

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

NICOLA FRANCHI, FRANCESCA BALLIN\*, AND LORIANO BALLARIN

*Department of Biology, University of Padova, Via Ugo Bassi 58/B, 35121 Padova, Italy*

**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),  $\gamma$ -glutamyl-cysteine ligase modulatory subunit (GCLM), gluta-

thione 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.

## 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 ( $\cdot\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), peroxy radicals ( $\cdot\text{RO}_2$ ), and hydroxyl radicals ( $\cdot\text{OH}$ ), 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.

Received 25 July 2016; accepted 30 January 2017; Published online 3 April 2017.

\*To whom correspondence should be received. E-mail: ballin.fra@gmail.com

**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,  $\gamma$ -glutamyl-cysteine ligase; GCLC, catalytic subunit of  $\gamma$ -glutamyl-cysteine ligase; GCLM, modulatory subunit of  $\gamma$ -glutamyl-cysteine ligase; GPx, glutathione peroxidase; GS, glutathione synthase; GSH, glutathione; GSSG, oxidized glutathione; GT, guanine thimine (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.

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. Ascidiaceans 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 *Halocynthia 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),  $\gamma$ -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- $\mu$ m 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  $5 \times 10^5$  hemocytes/ml.

#### Exposure to cadmium

A storage solution was prepared by dissolving  $\text{CdCl}_2$  in distilled water, whose concentration was determined by atomic absorption spectrometry, using a PerkinElmer 4000 spectrometer (PerkinElmer, Watham, MA), resulting in  $45 \text{ mmol l}^{-1}$ . It was subsequently diluted in FSW to obtain a working solution with a final concentration of  $0.2 \text{ } \mu\text{mol l}^{-1}$ . 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; Koutsoyianni *et al.*, 2015; Mùgica *et al.*, 2015).

Nine colonies of comparable size (around 25 zooids each) were exposed to  $0.2 \text{ } \mu\text{mol l}^{-1}$   $\text{CdCl}_2$  in FSW, in 3 9-l aquaria (3 colonies per aquarium), at  $16 \text{ } ^\circ\text{C}$ . 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  $-80 \text{ } ^\circ\text{C}$  until use.

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

Our EST collection was aligned on the *Botryllus* genome already available online (Voskoboinik *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 2<sup>nd</sup> Generation (Roche Molecular Systems, Inc., Pleasanton, CA).

**Table 1**

PCR primers used and relative melting temperatures ( $T_m$ )

Primer	$T_m$ ( $^\circ\text{C}$ )	Sequence 5'-3'
BsGCLF	53	GTTGGAAGAATCGGTAGGG
BsGCLFR	57.4	GCTTGAATGACTTCTCAGGGAG
BsGCLF-RT	53.9	CGAAAGCGTTGAGTGTATGG
BsGCLM-RT	56.9	CAAAATCAGTCACGCCGATGTG
BsGSF	55.2	CGAAGCCAACATCATCCGA
BsGSR	55	CTCGGTCGCTCTCATCTG
BsGSF-RT	60	CATGCGATCAGTCAAGATCC
BsGSR-RT	60	TTGCCATTGCAGTCTTCTTG
BsGPx5F	57.8	CATTGCTTGTTCGAGTGC
BsGPx5R	57	GCCACCAGAGTGTCCAATA
BsGPx5F-RT	60	GGAAATGGATGGACGCCGCA
BsGPx5R-RT	60	CCTAACTCTTCGGTGTATGCCGGAC
BsGPx3F	58	CGTCCGTACAAGACAAGGTGG
BsGPx3R	55	ACATCTCCAACGCAAGTCC
BsGPx3R-RT	59.3	GGAAGCCACGACACCTTGC
BsSODF	58.4	CCACGGGTTTCACATTACAGAG
BsSODR	60.9	AATCCAATCACGCCACACGCC
BsSODF-RT	60	CTGTGCAAGGACTGACTCCA
BsSODR-RT	60	CCGGCATGATCAACCTTAGT
BsACTF-RT	60	ACTGGGACGACATGGAGAAG
BsACTR-RT	60	GCTTCTGTGAGGAGGACAGG
M13F	55	TTGTAACACGACGGCCAGT
M13R	50	CAGGAAACAGCTATGACC
dT Anchor	57	ACCACGCGTATCGATGTCTG (dT)16
Anchor	57	ACCACGCGTATCGATGTCTG

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  $1 \text{ } \mu\text{g}$  of total RNA according to the Improm II manual (Promega Corp.). cDNA amplification was performed with Go-Taq Polymerase (Promega;  $5 \text{ U}/\mu\text{l}$ ), using the following cycling parameters:  $94 \text{ } ^\circ\text{C}$  for 2 min, 40 cycles of  $94 \text{ } ^\circ\text{C}$  for 30 s, melting temperature ( $T_m$ ) for 30 s ( $T_m$ s for the various primers are shown in Table 1),  $72 \text{ } ^\circ\text{C}$  for 1 min, and, a last step, at  $72 \text{ } ^\circ\text{C}$  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-5 $\alpha$  *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  $0.2 \mu\text{mol l}^{-1}$  CdCl<sub>2</sub> 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 Bs $\beta$ -actin (BsACTF-RT and BsACTR-RT) transcripts—the last one (Bs $\beta$ -actin) used as a housekeeping gene—were synthesized by Sigma-Aldrich (St. Louis, MO) (Table 1). The stable expression of Bs $\beta$ -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  $\beta$ -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; Sourdiss 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 ( $0.1 \text{ mol l}^{-1}$  MOPS,  $1 \text{ mmol l}^{-1}$  MgSO<sub>4</sub>,  $2 \text{ mmol l}^{-1}$  EGTA, and  $0.5 \text{ mol l}^{-1}$  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 ( $2 \mu\text{g/ml}$  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<sub>2</sub>O<sub>2</sub> in phosphate-buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH<sub>2</sub>PO<sub>4</sub>, 1.15 g/l Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2) for 10 min. Colonies were then dehydrated, included in Paraplast Plus Xtra (Sigma-Aldrich), and 7- $\mu\text{m}$  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 $\times$  magnification.

### Statistical analyses

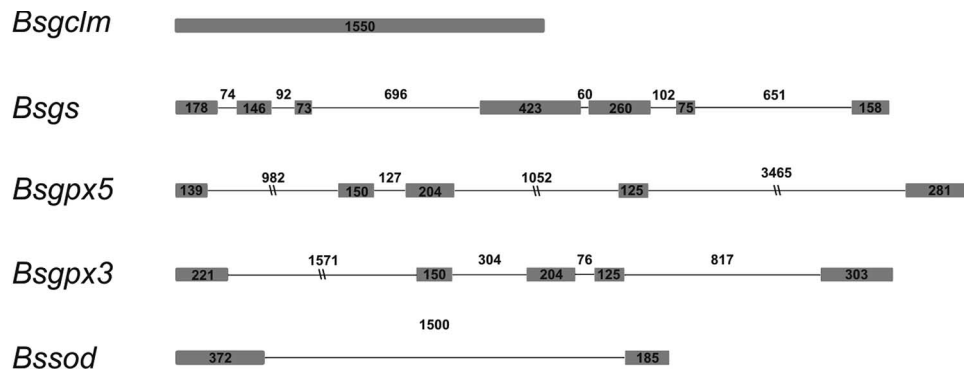
Each experiment was replicated three times with three independent colonies ( $n = 3$ ); data are expressed as means  $\pm$  SD. Multiple comparisons were carried out with ANOVA; means were compared using Duncan's test (Snedecor and Cochran, 1980).

## 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  $\gamma$ -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 thymine (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.

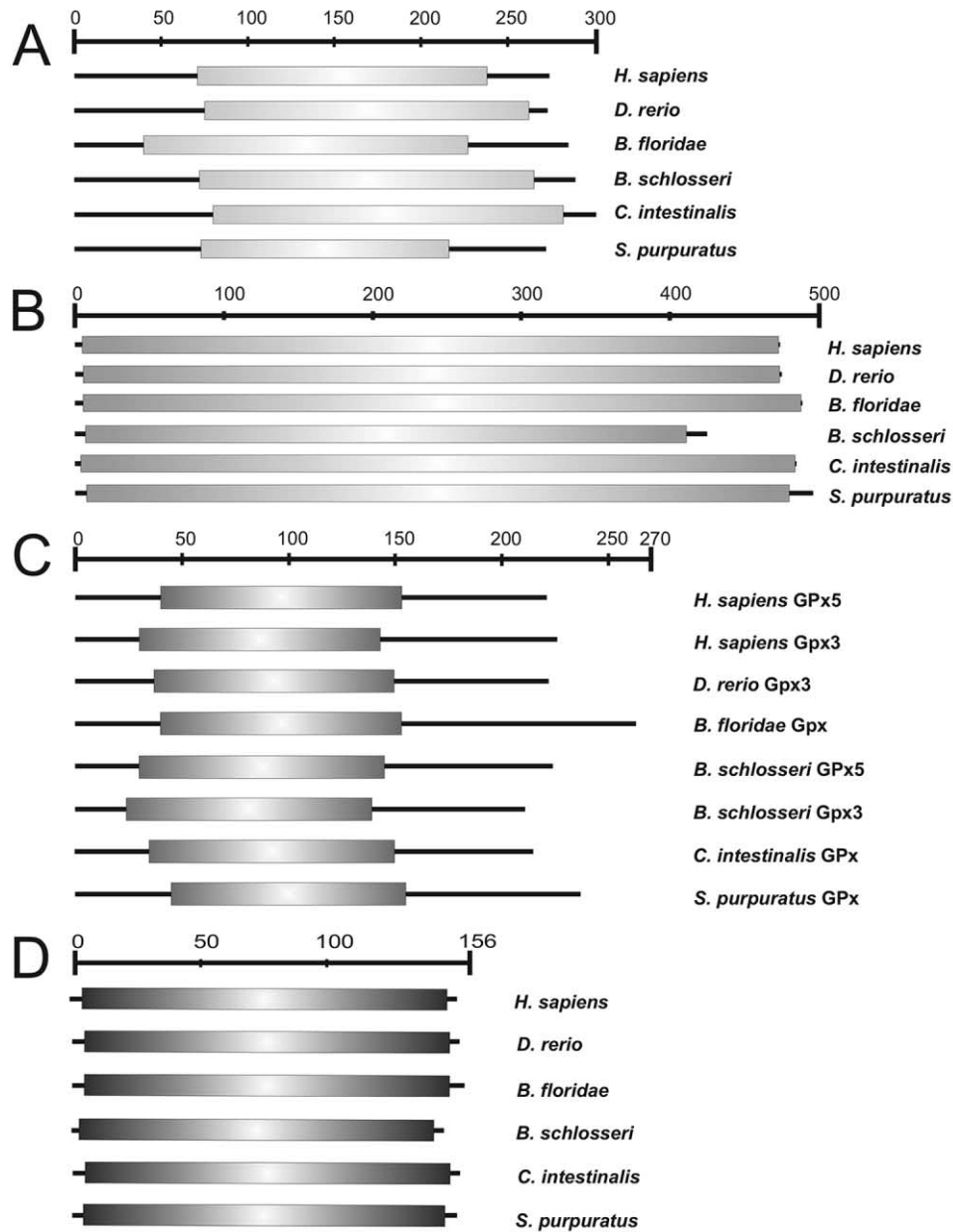
BsSODF and BsSODR amplified a sequence of 322 bp, similar to Cu/Zn SOD from other deuterostomes. The coding sequence of this transcript spans 447 bp in length and is flanked by 5' UTR and 3' UTR regions of 305 bp and 305 bp, respectively (GenBank accession no. KT120028). The structure of the gene was 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  $\gamma$ -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<sup>129</sup>, Ile<sup>143</sup>, Lys<sup>309</sup>, Asn<sup>368</sup>, Tyr<sup>370</sup>, the MEKI motif, Glu<sup>427</sup>, Lys<sup>454</sup>); the ATP-binding amino acids (Ile<sup>143</sup>, Lys<sup>309</sup>, Val<sup>365</sup>, Lys<sup>367</sup>, the MEKI motif, Glu<sup>427</sup>, Lys<sup>454</sup>); the magnesium-binding sites (Glu<sup>144</sup>, Asn<sup>146</sup>, Glu<sup>371</sup>); and the GSH-binding sites (Arg<sup>123</sup>, Ala<sup>148</sup>, Ser<sup>150</sup>, Glu<sup>215</sup>, Asn<sup>217</sup>, Gln<sup>221</sup>, Arg<sup>452</sup>, Val<sup>463</sup>, Ala<sup>464</sup>), 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* GPx) to 24.8% (*B. floridae*). By comparing multiple alignments of



**Figure 2.** Schematic deuterostome domain organization of the modulatory subunit of  $\gamma$ -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<sup>95</sup>, Trp<sup>173</sup>). 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<sup>161</sup>, Gln<sup>95</sup>, Trp<sup>173</sup>) (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<sup>41</sup>, His<sup>43</sup>, His<sup>115</sup>), zinc (His<sup>66</sup>, His<sup>75</sup>), zinc and cadmium (His<sup>58</sup>), as well as those involved in antioxidant reactions (Thr<sup>132</sup>, Arg<sup>138</sup>) (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).

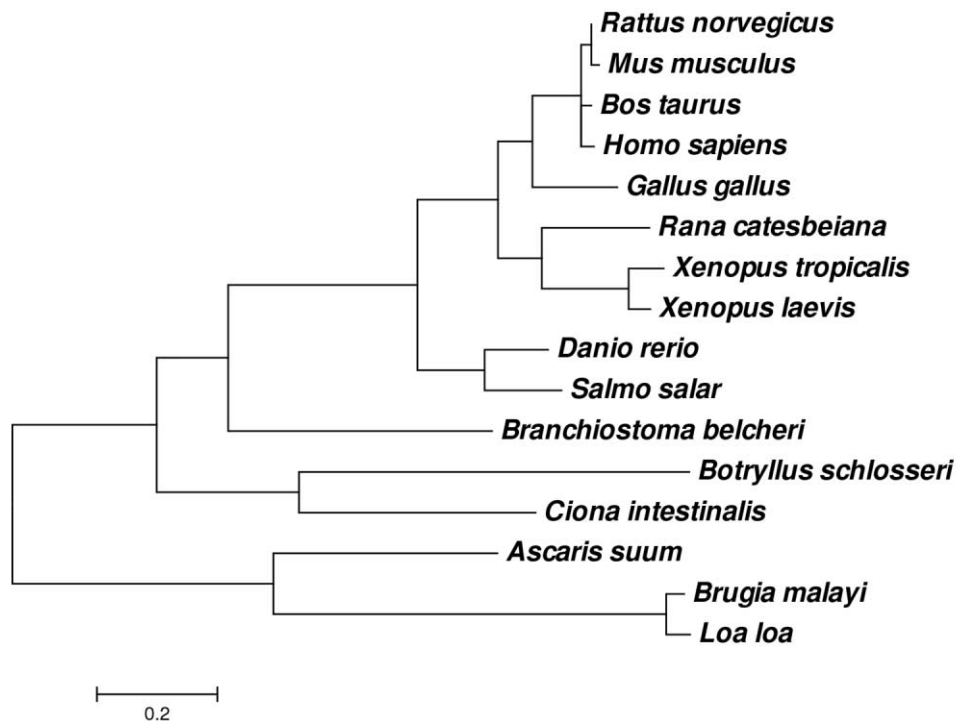
As regards the phylogenetic reconstruction of deuterostome GPxs, BsGPx5 clusters together with *C. intestinalis* GPxc and *Xenopus laevis*, *Xenopus tropicalis*, and *Danio rerio* GPx3, as

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).

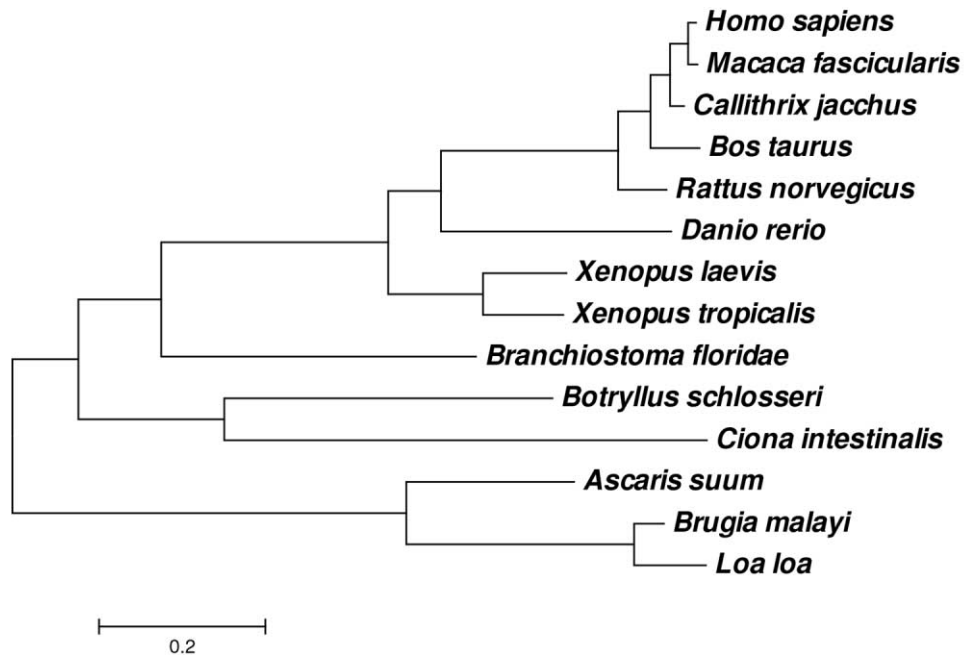
### qRT-PCR

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  $\gamma$ -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

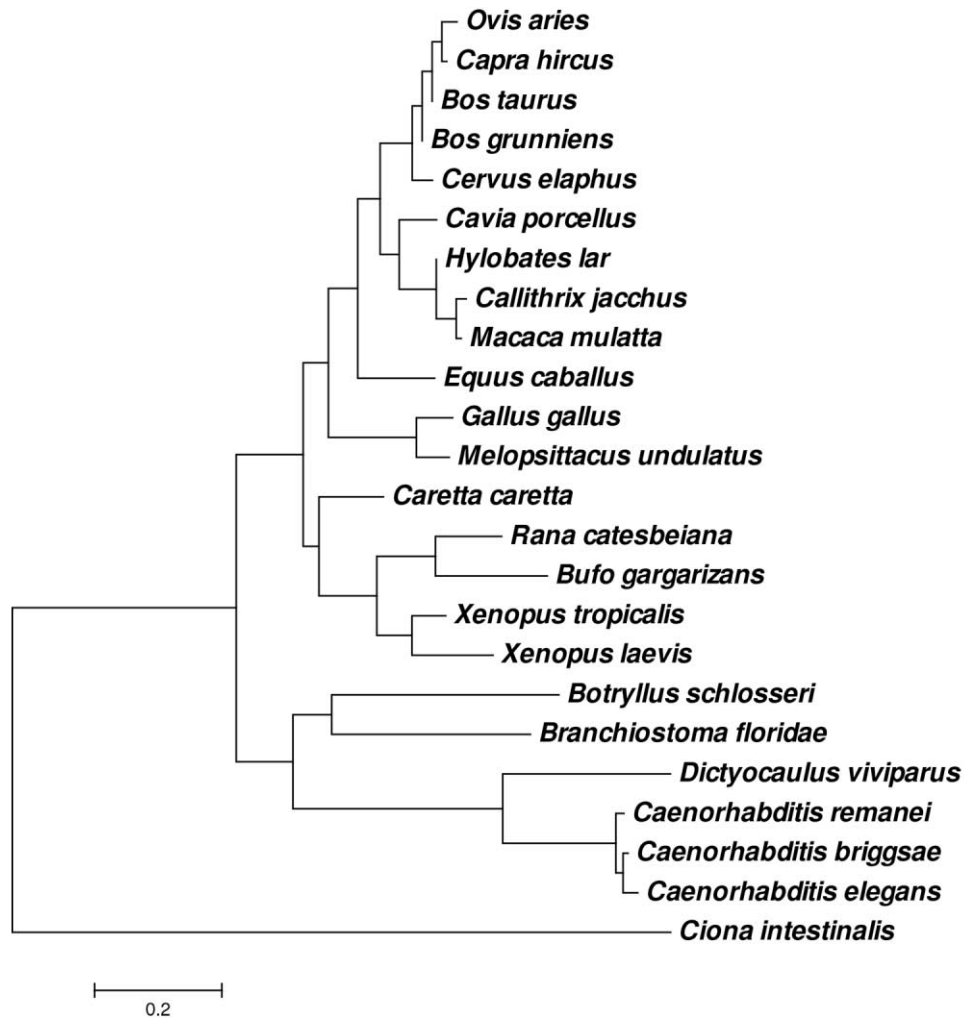
In colony sections, only hemocytes contained detectable levels of transcripts for BsGCLM, BsGS, BsCu/ZnSOD, BsGPx3, and BsGPx5 (data not shown). A more detailed analysis of hemocyte smears revealed that only immunocytes were labeled. In the presence of the specific riboprobes for BsGCLM and BsGPx3, cytotoxic 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-



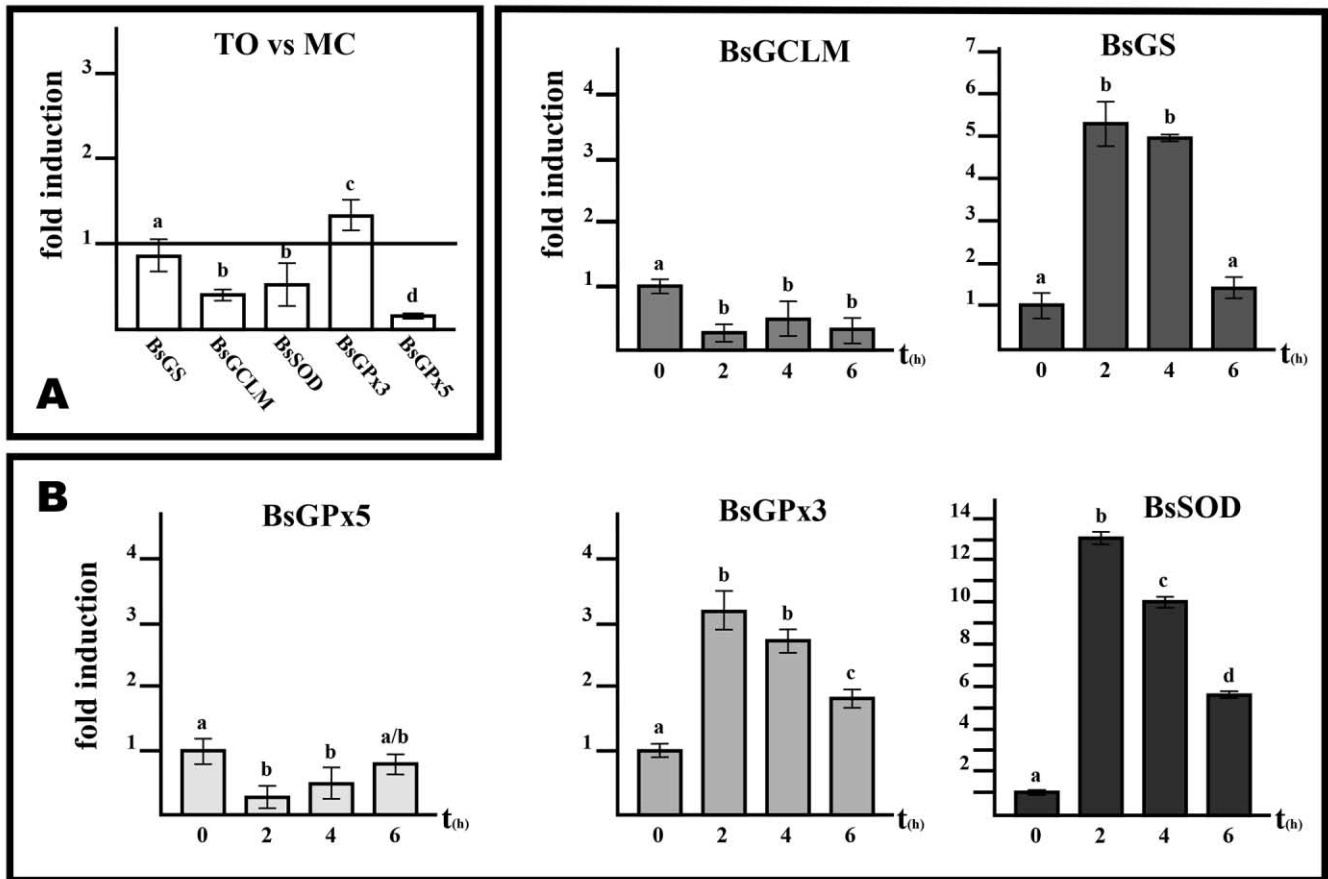
**Figure 5.** Evolutionary relationships (maximum likelihood; ML) among deuterostome glutathione peroxidase (GPxs). Similar topologies were obtained with neighbor-joining (NJ), minimum evolution (ME), and unweighted pair groups with arithmetic mean (UPGMA). Branch length scale = 0.2 substitutions per 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 ( $\gamma$ -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  $\gamma$ -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  $\gamma$ -glutamylcysteine. The transcripts for both genes are present in *Botryllus* immunocytes, either in phagocytes or morula cells. The amount of transcripts for BsGCLM decreases dur-

ing 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



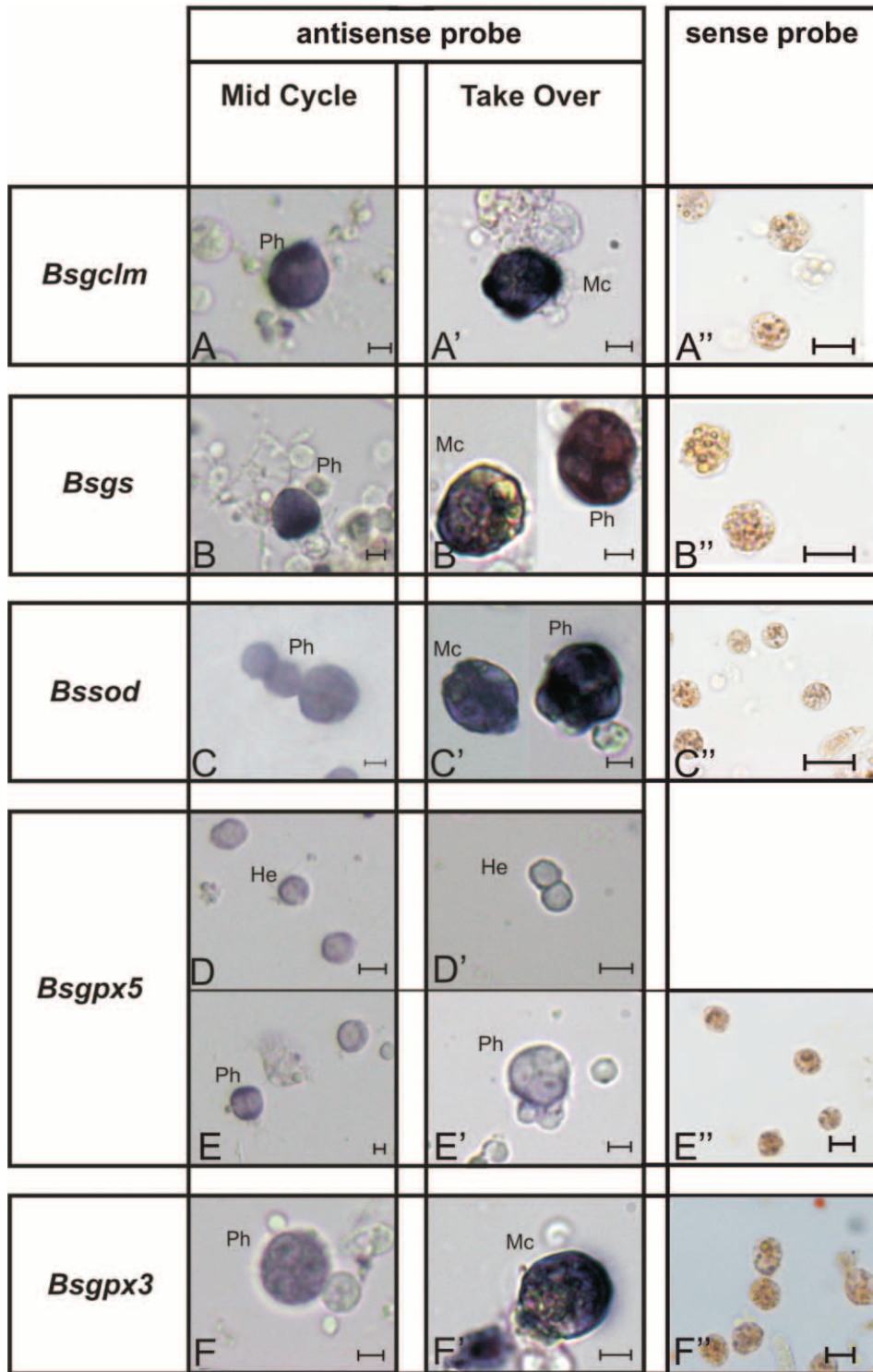
**Figure 6.** (A) Relative levels of mRNA in five *Botryllus schlosseri* enzymes: glutathione synthase (BsGS), *B. schlosseri* subunit of  $\gamma$ -glutamyl-cysteine ligase (BsGCLM), *B. schlosseri* Cu-Zn superoxide dismutase (BsCu/ZnSOD), and *B. schlosseri* glutathione peroxidase 3 (BsGPx3) and 5 (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  $0.2 \mu\text{mol l}^{-1}$   $\text{CdCl}_2$  with respect to unexposed colonies (Cd concentration = 0; reference control set to 1). Normalization of expression was achieved using endogenous  $\beta$ -actin as housekeeping gene. Each histogram bar corresponds to the average of three independent experiments, each with a different colony ( $n = 3$ )  $\pm$  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 ob-

tained 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  $10 \mu\text{mol l}^{-1}$   $\text{CdCl}_2$  (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  $\mu$ m.

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<sub>2</sub> 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

This work was supported by the Italian Ministry of Education, Universities and Research (MIUR; Prin 2010/11, 20109XZEPR).

### Literature Cited

- Abe, Y., G. Ishikawa, H. Satoh, K. Azumi, and H. Yokosawa. 1999. Primary structure and function of superoxide dismutase from the ascidian *Halocynthia roretzi*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **122**: 321–326.
- Arthur, J. R. 2000. The glutathione peroxidases. *Cell. Mol. Life Sci.* **57**: 1825–1835.
- Ballarin, L., and F. Cima. 2005. Cytochemical properties of *Botryllus schlosseri* haemocytes: indications for morpho-functional characterisation. *Eur. J. Histochem.* **49**: 255–264.
- Ballarin, L., F. Cima, and A. Sabbadin. 1994. Phagocytosis in the colonial ascidian *Botryllus schlosseri*. *Dev. Comp. Immunol.* **18**: 467–481.
- Ballarin, L., A. Franchini, E. Ottaviani, and A. Sabbadin. 2001. Morula cells as the major immunomodulatory hemocytes in ascidians: evidence from the colonial species *Botryllus schlosseri*. *Biol. Bull.* **201**: 59–64.
- Ballarin, L., F. Cima, M. Floreani, and A. Sabbadin. 2002. Oxidative stress induces cytotoxicity during rejection reaction in the compound ascidian *Botryllus schlosseri*. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **133**: 411–418.
- Ballarin, L., P. Burighel, and F. Cima. 2008a. A tale of death and life: natural apoptosis in the colonial ascidian *Botryllus schlosseri* (Urochordata, Ascidiacea). *Curr. Pharm. Des.* **14**: 138–147.
- Ballarin, L., A. Menin, L. Tallandini, V. Matozzo, P. Burighel, G. Basso, E. Fortunato, and F. Cima. 2008b. Haemocytes and blastogenetic cycle in the colonial ascidian *Botryllus schlosseri*: a matter of life and death. *Cell Tissue Res.* **331**: 555–564.
- Ballarin, L., F. Schiavon, and L. Manni. 2010. Natural apoptosis during the blastogenetic cycle of the colonial ascidian *Botryllus schlosseri*: a morphological analysis. *Zool. Sci.* **27**: 96–102.
- Brigelius-Flohé, R. 1999. Tissue-specific functions of individual glutathione peroxidases. *Free Radic. Biol. Med.* **27**: 951–965.
- Brigelius-Flohé, R., and M. Maiorino. 2013. Glutathione peroxidases. *Biochim. Biophys. Acta* **1830**: 3289–3303.
- Campagna, D., F. Gasparini, N. Franchi, N. Vitolo, F. Ballin, L. Manni, G. Valle, and L. Ballarin. 2016. Transcriptome dynamics in the asexual cycle of the chordate *Botryllus schlosseri*. *BMC Genomics* **17**: 275.
- Canesi, L. 2015. Pro-oxidant and antioxidant processes in aquatic invertebrates. *Ann. N. Y. Acad. Sci.* **1340**: 1–7.
- Cima, F., and L. Ballarin. 2009. Apoptosis and pattern of Bcl-2 and Bax expression in the alimentary tract during the colonial blastogenetic cycle of *Botryllus schlosseri* (Urochordata, Ascidiacea). *Ital. J. Zool.* **76**: 28–42.
- Cima, F., G. Basso, and L. Ballarin. 2003. Apoptosis and phosphatidylserine-mediated recognition during the take-over phase of the colonial life-cycle in the ascidian *Botryllus schlosseri*. *Cell Tissue Res.* **312**: 369–376.
- Cima, F., A. Sabbadin, and L. Ballarin. 2004. Cellular aspects of allorecognition in the compound ascidian *Botryllus schlosseri*. *Dev. Comp. Immunol.* **28**: 881–889.
- Cima, F., L. Manni, G. Basso, E. Fortunato, B. Accordi, F. Schiavon, and L. Ballarin. 2010. Hovering between death and life: natural apoptosis and phagocytes in the blastogenetic cycle of the colonial ascidian *Botryllus schlosseri*. *Dev. Comp. Immunol.* **34**: 272–285.
- De la Fuente, M., and V. M. Victor. 2000. Anti-oxidants as modulators of immune function. *Immunol. Cell Biol.* **78**: 49–54.
- Delsuc, F., H. Brinkmann, D. Chourrout, and H. Philippe. 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* **439**: 965–968.
- Dickinson, D. A., and H. J. Forman. 2002. Cellular glutathione and thiols metabolism. *Biochem. Pharmacol.* **64**: 1019–1026.
- Ferro, D., N. Franchi, V. Mangano, R. Bakiu, M. Cammarata, N. Parrinello, G. Santovito, and L. Ballarin. 2013. Characterization and metal-induced gene transcription of two new copper zinc superoxide dismutases in the solitary ascidian *Ciona intestinalis*. *Aquat. Toxicol.* **140–141**: 369–379.
- Franchi, N., and L. Ballarin. 2013. Influence of cadmium on the morphology and functionality of haemocytes in the compound ascidian *Botryllus schlosseri*. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **158**: 29–35.
- Franchi, N., F. Boldrin, L. Ballarin, and E. Piccini. 2011. CiMT-1, an unusual chordate metallothionein gene in *Ciona intestinalis* genome: structure and expression studies. *J. Exp. Zool. A Ecol. Genet. Physiol.* **315A**: 90–100.
- Franchi, N., D. Ferro, L. Ballarin, and G. Santovito. 2012. Transcription of genes involved in glutathione biosynthesis in the solitary

- tunicate *Ciona intestinalis* exposed to metals. *Aquat. Toxicol.* **114**–115: 14–22.
- Franchi, N., E. Piccinni, D. Ferro, G. Basso, B. Spolaore, G. Santovito, and L. Ballarin. 2014. Characterization and transcription studies of a phytochelatin synthase gene from the solitary tunicate *Ciona intestinalis* exposed to cadmium. *Aquat. Toxicol.* **152**: 47–56.
- Franchi, N., L. Ballarin, and F. Cima. 2015. Insights on cytotoxic cells of the colonial ascidian *Botryllus schlosseri*. *Invertebr. Surviv. J.* **12**: 109–117.
- Franchi, N., F. Ballin, L. Manni, F. Schiavon, G. Basso, and L. Ballarin. 2016. Recurrent phagocytosis-induced apoptosis in the cyclical generation change of the compound ascidian *Botryllus schlosseri*. *Dev. Comp. Immunol.* **62**: 8–16.
- Fridovich, I. 1986. Superoxide dismutases. *Adv. Enzymol. Relat. Areas Mol. Biol.* **58**: 61–97.
- Gasparini, F., L. Manni, F. Cima, G. Zaniolo, P. Burighel, F. Caicci, N. Franchi, F. Schiavon, F. Rigon, D. Campagna, and L. Ballarin. 2015. Sexual and asexual reproduction in the colonial ascidian *Botryllus schlosseri*. *Genesis* **53**: 105–120.
- Ghyselinck, N. B., I. Dufaure, J. J. Lareyre, N. Rigaudière, M. G. Mattéi, and J. P. Dufaure. 1993. Structural organization and regulation of the gene for the androgen-dependent glutathione peroxidase-like protein specific to the mouse epididymis. *Mol. Endocrinol.* **7**: 258–272.
- Griffith, O. W. 1999. Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radic. Biol. Med.* **27**: 922–935.
- Guindon, S., and O. Gascuel. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* **52**: 696–704.
- Hughes, S. L., J. G. Bundy, E. J. Want, P. Kille, and S. R. Sturzenbaum. 2009. The metabolomic responses of *Caenorhabditis elegans* to cadmium are largely independent of metallothionein status, but dominated by changes in cystathionine and phytochelatin. *J. Proteome Res.* **8**: 3512–3519.
- Jeppé, K. J., M. E. Carew, S. M. Long, S. F. Lee, V. Pettigrove, and A. A. Hoffmann. 2014. Genes involved in cysteine metabolism of *Chironomus tepperi* are regulated differently by copper and by cadmium. *Comp. Biochem. Physiol.* **162C**: 1–6.
- Kaloyianni, M., S. Dailianis, E. Chrisikopoulou, A. Zannou, S. Koutsogiannaki, D. H. Alamdari, G. Koliakos, and V. K. Dimitriadis. 2009. Oxidative effects of inorganic contaminants on haemolymph of mussels. *Comp. Biochem. Physiol.* **149C**: 631–639.
- Koutsogiannaki, S., S. Franzellitti, S. Kalogiannis, E. Fabbri, V. K. Dimitriadis, and M. Kaloyianni. 2015. Effects of cadmium and 17 $\beta$ -estradiol on *Mytilus galloprovincialis* redox status. Prooxidant-antioxidant balance (PAB) as a novel approach in biomonitoring of marine environments. *Mar. Environ. Res.* **103**: 80–88.
- Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez *et al.* 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947–2948.
- Lauzon, R. J., K. J. Ishizuka, and I. L. Weissman. 1992. A cyclical, developmentally regulated death phenomenon in a colonial urochordate. *Dev. Dyn.* **194**: 71–83.
- Lauzon, R. J., C. W. Patton, I. L. Weissman. 1993. A morphological and immunohistochemical study of programmed cell death in *Botryllus schlosseri* (Tunicata, Ascidiacea). *Cell Tissue Res.* **272**: 115–127.
- Lesser, M. P. 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. *Annu. Rev. Physiol.* **68**: 253–278.
- Liu, J., Z. X. Zhou, W. Zhang, M. W. Bell, and M. P. Waalkes. 2009. Changes in hepatic gene expression in response to hepatoprotective levels of zinc. *Liver Int.* **29**: 1222–1229.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>- $\Delta\Delta C_T$</sup>  method. *Methods* **25**: 402–408.
- Manni, L., G. Zaniolo, F. Cima, P. Burighel, and L. Ballarin. 2007. *Botryllus schlosseri*: a model ascidian for the study of asexual reproduction. *Dev. Dyn.* **236**: 335–352.
- Mùgica, M., U. Izagirre, and I. Marigómez. 2015. Lysosomal responses to heat-shock of seasonal temperature extremes in Cd-exposed mussels. *Aquat. Toxicol.* **164**: 99–107.
- Puppel, K., A. Kapusta, and B. Kuczyńska. 2015. The etiology of oxidative stress in the various species of animals, a review. *J. Sci. Food Agric.* **95**: 2179–2184.
- Rzhetsky, A., and M. Nei. 1992. Statistical properties of the ordinary least-squares, generalized least squares, and minimum-evolution methods of phylogenetic inference. *J. Mol. Evol.* **35**: 367–375.
- Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425.
- Sneath, A., and R. Sokal. 1973. *Numerical Taxonomy: the Principles and Practice of Numerical Classification*. Freeman, San Francisco, CA.
- Snedecor, G. W., and W. G. Cochran. 1980. *Statistical Methods*, 7th ed. Iowa State University Press, Ames.
- Sourdis, J., and M. Nei. 1988. Relative efficiencies of the maximum parsimony and distance-matrix methods in obtaining the correct phylogenetic tree. *Mol. Biol. Evol.* **5**: 298–311.
- Sunde, R. A., and W. G. Hoekstra. 1980. Structure, synthesis and function of glutathione peroxidase. *Nutr. Rev.* **38**: 265–273.
- Tamura, K., G. Stecher, D. Peterson, A. Filipinski, and S. Kumar. 2013. MEGA6: Molecular Evolutionary Genetics analysis Version 6.0. *Mol. Biol. Evol.* **30**: 2725–2729.
- Tang, X., Y. Nakata, H. O. Li, M. Zhang, H. Gao, A. Fujita, O. Sakatsume, T. Ohta, and K. Yokoyama. 1994. The optimization of preparations of competent cells for transformation of *E. coli*. *Nucleic Acids Res.* **22**: 2857–2858.
- Tomanek, L. 2014. Proteomics to study adaptations in marine organisms to environmental stress. *J. Proteomics* **105**: 92–106.
- Toppo, S., S. Vanin, V. Bosello, and S. C. Tosatto. 2008. Evolutionary and structural insights into the multifaceted glutathione peroxidase (Gpx) superfamily. *Antioxid. Redox Signal.* **10**: 1501–1514.
- Voskoboinik, A., N. F. Neff, D. Sahoo, A. M. Newman, D. Pushkarev, W. Koh, B. Passarelli, H. C. Fan, G. L. Mantalas, K. J. Palmeri *et al.* 2013. The genome sequence of the colonial chordate, *Botryllus schlosseri*. *Elife* **2**: e00569.
- Zeeshan, H. M., G. H. Lee, H. R. Kim, and H. J. Chae. 2016. Endoplasmic reticulum stress and associated ROS. *Int. J. Mol. Sci.* **17**: 327.

# A: Amino acid alignment of glm sequences obtained by CLUSTAL W

```

Rattus norvegicus  ----MGTDSDRAAGALLARASTLHLQGTGNLLNWGRLRKKCPSTHSEELRDCIQKTLNEWSS 56
Mus musculus      ----MGTDSDRAAGALLARASTLHLQGTGNLLNWGRLRKKCPSTHSEELRDCIQKTLNEWSS 56
Bos taurus        ----MGTDSDRAAGALLARASTLHLQGTGNLLNWGRLRKKCPSTHSEELRDCIQKTLNEWSS 56
Homo sapiens      ----MGTDSDRAAKALLARARTLHLQGTGNLLNWGRLRKKCPSTHSEELHDCIQKTLNEWSS 56
Gallus gallus     ----MGTEG--ARALLERARTLNLTGNLLSWGCGRKKCPVTPSEEVDRDCIQKTLTEWSS 54
Xenopus tropicalis ----MGTDSTAARTLLDKADKLVLTGNLLNWGCLKKKCPSTPSEELQDCIRTTLNEWSQ 56
Xenopus laevis    ----MGTDSTAARTLLDKADKLVLTGNLLNWGCLKKKCPSTPSEELQDCIRTTLNEWNQ 56
Rana catesbeiana  ----MGTD--ARSLLSAARTLILHTGNLMNWGRLKKKCPVTPGEEELGDCIRITLSEWSS 54
Danio rerio       ----MEHHVN-AKRLFSHATTLKLTHTGNLVNRSRLKKKCPSSPSEELQDCIQGTLSEWFT 55
Salmo salar       ----METHHTGAKVLLNHATTLNLHTGNLVNRSRLKKKCPSSPSEEIQCDCVRATLSEWFA 56
Branchiostoma belcheri --MSAVPVEEIIARLMLSSSTNNLTHTGNIINWNHLKRRKTSQNAAGEEVLECLSATLNVWSS 58
Botryllus schlosseri -----MDXAQMXVDQADTVVIXSGNIINSYSLKKRVGQKSSDELADSIKFTTSWKE 52
Brugia malayi     MSTKVNHSMEIISQVQKSSAVRLNTGNIQRHPELFRQMFNRSAEELVAALAEHLQ----D 56
Loa loa           MSTKVSQSSVEIMSQVQKSSAVCLNTGNIQRHPELFRQLFKNSAEELVAALAEHLQ----D 56
Ascaris suum      MESKAYNK-----SELR---VFRLNTGNINSCGELKRRACKDSAEELVAALQIEL-----N 48

```

```

Rattus norvegicus  QIS-PDLVREF-PDVLECTMSHAVEKINPDEREEMKVSAKLFIVGSNSSSSTRNAVDMAC 114
Mus musculus      QIS-PDLVREF-PDVLECTMSHAVEKINPDEREEMKVSAKLFIVGSNSSSSTRNAVDMAC 114
Bos taurus        QIS-PDLVREF-PDVLECTMSHAVEKINPDEREEMKVSAKLFIVGSNSSSSTRNAVDMAC 114
Homo sapiens      QIN-PDLVREF-PDVLECTVSHAVEKINPDEREEMKVSAKLFIVESNSSSSTRNAVDMAC 114
Gallus gallus     KVG-RDQNEQEM-VEVLECTVAQAVEKMNPEERDELKVSAKLFIVGSNSSS-IRDAVDMAC 111
Xenopus tropicalis KFS-PELVKEI-PQTLCTVQAMETINLDEREEMKVSVKLFIVSPSHSS-VTQAIDMAC 113
Xenopus laevis    KIS-PELVKDI-SQTLCTVQAMETINLDEREEMKVSVKLFIVSPSLSS-VTEAIDMAC 113
Rana catesbeiana  KAS-PEHIQDS-SQTLCAVNQALETINPEEREVMKVSAKLFIVGLDSS-IGHAVDMAC 111
Danio rerio       AMP-SSIDSEL-PSVLDCSIPENTEAITPEEREELKVSVKLFLTEWDCSS-IRSAVDMAC 112
Salmo salar       TIPPTPTDL-PDTIDCSIPOATEAITPEEREELKVSVKLFIQCEADLSS-IKDAVDMAC 114
Branchiostoma belcheri TVDPSWNNEES-NNALIITNPNQMERISEDESKLKVSVKIFLKLWDPDL-VVEAVDQVC 116
Botryllus schlosseri SVGPEYMNIPPEIHTLVNTDALTSWHTDEIRSDLKISAKIFLSEVEPQL-VRNAVXNVC 111
Brugia malayi     GPNGPDLAMRP-DGTIMLTRDSENCRLNTEKNDMKITLKVFMSSLDLKQ-VEESIDTAL 114
Loa loa           GPNGPDLAMRP-DGTIVMLARDENCRLNTEKADMKITLKVFMSSLDLKQ-VEESIDTAL 114
Ascaris suum      KRDL--DLHFAN-DQTIISLAAMET----NPNYPRNDLKITLKVFMSSSDFSQ-VTDCLNATK 100

```

```

      . : . : . : * : * . . : : .

```

```

Rattus norvegicus  SVLGVAQLDSVIMASPPIEDGV-----NLSLEHLQPYWEELENLVQSKKIVAIGTS 165
Mus musculus      SVLGVAQLDSVIMASPPIEDGV-----NLSLEHLQPYWEELENLVQSKKIVAIGTS 165
Bos taurus        SVLGVAQLDSVIMASPPIEDGV-----NLSLEHLQPYWEELENLVQSKKIVAIGTS 165
Homo sapiens      SVLGVAQLDSVIMASPPIEDGV-----NLSLEHLQPYWEELENLVQSKKIVAIGTS 165
Gallus gallus     SSLGVAQLDSVIMASPPIEDGV-----SLSLEYLQPYWEELETLVENKIVAIGTS 162
Xenopus tropicalis STLGVAQIDSMI IAPPPLEDGR-----SFSLEDLQPYWEELESVRNGKVVIGTS 164
Xenopus laevis    STLGVAQIDSVI IAPPPLEDGR-----SVSLENLQPYWEELELVRDAKVVIGTS 164
Rana catesbeiana  STLAVGQLDSVIMASPPIEDGV-----ILTLEHLQPYWEELESVLQDNKVVIGTS 162
Danio rerio       LSLGVSQQLDSVIMASPPLEDGE-----AQTLLHLQPLWQELLESVLQSKIAAIGTS 163
Salmo salar       QALAVSGLDSVIMASPPALPEEE-----SQTLANLQPAWRELEGLVQSHKIATIGTS 165
Branchiostoma belcheri NELDIGVVDVSLVLLAPPLEAEMG-----EELTVNHLPMWPEMERLYDVERVSAIGTS 169
Botryllus schlosseri KTLGVXYLDSAVVFXFPXXPEAY-----VEGSFSKALSVMREAMEAMTKGLVKHIGVT 164
Brugia malayi     HEINTKISISQLIIAFPPDDKLDIDPSVPTVEEWLSHILPFWTQLETLVTRTHKINTLQVA 174
Loa loa           REINTKISISQLIIAFPPDDKLDIDLSVPSEVQRWLSHILPFWKQLETLVTRTHKINTLQVA 174
Ascaris suum      SLLGVESIEQLIMSFN-NFEPESDSEDKELKNWVENVISVWEKIEALVKNGEISTVQVA 159

```

```

      : .. : : . . * : : : : * :

```

```

Rattus norvegicus  DLDKTQLEQLYQW-AQVKPNSQVNLASCCVMPDLTAFQKQFDIQLLTHNDPKELLSEA 224
Mus musculus      DLDKTQLEQLYQW-AQVKPNSQVNLASCCVMPDLTAFQKQFDIQLLTHNDPKELLSEA 224
Bos taurus        DLDKTQLEQLYQW-AQVKPNSQVNLASCCVMPDLTAFQKQFDIQLLTHNDPKELLSEA 224
Homo sapiens      DLDKTQLEQLYQW-AQVKPNSQVNLASCCVMPDLTAFQKQFDIQLLTHNDPKELLSEA 224
Gallus gallus     DLDKTLEQLYQW-AQVKPNSQVNLASCCVMPDLTAFQKQFDIQLLTHNDPKELLSEA 221
Xenopus tropicalis DLKALLEQLYLW-SQVKPASQVNLASCCIMPPDLTEFAKQFDIQLLTHNDPKELLSEE 223
Xenopus laevis    DLKALLEQLYLW-SQVKPASQVNLASCCIMPPDLTEFAKQFDIQLLTHNDPKELLSEE 223
Rana catesbeiana  DLDKPALLEQLYLW-AQIKPSSQVNLASCCIMPPDLTAFQKQFDIQLLTHSDSKELISEE 221
Danio rerio       DLDKTLEQLYNW-AQIKPSSQVNLASCCVMPDLTAFQKQFDIQLLTHSDPKELISAA 222
Salmo salar       DLDKELLEQLYNW-AQVKPSSQVNLASCCVMPDLTAFQKQFDIQLLTHNDPKELITAA 224
Branchiostoma belcheri DLDKEMLEQTHGM-ARVKPTINQVNVSCCVIPPELTAFQKQFDIQLLTHSDPRDVLPTP 228
Botryllus schlosseri DFDKTAQNLYRC-ATVKPTIDQIKTGHDFNVDDLIKLSSEYDIELSHDDAADMLPEK 223
Brugia malayi     DLQYEQKALYESTNDHRPMIDHYSTEHCCTVPELREYAKQKDIQLLTHNDPNLHISNE 233
Loa loa           DFDYEQKALYESTDDHRPMIDHYSTEHCCTVPELREYAKQKDIQLLTHNDPN-LYSIS 233
Ascaris suum      DFLDNHLKALYDG-AEIKPRIAHFNIAAGCCSVPKDLQDYARENDIQLLTHNDPK-PFVTA 217

```

```

* : * : : : * : . : * : * : : : * : * : * : * :

```

```

Rattus norvegicus  SFQEALQESIP-DIEAQEWVPLWLLRYSVIVKSRGIKSKGYILQAKRKG----- 274
Mus musculus      SFQEALQESIP-DIEAQDWVPLWLLRYSVIVKSRGIKSKGYILQAKRRG----- 274
Bos taurus        SFQEALQESIP-DIRAHEWVPLWLLRYSVIVKSRGIKSKGYILQAKRKG----- 274
Homo sapiens      SFQEALQESIP-DIQAHEWVPLWLLRYSVIVKSRGIKSKGYILQAKRRG----- 274
Gallus gallus     SFQEVLQESIQ-NVKAHEWVPLWILRYSVIVKSRGIKSKGYIMQAKRNAS----- 271
Xenopus tropicalis AFQEALKESAQ-ECHSSAWSPIWLLRYSVIVKTRGIIKLGKGYILQAQRKGS----- 274
Xenopus laevis    DFQEALKESVP-ECHFNWSPWVWVLRYSVIVKTRGIIKLGKGYILQAKRKG----- 274
Rana catesbeiana  SFQEVLRSVVE-GSDAWEVSPVWVLRYSVIVKTRGIIKSKGYIVQAKRKA----- 272
Danio rerio       GFQEAVQGSQ-ELQVDDWLEWVLRYSIIVKSRGIKAKGYIVHAKKSNH----- 273
Salmo salar       SFQEAVQESTQ-DLKVADWLEWVLRYSIIVKSRGIKAKGYIVHAKKSS----- 274
Branchiostoma belcheri TFQEILRGSSH-DDHVDEWQPFVWVLRYSVIVKTRGIIKAKGYIVNGRRHATDMLPSVA-- 285
Botryllus schlosseri SFQAIMEDNPNSTASAEWLPYSYAVRYSAIARCRGIVRSKGYIIVEAGKRLSNGYTTVTG 283
Brugia malayi     ERLDATT---RKLFGNEHFDLLFIARLTVWLRYSIIVKSGYIILKFMKRKIS----- 281
Loa loa           ERLDATT---RKLFGNEHFDLLFIARLTVWLRYSIIVKSGYIILKFLRKI----- 280
Ascaris suum      DGLKIDICNNEKYLPCDHDYKPSWASRYTVVWVGRSIIAAGKGMVQFERS----- 266

```

```

      : .

```

<i>Rattus norvegicus</i>	-----
<i>Mus musculus</i>	-----
<i>Bos taurus</i>	-----
<i>Homo sapiens</i>	-----
<i>Gallus gallus</i>	-----
<i>Xenopus tropicalis</i>	-----
<i>Xenopus laevis</i>	-----
<i>Rana catesbeiana</i>	-----
<i>Danio rerio</i>	-----
<i>Salmo salar</i>	-----
<i>Branchiostoma belcheri</i>	-----
<i>Botryllus schlosseri</i>	GEGYID 289
<i>Brugia malayi</i>	-----
<i>Loa loa</i>	-----
<i>Ascaris suum</i>	-----



Homo sapiens -IAVVYFRDGYMPRQYS-LQNWEARLLERSHAAKCPDIATQLAGTKKVVQELSRPGMLE 318  
Macaca fascicularis -IAVVYFRDGYMPRQYS-LQNWEARLLERSCAAKCPDIATQLAGTKKVVQELSRPGMLE 318  
Callithrix jacchus -IAVVYFRDGYMPCQYS-LQNWEARLLERSCAVKCPDIATQLAGTKKVVQELSRAGMLE 318  
Bos taurus -IAVVYFRDGYMPGHYS-LQNWEARLLERSCAVKCPDIATQLAGTKKVVQELSRVGLLE 318  
Rattus norvegicus -VAVVYFRDGYMPSQYN-AQNWEARLLERSCAAKCPDIATQLAGTKKVVQELSRVGLLE 318  
Xenopus laevis -VAVYFRDGYVPQDYT-EQDWEARLMLERSRAVKCPDVPTQLVGTKKVVQELSRPQILE 318  
Xenopus tropicalis -IAVYFRDGYVPQDYT-EQDWEARLMLERSRATKCPDIATQLVGTKKIQQELCRPHILE 318  
Danio rerio -VAIVYFRNGYMPQNYTSEQSWEVRLMERSVAVKCPDISTHLAGTKKVVQELARPGVLE 319  
Branchiostoma floridae -VAVYFRAGYIPADYPTQEWARTARLKI EQSKAIKPCISYHLAGTKKVVQELAQPGVLE 337  
Botryllus schlosseri -VGFVYFRAGYS PDHYFSEKEWDARLLETSTAINCPVAHQVLGAKKMQIILSQPNMVE 322  
Brugia malayi RVAVVYFRAGYSPSNYPTMEWARTARRIELSDAVKCPWIGLQLANTKKVQVLSSENGVLE 339  
Loa loa RVAVVYFRAGYSPNNYPTAEWARTARRIELSDAVKCPWIGLQLANTKKIQQVLSSENGVLE 339  
Ascaris suum RVSIVYFRAGYSPDNYCSELEWARTMELSNVAVKCPWIGLQLANTKKVQVLAACDQGLE 344  
:..\*:\* \*\* \* .\* . \* . \* : \* \* \* : \* : \* : \* : \* : \* : \*

Homo sapiens MLLPGQPEAVARLRATFAGLYSLDVGEE-GDQAI AEALAAPSRFVLPQREGGNNLYGE 377  
Macaca fascicularis MLLPGQPEAVARLRATFAGLYSLDMGEE-GDQAI AKALAAPSRFVLPQREGGNNLYGE 377  
Callithrix jacchus MLLPGQPEAVARLRATFAGLYSLNMGEE-GDQAI AEALAAPSRFVLPQREGGNNLYGE 377  
Bos taurus SFLPGQPEAVARLRATFAGLYSLDLGEE-GDQAI TKAIAAPSCFVLPQREGGNNLYGE 377  
Rattus norvegicus ALLPGQPEAVARLRATFAGLYSLDMGEE-GDQVAEALAAPSHFVLPQREGGNNFYGE 377  
Xenopus laevis KFLLPDQPEAVARIRETFTGLYSLDIGEE-GDEAVRVALANPDQFVLPQREGGNNLYGE 377  
Xenopus tropicalis KFLLPDNPEAVARIRETFTAGLYSLDTGEE-GDEAVKAALANPDQFVLPQREGGNNLYGE 377  
Danio rerio CFFPDEPETVVSQIRATFAGLYTLDGEE-GDNTVAMALANPDQYVLPQREGGNNIYGS 378  
Branchiostoma floridae KFLLKD-AEAVKVRATFAGQYTLLELGAE-GDRTVQMVTSDPGRFVMPQREGGNNIFGE 395  
Botryllus schlosseri RFIKD-RNSVKRIRDTFVGFYGLEMGST-GDESIQKVLQHPENYVLPQREGGNNLYND 380  
Brugia malayi KYITDDKM-CARIRQTFAGMWGLENDDEKQRI IQDAIAHPEKFLVLPQREGGNNIYK 398  
Loa loa RYITDGRM-STRIRKTFAGMWGLENDDRQKIIQDAVTHPEKFLVLPQREGGNNIYK 398  
Ascaris suum RFLPELKDCERIRATFAGLWGLESDDEETQIILKEAIEYPERFVLPQREGGNNIYGS 404  
: \* \*\* \* : \* : . : : . \* : \* : \* : \* : \* : \* : \* : \* : \*

Homo sapiens EMVQALKQLKDSERASYILMEKIEPEPFENCLLRPGSPARVVQCISELGIFGVYVRQ-- 435  
Macaca fascicularis EMVQALKQLKDSERASYILMEKTEPEPFENCLLRPGSPAQVVQCISELGIFGVYVRH-- 435  
Callithrix jacchus EMVQALKQLKDSERASYILMEKIEPEPFENCLLRPGSPVQVVQCISELGIFGVYVRQ-- 435  
Bos taurus EMVQALERLKDSEERASYILMEKIEPEPFNRCLLRPGSPARVIQCISELGIFGVYVRE-- 435  
Rattus norvegicus EMVHALEQLKDSERASYILMEKIEPEPFNRCLLRPGSPAQVVQCISELGIFGVYVRQ-- 435  
Xenopus laevis ELKEKLECKDSEERTSYILMDKINPKPLKNCLLRAGGRVQISECISELGMFGVYVRH-- 435  
Xenopus tropicalis ELKAKLECKDSEERNYSIILMDKINPKTKNCLLRAGGPVQISECISELGMFGVYVRR-- 435  
Danio rerio EICEVLEKLNSSERTAYILMDKI QPVPVQNI LLRPGAPLKVSSCLSELGAFGAYVRK-- 436  
Branchiostoma floridae DIPALNNMADV KERTAYIVMDRIRPAVVSNYAVRPGREPALTEAVSELGIFGVFIK-- 453  
Botryllus schlosseri EMVAKLKEVGEDDRCSYIVMEKY-----VRCQCSLSSYGPQKQAT-- 420  
Brugia malayi EVAEKLKTMNR-DEMAAYIIMERI TPMVVKNYVIRPQEEPLMDVVGELGVYAYLYGSAA 457  
Loa loa EVAEKLKTMNR-DEMAAYIIMERI TPMVVKNYVIRPQEPVLMVVGELGIYAYLYGSPA 457  
Ascaris suum EVAEKLKMSR-DERAAHILMERIQPMRVKNYLVRPFEEVTLGEEVVGELGIYGLYAEPA 463  
: \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \*

Homo sapiens -----EKTLMNKHVGHLLT KAIEHADGGVAAGVAVLDNPYPV 474  
Macaca fascicularis -----EKTLMNKHVGHLLT KAIEHADGGVAAGVAVLDNPYPV 474  
Callithrix jacchus -----GKTLVMNKHVGHLLT KAIEHADGGVAAGVAVLDNPYPV 474  
Bos taurus -----GKTLVMNKHVGHLLT KAIEHADGGVAAGVAVLDNPYPV 474  
Rattus norvegicus -----GTTLMNKHVGHLLT KAIEHADGGVAAGVAVLDNPYPV 474  
Xenopus laevis -----RDQMIYYDQVGHLLT KAIEHSDGGVAAGVAVLDNPYLV 474  
Xenopus tropicalis -----SGEMIYNEQVGHLLT KAIEHADGGVAAGVAVLDNPYLV 474  
Danio rerio -----GSELVNLNECVGHLLT KSSEHADGGVAAGVAVLDNPLL 475  
Branchiostoma floridae -----GDEVILNQEGGHLLT KPSSTEDGGVAAGNALLDSPYLI 492  
Botryllus schlosseri -----TNQLL----- 425  
Brugia malayi VDNIIIVENIMKNHVSGHIIISKDKSVDKGGVAIGAVVIDSPYLF 501  
Loa loa VDYIPAENVITNYVSGHIIISKDKNVDKGGVAIGAVVIDSPYLF 501  
Ascaris suum FDRG-CEKVYKNLAHGHIISKAANVDKGGVAVGAVVIDSPFLF 506  
: :

**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.**

```

Bos taurus_GPx6      MILQLWASCLFPLFLVGLAQLTPKQQQMKVDCYKGVGTGTIYEYGALTLNGEEYIQFKQYV 60
Sus scrofa_GPx6      MTPQFWASCLFSLCLVGFQAQLIPKEQKMKMDCYKGVGTGTIYEYGALTLNGEEYIPFKQYA 60
Equus caballus_GPx6  MIRQLWASSLFPFLFLVGFQAQLTPESQKMKMDCYKGVGTGTIYEYGALTLNGEEYIQFKQYA 60
Homo sapiens_GPx6    MFQQFQASCLVLLFLVGFQAQLTPKQNRKVDNCKGVGTGTIYEYGALTLNGEEYIQFKQYA 60
Pan troglodytes_GPx6 MFRQFQASCLVLLFLVGFQAQLILKPNRNVDCNKGVTGTIYEYGALTLNGEEYIQFKQYA 60
Mus musculus_GPx5    MVTELRVFLVPLLLASVYQTTTPRPEKMKMDCYKDVKGTTIYDEYALSLNGKEHIPFKQYA 60
Bos taurus_GPx5      MTIQLRASCLFLLFLLAGFVQTNLSNLE--KMDCYKDVKGTTIYDYDAFTLNGKEHIPFKQYA 58
Canis lupus_GPx3     MARLLRASCLLSLLLAGFVPPSRGQEKSTDCCHGGVSGTIYEYGALTLNGEEYIPFKQYA 60
Pongo abelii_GPx3    MARLLQASCLLSLLLAGFVPPSRGQEKSKMDCHGGISGTIYEYGALTLNGEEYIPFKQYA 60
Sus scrofa_GPx3      MARFFRASCLISLLLAGFLLPSPRGQEKSTDCYRGSVGTIYEYGALTLNGEEYIPFKQYA 60
Gallus gallus_GPx3   --MGCRAACVLAVLLAGLVPLGQQQERKVKCYDSVRGTIYDYGALTLNGEEYIPFKYA 58
Xenopus tropicalis_GPx3 MGVKLRGLLMLPCFLAALIAHQNEMDQKSVDCYSSIDGTIYDYGATTLTDLGTQFIPFKAYQ 60
Xenopus laevis_GPx3  MGVKFRGLLMLPCILAAFIHAQTVDVQKSVDCYSSADGSIYDYGATTLTDLGSDQFIPFKAYQ 60
Danio rerio_GPx3     MGTQSNPWTSVVVLLLA--LMHKIAALSNTQACNSAAGDSFHNYGAKTINGTQFIPFSHYA 58
Perca flavescens_GPx3 -----
Branchiostoma floridae_GPx MNGPAHHGHGRSLSHSLHHAAPHPIELNTPCLCPASVVGSSFFELSABEALGG-EMIPFSTY 59
Ciona intestinalis_GPx c -----MHQKGAVPALLVLLVGLTAVATSIENSTCFHNTFSVYDHSFPTLSG-ETVNLGEFR 54
Botryllus schlosseri_GPx5 -----MLWALLVASAALVAADDASQCLSQGLTGTVYDSTEATLAG-TNVDLTQLF 49
Ciona intestinalis_GPx b -----MRSVLLWLVGAVGVVHSMVPCYNSTN-YSVYSNQVFNLHK-QVNVLSRFH 48

Bos taurus_GPx6      GKHVLFVNVATYCG-LTAQYPELNALQEEELKPFVGVVLLGFPCNDFGKQEPFKNSEILMGL 119
Sus scrofa_GPx6      GKHVLFVNVATYCG-LTAQYPELNALQEEELKPFVGVVLLGFPCNDFGKQEPFKNSEILMGL 119
Equus caballus_GPx6  GKHVLFVNVATYCG-LTAQYPELNALQEEELKPFVGVVLLGFPCNDFGKQEPFKNSEILMGL 119
Homo sapiens_GPx6    GKHVLFVNVATYCG-LAAQYPELNALQEEELKPFVGVVLLGFPCNDFGKQEPFKNSEILMGL 119
Pan troglodytes_GPx6 GKHVLFVNVATYCG-LTAQYPELNALQEEELKPFVGVVLLGFPCNDFGKQEPFKNSEILMGL 119
Mus musculus_GPx5    GKHVLFVNVATYCG-LTIQYPELNALQEEELKPFVGVVLLGFPCNDFGKQEPFKNSEILMGL 119
Bos taurus_GPx5      GKHVLFVNVATYCG-LTAQYPELNALQEEELKPFVGVVLLGFPCNDFGKQEPFKNSEILMGL 117
Canis lupus_GPx3     GKHYLVFNVASYCG-LTGQYIELNALQEEELAPFGLVILGFPCNDFGKQEPFKNSEILMGL 119
Pongo abelii_GPx3    GKHYLVFNVASYCG-LTGQYIELNALQEEELAPFGLVILGFPCNDFGKQEPFKNSEILMGL 119
Sus scrofa_GPx3      GKHYLVFNVASYCG-LTGQYVELNALQEEELAPFGLVILGFPCNDFGKQEPFKNSEILMGL 119
Gallus gallus_GPx3   GKMYLVFNVASYCG-LTFQYVELNALQEEELAPFGLVILGFPCNDFGKQEPFKNSEILMGL 117
Xenopus tropicalis_GPx3 GKHYLVFNVASYCG-LTMQYVELNALQEEELKNNFVILGFPCNDFGKQEPFKNSEILMGL 119
Xenopus laevis_GPx3  GKHYLVFNVASYCG-LTMQYQEMNALHEELKSNDFVILGFPCNDFGKQEPFKNSEILMGL 119
Danio rerio_GPx3     GKHYLVFNVASYCG-LTFQYVELNALHEELRHLGFTILGFPCNDFGKQEPFKNSEILMGL 117
Perca flavescens_GPx3 -----MKPLGLTLLGFPCNDFGKQEPFKNSEILMGL 31
Branchiostoma floridae_GPx3 GKVVLVNVSASEHTTREYLQNLNDLVATYGPKGLVILGFPCNDFGKQEPFKNSEILMGL 119
Ciona intestinalis_GPx c GNVSMVNVATYCG-GATVPEYKAMNALSEEYQSSFTLAFPCNDFGKQEPFKNSEILMGL 114
Botryllus schlosseri_GPx5 GYVSVVMNPGVYSEWTSQMMTGMNTLLEKYESDGVVGLGFPCNDFGKQEPFKNSEILMGL 109
Ciona intestinalis_GPx b NEVTLINVATYCG-LTVAQYTLGNALLTHFNGRNFSVLAFFPCNDFGKQEPFKNSEILMGL 108
..  * . : * * : *

Bos taurus_GPx6      KYVRPGGGFVFN--FQLFEKGDVNGEKEQKVFTFLKNACPPTS---LLGSSS-QLFWEP 173
Sus scrofa_GPx6      KYVRPGGGFVFN--FQLFEKGDVNGEKEQKVFTFLKNACPPTS---LLGSSS-QLFWEP 173
Equus caballus_GPx6  KYVRPGGGFVFN--FQLFEKGDVNGEKEQKVFTFLKNACPPTS---LLGSPK-QLFWEP 173
Homo sapiens_GPx6    KYVCPGSGFVFN--FQLFEKGDVNGEKEQKVFTFLKNACPPTS---LLGSSS-QLFWEP 173
Pan troglodytes_GPx6 KYVCPGSGFVFN--FQLFEKGDVNGEKEQKVFTFLKNACPPTS---LLGSSS-QLFWEP 173
Mus musculus_GPx5    KYVRPCKGFLPN--FQLFAKGDVNGEKEQKVFTFLKRSCHPSPS---TVVMSK-HTFWEP 173
Bos taurus_GPx5      KYVRPGGGFVFN--FQLFKKGDVNGEKEQKVFTFLKQSCPHPS-----WEP 161
Canis lupus_GPx3     KYVRPGGGFVFN--FQLFEKGDVNGEKEQKVFTFLKNACPPTS---LLGSPG-RLFWEF 173
Pongo abelii_GPx3    KYVRPGGGFVFN--FQLFEKGDVNGEKEQKVFTFLKNACPPTS---LLGTS-RLFWEF 173
Sus scrofa_GPx3      KYVRPGGGFVFN--FQLFEKGDVNGEKEQKVFTFLKNACPPTS---LLGSPS-RLFWEF 173
Gallus gallus_GPx3   KYVRPGGGFVFN--FQLFKKGDVNGEKEQKVFTFLKNACPPTS---LLGSPG-RLFWEF 171
Xenopus tropicalis_GPx3 EYVRPCKGFLPN--FQLFEKGDVNGEKEQKVFTFLKNACPPTS---LLGSPG-RLFWEF 174
Xenopus laevis_GPx3  RYVRPCKGFLPN--FQLFEKGDVNGEKEQKVFTFLKNACPPTS---LLGSPG-RLFWEF 174
Danio rerio_GPx3     KYVRPCKGFLPN--FQLFEKGDVNGEKEQKVFTFLKNACPPTS---LLGSPG-RLFWEF 172
Perca flavescens_GPx3 KHVRPCKGFLPN--FLLFEKGDVNGEKEQKVFTFLKNACPPTS---VLLGNT-RLFWEF 85
Branchiostoma floridae_GPx RAVRPGNRYSPN--FQLFSKVDINGKHGHIYVEYLKLLKLPFPPSDNAATMAQEHLDICWSP 177
Ciona intestinalis_GPx c MYVRPCKGFLPNKKIYFSSKTVNGGSEDLFTSFKASCPPTS---NIGITS-ELYWTP 170
Botryllus schlosseri_GPx5 KYLRPCKGFLPNKQNFHLFTVTEVNGDTASDIFKFLRSACPPTS---ELGSKA-SFYWDT 165
Ciona intestinalis_GPx b MYVRPCKGFLPNKIKVNGRHEHTIYKVKASCPPTS---NLGSTR-NMYWNP 164
: * * : * : * : * : * : * : * : *

Bos taurus_GPx6      MKVHDIRNFEKFLVGPDPGVPMRWYHRASVSTVKSMDLEYLQFKSE----- 221
Sus scrofa_GPx6      MKVHDIRNFEKFLVGPDPGVPMRWYHRASVSTVKSMDIMEYLKQFKSE----- 221
Equus caballus_GPx6  MKVHDIRNFEKFLVGPDPGVPMRWYHRASVSTVKSMDILEYLKQFTPE----- 221
Homo sapiens_GPx6    MKVHDIRNFEKFLVGPDPGVPMRWYHRASVSTVKSMDILEYLKQFNTH----- 221
Pan troglodytes_GPx6 MKVHDIRNFEKFLVGPDPGVPMRWYHRASVSTVKSMDILEYLKQFNTH----- 221
Mus musculus_GPx5    IKVHDIRNFEKFLVGPDPGVPMRWYHRASVSTVKSMDIMAYLSHFHTI----- 221
Bos taurus_GPx5      IMVRDIRNFEKFLVGPDPGVPMRWYHRASVSTVKSMDILEYLKQFKTK----- 209
Canis lupus_GPx3     MKVHDIRNFEKFLVGPDPGIPIMRWYHRASVSTVKSMDILEYLKQFKTK----- 226
Pongo abelii_GPx3    MKVHDIRNFEKFLVGPDPGIPIMRWYHRASVSTVKSMDILEYLKQFKTK----- 226
Sus scrofa_GPx3      MKVHDIRNFEKFLVGPDPGVPMRWYHRASVSTVKSMDILEYLKQFKTK----- 226
Gallus gallus_GPx3   LRNHDIRNFEKFLVGPDPGVPMRWYHRASVSTVKSMDILEYLKQFKTK----- 219
Xenopus tropicalis_GPx3 IKVNDVKNFEKFLVGPDPGVPMRWYHRASVSTVKSMDILEYLKQFKTK----- 233
Xenopus laevis_GPx3  LRVNDIKNFEKFLVGPDPGRVAVKRWHPRTSVAQVRRREIVSYIKLQOQTQRLMLGLEQK- 233
Danio rerio_GPx3     LKVNDIRNFEKFLVGPDPGVPMRWYHRASVSTVKSMDILEYLKQFKTK----- 222
Perca flavescens_GPx3 VKLSDIKNFEKFLVGPDPGVPMRWYHRASVSTVKSMDILEYLKQFKTK----- 137
Branchiostoma floridae_GPx VRRTDVSNGNFEKFLVADSQPYRRYSWRAPEDLCKDIEELLKRVTRTNRQNGTAKND 237
Ciona intestinalis_GPx c IKANDIYNWNKFLLDKNGMIRYRFSAVTATQLKPWIDQLLNEK----- 215
Botryllus schlosseri_GPx5 LVARDLRGTYEKFLVDKNGKPRYRFPANVAMTEIYPIYIDELLAETPIQPTQFPFVEAGN- 224
Ciona intestinalis_GPx b VKSTDITNFNKFLLDKNGVPRYRISSDASPTSLIPYITMLSE----- 208
: * : * : * : * : * : * : * : * : *

```

<i>Bos taurus</i> _GPx6	-----	
<i>Sus scrofa</i> _GPx6	-----	
<i>Equus caballus</i> _GPx6	-----	
<i>Homo sapiens</i> _GPx6	-----	
<i>Pan troglodytes</i> _GPx6	-----	
<i>Mus musculus</i> _GPx5	-----	
<i>Bos taurus</i> _GPx5	-----	
<i>Canis lupus</i> _GPx3	-----	
<i>Pongo abelii</i> _GPx3	-----	
<i>Sus scrofa</i> _GPx3	-----	
<i>Gallus gallus</i> _GPx3	-----	
<i>Xenopus tropicalis</i> _GPx3	-----	
<i>Xenopus laevis</i> _GPx3	-----	
<i>Danio rerio</i> _GPx3	-----	
<i>Perca flavescens</i> _GPx3	-----	
<i>Branchiostoma floridae</i> _GPx	RPVLFNSKKYMQESITRSTVRDDAAY	263
<i>Ciona intestinalis</i> _GPx c	-----	
<i>Botryllus schlosseri</i> _GPx5	-----	
<i>Ciona intestinalis</i> _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          MGTQSNPWTSVVLALLA--LMHKIAALSNTQACNSAAGDSFHNYGAKTINGTQFIPFISHYA 58
Perca flavescens_GPx3   -----
Xenopus tropicalis_GPx3 MGVKLRGLLMLPCFLAALIAHQNEMDQKSVDCYSSIDGTIYDYGATTLDTGTFIPFKAYQ 60
Xenopus laevis_GPx3    MGVKFRGLLMLPCILAAFIHAQTVDVQKSVDCYSSADGSIYDYGATTLDTGTSQFIPFKAYQ 60
Canis lupus_GPx3       MARLLRASCLLSLLLAGFVPPSRGQEKSKTDCHGGVSGTIYEGALTINGEEYIPFKQYA 60
Pongo abelii_GPx3      MARLLQASCLLSLLLAGFVPPSRGQEKSKMDCHGGISGTIYEGALTINGEEYIPFKQYA 60
Sus scrofa_GPx3        MARFFRASCLISLLLAGFLLPSPRGQEKSKTDCYRGSVSGTIYEGALTINGEEYIPFKQYA 60
Bos Taurus_GPx6        MILQLWASCLFLPLFLVGLAQLTPKQQQMKVDCYKGVGTGTIYEGALTINGEEYIQFKQYV 60
Sus scrofa_GPx6        MTPQFWASCLFSLCLVGFQAQLIPKEQKMKMDCYKGVGTGTIYEGALTINGEEYIPFKQYA 60
Equus caballus_GPx6    MIRQLWASSLFLPLFLVGFQAQLTPESQKMKMDCYKGVGTGTIYEGALTINGEEYIQFKQYA 60
Homo sapiens_GPx6      MFQQFQASCLVLLFLVGFQAQQTLKPNRKRVDNKGVTGTIYEGALTINGEEYIQFKQYA 60
Pan troglodytes_GPx6   MFRQFQASCLVLLFLVGFQAQQTLKPNRKRVDNKGVTGTIYEGALTINGEEYIQFKQYA 60
Mus musculus_GPx5      MVTELRVFLVPLLLASVYQVTPRPEKMKMDCYKDVKGTIYDEALSLNGKEHIPFKQYA 60
Bos taurus_GPx5        MTIQLRASCLFLFFLAGFVPPSRGQEKSKTDCYRGSVSGTIYEGALTINGEEYIQFKQYA 58
Gallus gallus_GPx3     --MGCRAACVLAVLLAGLVLGQGEREKVKCYDSVRGTIYDYGALTINGEEYIPFKQYA 58
Branchiostoma floridae_GPx -----MVTDNG----- 6
Ciona intestinalis_GPx b -----MRSVLLWLG-VAGVVHSMVP--CYNSTNYSVYSNQVFNLHKQN-VNLSRFH 48
Ciona intestinalis_GPx c ---MHQKGAVPVLLVGLG-TLAVATSIENSTCFHNT-FSVYDHSFPTLSGET-VNLGEFR 54
Botryllus schlosseri_GPx3 -----MVTMKNVPLLLCSWISAIHALKVVSTCRYSE-KATIEYVPVTKLDGSP-FTLSSLQ 53

```

```

Danio rerio_GPx3          GKHVLFVNVATYGLTF-QYVELNALHEELRHLGFTILGFPCDIFGKQEPGENNEILSAL 117
Perca flavescens_GPx3   -----MKPLGLTLLGFPCNDFGKQEPGTNHEILPGL 31
Xenopus tropicalis_GPx3 GKYLFLVNVATYGLTM-QYQELNALQEEELKNNNFVILGFPSNDFGKQEPGRNDEILLGL 119
Xenopus laevis_GPx3    GKYLFLVNVATYGLTM-QYQEMNALHEELKNSDNFVILGFPCNDFGKQEPGRNDEIPLGL 119
Canis lupus_GPx3       GKYLFLVNVASYGLTG-QYLELNALQEEELAPFGLVILGFPCNDFGKQEPGENSEILPSL 119
Pongo abelii_GPx3      GKYLFLVNVASYGLTG-QYIELNALQEEELAPFGLVILGFPCNDFGKQEPGENSEILPTL 119
Sus scrofa_GPx3        GKYLFLVNVASYGLTG-QYVELNALQEEFEFGLVILGFPCNDFGKQEPDENSEILSTL 119
Bos taurus_GPx6        GKHVLFVNVATYGLTA-QYPELNALQEEELKPFVGVVVLGFPCNDFGKQEPAKNSEILLGL 119
Sus scrofa_GPx6        GKHVLFVNVATYGLTA-QYPELNALQEEELKPFVGVVVLGFPCNDFGKQEPAKNSEILLGL 119
Equus caballus_GPx6    GKHVLFVNVATYGLTA-QYPELNALQEEELKPFVGVVVLGFPCNDFGKQEPKNSSEILSG 119
Homo sapiens_GPx6      GKHVLFVNVAAAYGLAA-QYPELNALQEEELKPFVGVVLAFFPCNDFGKQEPGTNSEILLGL 119
Pan troglodytes_GPx6   GKHVLFVNVATYGLAA-QYPELNALQEEELKPFVGVVLAFFPCNDFGKQEPGTNSEILLGL 119
Mus musculus_GPx5      GKHVLFVNVATYGLTI-QYPELNALQEEELKPFVGVVVLGFPCNDFGKQEPDNLLEILLGL 119
Bos taurus_GPx5        GKHVLFVNVATYGLTA-QYPELNALQEEELKPFVGVVVLGFPCNDFGKQEPGENSEILLGL 117
Gallus gallus_GPx3     GKMLVLFVNVATYGLTL-QYLELNALQEEELKPFVGVVVLGFPCNDFGKQEPQNSEILLGL 117
Branchiostoma floridae_GPx -----HREILGFPTNDFGLQEPAPNSKILDCV 34
Ciona intestinalis_GPx b NEVTLINVATYGLTVAQYTGILNALLTHFNGRNFSVLAFFPCDIFHLEEPGEDSEILNGL 108
Ciona intestinalis_GPx c GNVSMVINVATYGLATVPQYKAMNALSEEYTOSSFVTLAFLPCNDFGLQEPANDEILNGV 114
Botryllus schlosseri_GPx3 DKVAVIINTACFGETWTQLPGMNALLQKYSKQGVVASGFPCDIFELQEPGLPREILPCY 113

```

```

Danio rerio_GPx3          KYVRPGNGFVFN--FQLFEKGDVNGDGEQALFTFLKNACPPVGESFGATSNRLFWEPLKV 175
Perca flavescens_GPx3   KHVRPGNGFVFN--FLLFEKGDVNGKDEQEVFTFLKNACPPVGDVVLGNPT-RMFWDPVKL 88
Xenopus tropicalis_GPx3 EYVRPGGKFVFN--FQLFEKGDINGRKEQKQFYTFLLKNSCPPVGDNFGSATNRLMWFPIKV 177
Xenopus laevis_GPx3    KYVRPGGFIIPN--FQLFEKGDVNGRKEQKQFYTFLLKNSCPPVGDTFGNPAFRNLWEPFLRV 177
Canis lupus_GPx3       KYVRPGGGFVFN--FQLFEKGDVNGEKEQKQFYTFLLKNSCPPTSELLGSPG-RLFWEPMKV 176
Pongo abelii_GPx3      KYVRPGGGFVFN--FQLFEKGDVNGEKEQKQFYTFLLKNSCPPTSELLGTS-RLFWEPMKV 176
Sus scrofa_GPx3        RYVRPGGGFIIPN--FQLFEKGDVNGEKEQKQFYTFLLKNSCPPTSELLGSPS-RLFWEPMKV 176
Bos taurus_GPx6        KYVRPGGGFVFN--FQLFEKGDVNGEKEQKQFYTFLLKNSCPPTSDLLGSSS-QLFWEPMKV 176
Sus scrofa_GPx6        KYVRPGGGFVFN--FQLFEKGDVNGEKEQKQFYTFLLKNSCPPTSDLLGSSN-QLFWEPMKV 176
Equus caballus_GPx6    KYVRPGGGFVFN--FQLFEKGDVNGEKEQKQFYTFLLKNSCPPTSDLLGSPK-QLFWEPMKV 176
Homo sapiens_GPx6      KYVCPGSGFVPS--FQLFEKGDVNGEKEQKQFYTFLLKNSCPPTSDLLGSSS-QLFWEPMKV 176
Pan troglodytes_GPx6   KYVCPGSGFVPS--FQLFEKGDVNGEKEQKQFYTFLLKNSCPPTSDLLGSSS-QLFWEPMKV 176
Mus musculus_GPx5      KYVRPGGKFLPN--FQLFEKGDVNGENEQKIFTFLLKRSCHPSETVVMMSK-HTFWEPPIKV 176
Bos taurus_GPx5        KYVRPGGGYVFN--FQLFKKGDVNGETEOKQVFTFLKQSCPHPS-----WEPIMV 164
Gallus gallus_GPx3     KYVRPGGGFVFN--FQLFQKGDVNGAKEQKQVYSFLKNSCPPVAEEFGNPK-NLFWEPFLRN 174
Branchiostoma floridae_GPx KHVNPNNGYVFN--FPMFQKADCNVNEQAFYTYMKSCCPAISDVFSKI-RLYWDFIKN 91
Ciona intestinalis_GPx b MYVRPGNGYVPHPKLNI FGKIKVNGRHEHTIYKNVKASCPTTLNLGSTR-NMYWNPVKS 167
Ciona intestinalis_GPx c MYVRPGHGFPVPHKNIYFFSKTQVNGGSEDFLTSIKASCPTTNIGITS-ELYWTFPIKA 173
Botryllus schlosseri_GPx3 KYVRPGKGIWIPHNFEHLNKTIVNGKDNLSYAHLLKSVCPQVTEIGTRS-EMYWDFPVK 172

```

```

Danio rerio_GPx3          NDIKLNFEKFLLDPDGPRVPMRWFPRVNVSEVRADILKYFHQLLQTAQ----- 222
Perca flavescens_GPx3   SDIKLNFEKFLVGPDGKPVPMRWHPSVNI SVVQADIRKYLQLYTTQQIFN----- 137
Xenopus tropicalis_GPx3 NDVKLNFEKFLVGPDGPRVPRKRWLPRTPVAQVREIMSYMKLQPGTQRLLMLGLEQK---- 233
Xenopus laevis_GPx3    NDIKLNFEKFLVGPDGRAVKRWHPRTSVAQVREIVSYIKLQQTQRLLMLGLEQK---- 233
Canis lupus_GPx3       HDIRLNFEKFLVGPDPGIPIMRWYHRTTVSTVKMDILAYMRRQAALAIKKG----- 226
Pongo abelii_GPx3      HDIRLNFEKFLVGPDPGIPIMRWYHRTTVSNVMDILSYMRRQAALGVKRR----- 226
Sus scrofa_GPx3        HDIRLNFEKFLVGPDPGVPVPMRWYHRTTINTVKLDILAYMRRRAALEAKRQ----- 226
Bos taurus_GPx6        HDIRLNFEKFLVGPDPGVPVPMRWYHRASVSTVKSMDILEYLKQFKSE----- 221
Sus scrofa_GPx6        HDIRLNFEKFLVGPDPGVPVPMRWYHRASVSTVKSMDIMEYLKQFKSE----- 221
Equus caballus_GPx6    HDIRLNFEKFLVGPDPGVPVPMRWYHRASVSTVKSMDILEYLKQFTPE----- 221
Homo sapiens_GPx6      HDIRLNFEKFLVGPDPGVPVPMRWYHRASVSTVKSMDILEYLKQFNTH----- 221
Pan troglodytes_GPx6   HDICLNFEKFLVGPDPGVPVPMRWYHRASVSTVKSMDILEYLKQFNTH----- 221
Mus musculus_GPx5      HDIRLNFEKFLVGPDPGVPVPMRWYHRASVSTVKSMDIMAYLSHFKTI----- 221
Bos taurus_GPx5        RDIRLNFEKFLVGPDPGIPIMRWYHRTTVSTVKTDLILAYMKQFKTK----- 209
Gallus gallus_GPx3     HDIKLNFEKFLVGTDPGVPVPMRWYHRANIATVKNDIIAYMRQQRGQ----- 219
Branchiostoma floridae_GPx TDIRLNFEKFLVDPAGKAVKRFSSVYTPGDLETVIDDFIKNWNDRDSDTSGARSSRNSG 151
Ciona intestinalis_GPx b TDITLNFNKFLLDKNGVPRYRISSDASPTSLIPYITMLSE----- 208
Ciona intestinalis_GPx c NDIIYNWNKFLLDKNGMIRYRFGSAVTATQLKFWIDQLLNEK----- 215
Botryllus schlosseri_GPx3 SDITLNFEKFLIDRKGKPRYRFGSPVLPSEIEPYIDSIL----- 211

```

```

*: **::***: * : . : : :

```

<i>Danio rerio</i> _GPx3	-----
<i>Perca flavescens</i> _GPx3	-----
<i>Xenopus tropicalis</i> _GPx3	-----
<i>Xenopus laevis</i> _GPx3	-----
<i>Canis lupus</i> _GPx3	-----
<i>Pongo abelii</i> _GPx3	-----
<i>Sus scrofa</i> _GPx3	-----
<i>Bos taurus</i> _GPx6	-----
<i>Sus scrofa</i> _GPx6	-----
<i>Equus caballus</i> _GPx6	-----
<i>Homo sapiens</i> _GPx6	-----
<i>Pan troglodytes</i> _GPx6	-----
<i>Mus musculus</i> _GPx5	-----
<i>Bos taurus</i> _GPx5	-----
<i>Gallus gallus</i> _GPx3	-----
<i>Branchiostoma floridae</i> _GPx	FLSWL 156
<i>Ciona intestinalis</i> _GPx b	-----
<i>Ciona intestinalis</i> _GPx c	-----
<i>Botryllus schlosseri</i> _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.

```

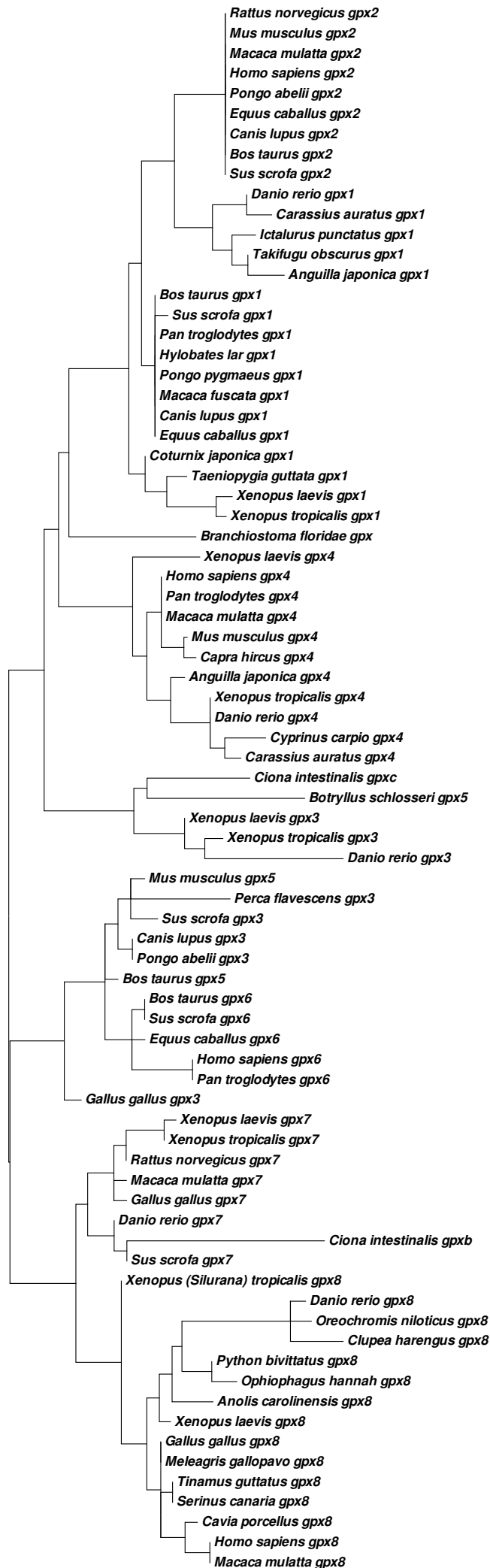
Ovis aries                -----MATKAVC-----VLKGDG-----PVQGTIRFEAK- 24
Capra hircus              -----MATKAVC-----VLKGDG-----PVQGTIHFEAK- 24
Bos taurus                -----MATKAVC-----VLKGDG-----PVQGTIHFEAK- 24
Bos grunniens            -----MATKAVC-----VLKGDG-----PVQGTIHFEAK- 24
Cervus elaphus           -----MATKAVC-----VMKGDG-----PVQGTIRFEAK- 24
Callithrix jacchus       -----MAMKAVC-----VLKGDG-----PVQGTINFEQKE 25
Macaca mulatta           -----MAMKAVC-----VLKGDG-----PVQGTINFEQKE 25
Hylobates lar            -----MAMKAVC-----VLKGDG-----PVQGTINFEQKE 25
Cavia porcellus          -----MATKAVC-----VLKGDG-----PVQGTIHFEQK- 24
Equus caballus           -----MALKAVC-----VLKGDG-----PVHGVIHFEQKQ 25
Gallus gallus            -----MATLKAVC-----VMKGDG-----PVEGVIHFEQKQ- 25
Melopsittacus undulatus  -----MATLKAVC-----VMKGDG-----PVQGTIHFEQKQ- 25
Caretta caretta         -----MATVAVC-----VLKGDG-----PVQGTINFEQKE 25
Rana catesbeiana        -----MKAIC-----VLKGDG-----EVTGVVRFEEQE 23
Bufo gargarizans        -----MVKAVC-----VLKGDG-----PVHGVVGFNQD- 23
Xenopus tropicalis      -----MVRVAVC-----VLKGDG-----DVKGVVHFQKQD 24
Xenopus laevis          -----MVKAVC-----VLKGDG-----DVKGVVHFQKQD 24
Wuchereria bancrofti    -----MSANAIA-----VLRGD-----NVSGIRFKQEK 24
Brugia malayi           -----MSANAIA-----VLRGD-----NVNGIRFKQEK 24
Loa loa                 -----MNAIA-----VLRGD-----TVSGIRFKQEK 22
Ascaris suum            -----MTRAVAVC-----VLRGD-----DVRGVVYLTQSK 25
Trichinella pseudospiralis -----MPFKAIC-----VIRGE-----NVITGVIFKQNT 24
Branchiostoma floridae  -----MSAVC-----NLEGD-----VSGTIRFVQE- 20
Botryllus schlosseri    -----MSAVC-----NLEGD-----VSGTIRFVQE- 20
Caenorhabditis briggsae MKNRVILVLA-LLACTEAAAS-----EVIRARAYIFKAVEGQIPTELIGTIDFDQS- 49
Caenorhabditis remanei  MKNRVILVLA-LFACIEAAS-----EVIRARAYIFKAVEGQIPTELIGTIDFDQS- 49
Caenorhabditis elegans  MKTRVVLILA-LSVCEAAS-----EVIRARAYIFKAEAGKIPTELIGTIDFDQS- 49
Dictyocaulus viviparus  MILHISLIISTILLGVHAHGNLCRNGAFMNVVKARAYMFEAVPDGDPQKLGIDFVQY- 59
                                : . : * : :

Ovis aries                -GDKVVVTGSIITGLTEGDHGFHVFQFGDNTQGCTSAAGPHFNPLSKKHGGPKDEERHVGDL 83
Capra hircus              -GDKVVVTGSIITGLTEGDHGFHVFQFGDNTQGCTSAAGPHFNPLSKKHGGPKDEERHVGDL 83
Bos taurus                -GDTVVVTGSIITGLTEGDHGFHVFQFGDNTQGCTSAAGPHFNPLSKKHGGPKDEERHVGDL 83
Bos grunniens            -GDTVVVTGSIITGLTEGDHGFHVFQFGDNTQGCTSAAGPHFNPLSKKHGGPKDEERHVGDL 83
Cervus elaphus           -GNTVVVTGSIITGLTEGDHGFHVFQFGDNTQGCTSAAGPHFNPLSKKHGGPKDEERHVGDL 83
Callithrix jacchus       SNGPVKVVWGSITGLAEGLHGFHVFQFGDNTQGCTSAAGPHFNPLSRKHGGPDEERHVGDL 85
Macaca mulatta           SNGPVKVVWGSITGLTEGLHGFHVFQFGDNTQGCTSAAGPHFNPLSRKHGGPDEERHVGDL 85
Hylobates lar            SNGPVKVYGRITGLTEGLHGFHVFQFGDNTQGCTSAAGPHFNPLSRKHGGPDEERHVGDL 85
Cavia porcellus          ANGPVVVKGRITGLVEGKHGFHVFQFGDNTQGCTSAAGPHFNPLSKKHGGPQDEERHVGDL 84
Equus caballus           EGGPVVLKGFIEGLTKGDHGFHVFQFGDNTQGCTTAGAHFNPLSKKHGGPKDEERHVGDL 85
Gallus gallus            GSGPVKVTGKITGLSDGDHGFHVFQFGDNTNGCTSAAGPHFNPEGKHGGPKDADRHVGDL 85
Melopsittacus undulatus  GNGPVKVTGKISGLADGDHGFHVFQFGDNTNGCTSAAGPHFNPEGKHGGPDAERHVGDL 85
Caretta caretta         GNGPVTLSGSIITGLTEGKHGFHVFQFGDNTNGCTSAAGPHFNPPGKNHGGPQDNERHVGDL 97
Rana catesbeiana        -DGPVTVTQITGLTDGKHGFHVFQFGDNTNGCTSAAGPHFNPPGKNHGGPQDNERHVGDL 82
Bufo gargarizans        -GGEVTVKGTINGLTDGLHGFHVFQFGDNTNGCTSAAGPHFNPHKSHGAPDEERHVGDL 82
Xenopus tropicalis      -EGPVTVEGKIYGLTDGKHGFHVFQFGDNTNGCTSAAGPHFNPEKSHGAPEDAVRHVGDL 83
Xenopus laevis          -EGAVSVEGKIEGLTDGLHGFHVFQFGDNTNGCTSAAGPHFNPEKSHGAPGDDRHRVGDL 83
Wuchereria bancrofti    EGLPTTISGEIKGLTPGLHGFHVFQYGDNTNGCTSAAGPHFNPNKTHGGPTDEMRHVGDL 84
Brugia malayi           EGSPTTISGEIKGLTPGLHGFHVFQYGDNTNGCTSAAGPHFNPNKTHGGPTDEMRHVGDL 84
Loa loa                 ESSPTAINGEIKGLTPGLHGFHVFQYGDNTNGCTSAAGPHFNPHKTHGGPTDEIRHVGDL 82
Ascaris suum            EDEPTTILKGEISGLTPGLHGFHVFQYGDNTNGCTSAAGPHFNPFKTHGGPTDEERHVGDL 85
Trichinella pseudospiralis -----ACFREVTVGLTEGPHGFHVFQFGDNTNGCTSAAGPHFNPNKTHGGPTDTRHVGDL 84
Branchiostoma floridae  -----ACFREVTVGLTEGPHGFHVFQFGDNTNGCTSAAGPHFNPNKTHGGPTDTRHVGDL 70
Botryllus schlosseri    -GTDCVITGTVQGLTPGNHGFHVFQFGDRTGCTSTGGHNPNTKVDHAGPDDNPRHFGDL 79
Caenorhabditis briggsae -GSFLKLNGLSVSGLAAGKHGFHVFQYGDNTNGCTSAAGPHFNPHKSHGAPDSDNRHVGDL 108
Caenorhabditis remanei  -GSFLKLNGLSVSGLAAGKHGFHVFQYGDNTNGCTSAAGPHFNPHKSHGAPDSDNRHVGDL 108
Caenorhabditis elegans  -GSFLKLNGLSVSGLAAGKHGFHVFQYGDNTNGCTSAAGPHFNPHKSHGAPDSDNRHVGDL 108
Dictyocaulus viviparus  -RSLVKNLNGTVSGLKSLHGFHVFQYGDNTNGCTSAAGPHFNPNKTHGGPDSNRHVGDL 118
                                : ** * ***** * : ** * * ** * . * * ** **

```

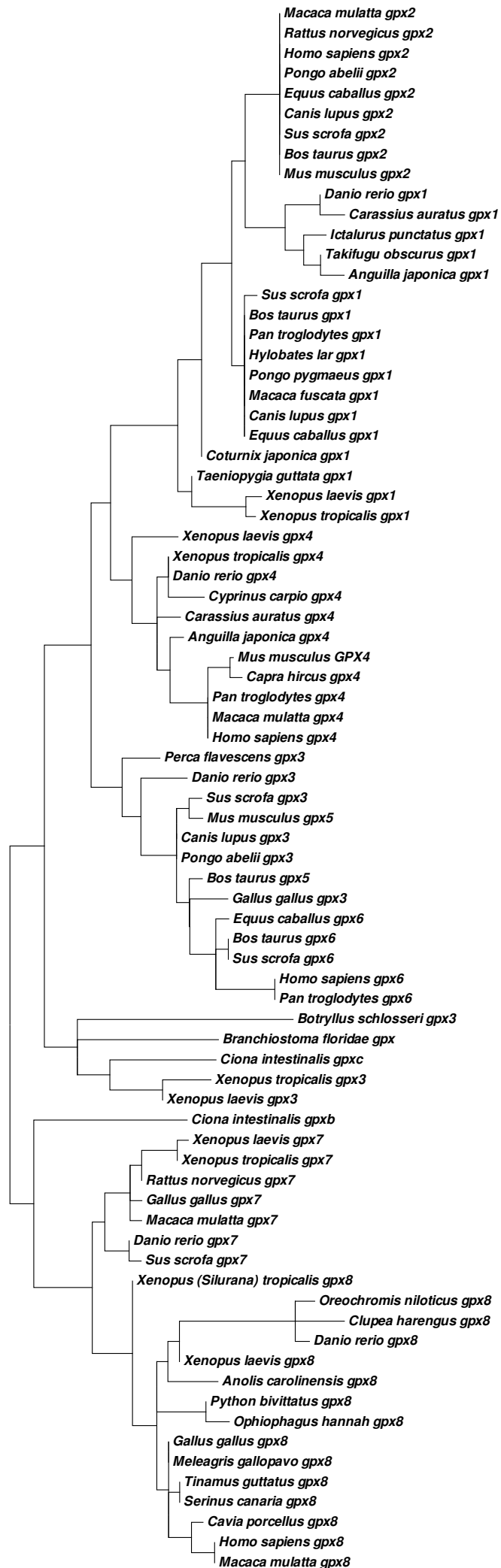
*Ovis aries* GNVKADKNGVAIVDIVDPLISLSGSEYSIIIGRTMVVHERPDDLGRG-GN--EESTKTGNAG 140  
*Capra hircus* GNVKADKNGVAIVDIVDPLISLSGSEYSIIIGRTMVVHEKPDDLGRG-GN--EESTKTGNAG 140  
*Bos taurus* GNVTADKNGVAIVDIVDPLISLSGSEYSIIIGRTMVVHEKPDDLGRG-GN--EESTKTGNAG 140  
*Bos grunniens* GNVTADKNGVAVVDIVDSLISLSGSEYSIIIGRTMVVHEKPDDLGRG-GN--EESTKTGNAG 140  
*Cervus elaphus* GNVTADKNGVAKVDIVDSLISLSGSEHSIIIGRTMVVHEKPDDLGRG-GN--EESTKTGNAR 140  
*Callithrix jacchus* GNVTAGKDGVAKVSIEDSVISLSGDHSIIIGRTLTVVHEKADDLGKG-GN--EESKKTGNAG 142  
*Macaca mulatta* GNVTAGKDGVAKVSIEDSVISLSGDHSIIIGRTLTVVHEKADDLGKG-GN--EESKKTGNAG 142  
*Hylobates lar* GNVTADKDGVAKVSIEDSVISLSGDHSIIIGRTLTVVHEKADDLGKG-GN--EESTKTGNAG 142  
*Cavia porcellus* GNVTAGADGVANVSIEDSLISLSGANSIIIGRTMVVHEKPDDLGRG-GN--EESTKTGNAG 141  
*Equus caballus* GNVTADENKADVDMKDSVISLSGKHSIIIGRTMVVHEKQDDLGRG-GN--EESTKTGNAG 142  
*Gallus gallus* GNVTA-KGGVAEVEIEDSVISLTGPHCIIGRTMVVHAKSDDLGRG-GD--NESKLTGNAG 141  
*Melopsittacus undulatus* GNVTA-KGGVAEVAIEDSIIISLSGPHSIVGRTMVVHEKCDLGRG-GD--NESKLTGNAG 141  
*Caretta caretta* GNVIANKEGVAEVCIKDSLISLTGSQSIIGRTMVVHEKEDDLGRG-GN--DES LKTGNAG 154  
*Rana catesbeiana* GNVTS-AGGVADINIKDKLISLKGESIIIGRTAVVHEKEDDLGRG-GD--NESLITGNAG 138  
*Bufo gargarizans* GNITS-KDGVAEFEFKDKIISLEGEHNIIGRTAVVHEKADDLGKG-GD--NESKVTGNAG 138  
*Xenopus tropicalis* GNVTA-KDGVAEFKLTDLSISLKNHSIIIGRCVAVVHEKEDDLGRG-GN--DES LKTGNAG 139  
*Xenopus laevis* GNVTA-EGGVAQFKITDLSISLKGPNIIIGRTAVVHEKADDLGKG-GN--DES LKTGNAG 139  
*Wuchereria bancrofti* GNIVAEGDGTAHINISDKHVQLLGNPNSIIIGRSIVVHADQDDLGRGVDKDKDES LKTGNAG 144  
*Brugia malayi* GNIVAGADGTAHIDISDKHVQLLGNPNSIIIGRSIVVHADQDDLGRGVDKDKDES LKTGNAG 144  
*Loa loa* GNIVAGADGTAHIDMSDKHVQLSFPNSIIIGRSIVVHADQDDLGRGVDKDKDES LKTGNAG 142  
*Ascaris suum* GNVEADANGIAKFIQVDKLVQLHGKYSVIGRSVIVVHVEDDLGRGVDKDKDES LKTGNAG 145  
*Trichinella pseudospiralis* GNIVAGSDGVAKIDIVDDQIKLTGEHSIIIGRTMVVHIQEDDLGRG-GD--DES LKTGNAG 141  
*Branchiostoma floridae* GNIVADASGTASVDITDNHLSLVGENSIIIGRGVHVHAEDEDLGRG-GH--ELSDTTGNAG 127  
*Botryllus schlosseri* GNITADANGKAENVITDKLVTLHGEYSVIGRAVVVHAGEDDLGLG-GF--PDSKTGHAG 136  
*Caenorhabditis briggsae* GNIESPASGDTAISVSDSLASLSGQYSIIIGRSVVIHEKTDDLGRGNSD---QSKTTGNAG 165  
*Caenorhabditis remanei* GNIESPTSGDTAISVSDSLASLSGQYSIIIGRSVVIHEKTDDLGRGNSD---QSKTTGNAG 165  
*Caenorhabditis elegans* GNIESPASGDTLISVSDSLASLSGQYSIIIGRSVVIHEKTDDLGRGTSD---QSKTTGNAG 165  
*Dictyocaulus viviparus* GNIVTSANGETVISISDPVITLNGYHSVIGRAVVIHADADDLGLGRSE---MSKSTGNAG 175  
 \*\*: : \* : . . \* \* \* : : \*\* \* : \* \* \* \* \* . \* \*\* :

*Ovis aries* GRLACGVIGIAP----- 152  
*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  
*Gallus gallus* PRLACGVIGIAK----- 154  
*Melopsittacus undulatus* PRLACGVIGIAK----- 154  
*Caretta caretta* SRLACGVVGIACL----- 167  
*Rana catesbeiana* GRLACGVIGICQ----- 150  
*Bufo gargarizans* GRLACGVIGICQ----- 150  
*Xenopus tropicalis* GRLACGVIGLCQ----- 151  
*Xenopus laevis* GRLACGVIGYSP----- 151  
*Wuchereria bancrofti* ARVACGIVAVSAAS----- 158  
*Brugia malayi* ARVACGIVAIGAAS----- 158  
*Loa loa* ARVACGIVALSAT----- 156  
*Ascaris suum* ARAACGIVAAPCEH----- 161  
*Trichinella pseudospiralis* ARVCGVIGIANPAA----- 156  
*Branchiostoma floridae* GRLACGIIGITK----- 139  
*Botryllus schlosseri* GRVACGVIGFGK----- 148  
*Caenorhabditis briggsae* ARLACGTI----- 173  
*Caenorhabditis remanei* ARLACGTIGKY----- 176  
*Caenorhabditis elegans* SRLACGTIGIVEERILETTTASLPPVTSQSPIGSSSYSTFYLPILYFLLSRIL 221  
*Dictyocaulus viviparus* ARVACGVIGIV----- 186  
 .\*\* :



0.1

**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).



0.2

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

	NP_001108609.2	<i>Macaca mulatta</i>
	NP_001125093.3	<i>Pongo abelii</i>
	EAW80889.1	<i>Homo sapiens</i>
	NP_001159953.1	<i>Equus caballus</i>
	NP_899653.2	<i>Rattus norvegicus</i>
	NP_109602.2	<i>Mus musculus</i>
<b>Vertebrate GPx3</b>	ACQ99329.1	<i>Perca flavescens</i>
	NP_001085319.2	<i>Xenopus laevis</i>
	NP_988961.2	<i>Xenopus tropicalis</i>
	NP_001131027.1	<i>Danio rerio</i>
	NP_001157926.1	<i>Canis lupus</i>
	NP_001124645.1	<i>Pongo abelii</i>
	NP_001108627.1	<i>Sus scrofa</i>
	NP_001156704.1	<i>Gallus gallus</i>
<b>Vertebrate GPx4</b>	NP_001112361.1	<i>Macaca mulatta</i>
	AAH46163.1	<i>Homo sapiens</i>
	NP_001272641.1	<i>Capra hircus</i>
	NP_032188.3	<i>Mus musculus</i>
	NP_001139295.1	<i>Pan troglodytes</i>
	NP_001165215.2	<i>Xenopus laevis</i>
	ACN78879.1	<i>Anguilla japonica</i>
	ABO36294.1	<i>Carassius auratus</i>
	ACR33821.1	<i>Cyprinus carpio</i>
	NP_001291701.1	<i>Xenopus tropicalis</i>
	ABW76146.1	<i>Danio rerio</i>
<b>Vertebrate GPx5</b>	AAI00750.1	<i>Mus musculus</i>
	NP_001020506.2	<i>Bos taurus</i>
<b>Vertebrate GPx6</b>	AAAY68223.1	<i>Homo sapiens</i>
	NP_001139297.1	<i>Pan troglodytes</i>
	NP_001131079.1	<i>Sus scrofa</i>
	NP_001156614.1	<i>Bos taurus</i>
	NP_001159955.1	<i>Equus caballus</i>
<b>Vertebrate GPx7</b>	NP_001088904.1	<i>Xenopus laevis</i>
	NP_001072404.1	<i>Xenopus tropicalis</i>
	NP_001156717.1	<i>Gallus gallus</i>
	NP_001018337.1	<i>Danio rerio</i>
	XP_013847458.1	<i>Sus scrofa</i>
	NP_001152840.1	<i>Macaca mulatta</i>
	NP_001100143.1	<i>Rattus norvegicus</i>
<b>Vertebrate GPx8</b>	NP_001088474.1	<i>Xenopus laevis</i>
	XP_012811797.1	<i>Xenopus tropicalis</i>
	NP_956516.1	<i>Danio rerio</i>
	XP_003455356.1	<i>Oreochromis niloticus</i>
	XP_012671344.1	<i>Clupea harengus</i>
	XP_007421400.1	<i>Python bivittatus</i>
	ETE72807.1	<i>Ophiophagus hannah</i>
	XP_003216232.3	<i>Anolis carolinensis</i>
	XP_423834.1	<i>Gallus gallus</i>
	XP_010723716.1	<i>Meleagris gallopavo</i>
	XP_010225014.1	<i>Tinamus guttatus</i>
	XP_009094073.1	<i>Serinus canaria</i>
	XP_003470318.1	<i>Cavia porcellus</i>
	NP_001008398.2	<i>Homo sapiens</i>
	XP_001098032.1	<i>Macaca mulatta</i>

<b>Invertebrate GPxs</b>	XP_002587571.1	<i>Branchiostoma floridae</i>
	NP_001177268.1	<i>Ciona intestinalis</i>
	NP_001177274.1	<i>Ciona intestinalis</i>
<b>Cu/Zn SOD</b>	NP_001138657.1	<i>Ovis aries</i>
	NP_001272479.1	<i>Capra hircus</i>
	NP_777040.1	<i>Bos taurus</i>
	Q52RN5.3	<i>Bos grunniens</i>
	AAB88116.1	<i>Cervus elaphus</i>
	XP_003467296.1	<i>Cavia porcellus</i>
	Q8HXQ3.3	<i>Hylobates lar</i>
	XP_002761406.1	<i>Callithrix jacchus</i>
	NP_001027976.1	<i>Macaca mulatta</i>
	NP_001075295.1	<i>Equus caballus</i>
	AAB25456.1	<i>Caretta caretta</i>
	NP_990395.1	<i>Gallus gallus</i>
	NP_001268474.1	<i>Melopsittacus undulatus</i>
	ACO51906.1	<i>Rana catesbeiana</i>
	ABD75370.1	<i>Bufo gargarizans</i>
	NP_001016252.1	<i>Xenopus tropicalis</i>
	CAA34602.1	<i>Xenopus laevis</i>
	XP_002590336.1	<i>Branchiostoma floridae</i>
	KJH46452.1	<i>Dictyocaulus viviparus</i>
	NP_001255002.1	<i>Caenorhabditis elegans</i>
	XP_002632365.1	<i>Caenorhabditis briggsae</i>
	XP_003113480.1	<i>Caenorhabditis remanei</i>
	XP_002122526.1	<i>Ciona intestinalis</i>

February 2017

Volume 232 • Number 1

# THE BIOLOGICAL BULLETIN



Marine  
Biological  
Laboratory

Published by the University of Chicago Press

## 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