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Characterization of cytosolic glutathione peroxidase and phospholipid-hydroperoxide glutathione peroxidase genes in rainbow trout (*Oncorhynchus mykiss*) and their modulation by *in vitro* selenium exposure

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ABSTRACT

Selenium (Se) is an oligonutrient with both essential biological functions and recognized harmful effects. As the selenocysteine (SeCys) amino acid, selenium is integrated in several Se-containing proteins (selenoproteins), many of which are fundamental for cell homeostasis. Nevertheless, selenium may exert toxic effects at levels marginally above those required, mainly through the generation of reactive oxygen species (ROS). The selenium chemical speciation can strongly affect the bioavailability of this metal and its impact on metabolism, dictating the levels that can be beneficial or detrimental towards an organism.

Glutathione peroxidase (GPxs) is the largest and the most studied selenoprotein family. Cytosolic glutathione peroxidase (cGPx, GPx1) and phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) are widely distributed throughout tissues, and play a pivotal role in regulating the oxidative status in the cell. In this study we have cloned GPx1 and GPx4 genes in rainbow trout (*Oncorhynchus mykiss*). The constitutive mRNA expression of these GPx genes was examined in 18 trout tissues and their responsiveness to Se availability was analysed using a rainbow trout liver cell line (RTL). An inorganic (sodium selenite, Na_2SeO_3) and organic (selenocysteine, Cys-Se-Se-Cys) selenocompound have been used as Se sources. GPx1 activity was also tested to verify the impact of transcript changes on the enzymatic function of these molecules. To understand if the results obtained from the transcript expression analysis were due to Se bioavailability or generation of ROS, the cytoxicity of the two selenocompounds was tested by measuring the impact of Se on cell membrane integrity. Lastly, Se availability was quantified by mass spectrophotometry to determine the amount of Se in the cell culture media, the Se background due to the foetal calf serum supplement and the contribution from the two selenocompounds used in the treatments.

Three isoforms of genes for both *GPx1* (*GPx1a*, *1b1* and *1b2*) and *GPx4* (*GPx4a1*, *a2* and *b*) have been identified. The discovery of a third gene encoding for GPx1 and GPx4 hints that salmonids may have the biggest selenoproteome amongst all vertebrates. Transcripts of GPx4 genes were more highly expressed in most tissues examined *in vivo* (except blood, head kidney and spleen), whereas those of the GPx1 genes were more responsive to selenium exposure *in vitro*, especially to the organic form. Interestingly, *GPx1a* was the most sensitive to selenium availability in non stressful conditions, whereas *GPx1b1* and *GPx1b2* were highly induced by exposure to selenium levels that had some toxic effects on the cells. Although the different concentrations tested of the two selenocompounds modulate GPx1 transcript expression to various degrees, no significant change of GPx1 enzymatic activity was detectable. Our results lead us to conclude that trout *GPx1* transcripts expression level may represent a sensitive biomarker for selenium intake, helping to evaluate if selenium concentration and chemical speciation impact on cell homeostasis.

1. Introduction

Selenium (Se) is an essential trace element, required as an integral part of diverse Se-containing proteins, called selenoproteins (Burk and Hill, 1993). Through its incorporation into selenoproteins as the selenocysteine (SeCys) amino acid, selenium exerts its biological effects primarily by regulating the antioxidant

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system and redox enzyme activities within the cell (McKenzie et al., 2002). Moreover, this trace element is involved in gene transcription and cell signalling cascades, thyroid hormone metabolism, immune responses and reproduction (Rayman, 2000; Hefnawy and Tórtora-Pérez, 2010). However, Se toxicity can be reached by levels marginally above those which are required. Among the essential trace elements, Se presents the narrowest range between essentiality and toxicity, and for this reason it is often difficult to establish which concentrations are beneficial and which become detrimental towards an organism's health (NRC, 1980; Foster and Sumar, 1997).

Diet and water are the two sources of Se. In the aquatic environment, Se can be absorbed through the gills, gut or epidermis; however diet is considered the primary source of Se for animals and the intestine the principal route of assimilation (Dallinger et al., 1987; Hamilton, 2004; Janz, 2011). The immediate bioavailability of Se depends on its chemical form, which determines the metabolic and toxic potential (Jonnalagadda and Prasada Rao, 1993; Jackson, 1997; Finley, 2006). Se exists in the environment and in biological systems as both inorganic and organic forms. The inorganic salts, selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}), contain Se in oxidized forms (Se(IV) and Se(VI) respectively), whereas organic forms contain Se in the reduced state (selenide: Se(−II)) (Thomas et al., 1990; Birringer et al., 2002). Generally, selenocysteine (SeCys) and selenomethionine (SeMet) are the most abundant selenocompounds present in the diet (Suzuki, 2005; Dumont et al., 2006). The diverse speciation of selenium suggests that the cellular uptake of this trace element is likely to occur *via* multiple membrane transporters (Misra et al., 2012b). Previous studies, focused on the mechanisms of intestinal absorption of selenocompounds in mammals, suggest that SeO_3^{2-} absorption occurs by simple diffusion, whilst SeO_4^{2-} may be transported by a common transport mechanism with sulphate (Arduser et al., 1985; Wolffram et al., 1986). SeMet appears to be absorbed by an active transport system shared with methionine (McConnell and Cho, 1965; McConnell and Cho, 1967; Wolffram et al., 1989), whereas SeCys has been reported to be either passively absorbed (McConnell and Cho, 1965) or absorbed *via* a competitive transport with cysteine in brush-border membranes (Wolffram et al., 1989; Leblondel et al., 2001). Currently, the mechanisms of Se transport in fish, particularly at the cellular level, are largely unknown. However, it is believed that the conversion of selenocompounds into metabolically active forms is the rate–limiting step for Se bioavailability (Contempre et al., 1996). In vertebrates, inorganic selenocompounds react spontaneously with glutathione (GSH) to form selenodi-diglutathione (GSSeSG) (Ganther, 1968), which is further converted to hydrogen selenide (H_2Se) (Steve Hsieh and Ganther, 1975). This central selenide pool serves either as the basis for methylated derivates that are excreted or as a precursor for further transformation into SeCys (Ganthert, 1966; Mozier et al., 1988; Ip et al., 1991; Berry et al., 1993; Guimarães et al., 1996). Vertebrates are also able to synthesize SeCys but not SeMet, which is usually metabolised through the *trans*-sulfuration pathways of the sulphur amino acids and then converted into SeCys (Beilstein and Whanger, 1992).

The mechanism of Se toxicity has not been clearly elucidated. It is hypothesised that it is related to the generation of reactive oxygen species (ROS) caused by the depletion of glutathione and protein-bound sulphydryl groups that occur during the metabolism of inorganic selencompounds (Anundi et al., 1984; Ganther, 1986). Also, SeMet can be transformed through an alternative pathway involving methylselenol, which is subsequently metabolized in the GSH-mediated redox cycle with the production of ROS (Chaudiere et al., 1992). Finally, Se may interfere with sulphur metabolism, which can occur under both toxic and non-toxic conditions (Combs and Combs, 1986). Indeed, SeMet can be incorporated non-specifically into proteins instead of methionine, leading to significant alterations in protein structure and consequently protein function (Schrauzer, 2000).

SeCys is the most relevant selenocompound, as the naturally occurring amino acid (the 21st) that provides the catalytic site for the enzymatic selenoproteins (Stadtman, 1996). SeCys is characteristically different from the remaining amino acids as it is encoded by one of the three stop codons (UGA) and is inserted into the selenoproteins by the $tRNA^{[SeCys]}$, which has specific features that distinguish it from all other tRNAs (Bock et al., 1991). The UGA codon is made to encode SeCys by the presence of a *cis*-acting stem-loop structure, designated the SeCys insertion sequence (SECIS) element, present in the 3′ un-translated regions (3′-UTRs) in the mRNA (Walczak et al., 1996). In addition, other factors are required for the incorporation of SeCys into the mature protein, namely SECIS-binding protein 2 (SBP2) (Copeland et al., 2000), and SeCys-specific elongation factor (EFsec, also called mSelB) (Fagegaltier et al., 2000). SECIS elements are present in the 3′ un-translated regions (3′-UTRs) of all eukaryotic selenoprotein genes (Berry et al., 1991). The general structure of the SECIS element includes two helices separated by an internal loop, a SECIS core structure located at the base of helix 2 and one or two apical loops; its configuration can be selenoprotein- and species-specific. The presence of a SECIS dictates any in-frame UGA codon within the coding region to serve as SeCys when a minimal spacing requirement between UGA and the SECIS element (51 to 111 nucleotides) is met (Low and Berry, 1996; Fletcher et al., 2001). A set of selenoproteins in an organism is known as the selenoproteome. To date 25 selenoprotein genes have been indentified in humans (Kryukov et al., 2003). Recent studies on the vertebrate selenoproteome showed that bony fish, with 38 selenoprotein genes identified in zebrafish, have a larger set of selenoproteins than humans and all terrestrial vertebrates (Mariotti et al., 2012).

Glutathione peroxidase (GPxs) is the largest and the most studied selenoprotein family (Flohé and Brigelius-Flohé, 2012). GPxs, together with other antioxidant enzymes (*i.e.* catalase, glutathione reductase and superoxide dismutase), regulate the oxidative status in the cell reducing either free or membrane-bound hydroperoxides (Apel and Hirt, 2004). Proteins within the GPx family differ in structure, substrate specificity and cellular location, with some having a tissue-specific distribution (Arthur, 2000). Eight GPx isoforms have currently been characterized in humans, six of which (GPx1, GPx2, GPx3, GPx4, GPx6, GPx8) are SeCys-containing proteins, the remaining two (GPx5 and GPx7) have a cysteine residue instead of selenium (Mariotti et al., 2012; Nauser et al., 2012). Among the GPx proteins, GPx1 and GPx4 were the first discovered and are the best characterized (Flohé et al., 1973; Rotruck et al., 1973; Ursini et al., 1982; Toppo et al., 2008). GPx1 (also called cytosolic glutathione peroxidase, cGPx) and GPx4 (phospholipid hydroperoxide glutathione peroxidase, PHGPx) are widely distributed throughout tissues and are highly conserved across all species (Margis et al., 2008). GPx1, is a homotetramer localised in the cytosol, nucleus and mitochondria, where it acts to neutralise hydrogen peroxide (H_2O_2) (Flohé et al., 1973). The kinetic properties of GPx1 imply that under physiological substrate concentrations, the maximum rate is never reached, *i.e.* the actual reaction rate is always under the control of the amount of enzyme (Wendel, 1981). Contrary to the other members of the GPxs family, GPx4 possesses a monomeric structure and hydrophobic surface (Ursini et al., 1985), which underlies a unique catalytic activity capable of reducing complex membrane bound hydroperoxides and lipid peroxides, such as phospholipid and cholesterol hydroperoxides (Thomas et al., 1990; Roveri et al., 1994; Schnurr et al., 1996). GPx4 localises primarily to cellular membranes, and its main function is the protection of plasma and lysosomal membranes from ROS-induced damage (Ursini et al., 1985).

Table 1
Primers used for cloning and real time PCR for trout GPx genes.

Gene name	Primer name	Primer sequence (5′→3′)	Application
Glutathione peroxidase 1a	F1	GGGTCAGTTGCTGTACAAAAGC	3′-RACE
	F2	GGTATCCGAAAGTTAGACGTCGTTG	3′-RACE
	F	ATGAAATGGCTGGGAAAATAAAGA	qPCR
	R	TCATCATTCTTACAATTCTCCTGATG	qPCR
Glutathione peroxidase 1b1	F1	AGCCCTGTTTAGCAGACAGAAAAAGC	3′-RACE
	F2	AACATGTCTGGAAGTGAGTTCTACAACA	3′-RACE
	F	CAACATGTCTGGAAGTGAGTTCTACAACA	qPCR
	R	TTCGTTATTGCAGTTCTCCTGATGTC	qPCR
Glutathione peroxidase 1b2	F1	AGAGAGAAGCGGGGAGTGACA	3′-RACE
	F2	ATGGCTGTATGTAAGATGTTCTACGACC	3′-RACE
	F	ACCAGGCAAATGGCTGTATGTAAGAT	qPCR
	R	CTTCGTTCTTGCAGTTCTCCTGATG	qPCR
Glutathione peroxidase 4a1	F1	GATTTGAGTACGCAGTACGTTTCCCA	3′-RACE
	F2	CCAACAAACCCTGCAGACGG	3′-RACE
	F	GTAAGATTGACGTGAACGGGGATAA	qPCR
	R	GAAATTCCACTTGATGCCATTTCC	qPCR
Glutathione peroxidase 4a2	F1	GACTTCTCTGCAAAGGATATTGATGG	3′-RACE
	F2	ACCCCAGTCAACTACTCTCAGTTTGC	3′-RACE
	F	CCTCGAGAAATACAGGGGCGA	qPCR
	R	TGAAATTCCACTTGATGCCATTTC	qPCR
Glutathione peroxidase 4b	F1	CTACTTAAACAGCTTGGTCACAGGATG	3′-RACE
	F2	GGATCGTAACGCGTGGGTTAG	3′-RACE
	F	AGGTGGAAATCAAGGAGTTTGTCAAAC	qPCR
	R	CTTTTGTGAAATTCCATTTGATGTTATTTCC	qPCR
Elongation factor-1α	F	CAAGGATATCCGTCGTCGTGGCA	qPCR
	R	ACAGCGAAACGACCAAGAG	qPCR

In this study we have first cloned and characterized the homologues of mammalian GPx1 and GPx4 genes in rainbow trout (*Oncorhynchus mykiss*). The transcript expression of these GPx genes was then examined in different trout tissues. Next, we established the rainbow trout liver cell line (RTL) as a model to analyse the responsiveness of GPx genes to Se, as a means to study Se effects in a controlled system prior to future mechanistic studies in whole animal experiments. The liver is known to be the principal tissue involved in Se metabolism and RTL are a well established model to determine the effect of various exogenous substances on fish cells (Lee et al., 1993). To verify whether different effects on transcript expression of GPx gene are induced by inorganic and organic selenocompounds, sodium selenite (Na_2SeO_3) and selenocysteine (Cys-Se-Se-Cys) were both used as Se sources at different concentrations. Na_2SeO_3 is the most common inorganic Se form and a commonly used compound in the study of the effects of this trace element both *in vitro* and *in vivo* (Spallholz, 1997; Valdiglesias et al., 2010). Cys-Se-Se-Cys is a stable form of SeCys, and consists of two oxidized selenocysteine residues that form a diselenocompound (Huber and Criddle, 1967). Lately, Cys-Se-Se-Cys has received more attention due to its potential use as a food/feed additive and its pharmacological applications. Moreover, recent studies have shown that Cys-Se-Se-Cys is the principal metabolite in the liver of fish feed organic selenium compounds, as SeMet (Misra et al., 2012a). However, to date only few investigations have been conducted on the balance between the beneficial and toxic effects of this diselenocompound (El-Sayed et al., 2006; Santhosh Kumar et al., 2009). GPx1 genes were found to be the most sensitive to selenium exposure, thus we also analysed GPx1 enzymatic activity after Se treatment. Due to the dual nature of Se, as an essential component of antioxidant enzymes and pro-oxidant compound, the cytoxicity of the two selenocompounds was tested to elucidate if the effects seen at the transcript expression level could be due to Se bioavailability or Se toxicity. Hence cell viability was determined after treatment with different concentrations of the two selenocompounds studied. Lastly, Se availability was quantified by mass spectrophotometry to determine the amount of Se in the cell culture media, the Se background due to the foetal calf serum supplement and the contribution from the two selenocompounds used in the treatments.

2. Materials and methods

2.1. Cloning and sequencing of trout GPx1 and GPx4

BLAST search (Altschul et al., 1997) at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the salmonid EST database using the zebrafish *GPx1a* (Acc. No. NM_001007281), *GPx1b* (Acc. No. NM_001004634), *GPx4a* (Acc. No. NM_001007282) and *GPx4b* (Acc. No. NM_001030070) as baits, identified a salmon partial sequence encoding for *GPx4* (Acc. No. BT044014), a partial trout sequence encoding for *GPx1* (Acc. No. AF281338) and over 30 expressed sequence tags (ESTs) from an *Oncorhynchus mykiss* cDNA library. The ESTs were assembled with the AlignIR programme (Li-COR, Inc.) and 3 contigs for each of *GPx1* and *GPx4* were detectable that contained the 5′-untranslated region (5′-UTR) and part of the open reading frame (ORF). Primers F1 and F2 were designed at the 5′-UTR (Table 1) of each contig, to obtain the full ORF and the 3′-untranslated region (3′-UTR). A 3′-RACE PCR was carried out using SMART cDNA from liver and spleen as described before (Wang and Secombes, 2003; Wang et al., 2008). The nested RACE products were ligated into pGEM-T easy vector (Promega) and transformed into *E. coli* TAM competent cells (ActiveMotif), following the manufacturer's protocol. Positive clones were screened by standard colony PCR and cultured at 37 °C overnight in a shaking incubator (200 rpm) before plasmid isolation. Plasmid DNA from at least four clones per PCR was extracted using a Qiagen minipreparation kit and sequenced by MWG Biotech (Germany).

2.2. Sequence analysis

The nucleotide sequences generated were assembled and analyzed with the AlignIR program (version 2.0; LI-COR). The

intron positions were predicted using the Spidey program at the NCBI (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/). The SECIS elements were predicted using SECISearch (version 2.19; http://genome.unl.edu/SECISearch.html) (Kryukov et al., 2003). Each preliminary cDNA sequence was translated using the ExPASy translate tool (http://web.expasy.org/translate/) (Gasteiger et al., 2005). To obtain the similarity and identity of the sequences the software MatGat (version 2.02) was used (Campanella, 2003). Multiple sequence alignments were generated using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Chenna, 2003) and shaded using BOXSHADE (version 3.21; http://www.ch.embnet.org/software/BOX_form.html). Based on a ClustalW multiple alignment phylogenetic trees were created by the neighbour-joining method using the MEGA program (version 5.0) (Tamura et al., 2011), using Pairwise Deletion of gaps/missing data and JTT amino acid matrix, and bootstrapped 10,000 times.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of tissue that had been homogenised in TRIZol (Invitrogen) using tungsten carbide beads (3 mm, Qiagen) and the TissueLyser II system (Qiagen) following the manufacturer's instructions. The RNA pellet was washed twice with 70% ethanol, air dried and resuspended in RNase free H_2O. The concentration and purity was determined by spectrophotometry (Nanodrop). The RNA was stored at −80 °C until required for cDNA synthesis. cDNA was synthesized from total RNA: 2 μg of total RNA was denatured (70 °C, 3 min) in the presence of 1 μl (500 μg/ml) of an adaptor-dT_{17} (Table 1) and RNase free water in a volume of 11 μl, then the RNA was cooled on ice. The first strand cDNA was synthesized from the total RNA using 1 μl of RevertAid™ reverse transcriptase (10,000 U, Fermentas) in the presence of 5 μl of 5× Reaction Buffer, 1 μl of dNTP (deoxynucleoside triphosphate mix 25 mM each) (Bioline), made up to a final volume of 25 μl with water and incubated at 42 °C for 2 h.

2.4. Real time reverse transcriptase PCR (qPCR)

Real time PCR was performed with a LightCycler 480 (Roche) to quantify the transcript expression of the GPx genes and the common reference gene using the primers given in Table 1. The primers employed for real time PCR were designed with at least one primer across a predicted intron and pre-tested to ensure that each primer pair could not amplify genomic DNA using the real time PCR protocols. The real time PCRs were performed in duplicate for each sample, along with a 10-fold serial dilution of references consisting of an equimolar mix of purified PCR products of each gene amplified from cDNA. The transcript level was calculated using the quantitative fit points method in the integrated LightCycler 480 software. The relative transcript expression level of each gene in different tissues was expressed as arbitrary units that were calculated from the serial dilution of references run in the same 96-well plate and normalized against the transcript expression level of *EF-1α* gene. Fold changes were also calculated as the average expression level of each treatment group divided by that of the corresponding controls.

2.5. Tissue distribution of the transcript expression of the trout GPx1 and GPx4 isoforms

Juvenile rainbow trout (*Oncorhynchus mykiss*), weighing approximately 100 g, were maintained in 250 l freshwater tanks at the aquarium facilities in the School of Biological Sciences, University of Aberdeen. The fish were fed *ad libitum*. Tail fin, adipose fin, scales, skin, brain, thymus, gills, head kidney, spleen, blood, heart, ovary, liver, eosophagus, stomach, pyloric caeca, mid intestine and distal intestine were collected from six healthy fish after schedule 1 killing. RNA was subsequently extracted as described in Section 2.3, and the transcript expression of GPx1 and GPx4 gene isoforms was analyzed by real time PCR, as described in Section 2.4.

2.6. Transcript expression of trout GPx1 and GPx4 in RTL cells after Na_2SeO_3 and Cys-Se-Se-Cys treatment

RTL cells were used to assess the transcript expression of GPx1 and GPx4 genes in a characterized trout cell line (Lee et al., 1993). The cells were maintained in Leibovitz medium (L-15, Gibco) containing 10% foetal bovine serum (FBS) (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (P/S) at 20 °C. Four days before stimulation the cells were trypsinised, washed twice by centrifugation at 200 × *g* for 5 min and passaged at a concentration of 1×10^6 cells/ml into 12-well culture plates (Millipore), with 2 ml of fresh medium and incubated at 20 °C. After 2 days the medium was replaced with L-15 containing 1% FBS and P/S. One day later, the medium was replaced with fresh medium containing either an inorganic (sodium selenite, Na_2SeO_3) or an organic (Selenocysteine L-stereoisomer, Cys-Se-Se-Cys) selenocompound, both purchased from Sigma-Aldrich. The cells were incubated with six different concentrations of the two selenocompounds: 0 (control), 10 nM, 100 nM, 1 μM, 10 μM and 50 μM. These concentrations were deemed optimal for stimulation of RTL cells from our previous studies (data not shown). After 24 h, the treatments were terminated by dissolving the cells in TRIZol. Total RNA was then extracted from the cells and cDNA synthesized as described in Section 2.3. The dose-dependent effects of Se on the transcript expression of GPx1 and GPx4 genes were analyzed by real time PCR, as described in Section 2.4.

2.7. GPx1 enzymatic activity in RTL cells after Na_2SeO_3 and Cys-Se-Se-Cys treatment

Cells were treated with the two selenocompounds investigated as described in Section 2.6, with the exception that they were passaged into 6-well culture plate (Millipore), with 3 ml of fresh medium.

After 24 h of treatment, the cells were rinsed once with ice cold phosphate buffered saline followed by incubation for 10 min in ice cold lysis buffer, containing 10 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol (DTT) and 40% glycerol. A protease inhibitor cocktail tablet (Roche) was added to the lysis buffer immediately before use, following the manufacturer's protocol. Cells were scraped from the plates and cell lysates were centrifuged (Peqlab PerfectSpin) at 13,000 × *g*, for 15 min, at 4 °C. Protein concentration was determined using the Bradford technique (Bradford, 1976) with a BioRad protein kit. Whole cell lysates were stored at −80 °C.

GPx1 activity was determined at 30 °C, with values read in a Spectramax Plus384 (Molecular Devices). Conditions for the GPx activity assay were: 50 mM potassium phosphate buffer (pH 7.0), 0.4 mM EDTA, 0.15 mM β-NADPH, 1 unit glutathione reductase, and 1 mM reduced glutathione. Background changes in absorbance were measured with the above components as well as with the addition of 10 μl of cell lysate. The reaction was initiated by the addition of 0.007% H_2O_2 and the change in absorbance was measured at 340 nm for 2 min.

2.8. Membrane integrity of RTL cells after Na_2SeO_3 and Cys-Se-Se-Cys treatment

Cells were seeded into 96-well plates at a concentration of 2×10^5 cells/ml, with 200 μl of medium per well, and incubated at 20 °C. In all cases the first column of wells was left without cells (blank). After 48 h the medium was replaced with L-15 containing 1% FBS and P/S. After 24 h, the medium was replaced with fresh

medium containing Na_2SeO_3 and Cys-Se-Se-Cys, at a concentration of 10 nM, 100 nM, 1 μM, 10 μM and 50 μM. The cytotoxic effect of the selenocompounds was examined at 24 h, by analysing the lactate dehydrogenase (LDH) activity released from damaged cells into the supernatant (Korzeniewski and Callewaert, 1983). To eliminate effects of potential damaged or detached cells after treatment, the cell culture was first centrifuged (250 × *g*) for 10 min at room temperature at the end of the incubation period. 100 μl of supernatants were then transferred into a new 96-well plate and the LDH release determined using a cytotoxicity detection kit (Roche, Germany) according to the manufacturer's guidelines. Cells exposed to 1% Triton X-100 were used as the "high" control (100% lysis), while cells exposed to culture medium only acted as the "low" control. The changes in absorbance were recorded in a spectrophotometer (as above) at 490 nm, using 600 nm as a reference wavelength.

2.9. Chemical analysis

The total selenium concentration in the *in vitro* model used was measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Thermo), to ensure the absence of interference of Se contained in the FCS and to verify Se availability in the cell cultures treated with Na_2SeO_3 and Cys-Se-Se-Cys. The concentration of the two Se isotopes (^{77}Se and ^{78}Se) was measured in duplicate in undiluted FCS, L-15 medium, L-15 medium containing 1% FCS (control), and in the medium of the cell cultures treated with the different concentrations of Na_2SeO_3 and Cys-Se-Se-Cys (as listed in Section 2.6). De-ionized water (18 mohm, Mellipore Element System) was used for sample dilution (1:10) and standard preparation. Nitric acid (ultra pure, 70%, Australian Chemical Reagents), and analytical grade hydrogen peroxide (30%, Analar, BDH) were used for sample digestion. Duplicate samples were analyzed with calibration, reagent blanks and reference material (human urine, 68 μg/l of Se, Serenorm) to check quality assurance and quality control at the beginning and end of each ICP-MS run.

2.10. Statistical analysis

Real-time quantitative PCR measurements were analyzed using the SPSS package 18.0 (SPSS Inc. Chicago, Illinois) as described previously (Wang et al., 2011). To improve the normality of data distribution, a $\log_2$ transformation of the arbitrary units after normalization to the transcript expression level of *EF-1α* was performed. One way-analysis of variance (ANOVA) and the Least Significant Difference (LSD) *post hoc* test were used to analyze the expression data and the viability response in RTL cells after stimulation, with $p < 0.05$ between treatment groups and control groups considered significant. Paired-sample *t*-tests were employed to compare the expression levels across different tissues and different isoforms in the same tissue (as six sets of samples from six individual fish were used in this study), and the quantified levels of the two selenium isotopes (^{77}Se and ^{78}Se) measured by ICP-MS in duplicate in undiluted FCS, L-15 medium, L-15 medium containing 1% FCS (control), and in the medium of the cell cultures treated with the different concentrations of selenocompounds.

3. Results

3.1. Sequence analysis of trout GPx1 and GPx4 gene isoforms

Six cDNA sequences, each with a complete open reading frame, 5′-UTR and a 3′-UTR, including a polyadenylation signal and a poly A tail, have been cloned in this study (Supplementary Fig. S1). The sequence features are summarized in Table 2. The translations of three of the cDNA sequences (Acc. Nos. HE687021-3) shared similar identities to mammalian GPx1 and GPx2 (60.1–66%, Supplementary Table S1). However, since these molecules grouped in phylogenetic tree analysis (discussed later) with other fish molecules previously identified and termed GPx1's, we named the trout sequences as GPx1a, 1b1 and 1b2, respectively. The translations of the other three cDNA sequences (Acc. Nos. HE687024-6) shared high identities (59.5–78%) to GPx4 from zebrafish and mammals (Supplementary Table S1) and were designated as GPx4a1, 4a2 and 4b. The designation of the trout sequences was further studied by phylogenetic tree analysis (Fig. 1) using the MEGA 5 program (Tamura et al., 2011). The Se-dependent GPx proteins (GPx1, GPx2, GPx3 and GPx4) from fish and selected mammals were employed for this analysis as GPx6 and GPx8 have not yet been characterized in fish. MEGA 5 does not recognize the SeCys residue. Thus it was substituted with a cysteine residue, which normally replaces the SeCys position in the Se-independent GPxs, and in all the non-SeCys-containing homologues of selenoproteins across the different phyla. As shown in Fig. 1, three independent clades are apparent, for GPx1/2, GPx3 and GPx4. In the GPx1/2 clade, the trout GPx1a grouped with zebrafish GPx1a and fugu GPx1a to form a branch; and the trout GPx1b1 and 1b2 grouped with zebrafish GPx1b and fugu GPx1b forming another branch. All the fish GPx1-like molecules grouped together to form a sub-clade separate from both the mammalian GPx1 and GPx2 sub-clades. In the GPx4 clade of the phylogenetic tree, two sub-groups of fish GPx4, containing respectively trout GPx4a1 and 4a2 in one group and trout GPx4b in the other, grouped with mammalian GPx4 with high bootstrap support (100%). The tree topology suggests that the fish GPx1 molecules are not strictly orthologues of mammalian GPx1 or GPx2, and are more likely related to an ancestral molecule that gave rise to GPx1 and GPx2 later in vertebrate evolution. In contrast, the fish GPx4 molecules appear to be orthologues of mammalian GPx4, although multiple paralogs are present.

Each cDNA sequence of the trout GPxs obtained in this study contains an in-frame stop codon "TGA" in the ORF encoding for the selenocysteine amino acid (SeCys, U; Supplementary Fig. S1), possibly with the exception of *GPx4b* that may have two additional selenocysteine codons (Supplementary Fig. S1F). The TGA codon is made to encode selenocysteine by the presence of a SECIS element in the 3′-UTR of the mRNA (Hatfield, 2002). Indeed, a SECIS element region can be identified in each trout cDNA sequence within its 3′-UTR using the SECISearch programme (Fig. 2). The SECIS elements in trout GPx1/2 genes contain the non-Watson-Crick base pairs 5′TGAA3′:5′TGAT3′, whilst 5′TGAC3′:5′TGAT3′ is present in the trout *GPx4* isoforms. All the *GPx* isoforms possess two apical loops and hence they belong to the type II group of selenoproteins (Latrèche, 2009).

The predicted polypeptides of the trout GPxs were aligned with GPx proteins from fish and mammals. As shown in Fig. 3, the "L(V/I)VN(VT)ASx(**U**)G(L/F)TxxxYxxLxxL" motif surrounding the reactive selenocysteine residue was well conserved. Similarly, the "G(L/F)x(V/I)L(G/A)FPCNQFxx**Q**EP" and "**WN**FxxKFL(V/I)" sequences that surround the glutamine, tryptophan and asparagine residues also involved in the catalytic site were conserved.

3.2. Tissue distribution of the expression of GPx1 and GPx4 isoforms

The constitutive transcript expression of the GPx1 and GPx4 gene isoforms was comparatively studied in 18 tissues from healthy fish, by qPCR (Fig. 4).

GPx1b2 showed the highest expression level in the head kidney, spleen and in the blood, although only in the blood was the expression significantly higher compared to the other two gene isoforms. The expression of *GPx1b1* was significantly higher in the different portions of the gastrointestinal tract (eosophagus, stomach, pyloric cecae, mid intestine). The expression of *GPx1a* was comparable or

Table 2
Summary of trout *GPx1* and *GPx4* isoform features.

Gene name	Length (bp)	5′-UTR (bp)	ORF (bp)	3′-UTR (bp)	SeCys position	Poly(A) tail position	Amino acid (aa)	Acc. No.
Glutathione peroxidase 1a	1062	70	573	419	188–190	1020–1025	190	HE687021
Glutathione peroxidase 1b1	1044	35	570	439	147–149	995–1000	189	HE687022
Glutathione peroxidase 1b2	1061	34	573	454	149–151	1013–1018	190	HE687023
Glutathione peroxidase 4a1	1014	69	603	342	295–297	964–969	200	HE687024
Glutathione peroxidase 4a2	966	61	603	302	287–289	917–922	200	HE687025
Glutathione peroxidase 4b	1038	27	576	435	226–228	986–992	191	HE687026

lower compared to the expression of the other two gene isoforms in all tissues except scales (Fig. 4A).

The transcript expression of GPx4a1 gene was the highest detected, and significantly so compared to the other two isoforms, in the gastro-intestinal tract, tail fin, scales and brain. *GPx4a1* showed the highest transcript expression also in comparison to *GPx1* isoforms, especially in the final portions of the gastro-intestinal tract (pyloric cecae, mid intestine and distal intestine). The transcript expression of *GPx4a2* was significantly lower in all tissues, relative to the other two isoforms, as seen in the gastro-intestinal tract, tail fin, scales and brain. GPx4a2 gene showed the lowest transcript expression level also in comparison to the *GPx1* isoforms. In contrast, the transcript expression of *GPx4b* was similar to that of *GPx4a1*, except in the gastro-intestinal tract, tail fin, scales and brain (Fig. 4B).

The ratio of the average level of *GPx1* and *GPx4* transcript expression in different tissues (Fig. 4) showed a higher constitutive transcript expression of the *GPx4* isoforms in all tissue examined, with the exception of head kidney, spleen and blood. The transcript expression of *GPx4* isoforms in the eosophagus was the highest detected, followed by stomach, pyloric cecae and gills.

3.3. Se availability in vitro

The cells were exposed to an organic (Cys-Se-Se-Cys) and inorganic (Na_2SeO_3) selenium compound at five different concentrations ranging from 10 nM to 50 μM, for 24 h. Total selenium concentration of the media in the *in vitro* model was determined by using ICP-MS before the start of the incubation period (Table 3). Two isotopes (^{77}Se and ^{78}Se) were measured and the resulting concentrations for total selenium showed no significant differences, hence no interference was recorded. The level of Se in the L-15 medium itself, L-15 medium containing 1% FCS, and the medium containing 10 nM of Na_2SeO_3 and Cys-Se-Se-Cys was below the limit of quantification (ten times the standard deviation of the blank level – 1 μg Se/L). Since Cys-Se-Se-Cys is a diselenocompound, the level of selenium into the organic Se treatment was 2.17 ± 0.35 higher

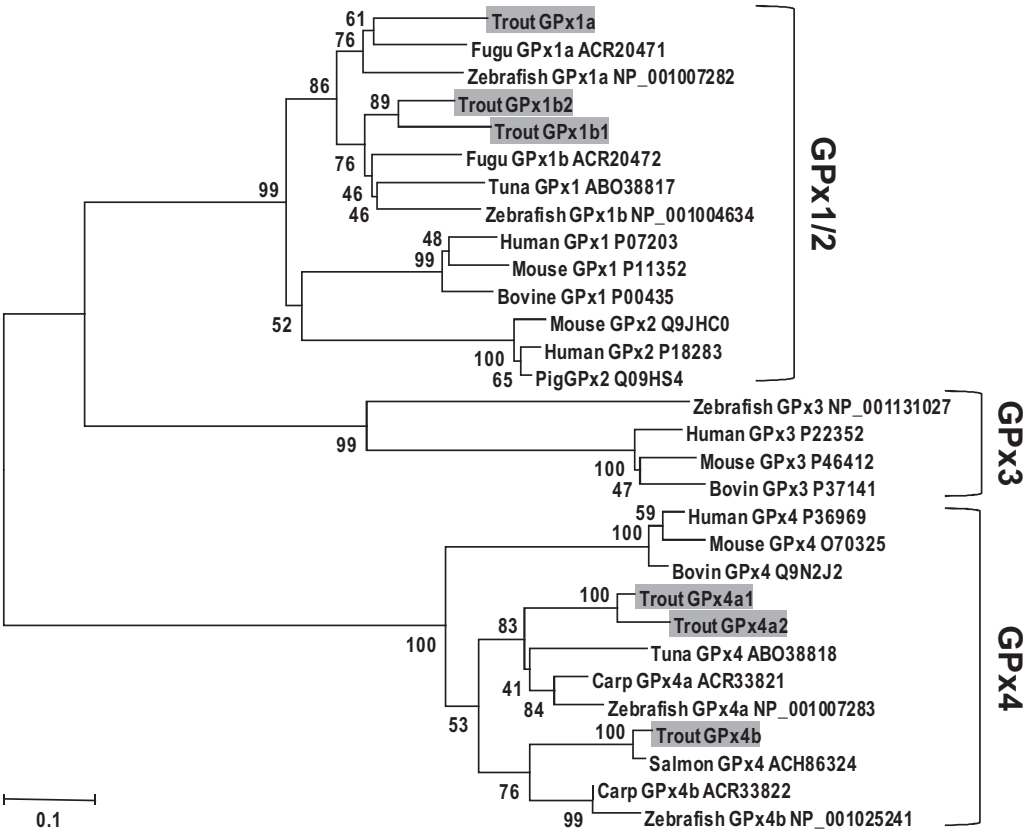


Fig. 1. Phylogenetic tree showing the relationship of the predicted trout GPx1 and GPx4 amino acid sequences with other known members of the Se-dependent GPx families from fish and mammals. The tree was constructed using ClustalW and MEGA (V.5.0) and inferred using the neighbour-joining method. Node values represent percent bootstrap confidence derived from 10,000 replicates. The common species name and the name of the molecule is followed by the accession number. The sequences reported in this report are shaded and without an accession number.

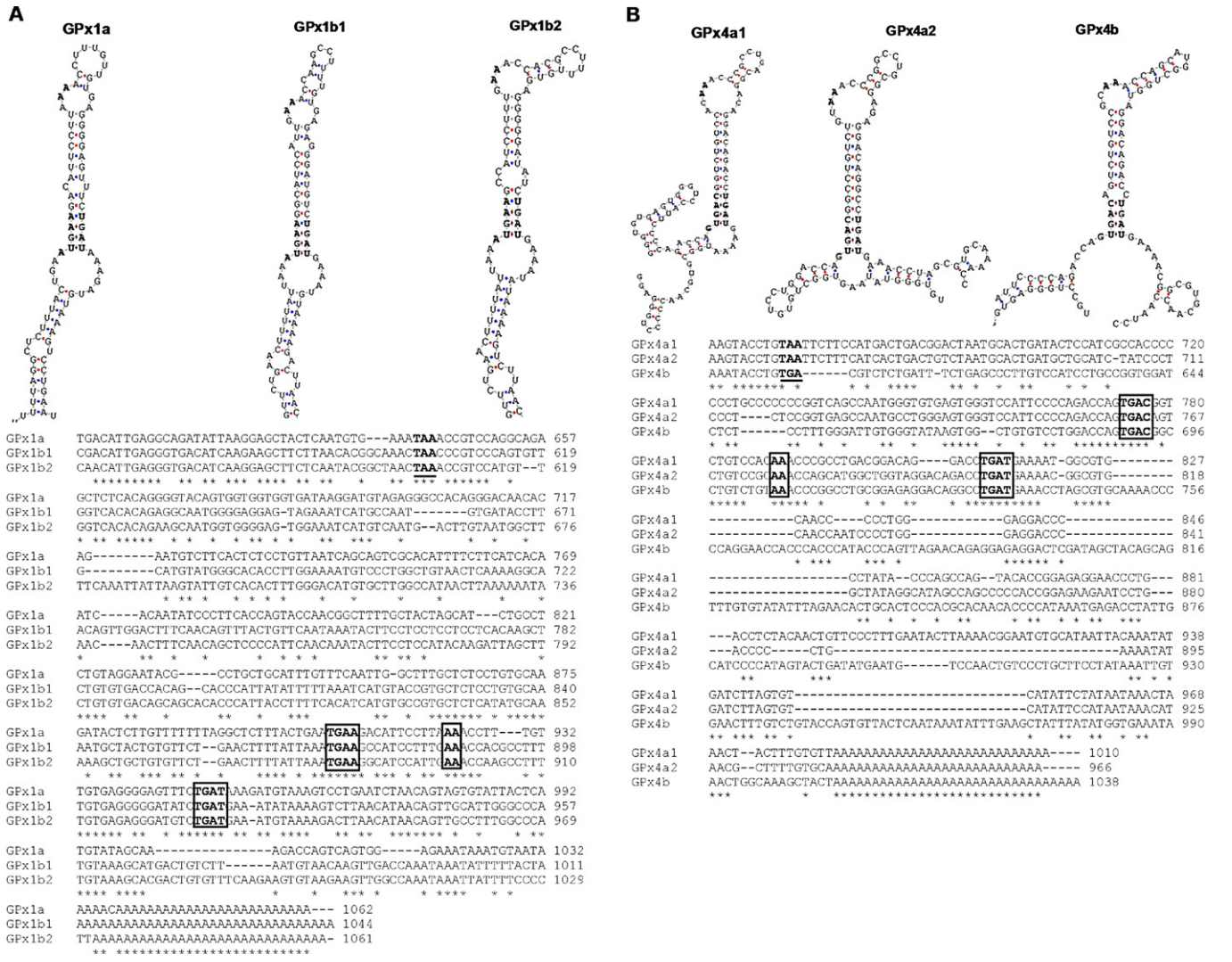


Fig. 2. SECIS elements and multiple alignment of the 3′-UTR of trout *GPx1* (A) and *GPx4* (B) isoforms. The SECIS elements were generated with SECISearch (V.2.19) and visualized with RNAnice. The multiple alignment of the 3′-UTR sequences was produced using ClustalW2. The stop codons are underlined, the nucleotides in the quartet and apical loop are boxed and conserved nucleotides are denoted with an asterisk.

Table 3
Quantification of the selenium content of FCS, L-15 medium, L-15 medium plus 1% FCS (control) and the medium plus different selenium treatments. The concentration of selenium was quantified by ICP-MS in each sample. Data are presented as the mean and SEM of the amount of two selenium isotopes (^{77}Se and ^{78}Se) measured in duplicate, and the *p* value of a paired-sample *t*-test comparing them. <1 μg/L represents data below the detectable level of this method.

Samples	Selenium measured (μg/L)		
	Average	SEM	*p*-value
L-15	<1	–	–
FCS	11.15	0.29	0.42
L-15 + FCS 1%	>1	–	–
L-15 + FCS 1% + Na_2SeO_3 10 nM	>1	–	–
L-15+ FCS 1% + Cys-Se-Se-Cys 10 nM	>1	–	–
L-15 + FCS 1% + Na_2SeO_3 100 nM	5.40	0.67	0.21
L-15 + FCS 1% + Cys-Se-Se-Cys 100 nM	14.04	0.13	4.30
L-15 + FCS 1% + Na_2SeO_3 1 μM	71.73	0.46	0.49
L-15 + FCS 1% + Cys-Se-Se-Cys 1 μM	137.77	6.83	0.97
L-15 + FCS 1% + Na_2SeO_3 10 μM	749.74	6.46	1.00
L-15 + FCS 1% + Cys-Se-Se-Cys 10 μM	1734.78	47.71	0.99
L-15 + FCS 1% + Na_2SeO_3 50 μM	4005.65	98.31	0.72
L-15 + FCS 1% + Cys-Se-Se-Cys 50 μM	7403.90	295.98	0.73

than the level in the inorganic Se treatment, at every concentration tested.

3.4. Response of trout GPx1 and GPx4 in RTL cells exposed to Na_2SeO_3 and Cys-Se-Se-Cys

The transcript expression of trout *GPx1* and *GPx4* was examined in the trout RTL cell line (Fig. 5). All the *GPx1* isoforms had a higher sensitivity to the organic Se, and were all significantly transcriptionally induced at the lower concentrations (10 nM and 100 nM) compared to the control and were more highly induced compared to the inorganic Se treatment. In particular, *GPx1a* appeared to be the most responsive gene at the lower (10 nM and 100 nM) and intermediated (1 μM) concentrations, especially within the cells treated with organic Se. *GPx1b1* and *GPx1b2* induction was significantly higher in the cells treated with organic Se at every concentration used, relative to that seen with the inorganic form, and notably a significantly higher transcriptional up-regulation of these two genes was seen in the cells exposed to 50 μM of both the compounds tested in contrast to the results with *GPx1a*. *GPx1b2* had the highest induction detected of the *GPx1* isoforms, at concentrations of 10 μM and 50 μM of organic Se.

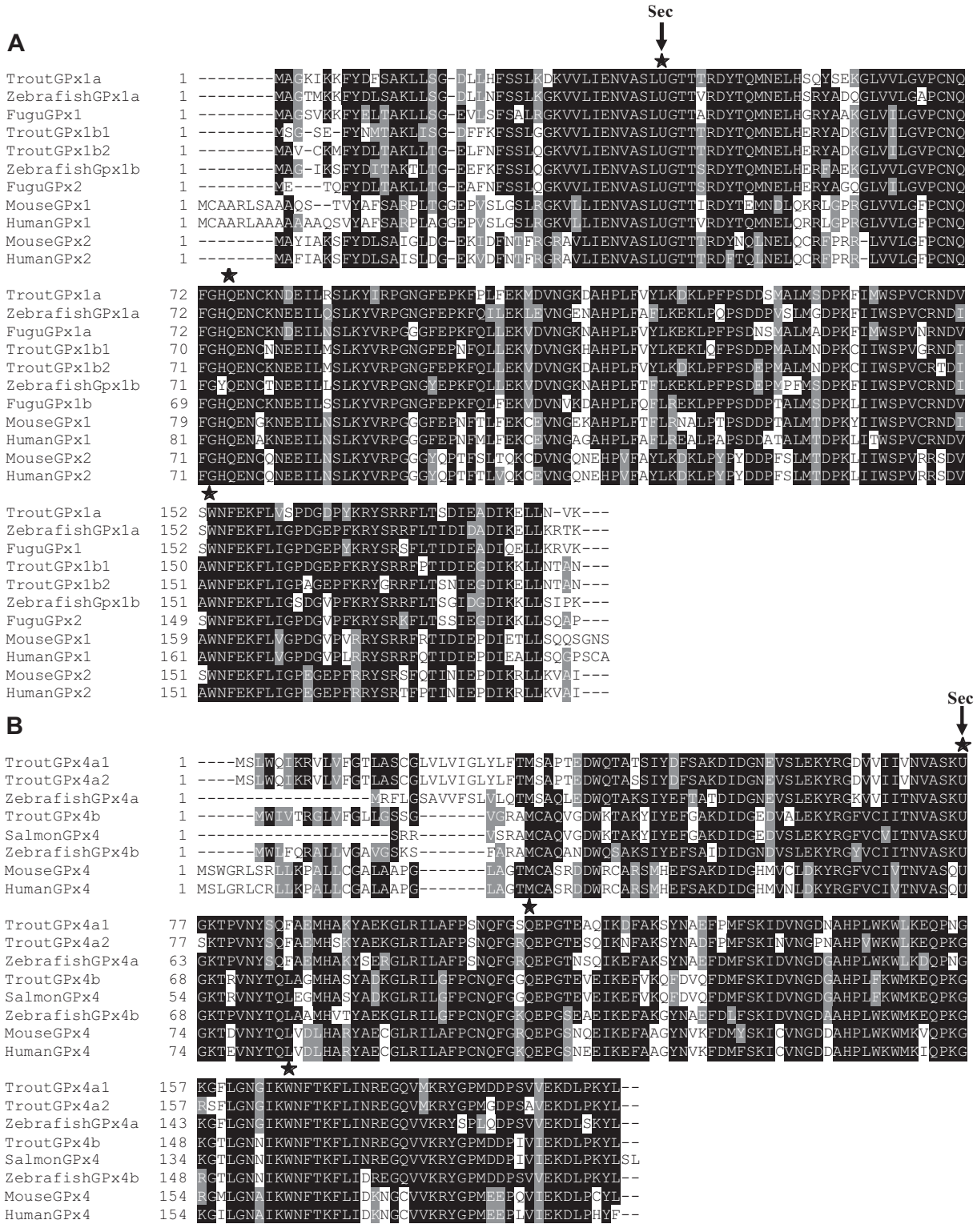
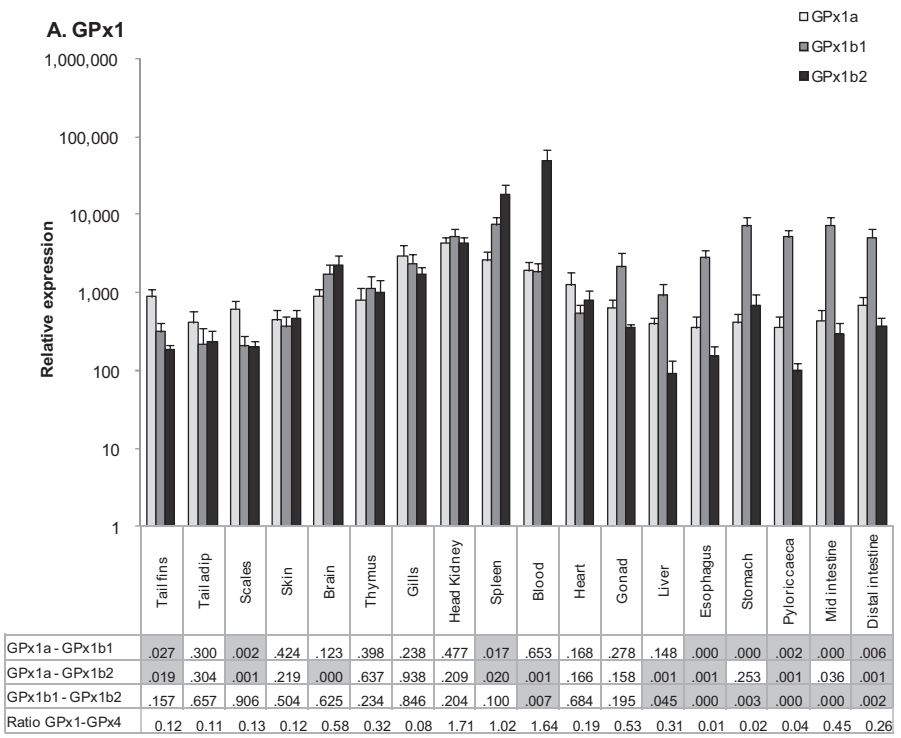
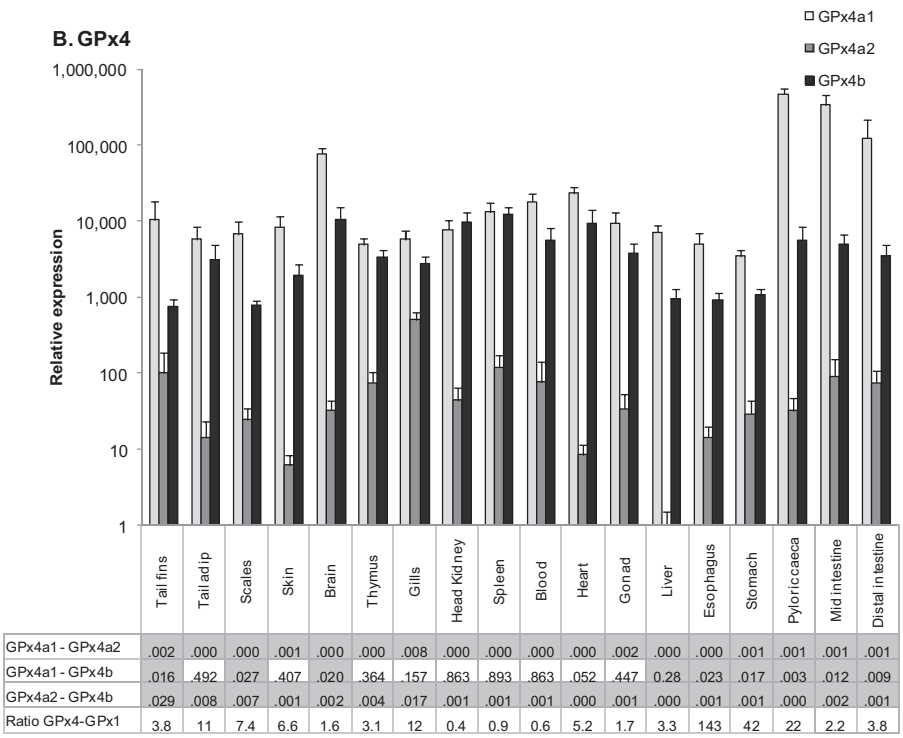


Fig. 3. Multiple alignment of the deduced amino acid sequences of trout GPx1 isoforms with known fish GPx1 molecules and selected mammalian GPx1 and GPx2 molecules (A), and trout GPx4 isoforms with selected GPx4 molecules from fish, and mammals (B). The multiple alignment was produced using ClustalW2 and conserved amino acids shaded using BOXSHADE (V3.21). The accession numbers for sequences used in this analysis are given in Fig. 1. Amino acid residues of the catalytic triad are marked with an asterisk, with the seleocysteine (SeCys) residue (U) marked with an arrow. Please, note that the salmon GPx4 is from an EST with an incompletely sequenced N-terminal region, and a possible error at the C-terminus.

Interestingly, comparing the transcriptional induction of *GPx1* isoforms within the Cys-Se-Se-Cys treatments with the induction of the same genes in cells treated with ten times higher concentrations of Na_2SeO_3 (since the organic form has a higher Se content relative to the inorganic form) all the *GPx1* isoforms were still more highly induced by the organic Se. For example, comparing *GPx1a* at 100 nM Cys-Se-Se-Cys *versus* 1 μM Na_2SeO_3 and at 1 μM Cys-Se-Se-Cys *versus* 10 μM Na_2SeO_3 it is clear that the organic



	Tail fins	Tail adip	Scales	Skin	Brain	Thymus	Gills	Head Kidney	Spleen	Blood	Heart	Gonad	Liver	Esophagus	Stomach	Pyloric caeca	Mid intestine	Distal intestine
GPx1a - GPx1b1	.027	.300	.002	.424	.123	.398	.238	.477	.017	.653	.168	.278	.148	.000	.000	.002	.000	.006
GPx1a - GPx1b2	.019	.304	.001	.219	.000	.637	.938	.209	.020	.001	.166	.158	.001	.001	.253	.001	.036	.001
GPx1b1 - GPx1b2	.157	.657	.906	.504	.625	.234	.846	.204	.100	.007	.684	.195	.045	.000	.003	.000	.000	.002
Ratio GPx1-GPx4	0.12	0.11	0.13	0.12	0.58	0.32	0.08	1.71	1.02	1.64	0.19	0.53	0.31	0.01	0.02	0.04	0.45	0.26



	Tail fins	Tail adip	Scales	Skin	Brain	Thymus	Gills	Head Kidney	Spleen	Blood	Heart	Gonad	Liver	Esophagus	Stomach	Pyloric caeca	Mid intestine	Distal intestine
GPx4a1 - GPx4a2	.002	.000	.000	.001	.000	.000	.008	.000	.000	.000	.000	.002	.000	.000	.001	.001	.001	.001
GPx4a1 - GPx4b	.016	.492	.027	.407	.020	.364	.157	.863	.893	.863	.052	.447	0.28	.023	.017	.003	.012	.009
GPx4a2 - GPx4b	.029	.008	.007	.001	.002	.004	.017	.001	.001	.001	.000	.001	.000	.001	.001	.000	.002	.001
Ratio GPx4-GPx1	3.8	11	7.4	6.6	1.6	3.1	12	0.4	0.9	0.6	5.2	1.7	3.3	143	42	22	2.2	3.8

Fig. 4. Relative transcript expression of trout *GPx1* and *GPx4* isoforms *in vivo*. The transcript expression level of *GPx1a*, *GPx1b1* and *GPx1b2* (A), and *GPx4a1*, *GPx4a2* and *GPx4b* (B) was determined by real time PCR in 18 tissues from six fish and expressed as arbitrary units that were first normalized against the transcript expression level of *EF-1α*. For comparison, the average transcript expression level of *GPx4b1* in liver, which has the lowest level among the six GPx genes in all the tissues examined, was defined as 1. The results were presented as the mean + SEM. The *p* value of a paired-sample *t* test comparing the expression of different isoforms of *GPx1* or *GPx4* is shown below, where significant differences between pairs of genes are shaded. The ratio of the average expression of the *GPx1* and *GPx4* isoforms in each tissue is also shown below.

form is more effective. Similarly, *GPx1b1* was more highly induced at 10 nM Cys-Se-Se-Cys *versus* 100 nM Na_2SeO_3, at 1 μM Cys-Se-Se-Cys *versus* 10 μM Na_2SeO_3 and at 10 μM Cys-Se-Se-Cys *versus* 50 μM Na_2SeO_3. Transcript expression of GPx1b2 gene was the most sensitive to organic Se, where the level of induction detected at 10 nM Cys-Se-Se-Cys was equivalent to that reached in the cells treated with 10 μM Na_2SeO_3. Among the *GPx1* isoforms, *GPx1b1* was the only form not responsive to inorganic selenium at the lower concentrations of 10 nM and 100 nM.

The *GPx4* isoforms appeared to be less sensitive to Se exposure. Amongst these *GPx4a1* was the most sensitive, in that it was the only isoform significantly induced by Se at the lowest

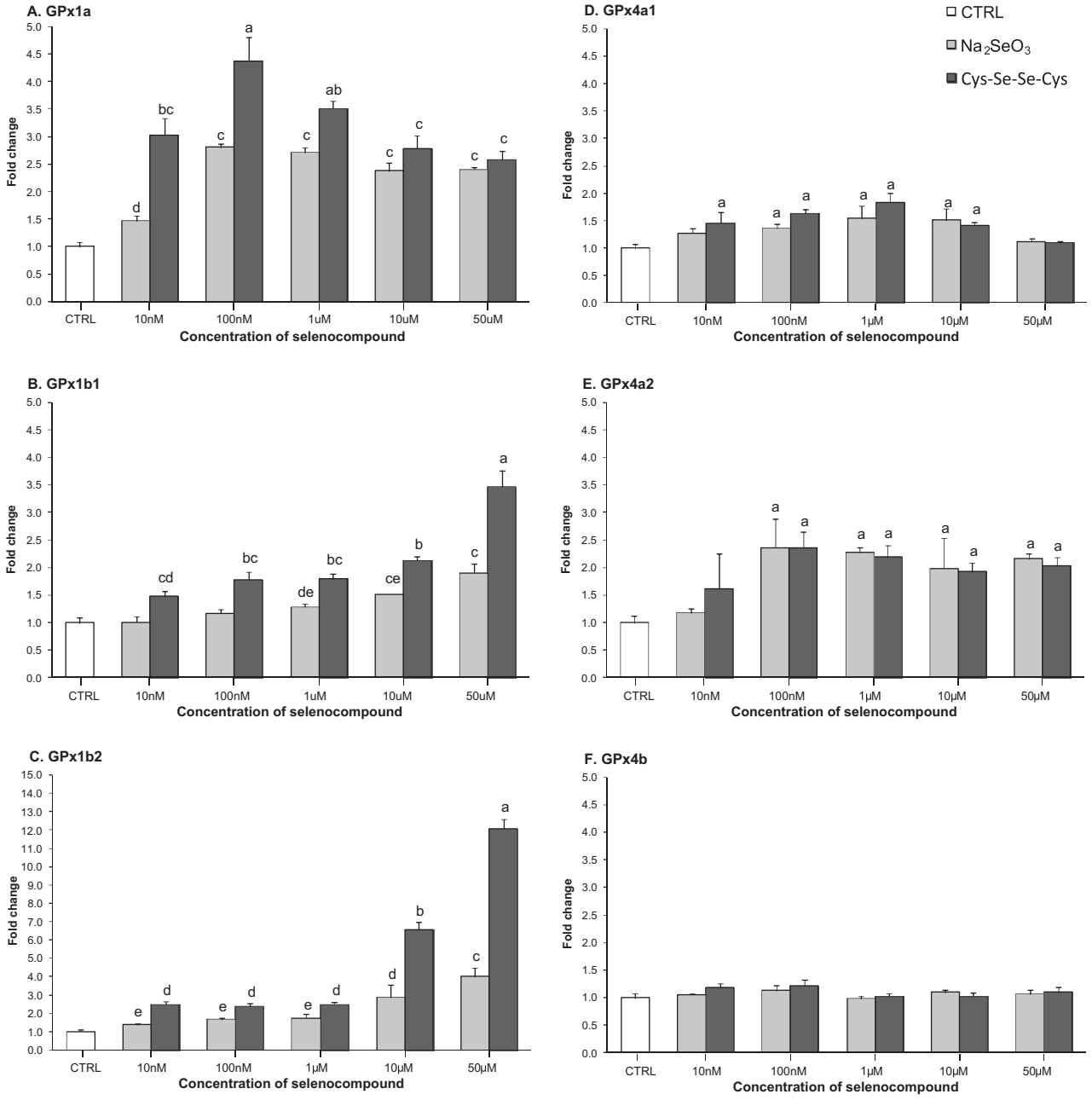


Fig. 5. Transcriptional modulation of trout *GPx1a* (A), *GPx1b1* (B), *GPx1b2* (C), *GPx4a1* (D), *GPx4a2* (E) and *GPx4b* (F) in the RTL cell line stimulated with Na_2SeO_3 and Cys-Se-Se-Cys at different concentrations, or medium as control. The treatments were terminated at 24 h post-stimulation and total RNA was isolated. The transcript expression of the GPx genes in RTL cells was quantified by real time PCR and normalised to the transcript expression level of *EF-1α* from the same sample, and then used for statistical analysis. A fold change, calculated as the average expression level of stimulated samples divided by that of the controls, is presented. The results represent the mean + SEM from four independent cultures. Values are statistically significant from the controls ($p < 0.05$) where marked with a letter, with different letters indicating significant differences between the treatment groups.

concentration (10 nM) used of the organic form. However, its induction returned to control levels at the highest concentration (50 μM) tested. At concentrations of 100 nM, 1 μM and 10 μM both *GPx4a1* and *GPx4a2* were similarly induced by the inorganic and organic Se treatments, with maximal increases of approx. 2-fold. *GPx4a2* was the only responsive gene at the highest concentration used. In contrast, *GPx4b* did not show any significant difference in expression level at any concentration tested relative to the control cells.

Since GPx1 genes were the most responsive under the conditions tested, the GPx1 enzymatic activity was also analyzed, to see if these increases in transcript level were reflected in the enzymatic activity detectable in the cell lysates. Although the three GPx1 genes showed different patterns of responsiveness to exposure to the two selencompounds studied, the increases in transcript level did not coincide with any significant increase in GPx1 activity (Supplementary Fig. S2).

3.5. *Effect of Na_2SeO_3 and Cys-Se-Se-Cys on cell viability*

Cell viability was evaluated by determining the cell membrane integrity, using the LDH assay (Fig. 6). The cell membrane integrity was comparable to control values in cells treated with

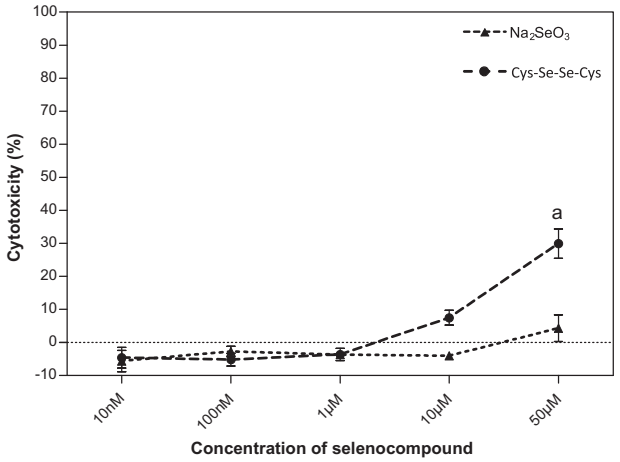


Fig. 6. Viability of RTL cells incubated with Na_2SeO_3 and Cys-Se-Se-Cys at different concentrations. The treatments were terminated at 24 h post-exposure and the cell viability was determined by analysing cell membrane integrity, using the LDH assay. Data represent the mean expressed as percentage of the control (0 nM) ± SEM of three different cultures. Values are statistically significant from the controls ($p < 0.05$) where marked with a letter, with different letters indicating significant differences between the treatment groups.

both inorganic and organic Se up to a concentration of 1 μM. At 10 μM and over, a marked difference between the two treatments was recorded. Integrity was slightly reduced (by 4.5%) using the inorganic Se at a concentration of 50 μM, but with the organic Se treatment membrane integrity was reduced by 7.5% and 30% at concentrations of 10 μM and 50 μM respectively, although only this last result was statistically significant from the control.

4. Discussion

It is recognized that selenium is an essential component for several biological pathways at the molecular, cellular and systemic level in every living organism (Neve, 1991). Selenium deficiency can heavily compromise the health of an organism, leading to cardiovascular pathology, disruption of reproduction, impairment of immune defences and as a consequence of this pathogen outbreaks (Rayman, 2000). Due to the importance of this trace element in biological systems, the use of selenium as a food and feed supplement in animal farming, including aquaculture, is increasing (Watanabe et al., 1997; Kiron, 2012). In the aquatic environment, selenium is widely distributed and a concern regarding elevated levels of Se for the biota is its ability to bioaccumulate through the food. Thus, at levels above those required selenium can have adverse effects. In animals, Se toxicity produces conditions know as "blind staggers" and "alkali disease", that result in tissue lesions, reproductive abnormalities and even death (O'Toole and Raisbeck, 1995; O'Toole et al., 1996).

Dietary requirements for fish have been reported to range between 0.1–0.5 μg Se g^{-1} (dry mass) (Hodson and Hilton, 1983; Gatlin and Wilson, 1984). However, recent investigations had demonstrated that the Se required level may increase up to 4.0 μg Se g^{-1} (dry mass) in fish subject to stress conditions (KüÜkbay et al., 2009; Rider et al., 2009). Previous studies carried out in trout, reported that this species in normal condition could regulate Se accumulation through the excretion of up to 3.0 μg Se g^{-1} (dry mass), however over this level Se rapidly bioaccumulated and caused chronic toxicity to fish. For example, elevated mortality has been recorded in fish fed over 13.0 μg Se g^{-1} (Hilton et al., 1980). It has been documented that crustacean prey of salmonids may contain concentrations ≥3.0 μg Se g^{-1} and the normal feed diet used in salmonid farming provide a range between 1.5–3.3 μg Se g^{-1} (dry mass) (Julshamn et al., 1990; Lorentzen et al., 1994). Waterborne Se can also contribute to Se bioaccumulation in fish. Usually the concentration of Se found in the water column is relatively low, approximately 0.1–0.4 μg Se l^{-1}, but in polluted conditions concentrations of 40–130 μg Se l^{-1} can exert toxicity (Watanabe et al., 1997).

A wide range of levels for Se requirement/toxicity in fish has been reported (Hamilton, 2004). This range is likely due to the various types of selenocompounds assimilated by the fish. Recent studies have demonstrated that organic selenocompounds exhibit several advantages over inorganic forms, such as increased Se retention, higher resistance to stress (*e.g.* crowding conditions, hypoxia and handling), lower induction of lipid peroxidation and increased antioxidant capacity (KüÜkbay et al., 2009; Rider et al., 2009). In addition, different required/tolerated levels are often influenced by the experimental conditions and which tissues and biomarkers are used. In fish the anterior intestine is believed to be the principal route for dietary Se assimilation, and the liver the main organ for metabolism. The kidney can also accumulate significant amounts of selenium during the process of excretion (Hodson and Hilton, 1983). A recent meta-analysis study reviewed the toxic thresholds of Se in the diet and body, and highlighted the importance of considering ovary-based threshold levels (DeForest et al., 1999). Indeed, the primary effect of selenium on fish populations is manifested *via* maternal transfer from the ovaries to the eggs (Gillespie and Baumann, 1986; Woock et al., 1987; Schultz and Hermanutz, 1990; Hermanutz et al., 1992), with the dietary pathway being the most important exposure route for juvenile and adult fish (Sandholm et al., 1973; Bertram and Brooks, 1986; Besser et al., 1993).

The symptoms of Se toxicity in fish have been extensively reviewed (Lemly, 1998, 2002). Most of the studies have determined the required and toxic range of Se in fish, based on mortality, growth abnormalities and histological survey data. Also, the activity of antioxidant enzymes, such as GPx and other markers of oxidative stress, have been extensively used in fish both *in vitro* and *in vitro* (Dörr et al., 2008; Misra and Niyogi, 2009; Rider et al., 2009, 2010; Elia et al., 2011; Misra et al., 2012a). Nevertheless, more in depth investigations on the mechanisms of Se uptake and its metabolism at the cellular level in fish are warranted. Although the metabolism of selenocompounds in zebrafish (*Danio rerio*) has been described in the KEGG (Kyoto Encyclopedia of Genes and Genome) metabolic pathways database, functional characterization at the metabolite level has not been completed (Misra et al., 2010). Without this information on Se metabolism, it is still difficult to be sure of the optimal Se requirement in the diet and to evaluate which form is the most bioavailable and least toxic. Identification and characterization of genes associated with Se metabolism (*i.e.* selenoprotein genes) in fish may allow a better understanding of the impact of Se on homeostasis in these animals. Also, the analysis of Se-related gene expression can represent a sensitive biomarker to monitor Se intake in fish and potentially redefine the threshold of requirement *vs* toxicity for different selenocompounds.

In this study we have cloned and characterized several rainbow trout, *Oncorhynchus mykiss*, GPx1 and GPx4 genes. The clustering of the sequences in the phylogenetic tree confirmed the association of the sequences characterized in trout with the respective GPx clades. Interestingly three isoforms for both *GPx1* (*GPx1a*, *1b1* and *1b2*) and *GPx4* (*GPx4a1*, *a2* and *b*) genes have been identified in this species. Previously two isoforms of *GPx1* (*GPx1a* and *1b*) and *GPx4* (*GPx4a* and *b*) have been found in teleost fish, such as *Danio rerio* (Kryukov and Gladyshev, 2000). The constructed phylogenetic tree indicates the fish GPx4 is an orthologue of the mammalian molecule, whereas the fish GPx1 molecules are not clear orthologues of mammalian GPx1 or GPx2. GPx2 (gastro-intestinal glutathione peroxidase,

GI-GPx) is characterized only in mammalian models so far (Chu et al., 1993) and it is predominantly expressed in proliferative zones of the intestinal mucosa (Florian et al., 2001). If the fish GPx1 molecule is related to an ancestral molecule that subsequently gave rise to mammalian GPx1 and GPx2 it is possible that functional divergence of the different fish paralogs may have occurred in an analogous manner. In this context, our results from the tissue distribution analysis of the trout GPx1 isoforms perhaps suggest that the trout *GPx1b1* isoform may be more related functionally to GPx2.

It is recognized that teleost fish possess a large selenoproteome (Lobanov et al., 2009) and our results hint that salmonids may have the biggest set of selenoproteins amongst all vertebrates. The presence of two copies of *GPx1* (*GPx1a* and *GPx1b*) and *GPx4* (*GPx4a* and *GPx4b*) genes in zebrafish and other teleosts is likely the result of a third-round whole genome duplication (WGD) event that occurred during teleost evolution (Dehal and Boore, 2005; Volff, 2005). Afterwards, a fourth round of WGD occurred in Salmonid fish, which underwent a diploidization of their chromosomic set. As pseudotetraploid organisms, Salmonids (*e.g.* rainbow trout and Atlantic salmon) may possess three to four copies of genes present as a single copy in mammals and in duplicate in other bony fish (Ohno et al., 1968; Allendorf and Thorgaard, 1984). Sequence analysis confirmed the presence of the catalytic triad, including the selenocysteine residue, within the trout GPx1 and GPx4 proteins, which are universally conserved in all phyla (Herbette et al., 2007). The presence of a SECIS element within the 3′-UTR was also confirmed in all the sequences identified. In the *GPx1* isoforms, the SECIS element was formed by the motif 5′TGAA3′:5′TGAT3′, whilst in *GPx4* 5′TGAC3′:5′TGAT3′ was used.

As GPx1 and GPx4 are important components of the intracellular antioxidant defences of many cell types, we analysed where these genes are tanscribed in a wide range of tissues. *GPx4* isoforms were highly transcribed in all tissues examined compared with *GPx1* isoforms, with the exception of blood, head kidney and spleen. The higher constitutive transcription of *GPx1* in the two main hematopoietic tissues in fish (head kidney and spleen) (Catton, 1951; Ellis et al., 1978) may be associated to the high amount of ROS (especially H_2O_2) produced during hematopoiesis and their essential role in the regulation of hematopoietic growth factor (HGF) signalling (Sattler et al., 1999; Ghaffari, 2008). The high level of GPx1 in the blood may be associated with the high oxidative stress that occurs in erythrocytes, being continuously exposed to ROS as the main oxygen carriers in the organism (Mills, 1957). The transcript expression of GPx4 (*GPx4a1*) was particularly high in the gut, a site that is likely to be continuously exposed to pro-oxidant compounds and oxidative species in water coming from the external environment, with the potential to damage cell membranes (Wilhelm et al., 1993; Wilhelm, 2007). In addition, the stomach and pyloric cecae are involved in the digestion and absorption of nutrients, which may also easily contain exogenous compounds with oxidative properties, such as iron, that can react with ascorbate released during digestion to give rise to ROS formation through Fenton chemistry (Kadiiska et al., 1995; Halliwell and Gutteridge, 1999). Whilst trout *GPx1* and *GPx4* isoforms were expressed in every tissue examined, *GPx4a2* showed the lowest transcript expression, with the lowest transcript expression level in the liver.

The effect of Se availability on the transcript expression of GPx1 and GPx4 genes was tested *in vitro*. Preliminary experiments were conducted using a wide range of concentrations (from 1 nM to 200 μM, in 1:10 serial dilution) to define an optimal range where effects on gene transcription following Se exposure could be detected but severe adverse effect on cell viability were avoided. The *GPx1* isoforms showed a relevant induction in all the selenium treatments. *GPx1a* was more induced by selenium at low/intermediate concentrations up to 1 μM, whereas *GPx1b1* and *GPx1b2* showed a higher up-regulation at increasing concentrations, with maximum transcript expression reached at the highest concentration used (50 μM) for both organic and inorganic selenium. The transcriptional up-regulation of GPx1 genes was significantly higher with the organic selenium treatments at most of the concentrations tested, even when comparing the induction by Cys-Se-Se-Cys treatment to cells exposed to a ten times higher concentration of Na_2SeO_3. The organic form had more effects on transcript expression of *GPx1* isoform and *GPx1b2* was clearly the most induced by organic selenium exposure.

The viability assay showed a reduction of membrane integrity at the highest concentration of Cys-Se-Se-Cys. The present results suggest that Cys-Se-Se-Cys can be toxic to fish cells *in vitro* above a concentration of 10 μM, whereas Na_2SeO_3 was only found to have a toxic effect (albeit small) at 50 μM. The common assumption in selenium cytoxicity studies is that inorganic selenocompounds are less bioavailable and more toxic at higher concentrations, whereas organic forms are more bioavailable and tolerated (Hoefig et al., 2011). However, it is also possible that Cys-Se-Se-Cys being more bioavailable can reach higher concentrations within a cell, which ultimately induce selenium toxic effects. The presence of two selenium residues also can contribute to its adverse effects on cell viability. Previous studies *in vivo* have shown that Cys-Se-Se-Cys is able to induce toxicity in mice hepatocytes, by inducing the formation of ROS and inhibiting the suppression of the Se methylation (the main mechanism to metabolise and detoxify selenocompounds in cells) (Hasegawa et al., 1996). Another study conducted *in vitro* showed that Cys-Se-Se-Cys had cytotoxic effects on splenic lymphocytes isolated from mice in the concentration range 250 nM to 100 μM (Santhosh Kumar et al., 2009). To date few studies have been conducted to determine the therapeutic properties *versus* the toxic effects of Cys-Se-Se-Cys, especially in fish models (Bjerregaard et al., 2011; Huang et al., 2012a; Huang et al., 2012b). Further studies are clearly required to better elucidate the metabolism and biological effects of this diselenocompound, both *in vitro* and *in vitro*.

A comparison of the results obtained from the expression analysis and the viability assay suggests that the *GPx1a* up-regulation detected at concentrations ranging from 10 nM to 1 μM is only related to the selenium availability, whereas the notable up-regulation of *GPx1b1* and *GPx1b2* at the highest concentrations used (10 and 50 μM) may be a direct effect of cell stress caused by selenium. Since trout have a larger number of GPx1 genes, different roles of the three isoforms is likely through subfunctionalisation, with *GPx1b1* and *GPx1b2* apparently induced preferentially in response to oxidative stress.

The higher transcript response to Se of trout GPx1 compared to the GPx4 genes supports the hypothesis that within the selenoproteome and even within the same selenoprotein family there are proteins with an essential role in cellular function but that are less responsive to selenium availability, as well as proteins that are non essential but are more involved in protection from environmental stress and selenium availability (Carlson et al., 2005). Indeed, gene knock out studies in mice have shown that GPx4 (Yant et al., 2003), along with other selenoproteins such as TrxR3 and DIO2 (Schneider et al., 2001; Conrad et al., 2004), has an essential role in cellular function and removal is lethal or results in an abnormal phenotype. In contrast, mammalian GPx1 (Cheng et al., 1998) and GPx2 (Esworthy et al., 2001) probably have non-essential functions since their removal in knockout mice manifests little or no phenotypic modification. In selenium deficient rats GPx1 activity is reduced to 1% of normal levels in liver, and to $\sim 4 \pm 9\%$ in kidney, heart and lung, whilst GPx4 activity is decreased to $25 \pm 50\%$ in the same tissues and is unaffected by selenium deprivation in testes. The dramatic decline in GPx1 activity upon selenium deprivation is due in large part to a rapid turnover of the mRNA for

this protein, and transcriptional control of expression (Saedi et al., 1988; Christensen and Burgener, 1992; Xin et al., 1995).

Although the transcript expression of *GPx1* isoform was significantly modulated in this study by the two selenocompounds, no significant change was seen on the enzymatic activity of the two different treatments at the concentrations tested. The absence of any induction of GPx1 activity upon exposure to Na_2SeO_3 at concentrations up to 50 μM confirmed previous results obtained in trout isolated hepatocytes exposed to different concentrations (ranging from 50 to 200 μM) of Na_2SeO_3 for 24 h (Misra and Niyogi, 2009). However, studies of the effect of Cys-Se-Se-Cys on GPx1 activity *in vitro* are lacking. A study conducted on trout isolated hepatocytes exposed to different concentrations of selenomethionine (SeMet) for 24 h showed that the GPx1 activity was slightly induced only at a concentration equal to 1000 μM (Misra et al., 2012a). However, it is not possible to compare SeMet and Cys-Se-Se-Cys because firstly they are a mono- and di-selenocompound, respectively, and secondly (as discussed above) every selenocompound may have different bioavailability and bioactivity.

The reason why there was no change in GPx1 activity may relate to it being a homotetramer, with even higher fold-increases in transcript level being needed to influence the activity of the enzyme. Moreover, GPx1 contributes only partially to the global enzymatic activity and this could explain the differences observed between mRNA level and enzymatic activity. Furthermore, a different sensitivity towards the inducers at the transcript level, mRNA processing, or transport and protein stability may lead to discrepancies between transcript expression and enzyme activity (Okey, 1990). Finally, in this particular study the different GPx1 isoforms showed two different kinds of responses, one related to the Se availability and the other to Se-induced stress. These two different patterns of transcript expression make it difficult to define a trend at the level of enzymatic activity. However, our results are consistent with Ideker et al. (2001) and Griffin et al. (2002) who found a low correlation coefficient ($r^2 = 0.61$) between mRNA and protein level changes depending on the gene investigated. Tukey and Johnson (1990) also postulated a different turnover and duration of induction between gene expression and enzyme activity. To our knowledge, only one previous study has investigated the correlation between GPx1 transcript level and enzymatic activity in a fish model, and in agreement with our results they did not detect any change at the activity level despite a significant modulation of transcription (Doyen et al., 2012). Thus, further studies are required to quantify at what point the level of gene transcription influences the activity of the protein. Also, at this stage it is not possible to determine if the different isoforms contribute equally, or disproportionately, to the synthesis and activity of the protein.

5. Conclusions

In summary we have cloned three paralogs of GPx1 (*GPx1a*, *GPx1b1* and *GPx1b2*) and GPx4 (*GPx4a1*, *GPx4a2* and *GPx4b*) in rainbow trout. The sequences have been analyzed with bioinformatics tools to confirm their identification as genes encoding GPx proteins; by homology and phylogenetic tree analysis, the conservation of the catalytic sites and the presence of a SECIS element in the 3′-UTR, fundamental characteristics of every selenoprotein. Constitutive transcript expression was found in 18 different tissues studied, with GPx4 more highly transcribed in most tissues examined (except blood, head kidney and spleen). With the exception of *GPx4b*, the GPx genes, and especially *GPx1* isoforms, were responsive to selenium exposure. *GPx1a* was the most sensitive to selenium availability in non stressful conditions, whereas *GPx1b1* and *GPx1b2* were highly induced by exposure to selenium levels that had some toxic effects on the cells. However, no significant effect on GPx1 enzymatic activity was detected. Thus, we conclude that transcript expression of trout GPx1 gene may be a sensitive biomarker of selenium exposure *in vitro*, and may aid the evaluation of the impact of selenium concentration and chemical speciation on cell homeostasis. In addition, the *in vitro* approach used here has allowed us to describe, characterize and delimit Se effects, and gives complementary information for further investigations *in vivo*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2012.12.020.

References

Allendorf, F.W., Thorgaard, G.H., 1984. Tetraploidy and the evolution of salmonid fishes. Evolutionary Genetics of Fishes, 1–53.
Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST A new generation of protein database search programs. Nucleic Acids Research 25, 3389–3402.
Anundi, I., Stahl, A., Hogberg, J., 1984. Effects of selenite on O2 consumption glutathione oxidation and NADPH levels in isolated hepatocytes and the role of redox changes in selenite toxicity. Chemico-Biological Interactions 50, 277–288.
Apel, K., Hirt, H., 2004. Reactive oxygen species Metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology 55, 373–399.
Arduser, F., Wolffram, S., Scharrer, E., 1985. Active absorption of selenate by rat ileum. Journal of Nutrition 115, 1203–1208.
Arthur, J.R., 2000. The glutathione peroxidases. Cellular and Molecular Life Sciences 57, 1825.
Beilstein, M.A., Whanger, P.D., 1992. Selenium metabolism and glutathione peroxidase activity in cultured human lymphoblasts Effects of transsulfuration defects and pyridoxal phosphate. Biological Trace Element Research 35, 105–118.
Berry, M.J., Banu, L., Chen, Y., Mandel, S.J., Kiefer, J.D., Harney, J.W., Larsen, P.R., 1991. Recognition of UGA as a selenocysteine codon in Type I deiodinase requires sequences in the 3' untranslated region. Nature 353, 273–276.
Berry, M.J., Banu, L., Harney, J.W., Larsen, P.R., 1993. Functional characterization of the eukaryotic SECIS elements which direct selenocysteine insertion at UGA codons. EMBO Journal 12, 3315–3322.
Bertram, P.E., Brooks, A.S., 1986. Kinetics of accumulation of selenium from food and water by fathead minnows. Water Research 20, 877–884.
Besser, J.M., Canfield, T.J., La Point, T.W., 1993. Bioaccumulation of organic and inorganic selenium in a laboratory food chain. Environmental Toxicology and Chemistry 12, 57–72.
Birringer, M., Pilawa, S., Flohé, L., 2002. Trends in selenium biochemistry. Natural Product Reports 19, 693–718.
Bjerregaard, P., Fjordside, S., Hansen, M.G., Petrova, M.B., 2011. Dietary selenium reduces retention of methyl mercury in freshwater fish. Environmental Science & Technology 45, 9793–9798.
Bock, A., Forchhammer, K., Heider, J., Baron, C., 1991. Selenoprotein synthesis: an expansion of the genetic code. Trends in Biochemical Sciences 16, 463–467.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry 72, 248–254.
Burk, R.F., Hill, K.E., 1993. Regulation of selenoproteins. Annual Review of Nutrition 13, 65–81.
Campanella, J., 2003. MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences. BMC Bioinformatics 4, 29.
Carlson, B.A., Xu, X., Gladyshev, V.N., Dolph, L.H., 2005. Selective rescue of selenoprotein expression in mice lacking a highly specialized methyl group in selenocysteine tRNA. Journal of Biological Chemistry 280, 5542–5548.
Catton, W.T., 1951. Blood cell formation in certain teleost fishes. Blood 6, 39–60.
Chaudiere, J., Courtin, O., Leclaire, J., 1992. Glutathione oxidase activity of selenocystamine A mechanistic study. Archives of Biochemistry and Biophysics 296, 328–336.
Cheng, W., Ho, Y., Valentine, B.A., Ross, D.A., Combs Jr., G.F., Lei, X.G., 1998. Cellular glutathione peroxidase is the mediator of body selenium to protect against paraquat lethality in transgenic mice. Journal of Nutrition 128, 1070–1076.
Chenna, R., 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Research 31, 3497–3500.
Christensen, M.J., Burgener, K.W., 1992. Dietary selenium stabilizes glutathione peroxidase mRNA in rat liver. Journal of Nutrition 122, 1620–1626.

Chu, F., Doroshow, J.H., Esworthy, R.S., 1993. Expression, characterization, and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. Journal of Biological Chemistry 268, 2571–2576.
Combs Jr., G.F., Combs, S.B., 1986. The Role of Selenium in Nutrition. Academic Press Inc, New York.
Conrad, M., Jakupoglu, C., Moreno, S.G., Lippl, S., Banjac, A., Schneider, M., Beck, H., Hatzopoulos, A.K., Just, U., Sinowatz, F., Schmahl, W., Chien, K.R., Wurst, W., Bornkamm, G.W., Brielmeier, M., 2004. Essential role for mitochondrial thioredoxin reductase in hematopoiesis heart development, and heart function. Molecular and Cellular Biology 24, 9414–9423.
Contempre, B., Le Moine, O., Dumont, J.E., Denef, J., Many, M.C., 1996. Selenium deficiency and thyroid fibrosis A key role for macrophages and transforming growth factor β (TGF-β). Molecular and Cellular Endocrinology 124, 7–15.
Copeland, P.R., Fletcher, J.E., Carlson, B.A., Hatfield, D.L., Driscoll, D.M., 2000. A novel RNA binding protein SBP2, is required for the translation of mammalian selenoprotein mRNAs. EMBO Journal 19, 306–314.
Dallinger, R., Prosi, F., Segner, H., Back, H., 1987. Contaminated food and uptake of heavy metals by fish: a review and a proposal for further research. Oecologia 73, 91–98.
DeForest, D.K., Brix, K.V., Adams, W.J., 1999. Critical review of proposed residue-based selenium toxicity thresholds for freshwater fish. Human and Ecological Risk Assessment 5, 1187–1228.
Dehal, P., Boore, J.L., 2005. Two rounds of whole genome duplication in the ancestral vertebrate. PLoS Biology 3, e314.
Dörr, A.J.M., Pacini, N., Abete, M.C., Prearo, M., Elia, A.C., 2008. Effects of a selenium-enriched diet on antioxidant response in adult crayfish (*Procambarus clarkii*). Chemosphere 73, 1090–1095.
Doyen, P., Rodius, F., L'Hoste, L., Hoffmann, L., Cauchie, H., 2012. Identification and mRNA expression of pi-class glutathione S-transferase and selenium-dependent glutathione peroxidase in the gudgeon Gobio gobio exposed to PCB 77. Comparative Biochemistry and Physiology C 155, 300–306.
Dumont, E., Vanhaecke, F., Cornelis, R., 2006. Selenium speciation from food source to metabolites A critical review. Analytical and Bioanalytical Chemistry 385, 1304–1323.
Elia, A.C., Prearo, M., Pacini, N., Dörr, A.J.M., Abete, M.C., 2011. Effects of selenium diets on growth accumulation and antioxidant response in juvenile carp. Ecotoxicology and Environmental Safety 74, 166–173.
Ellis, A.E., Roberts, R.J., Tytler, P., 1978. The anatomy and physiology of teleosts. Fish Pathology, 13–54.
El-Sayed, W.M., Aboul-Fadl, T., Lamb, J.G., Roberts, J.C., Franklin, M.R., 2006. Effect of selenium-containing compounds on hepatic chemoprotective enzymes in mice. Toxicology 220, 179–188.
Esworthy, R.S., Aranda, R., Martín, M.G., Doroshow, G.H., Binder, S.W., Chu, F.F., 2001. Mice with combined disruption of Gpx1 and Gpx2 genes have colitis. American Journal of Physiology – Gastrointestinal and Liver Physiology 281, G848–G855.
Fagegaltier, D., Hubert, N., Yamada, K., Mizutani, T., Carbon, P., Krol, A., 2000. Characterization of mSelB a novel mammalian elongation factor for selenoprotein translation. EMBO Journal 19, 4796–4805.
Finley, J.W., 2006. Bioavailability of selenium from foods. Nutrition Reviews 64, 146–151.
Fletcher, J.E., Copeland, P.R., Driscoll, D.M., Krol, A., 2001. The selenocysteine incorporation machinery: Interactions between the SECIS RNA and the SECIS-binding protein SBP2. RNA 7, 1442–1453.
Flohé, L., Brigelius-Flohé, R., 2012. Selenoproteins of the Glutathione Peroxidase Family, third ed. Springer New York.
Flohé, L., Gunzler, W.A., Schock, H.H., 1973. Glutathione peroxidise: A selenoenzyme. FEBS Letters 32, 132–134.
Florian, S., Wingler, K., Schmehl, K., Jacobasch, G., Kreuzer, O.J., Meyerhof, W., Brigelius-Flohé, R., 2001. Cellular and subcellular localization of gastrointestinal glutathione peroxidase in normal and malignant human intestinal tissue. Free Radical Research 35, 655–663.
Foster, L.H., Sumar, S., 1997. Selenium in health and disease: a review. Critical Reviews in Food Science and Nutrition 37, 211–228.
Ganther, H.E., 1986. Pathways of selenium metabolism including respiratory excretory products. Journal of the American College of Toxicology 5, 1–5.
Ganther, H.E., 1968. Selenotrisulfides Formation by the reaction of thiols with selenious acid. Biochemistry 7, 2898–2905.
Ganthert, H.E., 1966. Enzymic synthesis of dimethyl selenide from sodium selenite in mouse liver extracts. Biochemistry 5, 1089–1098.
Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., Bairoch, A., 2005. Protein Identification and Analysis Tools on the ExPASy Server, The Proteomics Protocols Handbook, Humana Press, pp. 571–607.
Gatlin, D.M., Wilson, R.P., 1984. Dietary selenium requirement of fingerling channel catfish. Journal of Nutrition 114, 627–633.
Ghaffari, S., 2008. Oxidative stress in the regulation of normal and neoplastic hematopoiesis. Antioxidants & Redox Signaling 10, 1923–1940.
Gillespie, R.B., Baumann, P.C., 1986. Effects of high tissue concentrations of selenium on reproduction by bluegills. Transactions of the American Fisheries Society 115, 208–213.
Griffin, T.J., Gygi, S.P., Ideker, T., Rist, B., Eng, J., Hood, L., Aebersold, R., 2002. Complementary profiling of gene expression at the transcriptome and proteome levels in *Saccharomyces cerevisiae*. Molecular & Cellular Proteomics 1, 323–333.
Guimarães, M.J., Peterson, D., Vicari, A., Cocks, B.G., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Ferrick, D.A., Kastelein, R.A., Bazan, J.F., Zlotnik, A., 1996. Identification of a novel selD homolog from Eukaryotes, Bacteria, and Archaea: Is there an autoregulatory mechanism in selenocysteine metabolism? Proceedings of the National Academy of Sciences of the United States of America 93, 15086–15091.
Halliwell, B.,Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine, Third ed. Oxford University Press.
Hamilton, S.J., 2004. Review of selenium toxicity in the aquatic food chain. Science of the Total Environment 326, 1–31.
Hasegawa, T., Mihara, M., Nakamuro, K., Sayato, Y., 1996. Mechanisms of selenium methylation and toxicity in mice treated with selenocystine. Archives of Toxicology 71, 31–38.
Hatfield, D.L., 2002. How selenium has altered our understanding of the genetic code. Molecular and Cellular Biology 22, 3565–3576.
Hefnawy, A.E.G., Tórtora-Pérez, J.L., 2010. The importance of selenium and the effects of its deficiency in animal health. Small Ruminant Research 89, 185–192.
Herbette, S., Roeckel-Drevet, P., Drevet, J.R., 2007. Seleno-independent glutathione peroxidases: More than simple antioxidant scavengers. FEBS Journal 274, 2163–2180.
Hermanutz, R.O., Allen, K.N., Roush, T.H., Hedtke, S.F., 1992. Effects of elevated selenium concentrations on bluegills (*Lepomis macrochirus*) in outdoor experimental streams. Environmental Toxicology and Chemistry 11, 217–224.
Hilton, J.W., Hodson, P.V., Slinger, S.J., 1980. The requirement and toxicity of selenium in rainbow trout (*Salmo gairdneri*). Journal of Nutrition 110, 2527–2535.
Hodson, P.V., Hilton, J.W., 1983. The Nutritional Requirements and Toxicity to Fish of Dietary and Waterborne Selenium. Ecological Bulletins 35, 335–340.
Hoefig, C.S., Renko, K., Köhrle, J., Birringer, M., Schomburg, L., 2011. Comparison of different selenocompounds with respect to nutritional value vs. toxicity using liver cells in culture. Journal of NutritionalBiochemistry 22, 945–955.
Huang, S.S.Y., Strathe, A.B., Hung, S.S.O., Boston, R.C., Fadel, J.G., 2012a. Selenocompounds in juvenile white sturgeon: Estimating absorption disposition, and elimination of selenium using Bayesian hierarchical modeling. Aquatic Toxicology 109, 150–157.
Huang, S.S.Y., Strathe, A.B., Wang, W., Deng, D., Fadel, J.G., Hung, S.S.O., 2012b. Selenocompounds in juvenile white sturgeon: Evaluating blood tissue, and urine selenium concentrations after a single oral dose. Aquatic Toxicology 109, 158–165.
Huber, R.E., Criddle, R.S., 1967. Comparison of the chemical properties of selenocysteine and selenocystine with their sulfur analogs. Archives of Biochemistry and Biophysics 122, 164–173.
Ideker, T., Thorsson, V., Ranish, J.A., Christmas, R., Buhler, J., Eng, J.K., Bumgarner, R., Goodlett, D.R., Aebersold, R., Hood, L., 2001. Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. Science 292, 929–934.
Ip, C., Hayes, C., Budnick, R.M., Ganther, H.E., 1991. Chemical form of selenium critical metabolites, and cancer prevention. Cancer Research 51, 595–600.
Jackson, M.J., 1997. The assessment of bioavailability of micronutrients: introduction. European Journal of Clinical Nutrition 51 (Suppl. 1), S1L S2.
Janz, D.M., 2011. 7–Selenium. In Fish Physiology, Volume 31, Part A. Academic Press, pp. 327–374.
Jonnalagadda, S.B., Prasada Rao, P.V.V., 1993. Toxicity, bioavailability and metal speciation. Comparative Biochemistry and Physiology B 106, 585–595.
Julshamn, K., Sandnes, K., Lie, O., Waagbo, R., 1990. Effects of dietary selenium supplementation on growth, blood chemistry and trace element levels in serum and liver of adult Atlantic salmon (*Salmo salar*). Fiskeridirektoratets skrifter. Serie havundersøkelser 3, 47–58.
Kadiiska, M.B., Burkitt, M.J., Xiang, Q., Mason, R.P., 1995. Iron supplementation generates hydroxyl radical in vivo An ESR spin- trapping investigation. Journal of Clinical Investigation 96, 1653–1657.
Kiron, V., 2012. Fish immune system and its nutritional modulation for preventive health care. Animal Feed Science and Technology 173, 111–133.
Korzeniewski, C., Callewaert, D.M., 1983. An enzyme-release assay for natural cytotoxicity. Journal of Immunological Methods 64, 313–320.
Kryukov, G.V., Castellano, S., Novoselov, S.V., Lobanov, A.V., Zehtab, O., Guigó, R., Gladyshev, V.N., 2003. Characterization of mammalian selenoproteomes. Science 300, 1439–1443.
Kryukov, G.V., Gladyshev, V.N., 2000. Selenium metabolism in zebrafish: Multiplicity of selenoprotein genes and expression of a protein containing 17 selenocysteine residues. Genes to Cells 5, 1049–1060.
KüÜkbay, F.Z., Yazlak, H., Karaca, I., Sahin, N., Tuzcu, M., Cakmak, M.N., Sahin, K., 2009. The effects of dietary organic or inorganic selenium in rainbow trout (*Oncorhynchus mykiss*) under crowding conditions. Aquaculture Nutrition 15, 569–576.
Latrèche, L., 2009. Novel structural determinants in human SECIS elements modulate the translational recoding of UGA as selenocysteine. Nucleic Acids Research 37, 5868–5880.
Leblondel, G., Mauras, Y., Cailleux, A., Allain, P., 2001. Transport measurements across Caco-2 monolayers of different organic and inorganic selenium. Biological Trace Element Research 83, 191–206.
Lee, L.E.J., Clemons, J.H., Bechtel, D.G., Caldwell, S.J., Han, K.B., Pasitschniak-Arts, M., Mosser, D.D., Bols, N.C., 1993. Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. Cell Biology and Toxicology, 9, 279–294.
Lemly, A.D., 2002. Symptoms and implications of selenium toxicity in fish The Belews Lake case example. Aquatic Toxicology 57, 39–49.
Lemly, A.D., 1998. Pathology of selenium poisoning in fish. In Environmental Chemistry of Selenium. Marcel Dekker, pp. 281–354.
Lobanov, A.V., Hatfield, D.L., Gladyshev, V.N., 2009. Eukaryotic selenoproteins and selenoproteomes. Biochimica et Biophysica Acta 1790, 1424–1428.

Lorentzen, M., Maage, A., Julshamn, K., 1994. Effects of dietary selenite or selenomethionine on tissue selenium levels of Atlantic salmon (*Salmo salar*). Aquaculture 121, 359–367.
Low, S.C., Berry, M.J., 1996. Knowing when not to stop Selenocysteine incorporation in eukaryotes. Trends in Biochemical Sciences 21, 203–208.
Margis, R., Dunand, C., Teixeira, F.K., Margis-Pinheiro, M., 2008. Glutathione peroxidase family—An evolutionary overview. FEBS Journal 275, 3959–3970.
Mariotti, M., Ridge, P.G., Zhang, Y., Lobanov, A.V., Pringle, T.H., Guigo, R., Hatfield, D.L., Gladyshev, V.N., 2012. Composition and evolution of the vertebrate and mammalian selenoproteomes. PLoS One 7.
McConnell, K.P., Cho, G.J., 1967. Active transport of L-selenomethionine in the intestine. American Journal of Physiology 213, 150–156.
McConnell, K.P., Cho, G.J., 1965. Transmucosal movement of selenium. American Journal of Physiology 208, 1191–1195.
McKenzie, R.C., Arthur, J.R., Beckett, G.J., 2002. Selenium and the regulation of cell signaling, growth, and survival Molecular and mechanistic aspects. Antioxidants & Redox Signaling 4, 339–351.
Mills, G.C., 1957. Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. Journal of Biological Chemistry 229, 189–197.
Misra, S., Hamilton, C., Niyogi, S., 2012a. Induction of oxidative stress by selenomethionine in isolated hepatocytes of rainbow trout (*Oncorhynchus mykiss*). Toxicology in Vitro 26, 621–629.
Misra, S., Kwong, R.W.M., Niyogi, S., 2012b. Transport of selenium across the plasma membrane of primary hepatocytes and enterocytes of rainbow trout. Journal of Experimental Biology 215, 1491–1501.
Misra, S., Niyogi, S., 2009. Selenite causes cytotoxicity in rainbow trout (*Oncorhynchus mykiss*) hepatocytes by inducing oxidative stress. Toxicology in Vitro 23, 1249–1258.
Misra, S., Peak, D., Niyogi, S., 2010. Application of XANES spectroscopy in understanding the metabolism of selenium in isolated rainbow trout hepatocytes: Insights into selenium toxicity. Metallomics 2, 710–717.
Mozier, N.M., McConnell, K.P., Hoffman, J.L., 1988. S-adenosyl-L-methionine:thioether S-methyltransferase, a new enzyme in sulfur and selenium metabolism. Journal of Biological Chemistry 263, 4527–4531.
Nauser, T., Steinmann, D., Koppenol, W.H., 2012. Why do proteins use selenocysteine instead of cysteine? Amino Acids 42, 39–44.
Neve, J., 1991. Physiological and nutritional importance of selenium. Cellular and Molecular Life Sciences 47, 187–193.
NRC, 1980. Mineral Tolerance of Domestic Animals. Academic Press.
Ohno, S., Wolf, U., Atkin, N.B., 1968. Evolution from fish to mammals by gene duplication. Hereditas 59, 169–187.
Okey, A.B., 1990. Enzyme induction in the cytochrome P-450 system. Pharmacology & Therapeutics 45, 241–298.
O'Toole, D., Raisbeck, M.F., 1995. Pathology of experimentally induced chronic selenosis (alkali disease) in yearling cattle. Journal of Veterinary Diagnostic Investigation 7, 364–373.
O'Toole, D., Raisbeck, M., Case, J.C., Whitson, T.D., 1996. Selenium-induced blind staggers and related myths A commentary on the extent of historical livestock losses attributed to selenosis on Western US rangelands. Veterinary Pathology 33, 109–116.
Rayman, M.P., 2000. The importance of selenium to human health. Lancet 356, 233–241.
Rider, S.A., Davies, S.J., Jha, A.N., Clough, R., Sweetman, J.W., 2010. Bioavailability of co-supplemented organic and inorganic zinc and selenium sources in a white fishmeal-based rainbow trout (*Oncorhynchus mykiss*) diet. Journal of Animal Physiology and Animal Nutrition 94, 99–110.
Rider, S.A., Davies, S.J., Jha, A.N., Fisher, A.A., Knight, J., Sweetman, J.W., 2009. Supra-nutritional dietary intake of selenite and selenium yeast in normal and stressed rainbow trout (*Oncorhynchus mykiss*): Implications on selenium status and health responses. Aquaculture 295, 282–291.
Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G., Hoekstra, W.G., 1973. Selenium: Biochemical role as a component of glatathione peroxidase. Science 179, 588–590.
Roveri, A., Maiorino, M., Ursini, F., 1994. Enzymatic and immunological measurements of soluble and membrane-bound phospholipid-hydroperoxide glutathione peroxidase. Methods in Enzymology 233, 202–212.
Saedi, M.S., Smith, C.G., Frampton, J., Chambers, I., Harrison, P.R., Sunde, R.A., 1988. Effect of selenium status on mRNA levels for glutathione peroxidase in rat liver. Biochemical and Biophysical Research Communications 153, 855–861.
Sandholm, M., Oksanen, H.E., Pesonen, L., 1973. Uptake of selenium by aquatic organisms. Limnology & Oceanography 18, 496–499.
Santhosh Kumar, B., Kunwar, A., Ahmad, A., Kumbhare, L.B., Jain, V.K., Priyadarsini, K.I., 2009. In vitro radioprotection studies of organoselenium compounds Differences between mono- and diselenides. Radiation and Environmental Biophysics 48, 379–384.
Sattler, M., Winkler, T., Verma, S., Byrne, C.H., Shrikhande, G., Salgia, R., Griffin, J.D., 1999. Hematopoietic growth factors signal through the formation of reactive oxygen species. Blood 93, 2928–2935.
Schneider, M.J., Fiering, S.N., Pallud, S.E., Parlow, A.F., St Germain, D.L., Galton, V.A., 2001. Targeted disruption of the type 2 selenodeiodinase gene (DIO2) results in a phenotype of pituitary resistance to T4. Molecular Endocrinology 15, 2137–2148.
Schnurr, K., Belkner, J., Ursini, F., Schewe, T., Kühn, H., 1996. The selenoenzyme phospholipid hydroperoxide glutathione peroxidase controls the activity of the 15-lipoxygenase with complex substrates and preserves the specificity of the oxygenation products. Journal of Biological Chemistry 271, 4653–4658.
Schrauzer, G.N., 2000. Selenomethionine: A review of its nutritional significance, metabolism and toxicity. Journal of Nutrition 130, 1653–1656.
Schultz, R., Hermanutz, R., 1990. Transfer of toxic concentrations of selenium from parent to progeny in the fathead minnow (*Pimephales promelas*). Bulletin of Environmental Contamination and Toxicology 45, 568–573.
Spallholz, J.E., 1997. Free radical generation by selenium compounds and their prooxidant toxicity. Biomedical and Environmental Sciences 10, 260–270.
Stadtman, T.C., 1996. Selenocysteine. Annual Review of Biochemistry 65, 83–100.
Steve Hsieh, H., Ganther, H.E., 1975. Acid-volatile selenium formation catalyzed by glutathione reductase. Biochemistry 14, 1632–1636.
Suzuki, K.T., 2005. Metabolomics of selenium: Se metabolites based on speciation studies. Journal of Health Science 51, 107–114.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5 molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28, 2731–2739.
Thomas, J.P., Geiger, P.G., Maiorino, M., Ursini, F., Girotti, A.W., 1990. Enzymatic reduction of phospholipid and cholesterol hydroperoxides in artificial bilayers and lipoproteins. Biochimica et Biophysica Acta 1045, 252–260.
Toppo, S., Vanin, S., Bosello, V., Tosatto, S.C.E., 2008. Evolutionary and structural insights into the multifaceted glutathione peroxidase (Gpx) superfamily. Antioxidants & Redox Signaling 10, 1501.
Tukey, R.H., Johnson, E.F., 1990. Molecular aspects of drug metabolizing enzymes. In Principles of Drug Action, 423–468.
Ursini, F., Maiorino, M., Valente, M., 1982. Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides. Biochimica et Biophysica Acta 710, 197–211.
Ursini, F., Maiorino, M., Gregolin, C., 1985. The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. Biochimica et Biophysica Acta (BBA) – General Subjects 839, 62–70.
Valdiglesias, V., Pásaro, E., Méndez, J., Laffon, B., 2010. In vitro evaluation of selenium genotoxic, cytotoxic, and protective effects. A review. Archives of Toxicology 84, 337–351.
Volff, J., 2005. Genome evolution and biodiversity in teleost fish. Heredity 94, 280–294.
Walczak, R., Westhof, E., Carbon, P., Krol, A., 1996. A novel RNA structural motif in the selenocysteine insertion element of eukaryotic selenoprotein mRNAs. RNA 2, 367–379.
Wang, T., Hanington, P.C., Belosevic, M., Secombes, C.J., 2008. Two macrophage colony-stimulating factor genes exist in fish that differ in gene organization and are differentially expressed. Journal of Immunology 181, 3310–3322.
Wang, T., Diaz-Rosales, P., Costa, M.M., Campbell, S., Snow, M., Collet, B., Martin, S.A., Secombes, C.J., 2011. Functional characterization of a nonmammalian IL-21: rainbow trout Oncorhynchus mykiss IL-21 upregulates the expression of the Th cell signature cytokines IFN-gamma, IL-10, and IL-22. Journal of Immunology 186, 708–721.
Wang, T., Secombes, C.J., 2003. Complete sequencing and expression of three complement components, C1r, C4 and C1 inhibitor, of the classical activation pathway of the complement system in rainbow trout *Oncorhynchus mykiss*. Immunogenetics 55, 615–628.
Watanabe, T., Kiron, V., Satoh, S., 1997. Trace minerals in fish nutrition. Aquaculture 151, 185–207.
Wendel, A., 1981. Glutathione peroxidase. Methods in Enzymology 77, 325–333.
Wilhelm, F.D., Giulivi, C., Boveris, A., 1993. Antioxidant defences in marine fish–I. Teleosts. Comparative Biochemistry and Physiology C 106, 409–413.
Wilhelm, F.D., 2007. Reactive oxygen species, antioxidants and fish mitochondria. Frontiers in Bioscience 12, 1229–1237.
Wolffram, S., Anliker, E., Scharrer, E., 1986. Uptake of selenate and selenite by isolated intestinal brush border membrane vesicles from pig, sheep, and rat. Biological Trace Element Research 10, 293–306.
Wolffram, S., Berger, B., Grenacher, B., Scharrer, E., 1989. Transport of selenoamino acids and their sulfur analogues across the intestinal brush border membrane of pigs. Journal of Nutrition 119, 706–712.
Woock, S.E., Garrett, W.R., Partin, W.E., Bryson, W.T., 1987. Decreased survival and teratogenesis during laboratory selenium exposures to bluegill Lepomis macrochirus. Bulletin of Environmental Contamination and Toxicology 39, 998–1005.
Xin, G.L., Evenson, J.K., Thompson, K.M., Sunde, R.A., 1995. Glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase are differentially regulated in rats by dietary selenium. Journal of Nutrition 125, 1438–1446.
Yant, L.J., Ran, Q., Rao, L., Van Remmen, H., Shibatani, T., Belter, J.G., Motta, L., Richardson, A., Prolla, T.A., 2003. The selenoprotein GPX4 is essential for mouse development and protects from radiation and oxidative damage insults. Free Radical Research Communications 34, 496–502.