Amphibian transition to the oxidant terrestrial environment affects the expression of glutathione S-transferases isoenzymatic pattern

Fernanda Amicarelli[a,*], Stefano Falone[b], Franca Cattani[b], Marina T. Alamanou[a], Antonella Bonfigli[a], Osvaldo Zarivi[a], Michele Miranda[a], Anna M. Ragnelli[a], Carmine Di Ilio[b]

[a]*Department of Basic and Applied Biology, Faculty of Sciences, University of L'Aquila, Via Vetoio Loc, Coppito 67100 (AQ), Italy*
[b]*Department of Biomedical Sciences, Faculty of Medicine, University "G. D'Annunzio", Via dei Vestini, 66013 Chieti (CH), Italy*

Abstract

It has been postulated that glutathione S-transferases (GST; EC 2.5.1.18) may play a role in protecting against oxidative stress.

In previous studies, we have purified and characterised from *Bufo bufo* embryos a GST isoenzyme (BbGSTP1-1), which falls at very low level in the adult liver, where a novel isoform (BbGSTP2-2), starts to be highly expressed. During transition to adult life, *B. bufo* leaves the aquatic environment to live predominantly in the terrestrial environment, characterised by higher oxygen concentration.

It has been found that BbGSTP2-2 is more efficient in scavenging from organic hydroperoxides.

Therefore, the appearance of BbGSTP2-2 may respond to the necessity of providing the adult toad with a more suitable protection against oxygen toxic by-products. In this work, we performed experiments aimed at verifying if oxidative stress (hyperoxic and H_2O_2 treatments) could act as a modulator of BbGSTP2-2 expression. Results show that: (a) BbGSTP2 mRNA starts to be expressed in the late embryonic period, while protein appears during metamorphosis; (b) oxidative stress induces anticipation of BbGSTP2 gene expression at both transcriptional and translational levels.

These findings seem to indicate that the appearance of BbGSTP2-2 is aimed at endowing the adult toad with more efficient antioxidant defence in the terrestrial atmosphere.
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1. Introduction

The ability to withstand toxic chemicals and oxidative stress is essential for the survival of all organisms. Various mechanisms have evolved to protect cells against foreign compounds and reactive oxygen species (ROS), including efflux pumps, antioxidant proteins, reduced glutathione (GSH), drug metabolism, sequestration of toxins and DNA repair systems [1,2].

Cytosolic glutathione S-transferases (GST; EC2.5.1.18) are a superfamily of multifunctional dimeric proteins highly involved in detoxification processes as they catalyze the conjugation of a large variety of electrophilic toxic compounds to GSH [3–6]. GSTs also play a key role in the reduction of organic hydroperoxides and it has been postulated that they are part of a detoxifying system evolved to protect cells against potentially toxic by-products from oxidative metabolism [2,7,8]. GSTs are widely distributed in nature, being found in all eukaryotes and in many bacteria [3–5]. The structure and functions of mammalian GSTs have been largely investigated and the multitude of forms so far characterised can be grouped into at least 12 different classes, namely, Alpha, Beta, Delta, Kappa, Mu, Omega, Phi, Pi, Sigma, Tau, Theta and Zeta, according to their homologies and properties [9–19].

By comparison with mammalian GSTs, relatively little is known about amphibian GSTs.

In previous studies, we have purified and characterised a GST isoenzyme from the common toad (*Bufo bufo*) embryos at an early stage of development [20,21]. On the basis of

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its sequence homology, the *B. bufo* embryonic enzyme has been included into the Pi class and named BbGSTP1-1 [22]. Further studies have demonstrated that this isoform, expressed continuously up to the end of development, falls at very low levels in the adult liver, where a different isoform, i.e. BbGSTP2-2, comes to be expressed at elevated amount anyway [20,23,24]. BbGSTP2-2 never appears during embryonic development, while BbGSTP1-1 is the unique isoform present, always expressed and widespread [20,25]. Moreover, these two Pi isoenzymes are the major forms present in the adult [26].

With the transition to adult life, *B. bufo* leaves the aquatic environment to live predominantly in the terrestrial environment, characterised by the presence of higher oxygen concentration, which may cause an increase in the endogenous production of ROS with risk of oxidative injury. Therefore, this dramatic change in GST isoform expression may respond to the necessity of providing the adult toad with a more suitable protection against oxygen toxicity. Alternatively, it could be attributed to changes in feeding style, no longer dependent from maternal yolk, but from exogenous resources of food. However, studies performed on *Salmo iridaeus* and *Xenopus laevis*, who never leave the aquatic environment, showed that no changes occurred in GST expression pattern during the transition from embryonic to adult life [27,28], thus suggesting that the first hypothesis could be more likely.

On the basis of these results, to better address the physiological role of the adult isoform, in this work, we performed experiments aimed at verifying if oxidative stress could act as a modulator of BbGSTP2-2 expression. To this purpose, *B. bufo* embryos have been made to develop under oxidative stress conditions (hyperoxic and H_2O_2 treatment) and mRNA and protein expression of both isoforms were monitored during embryonic and metamorphic development. Results show that oxidative stress causes a significant anticipation in the expression of both transcript and protein BbGSTP2-2 products. This finding strongly supports the assumption that the appearance of the novel GST is aimed at endowing the adult toad with a more efficient antioxidant defence to better counteract oxidative challenges in the terrestrial atmosphere.

2. Materials and methods

2.1. Collection of embryos and larvae

Adult *B. bufo* were collected near L'Aquila (Italy). Ovulation and fertilization occurred in the laboratory. After deposition, the embryonic jelly was removed at a very early developmental stage by treatment with 1 volume 1% (v/v) thioglycolate (pH 8.0). Embryos were then cultured in tap water that was renewed daily. Embryos and larvae were staged according to Shumway or Taylor and Kollros, respectively [29].

2.2. Hyperoxic treatment of the embryos

The aim of this treatment was to try to obtain an oxidative stress condition resembling, as much as possible, the one occurring in the transition from the aquatic to the terrestrial environment. To this end, embryos deriving from the same deposition were divided into two groups (control and treated) at a very early embryonic stage (stage 7, morula) and developed in two plexiglass incubators in tap water at the temperature of 18 °C. For the treated group, the chamber was filled with 98% oxygen (v/v), at a constant pressure of 1 atm. In accordance with Henry's law, under this hyperoxic condition, the oxygen dissolved in 1 kg of water was 8.49 mg, five times higher with respect to the normoxic condition, namely almost the same increase in oxygen concentration occurring in the transition from the aquatic to the terrestrial environment [30]. The CO_2 produced by the embryo metabolism was continuously removed by Baralyme® (Chemetron Medical Division, Allied Healthcare Products, St. Louis, MO). At the following developmental stages: 9 (blastula), 16 (neurula), 20 (gill circulation), 22 (tail fin circulation), 25 (operculum complete), control and treated embryos not presenting morphological malformations under stereomicroscopic observation were collected and immediately processed or frozen at −80 °C until analysed. The experiment was repeated in quadruplicate, always starting from the same deposition divided into two groups (control and treated) at the morula stage. We could not extend our investigation to the metamorphic period, as the high oxygen concentration caused extensive mortality of the embryos just before entering into metamorphic development.

2.3. H_2O_2 treatment of the embryos

In order to verify that data obtained from hyperoxic embryonic treatment would be due to oxidative stress-induced GST modulation and not to oxygen toxicity, we thought to perform a less drastic oxidative stress treatment that, anyway, could be high enough to mimic the stress occurring in the transition to the terrestrial environment. To this purpose, we tested the effect of various H_2O_2 concentrations (from 50 to 800 μM) on embryo development. Results indicated that 400 μM H_2O_2 as the highest concentration allowing embryos both to perform development entirely and undergo a mortality rate not significantly different from that occurring in control embryos (not shown). On the basis of this information, embryos at the morula stage, belonging to the same deposition, were divided into two groups and developed at 18 °C in: (a) tap water (control group), and (b) 400 μM H_2O_2 (treated group). Hydrogen peroxide concentration was monitored spectrophotometrically to evaluate the rate of its degradation. On this basis, appropriate H_2O_2 aliquots were added at fixed times in order to assure a constant concentration during all the developmental period. Normally developed

embryos were collected and processed at the developmental stages: 9 (blastula), 16 (neurula), 20 (gill circulation), 22 (tail fin circulation), 25 (operculum complete). In addition, the following metamorphic stages: II–IV, VII, X, XV, XXV were also analysed. The experiment was repeated in quadruplicate, always starting from the same deposition divided into two groups (control and treated) at the morula stage.

2.4. RNA extraction

Total RNA extraction from control and treated *B. bufo* embryos and larvae was performed by RNAqueous kit (Ambion). Contaminant DNA was eliminated by treatment with DNase I (Roche) at 37 °C for 2 h. DNase was then inactivated by heating at 75 °C for 5 min. The quantity and quality of RNA extracted was assessed spectrophotometrically and by native 1% agarose gel electrophoresis in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8), running at 50 V for 2 h and staining with ethidium bromide (EtBr). The resulting RNA was then used for end point RT-PCR analysis.

2.5. Primer designing and end point RT-PCR

RT-PCR was realized using two primer pairs specific for amplification of BbGSTP1 and BbGSTP2 mRNAs, starting from the sequences of the two cDNAs, recently cloned in our laboratory [GenBank accession nos. AF533980 and AF533979]. Primers were designed using the software Biosoft Primer Premier and synthesized by Sigma-Genosys.

Primers sequences:

BbGSTP1

forward 5′-CTAATAATGAGGGGAAGGGGTTTG-3′ Tm 66.0 °C
reverse 5′-GGGACGGCTACTAATGCGTTTGAC-3′ Tm 70.0 °C

product length: 158 bp.

BbGSTP2

forward 5′-AGGGCGTGAGCTGGATTGATG-3′ Tm 70.6 °C
reverse 5′-TTCCTTGTTTGCCTCGTTGTC-3′ Tm 66.2 °C

product length: 284 bp.

RNA template was always 250 ng. RT was performed at 48 °C for 50 min and PCR conditions were 30 cycles at 94 °C for 45 s, 65 °C for 1 min and 72 °C for 1 min. Thermal cycler was a Hybaid PCR Sprint. To be sure that RT-PCR products were from mRNA, we used RT minus controls for each condition.
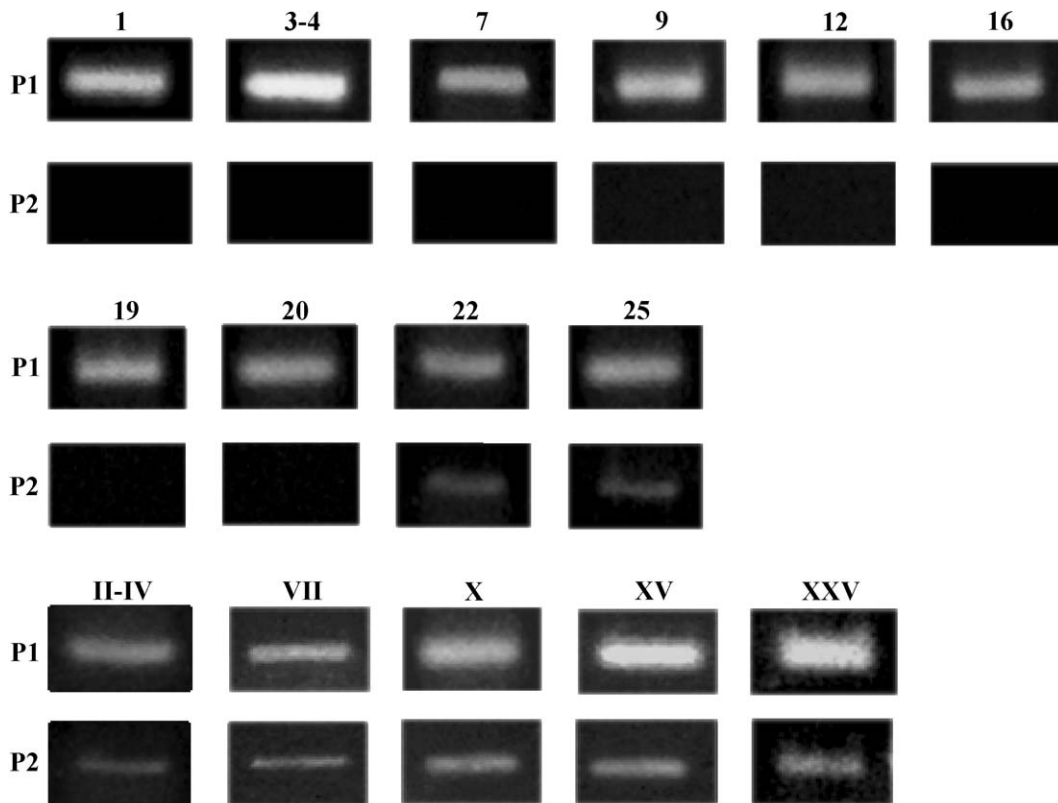

Fig. 1. Expression pattern of BbGSTP1 and BbGSTP2 mRNAs during *B. bufo* development. Post RT-PCR EtBr-stained agarose gel electrophoresis showing normoxic expression pattern of BbGSTP1 and BbGSTP2 mRNAs during embryonic (arabic numbers) and metamorphic (roman numbers) development. Gels photographed and scanned by Epson GT5500-Adobe Photoshop v.7.
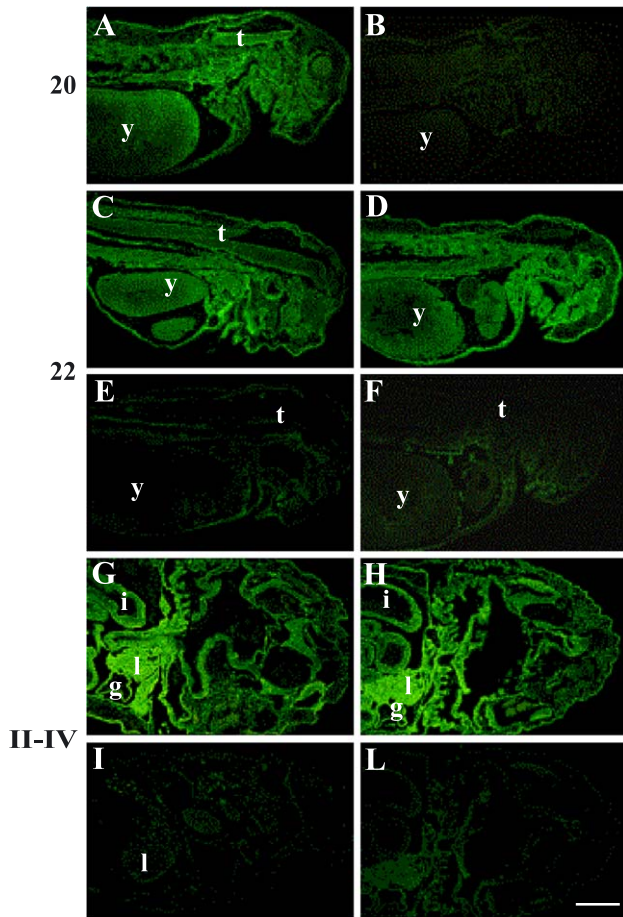
Fig. 2. Localization of BbGSTP1 and BbGSTP2 transcripts during *B. bufo* development. In situ hybridization of BbGSTP1 (A, C, G) and BbGSTP2 (B, D, H) transcripts in *B. bufo* embryos and larvae at the embryonic developmental stages 20 (A–B), 22 (C–F) and metamorphic stage II–IV (G–L). (E, I) Sense probe for BbGSTP1, (F, L) sense probe for BbGSTP2. g, gall bladder; i, intestine; l, liver; t, neural tube; y, yolk. Photos scanned by CanoScan FS2710-Adobe Photoshop v.7. Bar = 500 μm.

Amplification products were resolved by 4% agarose gel electrophoresis in TAE buffer (running conditions: 50 V for 2 h) and stained with EtBr. Band sizes were checked using Sigma PCR Low Ladder (100–1000 bp).

2.6. In situ hybridization

Control and H_2O_2-treated *B. bufo* embryos and larvae at all the considered stages were fixed for 2 h at 4 °C in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, dehydrated in ethanol, cleared in xylene and embedded in paraffin. Sections (7–8 μm thick) were cut and mounted on super Frost/Plus slides. Developmental and metamorphic stages considered were the following: 20–22 (embryonic); II–IV, VII, X, XV, XXV (metamorphic). The assay was made according to Rattray and Michael [31].

The probes used, designed and checked with Biosoft Primer Premiere software, were the following:

1. oligonucleotide probe specific for BbGSTP1 mRNA: 5′ GCTTGGGGACGGCTACTAATGCGTTTGAC 3′;
2. oligonucleotide probe specific for BbGSTP2 mRNA: 5′ TTCCTTGTTTGCCTCGTTGTCCCAGA 3′.

Synthesis and 5′ labelling with digoxigenin was performed by Sigma-Genosys. After deparaffinizing and rehydration, sections (7–8 μm thick) were rinsed in phosphate-buffered saline (PBS) (50 mM Na-phosphate buffer pH 7.4), for 5 min and post-fixed in 4% paraformaldehyde for 10–15 min at room temperature. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 1 h at room temperature. Sections were then permeabilized for 5 min at 37 °C with 5–10 μg/ml proteinase K (Sigma) in 100 mM Tris–HCl pH 7.5, 50 mM ethylenediaminetetraacetate (EDTA), washed in 2 mg/ml PBS-glycine and acetylated for 10 min in 0.25%
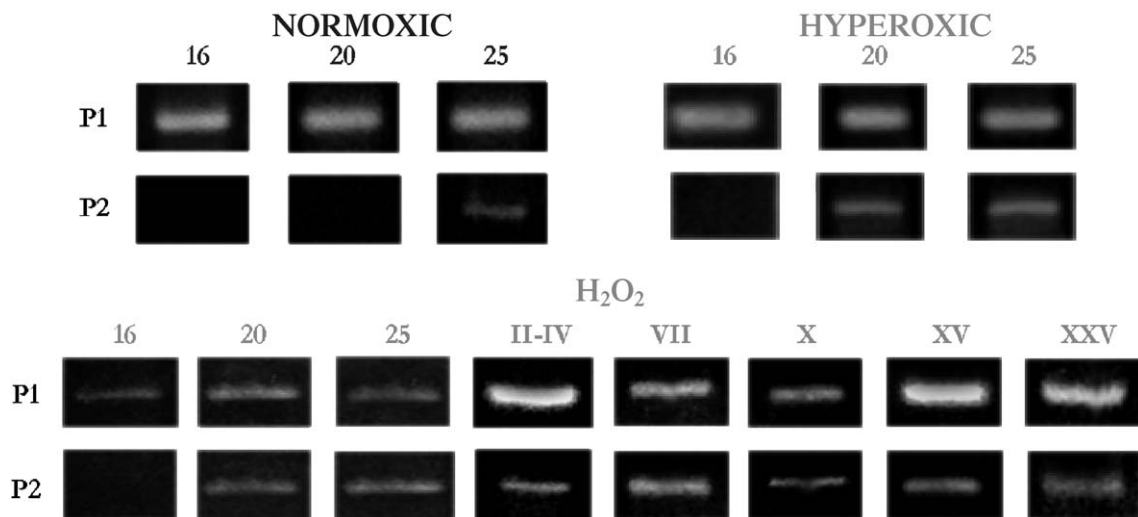

Fig. 3. Effect of oxidative stress on BbGSTP1 and BbGSTP2 mRNAs expression during *B. bufo* development. Post RT-PCR EtBr-stained agarose gel electrophoresis showing the effect of hyperoxic and H_2O_2 treatment on expression pattern of BbGSTP1 and BbGSTP2 mRNAs during *B. bufo* embryonic (arabic numbers) and metamorphic (roman numbers) development. Gels photographed and scanned by Epson GT5500-Adobe Photoshop v.7.
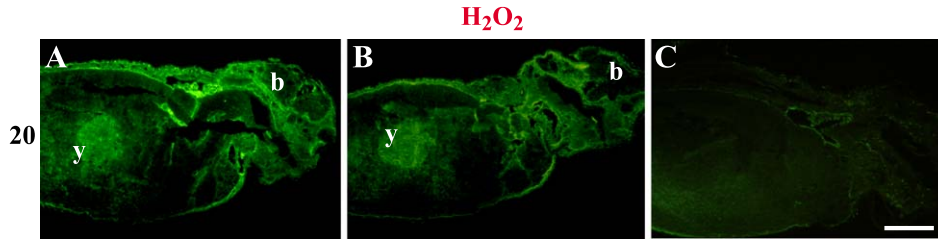
Fig. 4. Effect of oxidative stress on BbGSTP1 and BbGSTP2 transcript expression. In situ hybridization of BbGSTP1 (A) and BbGSTP2 (B) transcripts in H_2O_2-treated *B. bufo* embryos showing anticipation of BbGSTP2 mRNA at stage 20 of embryonic development. (C) Control section treated with RNase. b, brain; y, yolk. Photos scanned by CanoScan FS2710-Adobe Photoshop v.7. Bar = 500 μm.
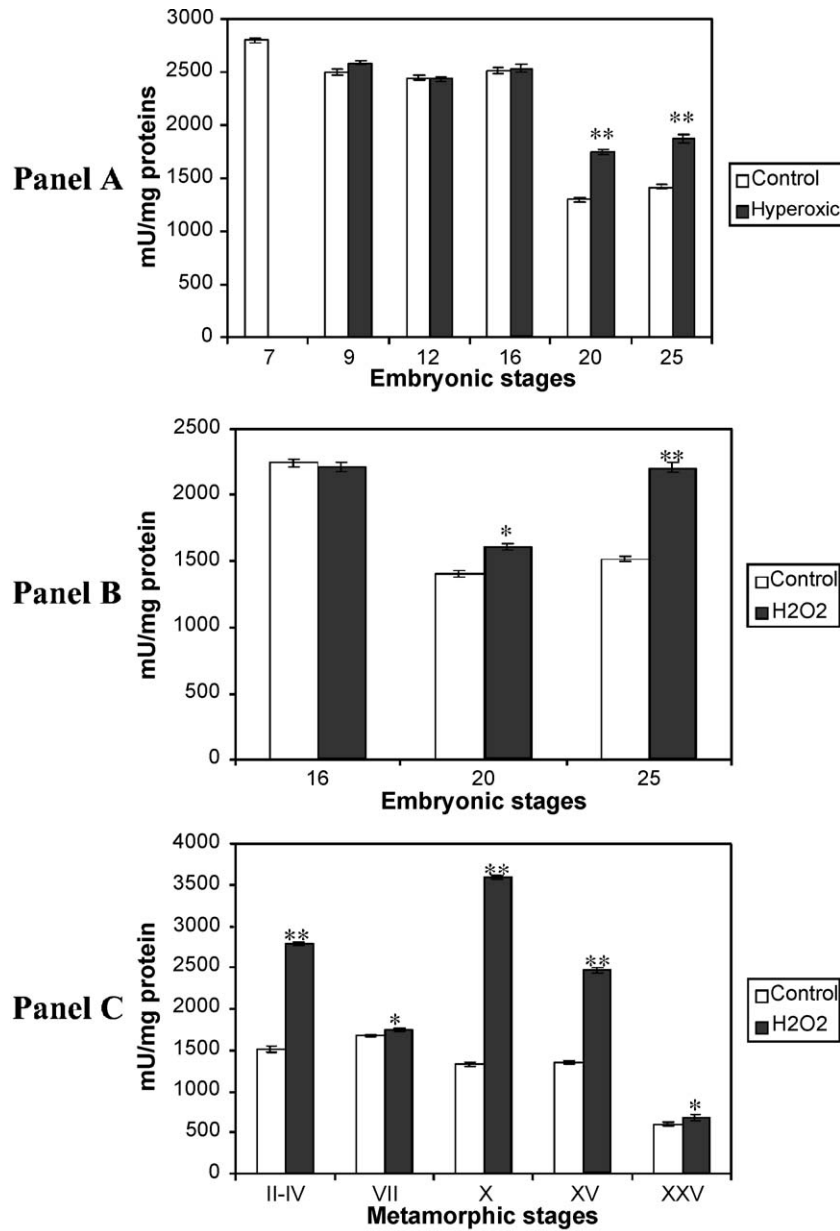

Fig. 5. Effect of oxidative stress on GST-specific activities during *B. bufo* development. GST-specific activities during *B. bufo* during development under hyperoxic (panel A) and H_2O_2 (panels B and C) treatments. Values are means ± S.E. of four different experiments. *$P < 0.05$ versus controls; **$P < 0.005$ versus controls.

acetic anhydride in 0.1 M triethanolamine pH 8.0 and dehydrated.

Probes (50 ng/ml in the hybridization buffer: $1\times$ Denhart's solution, $4\times$ standard saline citrate (SSC), 250 μg/ml salmon sperm DNA, 125 μg/ml tRNA, 10% dextran sulfate, 50% deionized formamide) were applied to sections and hybridized in a humidified box at 37 °C, overnight. Sections were then washed with: $1\times$ SSC, 2×15 min at RT; $1\times$ SSC 2×15 min at 45 °C; $0.2\times$ SSC, 1×5 min at 45 °C; $1\times$ SSC, 1×30 min at room temperature.

Probes were detected with anti-digoxigenin Fab fragments (Boehringer Mannheim) conjugated with horseradish peroxidase (POD), diluted 1:400 in 0.1 M Tris–HCl pH 7.5, 0.3 M NaCl, 1% bovine serum albumin (BSA). Amplification was performed with fluorescein TSA reagent (Perkin Elmer, Life Science, NEL 741) according to the manufacturer's instruction. Slides were then mounted with Citifluor and observed with a fluorescent microscope (Zeiss Axioplan 2MC 80). Negative controls were performed: (a) by treating the tissues with RNase (200 μg/ml for 1–2 h at 37 °C); (b) by omitting the probes from the hybridization reaction mixture; and (c) by using sense probes.

2.7. Cytosol preparation

Embryos and larvae at the selected developmental stages were suspended in 50 mM potassium phosphate buffer (pH 7.0), supplemented with 1.5 mM dithiothreitol and 1 mM EDTA in a 1:5 (v/v) ratio and homogenized with a Potter-Elvehjem homogenizer with 10 pestle strokes. The homogenates were centrifuged at $105,000\times g$ for 60 min at 4 °C in a Beckman Spinco L-70 centrifuge. The supernatants were recovered by syringe to avoid contamination by the upper lipid layer and used for GST enzymatic activity detection and Western blotting analysis.

Protein concentration was determined by the biuret method, using BSA as standard.

2.8. GST enzymatic assay

GST activity was recorded spectrophotometrically at 340 nm and 25 °C by the method described by Habig and Jakoby [32]. The standard assay mixture contained 0.1 M potassium phosphate buffer pH 6.5, 1 mM EDTA, 2 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene. One unit was defined as 1 μmol of GSH conjugated/min. Experiments were performed in quadruplicate.

2.9. Western blotting analysis

Antisera raised against *B. bufo* embryonic (BbGSTP1-1) and adult (BbGSTP2-2) GSTs were available in our laboratory and were the same used in previous works [20–22].

Discontinuous SDS-PAGE was performed by the method of Laemmli [33] in 12% polyacrylamide resolving gels. The
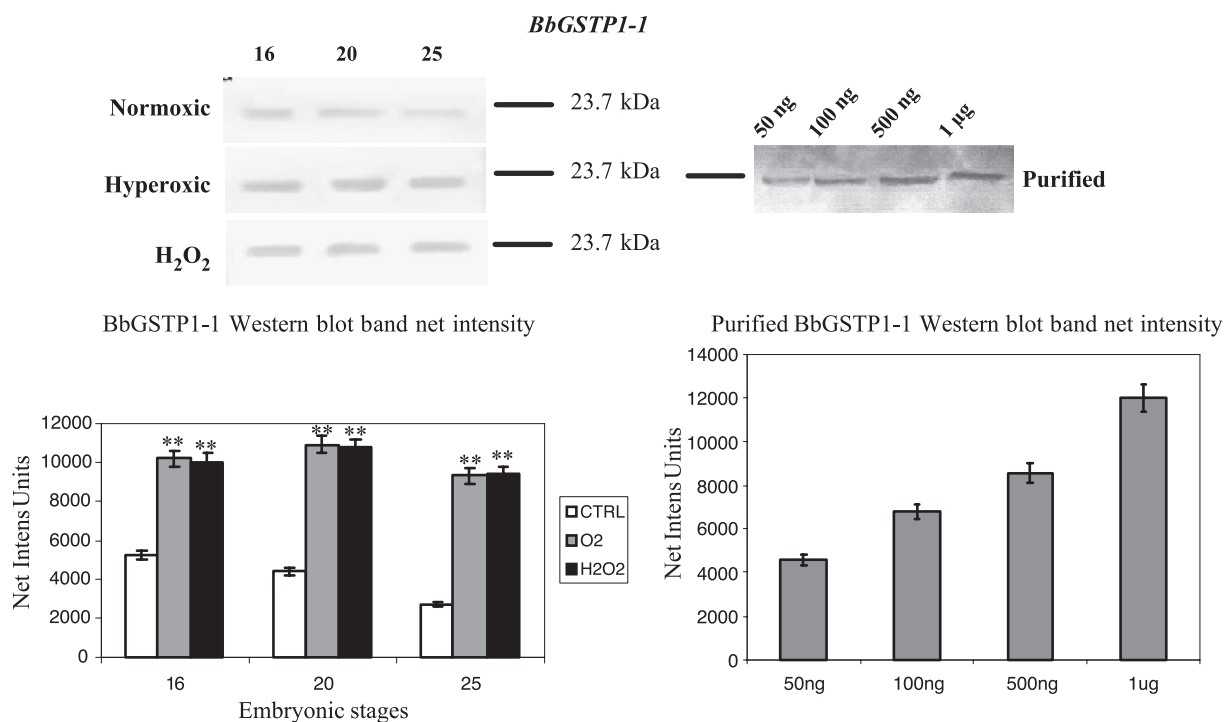

Fig. 6. Effect of oxidative stress on BbGSTP1-1 expression during *B. bufo* development. Western blotting of BbGSTP1-1 at the developmental stages 16, 20 and 25. Nitrocellulose scanned by Epson GT5500-Adobe Photoshop v.7. Densitometrical analysis performed using Kodak 1D Analsys software v3.6. Quantitative evaluation was performed by comparing sample densitometric data with those ones obtained from serial dilutions of known amounts of purified BbGSTP1-1. Values are means ± S.E. of four different experiments. **$P < 0.005$ versus controls.

protein resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes, using Bio-Rad Transblot system, according to Towbin et al. [34]. Electro-blotting was done for 1 h at 100 V (350 mA) in 25 mM Tris-base/192 mM glycine (pH 8.3) containing 20% (v/v) methanol and 0.1% (w/v) SDS. All incubations were performed at 25 °C with intermediate 5-min rinses in 10 mM Tris-base

buffer (pH 7.4), 155 mM NaCl, containing 0.01% (w/v) SDS (Tris-buffered saline (TBS)). Non-specific binding was blocked by placing nitrocellulose paper in TBS, supple-mented with 5% (w/v) non-fat-dried milk (Sigma). Papers were washed five times in TBS. Nitrocellulose papers were incubated with appropriately diluted antiserum (1:500) in TBS containing 5% (w/v) non-fat-dried milk at 25 °C
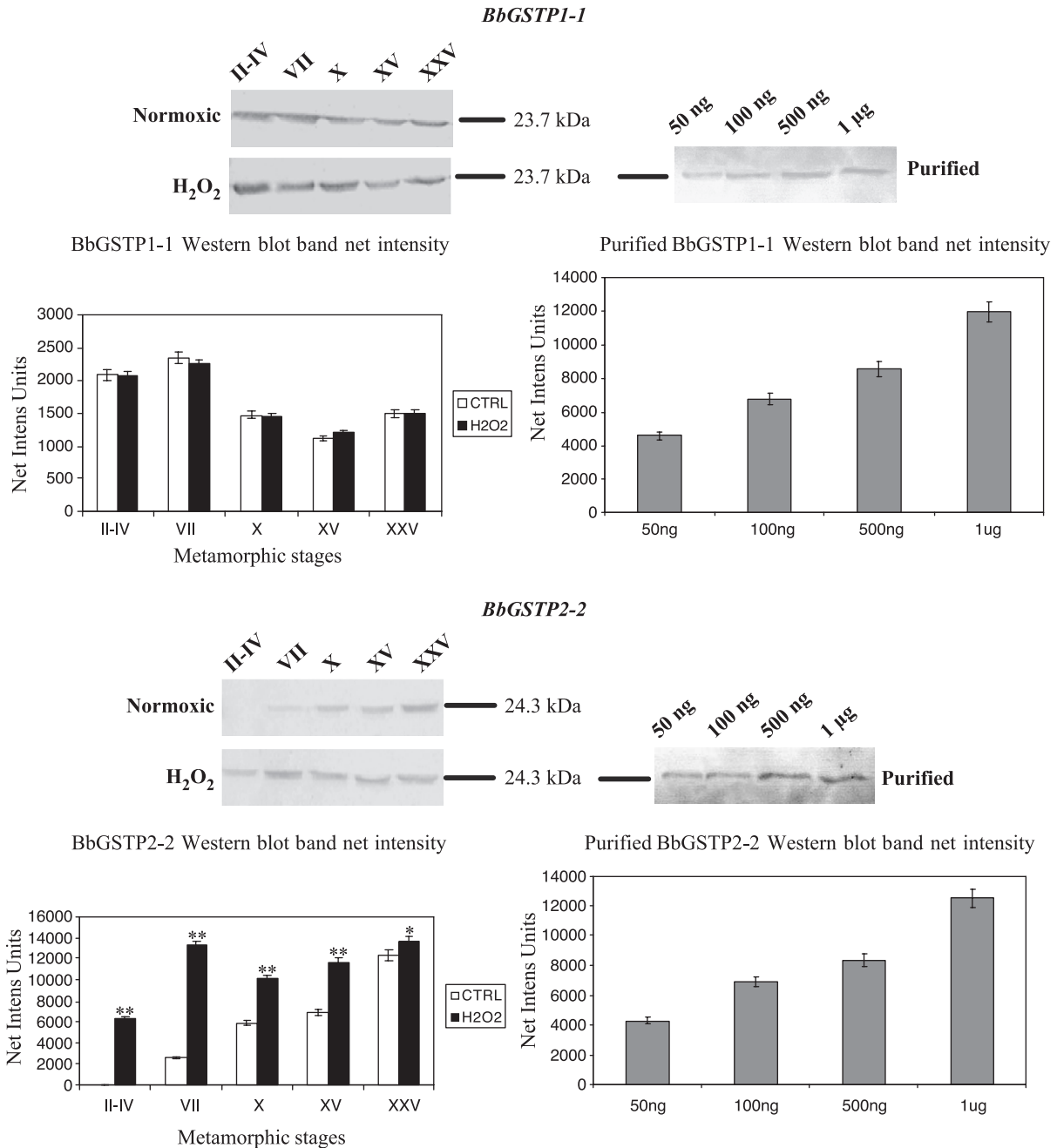

Fig. 7. Effect of oxidative stress on BbGSTP1-1 and BbGSTP2-2 expression during *B. bufo* metamorphosis. Western blotting of BbGSTP1-1 and BbGSTP2-2 in normoxic and H_2O_2-treated larvae at the following metamorphic stages: II–IV, VII, X, XV, XXV. Nitrocellulose scanned by Epson GT5500-Adobe Photoshop v.7. Densitometrical analysis performed using Kodak 1D Analysys software v3.6. Quantitative evaluation was performed by comparing sample densitometric data with those ones obtained from serial dilutions of known amounts of the two purified isoforms. Values are means ± S.E. of four different experiments. *$P < 0.05$ versus controls; **$P < 0.005$ versus controls.

overnight. The nitrocellulose paper was washed five times with TBS and then incubated for 2 h at 25 °C in the same buffer, containing 5% (w/v) non-fat-dried milk and peroxidase-conjugated anti-rabbit IgG antibody (Sigma) diluted 1:1000. After the treatment with peroxidase-conjugated antibodies, the nitrocellulose paper was washed five times with TBS, then soaked in development solution (20 ml of TBS containing 15 mg of 4-chloro-1-naphtol, 5.5 mg of 3,3′-diaminobenzidine tetrahydrochloride and 15 μl of 30% (v/v) H_2O_2). The blot was then washed three times with distilled water, air-dried and photographed.

Semiquantification of immunoreactive bands was performed using net intensities of bands, calculated by subtracting background levels in a preselected area set to include the largest band (Kodak 1D Analysis Software v3.6). Serial dilutions of known amounts of the two purified

isoforms, available in our laboratory and obtained as previously described [20–24], were blotted together with samples in order to correlate the level of protein detected with the net intensity of the relative band. Experiments were performed in quadruplicate.

2.10. Immunofluorescence analysis

Control and H_2O_2-treated embryos and larvae were fixed and embedded as described in the in situ hybridization paragraph. Sections (3–4 μm thick), were cut and laid on polylysine-coated slides. The sections, deparaffinized and rehydrated, were incubated for 1 h in PBS containing 5% (w/v) BSA.

Immunolabelling with purified anti-GST (BbGSTP1-1 or BbGSTP2-2) polyclonal antibodies, diluted 1:100 and
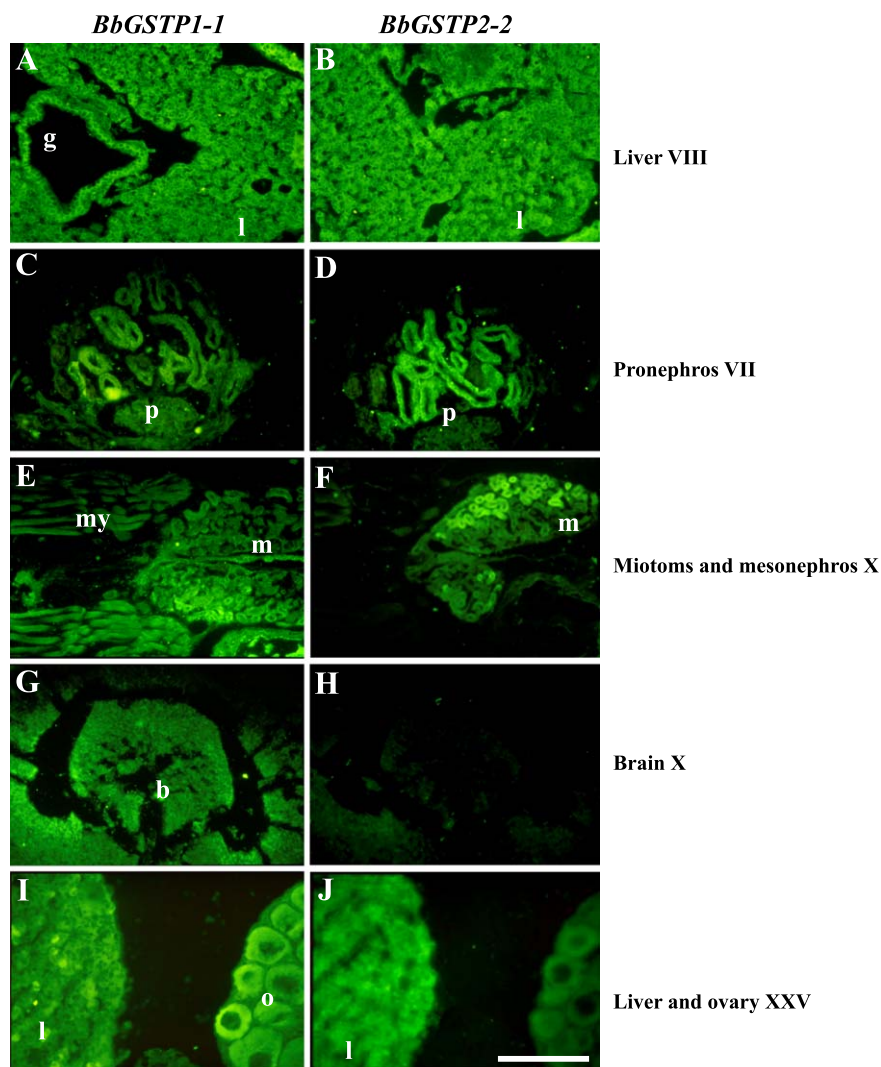
Fig. 8. Distribution of BbGSTP1-1 and BbGSTP2-2 in the organs of *B. bufo* larvae during metamorphosis. Immunolocalization of BbGSTP1-1 (A, C, E, G, I) and BbGSTP2-2 (B, D, F, H, J) proteins in *B. bufo* larvae at the following metamorphic stages: VII (A–D), X (E–H), XXV (I–J). b, brain; g, gall bladder; l, liver; m, mesonephros; my, myotomes; o, ovary; p, pronephros. Photos scanned by CanoScan FS2710-Adobe Photoshop v.7. Bar=800 μm (E, F), 200 μm (A, B, C, D, G, H, I, J).

1:300, in PBS containing 0.1% Tween 20 (v/v) (PBS-T) and 1% BSA (w/v) was carried out overnight at 4 °C in a humid chamber. After several washes in PBS-T, slides were incubated for 1 h at room temperature with anti-rabbit IgG conjugated with fluorescein isothiocyanate isomer (FITC) (Sigma), diluted 1:80 in PBS-T containing 1% (w/v) BSA. The slides were rinsed and mounted with citifluor (Agar). Control sections of all the samples were incubated with non-immune sera or omitting the primary antibody. The sections were observed and photographed with a fluorescence microscope (Zeiss Axioplan 2MC 80) equipped with epi-illumination and appropriate filters.

2.11. Statistical analysis

Results were expressed as means ± S.E. Statistical analysis was performed using Student's t-test. Differences were considered statistically significant for $P < 0.05$.

3. Results

3.1. Expression and localization of BbGSTP1 and BbGSTP2 transcripts during B. bufo development and effect of hyperoxic and H_2O_2 treatment

RT-PCR analysis reveals that BbGSTP1 transcript is always present during toad development, starting from the stage of unfertilized oocyte up to the end of metamorphosis (Fig. 1). In situ hybridization experiments show that the transcript is uniformly distributed all over the embryo at both embryonic and metamorphic stages (Fig. 2).

BbGSTP2 transcript appears at stage 22 of embryonic development and is well evident up to the end of metamorphosis (Fig. 1). In situ hybridization shows that BbGSTP2 transcript is distributed all over the embryo, starting from the embryonic stage 22 up to the end of metamorphosis (Fig. 2). Both hyperoxic and 400 μM H_2O_2 treatments cause an anticipation in the expression of BbGSTP2 transcript that appears at stage 20 of embryonic development, widely distributed at all the embryonic compartments (Figs. 3 and 4). Moreover, in the embryos treated with 400 μM H_2O_2, it can be observed that BbGSTP2 transcript is expressed up to the end of metamorphosis (Fig. 3), always widespread (not shown). All RT minus controls and negative controls relative to in situ hybridization experiments were always negative (not shown).

3.2. GST enzymatic activity during B. bufo development and effect of hyperoxic and H_2O_2 treatment

As it can be observed in Fig. 5, panel A, GST-specific activity shows the highest values at the early developmental

stages, to gradually decrease up to the end of embryonic development. At the earliest metamorphic stages, GST-specific activity shows values similar to those found at stage 25 of embryonic development, then it gradually decreases up to stage XXV (Fig. 5, panel C).

Under hyperoxic conditions, GST-specific activity assayed in the cytosols from embryo homogenates shows a significant increase with respect to controls starting from stage 20 up to the end of embryonic development (Fig. 5, panel A). Similar results were also obtained with embryos developed in the presence of 400 μM H_2O_2 (Fig. 5, panel B). During metamorphic development, H_2O_2 treatment still causes a significant increase in GST activity that peaks at stage X (Fig. 5, panel C).
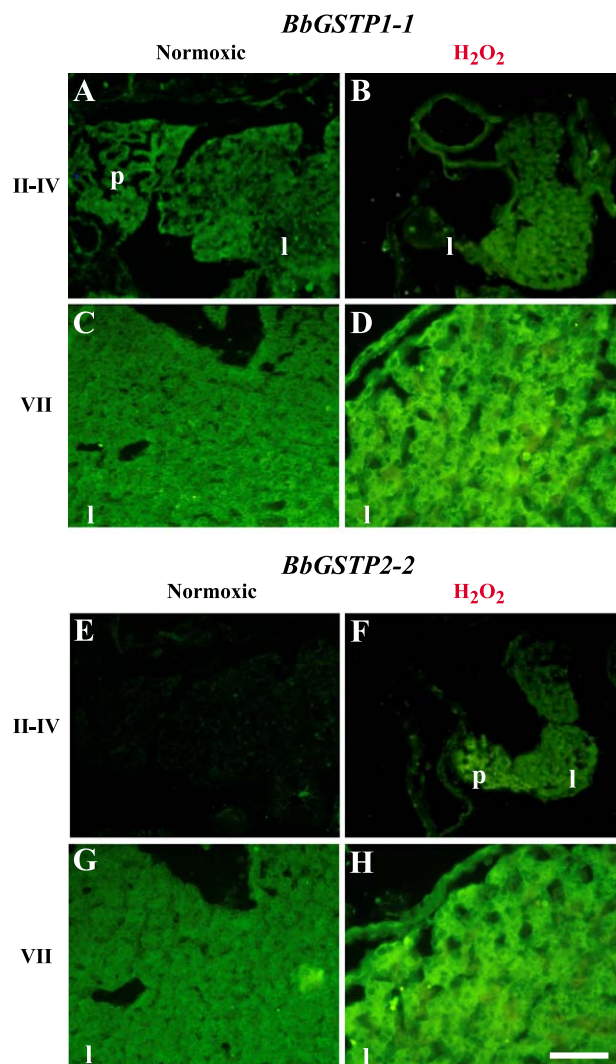
Fig. 9. Effect of oxidative stress on BbGSTP1-1 and BbGSTP2-2 distribution and expression during *B. bufo* metamorphosis. Immunolocalization of BbGSTP1-1 (A–D) and BbGSTP2-2 (E–H) proteins in normoxic (A, C, E, G) and H_2O_2-treated (B, D, F, H) *B. bufo* larvae at II–IV (A, B, E, F) and VII (C, D, G, H) metamorphic stages. l, liver; p, pronephros. Photos scanned by CanoScan FS2710-Adobe Photoshop v.7. Bar=200 μm (A, B, E, F), 100 μm (C, G), 50 μm (D, H).

3.3. Expression and localization of BbGSTP1-1 and BbGSTP2-2 proteins during B. bufo development and effect of hyperoxic and H_2O_2 treatment

Western blotting investigation shows that the BbGSTP1-1 protein is detectable all over embryonic development, confirming our previous studies (Fig. 6) [25]. Furthermore, BbGSTP1-1 protein is evident during all the metamorphic period, although in the early stages the signal is higher (Fig. 7). BbGSTP2-2 protein, on the contrary, can be detected starting from stage VII of metamorphosis, then the signal gradually increases up to the end of metamorphosis (Fig. 7).

Immunolocalization analysis reveals that BbGSTP1-1 is distributed all over the larvae at all the metamorphic stages (Fig. 7). On the contrary, the signal relative to BbGSTP2-2 is evident exclusively in the liver and kidney, starting from stage VII up to the end of metamorphosis (Fig. 8).

Under hyperoxic conditions Western blotting shows a significant increase in the expression of BbGSTP1-1 protein with respect to controls at stages 20 and 25 of embryonic development (Fig. 6), while no signal for BbGSTP2-2 protein is detectable at any embryonic developmental stages.

Similar results were also obtained with embryos developed in the presence of 400 μM H_2O_2 (Fig. 6).

As already stated, H_2O_2-treated embryos were able to perform development entirely, thus allowing to study the effect of oxidative stress on the expression of B. bufo GST proteins also during metamorphic development. From Western blotting analysis it can be observed that H_2O_2 treatment, does not affect BbGSTP1-1 expression during metamorphosis (Fig. 7). On the contrary, H_2O_2 treatment induces an anticipation of BbGSTP2-2 protein expression at stage II–IV of metamorphosis, as well as a significant increase in BbGSTP2-2 protein levels compared to controls at all the metamorphic developmental stages (Fig. 7).

Immunolocalization study confirms the H_2O_2-induced stage II–IV anticipation of BbGSTP2-2 protein expression and, furthermore, reveals that this treatment does not affect the localization of both embryonic and adult protein (Fig. 9).

4. Discussion

Our results show that BbGSTP2 transcript starts to be expressed at the embryonic stage 22; on the contrary, the protein appears quite later, at stage VII of metamorphosis. Therefore, as a first major point, our findings suggest that BbGSTP2-2 protein expression undergoes a post-transcriptional control mechanism, as it often occurs in vertebrates for proteins synthesized during development [35–38]. Moreover, the presence of BbGSTP2-2 protein exclusively in the liver and kidney throughout the metamorphic period, notwithstanding the widespread distribution of its transcript in all the embryonic and larval compartments, suggests that BbGSTP2 gene translational process is also subjected to a tissue-specific regulation.

As concerns the embryonic BbGSTP1-1 isoform, both transcript and protein are continuously expressed and widespread during both embryonic and metamorphic development, stressing the relevance of this isoenzyme for embryonic and larval detoxification processes. However, the appearance in the liver and kidney of the BbGSTP2-2 protein at stage VII of metamorphosis, followed by a gradual and constant increase in its expression up to the end of metamorphosis, suggests that metabolic activities occurring during this larval period may involve hepatic and renal detoxification processes for which adult GST isoform is more suitable. In this regard, it should be noted that during amphibian metamorphosis, hepatic tissue undergoes profound re-structuring and biochemical rearrangements, like induction of urea cycle enzymes and larval–adult haemoglobin switch, aimed at fulfilling new physiological requirements of the adult terrestrial mode of life [39–41].

As a second major point, our results show that oxidative stress modulates BbGSTP2 gene expression at both transcriptional and translational levels, as it causes significant anticipation of both transcript and protein expression. However, also under oxidative stress conditions, BbGSTP2-2 protein expression is still regulated, like in control conditions, by post-transcriptional mechanisms, as it appears quite later with respect to its transcript and only in the post-embryonic phase of development. Furthermore, the tissue-specific expression of BbGSTP2-2 protein remains unvaried, being localized in this condition only at the liver and kidney level up to the end of metamorphosis.

Oxidative stress also induces a significant increase in GST activity, during both embryonic and metamorphic development. Interestingly, GST activity increase is evident starting from stage 20 of embryonic development, namely, from that stage in which anticipation of BbGSTP2 transcript occurs. At this developmental stage (gill circulation), the differentiation of the circulatory system is almost complete and embryonic oxidative metabolism rises sharply, with consequent increase in the basal endogenous production of ROS. Thus, the observed oxidative stress-mediated increase in GST activity starting from stage 20 of embryonic development may be aimed at better protecting the embryo against further increase in ROS concentration.

An intriguing finding is that the oxidative stress-mediated modulation of GST activity, depending on the embryonic or metamorphic development, is associated with a differential increase in the level of embryonic or adult GST protein expression, respectively. In fact, while during embryonic development oxidative stress causes a significant increase in BbGSTP1-1 protein concentration (Fig. 6), the same stress in the metamorphic phase induces both antic-

ipation in the expression and increase in the levels of BbGSTP2-2, with BbGSTP1-1 not showing any significant variation with respect to control conditions (Fig. 7). It may be argued that some specific factors, occurring in the toad starting from the post-embryonic developmental period, are needed for BbGSTP2-2 protein expression. However, when the embryo has entered into this phase, which allows the expression of both toad GST proteins, oxidative stress induces selective activation of BbGSTP2-2 isoform that, most probably, may assure a better scavenging action towards reactive oxygen intermediates. Our previous studies showing a major reduction ability of BbGSTP2-2 versus organic hydroperoxides [23] are consistent with this conclusion. Moreover, the presence of this protein exclusively in the liver and kidney all over metamorphosis, even after oxidative stress-mediated stimulation, suggests that these organs are the main sites where antioxidant detoxifying processes occur. The embryonic–adult switch in GST protein expression occurring in the liver of adult toad, further supports the hypothesis that BbGSTP2-2 is more suitable for hepatic detoxifying activities of the adult mode of life. In this context, the almost contemporary appearance in the larval liver of both BbGSTP2-2 and urea cycle enzymes may support the hypothesis that adult toad GST expression might also be involved in the detoxification of compounds produced by urea-based excretion process, typical of adult terrestrial life. Further researches are needed to verify this hypothesis.

In summary, peculiar findings of this study are: (a) BbGSTP2-2 protein appears at stage VII of metamorphosis and only in the liver and kidney, while, widely diffused all over the embryo, BbGSTP2 transcript appears at stage 22 of embryonic development; (b) oxidative stress induces anticipation of BbGSTP2-2 gene expression at both transcriptional and translational level. Taken together, these data seem to indicate that the higher oxygen tension of the terrestrial atmosphere may play a pivotal role in the expression of the novel toad BbGSTP2-2.

Acknowledgements

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