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Characterization of Hsp70 gene in *Chironomus riparius*: Expression in response to endocrine disrupting pollutants as a marker of ecotoxicological stress

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ABSTRACT

We characterized the Hsp70 cDNA in *Chironomus riparius* and evaluated its expression profile under different environmental stressors. It is highly conserved, at both DNA and protein levels, displaying many of the hallmarks of Hsps and sharing 80–96% of overall amino acid identities with homologous sequences from other diptera. The changes are mainly concentrated in the C-terminal domain of the protein. Phylogenetic analysis was consistent with the known classification of insects. The Hsp70 gene was located by in situ hybridization in region III-3A at the third polytene chromosome, a locus activated upon heat shock as shown by RNA pol II binding. As *C. riparius* is widely used in aquatic ecotoxicology testing, we studied Hsp70 gene induction in fourth instar aquatic larvae submitted to heat shock and selected environmental pollutants classified as potential endocrine disruptors. RT-PCR analysis showed that Hsp70 mRNA levels increased significantly ($p<0.05$) after short-term acute exposures to a temperature shift (HS), cadmium chloride (Cd), butyl benzyl phthalate (BBP), diethylhexyl phthalate (DEHP), bisphenol A (BPA), 4-nonylphenol (NP) and ethinylestradiol (EE). However, neither pentachlorophenol (PCP) nor tributyltin (TBTO) treatments were able to activate the Hsp70 gene. The cognate form, Hsc70, was also analysed and, unlike Hsp70, was not altered by any of the different treatments assayed. Moreover, at the times tested, there was no significant mortality of the larvae. The rapid upregulation of the Hsp70 gene suggests that it is sensitive and selective for different environmental pollutants, and could be used as an early molecular endpoint in ecotoxicological studies.

1. Introduction

Several thousand anthropogenic chemicals are continuously released into the natural environment, and all organisms are challenged by events that cause acute or chronic stress. Gene–environment interactions play a critical role in these processes. Environmental toxicants can trigger biological effects at the organism level only after initiating biochemical and cellular events. The cellular response to stress is characterized by the activation of genes involved in cell survival to counteract the physiological disturbance induced by physical or chemical agents. Cells activate a set of genes, called heat-shock genes as they were discovered by temperature insults, which mediate protective responses to temperature, radiation and environmental contaminants (Morimoto, 1998). It is widely accepted that the family of heat-shock proteins counteracts cellular stress and its associated damage (Feder and Hoffman, 1999; Nolen and Morimoto, 2002). Hsps are suitable as an early warning bioindicator of environmental hazard, because of their sensitivity to even minor changes in cellular homeostasis and their conservation along the evolutionary scale. Currently, their potential use for predicting the toxicity of chemicals is being actively investigated (De Pomerai, 1996; Gupta et al., 2010). Attention is also now being focused on modulating the expression of this group of proteins for the treatment of a wide variety of human diseases (Powers and Workman, 2007).

Among Hsps, the Hsp70 family represents one of the most highly conserved proteins identified to date, and has constitutive as well as regulated members in all the organisms examined (Mayer and Bukau, 2005). Hsp70 is one of the most abundantly induced proteins under a variety of stress conditions, while Hsc70 members are constitutively expressed under normal growth conditions. Most experimental work on the Hsp70 family has aimed to clarify the molecular mechanism of the chaperon system, with considerable recent progress in understanding the family's diverse functions in cells related to signalling pathways and protein homeostasis (for a review see Young, 2010). In addition, it is worth noting the recent advances in the molecular description of Hsp70 genes in a variety of species, as well as their evaluation in response to environmental stressors and toxicants (Dang et al., 2010; Karouna-Renier and Rao, 2009; Rhee et al., 2009; Ming et al., 2010; Simoncelli et al., 2010; Sinha et al., 2010; Su et al.,

Abbreviations: aa, amino acids; BBP, butyl benzyl phthalate; bp, base pairs; BPA, bisphenol A; DEHP, diethylhexyl phthalate; EE, ethinylestradiol; NP, 4-nonylphenol; PCP, pentachlorophenol; RACE, rapid amplification of cDNA ends; TBTO, tributyltin oxide.

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2010; Waagner et al., 2010; Zhanng and Denlinger, 2010). It is now clear that compounds, including pesticides, metals and a variety of organic chemicals, are able to induce the production of some Hsps. However, depending upon the type of inducer there are variations in the pattern, magnitude, kinetics and duration of Hsp expression, which are still not clearly understood. Our knowledge of the underlying mechanisms governing the activation of Hsp70 genes is still far from complete. This environmental perspective adds further interest for studying the relevance of the Hsp70 gene as a toxicological endpoint after exposure to different environmental pollutants (Gupta et al., 2010).

Invertebrates, especially arthropods such as insects and crustaceans, constitute the vast majority of animal species on earth. Yet, they have received much less attention than vertebrates regarding the potential toxicity of most man-made chemicals. It is well known that aquatic insects are sensitive bioreporters of xenobiotic contamination, as exposure occurs during critical developmental stages, such as embryogenesis, larval development and pupation. The midge *Chironomus riparius* is an EPA- and OECD-approved test organism widely used in environmental toxicology (EPA, 1996; OCDE 2001). Chironomid larvae are employed in aquatic toxicity studies because of their ecological relevance in freshwater ecosystems and their association with benthic sediments, where the accumulation of many pollutants takes place. Survival tests and changes in developmental parameters are used in most studies to evaluate toxicity responses. Larval mouthpart deformities also function as indicators of anthropogenic stress (Martinez et al., 2003). Moreover, the giant polytene chromosomes from the salivary gland cells are a particularly suitable material for analysing the genotoxic effects of these compounds (Michailova et al., 2006). Although such studies have provided valuable data, novel molecular endpoints should also be used, in combination with classical reproductive endpoint and life-cycle testing, to increase our understanding of the mechanisms and modes of action of xenobiotics. The genes coding Hsps have recently acquired great relevance, and Hsp70 has been sequenced and evaluated as a biomarker of exposure to metals and insecticides in some species of chironomids, such as *Chironomus yoshimatsui* and *Chironomus dilutus* (Yoshimi et al., 2002; Karouna-Renier and Rao, 2009). The aim of the present study was to characterize the Hsp70 gene in *C. riparius* and to investigate transcriptional regulation of this gene under control and different stressful conditions, including temperature shifts and exposures to metals, insecticides and different organic chemicals classified as potential endocrine disrupting compounds (EU-Strategy for Endocrine Disruptors/Environment-Endocrine Disruptors Website). Developmental and reproductive impairments have been clearly demonstrated for these chemicals in a number of species, but relatively little is known about the subtle effects at the molecular level. Moreover, gene expression profiles could be a powerful new endpoint for ecotoxicological studies (Snell et al., 2003).

2. Material and methods

2.1. Animals and treatments

The experimental animals were fourth instar larvae from the midge *C. riparius*. They were obtained from laboratory cultures; larvae 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 (US-EPA, 1996; OECD, 2001). Larvae were grown from egg masses in an aqueous culture medium (0.5 mM $CaCl_2$, 1 mM NaCl, 1 mM $MgSO_4$, 0.1 mM NaHCO3, 0.025 mM KH_2PO_4, and 0.01 mM $FeCl_3$) supplemented with nettle leaves, commercial fish food, and cellulose tissue in polyethylene tanks (500 mL). Cultures were maintained under constant aeration at 20 °C and under standard light–dark periods (16L:8D). For experimental treatments, the larvae were exposed to the chemicals diluted in a culture medium for 24 h with constant aeration at 20 °C in glass recipients (200 mL). No food or substrate was provided during exposure. Dose selection was based on results from previous studies in *Chironomus* sp. and other arthropods. Fourth instar larvae were submitted to 10 mM cadmium chloride (Cd) (Fluka), 1 mg/L butyl benzyl phthalate (BBP) (Aldrich), 0.01 μg/L diethylhexyl phthalate (DEHP), 1 μM pentachlorophenol (PCP) (Aldrich), 3 mg/L bisphenol A (BPA) (Aldrich), 10 mg/L 4-nonylphenol (NP) (Fluka), 1 ng/L tributyltin oxide (TBTO) (Aldrich), and 5 mg/L ethinylestradiol (EE) (Sigma), nominal concentrations. For temperature treatments, larvae were heat-shocked at 35 °C for 120 min in a preheated and aerated cultured medium, as described previously (Morcillo et al., 1988). Each treatment consisted of at least 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.

2.2. 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. Briefly, frozen larvae were homogenated in one volume of Trizol and left for 5 min at room temperature. Then, 0.2 volumes of chloroform were added to each sample, mixed and left for 5 min at room temperature. Subsequently, the samples were centrifuged for 15 min at 4 °C and 15,000 *g*. Following transfer of the aqueous phase, the RNA was finally recovered by isopropyl alcohol precipitation (0.5 v/v), washed with 70% ethanol, and resuspended in DEPC water. The quality and quantity of total RNA were determined by agarose electrophoresis and absorbance spectrophotometry (Biophotomer Eppendorf), and purified RNA finally stored at −20 °C.

2.3. Amplification of HSP70

Total RNA extracted from *C. riparius* larvae, exposed for 2 h at 35 °C heat shock, was used for Hsp70 amplification. Based on the conserved sequences of Hsp70 genes from closely related species, two pairs of primers, Hsp70 (201-220) and Hsp70 (1006-987) (Table 1), were designed to amplify an Hsp70 cDNA fragment (806 bp) from *C. riparius*, while Hsp70 (852-873) and Hsp70 (2094-2076) were designed to amplify another Hsp70 cDNA fragment (1243 bp) 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, and 72 °C for 1.5 min, with a final extension step at 72 °C for 10 min. The PCR products were purified (ExoSAP-kit One Step PCR Clean-up no. US78200, GE Healthcare) and sequenced with the primers [Hsp70 (201-220), Hsp70 (242-226), Hsp70 (341-319), Hsp70 (852-873), Hsp70 (1006-987), Hsp70 (1974-2001) and Hsp70 (2094-2076)] detailed in Table 1.

2.4. 5′ and 3′ RACE

The full-length sequence of Hsp70 was determined using 5′ and 3′ RACE (Rapid Amplification of cDNA Ends), using commercial kits (Invitrogen) and following the procedures described by the manufacturer. New gene-specific primers were designed, based on sequence information obtained from the internal fragments. The sequences of all gene-specific primers used for RACE are given in Table 1.

For the 3′ end RACE PCR, a cDNA template was obtained as described above, and a PCR was performed with gene-specific primer Hsp703′ (1) and an adapter primer AUAP (RACE kit, Invitrogen). The PCR conditions were one cycle of 94 °C for 5 min, followed by 35 cycles

Table 1
Primers used for cDNA sequence and semi-quantitative RT-PCR of the HSP70 gene from *C. riparius*.

PCR objective	Oligo name	Primer DNA sequence
3′ RACE	Hsp703′ (1)	5′- GTGTACATCTGAATTATCATGGC-3′
	Hsp703′ (2)	5′-GAAGAATTTGAAGATCATTTGAAGG-3′
5′ RACE	Hsp705′ (1)	5′- TATTTTGTGGATTCATTGCAACC-3′
	Hsp705′ (2)	5′- AATCGTTCCGAGTCTGTGAATGC-3′
β actin	F	5′-GATGAAGATCCTCACCGAACG-3′
Semi-quantitative RT-PCR	R	5′-CCTTACGGATATCAACGTCGC-3′
Hsp70	F	5′-CATGTGAACGAGCCAAGAGA-3′
Semi-quantitative RT-PCR	R	5′-TTGCCACAGAAGAAATCTTG-3′
Hsc70	F	5′- CGTGCTATGACTAAGGACAA-3′
Semi-quantitative RT-PCR	R	5′- GCTTCATTGACCATACGTTC-3′
Hsp70	Hsp70 (201-220)	5′- GGTATTGATCTTGGCACAAC-3′
Sequence	Hsp70 (1006-987)	5′- GACAATGTTCTCTTGGCTCG-3′
	Hsp70 (852-873)	5′- GGTGATACACATTTGGGTGGTG-3′
	Hsp70 (2094-2076)	5′- CTTCCTCAACTGTTGGACC-3′
	Hsp70 (1974-2001)	5′- GTACAGCGAGTTTGTGGTCCAATAATGG-3′
	Hsp70 (341-319)	5′- TGCATCACCAATCAATCGTTCCG-3′
	Hsp70 (242-226)	5′- AAAGACACCAACACACG-3′
	Hsp70 (50-71)	5′- AAGTAAAAGAAAATTCAAAGCG-3′

at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s, and 72 °C for 10 min. The PCR products were purified and sequenced with the primers [Hsp70 (1974-2001), Hsp703′ (1), and Hsp703′ (2)] detailed in Table 1.

For the 5′ end RACE PCR, the RNA was transcribed by 5′ RACE kit (Invitrogen) with gene-specific primer Hsp705′ (1), and the cDNA was subsequently amplified with an adapter primer AAP (RACE kit, Invitrogen) and a gene-specific primer Hsp705′ (2). 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, 72 °C for 40 s; and 72 °C for 10 min. The PCR products were purified and sequenced with the primers [Hsp70 (242-226), Hsp70 (50-71), Hsp705′ (1) and Hsp705′ (2)] detailed in Table 1.

2.5. Sequence alignment, analysis and phylogenetic trees

Hsp70 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* HSP70 cDNA sequence was deposited in GenBank under Accession # ADL27420. In addition to the sequence reported here, we selected the following HSP70 family members for phylogenetic analysis: *Spodoptera exigua* (ACQ78180), *Mamestra brassicae* (BAF03555), *Bombyx mori* (NP_001037396), *Helicoverpa zea* (ACV32640), *Anatolica polita* (ABQ39970), *Liriomyza huidobrensis* (AAW32098), *Delia antiqua* (AAY28732), *Rhagoletis pomonella* (ABL06948), *Aedes aegypti* (ACJ64194), *Chironomus tentans* (AAN85117), *C. yoshimatsui* (BAD42358), *Culex quinquefasciatus* (XP_001864723), *Drosophila pseudoobscura* (XP_001358499), *Drosophila virilis* (XP_002056192), *Drosophila grimshawi* (XP_001990182), *Acyrthosiphon pisum* (XP_001949837), *Mus musculus* (AAA37864), *Gallus gallus* (AAA48825) and *Alligator mississippiensis* (BAF94142).

Sequence alignments of amino acid sequences from 20 inducible HSP70 family members were carried out using the Clustal X program, and confirmed by MAFFT version 6. A phylogenetic tree was generated by the MEGA 4 program using the neighbour-joining method. The Poisson model was used for distance estimation and bootstrap values were calculated with 1000 replicates.

2.6. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Following the instruction manual, 1 μg of total RNA with 0.5 μg Oligo dT_{20} (Invitrogen) was employed for reverse transcription with M-MLV enzyme (Invitrogen). The synthesized first-strand cDNA was stored at −20 °C until used as templates for PCR reactions. Multiplex reactions were carried out using gene-specific primers, with β-actin as reference gene. Gene-specific primers for actin and Hsc70 were designed from DNA sequences present in databases, following a search in the FASTA invertebrate database to detect conserved regions. The Hsc70 sequence from *C. yoshimatsui* (AF448434) was compared with sequences from *C. tentans* (AF448433), *Ceratitis capitata* (U20256), *Drosophila melanogaster* (AY084193), *Manduca sexta* (AF194819), *Locusta migratoria* (AY178988), *B. mori* (AB084922), *Chilo suppressalis* (AB206478), *Trichoplusia ni* (U23504) and *M. brassicae* (AB251896) and a highly conserved region was defined and used to design a set of primers (Table 1). The primers were designed to produce different-sized PCR products to allow simultaneous multiplex analysis with β-actin as an internal control. In notated cases, actin amplification was carried out using a mix of primers–dideoxy primers to obtain a similar signal with the cycles used. Sequences and fragment size of each gene-specific pair of primers are shown in Table 1. *C. riparius* gene fragments were cloned and sequenced to ensure that they matched the selected genes.

PCR was performed in 20 μL with 2 mM of $MgCl_2$, 0.2 mM dNTPs (Biotools, Spain), 0.4 μM of each primer and 0.2 μL of Taq polymerase (Biotools, Spain) under the following conditions: denaturation for 30 s, annealing for 30 s and elongation for 40 s. A MiniOpticon Thermocycler (Bio-Rad) was used. After several trials to ensure log-phase amplification, 32 cycles were carried out, with an annealing temperature of 54 °C, to amplify the fragments corresponding to Hsp70 and Hsc70. The amplified PCR products were run in a 9% acrylamide gel at 60 V for 3 h in 1× TGE buffer (40 mM Tris–Cl (pH 8.5), 200 mM glycine, and 2.5 mM EDTA), visualized after ethidium bromide staining and quantified with Chemigenius3 (Syngene), using GeneSnap 6.05 and GeneTools 3.06 software. Values of density across the whole bands were normalized against the actin standard, and the relative expression levels were then calculated. The level of expression in non-exposed control larvae was considered as background level or 1. To verify reproducibility, the analysis was repeated three times in independent experiments, with three replicates for each sample. The data were statistically analysed using SPSS software 11.5. The significant differences between groups were determined using the analysis of variance (ANOVA), with the Bonferroni method as the post hoc test. The level of statistical significance employed in all cases was $p<0.05$.

2.7. 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. The sequence of the Hsp70 probe was labelled by nick translation with Digoxigenin (Roche), denatured by boiling in water for

Fig. 1. (a) Nucleotide and amino acid sequences of *Chironomus riparius* HSP70. A long (167 bp) 5′ untranslated region (UTR) that is rich in adenosine (56%). A 211 bp 3′ UTR (not including the poly A tail) that contained one possible polyadenylation signal (**AATAAA**), and three AU-rich elements (ARE; ATTTA). Protein motifs are marked: **A**: three classical HSP signature motifs; □: ATP/GTP-binding site motif A (AEAYLGKT); A: cytosolic HSP70 character sequence (EEVD); **A**: bipartite nuclear targeting sequence (KRKFKKDITDNKRAVRR); **A**: non-organellar consensus motif (RARFEEM). (b) Schematic diagram of the protein domains of *Chironomus riparius* HSP70 showing a conserved ATPase domain in the N-terminal (aa 1–387); a linker region (aa 388–393); the peptide binding domain (aa 394–553); the variable region (aa 554–641) and the conserved C-terminal EEVD motif.

a

```
1    aatcatttcaaaaaagataactgaacaagaaagagctcagaagagtgacaagtaaaagaa
61   aattcaaagcgaatcaaaaagatttcaaagtttaaaagtgaaaaaaattgtgaaattgaa
121  taaaattaaatttttgtgaattgaaagtaaaataaaaataaaataaaaatgccagcaaaac
1                                                    M  P  A  K
181  agaaaacaggaacagcaattggtattgatcttggcacaacatactcgtgtgttggtgtct
5    Q  K  T  G  T  A  I  G  I  D  L  G  T  T  Y  S  C  V  G  V
241  ttcaacatggaaaagttgagatcatagccaatgagatgggaaatagaacaacaccaagtt
25   F  Q  H  G  K  V  E  I  I  A  N  E  M  G  N  R  T  T  P  S
301  atgttgcattcacagactcggaacgattgattggtgatgcagctaaaaatcaggttgcaa
45   Y  V  A  F  T  D  S  E  R  L  I  G  D  A  A  K  N  Q  V  A
361  tgaatccacaaaatacagtatttgatgcaaaacgattaattggacgtaaatttgatgatg
65   M  N  P  Q  N  T  V  F  D  A  K  R  L  I  G  R  K  F  D  D
421  aaaagattcaagctgatatgaaacattggccatttaaagtctataatgattgtggaaagc
85   E  K  I  Q  A  D  M  K  H  W  P  F  K  V  Y  N  D  C  G  K
481  caaagatccaagttgaatttaaaggagaacaaaagagatttgcaccagaagaaatcagtt
105  P  K  I  Q  V  E  F  K  G  E  Q  K  R  F  A  P  E  E  I  S
541  caatggtcttgacaaagatgaaagagactgctgaagcatatttaggacagaaagtcacag
125  S  M  V  L  T  K  M  K  E  T  A  E  A  Y  L  G  Q  K  V  T
601  atgcagttgtaactgtaccagcttatttcaatgactcgcaacgtcaagcaacaaaagatg
145  D  A  V  V  T  V  P  A  Y  F  N  D  S  Q  R  Q  A  T  K  D
661  caggtgctatcgccggtctgaatgtcttgagaatcatcaatgaaccaactgcagcagcac
165  A  G  A  I  A  G  L  N  V  L  R  I  I  N  E  P  T  A  A  A
721  tagcttatggtcttgataagaatcttaaaggagaacgaaatgttttaatttttgatcttg
185  L  A  Y  G  L  D  K  N  L  K  G  E  R  N  V  L  I  F  D  L
781  gtggtggaacttttgatgtctcaatcttaacaattgatgaaggttcattatttgaagtca
205  G  G  G  T  F  D  V  S  I  L  T  I  D  E  G  S  L  F  E  V
841  gatcaacagctggtgatacacatttgggtggtgaagactttgacaacagattagtaaatc
225  R  S  T  A  G  D  T  H  L  G  G  E  D  F  D  N  R  L  V  N
901  atttcattgaagaatttaagcgtaaacataaagcagacttaagcaagaatattcgtgcat
245  H  F  I  E  E  F  K  R  K  H  K  A  D  L  S  K  N  I  R  A
961  taagaagattaagaacagcatgtgaacgagccaagagaacattgtcatcatctacagaag
265  L  R  R  L  R  T  A  C  E  R  A  K  R  T  L  S  S  S  T  E
1021 cttcaattgaaattgatgcattacatgaaggagttgatttctattcaaagatcacaagag
285  A  S  I  E  I  D  A  L  H  E  G  V  D  F  Y  S  K  I  T  R
1081 caagatttgaagaaatgaatatggatttatttagatcaacacttgaaccagttgaacgtg
305  A  R  F  E  E  M  N  M  D  L  F  R  S  T  L  E  P  V  E  R
1141 cattaagagatgcaaaattcgacaagagtcaaattcatgatgttgttcttgttggtggat
325  A  L  R  D  A  K  F  D  K  S  Q  I  H  D  V  V  L  V  G  G
1201 caactcgaattccaaagattcagaaaatgcttcaagatttcttctctggcaaaagcttga
345  S  T  R  I  P  K  I  Q  K  M  L  Q  D  F  F  S  G  K  S  L
1261 acttttcaattaatccagatgaagcagttgcatatggagcggcagttcaagcagctattc
365  N  F  S  I  N  P  D  E  A  V  A  Y  G  A  A  V  Q  A  A  I
1321 taaccggtgatagtagctcaacaatccaagatgttttacttgttgatgtgacgccattat
385  L  T  G  D  S  S  S  T  I  Q  D  V  L  L  V  D  V  T  P  L
1381 cattgggaattgaaacagctggtggagtcatgacaaaacttattgaaagaaatgctcgaa
405  S  L  G  I  E  T  A  G  G  V  M  T  K  L  I  E  R  N  A  R
1441 ttccatgcaagcaacagaaaacatttacaacatattcagataatcagcctgcagttacta
425  I  P  C  K  Q  Q  K  T  F  T  T  Y  S  D  N  Q  P  A  V  T
1501 ttcaagtatttgaaggagaacgagcaatgacaaaagataataatttattgggaacattta
445  I  Q  V  F  E  G  E  R  A  M  T  K  D  N  N  L  L  G  T  F
1561 atttgactggaattgcaccagcaccaagaggaattccacaaattgaagtcacattcgatc
465  N  L  T  G  I  A  P  A  P  R  G  I  P  Q  I  E  V  T  F  D
1621 ttgatgctaatggcatcttgaatgtatcagccaaagatagttcaactggaaaacaagaga
485  L  D  A  N  G  I  L  N  V  S  A  K  D  S  S  T  G  K  Q  E
1681 caattacaatcaagaatgataaaggacgtctctcaaaggctgaaatcgatcgaatgcttt
505  T  I  T  I  K  N  D  K  G  R  L  S  K  A  E  I  D  R  M  L
1741 ctgaggctgaaaaatatcgtgacgaagatgaaaagcatcagcaacgaattcaagcaagaa
525  S  E  A  E  K  Y  R  D  E  D  E  K  H  Q  Q  R  I  Q  A  R
1801 atcaacttgaaagttacatctttggatgtaaacaagcagttgaagatgctccagctggca
545  N  Q  L  E  S  Y  I  F  G  C  K  Q  A  V  E  D  A  P  A  G
1861 aattgagtgaagatgacaagaaggtcgttcgtgataagtgtacatctgaattatcatggc
565  K  L  S  E  D  D  K  K  V  V  R  D  K  C  T  S  E  L  S  W
1921 ttgattccaacacacttgctgaaaaggaagaatttgaagatcatttgaaggatgtacagc
585  L  D  S  N  T  L  A  E  K  E  E  F  E  D  H  L  K  D  V  Q
1981 gagtttgtggtccaataatggctaaaatgcatggcggagcgggtgcacaaaaagctggtg
605  R  V  C  G  P  I  M  A  K  M  H  G  G  A  G  A  Q  K  A  G
2041 gatgtggtgcacaaagtggacagagagcatattcaggtccaacagttgaggaagtcgatt
625  G  C  G  A  Q  S  G  Q  R  A  Y  S  G  P  T  V  E  E  V  D
2101 aaattagtcaaatttaagagacaccaaatttgtcgtttatgctttaaagatactcaatt
2161 cgttaatcatattggatgtttgatgaataatttaaacacattatactaagtcaaagttag
2221 ggaacttatcgggctttttaaatttttatttactgctgtataaaatgttaaatattaag
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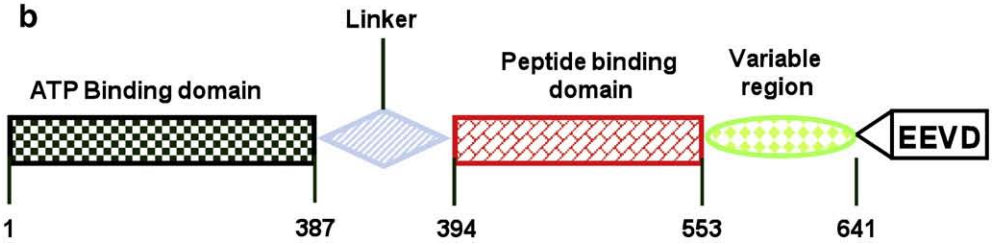
b



10 min and rapidly cooled on ice. The hybridization buffer was 50% deionized formamide and 4× SSC, 0.4% SDS. Each 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. For immunodetection of anti-RNA polymerase II antibodies, salivary gland cells were microdissected in Ringertz's solution (128 mM NaCl, 4.7 mM KCl, and 1.9 mM $CaCl_2$) under a Nikon stereomicroscope. The salivary glands were fixed for 10 min in 4% paraformalhehyde (Merck), 7 mM K_2HPO_4, 3 mM KH_2PO_4, 100 mM NaCl (PBS) and squashed in 50% acetic acid. The fixed tissues were then washed in PBS and incubated for 30 min in 20 mM glycine. After washing in PBS, they were permeabilised in a solution containing 1% Nonidet P40 and 0.5% sodium deoxycholate in PBS for 15 min. Slides were incubated in a PBS/BSA blocking solution or a TBST/Superblock solution for 1 h at room temperature. Thereafter, they were incubated in a humidified chamber at room temperature for 1 to 2 h in 1× PBS, 2% bovine serum albumin, 0.1% Tween (BTP) with the primary antibody, anti-RNA polymerase II monoclonal antibody (1:50) (Progen). After three intensive 10 min washes in PBST, they were incubated for 1 h with the appropriate anti-mouse antibodies conjugated with rhodamine or Texas Red (Sigma), diluted 1:100 in BTP. Slides were washed thoroughly three times for 15 min each with PBS 1×/0.01% Tween 20. The cells were then counterstained with 2 μg/mL DAPI (4′,6-diamino-2-phenylindole) for 5 min and mounted in anti-fading Vectashield (Vector Laboratories). 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 7.0.

3. Results

3.1. Sequence analysis, chromosomal localization and phylogenetic analysis of Hsp70

The length of the *C. riparius* Hsp70 open reading frame was 1935 bp encoding a protein with 644 amino acid residues (GenBank Accession no. HM769899). The full length of nucleotide sequence and the deduced amino acid sequence are shown in Fig. 1. The gene included a long 5′ untranslated region (UTR) (167 bp) that is rich in adenosine (56%), a characteristic feature of 5′ UTRs of previously identified HSPs, including *C. dilutus* Hsp70 and Hsp70′s from other species. The 211 bp 3′ UTR (not including the poly A tail) contained one possible polyadenylation signal (AATAAA) and three AU-rich elements (ARE; ATTTA) (Fig. 1a). Analysis of genomic DNA showed that the Hsp70 gene has no introns. Amino acid sequence analysis indicated a deduced molecular weight of 70.93 KDa for the HSP70 protein and conserved signature motifs. Classical HSP protein signature motifs include IDLGTTYS (aa 13–20), IFDLGGGTFDVSIL (aa 201–214) and VVLVGGSTRIPKIQN (AA 339–353). Three other typical motifs are also found in the *Cr*-Hsp70. The first is a deduced ATP–GTP-binding site, AEAYLGQK (aa 135–142). The second is a deduced bipartite nuclear localization signal, RKHKADLSKNIRALRR (aa 251–267). The third is a non-organellar consensus motif RARFEEM (aa 304–310). The sequence of the ATPase domain (aa 1–387) of Hsp70 is highly conserved, while the C-terminal domain is less conserved than the ATPase domain (Fig. 1b) (Su et al., 2010). This Hsp70 contains the conserved EEVD motif in the C-terminal, but lacks the conserved tetrapeptide repeat (GGMP) located at the C-terminal domain, which mediates the association of Hsp70 with Hsp90 into a multichaperone complex, found in other species.

The complete *C. riparius* Hsp70 amino acid sequence was deposited in GenBank under Accession # ADL27420. Multiple sequence alignment showed that the deduced amino acid sequence of *C. riparius* shared high similarity with other previously described Hsp70s. The *C. riparius* Hsp70 exhibited 96.6% and 95.2% identity at the amino acid level with *C. dilutus* and *C. yoshimatsui* respectively, 80% with *D. melanogaster*, and 75% with *Homo sapiens*. Using MEGA 4 programs, a phylogenetic tree was constructed, based on the sequence of Hsp70 from different species. The relationships displayed in the phylogenetic tree were consistent with the traditional taxonomy of insects and virtually identical between the protein-parsimony tree (not shown) and the neighbour-joining tree (Fig. 2).

Localization of the Hsp70 gene on polytene chromosomes from salivary gland cells, by in situ hybridization, showed a single locus located on region III-A3 at the third chromosome (Fig. 3a). This region was consistently puffed after exposure to high temperatures (2 h 35 °C), a condition that inhibited most chromosomal loci, while activating the heat-shock genes, as shown by immunostaining using anti-RNA polymerase II antibodies (Fig. 3b).

3.2. Transcriptional response of the Hsp70 gene under different experimental treatments

To assess the inducibility of the Hsp70 gene, mRNA expression in *C. riparius* fourth instar larvae was analysed by semi-quantitative RT-PCR, under normal larval growth conditions and various treatments. The different treatments assayed, summarized in Table 2, were selected on the basis of previous data obtained from the literature. All treatments were for short times and in acute doses. Mortality rates of larvae were determined for every toxicity assay. No significant differences in larval survival were found for the times and concentrations selected, for each chemical, or for the temperature shock applied (Table 2). In all cases, expression of Hsc70 was also analysed as a measurement of the effect of the different stressful treatments on a housekeeping gene, and to find out if there was differential behaviour by these two related Hsc70/Hsp70 genes. Following normalization to avoid random effects on sampling data, the expression of mRNA was analysed in multiplex RT-PCR in relation to actin mRNA levels. In each case, gene expression patterns were compared to those obtained from control cultures or, where required by chemicals in certain treatments, from control cultures exposed to the same concentration of solvents. To avoid differences being caused by experimental procedures or those intrinsic to the larvae being sampled, for each condition, three independent experiments from different cultures, as well as three replicates, were carried out. Fig. 4 shows the results obtained in Hsp70 and Hsc70 gene expression profiles for the different experimental treatments assayed. When larvae were submitted to a temperature shift of 15 °C above the normal temperature in the control culture, the expression of Hsp70 experienced a significant (four-fold) increase, whereas Hsc70 did not show significant changes. Similarly, in six of the eight experimental conditions assayed, Hsp70 mRNA abundance increased. Cadmium chloride (Cd), butyl benzyl phthalate (BBP), diethylhexyl phthalate (DEHP), bisphenol A (BPA), 4-nonylphenol (NP), and ethinylestradiol (EE) treatments resulted in a significant increase in Hsp70 mRNA ($p<0.05$), when compared with their respective control samples of untreated larvae exposed to the same concentration of solvent. However, there were no significant changes in the levels of Hsp70 mRNA after 24-hour treatments with pentachlorophenol (PCP) or tributyltin oxide (TBTO). Longer exposures to these two latter compounds provoked a high mortality of larvae (i.e. 48 h 1 μM PCP 5% survivals). In contrast to Hsp70, the cognate form Hsc70 was not significantly altered by any of the treatments assayed, with levels of mRNA Hsc70 nearly identical to those found in the corresponding control samples.

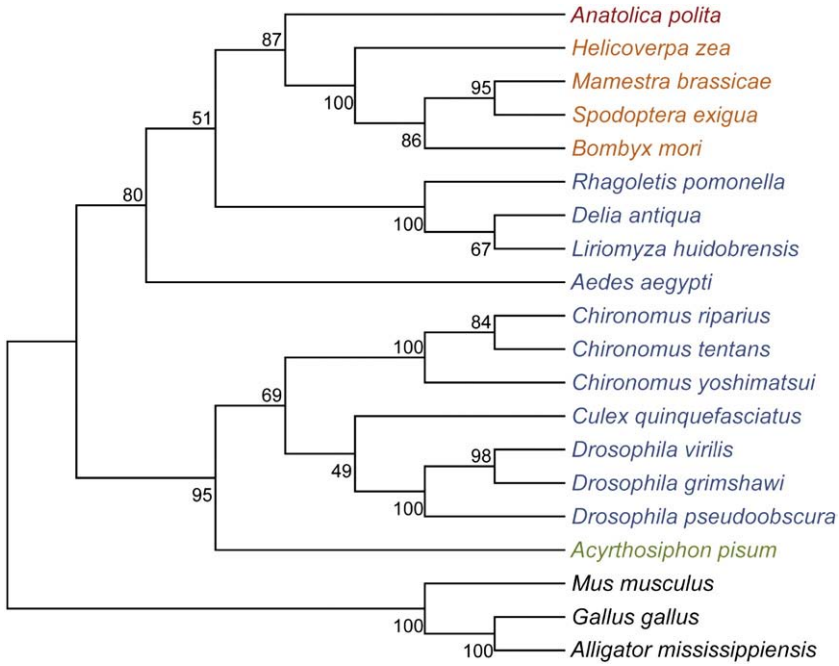


Fig. 2. Phylogenetic tree of HSP70 sequences, constructed by the neighbour-joining method. The values on the branches of the consensus tree indicate the number of times the partition of the species into the two sets, separated by that branch, occurred among the 1000 bootstrap replicates. The tree is based on alignment of the amino acid sequences of *Chironomus riparius* HSP70 and other proteins corresponding to four major insect taxa. In orange, Lepidoptera, *Spodoptera exigua* (ACQ78180), *Mamestra brassicae* (BAF03555), *Bombyx mori* (NP_001037396), and *Helicoverpa zea* (ACV32640). In red, Coleoptera, *Anatolica polita* (ABQ39970). In blue, Diptera, *Liriomyza huidobrensis* (AAW32098), *Delia antiqua* (AAY28732), *Rhagoletis pomonella* (ABL06948), *Aedes aegypti* (ACJ64194), *Chironomus tentans* (AAN85117), *Chironomus yoshimatsui* (BAD42358), *Chironomus riparius* (ADL27420), *Culex quinquefasciatus* (XP_001864723), *Drosophila pseudoobscura* (XP_001358499), *Drosophila virilis* (XP_002056192), and *Drosophila grimshawi* (XP_001990182). In green, Hemiptera, *Acyrthosiphon pisum* (XP_001949837). Vertebrate species were used as outgroups: *Mus musculus* (AAA37864), *Gallus gallus* (AAA48825) and *Alligator mississippiensis* (BAF94142).

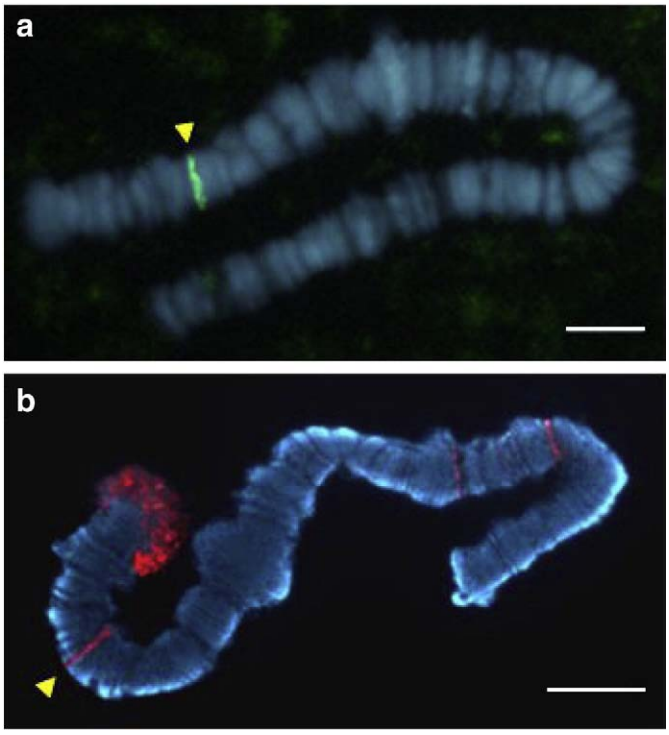


Fig. 3. (a) Localization of the hsp70 gene in the *Chironomus riparius* genome. Using digoxigenin-labelled probes, a single signal stained in green with FITC is detected by FISH at polytene location III-A3B on the right arm of third chromosome counterstained in blue with DAPI. (b) Transcription at locus IIA3 detected by antibodies against RNA polymerase II in polytene chromosomes of salivary glands from *Chironomus riparius* larvae heat-shocked at 35 °C. The heat-shock-induced locus at region III-A3 stained in red with Texas Red anti-RNA pol II antibodies is marked by arrowheads, while a typical induced telomeric puff (T-BRIII) is marked by a white arrow. Bar 10 μm.

4. Discussion

HSPs are among the most ancient and highly conserved of all proteins. Homologues of HSPs occur in every species in which they have been sought, and in all kingdoms of living things, even in the smallest genomes. HSPs have been found in every cell type under both normal and stressed conditions, as they play a basic role in fundamental cellular processes (Feder and Hoffman, 1999). In our study, the Hsp70 gene in *C. riparius* has been characterized and its activity evaluated under different experimental treatments, comparing its behaviour with that of the Hsc70 gene.

The sequence of the *C. riparius* Hsp70 gene and the deduced HSP70 protein conserved the typical motifs that have been previously described (Sonoda et al., 2006). At the amino acid level it shared a high level of identity with the HSP70 proteins of *C. yoshimatsui* and *C. tentans* (Yoshimi et al., 2002; Karouna-Renier and Rao, 2009). Different domains could be identified and, in particular, the ATPase domain was the most conserved. Interestingly, this HSP70 lacks the conserved tetrapeptide repeat (GGMP) located at the C-terminal domain, which mediates the association of HSP70 with HSP90 into a multichaperone complex and that has been found in other species (Su et al., 2010; Rhee et al., 2009; Sinha et al., 2010). The cytosolic HSP70-specific motif (EEVD) is located at the C-terminal. The Hsp70 gene was localized by in situ hybridization at region III-A3 of the right arm of the third polytenic chromosome, which suggests the presence of a single locus in the *C. riparius* genome. This contrasts with the situation for *D. melanogaster*, where two different loci (87A7/87C1) were identified in chromosome 3 (Gupta et al., 2010). This III-A3 region binds to RNA polymerase II, as shown by immunostaining, and corresponds to the locus that appeared *de novo* induced as a puff, along with other well characterized HS-puffs, after thermal or CO_2 treatments, which induced heat shock or anoxia stress, respectively (Barettino et al., 1988; Morcillo et al., 1988). As there is no genome project for most invertebrate aquatic species, gene sequence information adds a new

Table 2
Characterization of the different treatments assayed.

Stress treatment	Characteristics	Concentration	Time	Survival (%)	References
Temperature (HS)	+15 °C over control culture	35 °C	2 h	93	Morcillo et al. (1988)
Cadmium chloride (Cd)	Heavy metal	10 mM	24 h	82	Planelló et al. (2007)
Butyl benzyl phthalate (BBP)	Plasticizer	1 mg/L	24 h	83	Liu et al. (2009)
Di (2-ethylhexyl) phthalate (DEHP)	Plasticizer in PVC	0.01 mg/L	24 h	100	Park and Kwak (2008)
Bisphenol A (BPA)	In epoxy resins and polycarbonates	3 mg/L	24 h	96	Kang et al. (2007) Segner et al. (2003)
4-Nonylphenol (NP)	Industrial surfactant	10 mg/L	24 h	85	Ha and Choi (2008)
Pentachlorophenol (PCP)	Organochloride biocide	1 μM	24 h	98	Fernández-Freire et al. (2005)
Tributyltin oxide (TBTO)	Insecticide	1 ng/L	24 h	100	Hahn and Schulz (2002)
Ethinylestradiol (EE)	Synthetic estrogen contraceptive pills	5 mg/L	24 h	100	Dussault et al. (2008) Segner et al. (2003)

tool for studying gene–environment interactions. In the future, the use of siRNA, DNA microarray and proteomics will have considerable impact on the ecotoxicological assessment of pollutants, and on our understanding of their mechanism of action. Moreover, localization of Hsp70 in polytene chromosomes makes it possible to directly visualize the effect of pollutants by further cytogenetic analysis.

As *C. riparius* is one of the test species recommended by the OECD for acute toxicity testing, one of the aims of this work was to evaluate the early response of the Hsp70 gene under different environmental stressors, including temperature shifts and short-term exposures to different xenobiotics and hormone analogues. The chemical compounds selected are classified as potential endocrine disrupting compounds. To determine if the expression of Hsp70 was differentially regulated by these various stressors, mRNA levels were analysed by semi-quantitative RT-PCR. Accumulated evidence has shown that HS-proteins are induced by environmental and pathological stress, encouraging the current research into the toxicological, medical and therapeutic implications of these proteins. It is generally acknowledged that induction of the Hsp70 protein reflects transcriptional activation of the gene by specific transcription factors and DNA promoter elements (HSF and HSE, respectively). Nevertheless, it should be noted that analysis of mRNA levels is a more accurate approach for evaluating the subtle changes provoked by potential stressors than that using methods based on protein levels (ELISA and Western), due to the low specificity of most antibodies for distinguishing between the different constitutive and inducible forms of 70 kD family proteins. In our study, using specific gene probes, the roles of Hsp70 and Hsc70 were simultaneously analysed to demonstrate the differential regulation of these two genes under the experimental treatments assayed. Although there is a great deal of data about Hsp70 gene activity under environmental stressors, little is known about the comparative function of these genes or about the differential regulation of their activity in the toxic response.

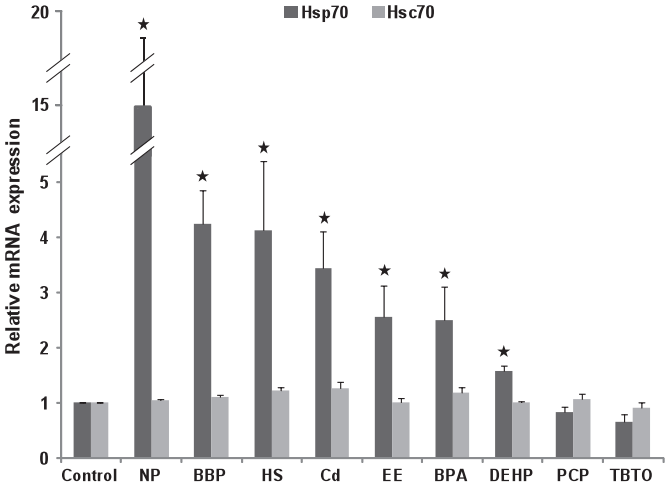


Fig. 4. Analysis of the expression of Hsp70 and Hsc70 genes under different stressful conditions in *C. riparius* fourth instar larvae: 4-nonylphenol (NP), butyl benzyl phthalate (BBP), heat-shock (HS), cadmium (Cd), ethinylestradiol (EE), bisphenol A (BPA), diethylhexyl phthalate (DEHP), pentachlorophenol (PCP), and tributyltin oxide (TBTO). RT-PCR experiments were performed using gene-specific primers for Hsp70 and Hsc70 and for the constitutively expressed gene β-actin, which was used as an internal control (Table 1). The primers were designed to produce different-sized PCR products to allow for simultaneous analysis. The raw values were normalized against β-actin, and the data used to calculate relative expression levels. The relative values are represented; the expression level under control conditions was set to 1. The average and standard errors of measurements taken in three independent experiments with three sample replicates for each stage are shown. *Significant differences ($p \leq 0.05$).

As expected, the Hsp70 gene was significantly upregulated by a temperature shock, showing levels of mRNA up to four-fold higher than those of controls after 2 h at 35 °C. Surprisingly, in contrast, Hsc70 maintained a constitutive expression level, it was not up- or down-regulated despite a sudden shift of 15 °C above the normal growth temperature. At this increased temperature, there was a drastic reduction in the transcriptionally active sites in polytene chromosomes, with the Hsp70 locus and other HS-loci being the only ones brightly stained by RNA polymerase II antibodies (Fig. 3), while it has been shown that cultures can survive up to 12 h (Morcillo et al., 1988).

In addition, most of the chemical treatments tested, except pentachlorophenol (PCP) and tributyltin oxide (TBTO), also triggered activation of the Hsp70 gene at the chosen concentrations. However, none of the experimental treatments assayed provoked a significant alteration in the expression level of the Hsc70 gene. Butyl benzyl phthalate (BBP), 4-nonylphenol (NP), cadmium chloride (Cd), ethinylestradiol (EE), bisphenol A (BPA) and diethylhexyl phthalate (DEHP) produced a significant increase in Hsp70 mRNA levels early after exposure. Remarkable differences were found in the levels of activation between the different compounds, with NP inducing the highest increase (of up to 15 fold) at the dose assayed. Nonylphenol is a stable and persistent derivate of nonylphenol ethoxylate, widely used as a component of detergents, paints, pesticides and many other products. The potency of NP as an estrogen receptor agonist has been demonstrated *in vitro* and *in vivo* in fish and mammals (Folmar et al., 2002), but little is known about its endocrine disrupting effects in invertebrate organisms (Lye et al., 2008). Despite, or due to, the drastic activation of Hsp70 observed in *C. riparius*, NP is not a very toxic compound, surviving to doses up to 100 mg/L. Similar concentrations of NP were found to alter the expression of some haemoglobin genes in this organism (Ha and Choi, 2008). DEHP induced a lower (1.6×) but significant increase in Hsp70, and has also previously been shown to increase Hsp90 and Hsp40 levels in *C. riparius* (Park and Kwak, 2008). DEHP is a widely used plasticizer often found in freshwater systems and dominated the phthalate concentrations (up to 8.44 mg/kg in sediments) evaluated in water samples from Germany (Fromme et al., 2002). At the doses and

temperature tested, the rest of the compounds induced an increase in Hsp70 gene activity equivalent to that of a heat shock, the classical inducer of these genes. Our study reinforces the interest of the Hsp70 gene as a broad range marker of cellular stress (induced by metals, EDCs, herbicides and antibiotics), confirming previous data from our and other laboratories (Lee et al., 2006; Karouna-Renier and Rao, 2009; Park and Kwak, 2008; Planelló et al., 2008, 2010) in different species of chironomids. Heavy metals are considered to be among the most consistent inducers of Hsp70 in the different organisms studied to date (Gupta et al., 2010). Nevertheless, it is worth pointing out that there are some differences in the literature about the effect of other chemical compounds on Hsp70 in different experimental systems. For example, and contrary to our data, NP was found to downregulate the Hsp70 gene in the copepod *Tigriopus* whereas, as in our study, this gene was induced by BPA (Rhee et al., 2009). For a particular chemical, a similar mode of action at the cellular level is most likely, at least in related organisms; the different induction patterns might reflect differences in the previous basal levels of Hsps, due to culture and environmental conditions rather than a different mode of action. Induced thermotolerance and/or cross-tolerance to other stressors should also be considered, as this has long been demonstrated for heat-shock proteins in other species (Carretero et al., 1991).

Although the sensitivity of the cellular stress response makes the Hsp70 gene attractive in ecotoxicology studies, the screening of a particular Hsp may not provide a sufficiently sensitive bioindicator, as for example in the cases of PCP and TBTO. Pentachlorophenol (PCP) belongs to the chlorophenol family; it is highly toxic and persists in the environment. It has been widely used as a general biocide and, more particularly, as a wood preservative. Although there is evidence of the effect of PCP on oxidative metabolism, no studies have yet examined its direct effect on gene expression. PCP did not alter Hsp70 gene levels in *C. riparius* after 24 h of exposure, although after a further 24 h the larvae died (8% survival after 48 h). In a similar way, it has been shown that HSP70 protein levels (measured by ELISA) did not change in *Raphidocelis subcapitata* (Bierkens et al., 1998). Moreover, the TBTO treatments assayed did not activate the Hsp70 gene, even though acute endocrine disrupting effects have been previously reported for this compound in this species, differentially affecting ecdysteroid synthesis and development in males and females (Hahn and Schulz, 2002). Although it is tempting to speculate about the possible relationship between a lack of HSP70 and the high sensitivity and mortality induced by these two biocides, more in depth research is required to assess a causal relationship. In recent years, it has been suggested that activation of heat-shock proteins, and in particular HSP70, in response to environmental insults may play an important role in protecting cells against a broad spectrum of potentially lethal pollutants (Gupta et al., 2010). Despite extensive research, new data are still required to be able to generate a complete picture of Hsp70 regulation and its relationship to cellular toxicity.

In contrast to the differential effects observed in Hsp70 activity, none of the experimental treatments assayed provoked a significant alteration in the expression levels of the Hsc70 gene. These results suggest that the treatments assayed were not sufficiently severe to be able to inhibit a normally active gene, which seems to have a robust resistance to the effects of toxicants, and appears to be differentially regulated than Hsp70. Constitutive 70 kD proteins have "housekeeping" functions within a cell, related to protein assembly, folding, translocation and denaturation (Feder and Hoffman, 1999). It has been shown that some metals, such as copper, upregulate both Hsp70/Hsc70 genes in *C. tentans* (Karouna-Renier and Zehr, 2003; Karouna-Renier and Rao, 2009).

Our results show that the Hsp70 gene was induced by the synthetic steroid ethinylestradiol (EE) in *Chironomus*, which is consistent with some reports of heat-shock protein expression induced by ecdysone in *Drosophila* and estrogen in mammals (Ryan and Hightower, 1998). It has been suggested that there may be a functional relationship between steroid hormones (such as ecdysone in insects and estrogen in mammals) and heat-shock proteins. Indeed, it has been shown that three proteins, HSP90, HSC70 and HSP70, are components of the hormone nuclear receptor complex (Nolen and Morimoto, 2002).

A major advantage for using subcellular biomarkers, such as stress protein genes, is an increased sensitivity and earlier response, when compared to these characteristics for conventional parameters at higher levels of biological organization. However, the predictive power may be limited to the toxicant employed and the model system under defined environmental conditions. As marked differences were seen in the ability of relevant toxicants to induce Hsp70, a question mark has been raised about the universal response of the Hsp70 gene to cytotoxicity. Different stressors can induce distinct stress response pathways by targeting specific genes. More extensive research is required to increase the utility of heat-shock proteins in studies predicting the level of toxicity based on stress gene expression in model organisms.

In summary, we found that Hsp70 gene expression in the aquatic larvae of *C. riparius* is upregulated during exposure to a broad sample of environmental pollutants classified as endocrine disruptors, including metals such as cadmium (Cd), organic man-made compounds, such as polyphenols and phthalates (butyl benzyl phthalate, BBP; diethylhexyl phthalate, DEHP; bisphenol A, BPA; and nonylphenol, NP), and synthetic steroids (ethinylestradiol, EE), where neither the survival of the larvae is affected nor the Hsc70 expression is altered by the exposure conditions. These results suggest that this gene could be useful as a rapid and sensitive marker of exposure in ecotoxicological testing. However, other compounds assayed, such as the biocide pentachlorophenol (PCP) and tributyltin oxide (TBTO), did not increase Hsp70 gene expression, nor alter Hsc70 mRNA basal levels, but they were able to kill the larvae some hours later. In conclusion, the Hsp70 gene in *C. riparius* showed a different susceptibility to the toxic effects of different environmental pollutants. Its potential use for predicting the toxicity of chemicals merits further research to validate its sensitivity and specificity. Finally, gene expression studies can provide important insights into how genomes react to stressful events and environmental hazards, which are likely to be even more frequent as aquatic systems become increasingly polluted.

Acknowledgements

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