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ORIGINAL PAPER

Characterization of the small heat shock protein *Hsp27* gene in *Chironomus riparius* (Diptera) and its expression profile in response to temperature changes and xenobiotic exposures

Pedro Martínez-Paz · Mónica Morales · Raquel Martín · José Luis Martínez-Guitarte · Gloria Morcillo

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Abstract Small heat shock proteins constitute the most diverse and least conserved group within the large family of heat shock proteins, which play a crucial role in cell response to environmental insults. *Chironomus riparius* larvae are widely used in environmental research for testing pollutant toxicity in sediments and freshwater environments. Different genes, such as *Hsp70*, *Hsc70*, *Hsp90*, and *Hsp40*, have been identified in this species as sensitive biomarkers for xenobiotics, but small *Hsps* genes remain largely unknown. In this study, the *Hsp27* has been characterized in *C. riparius* and its transcriptional response evaluated under several environmental stimuli. The *Hsp27* gene was mapped by FISH on polytene chromosomes at region I-C4 and was found to encode a 195 aa protein, which contains an α-crystallin domain bounded by three conserved regions. This protein shows homology with *Drosophila melanogaster* HSP27, *Ceratitis capitata* HSP27, and *Sarcophaga crassipalpis* HSP25. Real-time reverse transcriptase–polymerase chain reaction analysis showed that heat shock (35 °C) and cadmium dramatically upregulate this gene. Moreover, exposures to triclosan and bisphenol A were able to significantly increase mRNA levels. However, neither nonylphenol nor tributyltin altered *Hsp27* gene expression. The transcriptional activity of *Hsp27* gene was modulated during cold stress. Interestingly, cold shock (4 °C) significantly reduced *Hsp27* transcripts, but this gene was significantly overexpressed during the recovery time at the normal growing temperature. These results show that the *Hsp27* gene is sensitive to different environmental stimuli, including endocrine-disrupting pollutants, suggesting its potential as a suitable biomarker for ecotoxicological studies in aquatic systems.

Keywords Cold heat shock · Cadmium · Bisphenol A (BPA) · 4-Nonylphenol (NP) · Tributyltin (TBT) · Triclosan (TCS)

P. Martínez-Paz · M. Morales · R. Martín · J. L. Martínez-Guitarte · G. Morcillo
Grupo de Biología y Toxicología Ambiental, Facultad de Ciencias, Universidad Nacional de Educación a Distancia, UNED, Senda del Rey 9, 28040 Madrid, Spain

G. Morcillo (✉)
Facultad de Ciencias, UNED, Senda del Rey 9, 28040 Madrid, Spain
e-mail: gmorcillo@ccia.uned.es

Introduction

Heat shock proteins (HSPs) are a family of highly conserved proteins from prokaryotes to eukaryotes and ubiquitous in all cell types, two facts that prove their essential role in cells. They have important functions in many physiological processes, and are particularly relevant when cells are under stressful conditions as chaperones for the maintenance of correct protein folding (Nolen and Morimoto 2002; Silver and Noble 2012). HSPs were first discovered under heat shock and were later found to be highly abundant in cells submitted to many other chemical and physical stimuli in a wide range of species. In general, *Hsp* genes are expressed at low levels under normal growing conditions, but their expression increases considerably in a rapid response to different forms of stressors including heat, hypoxia, chemical exposures, infections, magnetic fields, and tumorigenesis (Parsell and Lindquist 1993; Feder and Hofmann 1999; Bierkens et al. 1998). In arthropods, they are induced by environmental stressors such as heat, desiccation, and heavy metals (Hoffmann and Parsons 1991; Tammariello et al. 1999; Hoffmann et al. 2003). It is known that *Hsp* genes constitute a subset of a larger group of genes coding for molecular chaperones. Chaperonins play an important role by assisting in the correct folding of nascent peptides, also acting when denatured proteins accumulate in cells, preventing them from irreversible aggregation and misfolding (Hartl and Hayer-

Hartl 2002; Sorensen et al. 2003). As HSP induction is critical to the maintenance of cellular homeostasis in response to changes in the environment, these proteins have been proposed as general biomarkers for environmental monitoring (Gupta et al. 2010). Given the large quantity and huge variety of contaminants of anthropogenic origin, the development of alternative methods to animal testing is becoming a real challenge, and the application of toxicogenomic tests is gaining acceptance as a rapid and efficient methodology (Snell et al. 2003). As sensitive stress-sensors, *Hsps* genes are suitable candidates not only to evaluate the damaging potential of chemicals, but also to analyze the effects of subtle alterations in physical abiotic parameters (i.e., temperature, radiation), which are increasingly acquiring importance as a consequence of the human impact on natural ecosystems. This environmental perspective adds further interest for studying the potential of the *Hsp27* gene as a toxicological endpoint.

The larvae of the midge *Chironomus riparius* (Diptera) have been extensively used as a model for testing pollutant toxicity in sediments and freshwater environments (EPA US 1996; OECD, Organisation for Economic Co-operation and Development 2001). They are abundantly distributed and have strong potency to survive in contaminated environments, even when many other aquatic organisms cannot. Ecotoxicological tests of chemicals have been traditionally performed in laboratory assays using mortality, growth rate, behavior, and reproduction as endpoints (Hatakeyama 1988; Williams et al. 1987). Larval mouthpart deformities are also indicators of anthropogenic stress (Martinez et al. 2003). Moreover, the giant polytene chromosomes from the salivary gland cells are also suitable for analyzing the genotoxic effects of pollutants (Michailova et al. 2006). More recently, chironomids are being used for toxicity testing using molecular endpoints. The utility of gene biomarkers for monitoring both environmental quality and the health of organisms inhabiting polluted ecosystems is gaining increasing attention. In recent years, some genes have been described as biomarkers for toxicant exposures including, among others, those for heat shock proteins (*Hsp70*, *Hsc70*, *Hsp90*, *Hsp40*), ribosomal proteins, cytochrome P450, and nuclear receptors (Martínez-Guitarte et al. 2007; Gopalakrishnan et al., 2011; Morales et al. 2011; Planelló et al. 2007; 2008; 2010; 2011; Park and Kwak 2008, 2009, 2010; Martínez-Paz et al. 2012; Ozáez et al. 2013). *Hsp70* has been sequenced and evaluated as a biomarker of exposure to metals and insecticides in other species of chironomids, such as *Chironomus yoshimatsui* and *Chironomus dilutus* (Yoshimi et al. 2002; Karouna-Renier and Rao 2009). However, there is still scarce DNA sequence information for these aquatic species and, in particular, the family of small *Hsps* genes has not yet been characterized.

Small heat shock proteins (sHSPs) are stress-inducible molecular chaperones that range in size from 10 to 30 kDa. A low degree of conservation is found among sHSPs, when compared to other proteins of the HSP family, with the exception of an α-crystallin domain of 80–100 amino acids (Denlinger et al. 2001). These proteins are involved in conditions of extreme temperatures, oxidation, UV irradiation, heavy metals, and chemical intoxication (Reineke 2005; Waters et al. 2008). The sHSPs are also involved in some important biological processes such as cell growth, apotosis, differentiation, diapause, lifespan, membrane fluidity, and starvation resistance in insects (Arrigo 1998; Gkouvitsas et al. 2008; Morrow et al. 2004; Tsvetkova et al. 2002; Hao et al. 2007). Yet, in insects, there is only limited information about the expression, regulation and function of sHSPs. Members of the α-crystallin/sHSP superfamily have recently been cloned from a few insect species: *Sarcophaga crassipalpis* (Rinehart et al. 2007), *Venturia canescens* (Reineke 2005), *Bombyx mori* (Sakano et al. 2006), *Plutella xylostella* (Sonoda et al. 2006), *Liriomyza* sp. (Huang and Kang 2007), *Locusta migratoria* (Wang et al. 2007), *Ceratitis capitata* (Kokolakis et al. 2008), *Mamestra brassicae* (Sonoda et al. 2007), *Sesamia nonagrioides* (Gkouvitsas et al. 2008), *Liriomyza sativae* (Huang et al. 2009), and *Macrocentrus cingulum* (Xu et al. 2010). In the flesh fly *S. crassipalpis*, three sHSPs are upregulated during diapause and it has been proposed that they contribute to the survival of this insect at low temperatures (Yocum et al. 1998; Li et al. 2007; Rinehart et al. 2007; Colinet et al. 2010). A slight upregulation of an sHSP has also been observed during diapause of the northern house mosquito, *Culex pipiens* (Robich et al. 2007). However, this is not the case in all insect species studied to date (Goto and Kimura 2004; Tachibana et al. 2005).

The aim of the present study was to characterize the *Hsp27* gene in *C. riparius* and to analyze the transcriptional regulation of this gene under different temperature shocks and xenobiotic exposures to chemicals frequently found as contaminants in the aquatic environment. Tributyltin (TBT), nonylphenol (NP), triclosan (TCS), bisphenol A (BPA), and cadmium (Cd) were selected. These compounds are all environmental chemicals with estrogenic activity. TBT is a toxic chemical, belonging to the organotin compounds or stannanes, used for disinfection, antifouling and preservation in industrial processes. Due to its widespread use, it is a common contaminant of marine and freshwater environments, and is included on the list of EU priority compounds in water because of its toxic, persistent, bioaccumulative, and endocrine disruptive characteristics (Antizar-Ladislao 2008). Nonylphenol results from the degradation of alkylphenol polyethoxylates used as surfactants in industrial products. It accumulates in environmental compartments with a high organic content, such as sewage sludge and river sediments. NP has been referred to on the list of priority substances in the Water Frame Directive of the EU, and has been classified as an endocrine disrupter (Soares et al. 2008). TCS, a halogenated phenol, is a nonionic broad spectrum

antimicrobial widely used as an ingredient in disinfectants, detergent, mouthwash, fabric, deodorant, shampoo, and plastic additives, as well as in innumerable other veterinary, industrial, and household products. The hormonal activity of TCS has been demonstrated by its capability to modulate thyroid hormone-related genes and anuran development (Veldhoen et al. 2006); it also has endocrine disruptive effects in fishes (Raut and Angus 2010). BPA is used in polycarbonate plastics and epoxy resin products, as well as an antioxidant in plasticizers and as an additive in other plastics. The total amount of BPA released into the environment has been estimated, and its predicted concentration in water and sediments has become a primary environmental concern (Cousins et al. 2002). Finally, Cd is a widespread pollutant known to be highly toxic, up to 20 times more toxic than other heavy metals, with numerous detrimental effects on most organisms. It has been ranked sixth on the European Union list of priority hazardous substances (Water Framework Directive 2008/105/EC). Although the effect caused by cadmium at the organism and population levels has been well documented, there are fewer data on sensitive molecular biomarkers for evaluating the impact of this contaminant on aquatic ecosystems. Our study reveals the potential role of the *C. riparius Hsp27* gene as a sensitive marker in response to temperature changes and exposure to chemicals in benthic invertebrates.

Material and methods

Animals and treatments

The experimental animals were the aquatic larvae from the midge *C. riparius*. They were originally collected from natural populations in a non-polluted area of Valencia (Spain), and reared under standard laboratory conditions according to toxicity testing guidelines (EPA US 1996; OECD, Organisation for Economic Co-operation and Development 2001). Larvae were grown from egg masses in aqueous culture medium (0.5 mM $CaCl_2$, 1 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $NaHCO_3$, 0.025 mM KH_2PO_4, 0.01 mM $FeCl_3$) supplemented with nettle leaves, commercial fish food (TetraMin), and cellulose tissue in polyethylene tanks (500 mL). Cultures were maintained under constant aeration at 20 °C and under standard light–dark periods (16:8). For experimental treatments, the larvae were exposed to the chemicals diluted in culture medium for 24 h with constant aeration at 20 °C. No food or substrate was provided during exposure. Fourth instar larvae were submitted to 0.1, 1, and 10 ng/L tributyltin oxide (TBTO) (Aldrich), 1, 10 and 100 μg/L NP (Fluka), 10, 100, and 1000 μg/L of TCS [5-chloro-2-(2,4-dichlorophenoxy)phenol] (Sigma), 0.5 and 3 mg/L BPA (Aldrich), and cadmium (Cd) 1 and 10 mM (Fluka). For temperature treatments, larvae were heat shocked at 35 °C for 2 h in preheated and aerated cultured medium, as described previously (Morcillo et al. 1988) and at 4 °C for 2 h in precooled and aerated cultured medium for cold shock. Thereafter, groups of larvae were transferred again to culture medium at 20 °C for 2, 4, and 6 h for recovery experiments after the cold shock. Each treatment consisted of three replicates, and three independent experiments were performed in each analysis using samples from three different control egg masses. The control larvae used in each case were exposed to the same concentration of solvent as the corresponding treatment, and were also measured in triplicate. Larvae were stored at −80 °C until RNA isolation was carried out.

RNA isolation

Total RNA was extracted from control and exposed fourth instar larvae (ten animals for each experiment) using a guanidine isothiocyanate-based method, performed with a commercial kit (Trizol, Invitrogen) according to the manufacturer's protocol. After, RNA was treated with RNase-free DNase (Roche), followed by phenolization. The quality and quantity of total RNA were determined by agarose electrophoresis and absorbance spectrophotometry (Biophotomer Eppendorf). Purified RNA was finally stored at −80 °C.

DNA isolation

Genomic DNA was isolated from 25 control larvae by homogenization in 100 mM NaCl, 25 mM EDTA, 10 mM Tris, pH 8, 0.5 % SDS. The homogenate was then treated with 20 mg/ml of proteinase K (Roche) at 65 °C overnight and 20 mg/ml of Rnase A (Roche) followed by incubation at 37 °C for 1 h. Two phenol/chloroform/isoamylalcohol (25/24/1) extractions were performed followed by ethanol precipitation, and DNA pellets were resuspended in 100 μl of Tris–EDTA (pH 8.0). The quality and quantity of total DNA were determined by agarose electrophoresis and absorbance spectrophotometry (Biophotomer Eppendorf). Purified DNA was finally stored at 4 °C.

Retrotranscription

Reverse transcription was performed with 0.5 μg of the isolated RNA. 0.5 μg oligo dT_{20} primer (Invitrogen) was used with M-MLV enzyme (Invitrogen), following the procedures described by the manufacturer.

Characterization and isolation of cDNA

Total RNA extracted from *C. riparius* larvae was used for *heat shock protein 27* amplification. The full-length sequence was determined using 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) with commercial kits (Invitrogen), following

the manufacturer's instructions. Primer Hsp27 3F was designed based on the conserved sequences of *Hsp27* gene from closely related species. The sequences of all gene-specific primers used for *Hsp27* amplification are given in Table 1. For 3′ end RACE polymerase chain reaction (PCR), a cDNA template was obtained, as described above, using Adapter Primer (RACE kit, Invitrogen) and PCR was performed with gene-specific primer Hsp27 3F and an adapter primer AUAP (RACE kit, Invitrogen). PCR conditions were one cycle of 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s, and finally 72 °C for 10 min. The PCR product was cloned with a TOPO TA Cloning Kit (Invitrogen) and plasmids were purified and sequenced with M13 Forward and M13 Reverse primers. For the 5′ end RACE PCR, new gene-specific primers were designed based on sequence information obtained from the 3′ end fragment. The RNA was transcribed by a 5′ RACE kit (Invitrogen) with gene-specific primer Hsp27 7R, and the cDNA was subsequently amplified with an adapter primer AAP (RACE kit, Invitrogen) and a gene-specific primer *Hsp27* 6R. The PCR conditions were one cycle of 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 40 s; and 72 °C for 10 min. The PCR products were purified and sequenced with hsp27 6R primer.

Hsp27 fragments were sequenced from both strands using ABI Big-Dye 3.1 dye chemistry and ABI 3730XL automated DNA sequencers (PE Biosystems). The complete *C. riparius Hsp27* cDNA sequence was deposited in GenBank under accession number # KC495957.

Bioinformatic and phylogenetic analyses

Molecular weight and theoretical isoelectric point were estimated with the Compute pI/Mw tool from ExPASy—Bioinformatics Resource Portal. Multiple sequence alignments and phylogenetic analyses were performed using the Clustal W2 software (http://www.ebi.ac.uk/tools/msa/clustalw2) and compared with *C. capitata* HSP27, *Drosophila melanogaster* HSP27 and *S. crassipalpis* HSP25. The accession numbers for sHsp used in this study are: *B. mori Hsp19.9* (AB195970), *B. mori Hsp20.4* (AF315318), *B. mori Hsp20.1* (AB195971), *B. mori Hsp20.8* (AF315317), *B. mori Hsp21.4* (AB195972), *B. mori Hsp23.7* (AB195973), *C. capitata Hsp27* (EU700493.1), *D. melanogaster Hsp23* α (AAF50286.1), *D. melanogaster Hsp23* β (AFH04365.1), *D. melanogaster Hsp26* (AAA28636.1), *D. melanogaster Hsp27* (AAA28638), *Liriomyza huidobrensis Hsp20* (DQ452370), *L. sativae Hsp21.3* (DQ452371), *L. migratoria Hsp20.5* (DQ355963), *L. migratoria Hsp20.6* (DQ355964), *L. migratoria Hsp20.7* (DQ355965), *S. crassipalpis Hsp23* (AF156162.1), *S. crassipalpis Hsp25* (EF103577), and *V. canescens Hsp35* (AY775544).

In situ hybridization and immunodetection

Salivary glands were dissected and fixed in acetic acid/ethanol (3:1), squashed in 50 % acetic acid and the slides dehydrated in absolute ethanol. Subsequently, FISH was carried out essentially as described previously (Martínez-Guitarte et al. 2007). The squashes were air-dried and treated with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) at 65 °C for 45 min, dehydrated in 50–100 % ethanol series for 10 min and treated with 0.07 N sodium hydroxide for 3 min. Finally, the slides were thoroughly washed with 1× PBS before applying the probe. As probe, we used an 874 bp fragment of *Hsp27* cDNA from *C. riparius* (amplified with Hsp27 9F and Hsp27 10R primers), it was labeled by nick translation with Digoxigenin (Roche), denatured by boiling in water for 10 min and rapidly cooled on ice. The hybridization buffer was 50 % deionized formamide and 4× SSC, 0.4 % SDS. Each

Table 1 Primers used for cDNA, genomic DNA sequence and real-time RT–PCR of genes from *C. riparius*

PCR objective	Oligo name	Primer DNA sequence	Amplification efficiency (%)
β actin real-time PCR	Actina F	5′-GATGAAGATCCTCACCGAACG-3′	104
	Actina 2R	5′-CGGAAACGTTCATTACCG-3′	
GAPDH real-time PCR	GAPDH F	5′-GGTATTTCATTGAATGATCACTTTG-3′	96.6
	GAPDH R	5′-TAATCCTTGGATTGCATGTACTTG-3′	
L13 real-time PCR	L13 F	5′-AAGCTGCTTTCCCAAGAC-3′	107.1
	L13 R	5′-TTGGCATAATTGGTCCAG-3′	
Hsp27 real-time PCR	Hsp27 rt F	5′-TCAACACACAGGACCG-3′	109.3
	Hsp27 rt R	5′-ATCCTTTATTGGTGATTAATTATG-3′	
Hsp27 sequence	Hsp27 3 F	5′-AAGGATGGCTTCCAGGTCTGTATGGA-3′	
	Hsp27 6R	5′-TGTCTTTACAGTAATTTCACTTGG-3′	
	Hsp27 7R	5′-GTTTTCCTTCAATAACCACC-3′	
	Hsp27 9F	5′-ATGTCATTAGTTCCTACTTTGTGGA-3′	
	Hsp27 9R	5′-AAGTCGAGCCGGTCCTGTG-3′	
	Hsp27 10R	5′-AAGGTCATCCTAAGAACCAACTAATC −3′	

slide was treated with 95 pmol of probe and incubated overnight at room temperature. After hybridization, the slides were washed twice in PBS, 0.1 % Tween 20 for 10 min. For detection of the probe, slides were incubated for 1 h in anti-digoxigenin IgG conjugated with fluorescein isothiocyanate (Roche) diluted 1:100 in PBS, 0.1 % Tween 20, and 1 % Blocking reagent (Roche). Following the washes, the slides were stained with 2 μg/ml DAPI (4′,6-diamino-2-phenylindole) for 3 min and mounted in ProLong (Invitrogen) anti-fading. All slides were examined under a Zeiss Axiohot photomicroscope equipped with an epifluorescence system and a Photometrics Cool Snap CCD camera. Images were processed with Adobe PhotoShop CS5.

Real-Time PCR

Quantitative real-time PCR (qRT–PCR) was used to evaluate the mRNA expression profile of *Hsp27* after chemical exposure and cold and heat shock treatments. *Actin*, *GAPDH*, and *L13* were employed as reference genes. Gene-specific primers used for *actin*, *GAPDH*, *ribosomal protein L13*, and *Hsp27* are listed in Table 1. The qRT–PCR was performed using SsoFast EvaGreen Supermix (BioRad) in a CFX96 real-time PCR Detection System (BioRad). Each gene efficiency reaction was carried out with template diluted 1:2 in five steps, and the slope of the regression curves was then calculated (Table 1). The qRT–PCR cycling conditions were: initial denaturation at 95 °C for 3 min, 35 cycles of 95 °C denaturation for 5 s, 58 °C annealing for 15 s and 65 °C elongation for 10 s. To verify the accuracy of each amplicon, a melting curve analysis was performed after amplification. BioRad CFX Manager 3.0 software was used to determine the mRNA levels by normalized gene expression ($2^{-\Delta\Delta Cq}$) against three endogenous reference genes. Each sample was run in duplicate wells and three independent replicates were performed in each experiment.

Amplification of genomic DNA by polymerase chain reaction

Based on the sequences of *Hsp27* cDNA, two primers, *Hsp27* 9F and *Hsp27* 9R (Table 1), were designed to amplify an *Hsp27* genomic DNA fragment from *C. riparius*. Cycling parameters for PCR amplification were one cycle of 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1.5 min, with a final extension step at 72 °C for 10 min. PCR products were purified and sequenced with the primers *Hsp27* 9F and *Hsp27* 9R detailed in Table 1.

Statistical analysis

The levels of *Hsp27* gene mRNA in each sample were normalized against the level of *actin*, *GAPDH*, and *L13* in the same samples based on standard curves. The normalized levels of the specific gene transcripts in treated groups were compared to those of the non-exposed controls using ANOVA followed by Dunnett's multiple comparison tests using SPSS 19® (IBM). A $p<0.05$ was considered to indicate statistical significance.

Results

Characterization and sequence analysis of *Hsp27*

The full length of the *C. riparius Hsp27* mRNA was 1,010 bp, containing a 588-bp open-reading frame that encodes a protein of 195 amino acid residues with a calculated molecular weight of 22.8 kDa and an isoelectric point of 6.17. The complete nucleotide and amino acid sequence was deposited in GenBank under accession number # KC495957. The gene included a 5′ untranslated region (UTR) (107 bp) that is rich in adenosine (53.3 %), a characteristic feature of the 5′ UTRs of previously identified HSPs. The 293 bp 3′ UTR (not including the poly A tail) contained three possible polyadenylation signals (AATAAA) and one AU-rich element (ARE; ATTT A). In addition, there was only one cysteine at position 91 in the entire protein sequence, which is consistent with previous data showing that cysteine residues were rarer in the sequences of molecular chaperones than in other protein families (Fu et al. 2003). Analysis of genomic DNA showed that *Hsp27* gene has no introns. A search, via the BLAST software program, showed that the protein belongs to the α-crystallin/sHSP superfamily. Multiple sequence alignment showed that the deduced amino acid sequence of *C. riparius* shared less similarity with other previously described HSP27s. The *C. riparius* HSP27 exhibited 47 % identity at the amino acid level with *C. capitata*, 45 % with *D. melanogaster*, and 48 % with *S. crassipalpis*. Sequence comparison revealed three domains of relative high homology between *D. melanogaster* HSP27, *S. crassipalpis* HSP25 and *C. capitata* HSP27 (Fig. 1a). The first domain (aa M_1–I_{11} in ChrHSP27) present in the amino-terminal region is hydrophobic and shows a 29 % amino acid sequence identity in the four proteins (Fig. 1b). The second domain (aa D_{37}–N_{49} in ChrHSP27) shows 48 % identity among the four HSP27 homologs, and it is specific for heat shock protein 27 (Fig. 1b). The third domain downstream of the α-crystalline domain contains 28 residues (aa K_{159}–K_{186} in ChrHSP27) and shows 48 % amino acid sequence identity among the four HSP27 homologs (Fig. 1b). This domain is also present in all main *Drosophila* sHsps (Southgate et al. 1983).

Localization by in situ hybridization of the *Hsp27* gene on polytene chromosomes from salivary gland cells showed a single locus located on region I-C4 of the right arm at the polytenic chromosome I (Fig. 2).

Using ClustalW2, a phylogenetic tree based on the sequence of sHSP from different species was constructed. The

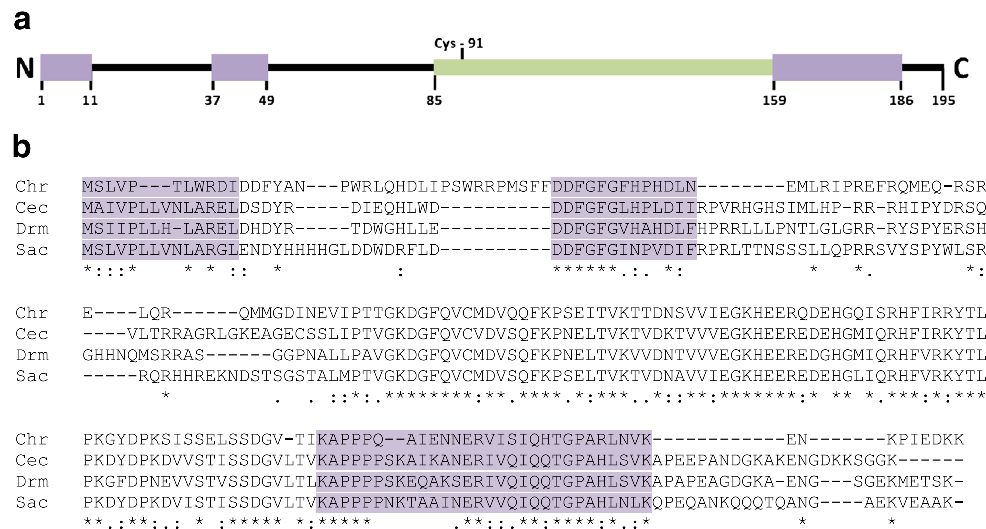

Fig. 1 **a** Schematic diagram of the protein domains of *C. riparius* HSP27 showing a conserved amino-terminal (*1–11*) and carboxy-terminal (*158–186*) region, a specific conserved region of HSP27 (*37–49*), α-crystallin domain (*85–158*) and the only cysteine (91). **b** Amino acid sequence comparison of heat shock protein 27 (HSP27) homologs from *C. riparius* (*Chr*), *C. capitata* (*Cec*), *D. melanogaster* (*Drm*) and *S. crassipalpis* (*Sac*). *Asterisks*, *double dots*, and *single dots* denote fully conserved, strongly conserved and weakly conserved amino acid residues, respectively. The three conserved regions characteristics of HSP27 are shown in *purple*

relationships displayed in the phylogenetic tree were consistent with the traditional taxonomy of insects (Fig. 3). *C. riparius* HSP27 was clustered in the same group with *D. melanogaster* HSP27, *C. capitata* HSP27 and *S. crassipalpis* HSP25, and separated from the HSP23 of *D. melanogaster* and *S. crassipalpis*. The sHSPs from *B. mori* and *L. migratoria* were separated from all other Dipteran sHSPs.

Effects of temperature in *Hsp27* mRNA levels

To examine the transcriptional response of *Hsp27* gene to cold shock, *C. riparius* larvae were submitted for 2 h at 4 °C. mRNA levels from control and cold-shocked samples were analyzed by quantitative qRT–PCR, following normalization to avoid random effects on sampling data in relation to *actin*, *GAPDH* and *rpL13* mRNA levels. A significant reduction in *Hsp27* mRNA levels was observed in larvae exposed to 4 °C (Fig. 4a). Nevertheless, when cold-shocked larvae were allowed to recover at control temperature, the expression of *Hsp27* gene was reactivated reaching higher mRNA levels than those found in untreated larvae. Within 4 h of recovery, after the shift to 4 °C, the *Hsp27* gene appeared to be significantly upregulated. Thereafter, the overexpressed *Hsp27* gene returned again to control level within 6 h of recovery at the normal growing temperature (Fig. 4b).

The effect of heat shock on the expression profile of the *Hsp27* gene was analyzed in *C. riparius* larvae submitted to 35 °C for 2 h. As shown in Fig. 4a, the *Hsp27* gene was clearly upregulated by the temperature shift with a significant increase in mRNA levels up to 400-fold higher than those found in control non-treated larvae.

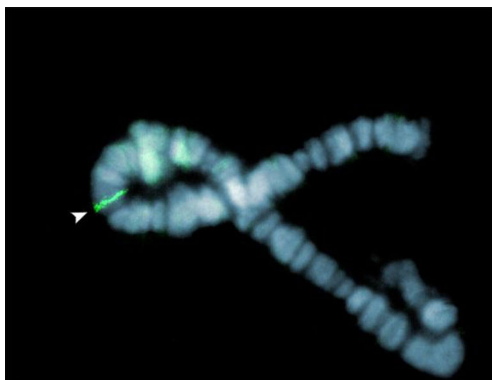

Fig. 2 Localization of the *Hsp27* gene in the *C. riparius* genome. Using digoxigenin-labeled probes, a single signal stained in green with FITC is detected by FISH at polytene location I-C4 on the right arm of the chromosome I counterstained in blue with DAPI. Chromosomes were obtained from salivary gland cells

Effect of different xenobiotics on the expression profile of *Hsp27* gene

To assess if the *Hsp27* gene is a xenobiotic-responsive gene in *C. riparius*, the expression profile was analyzed in larvae submitted to different chemicals. TBTO, NP, TCS, BPA, and Cd were assayed for short 24-h exposures at different concentrations. No significant differences in larvae survival were found for the times and concentrations selected for each chemical. The *Hsp27* mRNA level was analyzed by real-time RT–PCR, following normalization with reference genes. In each case, gene expression patterns were compared to those obtained from control cultures exposed to the same concentration of solvents. As shown in Fig. 5e, *C. riparius* larvae exposed to Cd showed a significant increase in the expression

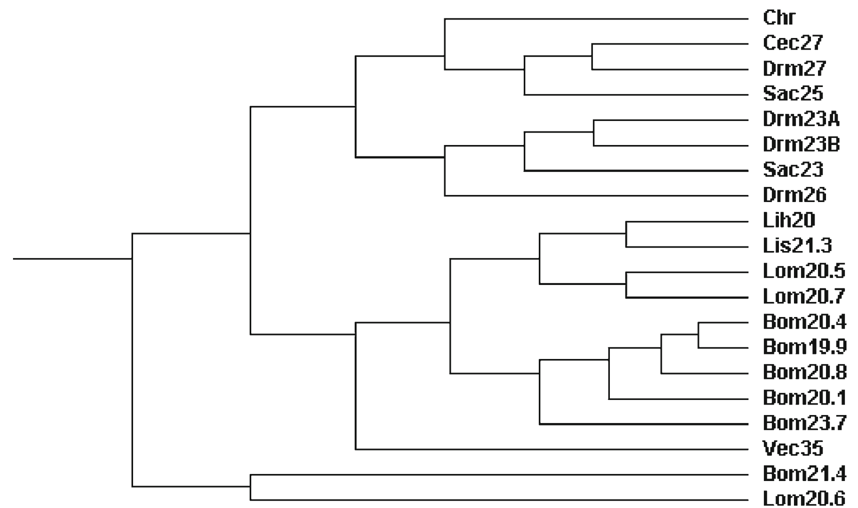

Fig. 3 Phylogenetic analysis of insect small heat shock protein (sHSP) sequences. The full names of the species are: *C. riparius* (*Chr*), *C. capitata* (*Cec*), *D. melanogaster* (*Drm*), *S. crassipalpis* (*Sac*), *L. huidobrensis* (*Lih*), *L. sativae* (*Lis*), *B. mori* (*Bom*), *V. canescens* (*Vec*), and *L. migratoria* (*Lom*)

of the *Hsp27* gene. The mRNA level for 1 and 10 mM Cd-treated larvae were, respectively, 25- and 35-fold higher than that found in untreated control. BPA exposures also resulted in a significant increase in *Hsp27* gene expression, doubling that found in control larvae after 24-h 0.5 mg/L BPA treatments (Fig. 5d). When larvae were submitted to TCS, there was also a significant increase in the expression of the *Hsp27* gene, after 24 h of exposure at 1,000 μg/L (Fig. 5c). However, neither 4-NP (Fig. 5b) nor TBT (Fig. 5a) altered *Hsp27* gene activity, because there were no significant changes in mRNA levels after any of the assayed treatments.

Discussion

In the present work, the *Hsp27* gene from *C. riparius* has been characterized and its activity evaluated under a variety of experimental treatments, showing for the first time that this gene is differentially regulated by temperature shifts and pollutants in this species. The *C. riparius Hsp27* gene is intronless, maps at a single chromosomal locus and the deduced protein contains the typical motifs that have been previously described (Sun and MacRae 2005). Amino acid sequence comparison revealed homology with other dipteran species, finding identities of around 50 % with *D. melanogaster* HSP27, *C. capitata* HSP27, and *S. crassipalpis* HSP 25. This is in contrast with that reported for the *C. riparius Hsp70* gene, which appeared highly conserved, at both DNA and protein levels, sharing 80–96 % of overall amino acid identities with homologous sequences from other diptera (Morales et al. 2011). Indeed, members of the sHSPs appear to be the least conserved, with the exception of the α-crystallin domain, when compared with other HSP families (Denlinger et al. 2001). In addition to the α-crystallin domain, three conserved domains were identified in HSP27 homologs of *D. melanogaster*, *S. crassipalpis*, *C. capitata*, and *C.*

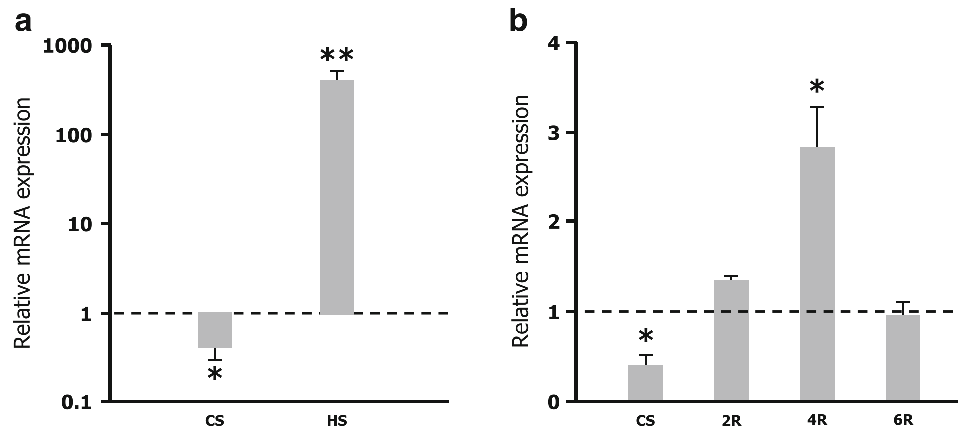

Fig. 4 **a** Relative levels of *Hsp27* mRNA in larvae exposure at 4 °C (*CS*) and 35 °C (*HS*) for 2 h measured by real-time RT–PCR. Note the logarithmic *y*-axis, where values>1 indicate upregulation and values<1 indicate downregulation of the transcript. **b** Expression of *Hsp27* mRNA in larvae exposed at 4 °C (CS) and recovery after exposure for 2 h (*2R*), 4 h (*4R*), and 6 h (*6R*) measured by real-time RT–PCR. The mean±SEM are shown of measurements taken in three independent biological samples, each with three replicates. The expression level of untreated control larvae was set to 1. Significant differences $*p \leq 0.05$, $**p \leq 0.01$

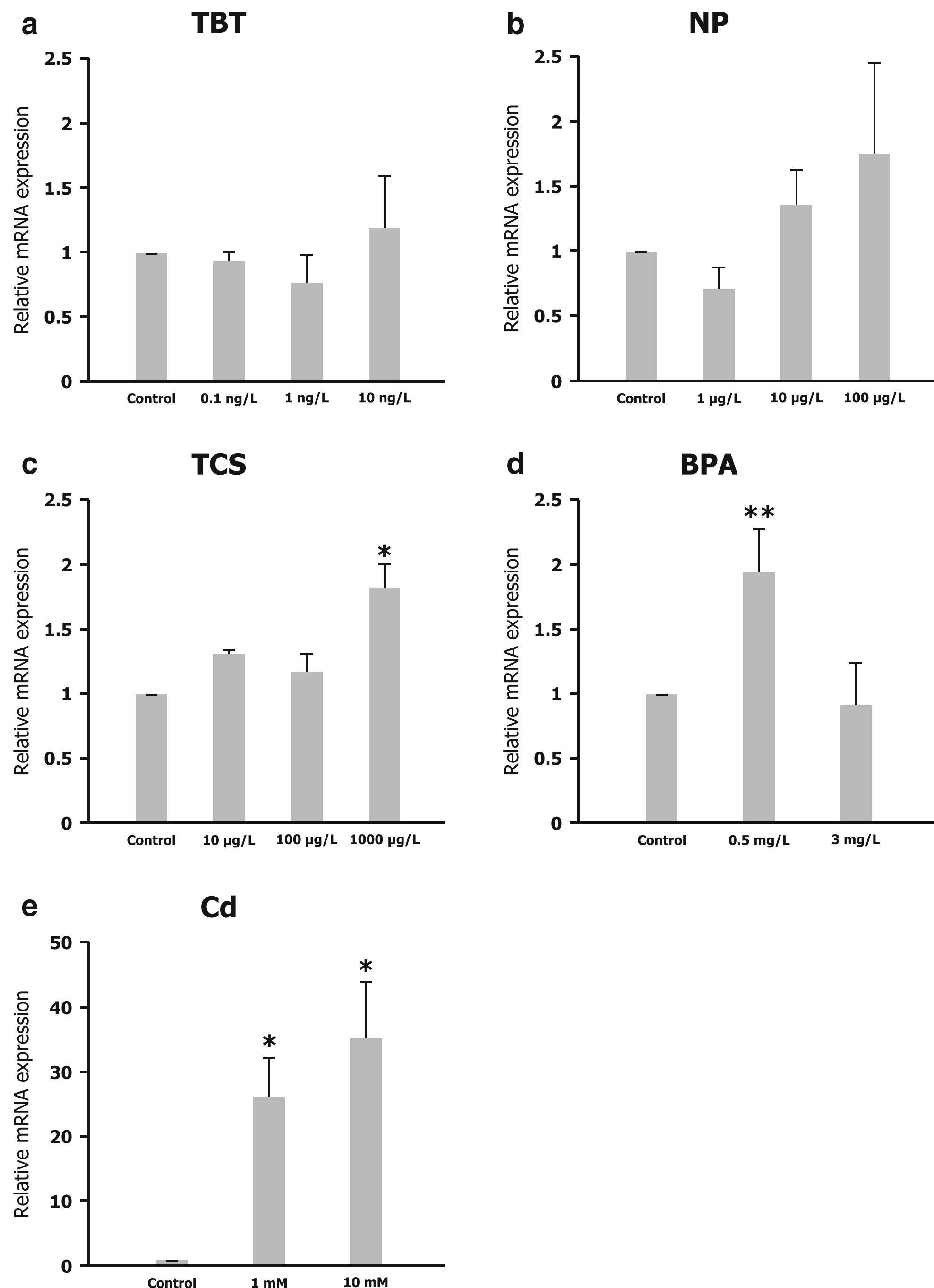

Fig. 5 Expression levels of the *Hsp27* gene in control larvae and treated larvae with 0.1, 1 and 10 ng/L of tributyltin (**a**), 1, 10 and 100 μg/L of nonylphenol (**b**), 10, 100 and 1000 μg/L of triclosan (**c**), 0.5 and 3 mg/L of bisphenol A (**d**), and 1 and 10 mM of $CdCl_2$ (**e**) measured by real-time RT–PCR with primers and reference genes as indicated in "Material and methods". The expression level of untreated control larvae was set to 1. Significant differences $*p \leq 0.05$, $**p \leq 0.01$

riparius (Fig. 2). The first domain, in the amino-terminal region of the protein (aa M_1–I_{11} in ChrHSP27), is very hydrophobic and is the least conserved, showing around 30 % amino acid sequence identity among these four homologs. This hydrophobic domain is also conserved in *Drosophila* HSP23 and HSP26, but not in *Drosophila* HSP22 (Southgate et al. 1983), and it has been suggested that it plays a role in oligomerization and chaperoning activity in several sHSPs (Sun and MacRae 2005). The second domain (aa D_{37}–N_{49} in ChrHSP27) shows around 50 % amino acid sequence identity among the four HSP27 homologs. This domain is very specific as it is only conserved in the HSP27 (Kokolakis et al. 2008), suggesting that it may play a distinct role in this protein. The third domain, located downstream to the α-crystallin domain, contains 28 amino acids (aa K_{159}–K_{186} in ChrHSP27) and shows around 50 % amino acid sequence identity among the four HSP27 homologs. This domain is present in all the main *Drosophila* sHSP (Southgate et al. 1983). In addition, *C. riparius* HSP27 has a conserved cysteine at position 91, which has been reported to have a regulatory role in the apoptotic signaling of HSP27 in human cells (Bruey et al. 2000). Future studies will determine if the

conserved cysteine of *C. riparius* HSP27 functions in a similar manner to that found for mammalian cell culture systems (Fu et al. 2003).

Previous phylogenetic analysis that included vertebrates and plants has already suggested that different sHSPs of the same species are phylogenetically closer than homologous sHSPs between species, pointing to the possibility that sHSPs might have evolved by gene duplication after species divergence (de Jong et al. 1993). However, more recent studies have indicated that several mammalian sHSPs have clearly recognizable orthologs in lower vertebrates (Franck et al. 2004), while the HSP27 homologs from *D. melanogaster*, *C. capitata*, and *S. crassipalpis* have been separated from other dipteran sHSPs (Kokolakis et al. 2008). In agreement with these data, we found that the HSP27 of *C. riparius* was also separated from other dipteran sHSPs, suggesting that the four proteins are orthologous and may have evolved before divergence of these species.

As *C. riparius* is one of the invertebrate aquatic species recommended by the OECD for acute toxicity testing, one of the aims of this work was to evaluate the early response of the *Hsp27* gene under different environmental stressors, including high and low temperature and short-term exposures to chemicals. Results are summarized in Table 2 and compared to those previously reported for *Hsps70* in this organism. In relation to temperature, the *Hsp27* gene appeared to be significantly upregulated by a heat shock at 35 °C, as expected since high temperature is the classic inducer of HSPs. We found levels of mRNA up to 400-fold higher than those from larvae at the normal growing temperature (20 °C). This response of the *Hsp27* gene is similar and concomitant with the activation of the *Hsp70* gene previously reported during the same temperature shift (Morales et al. 2011). In contrast, it is worth mentioning that the *Hsc70* gene maintained a constitutive expression, and it was neither upregulated nor downregulated after 35 °C exposures of *C. riparius* larvae (Morales et al. 2011). Several studies in *D. melanogaster* had previously reported that Hsp27 gene and proteins are highly induced over a broad temperature range (30-37 °C) (Lindquist 1980; Vazquez et al. 1993). It has been proposed that overexpression of either *Hsp26* or *Hsp27* genes extends lifespan in *D. melanogaster*, and also increases stress resistance in transgenic flies (Wang et al. 2004).

Table 2 *Hsp27* gene expression under different environmental stressors, including high and low temperature and short-term exposures to chemicals

	HS	CS	TBT	NP	TCS	BPA	Cd
Hsp70	+	nd	=	++	nd	+	+
Hsc70	=	nd	=	=	nd	=	=
Hsp27	++	−	=	=	+	+	++

HS heat shock; *CS* cold shock; *TBT* tributyltin; *NP* nonylphenol; *TCS* triclosan; *BPA* bisphenol A; *Cd* cadmium

+ upregulated, ++ strongly upregulated, − downregulated; = not altered, *nd* not determined

A common physiological response to thermal injury is the increased expression of heat shock proteins, but the response to cold stress has been far less studied in insects. In our study, a modulation of *C. riparius Hsp27* gene activity was found during cold exposure and also throughout the recovery period. While the *Hsp27* gene was significantly downregulated in response to cold exposure at 4 °C, a clear reactivation of the gene took place when larvae recovered at the normal growing temperature. Moreover, a significant overexpression took place 4 h after the cold exposure. It is worth noting that the upregulation of the gene occurred at the normal temperature of 20 °C and, therefore, it could be due to a retarded effect of the cold (4 °C) in activating the Hsp27 gene or, alternatively, due to a thermal shift effect (from 4 °C to 20 °C), even though the final temperature was the normal growing temperature and not strictly a heat shock for these organisms. Thereafter, the overexpressed *Hsp27* gene again returned to the control level within 6 h of recovery at the normal temperature. The recovery period is required before the *Hsp27* gene is activated, indicating that the gene is not involved in the immediate response to cold shock that leads to rapid cold hardening (Chen et al. 1987; Lee et al. 1987; Clark and Worland 2008). It is interesting to note that cold shock also did not induce the *Hsp70* gene in *C. riparius* (data not shown). These data suggest that some members of the HSP family behave in a different manner during high and low temperature stress. Although there is a consensus regarding the activation of *Hsp* genes by high temperatures, there is little agreement in their response to cold shocks and previous studies have shown notable differences. A reduction in expression levels of different Hsps genes (*Hsp19.7*, *Hsp20.7*, *Hsp70*, and *Hsp90*) was found in cultured cells of *M. brassicae* after 48-h exposures to 0 °C. The expression levels were reactivated during the recovery phases (Sonoda et al. 2007). Sinclair et al. 2007 did not observe any modulation of *Hsp23* during exposure to or recovery from a short cold stress in *D. melanogaster*. In the same species, the transcriptional activity of different s*Hsps* (*Hsp22*, *Hsp23*, *Hsp26*, and *Hsp27*) was not modulated by cold, but peaks of expression occurred during the recovery phase (Qin et al. 2005; Colinet et al. 2010). In contrast, Yocum et al. 1998 reported that expression of the *Hsp23* transcript of the nondiapausing *S. crassipalpis* was induced in response to cold and low temperature also significantly induced the *Hsp27* gene in fishes (Yang et al. 2012).

Regarding the chemical treatments, three out of five compounds tested were able to trigger activation of the *Hsp27* gene. All are known for their endocrine activity, and it is worth stressing that previous experiments have shown that the *Hsp27* gene is regulated by the hormone 20-hydroxyecdysone in a salivary gland culture of the fruit fly *C. capitata* (Kokolakis

et al. 2008). In *C. riparius*, the expression of *Hsp27* appeared to be differentially regulated by these hormonal disruptors. Tributyltin or nonylphenol did not alter the *Hsp27* gene after the exposures assayed in *C. riparius*. In addition, we have previously found that the *Hsp70* gene was not altered by TBT (Morales et al. 2011). Therefore, it seems that exposure to TBT does not elicit a stress response in insects, even though this compound has a high toxicity for the larvae, provokes significant DNA damage and alters the expression pattern of a suite of genes involved in the initial response to steroid hormonal signals (Morales et al. 2013). Yet, nonylphenol was able to induce *Hsp70* gene activity in *C. riparius* (Lee and Choi 2006; Morales et al. 2011). The differential sensitivity of *Hsp27* gene to specific chemicals was also reported in *Apis cerana*; while only some pesticides were able to upregulate this gene others did not alter it or even downregulate transcription (Liu et al. 2012).

In contrast, exposure to triclosan, bisphenol A and, particularly, cadmium significantly increased *Hsp27* mRNA levels in *C. riparius.* This is the first evidence of the ability of TCS, a widely used bactericide and common pollutant of waterways, to alter the activity of a small heat shock protein gene in invertebrates. To date, there are very few studies on the regulation of genes after TCS exposures in aquatic organisms, but it has previously been reported that TCS elicited a cellular stress response, as indicated by the altered *CAT* and *Hsp30* transcript levels in frogs (Hinther et al. 2011). We also found that BPA was able to increase *Hsp27* gene expression, with this compound also being an activator of the *Hsp70* gene in this organism (Planelló et al. 2008; Morales et al. 2011). Among the toxicants tested in the present study, cadmium was the most potent inducer of the *Hsp27* gene, which appeared to be highly overexpressed by up to 35-fold at the higher dose assayed. These data suggest that this gene might be a useful biomarker for assessing cadmium, a widely distributed industrial and environmental toxin, and should be assayed for other heavy metals. In *C. riparius*, we have previously found that cadmium altered ribosome biogenesis and activated the stress response, as indicated by upregulation of *Hsp70* (Martínez-Guitarte et al. 2007; Morales et al. 2011). The expression of *Hsp19.7*, *Hsp20.7*, *Hsp70*, and *Hsp90* genes has been induced by cadmium exposure in *M. brassicae* (Sonoda et al. 2007). Accumulation of the HSP27 protein was detected in human renal epithelial cells after chronic treatment with low doses of cadmium (Bonham et al. 2003) and, in fishes, the *Hsp27* gene also experienced a dramatic upregulation after a combined stress of temperature and cadmium (Yang et al. 2012).

In conclusion, the experimental data from our study indicate that the *Hsp27* gene in *C. riparius* is capable of sensing the cellular stress caused by heat and cold, as well as that caused by various environmental pollutants. The application of HSPs in environmental risk assessment has gained momentum during the last decade and, in particular, the induction of HSP70 has been used as an environmental screening tool (Karouna-Renier and Zehr 2003). Despite increasing evidence to support the role of HSP27 as a biomarker for many diseases including cancer (Wang et al. 2009), more studies are required to address its usefulness in a toxicological context. The induction of the expression of *Hsps* as a bioindicator for environmental pollution is being advocated by a number of researchers (Gupta et al. 2010). Yet, to date, only a few studies have analyzed small *Hsps* genes in response to toxicants. Our data suggest that the small *Hsp27* gene might play an important role in xenobiotic responses, reflecting the integrated severity of the environmental stress. Its potential use for predicting the toxicity of chemicals merits further research to validate its sensitivity and specificity.

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