Effects of the human antiepileptic drug carbamazepine on the behavior, biomarkers, and heat shock proteins in the Asian clam *Corbicula fluminea*

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ABSTRACT

Carbamazepine (CBZ), an anticonvulsant and mood-stabilizing pharmaceutical, is a widespread contaminant in aquatic environments. In this study, the effects of chronic exposure to environmentally relevant CBZ concentrations were investigated in freshwater clams *Corbicula fluminea*. Adult *C. fluminea* were exposed to 0.5, 5, and 50 µg/L of CBZ for 30 days, after which siphoning behavior (filtration rates), biomarker levels, and heat shock protein expression were measured. The filtration rates were significantly decreased ($p < 0.05$) by 50 µg/L CBZ treatment, indicating a negative impact on *C. fluminea* health. Superoxide dismutase (SOD) and glutathione reductase (GR) activities were decreased, and catalase (CAT) activity and malondialdehyde (MDA) content were increased in the gills and digestive gland, suggesting that CBZ induced an oxidative effect. The levels of *Hsp22*, *Hsp40*, and *Hsp70* mRNAs were also markedly induced after 5 or 50 µg/L CBZ treatment ($p < 0.05$), whereas *Hsp60* and *Hsp90* mRNAs in gills and *Hsp60* mRNA in digestive gland were significantly repressed ($p < 0.05$). Finally, the expression of *Hsp70* protein was significantly increased ($p < 0.05$) by 5 and 50 µg/L exposure. In aggregate, these results confirm that environmentally relevant concentrations of CBZ can exert a negative effect on *C. fluminea* tissue at the molecular and protein level.

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1. Introduction

In the last years, a large and diverse collection of bioactive pharmaceutical chemicals are being used in human and veterinary medicine and in livestock farming (Kim et al., 2007). Recent studies have to document the presence of pharmaceuticals (i.e., hormonal, antiepileptic and neuroactive drugs) at the ng/L to µg/L in sewage treatment plant (STP) effluents, surface waters, groundwater, and even in drinking water sources (Daughton and Ternes, 1999; Fent et al., 2006; Blair et al., 2013; Daughton, 2014; Liu and Wong, 2013). The ecotoxicological risk that this contamination poses to non-target organisms however is still scarce (Fent et al., 2006; Gonzalez-Rey and Bebianno, 2013).

One heavily used pharmaceutical, carbamazepine (CBZ), is an anticonvulsant and mood-stabilizing drug used primarily for treating epilepsy and bipolar disorder, as well as trigeminal neuralgia (García-Morales et al., 2007). CBZ is not easily removed from waste streams by conventional waste water treatment plants (WWTP), with a removal efficiency of less than 10% (Zhang et al., 2008). Consequently, significant levels of CBZ are steadily discharged into receiving water bodies (Liu and Wong, 2013), and CBZ contamination has been widely documented in WWTP influents and effluents, surface water, and even drinking water (Fent et al., 2006). In fact, a recent study reported CBZ contamination in 95% of water samples collected from various locations throughout the USA, at an average concentration of 350 ng/L (Ferrer and Thurman, 2012). CBZ contamination has also been documented in WWTP influents and effluents in Shanghai, China, at concentrations ranging from 230 to 1110 ng/L and in the Yangtze River at concentrations up to 1090 ng/L (Liu and Wong, 2013).

Previous studies have reported that CBZ can induce chronic effects in non-target organisms at relevant environmental concentrations (Ferrari et al., 2004; Martin-Diaz et al., 2009; Contardo-Jara et al., 2011; Tsiaka et al., 2013). For example, Martin-Diaz et al. (2009) reported that CBZ exposure (0.1 and 10 µg/L) influenced the

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antioxidant enzyme system, cAMP pathway, and the mitoxantrone resistance protein (MXR) system in mussels (*Mytilus galloprovincialis*). Similarly, exposure to 100 or 1000 nM CBZ repressed the levels of *heat shock protein 70* (*Hsp70*) and *superoxide dismutase* (*SOD*) mRNA in the gills and metallothionein (MT) and *P*-glycoprotein (*P-gp*) in the digestive gland of zebra mussels (*Dreissena polymorpha*) (Contardo-Jara et al., 2011). In another study, Tsiaka et al. (2013) found that 0.01–100 µg/L CBZ exposure was cytotoxic to *M. galloprovincialis* cells *in vitro* and increased cellular superoxide anions and nitric oxide. These results all suggest that CBZ can significantly affect multiple oxidative and biochemical pathways in bivalves, with significant deleterious effects. Even so, neither the mechanism of CBZ action nor the behavioral effects of CBZ on aquatic organisms have been well investigated.

In this study, we sought to determine the toxic effects of CBZ on a freshwater bivalve (*Corbicula fluminea*) by measuring multiple toxicological endpoints considering environmentally relevant concentrations. *C. fluminea* is commonly known as the Asian clam and is native to Southeast Asia (Araujo et al., 1993). As a benthonic freshwater bivalve, *C. fluminea* has been commonly used in field and laboratory studies to measure environmental perturbations or contaminations (Vasconcelos et al., 2009; Chen et al., 2013; Ren et al., 2013). In the present study, adult *C. fluminea* were exposed to CBZ for 30 days, after which multi-endpoints were measured, including siphoning behavior, the levels of four stress biomarkers: SOD, catalase (CAT), glucocorticoid receptor (GR), and malondialdehyde (MDA), and the expression of heat shock protein mRNAs in gills and digestive gland. The fluctuation of antioxidant enzymes and heat shock proteins have been used successfully as oxidative stress and early-warning bio-indicators of cellular hazards in bivalves species (Contardo-Jara et al., 2011; Gonzalez-Rey and Bebianno, 2013; Gupta et al., 2010). Moreover, since the *Hsp70* is the most general biomarker used for cellular stress and more highly conserved across taxa (Del Rey et al., 2011; Gupta et al., 2010), the protein expressions of *Hsp70* in gills and digestive gland were also determined. The possible mechanism of CBZ action on aquatic organisms was examined based on the toxicological findings.

2. Materials and methods

2.1. Chemicals

Carbamazepine (CAS: 298-46-4, purity > 97%) was purchased from J&K Chemical Ltd. (USA). CBZ stock solutions were prepared in dimethylsulfoxide (DMSO) and stored in brown bottles.

2.2. Clams

C. fluminea (average length of 20.56 ± 2.05 mm) were collected from natural populations in Hongzehu Lake, China, and were transferred to the laboratory in fish tanks with continuous aeration. Once in the lab, they were housed in 50-L glass aquaria (30 individuals per aquarium) filled with aerated natural water maintained at a constant temperature (20 ± 1 °C), pH (7.8 ± 0.2), and oxygen saturation ($96\% \pm 2\%$). Photoperiod was set at 12:12 h (light:dark). The clams were fed single-celled *Chlorella vulgaris* and *Scenedesmus obliquus* algae daily.

2.3. CBZ exposure assay

The clams were acclimated to the exposure aquarium (10 L containing 8 L aerated natural water) for 7 days before initiate of the experiment. Then, the clams were randomly distributed into five

experimental groups, each group contained three replicate aquariums and each replicate aquarium included 20 clams. Nutrition, water, temperature and light conditions were maintained constant during the time of acclimatization and exposure. After the acclimatization, the clams were exposed for 30 days to dilutions of a CBZ/DMSO solution that provided final CBZ concentrations of 0.5, 5, or 50 µg/L (2.12, 21.2 or 212 nM). Final DMSO concentration was 0.001% (w/v) in the CBZ exposure groups. A group containing aerated natural water was used as control, and a treatment with 0.001% (w/v) DMSO was served as solvent control. All control or treated groups were in triplicate. The water and CBZ or DMSO was refreshed daily.

The mortality for all groups was recorded throughout the 30-day exposure period, and the gills and digestive gland were collected at the end of the study and stored individually at -80 °C for future analyses. At least three independent biological replicates were harvested for each treatment group. The clams in the control groups were immediately analyzed for biomarkers to assess their initial health status, and the results were not significantly different from those of clams exposed to the solvent control (0.001% DMSO) for 30 days (data not shown). A condition index (CI) was calculated based on individual weights of 10 *C. fluminea* from each group as follows (Gonzalez-Rey and Bebianno, 2013):

$$CI(\%) = \frac{W_s}{W_t} \times 100,$$

where W_s is the wet weight of the entire soft tissue, and W_t is the wet weight of the entire body, including the shell.

2.4. Water concentrations of CBZ

CBZ concentrations were quantified immediately after tanks were dosed each week using ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) according to a previously described method (Batt et al., 2008). Briefly, water samples were filtered through a 0.2-µm GH Polypro membrane filter (PALL, New York, USA) to remove any suspended matter and injected onto a Waters Acquity UPLC fitted with a BEH C18 column (50×2.1 mm, 1.7 µm; Waters, Milford, MA, USA) maintained at 30 °C. A tandem Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) ion source was used for CBZ detection, with all measurements taken in positive ESI mode. Multiple reaction monitoring (MRM) transitions were 237.1 > 194.2 (quantification) and 237.1 > 179.2 (confirmation), respectively. External standard solutions of CBZ were used to determine precise concentrations in test samples. The limit of detection and limit of quantification for the method were 0.01 and 0.02 µg/L, respectively. The actual determined CBZ concentrations (mean $\pm$ standard deviation; and % analyzed/nominal) during the exposure period were 0.45 ± 0.13 µg/L (90%) for the 0.5 µg/L treatments, 4.62 ± 0.88 µg/L (92%) for the 5 µg/L treatments, and 43.76 ± 2.35 µg/L (87%) for the 50 µg/L treatments. The nominal CBZ values are used throughout the remaining text.

2.5. Siphoning behavior

The siphoning rate was measured using a previously described method (Cooper and Bidwell, 2006) with slight modifications and is based on depletion of neutral red dye particles from the water due to filtration by the clams. Immediately after CBZ exposure, five *C. fluminea* from each CBZ concentration group and control group were placed in 200-mL beakers that contained 100 mL of a neutral red solution (1 mg/L in dechlorinated tap water with no added CBZ) and were allowed to siphon for 2 h. Just prior to placing the *C. fluminea* in the solution and just after the 2 h siphoning period, 1-mL aliquots of the water were removed from each beaker, and the

neutral red concentration was determined by measuring the optical density at 530 nm using a spectrophotometer. Standard solutions of neutral red were used to generate a standard curve from which the dye concentrations in each test sample were calculated using the equation of Cooper and Bidwell (2006):

$$m = \left[\frac{M}{nt} \right] \log \left(\frac{C_0}{C_t} \right),$$

where M is the volume of the test solution, n is the number of clams used, t is the time in hours, C_0 is the initial concentration of the dye, C_t is the concentration of the dye at time t , and m is the filtration rate (mL/animal/h).

2.6. Tissue preparation for biomarker and western blot analysis

Approximately 200 mg of each tissue sample was ground with a pestle in a 1-mL centrifuge tube in the presence of 0.5 mL radio-immunoprecipitation assay lysis buffer (Beyotime, China) on ice. The resulting homogenate was centrifuged at $11,000 \times g$ for 10 min, and the supernatant was collected into a new 1.5-mL tube. The protein content of the supernatant was determined using a BCA Protein Assay Kit (Beyotime, China), and the samples were frozen at -80°C until further analysis.

2.7. Biomarker measurements

The activities of SOD, CAT, and GR were analyzed in gills and digestive gland tissue samples. An aliquot of 20 μL supernatant from each tissue sample was used for each of the biomarker assays. SOD activity was determined using a SOD Assay Analysis Kit (Beyotime, China) according to the manufacturer's protocol, which is based on the method of McCord and Fridovich (1969). The SOD activity is expressed in units (U) per minute per mg protein. CAT activity was measured by monitoring the decrease in absorbance of hydrogen peroxide (H_2O_2) at 240 nm, with units of U/mg protein. A value of 1 U CAT activity was defined as 1 μmol H_2O_2 consumption per min at 25°C and pH 7.0. GR activity was measured using a Glutathione Reductase Assay Kit (Beyotime, China). One unit of GR activity was defined as the reduction of 1 μmol of oxidized glutathione (GSSG) at 25°C , pH 7.0, and GR activity is expressed as U/mg protein. MDA content was measured using a Lipid Peroxidation MDA Assay Kit (Beyotime, China) that relies on detecting thiobarbituric acid adduction to MDA. Precise concentrations were determined by comparison with a standard curve generated with prepared MDA standards. MDA concentrations are expressed as nmol/mg mg protein.

2.8. qRT-PCR

Total RNA was isolated from gill and digestive gland tissue samples by means of an SV Total RNA Isolation System (Promega, USA) according to the manufacturer's protocols. The mRNA expression was measured using an ABI 7500 real-time quantitative PCR system (Life Technology, USA) according to a method reported in a previous study (Chen et al., 2013). The sequences and PCR product sizes for each primer pair are shown in Table 1. The results were analyzed based on the $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak, 2008), with all experiments performed three times in triplicate, with similar results.

2.9. Electrophoresis and immunochemical assay of Hsp70 protein

The protein samples were dissolved in a sodium dodecyl sulfate (SDS) sample buffer at a concentration of 5 $\mu\text{g}/\mu\text{L}$ and boiled for 5 min. Aliquots corresponding to 50 μg total protein were analyzed by SDS-polyacrylamide gel electrophoresis (5% stacking and 10% separating) for 2 h at 80 V. After electrophoresis, the proteins were transferred to a nitrocellulose membrane via wet blotting (100 mA for 50 min), after which non-specific binding sites were blocked by immersing the membrane in TBST (0.02 M Tris-HCl, 0.5 M NaCl with 0.05% Tween-20, pH 7.5) that contained 5% non-fat dry milk for 1 h at 25°C . The membrane was then rinsed with TBST for 10 min and incubated at 4°C overnight in the presence of rabbit anti-Hsp70 (1:200 dilution, HSP70 (K-20)-R, Santa Cruz Biotechnology, USA) and rabbit anti- β -actin (1:1000 dilution, Cell Signaling Technology, Inc., USA) antibodies. The blots were washed twice with TBST for 10 min and once with TBS for 10 min, and the blots were then incubated with a second antibody (1:1000 dilutions, goat anti-rabbit IgG-HRP: sc-2004, Santa Cruz Biotechnology, USA) for 1 h at 28°C . After incubation, the bands were washed as in the previous step and visualized using an Enhanced Chemiluminescence (ECL) detection reagent kit (Beyotime, China), with the band optical densities quantified using VisionWorksLS Image Acquisition and Analysis Software 7.1 (UVP, USA). Relative optical densities were obtained by normalizing each band against the standard band from control clams.

2.10. Statistical analysis

All statistical analyses were performed using SPSS (version 16.0) and OriginPro (version 8.0) software. The experimental data were checked for homogeneity of variance by using Levene's test. All quantitative data are expressed as the mean $\pm$ standard error of the mean (SEM). The significance of the differences between exposed and control samples was determined using a one-way ANOVA ($p < 0.05$) followed by Dunnett's test for multiple comparisons.

Table 1
Primer sequences used in *Corbicula fluminea*.

Genes	Genes (accession no.)	Realtime-PCR primer sequence (5'-3')	Product size (bp)	Annealing temperatures($^\circ\text{C}$)	PCR efficiency (%)
β -actin	EF446608.1	F:CGCCATCCAGGCTGTGCTTTCA R:ATGGCGTGTGGAAGGGCGTA	123	55.0	97.0
Hsp22	KF218338	F:CTTCAACCTGGAGCATTTGTG R:CGTGATTTGTTTCGGACTG	154	57.0	96.1
Hsp40	KF218339	F:ACAAGCAGCATCCAGATTCC R:TCCAGTCAGAGCCTTTCCCA	82	56.5	99.9
Hsp60	KC979065	F:GGGACAACAGAAAGAACACCC R:CCGACACGACCAAGTCATT	120	55.0	97.9
Hsp70	KC979064	F:CGCCGACGCTGATTACCTTA R:AAACGGTTGATAGGACGCAAG	84	55.0	95.6
Hsp90	KC979063	F:ATGGTGCTGGAATCTCTCAGG R:GCGTCACAAAGGAAGGACTG	246	55.0	101.6

Fig. 1. Average ($n=5$) filtration rate (mL/animal/h) of *C. fluminea* in CBZ-contaminated water containing 1 mg/L neutral red solution following exposure to the indicated concentrations. The experiments were performed in triplicate and repeated three times with similar results. The bars display the mean $\pm$ SEM. A statistically significant difference between treatment group and control ($p < 0.05$, $n = 5$, ANOVA) is indicated by differences in the letters above the bars.

3. Results

3.1. Mortality and condition index (CI)

Over the course of the 30-day exposure to various CBZ concentrations, less than 3% mortality was observed at all CBZ concentrations (data not shown). Likewise, the CI was not significantly different among the treatments and the control (range from 32.8% to 39.2%, data not shown).

3.2. CBZ effects on siphoning behavior

There was no statistical difference observed in the siphoning behavior among the control and low concentration CBZ treatments (0.5 and 5 µg/L). The filtration rates of individual clams among these groups ranged from 16.52 to 19.62 mL/animal/h. However, the filtration rate at 50 µg/L CBZ (3.45 ± 0.82 mL/animal/h) was significantly decreased compared to the control group (19.27 ± 1.36 mL/animal/h) ($p < 0.05$; Fig. 1).

3.3. CBZ effects on biomarkers

The activities of SOD, CAT, and GR and the MDA content were measured in gill and digestive gland tissue isolated from all treatment groups. The SOD activity was higher in gills than in the digestive gland for all control and CBZ exposure groups (Fig. 2A and B). In the gills, SOD activity was significantly inhibited by 50 µg/L CBZ exposure compared to the control group ($p < 0.05$; Fig. 2A); no significant differences were observed at the 0.5 and 5 µg/L groups. In the digestive gland, inhibition of SOD activity was observed in both the 5 and 50 µg/L CBZ exposure groups compared to the control group ($p < 0.05$; Fig. 2B). In contrast to the SOD activity measurements, CAT activity was higher in digestive gland than in gills (Fig. 2C and D). Moreover, CAT activity was significantly up-regulated by all concentrations of CBZ in both the gills and in the digestive gland ($p < 0.05$; Fig. 2C). The GR activity was significantly decreased in the gills by all three CBZ concentrations ($p < 0.05$; Fig. 2E). No significant changes in GR activity were observed in the digestive gland in the 0.5 µg/L CBZ treatment, whereas significant down-regulation was observed after 5 and 50 µg/L CBZ exposure (Fig. 2F). Finally, MDA was significantly up-regulated in the gills

by 5 and 50 µg/L CBZ and in the digestive gland by 50 µg/L CBZ ($p < 0.05$; Fig. 2G and H). No significant differences in MDA levels were observed in either tissue type after 0.5 µg/L CBZ exposure (Fig. 2G and H).

3.4. CBZ effects on Hsp gene expression

The mRNA expression levels of five Hsp genes (*Hsp22*, *Hsp40*, *Hsp60*, *Hsp70*, and *Hsp90*) in the gills and digestive gland were evaluated after CBZ exposure (Fig. 3). In the gills, expression of *Hsp22*, *Hsp40*, and *Hsp70* transcripts was significantly increased (3.81-fold, 14.81-fold, and 3.52-fold, respectively) by 5 and 50 µg/L CBZ exposure compared to controls ($p < 0.05$), with the greatest increase observed after 50 µg/L CBZ exposure (Fig. 3A, C and G). In contrast, the expression of *Hsp60* and *Hsp90* was repressed in the gills after 5 and 50 µg/L CBZ exposure ($p < 0.05$), with the greatest inhibition observed in the 50 µg/L CBZ group (0.47- and 0.18-fold for *Hsp60* and *Hsp90*, respectively; Fig. 3E and I).

In the digestive gland, the expression of *Hsp22*, *Hsp40*, *Hsp70*, and *Hsp90* transcripts was significantly increased after 5 and 50 µg/L CBZ exposure ($p < 0.05$) as follows: *Hsp22*, 4.74-fold; *Hsp40*, 16.73-fold; *Hsp70*, 8.47-fold; and *Hsp90*, 8.21-fold; Fig. 3). In addition, *Hsp60* mRNA level was significantly decreased in the digestive gland after 5 and 50 µg/L CBZ exposure ($p < 0.05$; Fig. 3F).

3.5. CBZ effects on Hsp70 protein expression

The protein expression of Hsp70 was also determined by western blotting. Exposure to 5 or 50 µg/L CBZ significantly increased Hsp70 protein levels in both the gills and the digestive gland ($p < 0.05$; Fig. 4A and B). The largest increase was observed in the 50 µg/L CBZ exposure group, with a 2.36-fold increase in the gills and a 3.37-fold increase in the digestive gland ($p < 0.05$; Fig. 4A and B).

4. Discussion

C. fluminea lives in the sediment of waterways and filters suspended materials and colloids from large volumes of water. As such, it is a useful indicator species for assessing the toxicity of water contaminants (Vasconcelos et al., 2009). In the present study, we used *C. fluminea* to assess the effects of chronic exposure to CBZ by evaluating siphoning behavior, biomarkers, transcripts levels of Hsp genes, and the expression of Hsp70 protein in the gills and digestive gland.

Siphoning behavior and valve movement have both been utilized as indicators for continuous biomonitoring of water supplies and effluents (Chen et al., 2012). Bivalve siphons play important functional roles in nutritional physiology, defense, and reproduction (Moulton et al., 1996) and are therefore a signal of general health or stress. In fact, previous studies have demonstrated that bivalve siphoning can be influenced by metals or chlorpyrifos (Doherty et al., 1987; Milam and Farris, 1998; Cooper and Bidwell, 2006). Cooper and Bidwell (2006) reported that the valves of the clams were always closed during exposure to 3.13 mg/L chlorpyrifos treatments. A reduction in siphoning activity as a response to chemical stress has also been associated with ammonia accumulation in the tissue, a reduction in oxygen exchange, and reduced feeding (Moulton et al., 1996). Herein, we found that CBZ exposure decreased the filtration rates of clams, although no significant valve closure was observed. It is therefore reasonable to conclude that the decreased siphoning caused by CBZ exposure reflects an overall negative health impact and an indicator of chemical stress.

The effects of pollutants are often manifested as imbalances in antioxidant and fatty acid metabolism systems (Koehler, 2004). Previous studies have shown that exposure to CBZ, ibuprofen, or

Fig. 2. Antioxidant enzyme activities and MDA levels in different tissues from clams exposed to carbamazepine. SOD activity in gills (A) and digestive gland (B); CAT activity in gills (C) and digestive gland (D); GR activity in gills (E) and digestive gland (F); MDA levels in gills (G) and digestive gland (H). Bars display the mean $\pm$ SEM ($n=6$). A statistically significant difference between treatment groups and control ($p < 0.05$, $n=6$, ANOVA) is indicated by differences in the letters above the bars.

bezafibrate pharmaceuticals induced oxidative effects in the zebra mussel *D. polymorpha* (Contardo-Jara et al., 2011). Consistent with this, CBZ exposure significantly decreased SOD and GR activity and increased CAT activity in the gills and digestive gland of *C. fluminea*. These results indicate that environmentally relevant concentrations of CBZ enhanced the production of reactive oxygen species (ROS) in *C. fluminea*. It is most likely that an increase in superoxide anions stimulated the consumption of SOD, and that CAT activity was induced by H_2O_2 formation (Ren et al., 2013). The observed decrease in GR activity may also indicate increased ROS levels, since the role of GR is to regenerate the antioxidant glutathione from GSSG (Verlecar et al., 2008).

MDA levels were also significantly increased in the gills and digestive gland by exposure to higher CBZ concentrations, which further supports the notion of an increase in ROS. MDA is produced from the decomposition of unsaturated fatty acid peroxides that are generated by and rapidly degraded by ROS (Marnett, 1999; Stancliffe et al., 2011). Thus, if the ROS content exceeded the elimination capacity of antioxidant enzymes, the excess ROS could stimulate MDA production (Lushchak, 2011). A similar result was reported by Ren et al. (2013), in that cadmium exposure reduced SOD activity and enhanced CAT and MDA in the digestive gland of

C. fluminea (Ren et al., 2013). Moreover, Martin-Diaz et al. (2009) reported that 0.1 and 10 $\mu\text{g/L}$ CBZ exposure induced CAT activity in the digestive gland and MDA content in the gills of *M. galloprovincialis*. Finally, an *in vitro* study with *M. galloprovincialis* cells revealed an increase in MDA content after 0.01–100 $\mu\text{g/L}$ CBZ exposure (Tsiaka et al., 2013). Thus, previously reported results and our findings all indicate that CBZ can induce oxidative stress in bivalve species.

Heat shock proteins are also suitable early-warning bio-indicators of cellular hazards due to their sensitivity to even minor assaults (Santoro, 2000; Sørensen et al., 2003; Gupta et al., 2010). Previous studies have reported that various stressors (e.g., temperature, metal toxicity and infection) affect the expression of Hsp60, Hsp70, and Hsp90 transcript and protein levels (Luchmann et al., 2011; Liu et al., 2012; Zhang and Zhang, 2012; Jing et al., 2013). Likewise, Kim et al. (2011) reported that low doses of UV-B radiation (2 and 4 kJ/m^2) increased the expression of Hsp10, Hsp20, and Hsp27 mRNAs in *Brachionus* rotifers. Herein, we evaluated the effect of CBZ exposure on five Hsps, including Hsp22 and Hsp40, which belong to the heterologous sHsp family of 12–43 kDa Hsps (Hull et al., 2013). Hsp22 is a recently identified member of the small heat shock protein superfamily that can interact with mimics of phosphorylated

Fig. 3. Fold changes in the mRNA expression of the five Hsps in *C. fluminea* gills and digestive gland following CBZ exposure. *Hsp22* in gills (A) and digestive gland (B); *Hsp40* in gills (C) and digestive gland (D); *Hsp60* in gills (E) and digestive gland (F); *Hsp70* in gills (G) and digestive gland (H); *Hsp90* in gills (I) and digestive gland (J). The bars display the mean $\pm$ SEM ($n=6$). A statistically significant difference between treatment groups and control ($p < 0.05$, $n=6$, ANOVA) is indicated by differences in the letters above the bars.

Hsp27 (Benndorf et al., 2001). *Hsp40*, also known as chaperone DnaJ, is a molecular chaperone protein, which expressed in a wide variety of organisms from bacteria to humans. *Hsp40* can protect proteins from irreversible aggregation during synthesis or times of cellular stress, and also play a role in regulating the ATPase activity of *Hsp70* (Caplan et al., 1993). We found that exposure to 5 or 50 $\mu\text{g/L}$ CBZ significantly stimulated *Hsp22* and *Hsp40* mRNA expression in *C. fluminea* gills and digestive gland. This result mirrors a previous study in which we found that *Hsp22* and *Hsp40* mRNAs expression was stimulated by exposure to 0.5 or 5 $\mu\text{g/L}$ fluoxetine (Chen et al., 2013). Thus, like the biomarker study, the expression levels of *Hsp20* and *Hsp40* indicate that CBZ exposure caused significant stress.

The other three Hsps we evaluated, *Hsp60*, *Hsp70* and *Hsp90*, are very widely studied and are quite abundant in cells under normal physiological conditions (Feder and Hofmann, 1999; Hull et al., 2013). As such, they are important sentinels of chemical stress. Luchmann et al. (2011) reported that *Hsp60* was upregulated and *Hsp90* was repressed in the gills and digestive gland of *Crassostrea brasiliana* oysters after exposure to diesel fuel, with *Hsp60* being the most prominent biomarker in gills. Similarly, *Hsp90* was down

regulated in *Tanichthys albonubes* by exposure to cadmium (Liu et al., 2012). Herein, the mRNA levels of *Hsp90* and *Hsp60* were enhanced and repressed, respectively, after CBZ exposure. Similarly, the mRNA levels of *Hsp60*, *Hsp70*, and *Hsp90* were enhanced in the digestive gland of *C. fluminea* after 30 days of exposure to fluoxetine (Chen et al., 2013). Thus, the perturbation of the expression of five different Hsp mRNAs indicates that CBZ does cause significant chemical stress.

Among the five Hsps, *Hsp70* is often the most prominent and the first to be expressed following environmental assaults (Gupta et al., 2010). *Hsp70* mRNA levels were decreased in gills of the zebra mussel *D. polymorpha* after 7 days of exposure to 2.36, 23.6, or 263 $\mu\text{g/L}$ CBZ (Contardo-Jara et al., 2011), and Del Rey et al. (2011) reported that *Hsp70* protein levels in *Mytilus californianus* gills were initially attenuated and later up-regulated by exposure to 0.2 or 0.5 $\mu\text{g/L}$ caffeine. This result is similar to our findings, in which CBZ exposure up-regulated *Hsp70* transcripts levels in *C. fluminea*. Western blotting analysis of *Hsp70* protein levels gave results consistent with the increase in mRNA expression. In aggregate, our results confirm that CBZ can exert molecular-level stress on *C. fluminea* tissues and that Hsp

Fig. 4. Relative Hsp70 protein levels in gills (A) and digestive gland (B) of *C. fluminea* exposed to CBZ. Western blots were performed in the gills and digestive gland from *C. fluminea* exposed to CBZ and control groups. Equal amounts of the protein were electrophoresed in each gel lane, and representative immunoblots are shown. β-actin was used as a loading control. The ratios of respective proteins to β-actin were calculated by performing densitometric analysis using VisionWorksLS software. The bars display the mean ± SEM ($n = 6$). A statistically significant difference between treatment groups and control ($p < 0.05$, $n = 6$, ANOVA) is indicated by differences in the letters above the bars.

levels are useful sentinels of the stress caused by environmental contaminants.

5. Conclusions

Exposure to environmentally relevant concentrations of CBZ altered the siphoning behavior, biomarkers, and Hsp mRNA and protein levels in the gills and digestive gland of *C. fluminea*. The changes in the biomarkers suggest that the effect of CBZ is related to oxidative stress. Consistent with literature studies, our data indicate that Hsp expression is a useful biomarker of environmental stress. Finally, this study highlights the need for a better understanding of the effects of environmental pharmaceutical contaminants on non-target organisms.

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