 148
Xenopus laevis            FAIHKQVQQEDCTQEVFLGINRSDYMFDCRD-----DGTP----------ALKQIEINTI 148
Xenopus tropicalis        LKIQRQVKKEGCEQEVFLGINRSDYMFDCRD-----DGTP----------ALKQIEINTI 148
Danio rerio               FNIYKQVQQEGHAQKIVVGLNRSDYMLDHSP-----DGRT----------SLKQIEINTI 148
Branchiostoma floridae    FRIYEQVRREGVAQTVCLGLHRSDYMLDDPSRTGQENGEPEAKQPRLDLLQLKQIEINCI 162
Botryllus schlosseri      FEIYETALLE-KPKKTAMAIIRSDYMFNKTD-----ESEP----------SLKQIEVNMI 148
Brugia malayi             VEILNAVXTSNFCQKKTLLIQRNDYMCHEDS-----YGNR----------SLKQIEVNNI 161
Loa loa                   VEILNAVRTSDFCQRKTLMIQRNDYMCHEDN-----YGNR----------SLKQIEVNNI 161
Ascaris suum              VDILKRVHEAGIKQTKTLLIQRADYMCDGQR-----SDEF----------KLKQVEVNNI 165
                          . * . .      :   : : * *** .          .             ***:*:* :

Homo sapiens              SASFGGLASR-TPAVHRHVLSVLSKT--KEAGKILSNNPSKGLALGIAKAWELYGSPNAL 205
Macaca fascicularis       SASFGGLASR-TPAVHRHVLSVLSKT--KEAGKILSNNPSKGLALGIAKAWELYGSTNAL 205
Callithrix  jacchus       SASFGGLASR-TPAVHRHVLSVLSKT--KEAAKILSNNPSKGLALGIAKAWELYGSANAL 205
Bos taurus                SASFGGLASR-TPAVHRHVLSVLGKT--KEAAKILSNNPSKGLAMGIAKAWELYGSANAQ 205
Rattus norvegicus         SASFGGLASR-TPAVHRHVLNVLNKT--NEASKILSNNPSKGLALGIAKAWELYGSANAV 205
Xenopus laevis            AASFGGLASR-TPAVHQHVLKFLRKS--EESSSILTNDAVEGIGWGIAHAWALYGSVDAT 205
Xenopus tropicalis        AASFGGLSAR-VPEVHQHVLKVLKKS--EEASNILPNNPVEGIAWGIASSWAVYGSTSAV 205
Danio rerio               AASFGGLASR-TPDVHRHILKVANLP--DECSLVLDNNPAAGLAKGLAKAWELYGSKRAV 205
Branchiostoma floridae    ASSFGGLGTQ-MPGLHRHVLRLCGVSRFTADQQLPQNRAMSSLADGLAAAWELYGEQSAV 221
Botryllus schlosseri      ASSFGGIGTEKTRSLHDYTLRHAGFE--DVATSLPQNKALQNLAQSIVNAWDLYGDNSAV 206
Brugia malayi             AASMGXLAER-ATCVHKRTLETVQLPSKIIEKAIXDNHPTVTIARGIYEAWYDFGVPEAI 220
Loa loa                   AASMGSLAER-ATCVHRRTLETLQLPNKIIEKAILNNHPTVTVAKGICEAWYDYGVPEAI 220
Ascaris suum              AASMGWLSEM-ASCLHRRVLQDLNVPDDIIANALPENRPIDTVAKGIYDAWLDIGDQSAL 224
                          ::*:* :.      :*   *             :  * .   :. .:  :*   *   *

Homo sapiens              VLLIAQEKERNIFDQRAIENELLARN---IHVIRRTFEDISEKGSLD--QDRRLFVDGQE 260
Macaca fascicularis       VLLIAQEKERNIFDQRAIENELLARN---IHVIRRTFEDISEKGSLD--QDRRLFVDGQE 260
Callithrix  jacchus       VLLIAQEKERDIFDQRAIENELLARN---IHVIRRRFEAISEKGSLD--QDRRLFVDGQE 260
Bos taurus                VLLIAQEKERNIFDQRAIENELLARN---IHVIRRRFEDVSEKGSLD--QDRRLFMDGQE 260
Rattus norvegicus         VLLIAQEKERNIFDQRAIENELLDRK---IHVIRRRFEDVSERGSLD--QNRRLFMEDQE 260
Xenopus laevis            VMFLVENEQRNILDQRFIEAELCKRN---VRVIRRRLADVFERGTLD--EERHLFIDGYE 260
Xenopus tropicalis        VLFLVENVQRNILDQRCIENELMKRN---VRTIRRRLADVYERGSLD--KDRGLFLDGYE 260
Danio rerio               VLFLVENVQRNIYDHRYVENELWKRN---IPVIRRQFEDVFRTGSLD--ESKRLFVDGHE 260
Branchiostoma floridae    IVFVVQEENRNAMDQHWLEFAAMDRNP-SIRVRRYTLTQIHDRGELR--PDKTLLVDGEE 278
Botryllus schlosseri      IVFVITDIENNIFDQRALEFRICEIQP-DIVVKRYTLTQISELGDHD--DDGNMVINDQK 263
Brugia malayi             VLFVVEDANRNQIDQRHVEYCIDELSNRNARCLRITLTDGAKRLKLN-ESNHHLVLDNIL 279
Loa loa                   ILFVVEDANQNQIDQRHVEYCIDELSSRSIRCLRITLTDGAKRLKVN-ETNHYLVLDNML 279
Ascaris suum              ILFVVEEVNQMQVDQRHVEYRIDELSSRRAKCVRLTLTQCAERLSLGGRSGHDLMLDACR 284
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Homo sapiens              -IAVVYFRDGYMPRQYS-LQNWEARLLLERSHAAKCPDIATQLAGTKKVQQELSRPGMLE 318
Macaca fascicularis       -IAVVYFRDGYMPRQYS-LQNWEARLLLERSCAAKCPDIATQLAGTKKVQQELSRPGMLE 318
Callithrix  jacchus       -IAVVYFRDGYMPCQYS-LQNWEARLLLERSCAVKCPDIATQLAGTKKVQQELSRAGMLE 318
Bos taurus                -IAVVYFRDGYMPGHYS-LQNWEARLLLERSCAVKCPDIATQLAGTKKVQQELSRVGVLE 318
Rattus norvegicus         -VAVVYFRDGYMPSQYN-AQNWEARLLLERSCAAKCPDIATQLAGTKKVQQELSRVGLLE 318
Xenopus laevis            -VAVAYFRTGYVPQDYT-EQDWEARLMLERSRAVKCPDVPTQLVGTKKVQQELSRPQILE 318
Xenopus tropicalis        -IAVAYFRTGYVPQDYT-EQDWEARLMLERSRATKCPDIATQLVGTKKIQQELCRPHILE 318
Danio rerio               -VAIVYFRNGYMPQNYTSEQSWEVRLMMERSVAVKCPDISTHLAGTKEVQQELARPGVLE 319
Branchiostoma floridae    -VAVAYFRAGYIPADYPTEQEWTARLKIEQSKAIKCPCISYHLAGTKKVQQELAQPGVLE 337
Botryllus schlosseri      -VGFVYYRAGYSPDHYPSEKEWDARLLLETSTAINCPCVAHQLVGAKKMQQILSQPNMVE 322
Brugia malayi             RVAVVYFRAGYSPSNYPTEMEWTARRIIELSDAVKCPWIGLQLANTKKVQQVLSENGVLE 339
Loa loa                   RVAVVYFRAGYSPNNYPTEAEWTARRIIELSDAVKCPWIGLQLANTKKIQQVLSENGVLE 339
Ascaris suum              RVSIVYFRAGYSPDNYCSELEWNARLTMELSNAVKCPWIGLQLANTKKVQQVLACDGQLE 344
                           :...*:* ** * .*    .* .*  :* * * :** :  :*..:*::** *.    :*

Homo sapiens              MLLPGQPEAVARLRATFAGLYSLDVGEE-GDQAIAEALAAPSRFVLKPQREGGGNNLYGE 377
Macaca fascicularis       MLLPGQPEAVARLRATFAGLYSLDMGEE-GDQAIAKALAAPSRFVLKPQREGGGNNLYGE 377
Callithrix  jacchus       MLLPGQPEAVARLRATFAGLYSLNMGEE-GDQAIAEALAAPSRFVLKPQREGGGNNLYGE 377
Bos taurus                SFLPGQPEAVARLRATFAGLYSLDLGEE-GDQAITKAIAAPSCFVLKPQREGGGNNLYGE 377
Rattus norvegicus         ALLPGQPEAVARLRATFAGLYSLDMGEE-GDQAVAEALAAPSHFVLKPQREGGGNNFYGE 377
Xenopus laevis            KFLPDKPEAVARIRETFTGLYSLDIGEE-GDEAVRVALANPDQFVLKPQREGGGNNLYGE 377
Xenopus tropicalis        KFLPDNPEAVARIRETFAGLYSLDTGEE-GDEAVKAALANPDQFVLKPQREGGGNNLYGE 377
Danio rerio               CFFPDEPETVSQIRATFAGLYTLDMGEE-GDNTVAMALANPDQYVLKPQREGGGNNIYGS 378
Branchiostoma floridae    KFLKD-AEAVKRVRATFAGQYTLELGAE-GDRTVQMVTSDPGRFVMKPQREGGGNNIFGE 395
Botryllus schlosseri      RFIKD-RNSVKRIRDTFVGFYGLEMGST-GDESIQKVLQHPENYVLKPQLEGGGNNLYND 380
Brugia malayi             KYITDDKM-CARIRQTFAGMWGLENDDEKTQRIIQDAIAHPEKFVLKPQLEGGGNYYGK 398
Loa loa                   RYITDGRM-STRIRKTFAGMWGLENDDDRTQKIIQDAVTHPEKFVLKPQLEGGGNYYGK 398
Ascaris suum              RFLPELKEDCERIRATFAGLWGLESDEEETQIILKEAIEYPERFVLKPQLEGGGNYYGS 404
                            :        ::* **.* : *: .    :  :  .   *  :*:*** ****.* :..

Homo sapiens              EMVQALKQLKDSEERASYILMEKIEPEPFENCLLRPGSPARVVQCISELGIFGVYVRQ-- 435
Macaca fascicularis       EMVQALKQLKDSEERASYILMEKTEPEPFENCLLRPGSPAQVVQCISELGIFGVYVRH-- 435
Callithrix  jacchus       EMVQALKQLKDSEERASYILMEKIEPEPFENCLLRPGSPVQVVQCISELGIFGVYVRQ-- 435
Bos taurus                EMVQALERLKDSEERASYILMEKIEPEPFRNCLLRPGSPARVIQCISELGIFGVYVRE-- 435
Rattus norvegicus         EMVHALEQLKDSEERASYILMEKIEPEPFRNCLLRPGSPAQVVQCISELGIFGVYVRQ-- 435
Xenopus laevis            ELKEKLQECKDSEERTSYILMDKINPKPLKNCLLRAGGRVQISECISELGMFGVYVRH-- 435
Xenopus tropicalis        ELKAKLEECKDSEERNSYILMDKINPKTTKNCLLRAGGPVQISECISELGMFGVYVRR-- 435
Danio rerio               EICEVLEKLKNSSERTAYILMDKIQPVPVQNILLRPGAPLKVSSCLSELGAFGAYVRK-- 436
Branchiostoma floridae    DIPAALNNMADVKERTAYIVMDRIRPAVVSNYAVRPGREPALTEAVSELGIFGVFIGK-- 453
Botryllus schlosseri      EMVAKLKEVGEDDRRCSYIVMEKY------------------VRCQCSLSSYGPKQAT-- 420
Brugia malayi             EVAEKLKTMNR-DEMAAYIIMERITPMVVKNYVIRPQEEPLLMDVVGELGVYAYLYGSAA 457
Loa loa                   EVAEKLKTMSR-DEMAAYIIMERITPMVVKNYVIRPRQEPVLMDVVGELGIYAYLYGSPA 457
Ascaris suum              EVAEKLKEMSR-DERAAHILMERIQPMRVKNYLVRPFEEVTLGEVVGELGIYGCLYAEPG 463
                          ::   *:     ..  ::*:*::                        .*. :.

Homo sapiens              -----EKTLVMNKHVGHLLRTKAIEHADGGVAAGVAVLDNPYPV 474
Macaca fascicularis       -----EKTLVMNKHVGHLLRTKAIEHADGGVAAGVAVLDNPYPV 474
Callithrix  jacchus       -----GKTLVMNKHVGHLLRTKAIEHADGGVAAGVAVLDNPYPV 474
Bos taurus                -----GKTLVMNKHVGHLLRTKAIEHADGGVAAGVAVLDNPYPV 474
Rattus norvegicus         -----GTTLVMNKHVGHLLRTKAIEHADGGVAAGVAVLDNPYPV 474
Xenopus laevis            -----RDQMIYYDQVGHLLRTKAIEHSDGGVAAGVAVLDNPYLV 474
Xenopus tropicalis        -----SGEMIYNEQVGHLLRTKAIEHADGGVAAGVAVLDNPYLV 474
Danio rerio               -----GSELVLNECVGHLLRTKSSEHADGGVAAGVAVLDNPLLV 475
Branchiostoma floridae    -----GDEVILNQEGGHLLRTKPSSTEDGGVAAGNALLDSPYLI 492
Botryllus schlosseri      -----TNQLL------------------------------- 425
Brugia malayi             VDNIIVENIMKNHVSGHIIRSKDKSVDKGGVAIGAAVIDSPYLF 501
Loa loa                   VDYIPAENVITNYVSGHIIRSKDKNVDKGGVAIGAAVIDSPYLF 501
Ascaris suum              FDRG-CEKVYKNLAHGHIIRSKAANVDKGGVAVGAAVIDSPFLF 506
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C: Amino acid alignment of GPx5 sequences obtained by CLUSTAL W. Red residue correspond to selenocystein and green residues are amino acids with catalytic activity.

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Bos taurus_GPx6              MILQLWASCLFPLFLVGLAQLTPKQQQMKVDCYKGVTGTIYEYGALTLNGEEYIQFKQYV 60
Sus scrofa_GPx6              MTPQFWASCLFSLCLVGFAQLIPKEQKMKMDCYKGVTGTIYEYGALTLNGEEYIPFKQYA 60
Equus caballus_GPx6          MIRQLWASSLFPLFLVGFAQLTPESQKMKMDCYKGVTGTIYEYGALTLNGEEYIQFKQYA 60
Homo sapiens_GPx6            MFQQFQASCLVLLFLVGFAQQTLKPQNRKVDCNKGVTGTIYEYGALTLNGEEYIQFKQFA 60
Pan troglodytes_GPx6         MFRQFQASCLVLLFLVGFAQQILKPQNRKVDCNKGVTGTIYEYGALTLNGEEYIQFKQFA 60
Mus musculus_GPx5            MVTELRVFYLVPLLLASYVQTTPRPEKMKMDCYKDVKGTIYDYEALSLNGKEHIPFKQYA 60
Bos taurus_GPx5              MTIQLRASCLFLFFLAGFVQTNSNLE--KMDCYKDVKGTIYDYDAFTLNGKEHIQFKQYA 58
Canis lupus_GPx3             MARLLRASCLLSLLLAGFVPPSRGQEKSKTDCHGGVSGTIYEYGALTINGEEYIPFKQYA 60
Pongo abelii_GPx3            MARLLQASCLLSLLLAGFVPQSRGQEKSKMDCHGGISGTIYEYGALTIDGEEYIPFKQYA 60
Sus scrofa_GPx3              MARFFRASCLISLLLAGFLPPSRGQEKSKTDCYRGVSGTIYEYGALTIDGEEYIPFKKYA 60
Gallus gallus_GPx3           --MGCRAACVLAVLLAGLVPLGQGQEREKVKCYDSVRGTIYDYGALTIDGDEYIPFRKYA 58
Xenopus tropicalis_GPx3      MGVKLRGLLMLPCFLAALIHAQNEMDQKSVDCYSSIDGTIYDYGATTLDGTQFIPFKAYQ 60
Xenopus laevis_GPx3          MGVKFRGLLMLPCILAAFIHAQTDVDQKSVDCYSSADGSIYDYGATTLDGSQFIPFKAYQ 60
Danio rerio_GPx3             MGTQSNPWTSVVLLLA--LMHKIAALSNTQACNSAAGDSFHNYGAKTINGTQFIPFSHYA 58
Perca flavescens_GPx3        ------------------------------------------------------------
Branchiostoma floridae_GPx   MNGPAHHGHGRLSHSKLHHAAPHPIELNTPLCPASVVGSFFELSAEALGG-EMIPFSTYD 59
Ciona intestinalis_GPx c     -----MHQKGAVPAVLLVLGTLAVATSIENSTCFHNTFSVYDHSFPTLSG-ETVNLGEFR 54
Botryllus schlosseri_GPx5    ----------MLWALLVASAALVAADDASQCLSQGLTGTVYDSTEATLAG-TNVDLTQLF 49
Ciona intestinalis_GPx b     ----------MRSAVLLWLGVAGVVHSMVPCYNSTN-YSVYSNQVFNLHK-QNVNLSRFH 48

Bos taurus_GPx6              GKHVLFVNVATYUG-LTAQYPELNALQEELKPFGVVVLGFPCNQFGKQEPAKNSEILMGL 119
Sus scrofa_GPx6              GKHVLFVNVATYUG-LTAQYPELNALQEELKPFGVVVLGFPCNQFGKQEPAKNSEILLGL 119
Equus caballus_GPx6          GKHVLFINVATYUG-LTAQYPELNALQEELKPFGVVLLGFPCNQFGKQEPGKNSEILSGL 119
Homo sapiens_GPx6            GKHVLFVNVAAYUG-LAAQYPELNALQEELKNFGVIVLAFPCNQFGKQEPGTNSEILLGL 119
Pan troglodytes_GPx6         GKHVLFVNVATYUG-LAAQYPELNALQEELKNFGVIVLAFPCNQFGKQEPGTNSEILLGL 119
Mus musculus_GPx5            GKHVLFVNVATYCG-LTIQYPELNALQEDLKPFGLVILGFPCNQFGKQEPGDNLEILPGL 119
Bos taurus_GPx5              GKHVLFVNVATYCG-LTAQYPELNALQEELKPFGLVVLGFPCNQFGNQEPGENSEILPGL 117
Canis lupus_GPx3             GKYILFVNVASYUG-LTGQYLELNALQEELAPFGLVILGFPCNQFGKQEPGENSEILPSL 119
Pongo abelii_GPx3            GKYVLFVNVASYUG-LTGQYIELNALQEELAPFGLVILGFPCNQFGKQEPGENSEILPTL 119
Sus scrofa_GPx3              GKYVLFVNVASYUG-LTGQYVELNALQEEFEPFGLVILGFPCNQFGKQEPGDNSEILSTL 119
Gallus gallus_GPx3           GKMVLFVNVATYUG-LTLQYLELNALQNELGPYGLVVLGFPSNQFGKQEPGQNSEILPAL 117
Xenopus tropicalis_GPx3      GKYILFVNVATYUG-LTMQYQELNALQEELKNNNFVILGFPSNQFGMQEPGRNDEILLGL 119
Xenopus laevis_GPx3          GKYLLFVNVATFUG-LTMQYQEMNALHEELKSNDFVILGFPCNQFGLQEPGRNDEIPLGL 119
Danio rerio_GPx3             GKHVLVVNVATYUG-LTFQYVELNALHEELRHLGFTILGFPCDQFGKQEPGENNEILSAL 117
Perca flavescens_GPx3        ----------------------------MKPLGLTLLGFPCNQFGKQEPGTNHEILPGL 31
Branchiostoma floridae_GPx   GKVVLVVNVASASEHTTREYLQLNDLVATYGPKGLIVLGFCCNQFGNGEPFSNHEILRCL 119
Ciona intestinalis_GPx c     GNVSMVINVATYUGATVPQYKAMNALSEEYTQSSFVTLAFPCNQFGLQQPEANDEILNGV 114
Botryllus schlosseri_GPx5    GYVSVVMNPGVYSEWTSQMMTGMNTLLEKYESDGVVGLGFPCNQFNLEQPGSPGEILNAY 109
Ciona intestinalis_GPx b     NEVTLLINVATYUGLTVAQYTGLNALLTHFNGRNFSVLAFPCDQFHLEEPGEDSEILNGL 108
                                                      ..  *.* .:**   :*    **

Bos taurus_GPx6              KYVRPGGGFVPN--FQLFEKGDVNGEKEQKVFTFLKNACPPTSD---LLGSSS-QLFWEP 173
Sus scrofa_GPx6              KYVRPGGGFVPN--FQLFEKGDVNGEKEQKVFTFLKNSCPPTSD---LLGSSN-QLFWEP 173
Equus caballus_GPx6          KYVRPGGGFVPN--FQLFEKGDVNGEKEQKVFTFLKNSCPPTSD---LLGSPK-QLFWEP 173
Homo sapiens_GPx6            KYVCPGSGFVPS--FQLFEKGDVNGEKEQKVFTFLKNSCPPTSD---LLGSSS-QLFWEP 173
Pan troglodytes_GPx6         KYVCPGSGFVPS--FQLFEKGDVNGEKEQKVFTFLKNSCPPTSD---LLGSSS-QLFWEP 173
Mus musculus_GPx5            KYVRPGKGFLPN--FQLFAKGDVNGENEQKIFTFLKRSCPHPSE---TVVMSK-HTFWEP 173
Bos taurus_GPx5              KYVRPGGGYVPN--FQLFKKGDVNGETEQKVFTFLKQSCPHPS--------------WEP 161
Canis lupus_GPx3             KYVRPGGGFVPN--FQLFEKGDVNGEKEQKFYTFLKNSCPPTSE---LLGSPG-RLFWEP 173
Pongo abelii_GPx3            KYVRPGGGFVPN--FQLFEKGDVNGEKEQKFYTFLKNSCPPTSE---LLGTSD-RLFWEP 173
Sus scrofa_GPx3              RYVRPGGGFIPN--FQLFEKGDVNGEKEQKFYTFLKNACPPTSE---LLGSPS-RLFWEP 173
Gallus gallus_GPx3           KYVRPGGGFVPN--FQLFQKGDVNGAKEQKVYSFLKNSCPPVAE---EFGNPK-NLFWEP 171
Xenopus tropicalis_GPx3      EYVRPGGKFVPN--FQLFEKGDINGRKEQKFYTFLKNSCPPVGD---NFGSATNRLMWEP 174
Xenopus laevis_GPx3          RYVRPGGNFIPN--FQLFEKGDVNGRKEQKFYTFLKNSCPPVGD---TFGNPAFRLNWEP 174
Danio rerio_GPx3             KYVRPGNGFVPN--FQLFEKGDVNGDGEQALFTFLKNACPPVGE---SFGATSNRLFWEP 172
Perca flavescens_GPx3        KHVRPGNGFVPN--FLLFEKGDVNGKDEQEVFTFLKNSCPPVGD---VLGNPT-RMFWDP 85
Branchiostoma floridae_GPx   RAVRPGNRYSPN--FQLFSKVDINGKHGHIVFEYLKLKLPFPSDNAATMAQEHLDICWSP 177
Ciona intestinalis_GPx c     MYVRPGHGFVPNKKIYFFSKTQVNGGSEDPLFTSIKASCPPTTN---NIGITS-ELYWTP 170
Botryllus schlosseri_GPx5    KYLRPGNGWTPHQNFHLFTVTEVNGDTASDIFKFLRSACPAYTE---ELGSKA-SFYWDT 165
Ciona intestinalis_GPx b     MYVRPGNGYVPHPKLNIFGKIKVNGRHEHTIYKNVKASCPPTTL---NLGSTR-NMYWNP 164
                               : **  : *   : :*   .:**     .:  ::   *                * .

Bos taurus_GPx6              MKVHDIRWNFEKFLVGPDGVPVMRWYHRASVSTVKSDMLEYLKQFKSE------------ 221
Sus scrofa_GPx6              MKVHDIRWNFEKFLVGPDGVPVMRWYHRASVSTVKSDIMEYLKQFKSE------------ 221
Equus caballus_GPx6          MKVHDIRWNFEKFLVGPDGVPVMRWFHRASVSTVKSDILEYLKQFTPE------------ 221
Homo sapiens_GPx6            MKVHDIRWNFEKFLVGPDGVPVMHWFHQAPVSTVKSDILEYLKQFNTH------------ 221
Pan troglodytes_GPx6         MKVHDICWNFEKFLVGPDGVPVMRWFHQAPVSTVKSDILEYLKQFNTH------------ 221
Mus musculus_GPx5            IKVHDIRWNFEKFLVGPDGVPVMRWFHQAPVSTVKSDIMAYLSHFKTI------------ 221
Bos taurus_GPx5              IMVRDIRWNFEKFLVGPDGIPVMRWFHRTPVSTVKTDILAYMKQFKTK------------ 209
Canis lupus_GPx3             MKVHDIRWNFEKFLVGPDGIPIMRWYHRTTVSTVKMDILAYMRRQAALAIKGK------- 226
Pongo abelii_GPx3            MKVHDIRWNFEKFLVGPDGIPIMRWHHRTTVSNVKMDILSYMRRQAALGVKRK------- 226
Sus scrofa_GPx3              MKVHDIRWNFEKFLVGPDGVPVMRWYHRTTINTVKLDILAYMRRRAALEAKRQ------- 226
Gallus gallus_GPx3           LRNHDIKWNFEKFLVGTDGVPVMRWYHRANIATVKNDIIAYMRQQRGQ------------ 219
Xenopus tropicalis_GPx3      IKVNDVKWNFEKFLVGPDGRPVKRWLPRTPVAQVRREIMSYMKLQPGTQRLLMLGLEQK- 233
Xenopus laevis_GPx3          LRVNDIKWNFEKFLVGPDGRAVKRWHPRTSVAQVRREIVSYIKLQQGTQRLLMLGLEQK- 233
Danio rerio_GPx3             LKVNDIKWNFEKFLLDPDGRPVMRWFPRVNVSEVRADILKYFHQLLQTAQ---------- 222
Perca flavescens_GPx3        VKLSDIKWNFEKFLVGPDGKPVMRWHPSVNISVVQADIRKYLLQLYTQQIFN-------- 137
Branchiostoma floridae_GPx   VRRTDVSGNFEKFLVASDGQPYRRYSWRTAPEDLCKDIEELLRKVTRTNREQGNTGAKND 237
Ciona intestinalis_GPx c     IKANDIYWNWNKFLLDKNGMIRYRFGSAVTATQLKPWIDQLLNEK--------------- 215
Botryllus schlosseri_GPx5    LVARDLRGTYEKFVIDKNGKPRYRFAPNVAMTEIYPYIDELLAETPIQPTQFPGVEAGN- 224
Ciona intestinalis_GPx b     VKSTDITWNFNKFLLDKNGVPRYRISSDASPTSLIPYITTMLSE---------------- 208
                             :   *:  .::**::  :*    :    .    :   :   :
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Bos taurus_GPx6              --------------------------
Sus scrofa_GPx6              --------------------------
Equus caballus_GPx6          --------------------------
Homo sapiens_GPx6            --------------------------
Pan troglodytes_GPx6         --------------------------
Mus musculus_GPx5            --------------------------
Bos taurus_GPx5              --------------------------
Canis lupus_GPx3             --------------------------
Pongo abelii_GPx3            --------------------------
Sus scrofa_GPx3              --------------------------
Gallus gallus_GPx3           --------------------------
Xenopus tropicalis_GPx3      --------------------------
Xenopus laevis_GPx3          --------------------------
Danio rerio_GPx3             --------------------------
Perca flavescens_GPx3        --------------------------
Branchiostoma floridae_GPx   RPVLFNSKKYMQESITRSTVRDDAAY 263
Ciona intestinalis_GPx c     --------------------------
Botryllus schlosseri_GPx5    --------------------------
Ciona intestinalis_GPx b     --------------------------
```

D: Amino acid alignment of GPx3 sequences obtained by CLUSTAL W. Red residue correspond to selenocystein and green residues are amino acids with catalytic activity.

```
Danio rerio_GPx3              MGTQSNPWTSVVLLLA--LMHKIAALSNTQACNSAAGDSFHNYGAKTINGTQFIPFSHYA 58
Perca flavescens_GPx3         ------------------------------------------------------------
Xenopus tropicalis_GPx3       MGVKLRGLLMLPCFLAALIHAQNEMDQKSVDCYSSIDGTIYDYGATTLDGTQFIPFKAYQ 60
Xenopus laevis_GPx3           MGVKFRGLLMLPCILAAFIHAQTDVDQKSVDCYSSADGSIYDYGATTLDGSQFIPFKAYQ 60
Canis lupus_GPx3              MARLLRASCLLSLLLAGFVPPSRGQEKSKTDCHGGVSGTIYEYGALTINGEEYIPFKQYA 60
Pongo abelii_GPx3             MARLLQASCLLSLLLAGFVPQSRGQEKSKMDCHGGISGTIYEYGALTIDGEEYIPFKQYA 60
Sus scrofa_GPx3               MARFFRASCLISLLLAGFLPPSRGQEKSKTDCYRGVSGTIYEYGALTIDGEEYIPFKKYA 60
Bos Taurus_GPx6                    MILQLWASCLFPLFLVGLAQLTPKQQQMKVDCYKGVTGTIYEYGALTLNGEEYIQFKQYV 60
Sus scrofa_GPx6               MTPQFWASCLFSLCLVGFAQLIPKEQKMKMDCYKGVTGTIYEYGALTLNGEEYIPFKQYA 60
Equus caballus_GPx6           MIRQLWASSLFPLFLVGFAQLTPESQKMKMDCYKGVTGTIYEYGALTLNGEEYIQFKQYA 60
Homo sapiens_GPx6             MFQQFQASCLVLLFLVGFAQQTLKPQNRKVDCNKGVTGTIYEYGALTLNGEEYIQFKQFA 60
Pan troglodytes_GPx6          MFRQFQASCLVLLFLVGFAQQILKPQNRKVDCNKGVTGTIYEYGALTLNGEEYIQFKQFA 60
Mus musculus_GPx5             MVTELRVFYLVPLLLASYVQTTPRPEKMKMDCYKDVKGTIYDYEALSLNGKEHIPFKQYA 60
Bos taurus_GPx5               MTIQLRASCLFLFFLAGFVQTNSNLE--KMDCYKDVKGTIYDYDAFTLNGKEHIQFKQYA 58
Gallus gallus_GPx3            --MGCRAACVLAVLLAGLVPLGQGQEREKVKCYDSVRGTIYDYGALTIDGDEYIPFRKYA 58
Branchiostoma floridae_GPx    ---------------------------------------------MVTDNG---------- 6
Ciona intestinalis_GPx b      --------MRSAVLLWLG-VAGVVHSMVP--CYNSTNYSVYSNQVFNLHKQN-VNLSRFH 48
Ciona intestinalis_GPx c      ---MHQKGAVPAVLLVLG-TLAVATSIENSTCFHNT-FSVYDHSFPTLSGET-VNLGEFR 54
Botryllus schlosseri_GPx3     -----MVTMKNVLPLLLCSWISAIHALKVSTCRYSE-KAIYEYPVTKLDGSP-FTLSSLQ 53

Danio rerio_GPx3              GKHVLVVNVATYUGLTF-QYVELNALHEELRHLGFTILGFPCDQFGKQEPGENNEILSAL 117
Perca flavescens_GPx3         ----------------------------MKPLGLTLLGFPCNQFGKQEPGTNHEILPGL 31
Xenopus tropicalis_GPx3       GKYILFVNVATYUGLTM-QYQELNALQEELKNNNFVILGFPSNQFGMQEPGRNDEILLGL 119
Xenopus laevis_GPx3           GKYLLFVNVATFUGLTM-QYQEMNALHEELKSNDFVILGFPCNQFGLQEPGRNDEIPLGL 119
Canis lupus_GPx3              GKYILFVNVASYUGLTG-QYLELNALQEELAPFGLVILGFPCNQFGKQEPGENSEILPSL 119
Pongo abelii_GPx3             GKYVLFVNVASYUGLTG-QYIELNALQEELAPFGLVILGFPCNQFGKQEPGENSEILPTL 119
Sus scrofa_GPx3               GKYVLFVNVASYUGLTG-QYVELNALQEEFEPFGLVILGFPCNQFGKQEPGDNSEILSTL 119
Bos taurus_GPx6               GKHVLFVNVATYUGLTA-QYPELNALQEELKPFGVVVLGFPCNQFGKQEPAKNSEILMGL 119
Sus scrofa_GPx6               GKHVLFVNVATYUGLTA-QYPELNALQEELKPFGVVVLGFPCNQFGKQEPAKNSEILLGL 119
Equus caballus_GPx6           GKHVLFINVATYUGLTA-QYPELNALQEELKPFGVVLLGFPCNQFGKQEPGKNSEILSGL 119
Homo sapiens_GPx6             GKHVLFVNVAAYUGLAA-QYPELNALQEELKNFGVIVLAFPCNQFGKQEPGTNSEILLGL 119
Pan troglodytes_GPx6          GKHVLFVNVATYUGLAA-QYPELNALQEELKNFGVIVLAFPCNQFGKQEPGTNSEILLGL 119
Mus musculus_GPx5             GKHVLFVNVATYCGLTI-QYPELNALQEDLKPFGLVILGFPCNQFGKQEPGDNLEILPGL 119
Bos taurus_GPx5               GKHVLFVNVATYCGLTA-QYPELNALQEELKPFGLVVLGFPCNQFGNQEPGENSEILPGL 117
Gallus gallus_GPx3            GKMVLFVNVATYUGLTL-QYLELNALQNELGPYGLVVLGFPSNQFGKQEPGQNSEILPAL 117
Branchiostoma floridae_GPx    --------------------------------HRFEILGFPTNNFGLQEPAPNSKILDCV 34
Ciona intestinalis_GPx b      NEVTLLINVATYUGLTVAQYTGLNALLTHFNGRNFSVLAFPCDQFHLEEPGEDSEILNGL 108
Ciona intestinalis_GPx c      GNVSMVINVATYUGATVPQYKAMNALSEEYTQSSFVTLAFPCNQFGLQQPEANDEILNGV 114
Botryllus schlosseri_GPx3     DKVAVIINTACFUGETWTQLPGMNALLQKYSKQGVVASGFPCDQFELQEPGLPREILPCY 113
                                                              .   .** ::*  ::*    :*

Danio rerio_GPx3              KYVRPGNGFVPN--FQLFEKGDVNGDGEQALFTFLKNACPPVGESFGATSNRLFWEPLKV 175
Perca flavescens_GPx3         KHVRPGNGFVPN--FLLFEKGDVNGKDEQEVFTFLKNSCPPVGDVLGNPT-RMFWDPVKL 88
Xenopus tropicalis_GPx3       EYVRPGGKFVPN--FQLFEKGDINGRKEQKFYTFLKNSCPPVGDNFGSATNRLMWEPIKV 177
Xenopus laevis_GPx3           RYVRPGGNFIPN--FQLFEKGDVNGRKEQKFYTFLKNSCPPVGDTFGNPAFRLNWEPLRV 177
Canis lupus_GPx3              KYVRPGGGFVPN--FQLFEKGDVNGEKEQKFYTFLKNSCPPTSELLGSPG-RLFWEPMKV 176
Pongo abelii_GPx3             KYVRPGGGFVPN--FQLFEKGDVNGEKEQKFYTFLKNSCPPTSELLGTSD-RLFWEPMKV 176
Sus scrofa_GPx3               RYVRPGGGFIPN--FQLFEKGDVNGEKEQKFYTFLKNACPPTSELLGSPS-RLFWEPMKV 176
Bos taurus_GPx6               KYVRPGGGFVPN--FQLFEKGDVNGEKEQKVFTFLKNACPPTSDLLGSSS-QLFWEPMKV 176
Sus scrofa_GPx6               KYVRPGGGFVPN--FQLFEKGDVNGEKEQKVFTFLKNSCPPTSDLLGSSN-QLFWEPMKV 176
Equus caballus_GPx6           KYVRPGGGFVPN--FQLFEKGDVNGEKEQKVFTFLKNSCPPTSDLLGSPK-QLFWEPMKV 176
Homo sapiens_GPx6             KYVCPGSGFVPS--FQLFEKGDVNGEKEQKVFTFLKNSCPPTSDLLGSSS-QLFWEPMKV 176
Pan troglodytes_GPx6          KYVCPGSGFVPS--FQLFEKGDVNGEKEQKVFTFLKNSCPPTSDLLGSSS-QLFWEPMKV 176
Mus musculus_GPx5             KYVRPGKGFLPN--FQLFAKGDVNGENEQKIFTFLKRSCPHPSETVVMSK-HTFWEPIKV 176
Bos taurus_GPx5               KYVRPGGGYVPN--FQLFKKGDVNGETEQKVFTFLKQSCPHPS-----------WEPIMV 164
Gallus gallus_GPx3            KYVRPGGGFVPN--FQLFQKGDVNGAKEQKVYSFLKNSCPPVAEEFGNPK-NLFWEPLRN 174
Branchiostoma floridae_GPx    KHVNPGNGYVPN--FPMFQKADCNGVNEQAFYTYMKSCCPAISDVFISKI-RLYWDPIKN 91
Ciona intestinalis_GPx b      MYVRPGNGYVPHPKLNIFGKIKVNGRHEHTIYKNVKASCPPTTLNLGSTR-NMYWNPVKS 167
Ciona intestinalis_GPx c      MYVRPGHGFVPNKKIYFFSKTQVNGGSEDPLFTSIKASCPPTTNNIGITS-ELYWTPIKA 173
Botryllus schlosseri_GPx3     KYVRPGKGWIPHPNFHFLNKTKVNGKDENSLYAHLKSVCPQVTDEIGTRS-EMYWDPVKV 172
                               :* **  ::*   : :: * . **  *. .:  :*  **              * *:

Danio rerio_GPx3              NDIKWNFEKFLLDPDGRPVMRWFPRVNVSEVRADILKYFHQLLQTAQ------------- 222
Perca flavescens_GPx3         SDIKWNFEKFLVGPDGKPVMRWHPSVNISVVQADIRKYLLQLYTQQIFN----------- 137
Xenopus tropicalis_GPx3       NDVKWNFEKFLVGPDGRPVKRWLPRTPVAQVRREIMSYMKLQPGTQRLLMLGLEQK---- 233
Xenopus laevis_GPx3           NDIKWNFEKFLVGPDGRAVKRWHPRTSVAQVRREIVSYIKLQQGTQRLLMLGLEQK---- 233
Canis lupus_GPx3              HDIRWNFEKFLVGPDGIPIMRWYHRTTVSTVKMDILAYMRRQAALAIKGK---------- 226
Pongo abelii_GPx3             HDIRWNFEKFLVGPDGIPIMRWHHRTTVSNVKMDILSYMRRQAALGVKRK---------- 226
Sus scrofa_GPx3               HDIRWNFEKFLVGPDGVPVMRWYHRTTINTVKLDILAYMRRRAALEAKRQ---------- 226
Bos taurus_GPx6               HDIRWNFEKFLVGPDGVPVMRWYHRASVSTVKSDMLEYLKQFKSE---------------- 221
Sus scrofa_GPx6               HDIRWNFEKFLVGPDGVPVMRWYHRASVSTVKSDIMEYLKQFKSE---------------- 221
Equus caballus_GPx6           HDIRWNFEKFLVGPDGVPVMRWFHRASVSTVKSDILEYLKQFTPE---------------- 221
Homo sapiens_GPx6             HDIRWNFEKFLVGPDGVPVMHWFHQAPVSTVKSDILEYLKQFNTH---------------- 221
Pan troglodytes_GPx6          HDICWNFEKFLVGPDGVPVMRWFHQAPVSTVKSDILEYLKQFNTH---------------- 221
Mus musculus_GPx5             HDIRWNFEKFLVGPDGVPVMRWFHQAPVSTVKSDIMAYLSHFKTI---------------- 221
Bos taurus_GPx5               RDIRWNFEKFLVGPDGIPVMRWFHRTPVSTVKTDILAYMKQFKTK---------------- 209
Gallus gallus_GPx3            HDIKWNFEKFLVGTDGVPVMRWYHRANIATVKNDIIAYMRQQRGQ---------------- 219
Branchiostoma floridae_GPx    TDIRWNFEKFLVDPAGKAVKRFSSYVTPGDLETVIDDFIKNWNDTSRDDTSGARSSRNSG 151
Ciona intestinalis_GPx b      TDITWNFNKFLLDKNGVPRYRISSDASPTSLIPYITTMLSE-------------------- 208
Ciona intestinalis_GPx c      NDIYWNWNKFLLDKNGMIRYRFGSAVTATQLKPWIDQLLNEK------------------- 215
Botryllus schlosseri_GPx3     SDITWNYEKFLIDRKGKPRYRFGPSVLPSEIEPYIDSIL---------------------- 211
                               *: **::***:.  *    :    .    :   :   :
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Danio rerio_GPx3              -----
Perca flavescens_GPx3         -----
Xenopus tropicalis_GPx3       -----
Xenopus laevis_GPx3           -----
Canis lupus_GPx3              -----
Pongo abelii_GPx3             -----
Sus scrofa_GPx3               -----
Bos taurus_GPx6               -----
Sus scrofa_GPx6               -----
Equus caballus_GPx6           -----
Homo sapiens_GPx6             -----
Pan troglodytes_GPx6          -----
Mus musculus_GPx5             -----
Bos taurus_GPx5               -----
Gallus gallus_GPx3            -----
Branchiostoma floridae_GPx    FLSWL 156
Ciona intestinalis_GPx b      -----
Ciona intestinalis_GPx c      -----
Botryllus schlosseri_GPx3     -----
```

E: Amino acid alignment of Cu/Zn SOD sequences obtained by CLUSTAL W. Fuxia highlight amino acids which coordinate copper in the catalytic sites and light blue highlight ion zinc. Green show histidine that bind copper and zinc and gray are the amino acid involved in antioxidant activity.

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Ovis aries                  -------------MATKAVC----------VLKGDG------------PVQGTIRFEAK- 24
Capra hircus                -------------MATKAVC----------VLKGDG------------PVQGTIHFEAK- 24
Bos taurus                  -------------MATKAVC----------VLKGDG------------PVQGTIHFEAK- 24
Bos grunniens               -------------MATKAVC----------VLKGDG------------PVQGTIHFEAK- 24
Cervus elaphus              -------------MATKAVC----------VMKGDG------------PVQGTIRFEAK- 24
Callithrix jacchus          -------------MAMKAVC----------VLKGDG------------PVQGTINFEQKE 25
Macaca mulatta              -------------MAMKAVC----------VLKGDS------------PVQGTINFEQKE 25
Hylobates lar               -------------MAMKAVC----------VLKGDS------------PVQGIINFEQKE 25
Cavia porcellus             -------------MATKAVC----------VLKGDG------------PVQGIIHFEQK- 24
Equus caballus              -------------MALKAVC----------VLKGDG------------PVHGVIHFEQQQ 25
Gallus gallus               ------------MATLKAVC----------VMKGDA------------PVEGVIHFQQQ- 25
Melopsittacus undulatus     ------------MATLKAVC----------VMKGEG------------PVQGVIHFQQQ- 25
Caretta caretta             -------------MATVKAVC----------VLKGEDPVKEPVKGPVKEPVKGIIYFEQQ- 37
Rana catesbeiana            ---------------MKAIC----------VLKGSS------------EVTGVVRFEQEE 23
Bufo gargarizans            --------------MVKAIC----------VLKGNG------------PVHGIVGFNQD- 23
Xenopus tropicalis          --------------MVRAVC----------VLAGSG------------DVKGVVHFQQQD 24
Xenopus laevis              --------------MVKAVC----------VLAGSG------------DVKGVVHFEQQD 24
Wuchereria bancrofti        -------------MSANAIA----------VLRGD-------------NVSGIIRFKQEK 24
Brugia malayi               -------------MSANAIA----------VLRGD-------------NVNGIIRFKQEK 24
Loa loa                     ---------------MNAIA----------VLRGD-------------TVSGIIRFKQDK 22
Ascaris suum                -------------MTSRAVA----------VLRGEG------------DVRGVVYLTQSK 25
Trichinella pseudospiralis  -------------MPFKAIC----------VIRGE-------------NVTGTVIFKQNT 24
Branchiostoma floridae      ------------------------------MLVGS--------------VIGTIFFEQQ- 15
Botryllus schlosseri        ---------------MSAVC----------NLEGD--------------VSGTIRFVQE- 20
Caenorhabditis briggsae     MKNRVILVLA-LLACTEAAS---------EVIRARAYIFKAVEGQIPTELIGTIDFDQS- 49
Caenorhabditis remanei      MKNRVILILA-LFACIEAAS---------EVIRARAYIFKAVEGQIPTELIGTIDFDQS- 49
Caenorhabditis elegans      MKTRVVLILA-LSVCIEAAS---------EVIRARAYIFKAEAGKIPTELIGTIDFDQS- 49
Dictyocaulus viviparus      MILHISLIISTILLGVHAHGNLCRNGAFMNVVKARAYMFEAVPDGDPQKLIGIIDFVQY- 59
                                                      : .             : * : :

Ovis aries                  -GDKVVVTGSITGLTEGDHGFHVHQFGDNTQGCTSAGPHFNPLSKKHGGPKDEERHVGDL 83
Capra hircus                -GDKVVVTGSITGLTEGDHGFHVHQFGDNTQGCTSAGPHFNPLSKKHGGPKDEERHVGDL 83
Bos taurus                  -GDTVVVTGSITGLTEGDHGFHVHQFGDNTQGCTSAGPHFNPLSKKHGGPKDEERHVGDL 83
Bos grunniens               -GDTVVVTGSITGLTEGDHGFHVHQFGDNTQGCTSAGPHFNPLSKKHGGPKDEERHVGDL 83
Cervus elaphus              -GNTVVVTGSITGLTEGDHGFHVHQFGDNTQGCTSAGPHFNPLSKKHGGPKDEERHVGDL 83
Callithrix jacchus          SNGPVKVWGSITGLAEGLHGFHVHQFGDNTQGCTSAGPHFNPLSRKHGGPEDEERHVGDL 85
Macaca mulatta              SNGPVKVWGSITGLTEGLHGFHVHQFGDNTQGCTSAGPHFNPLSRQHGGPKDEERHVGDL 85
Hylobates lar               SNGPVKVYGRITGLTEGLHGFHVHQFGDNTQGCTSAGPHFNPLSRKHGGPKDEERHVGDL 85
Cavia porcellus             ANGPVVVKGRITGLVEGKHGFHVHEFGDNTQGCTSAGPHFNPLSKKHGGPQDEERHVGDL 84
Equus caballus              EGGPVVLKGFIEGLTKGDHGFHVHEFGDNTQGCTTAGAHFNPLSKKHGGPKDEERHVGDL 85
Gallus gallus               GSGPVKVTGKITGLSDGDHGFHVHEFGDNTNGCTSAGAHFNPEGKQHGGPKDADRHVGDL 85
Melopsittacus undulatus     GNGPVKVTGKISGLADGDHGFHVHEFGDNTNGCTSAGPHFNPEGKQHGGPSDAERHVGDL 85
Caretta caretta             GNGPVTLSGSITGLTEGKHGFHVHEFGDNTNGCTSAGAHFNPPGKNHGGPQDNERHVGDL 97
Rana catesbeiana            -DGPVTVTGQITGLTDGKHGFHIHTYGDNTDGCVSAGPHFNPQGKTHGGPDDEVRHVGDL 82
Bufo gargarizans            -GGEVTVKGTINGLTDGLHGFHIHVYGDNTNGCMSAGPHFNPHGKSHGAPEDEERHVGDL 82
Xenopus tropicalis          -EGPVTVEGKIYGLTDGKHGFHIHEFGDNTNGCISAGPHFNPESKTHGAPEDAVRHVGDL 83
Xenopus laevis              -EGAVSVEGKIEGLTDGLHGFHIHVFGDNTNGCMSAGSHFNPENKNHGAPGDTDRHVGDL 83
Wuchereria bancrofti        EGLPTTISGEIKGLTPGLHGFHVHQYGDTTNGCISAGPHFNPYNKTHGGPTDEMRHVGDL 84
Brugia malayi               EGSPTTISGEIKGLTPGLHGFHVHQYGDTTNGCISAGPHFNPYNKTHGGPTDEMRHVGDL 84
Loa loa                     ESSPTAINGEIKGLTPGLHGFHVHQYGDTTNGCISAGPHFNPHNKTHGGPTDEIRHVGDL 82
Ascaris suum                EDEPTILKGEISGLTPGLHGFHVHEYGDMTNGCISAGAHFNPFKKTHGGPTDEERHIGDL 85
Trichinella pseudospiralis  ENDKTTITGEIKGLTPGKHGFHVHEWGDNSMGCISAGAHYNPFGKTHGGPTDTVRHVGDL 84
Branchiostoma floridae      -----ACFREVTGLTEGPHGFHVHEFGDYRNGCTDMGAHYNPIGTNHGGPNDAVRHVGDL 70
Botryllus schlosseri        -GTDCVITGTVQGLTPGNHGFHIHEFGDRTEGCTSTGGHWNPTKVDHAGPDDNPRHFGDL 79
Caenorhabditis briggsae     -GSFLKLNGSVSGLAAGKHGFHIHEKGDTGNGCLSAGSHYNPHKLSHGAPDDSNRHIGDL 108
Caenorhabditis remanei      -GSFLKLNGTVSGLQAGKHGFHIHEKGDTGNGCLSAGGHYNPHKLSHGAPDDSNRHIGDL 108
Caenorhabditis elegans      -GSFLKLNGSVSGLAAGKHGFHIHEKGDTGNGCLSAGGHYNPHKLSHGAPDDSNRHIGDL 108
Dictyocaulus viviparus      -RSLVKLNGTVSGLKSGLHGFHVHEKGNLANGCLAAGGHYNPYKLMHGAPSDSNRHVGDL 118
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Cervus elaphus              GNVTADKNGVAKVDIVDSLISLSGEHSIIGRTMVVHEKPDDLGRG-GN--EESTKTGNAR 140
Callithrix jacchus          GNVTAGKDGVAKVSIEDSVISLSGDHSIIGRTLVVHEKADDLGKG-GN--EESKKTGNAG 142
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Gallus gallus               GNVTA-KGGVAEVEIEDSVISLTGPHCIIGRTMVVHAKSDDLGRG-GD--NESKLTGNAG 141
Melopsittacus undulatus     GNVTA-KGGVAEVAIEDSIISLSGPHSIVGRTMVVHEKCDDLGRG-GD--NESKLTGNAG 141
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Bufo gargarizans            GNITS-KDGVAEFEFKDKIISLEGEHNIIGRTAVVHEKADDLGKG-GD--NESKVTGNAG 138
Xenopus tropicalis          GNVTA-KDGVAEFKLTDSLISLKGNHSIIGRCAVVHEKEDDLGKG-GN--DESLKTGNAG 139
Xenopus laevis              GNVTA-EGGVAQFKITDSLISLKGPNSIIGRTAVVHEKADDLGKG-GN--DESLKTGNAG 139
Wuchereria bancrofti        GNIVAEGDGTAHINISDKHVQLLGPNSIIGRSIVVHADQDDLGKGVGDKKDESLKTGNAG 144
Brugia malayi               GNIVAGADGTAHIDISDKHVQLLGPNSIIGRSIVVHADQDDLGKGVGDKKDESLKTGNAG 144
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Trichinella pseudospiralis  GNIVAGSDGVAKIDIVDDQIKLTGEHSIIGRTMVVHIQEDDLGKG-GD--DESLKTGNAG 141
Branchiostoma floridae      GNIVADASGTASVDITDNHLSLVGENSIIGRGVVVHADEDDLGKG-GH--ELSDTTGNSG 127
Botryllus schlosseri        GNITADANGKAEVNITDKLVTLHGEYSVIGRAVVVHAGEDDLGLG-GF--PDSKTTGHAG 136
Caenorhabditis briggsae     GNIESPASGDTAISVSDSLASLSGQYSIIGRSVVIHEKTDDLGRGNSD---QSKTTGNAG 165
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Dictyocaulus viviparus      GNIVTSANGETVISISDPVITLNGYHSVIGRAVVIHADADDLGLGRSE---MSKSTGNSG 175
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Capra hircus                SCLACGVIGIAP------------------------------------------- 152
Bos taurus                  SRLACGVIGIAK------------------------------------------- 152
Bos grunniens               SRLACGVIGIAK------------------------------------------- 152
Cervus elaphus              NRLACGVIGIAQ------------------------------------------- 152
Callithrix jacchus          GRLACGVIGIAQ------------------------------------------- 154
Macaca mulatta              GRLACGVIGIAQ------------------------------------------- 154
Hylobates lar               SRLACGVIGIAQ------------------------------------------- 154
Cavia porcellus             SRLACGVIGIAQ------------------------------------------- 153
Equus caballus              SRLACGVIGIAP------------------------------------------- 154
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Caretta caretta             SRLACGVVGIAKL------------------------------------------ 167
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Xenopus tropicalis          GRLACGVIGLCQ------------------------------------------- 151
Xenopus laevis              GRLACGVIGYSP------------------------------------------- 151
Wuchereria bancrofti        ARVACGIVAVSAAS----------------------------------------- 158
Brugia malayi               ARVACGIVAIGAAS----------------------------------------- 158
Loa loa                     ARVACGIVALSATS----------------------------------------- 156
Ascaris suum                ARAACGVIAVAAPCEH--------------------------------------- 161
Trichinella pseudospiralis  ARVGCGVIGIANPAA------------------------------------------ 156
Branchiostoma floridae      GRLACGIIGITK------------------------------------------- 139
Botryllus schlosseri        GRVACGVIGFGK------------------------------------------- 148
Caenorhabditis briggsae     ARLACGTI----------------------------------------------- 173
Caenorhabditis remanei      ARLACGTIGKY-------------------------------------------- 176
Caenorhabditis elegans      SRLACGTIGIVEERILETTTASLPPVTQSQPIGSSSYYYSTFYLPIILYFLLSRIL 221
Dictyocaulus viviparus      ARVACGVIGIV-------------------------------------------- 186
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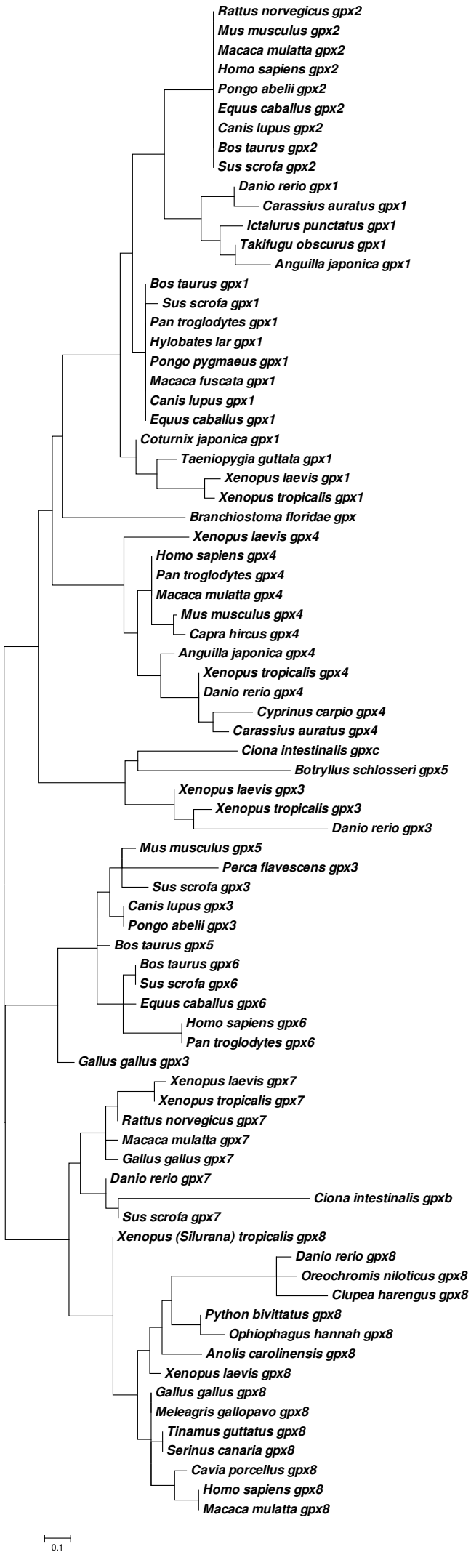
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Branchiostoma floridae gpx
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Anguilla japonica gpx4
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Danio rerio gpx4
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Carassius auratus gpx4
Ciona intestinalis gpxc
Botryllus schlosseri gpx5
Xenopus laevis gpx3
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Bos taurus gpx6
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Homo sapiens gpx6
Pan troglodytes gpx6
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Rattus norvegicus gpx7
Macaca mulatta gpx7
Gallus gallus gpx7
Danio rerio gpx7
Ciona intestinalis gpxb
Sus scrofa gpx7
Xenopus (Silurana) tropicalis gpx8
Danio rerio gpx8
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Clupea harengus gpx8
Python bivittatus gpx8
Ophiophagus hannah gpx8
Anolis carolinensis gpx8
Xenopus laevis gpx8
Gallus gallus gpx8
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Serinus canaria gpx8
Cavia porcellus gpx8
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Macaca mulatta gpx8
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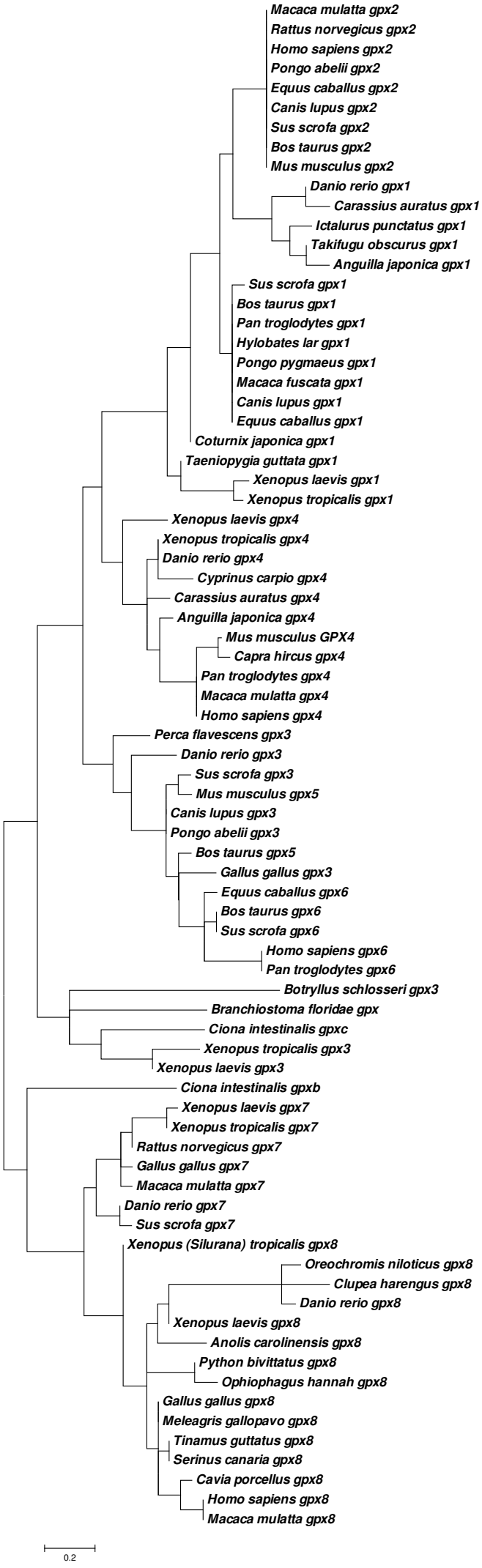
Figure S2. Evolutionary relationships (ML) among metazoan superoxide dismutases (SODs). Similar topologies were obtained with neighbor-joining (NJ), minimum evolution (ME), and unweighted pair group with arithmetic mean (UPGMA).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Bos taurus gpx2
Mus musculus gpx2
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Ictalurus punctatus gpx1
Takifugu obscurus gpx1
Anguilla japonica gpx1
Sus scrofa gpx1
Bos taurus gpx1
Pan troglodytes gpx1
Hylobates lar gpx1
Pongo pygmaeus gpx1
Macaca fuscata gpx1
Canis lupus gpx1
Equus caballus gpx1
Coturnix japonica gpx1
Taeniopygia guttata gpx1
Xenopus laevis gpx1
Xenopus tropicalis gpx1
Xenopus laevis gpx4
Xenopus tropicalis gpx4
Danio rerio gpx4
Cyprinus carpio gpx4
Carassius auratus gpx4
Anguilla japonica gpx4
Mus musculus GPX4
Capra hircus gpx4
Pan troglodytes gpx4
Macaca mulatta gpx4
Homo sapiens gpx4
Perca flavescens gpx3
Danio rerio gpx3
Sus scrofa gpx3
Mus musculus gpx5
Canis lupus gpx3
Pongo abelii gpx3
Bos taurus gpx5
Gallus gallus gpx3
Equus caballus gpx6
Bos taurus gpx6
Sus scrofa gpx6
Homo sapiens gpx6
Pan troglodytes gpx6
Botryllus schlosseri gpx3
Branchiostoma floridae gpx
Ciona intestinalis gpxc
Xenopus tropicalis gpx3
Xenopus laevis gpx3
Ciona intestinalis gpxb
Xenopus laevis gpx7
Xenopus tropicalis gpx7
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Macaca mulatta gpx7
Danio rerio gpx7
Sus scrofa gpx7
Xenopus (Silurana) tropicalis gpx8
Oreochromis niloticus gpx8
Clupea harengus gpx8
Danio rerio gpx8
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Anolis carolinensis gpx8
Python bivittatus gpx8
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Serinus canaria gpx8
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Macaca mulatta gpx8
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Figure S3. Evolutionary relationships (maximum likelihood; ML) among deuterostome glutathione peroxidases (GPxs). Similar topologies were obtained with neighbor-joining (NJ), minimum evolution (ME), and unweighted pair group with arithmetic mean (UPGMA).

Supplementary Table 1: Sequences, available in GenBank, used for phylogeny.

	GenBank ID	Species
GCLM	NP_059001.1	*Rattus norvegicus*
	NP_032155.1	*Mus musculus*
	NP_001033232.1	*Bos Taurus*
	NP_002052.1	*Homo sapiens*
	NP_001007954.1	*Gallus gallus*
	NP_001080413.1	*Xenopus laevis*
	NP_001016536.1	*Xenopus tropicalis*
	ACO52032.1	*Rana catesbeiana*
	NP_956139.1	*Danio rerio*
	NP_001167414.1	*Salmo salar*
	AAQ96653.1	*Branchiostoma belcheri*
	XP_002128824.1	*Ciona intestinalis*
	ERG80734.1	*Ascaris suun*
	XP_001899959.1	*Brugia malayi*
	XP_003139745.1	*Loa loa*
GS	XP_002125323.1	*Ciona intestinalis*
	NP_001270338.1	*Macaca fascicularis*
	NP_000169.1	*Homo sapiens*
	XP_008993693.1	*Callithrix jacchus*
	NP_001081013.1	*Xenopus laevis*
	NP_001008045.1	*Xenopus tropicalis*
	NP_001006104.1	*Danio rerio*
	XP_006811195.1	*Branchiostoma floridae*
	NP_001015630.1	*Bos taurus*
	NP_037094.1	*Rattus norvegicus*
	ERG80184.1	*Ascaris suun*
	XP_001892534.1	*Brugia malayi*
	XP_003142623.1	*Loa loa*
GPxs		
Vertebrate GPx1	NP_001007282.2	*Danio rerio*
	AGC50802.1	*Carassius auratus*
	ACR20471.1	*Takifugu obscurus*
	ACN78878.1	*Anguilla japonica*
	XP_017306443.1	*Ictalurus punctatus*
	NP_001130041.1	*Taeniopygia guttata*
	BAF95575.1	*Coturnix japonica*
	NP_999366.1	*Sus scrofa*
	NP_776501.1	*Bos taurus*
	NP_001108591.1	*Canis lupus*
	NP_001159951.1	*Equus caballus*
	BAC67247.1	*Macaca fuscata*
	XP_003776297.2	*Pongo pygmaeus*
	NP_001070980.2	*Pan troglodytes*
	Q4AEI2.2	*Hylobates lar*
	NP_001088896.2	*Xenopus laevis*
	NP_001015740.2	*Xenopus tropicalis*
Vertebrate GPx2	NP_001156611.1	*Bos taurus*
	NP_001108608.1	*Sus scrofa*
	NP_001108607.1	*Canis lupus*

	NP_001108609.2	*Macaca mulatta*
	NP_001125093.3	*Pongo abelii*
	EAW80889.1	*Homo sapiens*
	NP_001159953.1	*Equus caballus*
	NP_899653.2	*Rattus norvegicus*
	NP_109602.2	*Mus musculus*
Vertebrate GPx3	ACQ99329.1	*Perca flavescens*
	NP_001085319.2	*Xenopus laevis*
	NP_988961.2	*Xenopus tropicalis*
	NP_001131027.1	*Danio rerio*
	NP_001157926.1	*Canis lupus*
	NP_001124645.1	*Pongo abelii*
	NP_001108627.1	*Sus scrofa*
	NP_001156704.1	*Gallus gallus*
Vertebrate GPx4	NP_001112361.1	*Macaca mulatta*
	AAH46163.1	*Homo sapiens*
	NP_001272641.1	*Capra hircus*
	NP_032188.3	*Mus musculus*
	NP_001139295.1	*Pan troglodytes*
	NP_001165215.2	*Xenopus laevis*
	ACN78879.1	*Anguilla japonica*
	ABO36294.1	*Carassius auratus*
	ACR33821.1	*Cyprinus carpio*
	NP_001291701.1	*Xenopus tropicalis*
	ABW76146.1	*Danio rerio*
Vertebrate GPx5	AAI00750.1	*Mus musculus*
	NP_001020506.2	*Bos taurus*
Vertebrate GPx6	AAY68223.1	*Homo sapiens*
	NP_001139297.1	*Pan troglodytes*
	NP_001131079.1	*Sus scrofa*
	NP_001156614.1	*Bos taurus*
	NP_001159955.1	*Equus caballus*
Vertebrate GPx7	NP_001088904.1	*Xenopus laevis*
	NP_001072404.1	*Xenopus tropicalis*
	NP_001156717.1	*Gallus gallus*
	NP_001018337.1	*Danio rerio*
	XP_013847458.1	*Sus scrofa*
	NP_001152840.1	*Macaca mulatta*
	NP_001100143.1	*Rattus norvegicus*
Vertebrate GPx8	NP_001088474.1	*Xenopus laevis*
	XP_012811797.1	*Xenopus tropicalis*
	NP_956516.1	*Danio rerio*
	XP_003455356.1	*Oreochromis niloticus*
	XP_012671344.1	*Clupea harengus*
	XP_007421400.1	*Python bivittatus*
	ETE72807.1	*Ophiophagus hannah*
	XP_003216232.3	*Anolis carolinensis*
	XP_423834.1	*Gallus gallus*
	XP_010723716.1	*Meleagris gallopavo*
	XP_010225014.1	*Tinamus guttatus*
	XP_009094073.1	*Serinus canaria*
	XP_003470318.1	*Cavia porcellus*
	NP_001008398.2	*Homo sapiens*
	XP_001098032.1	*Macaca mulatta*

Invertebrate GPxs	XP_002587571.1	*Branchiostoma floridae*
	NP_001177268.1	*Ciona intestinalis*
	NP_001177274.1	*Ciona intestinalis*
Cu/Zn SOD	NP_001138657.1	*Ovis aries*
	NP_001272479.1	*Capra hircus*
	NP_777040.1	*Bos taurus*
	Q52RN5.3	*Bos grunniens*
	AAB88116.1	*Cervus elaphus*
	XP_003467296.1	*Cavia porcellus*
	Q8HXQ3.3	*Hylobates lar*
	XP_002761406.1	*Callithrix jacchus*
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	NP_001075295.1	*Equus caballus*
	AAB25456.1	*Caretta caretta*
	NP_990395.1	*Gallus gallus*
	NP_001268474.1	*Melopsittacus undulatus*
	ACO51906.1	*Rana catesbeiana*
	ABD75370.1	*Bufo gargarizans*
	NP_001016252.1	*Xenopus tropicalis*
	CAA34602.1	*Xenopus laevis*
	XP_002590336.1	*Branchiostoma floridae*
	KJH46452.1	*Dictyocaulus viviparus*
	NP_001255002.1	*Caenorhabditis elegans*
	XP_002632365.1	*Caenorhabditis briggsae*
	XP_003113480.1	*Caenorhabditis remanei*
	XP_002122526.1	*Ciona intestinalis*

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THE BIOLOGICAL BULLETIN

Marine Biological Laboratory

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Cover

Pictured is a grouping of encrusting organisms in the Venetian Lagoon, an enclosed bay in the Adriatic Sea. In the center is a solitary ascidian (*Styela plicata*) growing over a bed of mussels (*Mytilus galloprovincialis*), its tunic almost completely covered by a colony of the compound ascidian *Botryllus schlosseri*. Another colony, differing in pigmentation, appears on the right. Colonies of hydrozoans (likely the genus *Kirchenpaueria*) are also visible. *Botryllus schlosseri* is one of the species that characterizes the climax of the ecological succession in the Venetian Lagoon, and it is widely used as a model organism for evolutionary studies ranging from reproduction to immune defense.

On pages 45–57 of this issue of The Biological Bulletin, authors Franchi, Ballin, and Ballarin report the identification and characterization of transcripts for Cu/Zn superoxide dismutase (SOD), gamma glutamylcysteine ligase modulatory subunit (GCLM), glutathione synthase (GS), and two glutathione peroxidases (i.e., GPx3 and GPx5), all involved in protection from reactive oxygen species (ROS). The authors also studied the expression of antioxidant genes in the course of the blastogenetic cycle, assuring the cyclical generation change in *Botryllus* colonies. In addition, they investigated the effects of cadmium (Cd), an inducer of oxidative stress, on the transcription of the above-named genes.

Their results indicate that 1) gene transcription is modulated during the blastogenetic cycle and upon exposure to Cd, and 2) hemocytes synthesize both enzymatic and nonenzymatic antioxidants, in line with the idea that they represent a major detoxification system for ascidians.

Credits: Photo, Euichi Hirose, Faculty of Science Department of Chemistry, Biology, and Marine Science, University of the Ryukyus, Okinawa, Japan. Cover design, Sarah Gardiner, University of Chicago