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Oxidative stress, protein carbonylation and heat shock proteins in the black tiger shrimp, *Penaeus monodon*, following exposure to endosulfan and deltamethrin

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ABSTRACT

The impact of commonly used pesticides, endosulfan and deltamethrin, on the molecular stress level in black tiger shrimp *Penaeus monodon*, was assessed using classical oxidative stress biomarkers, protein carbonylation profiles, and levels of heat shock proteins. Results showed that 4 days exposure to 0.1 $\mu g\,L^{-1}$ deltamethrin significantly ($p < 0.05$) increased lipid peroxidation (LPO) level in gills (64.3 ± 3.2 compared to 34.2 ± 5.3 nmol MDA equiv. g^{-1} tissue at day 0). However, no pesticide treatment had significant effect on the activities of antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST). Carbonylated protein profiles were determined on gills following 2,4-dinitrophenylhydrazine derivatization and 2D-PAGE along with Western blotting. Immunoblotting with dinitrophenol-specific antibody revealed 17 protein spots carbonylated in response to 4 days exposure to 0.1 $\mu g\,L^{-1}$ deltamethrin while 24 protein spots specifically oxidized at day 0 were no longer detected after deltamethrin treatment. On the other hand, endosulfan exposure at 0.1 and 1 $\mu g\,L^{-1}$ induced up to 2.1-fold increase of HSP90 level in muscle. This approach is providing new insights into the molecular impacts of deltamethrin and endosulfan on an economically important crustacean. While deltamethrin has shown a pro-oxidant effect in gills, endosulfan exposure rather induced proteotoxic effects in muscles. This argues that LPO level, protein carbonylation specificities, and HSP90 levels may be potential discriminating biomarkers to assess the chemical stress level in farm shrimp.

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1. Introduction

In southern and eastern Asia, Latin America and other tropical regions, the shrimp culture rapidly expanded over the last 20 years. This industry has become an important economic sector in many Asian countries, including Vietnam. The species dominating the marine shrimp culture in Vietnam are penaeid shrimp, especially *Penaeus monodon*, commonly known as the black tiger shrimp. In 2004, the Vietnamese production of *P. monodon* reached 290 000 tonnes (MoFI, 2005). Moving from extensive to intensive shrimp culture system required additional effective antibacterial compounds and pesticides for prevention and for treatment of water quality, and to reduce disease problems (Tu et al., 2007). Those chemicals could have negative effects on the health of cultured shrimp, cause a risk for food safety, occupational health hazards, and/or have negative effects on adjacent ecosystems (Gräslund et al., 2003). Regarding the health of cultured shrimp, there is a general risk that exposure to chemicals can increase their stress level, and thereby decrease growth and/or increase their susceptibility to infections (Reyes et al., 1999; Le Moullac and Haffiner, 2000). Consequently, an inappropriate chemical use can lead to economic losses for producers. As a result there is an increasing awareness in the shrimp aquaculture industry about the necessity to develop sensitive diagnosis tools for assessing the stressful effect of commonly encountered chemicals on cultured shrimp.

Endosulfan and deltamethrin are commonly used pesticides in Vietnamese shrimp farms (Tu et al., 2007). The former, a polycyclic chlorinated hydrocarbon of the cyclodiene subgroup, has been classified as a hazardous chemical by the WHO (1984). It is used as a broad-spectrum insecticide mainly in agriculture and, in some countries, in public health. This well known insecticide is considered to be highly toxic to fish species (Srivastav et al., 1997; Capkin et al., 2006) and aquatic invertebrates (Lombardi et al., 2001; Wirth et al., 2001, 2002). Joshi and Mukhopadhyay (1990) estimated its 48 h LC50 value in juvenile black tiger shrimp to be 12.2 $\mu g\,L^{-1}$. At low concentration in water, sublethal effects such as altered energy metabolism (Mishra and Shukla, 1995) and ionic regulation (Singh et al., 2002) have been reported. The long-term ecological hazards associated with the use of organochloride, organophosphate, and carbamate pesticides propelled the introduction of a

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new generation of pesticides with a lesser degree of persistence. In this direction, the use of pyrethroids as insecticidal and antiparasitic formulations has markedly increased as a viable substitute and account for over 30% of insecticides used globally (Prasanthi et al., 2005). Deltamethrin is a synthetic type two pyrethroid, which has a wide range of application in industrial and agricultural purposes. It is also used as an alternative pesticide in animal health, in vector control, and in public health. It has been shown to be toxic for fish (Viran et al., 2003; Köprücü and Aydin, 2004; Ural and Saglam, 2005; Köprücü et al., 2006), aquatic arthropods (Ratushnyak et al., 2005), and honeybees (Vandame et al., 1995; Badiou et al., 2008) in laboratory tests. L'Hotellier and Vincent (1986) showed that its 96 h LC50 value in pink shrimp (*Penaeus duorarum*) was 0.35 $\mu g L^{-1}$. However, there is no data concerning its acute toxicity in black tiger shrimp, even if Smith and Stratton (1986) argued that lobster and shrimp are susceptible to all pyrethroids.

Comprehension of the mechanisms related to the sublethal effects caused by these pesticides upon shrimp metabolism would help to develop sensitive and precise diagnostic tools with a predictive capability in assessing the toxic effects, thus contributing to better pond management (Bainy, 2000). Among the most commonly used biomarkers, those related to oxidative stress assume an important position, being frequently used both in environmental monitoring and laboratory assays (Ahmad et al., 2000; Livingstone, 2001; Pandey et al., 2003). Rates or amounts of reactive oxygen species (ROS) production can be increased by the presence of a wide range of natural and man-made xenobiotics (Livingstone, 2001). In their review, Mohammad et al. (2004) concluded that the stimulation of free radical production, induction of lipid peroxidation, and disturbance of the total antioxidant capability of the body are mechanisms of toxicity for most pesticides, including organochlorines and pyrethroids. Consequently, the antioxidant defences are potentially interesting biomarkers to pesticides while enhanced lipid peroxidation, a consequence of oxidative deterioration of membrane lipids, is generally referred to an index of oxidative stress (Ribera et al., 1990).

Moreover, changes in protein carbonylation process are a biochemical perturbation resulting from oxidative stress (McDonagh et al., 2006). Exposure to ROS is known to cause a range of reversible and irreversible covalent modifications of amino acid side-chains of proteins (reviewed by Ghezzi and Bonetto, 2003) and thereby alter protein structure or function. Amino acid side-chains can be irreversibly modified into aldehyde or ketone groups (carbonylation) which can lead to protein aggregation, inactivation or degradation (Levine et al., 2000). While these effects have been intensively investigated in systems such as mammalian tissues, cell and yeast cultures (Conrad et al., 2000; Costa et al., 2002), they have been studied relatively recently in environmental contexts. It has been suggested that induction of protein carbonylation may be a powerful addition to the battery of biochemical endpoints in study of oxidative stress in the environment as it holds out the prospect of gaining insights to intracellular mechanisms resulting in specific targeting of proteins (McDonagh et al., 2005). At our knowledge, tissue-specific formation of protein carbonylation by environmental toxicants has been demonstrated by means of a proteomic approach in a few mollusc species only (Dowling et al., 2006; McDonagh et al., 2005, 2006; Chora et al., 2008).

Heat shock proteins (HSPs) are other commonly used biomarkers in aquatic organisms. They include a number of families induced during different stress conditions such as thermal stress, osmotic stress and exposure to xenobiotics. They mainly prevent protein denaturation, help the cell to recover damaged proteins by chaperoning their refolding and are considered to be general indicators of sub-lethal cellular protein damage (Sanders, 1993; Feder and Hofmann, 1999). Stress proteins HSP60, HSP70 and HSP90 appear to potentially be good indicators of some pesticides exposure, such as organochlorines and pyrethroids (Werner et al., 2002; Lee et al., 2006; Mukhopadhyay et al., 2006).

The present study investigated the effects of endosulfan and deltamethrin on oxidative stress status and on heat shock protein levels in different tissues of *P. monodon* and on their potential use as biomarkers. Besides, the effect of deltamethrin on protein carbonylation profile was investigated for the first time in a crustacean.

2. Materials and methods

2.1. Animals collection and maintenance

Experiment was conducted in 2006 at the College of Aquaculture and Fisheries, Can Tho University, Vietnam. Black tiger shrimp (*P. monodon*) of both sexes weighing 8.5 ± 0.4 g were obtained from an extensive shrimp farm in Ca Mau province, Vietnam. Upon arrival, animals were acclimated to laboratory conditions in composite tanks (capacity 2 m^3) filled with natural aerated saline water 15 ppt. Temperature was maintained at 30–32 °C with a natural light–dark cycle. Shrimp were held under these conditions for one week before pesticide exposure. During acclimation period, shrimp were fed five times a day with commercial shrimp food, Greenfeed™ (35% crude protein).

2.2. Pesticide exposure

Twenty-five acclimated shrimp were randomly distributed in each 18 composite tanks (capacity 500 L) filled with 200 L natural aerated saline water 15 ppt. Endosulfan (Riedel-de Haën, 36750) or deltamethrin (Sigma D9315) were added to water using acetone as a solvent, resulting in endosulfan nominal concentrations of 0.1 and 1 $\mu g L^{-1}$, and deltamethrin nominal concentrations of 0.01 and 0.1 $\mu g L^{-1}$. All treatments and the controls received the same acetone concentration (0.01%), which was below the NOEC of 0.1% reported by Mayer (1987). The concentrations were chosen to be under LC50 values estimated according to literature (L'Hotellier and Vincent, 1986; Joshi and Mukhopadhyay, 1990) and to a pre-test on black tiger shrimp which showed a significant mortality rate at 10 $\mu g L^{-1}$ for endosulfan and at 1 $\mu g L^{-1}$ for deltamethrin (data not shown). Each condition included three replicated tanks. Exposure duration was 4 days followed by a decontamination period of 7 days. During the contamination stage, water was gently siphoned out and replaced every day so that shrimp were exposed daily with the same freshly prepared concentrations of pesticides. Animals were not fed during contamination while they were fed as described above during decontamination. No mortality was observed during the experiment.

2.3. Sampling and biological material collection

Shrimp were sampled before treatment (day 0), after 4 days of contamination (day 4) and after 7 days of decontamination (days 4 + 7). At each sampling time, six individuals per tank were killed by decapitation. Hepatopancreas, gills and muscle were collected on ice, pooled per tank and kept at −80 °C until homogenization.

2.4. Oxidative stress markers

2.4.1. Sample preparation

Hepatopancreas and gills were homogenized 1:5 (w:v) in ice-cold 50 mM phosphate buffer pH 7.5 containing protease inhibitor cocktail (Sigma P2714). Five hundreds μL of homogenate were taken for lipid peroxidation. The rest of the homogenate was centrifuged at 10 000 × *g* for 10 min at 4 °C and the supernatant (post-mitochondrial fraction – PMF) was kept at −80 °C for antioxidant enzyme activities assays. Protein contents were assayed by the method of Lowry et al. (1951) using Folin's reagent and BSA as standard. For protein carbonylation analysis, proteins from gill tissue were extracted for shrimp exposed to 0.1 $\mu g L^{-1}$ deltamethrin at time 0 and after 4 days of exposure. Gills were homogenized by sonication for twice 15 s on ice in lysis buffer solution (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris–HCl, pH 8.5 and 0.25% protease inhibitor cocktail P2714, Sigma). Samples were then centrifuged for 15 min at 12 000 × *g* and the supernatant kept at −80 °C. Protein contents were calculated using the method of Bradford (1976) with BSA as a standard.

2.4.2. Lipid peroxidation level

LPO results in the production of malondialdehyde (MDA) consequently to free radicals production, and was assessed by the thiobarbituric acid reactive substances assay TBARS (Fatima et al., 2000). MDA reacts with thiobarbituric acid (TBA) and the product is read spectrophotometrically at 535 nm. Homogenate was added 1:1 (v:v) to 5% trichloroacetic acid (TCA), and incubated on ice for 15 min. The solution was then mixed in a ratio 2:1 with 0.67% TBA, and centrifuged at 2200 × *g* at 4 °C for 10 min. Whole supernatant was boiled for 10 min and refreshed at room temperature before the absorbance was recorded. A calibration curve with increasing MDA concentrations allowed the calculation of LPO expressed as nmol MDA equiv. g^{-1} tissue.

2.4.3. Antioxidant enzyme activities

Catalase (CAT) activity was estimated by the titanium oxysulphate ($TiOSO_4$) method (Baudhuin et al., 1964). Twenty-five μL of homogenate were pretreated with 0.02% (v:v) Triton X-100 during 2 min. They were then incubated at 0 °C during 6 min after addition of 0.2 M imidazol buffer (pH 7.0), 0.1% BSA, and 1.5 mM H_2O_2 in a volume of 1.25 mL. The reaction was stopped with 750 μL of a saturated solution of $TiOSO_4$ in 2N H_2SO_4. The change of absorbance after 6 min was recorded at 420 nm. One unit of activity is defined as the amount of enzyme causing the destruction of 90% of the substrate within 1 min in a volume of 50 mL in the assay condition and is expressed as U mg^{-1} protein.

Glutathione peroxidase (GPx) activity was determined using the method of Flohé and Günzler (1984) and modified by Mohandas et al. (1984). The reaction mixture consisted of 50 mM phosphate buffer (pH 7), 1 mM EDTA, 1 mM sodium azide, NaN_3, 2 U mL^{-1} glutathione reductase, GR, 2 mM reduced glutathione, GSH, 0.2 mM NADPH. After 10 min incubation at 37 °C, the overall reaction was initiated by adding 0.5 mM hydrogen peroxide, H_2O_2. Oxidation of NADPH was recorded spectrophotometrically at 340 nm during 5 min. The enzyme activity was calculated as nmol NADPH oxidized min^{-1} mg^{-1} protein.

Glutathione S-transferase (GST) activity was determined using the method of Habig et al. (1974). The reaction mixture consisted of 33 mM Hepes buffer (pH 7.5), 1.5 mM GSH, 1.5 mM 1-chloro-2,-dinitrobenzene, CDNB and water in a total volume of 1 mL. The conjugation of GSH with CDNB via GST activity was recorded spectrophotometrically at 340 nm during 3 min. The activity was expressed as nmol of CDNB conjugate formed min^{-1} mg^{-1} protein.

2.4.4. Proteomic analysis

Proteomic analysis was used to detect a potential protein carbonylation induction using two-dimensional gel electrophoresis. We decided to focus our attention on one specific condition for which we observed significant effects on lipid peroxidation, and for which literature indicated the most important protein carbonylation level, i.e. deltamethrin on gills (Parvez and Raisuddin, 2005). Carbonyls derivatives were detected following conjugation with DNPH (2,4-dinitrophenylhydrazine) which can be achieved by immunochemical assay (McDonagh et al., 2005).

Immobiline DryStrips (pH 4–7; 11 cm; GE Healthcare) were rehydrated for 20 h into 200 μL of a solution containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.28% DTT, 0.5% IPG buffer pH 4–7 and a trace amount of bromophenol blue. Homogenates were reduced 1:1 (v:v) in a solution containing 7 M urea, 2 M thiourea, 2% CHAPS, 2% DDT, 2% IPG buffer pH 4–7. After 15 min incubation time at room temperature, the solution was centrifuged at 12 000 × *g* for 10 min. One hundred and fifty μg of protein were then absorbed onto the strip. First-dimension isoelectric focusing was run on an IPGphor system (GE Healthcare) at 500 V for 1 h, 1000 V for 1 h, 6000 V for 2 h 30 and 6000 V for 30 min (total of 10 300 V h).

Following isoelectric focusing, oxidized proteins on the strip were derivatized with DNPH according to the protocol described by Conrad et al. (2000). Strips were derivatized in a solution containing 2 N HCl and 10 mM DNPH for 20 min at room temperature. Strips were then washed in 2 M Tris–base/30% glycerol for 15 min. Afterwards, they were equilibrated for twice 15 min in 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 50 mM Tris pH 8.8, 65 mM DTT and then 200 mM iodoacetamide replacing DTT. After equilibration, strips were loaded onto 12.5% SDS-PAGE gels, overlaid with 1% agarose in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS), and run at a constant voltage (45 mA/gel) until the dye front reached the end of the gel. Gels were then equilibrated for 5 min in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol) and electroblotted onto 0.45 μm nitrocellulose membranes (143 mA for 1 h 30). Membranes were soaked into stop solution containing 10 mM Trizma–HCl, 150 mM NaCl, 0.05% Nonidet, pH 7.5 (TBST) and 2.5% powdered skim milk overnight at 4 °C before washing three times for 10 min with TBST and 1 h incubation with the antibody solution consisting of 1:750 rabbit anti-DNP/HRP (polyclonal Rabbit Anti-DNP/HRP, P5102, Dako Cytomation) in TBST containing 2% powdered skim milk. Finally, blots were washed 3 × 15 min in TBST; 4 × 5 min in TBST and 1 × 5 min in TBS (TBST without nonidet) and developed with the Amersham ECL™ kit as recommended by the manufacturer. Continuous shaking was used during all incubation and washing steps.

Following exposure to the chemiluminescence kit, the membranes were scanned with a densitometer (ImageScanner™, GE Healthcare) and analyzed in a visual way by overlapping to match the carbonyl protein spots.

2.5. HSP assays

Muscle was chosen for HSP analysis due to the likely high stability of HSP expression over time and its potential usefulness to assess such biomarkers. Muscles were homogenized in 25:1 (v:w) buffer containing 50 mM Trizma–HCl pH 7.6, 1% Nonidet and 1% protease inhibitor cocktail (Sigma P2714). Samples were centrifuged at 12 000 × *g* (4 °C) for 15 min and the supernatant was kept at −80 °C. Total soluble protein contents were calculated using the method of Bradford (1976) with BSA as a standard.

Samples were then diluted in a buffer containing TBS, 0.1% SDS and 5 mM DTT, and the solution boiled 5 min. Three μg of proteins were directly loaded onto a nitrocellulose membrane (0.45 μm) with a slot blotter (Bio-Dot SF microfiltration apparatus from BioRad). Human purified proteins (StressGen SPP-755) were used as standards at increasing quantities (0.5–2.5–5 ng) for HSP70 quantification. Since the species-specific HSP70 protein is not available as positive control, the data are reported as μg equivalents HSP70 per mg of total soluble proteins (μg equiv. HSP70 mg^{-1} prot.). For HSP90 quantification, we could not find a good internal standard so that we expressed the results as relative HSP90 level related to a reference sample prepared from a mixture of shrimp muscle. Membranes were blocked for 1 h in 5% dry milk in TTBS, rinsed twice in TTBS and once in TBS for 5 min, and then incubated for 1 h with the primary antibody 1:1000 (Affinity BioReagents MA3-006 for HSP70 and Stressgen SPA-830D for HSP90) in TTBS plus 2% dry milk. The blots were rinsed twice in TTBS and once in TBS for 5 min and incubated for 1 h with the secondary antibody 1:2000 (horseradish peroxidase anti-mouse Ig, NA931 Amersham). Finally, blots were rinsed thrice in TTBS and once in TBS and developed with the Amersham ECL™ kit as recommended by the manufacturer. Films were scanned with a densitometer (ImageScanner™) and analysed using the software TotalLab (GE Healthcare). The optical density of each band was plotted versus purified human HSP70 or versus the reference HSP90 muscle sample.

As an organ directly in contact with the surrounding water and pollutants, gills were also tested for HSP70 and 90 levels following the same approach described above. However, technical issues (we obtained a very high variability among the conditions and a strong time-dependent effect) avoided drawing definitive conclusions (data not shown). Further technical setup is necessary to collect informative data for this organ in black tiger shrimp.

2.6. Statistical analysis

Data were expressed as mean ± S.D. and analysed using an analysis of variance (two-way ANOVA) with repeated measures following the general model summarised by Paine (1996). This model was chosen on account of measurements were made on the same replicates at several times (since the tank is the experimental unit). We assessed variations of LPO, CAT, GPx, GST and HSPs as a result of treatments (Endosulfan: control, 0.1 μg L^{-1}, 1 μg L^{-1} and deltamethrin: control, 0.01 μg L^{-1}, 0.1 μg L^{-1}), replicate tanks (within treatments), sampling times within replicates (day 0; day 4; days 4 + 7). The interaction between time and treatment implied that a specific time effect was observed depending upon the treatment and tested statistically the hypothesis that any change in dependent variables over time was caused by pesticide exposure. If an ANOVA with repeated measures detected a significant interaction effect, multiple comparison test (Fisher LSD test) at a 5% significant level was used to test differences between groups. All data were natural logarithm transformed to stabilize the variance and to approximate normal distribution prior to use in further statistical analysis. All tests were performed using the software Statistical 5.5 (StatSoft, Inc. 2000).

3. Results

3.1. Lipid peroxidation

In endosulfan exposed-shrimp, TBARS levels ranged between 119.9 ± 15.8 and 192.6 ± 38.1 nmol MDA equiv. g^{-1} tissue for hepatopancreas and between 26.0 ± 4.5 and 63.8 ± 34.3 nmol MDA equiv. g^{-1} tissue for gills. Even though the LPO values in gills tended to be higher after 4 days of exposure, endosulfan had no significant effect ($p > 0.05$) on LPO in any of the studied tissues (Fig. 1). Deltamethrin exposed-shrimp had TBARS levels ranging from 85.1 ± 8.5 to 175.4 ± 57.4 nmol MDA equiv. g^{-1} tissue in hepatopancreas and from 17.8 ± 0.3 to 64.3 ± 3.2 nmol MDA equiv. g^{-1} tissue in gills. Deltamethrin had no significant effect on LPO values in hepatopancreas, whereas 4 days of exposure to 0.1 μg L^{-1} resulted in a significant increase from 34.2 ± 5.3 to 64.3 ± 3.2 nmol MDA equiv. g^{-1} tissue in gills ($p < 0.05$) (Fig. 1). This deltamethrin effect was reversible since a significant decrease of LPO was recorded after 7 days of decontamination.

3.2. Antioxidant enzyme activities

The activities of antioxidant enzymes in hepatopancreas and gill tissues of control and exposed shrimp are depicted in Tables 1 and 2. Regardless of tissues, pesticide treatments did not induce significant interaction effect on the activities of studied antioxidant enzymes, namely, catalase, glutathione peroxidase, and glutathione S-transferase ($p > 0.05$). Only GPx activity was found to be slightly and none significantly induced in gills over time at the highest deltamethrin concentration tested (0.1 μg L^{-1}).

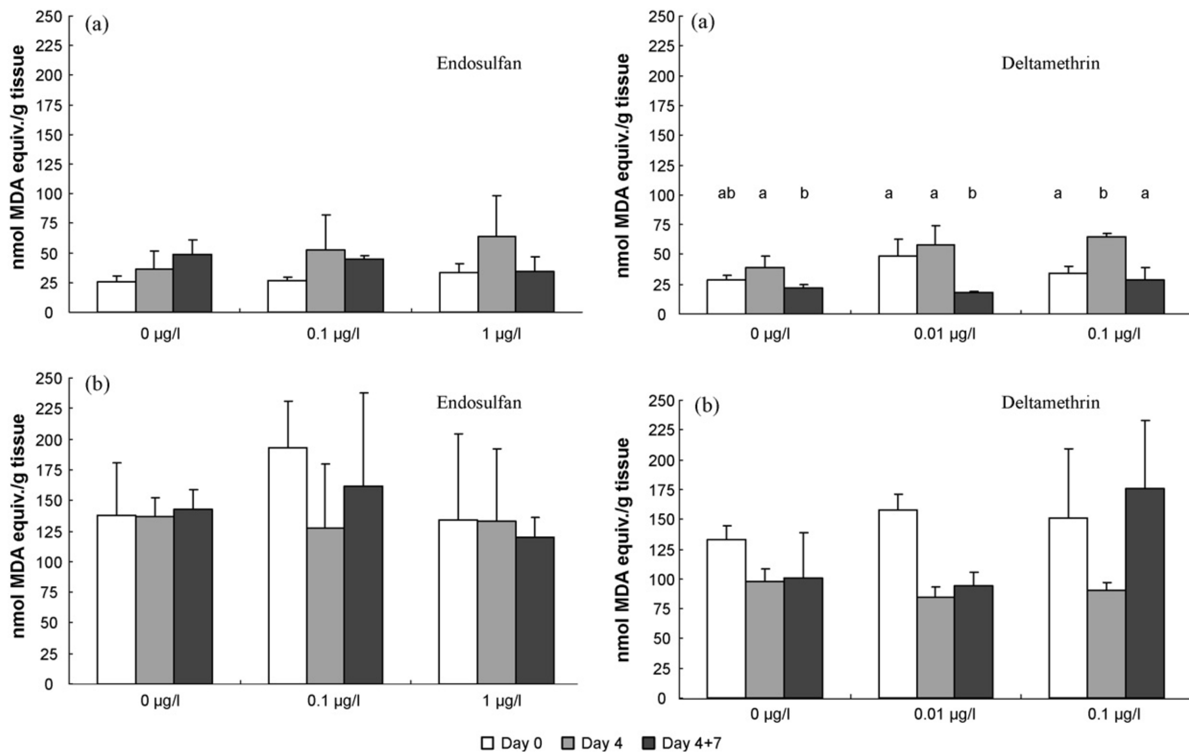

Fig. 1. LPO level (nmol MDA equiv. g^{-1} tissue) in gills (a) and hepatopancreas (b) of *Penaeus monodon* following 4 days of exposure to endosulfan or deltamethrin and 7 days of decontamination. Data are presented as mean ± S.D. ($n = 3$). If a significant time–treatment interaction effect was observed, different letters mean significant differences between sampling time within a given treatment ($p < 0.05$).

Table 1

Activities of antioxidant enzymes in hepatopancreas of *Penaeus monodon* following 4 days of exposure to endosulfan or deltamethrin and 7 days of decontamination.

Days	0			4			4 + 7		
Endosulfan (μg L^{-1})	0	0.1	1	0	0.1	1	0	0.1	1
CAT	0.015 ± 0.004	0.019 ± 0.001	0.016 ± 0.009	0.011 ± 0.002	0.013 ± 0.002	0.009 ± 0.002	0.015 ± 0.002	0.013 ± 0.004	0.012 ± 0.006
GPx	10.2 ± 3.2	13.0 ± 3.6	12.9 ± 7.9	7.9 ± 3.2	9.6 ± 1.7	9.1 ± 2.1	8.8 ± 1.6	7.2 ± 0.1	8.3 ± 1.6
GST	82.5 ± 41.0	94.8 ± 27.2	106.8 ± 13.6	39.0 ± 3.7	46.9 ± 11.6	49.7 ± 23.1	93.9 ± 32.8	93.2 ± 43.0	93.1 ± 10.7
Deltamethrin (μg L^{-1})	0	0.01	0.1	0	0.01	0.1	0	0.01	0.1
CAT	0.008 ± 0.002	0.014 ± 0.007	0.015 ± 0.003	0.013 ± 0.004	0.009 ± 0.003	0.013 ± 0.005	0.01 ± 0.004	0.013 ± 0.003	0.01 ± 0.001
GPx	7.9 ± 2.9	8.8 ± 0.8	7.4 ± 1.6	7.0 ± 1.2	5.2 ± 1.6	5.9 ± 1.2	8.4 ± 1.4	9.4 ± 1.8	8.5 ± 2.1
GST	57.6 ± 24.5	96.9 ± 16.2	88.3 ± 11.9	95.3 ± 22.3	72.8 ± 25.0	110.1 ± 11.7	93.7 ± 16.5	116.6 ± 16.4	107.7 ± 28.2

Values are presented as mean ± S.D. ($n = 3$). CAT: U mg^{-1} protein; GPx: nmol NADPH oxidized min^{-1} mg^{-1} protein; GST: nmol CDNB min^{-1} mg^{-1} protein.

Table 2

Activities of antioxidant enzymes in gills of *Penaeus monodon* following 4 days of exposure to endosulfan or deltamethrin and 7 days of decontamination.

Days	0			4			4 + 7		
Endosulfan (μg L^{-1})	0	0.1	1	0	0.1	1	0	0.1	1
CAT	0.036 ± 0.007	0.031 ± 0.008	0.039 ± 0.005	0.036 ± 0.008	0.026 ± 0.006	0.023 ± 0.011	0.037 ± 0.009	0.038 ± 0.012	0.03 ± 0.003
GPx	11.2 ± 2.2	15.3 ± 1.2	14.9 ± 2.3	10.1 ± 3.7	9.2 ± 3.5	8.7 ± 3.0	9.6 ± 1.0	8.5 ± 0.8	10.6 ± 1.2
GST	93.1 ± 7.7	132.0 ± 18.3	134.8 ± 9.4	144.0 ± 28.0	122.7 ± 20.5	147.0 ± 17.8	120.4 ± 14.5	133.6 ± 35.4	141.7 ± 34.9
Deltamethrin (μg L^{-1})	0	0.01	0.1	0	0.01	0.1	0	0.01	0.1
CAT	0.047 ± 0.007	0.045 ± 0.012	0.043 ± 0.012	0.023 ± 0.01	0.025 ± 0.011	0.032 ± 0.005	0.025 ± 0.003	0.023 ± 0.006	0.029 ± 0.012
GPx	9.3 ± 1.7	6.6 ± 4.0	2.7 ± 1.6	10.3 ± 5.3	6.3 ± 3.9	6.7 ± 2.8	4.6 ± 3.3	3.6 ± 2.1	8.8 ± 1.7
GST	115.3 ± 30.1	120.4 ± 5.6	110.1 ± 7.3	128.7 ± 13.9	116.7 ± 7.5	102.7 ± 13.1	95.4 ± 6.9	84.9 ± 8.4	97.7 ± 15.6

Values are presented as mean ± S.D. ($n = 3$). CAT: U mg^{-1} protein; GPx: nmol NADPH oxidized min^{-1} mg^{-1} protein; GST: nmol CDNB min^{-1} mg^{-1} protein.

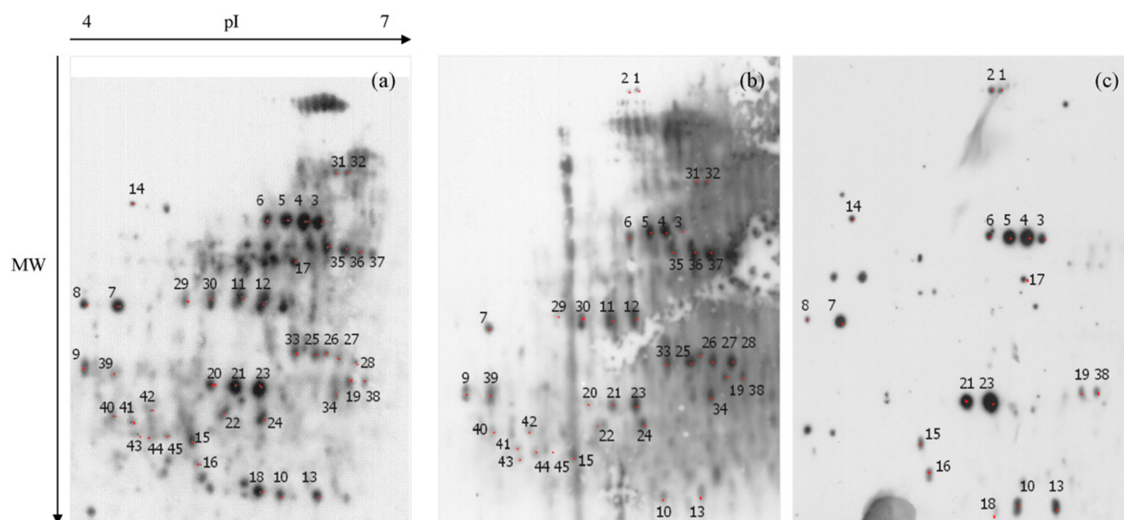

Fig. 2. 2D immunoblots of carbonylated proteins in gill samples from untreated *Penaeus monodon* (0-day). (a) replicate 1; (b) replicate 2; (c) replicate 3. MW: molecular weight; p*I*: isoeletric point.

3.3. Protein carbonylation

As the present report has shown significantly enhanced LPO in gills of *P. monodon* exposed to the highest tested concentration of deltamethrin, we decided to focus our attention on protein carbonyl induction in gills of 0.1 $\mu g\,L^{-1}$ deltamethrin-exposed shrimp. We analysed the pesticide response in gills of 0-day (control condition) and 4-day exposed individuals. Each condition included triplicates. In general, immunoblots of 2D SDS-PAGE carbonylated gill proteins displayed a similar profile between control (0-day) and deltamethrin treated animals (4-day) (Figs. 2 and 3). The triplicates of a same sampling day have been overlapped in order to match as many carbonyl protein spots as possible. Spots found at least in two out of three replicates were assigned by a number. In gills of untreated shrimp (0-day), 45 carbonyl protein spots were assigned while we could visualize 38 carbonyl protein spots after a 4-day exposure to 0.1 $\mu g\,L^{-1}$ deltamethrin. Composite gels, consisting of carbonyl protein spots found at least in two out of three immunoblot replicates for each sampling day were then drawn and compared (Fig. 4). Even if almost the same number of spots were observed in both conditions, some qualitative changes occurred: 17 protein spots have been carbonylated in response to deltamethrin treatment (# 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61 and 62) while 24 protein spots not visible following deltamethrin exposure were specifically oxidized at time 0-day (# 1, 2, 10, 13, 14, 17, 19, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44 and 45).

3.4. HSPs

Endosulfan had significant effect on HSP levels in muscle of *P. monodon* as observed in Fig. 5. While control organisms had HSP90 levels stable through all the experimental period, 0.1 and 1 $\mu g\,L^{-1}$ of the pesticide induced a 1.7-fold and a 2.1-fold increase, respectively ($p < 0.01$). However, this increase was observed after 7 days of decontamination only, indicating that HSP90 synthesis in response to endosulfan required more than 4 days. Moreover endosulfan seemed to slightly affect HSP70 expression. In controls and in organisms exposed to 0.1 $\mu g\,L^{-1}$, HSP70 levels dropped significantly after 7 days of decontamination (from 1.55 ± 0.16 to 0.66 ± 0.05 ng equiv. μg^{-1} prot. for controls and from 1.61 ± 0.13 to 0.89 ± 0.08 ng equiv. μg^{-1} prot. for 0.1 $\mu g\,L^{-1}$ group) while a slight and non significant decrease was observed in muscle from shrimp exposed to 1 $\mu g\,L^{-1}$ (from 1.46 ± 0.07 to 1.11 ± 0.21 ng equiv. μg^{-1} prot.). The time effect and the interaction were highly significant ($p < 0.01$) suggesting that HSP70 level in muscle is not stable through

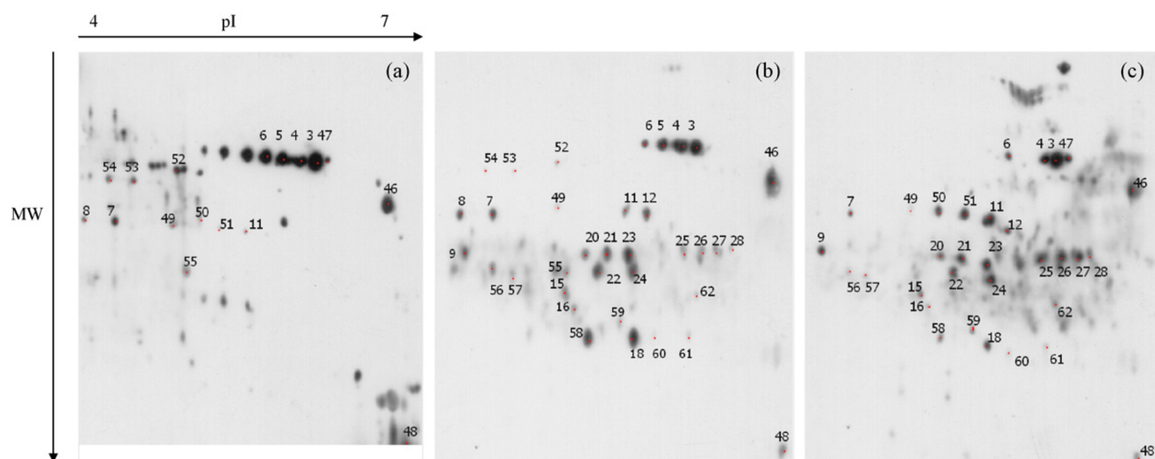

Fig. 3. 2D immunoblots of carbonylated proteins in gill samples from treated *Penaeus monodon* with 0.1 $\mu g\,L^{-1}$ deltamethrin (4-day). (a) replicate 1; (b) replicate 2; (c) replicate 3. MW: molecular weight; p*I*: isoeletric point.

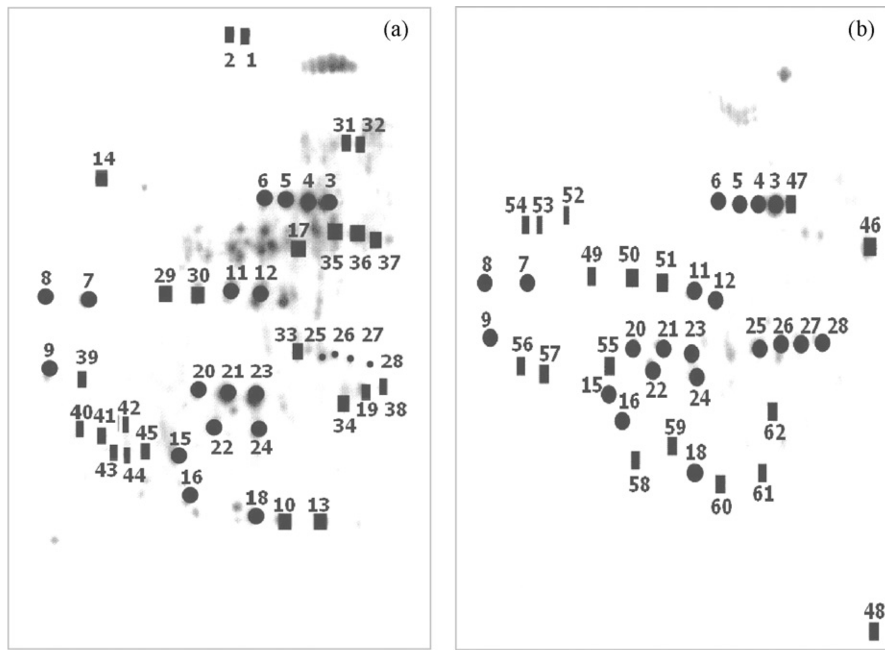

Fig. 4. Composite 2D immunoblots of carbonylated proteins in gill samples from *Penaeus monodon*. (a) 0-day composite gel; (b) 4-day composite day. (●) Common carbonylated protein spots; (■) Specifically carbonylated protein spots.

time and that exposure to endosulfan at 1 $\mu g\,L^{-1}$ compensates this time effect by increasing this protein level.

At the opposite, deltamethrin did not show any significant effect on HSP expression (Fig. 5). As observed previously for shrimp exposed to endosulfan, a significant time effect ($p < 0.01$) indicated that HSP70 level decreased over time whatever the pesticide exposure. This decrease was not different in controls and in exposed shrimp ($p > 0.5$ for interaction). For HSP90, the same level was observed for all treatments and for all sampling time.

4. Discussion

Sustainability in aquaculture has been proposed to be achieved using integrated aquaculture management. Components of a sound management plan should include the promotion of a research program for determining impacts from the shrimp industry and require an environmental impact assessment tool (Dierberg and Kiattisimkul, 1996). The issue of animal welfare in aquaculture is of growing interest and there is an increasing consumer demand for

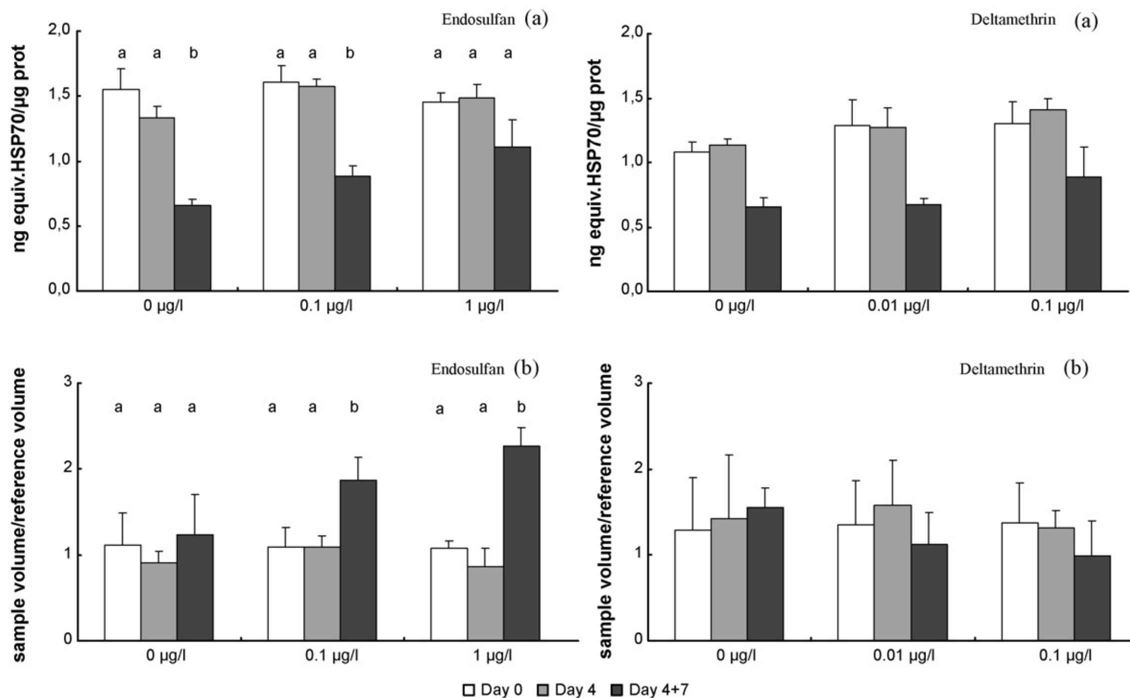

Fig. 5. HSP70 (a) and HSP90 (b) levels in muscles of *Penaeus monodon* following 4 days of exposure to endosulfan or deltamethrin and 7 days of decontamination. Data are presented as mean ± S.D. ($n = 3$). If a significant time–treatment interaction effect was observed, different letters mean significant differences between sampling time within a given treatment ($p < 0.05$).

documentation of safe and ethical food production. Consequently sustainable development of shrimp culture necessitates that animals are in good health condition, unstressed, and that as low amount of chemicals as possible are used. For this purpose the use of biomarkers in cultured shrimp can be of a great interest. Such assessment tool could ideally indicate whether the shrimp are becoming stressed, as an early warning signal in order to prevent excess use of chemicals. To avoid permanent damage, it is useful to have biomarkers capable of diagnosing a stress condition at its earliest onset to allow an immediate corrective intervention.

It is documented that pesticides may cause oxidative stress leading to the generation of free radicals and alterations in antioxidants or free oxygen radical scavenging enzyme systems (Almeida et al., 1997). The typical reaction during ROS-induced damage involves the peroxidation of unsaturated fatty acids. Estimation of lipid peroxidation has been found to have high predictive importance as revealed from a number of research papers describing its use as biomarkers of pollution (Ribera et al., 1990; Pandey et al., 2003). Moreover lipid peroxidation has been suggested as one of the molecular mechanisms involved in pesticide induced toxicity (Khrer, 1993). In the present study, neither deltamethrin nor endosulfan was able to stimulate the hepatic lipid peroxidation process in exposed shrimp. However, stimulation of lipid peroxidation by deltamethrin was observed in gills after 4 days of exposure to 0.1 $\mu g\,L^{-1}$, demonstrating that this pyrethroid has pro-oxidant properties in shrimp tissues. Several studies have already shown the capacity of endosulfan and deltamethrin to induce oxidative stress in fish, as depicted by elevated levels of LPO in various tissues (Pandey et al., 2001; Dorval et al., 2003; Sayeed et al., 2003; Atif et al., 2005; Parvez and Raisuddin, 2006). Nevertheless it is the first evidence of such an effect in a farmed crustacean. Vulnerability of gills to exposure to aquatic pollutants has been established in previous studies involving different fish species (Ahmad et al., 2000; Fatima et al., 2000). Gills are the primary sites for the absorption of aquatic pollutants, including pesticides. They are also reported to be weak in terms of their antioxidant potential compared to that of other organs (Arun and Subramanian, 1998; Sayeed et al., 2003).

Apart from lipid peroxidation, protein carbonylation has recently been reported as a biomarker of oxidative stress in several sentinel species (Almroth et al., 2005; McDonagh et al., 2005; Parvez and Raisuddin, 2005; Dowling et al., 2006; McDonagh et al., 2006; Chora et al., 2008). McDonagh et al. (2005) have applied a proteomic approach to mussels from a polluted site known to possess relatively high levels of sediment cadmium and zinc. Their data showed that gills and digestive gland are major sites of glutathionylation and carbonylation as a result of oxidative stress. Furthermore, global protein carbonyl induction as a biomarker of oxidative stress was used in laboratory studies to assess the toxic effects of pesticides in various tissues (gills, liver and kidney) of freshwater fish, *Channa punctatus*, exposed to deltamethrin, endosulfan and paraquat (Parvez and Raisuddin, 2005). Among these pesticides, deltamethrin showed the maximum level of induction and amongst the tissues gills were found to be the most vulnerable ones. However, the present proteomic analysis is the first to investigate the qualitative protein carbonylation profile in a crustacean species exposed to pesticides. When exposed to deltamethrin, the carbonylation pattern in gills did not seem to be higher compared to the control profile since about the same total number of protein spots were assigned in gills of untreated animals (45) and of shrimp exposed to deltamethrin (38). Although no quantitative difference in protein carbonylation was detected by two-dimensional gel electrophoresis analysis, a qualitative modification of the proteome oxidation status has been recorded. In response to the deltamethrin treatment, 17 protein spots were present and 24 absent compared to control. It indicates that general oxidation status of the proteome has been reorganized. It strengthens the observation that exposure to deltamethrin at 0.1 $\mu g\,L^{-1}$ during 4 days induces oxidative damages on lipids and proteins in cultured shrimp. McDonagh et al. (2005) highlighted that protein carbonylation profile modifications in response to ROS exposure was strongly evident in samples showing only modest effects on the general protein expression profile. In addition, the present study assumes that this approach can also discriminate different stress levels even though the general protein carbonylation level is only weakly modified. This strengthens the utility of proteomic-based detection of protein carbonylation as a potential useful and sensitive biomarker that can be used in field monitoring studies.

Catalase and glutathione peroxidase have complementary roles in hydrogen peroxide detoxication. In the present study, even though a slight non significant increase of GPx activity was observed in gills of shrimp exposed to the highest tested deltamethrin concentration, none of the tested pesticides have significant effect on CAT and GPx activities in hepatopancreas and in gills. This lack of GPx and CAT activities induction in gills after 4 days exposure to 0.1 $\mu g\,L^{-1}$ deltamethrin could explain, at least partly, that this experimental condition may induce oxidative damages on lipids and proteins. Low activity of GPx and CAT in gills of endosulfan exposed shrimp, as observed in the present study, suggests the inefficiency of this organ in neutralizing the impact of peroxides and thus resulting in increased LPO (Fatima et al., 2000). Several studies dealing with the responses of antioxidant enzymes to pesticides, which enhance reactive oxygen species production, have been reported. However, they were inconclusive and show wide individual differences (Oruç et al., 2004).

Using GST and other detoxification enzymes of phase II biotransformation as biomarkers of exposure to xenobiotics has gained credence for aquatic pollution monitoring (Livingstone, 1998). Glutathione S-transferases are a group of widely distributed enzymes that catalyze the conjugation of glutathione (GSH) with various electrophilic substances. GST-mediated conjugation is involved in the detoxification of many xenobiotics, which play an important role in protecting tissues from oxidative stress (Fournier et al., 1992). Nevertheless, in the present report, endosulfan and deltamethrin treatments were unable to affect GST activity in exposed shrimp. These results suggest that GST is not involved in the detoxification of these compounds in *P. monodon* under our experimental conditions. On the contrary, GST was found to be induced in response to exposure to endosulfan in fish (Pandey et al., 2001; Dorval et al., 2003) and in daphnia (Barata et al., 2005). Parallel induction of GST was noted in liver and kidney of fish exposed to deltamethrin (0.75 $\mu g\,L^{-1}$), while it was reduced in gills (Sayeed et al., 2003; Atif et al., 2005). Since several isoforms exist and can differently react to xenobiotic exposure (Hoarau et al., 2004; Silvestre et al., 2006) and since only one substrate was used in the present study, further investigations on possible isoform specific induction in shrimp tissue due to deltamethrin exposure should bring deeper informations.

Whereas deltamethrin induced oxidative damages in tissues of *P. monodon*, we could not observe a comparable effect for endosulfan exposed shrimp. However, exposure to this organochlorine induced heat shock protein responses, mainly an induction of HSP90 in muscle. For this biomarker, a positive dose/response relationship was observed since up to 2.1-fold induction was noted at the highest tested concentration. At our knowledge, it is the first evidence that endosulfan can induce HSP90 over-expression in aquatic organisms. Since most pesticides are lipophilic in nature, they easily diffuse into cells and may interfere with the hydrophobic core of protein, thus leading to denaturation of protein by exposing the lipophilic moieties to the aqueous environment (Meyer et al., 1995). The present results indicate that endosulfan has proteotoxic effects in muscle of exposed shrimp and that HSP90 must be synthesized to avoid or minimize protein denaturation and/or aggregation.

Contrarily, induction of HSP70 in organisms due to exposure to pesticides has previously been reported (Eder et al., 2004). HSP70 induction in fish has been reported following exposure to the phosphorothiate insecticide, chlorpyrifos via food (Varo et al., 2002). In prawn *Macrobrachium malcolmsonii*, endosulfan induced HSP70 over-expression after 96 h exposure in gills for concentrations of 0.32 and 0.016 $\mu g\,L^{-1}$. In *P. monodon*, endosulfan seems to induce over-expression of HSP70 in muscle. However we observed that at days 4 + 7, HSP70 level had significantly decreased compared to day 0 and day 4, for controls and for shrimp exposed to 0.1 $\mu g\,L^{-1}$ endosulfan. This decrease was slight and non significant for animals exposed to 1 $\mu g\,L^{-1}$, indicating a possible new synthesis of HSP70 due to pesticide exposure. On the contrary, no similar effect could be observed for shrimp exposed to deltamethrin since the same decrease was noted at days 4 + 7 for all concentrations tested. This instability of HSP70 level over the experimental duration may be due to external uncontrolled parameters that could have modified this protein expression. The use of HSP70 as biomarker of pesticide exposure must take this fact into account and requires high precautions.

Endosulfan and deltamethrin exhibited different mechanisms of toxic action in tissues of *P. monodon*. The difference in biological responses could allow discriminating both pesticide exposure if these responses are used as biomarkers in shrimp farms. Deltamethrin has shown a pro-oxidant effect in gills while endosulfan has shown HSP induction in muscle. Lipid peroxidation level, protein carbonylation specificities, HSP70 and HSP90 levels could be adapted for biomarker development, while antioxidant enzyme activities tested, i.e. CAT, GPx and GST, were never affected. However, some limitations exist. LPO response to deltamethrin seemed transient since its induction observed after 4 days exposure was not further observed after 7 days of decontamination. Similar time dependence effect could also diminish the usefulness of HSP90 and HSP70 as biomarkers. The former showed induction after 7 days of decontamination only, while the latter showed a decreased expression over experimental duration. These limitations suggest that care must be taken when analysing these biomarkers and that their induction and elimination kinetics should be known. Our complementary proteomic approach seems very promising but requires deeper analysis and protein identification. Undoubtedly, a set of biomarkers, using proteomics and more classical biochemical approaches, should be developed as an early warning signal in order to prevent excess use of chemicals in shrimp farming.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References

Ahmad, I., Hamid, T., Fatima, M., Chand, H.S., Jain, S.K., Athar, M., Raisuddin, S., 2000. Induction of hepatic antioxidants in freshwater catfish (*Channa punctatus* Bloch) is a biomarker of paper mill effluent exposure. Biochim. Biophys. Acta 1523, 37–48.

Almeida, M.G., Famini, F., Davino, S.C., Aznar, A.E., Koch, O.R., Barros, S.B.M., 1997. Pro-and anti-oxidant parameters in rat liver after short-term exposure to hexachlorobenzene. Hum. Exp. Toxicol. 16, 257–261.

Almroth, B.C., Sturve, J., Berglund, A., Forlin, L., 2005. Oxidative damage in eelpout (*Zoarces viviparus*), measured as protein carbonyls and TBARS, as biomarkers. Aquat. Toxicol. 73, 171–180.

Arun, S., Subramanian, P., 1998. Antioxidant enzymes in freshwater prawn *Macrobrachium malcolmsonii* during embryonic and larval development. Comp. Biochem. Physiol. B 121, 273–277.

Atif, F., Parvez, S., Pandey, S., Ali, M., Kaur, M., Rehman, H., Khan, H.A., Raisuddin, S., 2005. Modulatory effect of cadmium exposure on deltamethrine-induced oxidative stress in *Channa punctatus* Bloch. Arch. Environ. Contam. Toxicol. 49, 371–377.

Badiou, A., Meled, M., Belzunces, L.P., 2008. Honeybee *Apis mellifera* acetylcholinesterase—a biomarker to detect deltamethrin exposure. Ecotoxicol. Environ. Saf. 69, 246–253.

Bainy, A.C.D., 2000. Biochemical responses in penaeids caused by contaminants. Aquaculture 191, 163–168.

Barata, C., Varo, I., Navarro, J.C., Arun, S., Porte, C., 2005. Antioxidant enzymes activities and lipid peroxidation in freshwater cladoceran *Daphnia magna* exposed to redox cycling compounds. Comp. Biochem. Physiol. C 140, 175–186.

Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O.Z., Wattiaux, R., Jacques, P., De Duve, C., 1964. Tissue fractionation studies. 17. Intracellular distribution of monoamine axidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue. Biochem. J. 92, 179–184.

Bradford, M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principal of protein-dye binding. Anal. Biochem. 72, 248–254.

Capkin, E., Altinok, I., Karahan, S., 2006. Water quality and fish size affect toxicity of endosulfan, an organochlorine pesticide, to rainbow trout. Chemosphere 64, 1793–1800.

Chora, S., McDonagh, B., Sheehan, D., Starita-Geribaldi, M., Roméo, M., Bebianno, M.J., 2008. Ubiquination and carbonylation as marker of oxidative-stress in *Ruditapes decussates*. Mar. Environ. Res. 66, 95–97.

Costa, V.M.V., Amorim, M.A., Quintanilha, A., Moradas-Ferreira, P., 2002. Hydrogen peroxide-induced carbonylation of key metabolic enzymes in *Saccharomyces cerevisiae*: The involvement of the oxidative stress response regulators Yap1 and Skn7. Free Radic. Biol. Med. 33, 1507–1515.

Conrad, C.C., Marshall, P.L., Talent, J.M., Malakowsky, C.A., Choi, J., Gracy, R.W., 2000. Oxidized proteins in Alzheimer's plasma. Biochem. Biophys. Res. Commun. 275, 678–681.

Dierberg, E., Kiattisimkul, W., 1996. Issues, impacts, and implications of shrimp aquaculture in Thailand. Environ. Manage. 20, 649–666.

Dorval, J., Leblond, V.S., Hontela, A., 2003. Oxidative stress and loss of cortisol secretion in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*) exposed in vitro to endosulfan, an organochlorine pesticide. Aquat. Toxicol. 63, 229–241.

Dowling, V., Hoarau, P.C., Roméo, M., O'Halloran, J., van Pelt, F., O'Brien, N., Sheehan, D., 2006. Protein carbonylation and heat shock response in *Ruditapes decussates* following *p,p′*-dichlorodiphenyldichloroethylene (DDE) exposure: a proteomic approach reveals that DDE causes oxidative stress. Aquat. Toxicol. 77, 11–18.

Eder, K.J., Leutenegger, G.M., Wilson, B.W., Werner, I., 2004. Molecular and cellular biomarker responses to pesticide exposure in juvenile Chinook salmon (*Oncorhynchus tshawytscha*). Mar. Environ. Res. 58, 809–813.

Fatima, M., Ahmad, I., Sayeed, I., Athar, M., Raisuddin, S., 2000. Polluted-induced over activation of phagocytes is concomitantly associated with peroxidative damage in fish tissues. Aquat. Toxicol. 49, 243–250.

Feder, M.E., Hofmann, G.E., 1999. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and Ecological Physiology. Annu. Rev. Physiol. 61, 243–282.

Flohé, L., Günzler, W.A., 1984. Assays of glutathione peroxidase. Methods Enzymol. 105, 114–121.

Fournier, D., Bride, J.M., Poirie, M., Berge, J.B., Plapp, F.W., 1992. Insect glutathione S-transferases. Biochemical characteristics of the major forms of houseflies susceptible and resistant to insecticides. J. Biol. Chem. 267, 1840–1845.

Ghezzi, P., Bonetto, V., 2003. Redox proteomics: identification of oxidatively modified proteins. Proteomics 3, 1145–1153.

Gräslund, S., Holmström, K., Wahlström, A., 2003. A field survey of chemicals and biological products used in shrimp farming. Mar. Pollut. Bull. 46, 81–90.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferase. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Hoarau, P., Garello, G., Gnassia-Barelli, M., Roméo, M., Girard, J.P., 2004. Effect of three xenobiotic compounds on Glutathione S-Transferase in the clam *Ruditapes decussates*. Aquat. Toxicol. 68, 87–94.

Joshi, H.C., Mukhopadhyay, M.K., 1990. Toxicity of quinalphos and endosulfan to different life-stages of tiger prawn (*Penaeus monodon*). Environ. Conserv. 17, 266–267.

Khrer, J.P., 1993. Free radicals as mediator of tissue injury and disease. Crit. Rev. Toxicol. 23, 21–48.

Köprücü, K., Aydin, R., 2004. The toxic effects of pyrethroid deltamethrin on the common carp (*Cyprinus carpio* L.) embryos and larvae. Pestic. Biochem. Physiol. 80, 47–53.

Köprücü, S.S., Köprücü, K., Ural, M.S., 2006. Acute toxicity of the synthetic pyrethroid deltamethrin to fingerling European catfish, *Silurus glanis* L. Environ. Contam. Toxicol. 76, 59–65.

Lee, S.M., Lee, S.B., Park, C.H., Choi, J., 2006. Expression of heat shock protein and hemoglobin genes in *Chironomus tentans* (Diptera, chironomidae) larvae exposed to various environmental pollutants: apotential biomarker of freshwater monitoring. Chemosphere 65, 1074–1081.

Le Moullac, G., Haffiner, P., 2000. Environmental factors affecting immune responses in Crustacean. Aquaculture 191, 121–131.

Levine, R.L., Wehr, N., Williams, J.A., Stadman, E.R., Shacter, E., 2000. Determination of carbonyl groups in oxidized proteins. Methods Mol. Biol. 99, 15–24.

L'Hotellier, M., Vincent, P., 1986. Assessment of the impact of deltamethrin on aquatic species. In: British Crop Protection Conference—Pests and Disease, pp. 1109–1116.
Livingstone, D.R., 1998. The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative difference in biotransformation by invertebrates and fish. Comp Biochem. Physiol. A 120, 43–49.
Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666.
Lombardi, J.V., Machado-Neto, J.G., Brossi-Garcia, A.L., Marques, H.L.A., Kubo, E., 2001. Acute toxicity of the pesticides endosulfan and ametryne to the freshwater prawn *Macrobrachium rosenbergii* De Man. Environ. Contam. Toxicol. 67, 665–671.
Lowry, O., Rosebrough, N., Farr, N., Randall, R., 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Mayer, F., 1987. Acute toxicity handbook of chemicals to estuarine organisms. U.S. Environmental Protection Agency, Environmental Research Laboratory, Gulf Breeze, FL. EPA/600/8-87/017. April. 274 pp.
McDonagh, B., Tyther, R., Sheehan, D., 2005. Carbonylation and glutathionylation of proteins in the blue mussel *Mytilus edulis* detected by proteomic analysis and Western blotting: actin as a target for oxidative stress. Aquat. Toxicol. 73, 315–326.
McDonagh, B., Tyther, R., Sheehan, D., 2006. Redox proteomics in the mussel, *Mytilus edulis*. Mar. Environ. Res. 62, 101–104.
Meyer, U., Schweim, P., Fracella, F., Rensing, L., 1995. Close correlation between heat shock response and cytotoxicity in *Neurospora crassa*-treated with aliphatic alcohols and phenols. Appl. Environ. Microbiol. 61, 979–984.
Ministry of Fisheries. Annual Report on the State Achievements of 2004 and Action Plan of 2005 of the Fisheries Sector. 25 pp. (in Vietnamese).
Mishra, R., Shukla, S.P., 1995. Effects of endosulfan on bioenergetic properties of skeletal muscle mitochondria from the freshwater catfish, *Clarias batrachus*. Comp. Biochem. Physiol. C 112, 153–161.
Mohammad, A., Akram, R., Shahin, S., Sherkoufeh, N., Ali, R., 2004. Pesticides and oxidative stress: a review. Med. Sci. Mon. 10, 141–147.
Mohandas, J., Marshall, J.J., Duggin, G.G., Horvath, J.S., Tiller, D.J., 1984. Low activities of glutathione-related enzymes as factors in the genesis of urinary bladder cancer. Cancer Res. 44, 5086–5091.
Mukhopadhyay, I., Siddique, H.R., Bajpai, V.K., Saxena, D.K., Chowdhuri, D.K., 2006. Synthetic pyrethroid cypermetrhin induced cellular damage in reproductive tissues of *Drosophilia melanogaster*: Hsp 70 as a marker of cellular damage. Arch. Environ. Contam. Toxicol. 51, 673–680.
Oruç, O.E., Sevgiler, Y., Üner, N., 2004. Tissue-specific oxidative stress responses in fish exposed to 2,4-D and azinphosmethyl. Comp. Biochem. Physiol. C 137, 43–51.
Paine, M.D., 1996. Repeated measures designs. Environ. Toxicol. Chem. 15, 1439–1441.
Pandey, S., Ahmad, S., Parvez, B., Bin-Hafeez, R., Haque, R., Raisuddin, S., 2001. Effect of endosulfan on antioxidants of freshwater fish *Channa punctatus* Bloch. 1. Protection against lipid peroxidation in liver by copper preexposure. Arch. Environ. Contam. Toxicol. 41, 345–352.
Pandey, S., Parvez, S., Sayeed, I., Haque, R., Bin-Hafeez, B., Raisuddin, S., 2003. Biomarkers of oxidative stress: a comparative study of river Yamuna fish *Wallago attu* (Bl. & Schn.). Sci. Total. Environ. 309, 105–115.
Parvez, S., Raisuddin, S., 2005. Protein carbonyls: novel biomarkers of exposure to oxidative stress-inducing pesticides in freshwater fish *Channa punctata* (Bloch). Environ. Toxicol. Pharmacol. 20, 112–117.
Parvez, S., Raisuddin, S., 2006. Copper modulates non-enzymatic antioxidants in the freshwater fish *Channa punctatus* (Bloch) exposed to deltamethrin. Chemosphere 26, 1324–1332.
Prasanthi, K., Muralidhara, Rajini, P.S., 2005. Fenvalerate-induced oxidative damage in rat tissues and its attenuation by dietary sesame oil. Food Chem. Toxicol. 43, 299–306.
Ratushnyak, A., Andreeva, M.G., Trushin, M.V., 2005. Effects of type II pyrethroids on *Daphnia magna*: dose and temperature dependences. Riv. Biol. 98, 349–357.
Reyes, G.G., Villagrana, L.C., Alvarez, G.L., 1999. Environmental conditions and pesticide pollution of two coastal ecosystems in the Gulf of California. Mexico. Ecotoxicol. Environ. Saf. 44, 280–286.
Ribera, D., Narbonne, J.F., Livingstone, D.R., O'Hara, S., 1990. Responses of antioxidants and lipid peroxidation in mussels to oxidative damage exposure. Comp. Biochem. Physiol. C 100, 177–181.
Sanders, B.M., 1993. Stress proteins in aquatic organisms: an environmental perspective. Crit. Rev. Toxicol. 23, 49–75.
Sayeed, I., Parvez, S., Pandey, S., Bin-Hafeez, B., Haque, R., Raisuddin, S., 2003. Oxidative stress biomarkers of exposure to deltamethrin in freshwater fish, *Channa punctatus* Bloch. Ecotoxicol. Environ. Saf. 56, 295–301.
Silvestre, F., Dierick, J.F., Dumont, V., Dieu, M., Raes, M., Devos, P., 2006. Differential protein expression profiles in anterior gills of *Eriocheir sinensis* during acclimation to cadmium. Aquat. Toxicol. 76, 46–58.
Singh, N.N., Das, V.K., Srivastava, A.K., 2002. Insecticides and ionic regulation in teleosts: a review. Zool. Poloniae 47, 21–36.
Srivastav, A.K., Srivastava, S.K., Srivastav, S.K., 1997. Impact of deltamethrin on serum calcium and inorganic phosphate of freshwater catfish, Heteropneustes fossilis. Bull. Environ. Contam. Toxicol. 59, 841–846.
Smith, T.M., Stratton, G.W., 1986. Effect of synthetic pyrethroid insecticides on non-target organisms. Residue Rev. 97, 93–120.
Tu, H.T., Silvestre, F., Bernard, A., Douny, C., Phuong, N.T., Tao, C.T., Maghuin-Rogister, G., Kestemont, P., 2007. Oxidative stress response of black tiger shrimp (*Penaeus monodon*) to enrofloxacin and to culture system. Aquaculture 285, 244–248.
Ural, M.S., Saglam, N., 2005. A study on the acute toxicity of pyrethroid deltamethrin on the fry rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792). Pestic. Biochem Physiol. 83, 124–131.
Vandame, R., Meled, M., Colin, M.E., Belzunces, L.P., 1995. Alteration of the homing-flight in the honey bee *Apis mellifera* L. exposed to sublethal dose of deltamethrin. Environ. Toxicol. Chem. 14, 855–860.
Varo, I., Serrano, R., Pitarch, E., Amat, F., Lòpez, F.J., Navarro, J.C., 2002. Bioaccumulation of chlorpyrifos through an experimental food chain: study of protein HSP70 as biomarker of sublethal stress in fish. Arch. Environ. Contam. Toxicol. 42, 229–235.
Viran, R., Erkoc, F.U., Polat, H., Kocak, O., 2003. Investigation of acute toxicity of deltamethrin on guppies (*Poecilia reticulata*). Ecotoxicol. Environ. Saf. 55, 82–85.
Werner, I., Geist, J., Okihino, M., Rosenkranz, P., Hinton, D.E., 2002. Effects of dietary exposure to teh pyrethroid pesticide esfenvalerate on medake (*Oryzias lapites*). Mar. Environ. Res. 54, 609–614.
Wirth, E.F., Lund, S.A., Fulton, M.H., Scott, G.I., 2001. Determination of acute mortality in adults and sublethal embryo responses of *Palaemonetes pugio* to endosulfan and methoprene exposure. Aquat. Toxicol. 53, 9–18.
Wirth, E.F., Lund, S.A., Fulton, M.H., Scott, G.I., 2002. Reproductive alterations in adult grass shrimp, *Palaemonetes pugio* following sublethal, chronic endosulfan exposure. Aquat. Toxicol. 59, 93–99.
World Health Organisation Endosulfan (Environmental Health Criteria No. 40), 1984, Geneva http://www.inchem.org/documents/ehc/ehc/ehc40.htm.