Bull Environ Contam Toxicol (2008) 81:553–557
DOI 10.1007/s00128-008-9534-2

Biochemical Responses of Cnidarian Larvae to Mercury and Benzo(a)pyrene Exposure

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Received: 19 November 2007 / Accepted: 22 August 2008 / Published online: 27 September 2008

Abstract The biochemical responses of planulae from the coral *Porites astreoides* exposed to 10 μg/L of benzo(a)pyrene (B(a)P) and to 10 μg/L of mercury (Hg) was evaluated. The survivorship of larvae only dropped significantly after 48 h of B(a)P exposure, whereas it remained at 98% for Hg exposure and up to 96 h. Exposure to B(a)P significantly increased free thiols, and the activity of glutathione-*S*-transferase and catalase were unaltered under exposure of any of the contaminants. This study is the first contribution of the biochemical effects in cnidarian larvae exposed to contaminants.

Keywords Coral · Larvae · Metals · PAH's

Larvae have important roles in the life cycle of marine invertebrates, as during this phase there are dramatic morphological and physiological changes that lead to the adult form (Edmunds et al. 2001); and, they constitute the dispersion phase of many sessile species. Despite their importance, data concerning the toxicity of metals and organic compounds in larvae is scarce (Bellas et al. 2005), even though it is often assumed that during this phase organisms are less tolerant than adults to these contaminants.

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Biotransformation as well as antioxidant enzymes are involved in the response of organisms to contaminants. Biotransformation enzymes (phase I and II) respond to the presence of endogenous and exogenous organic compounds, while antioxidants are generalized stress enzymes that maintain the balance of free radicals in the cells. Both types of enzymes have been found in aquatic invertebrates (Livingstone 2001), and vary in concentration throughout their life cycle (e.g., Peters and Livingstone 1996). Despite these findings, the response of early life stages to contaminants is relatively unknown for many marine invertebrates.

The sensitivity of early development stages to trace metals has been frequently reported for sea urchins and bivalves (reviewed in Bellas et al. 2005). In coral larvae, the effects of metals (Reichelt-Brushett and Harrison 2000) and organic contaminants (e.g., Epstein et al. 2000) have been measured using rates of survivorship and settlement. However, adult scleractinian corals have biotransformation enzymes such as cytochrome P450 and GST (Gassman and Kennedy 1992; García et al. 2005) and antioxidative responses (Yakovleva et al. 2004; Ramos and García 2007). Thus, in this study we examined the tolerance and biochemical response of larvae of the coral *Porites astreoides* living in coastal habitats with anthropogenic development, by using short-term exposure bioassays to an organic contaminant, benzo(a)pyrene, and to an inorganic metal, mercury.

Materials and Methods

Colonies of *P. astreoides* were collected in Cayo Paiclá at National Park Morrocoy, Venezuela (10°48′83″ N, 68°16′16″ O) and transported with aeration to the laboratory within 3 h. Each pair of colonies was set in a 14 L plexiglass

aquarium (20 × 50 × 30 cm) with aeration. All planulae released the next day were collected and set in groups of 30 larvae per beaker with 0.7 L of seawater. Seawater was renewed daily and filtered through fiberglass wool, activated carbon, a phytoplankton net and Whatman no. 1 filter paper. Three beakers were used as replicates for each of the following treatments: Control (no contaminant added), Hg (10 μg Hg/L) and B(a)P (10 μg B(a)P/L), hereafter referred to as Control, Hg and B(a)P treatments. We select these concentrations as non-lethals based on previous bioassays with adult coral colonies (Bastidas and García 2004, Ramos and García 2007). To obtain these concentrations, we used a stock solution of 1,000 mg Hg^{2+}/L and one of 100 mg B(a)P/L solution, prepared from $HgCl_2$ (AnalaR®) and benzo(a)pyrene (Sigma-Aldrich), respectively. The stock solutions were administered directly to the beakers at each seawater renewal (24 h). The beakers were covered with translucent plastic to minimize water evaporation. During the bioassay the mean seawater temperature was 27.23 ± 0.52°C. Every 24 h, live planulae were counted to estimate survivorship. At the end of the bioassay (96 h), the surviving larvae were preserved with 20% glycerol in liquid nitrogen. The protein extractions were performed with a 0.05 M Tris–HCl buffer (pH 8.6), containing 0.15 M of NaCl, 0.15 M of sucrose, 1% of Triton X100, 5 mM of β-mercaptoethanol and a mixture of protease inhibitors including 1 mM of EDTA, 1 mM of phenylmethylsulfonyl fluoride, 0.1 mM of iodoacetic acid, 1 mM of benzamidine HCl, 0.1 mM of chloroquine, 1 μM of pepstatin A and 10 μM of leupeptin. The total protein concentrations were measured by Bradford (1976) technique, with bovine serum albumin used as the standard. Total thiols (Total SH) and free thiols (Free SH) were determined from the Ellman reaction (Ellman 1959) with DTNB (5,5′-dithiobis-2-nitrobenzoic acid), registering the absorbance at 412 nm and *N*-acetyl cysteine as standard. Proteic thiols (Proteic SH) were obtained by subtracting the Free SH from Total SH. The catalase enzymatic activity was measured following Aebi (1974), with 150 mM of H_2O_2 in 80 mM of potassium phosphate buffer (pH 7.0). Glutathione-*S*-transferase (GST) enzymatic activity was measured following Habig et al. (1974), with 1 mM of 1-chloro-2,4-dinitrobenzene, 1 mM of GSH in 0.1 M of potassium phosphate buffer (pH 6.5). One-way ANOVA analyses were used to evaluate differences in biochemical variables among treatments, with prior verification of the normality and homoscedasticity of the data, using SPSS 12.0.

Results and Discussion

The survivorship of *P. astreoides* larvae after 96 h exposure to inorganic Hg was significantly higher than that of larvae exposed to the same concentration of B(a)P (98% vs. 72%, G-value = 26.07 $p < 0.01$; Fig. 1). This 1.4-fold difference in survivorship was even more remarkable considering that the exposure concentration of Hg is higher when it is expressed in molar units: 0.049 μM for Hg vs. 0.039 μM for B(a)P. Also, the onset of larvae mortality due to the contaminant occurred earlier when exposed to B(a)P (48 h) compared to Hg (72 h).

The survivorship of the larvae exposed to Hg was similar to that of larvae in the control treatment. Therefore, for the larvae of *P. astreoides* from Morrocoy National Park, an exposure to 10 μg/L of Hg for 96 h had no toxic effect. In contrast, in other studies, an equal concentration of Hg was considered an average EC50 (Median Effective Concentration) for bivalve larvae (His et al. 1997) and various species of decapods had a LC50 (Median Lethal Concentration) below 74 μg/L (Mariño-Balsa et al. 2000). Also, Hg concentrations below 10 μg/L caused 50% of larval anomalies in *Mytilus edulis* and 50% mortality in larvae of *Cancer magister* (Martin et al. 1981).

Thus, *P. astreoides* larvae from Morrocoy exposed to 0.049 μM of Hg (or 10 μg Hg/L) had the largest tolerance to Hg amongst marine invertebrates reported up to now. Damiens et al. (2006) suggested that larvae born from adults living in contaminated environments could be less sensitive to pollution than those from parents of less contaminated environments. This could help to explain the higher tolerance of *P. astreoides* larvae in this study, as the parental colonies were collected from a relatively contaminated environment, where sediments had from 5.40 up to 184.60 μg Hg/kg and waters had a mean of 0.40 μg/L. Also, the incubation of *P. astreoides* larvae could contribute to a higher tolerance, given that they are more mature when released in comparison to larvae with planktonic development. However, the relative importance of different stages of life cycle and pre-exposure of organisms is unclear and could vary according to the nature of the xenobiotic (Weis 2002).

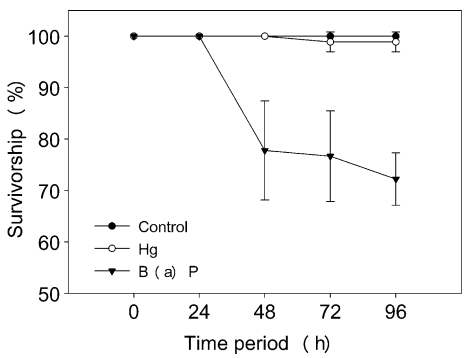

Fig. 1 Survivorship rate (%) of *P. astreoides* larvae after 96 h of exposure to Hg and B(a)P. Error bars represent standard deviation from the mean

Various components of the system of enzymatic biotransformation (such as Cytocrome P450) related to the toxicity of B(a)P have been reported for different phyla of aquatic invertebrates (Solé and Livingstone 2005) but only for adults. Also, the effect of other organic compounds such as insecticides, organometallic antifoulants and surfactants, has been studied in embryos and larvae of marine invertebrates (Bellas et al. 2005). To the best of our knowledge, there has been only one laboratory assay published with B(a)P in adult cnidaria (the anemone *Bunodosoma cavernata*; Winston et al. 1998), and the toxicity of this compound for larval stages in cnidarians has not been reported. Therefore, we were unable to compare any further our results of B(a)P exposure in the lab with larvae of other marine invertebrate species.

The total content of proteins in larvae of *P. astreoides* was similar among treatments (control: 242.96 ± 113.25, Hg: 190.73 ± 61.77 and B(a)P: 155.03 ± 52.16 µg/mL; $p = 0.452$). Also, catalase (CAT) activity was unaltered in the presence of these two contaminants compared to the control (Control: 8.26 ± 4.10; B(a)P: 7.30 ± 2.53; and Hg: 12.16 ± 4.50 µmol/min/mg protein; $p = 0.324$). This was consistent to the lack of change in CAT activity found by Quiniou et al. (2007) for larvae of *Crassostrea gigas* when comparing sites with different degrees of contamination. However, the response of CAT activity is highly variable for organic and inorganic contaminants. For example, our results contrasted with (1) a decrease in the CAT activity of the gastropod *Achatina fulica* exposed to 0.5 mg/L of Cd and 1 mg/L of Zn (Chandran et al. 2005); and (2) its increase in *Crassostrea angulata* in response to 8 µg/L Cu and 21 µg/L Zn (Funes et al. 2006).

CAT activity obtained in larvae of *P. astreoides* was 10-fold higher than in adult colonies exposed to the same concentration of Hg (1.54 ± 0.66 µmol/min/mg protein, unpublished data). A decrease of CAT activity in the course of ageing has been extensively reported for other marine invertebrates, and this change in enzymatic activity during development has been attributed to intrinsic changes in the oxygen consumption during the lifetime of organisms (e.g., Correia et al. 2003).

In *P. astreoides* larvae, GST activity was similar between Hg exposed and control larvae (control: 0.33 ± 0.15, Hg: 0.64 ± 0.43 µmol/min/mg protein, $p = 0.213$). On the other hand, with B(a)P exposure, the GST activity was only detected in one out of three replicates, each of them with 30 larvae (1.56 µmol/min/mg protein). Thus, although GST activity was 5-fold higher in that replicate than in controls, there is no statistical analysis associated to this effect of B(a)P exposure. The altered values of GST in coral larvae suggest a perturbation of the redox status induced by chemical exposure, in concordance with the increased GST activity found in other marine invertebrates in the presence of metals (Funes et al. 2006) and B(a)P (e.g., Cheung et al. 2004).

Mean activity of GST for control larvae in this study was 3–30 times higher than that reported for adult colonies of the corals of *Favia fragum* (0.015 ± 0.008 µmol/min/mg protein; Gassman and Kennedy 1992); *Siderastrea siderea* and *Montastraea faveolata* (0.011 ± 0.059 and 0.107 ± 0.535 µmol/min/mg protein respectively; Ramos and García 2007). In *P. astreoides*, mean GST activity in larvae was 3.5 times higher than in adult colonies exposed both to 10 µg Hg/L (0.64 ± 0.43 vs. 0.18 ± 0.15 µmol/min/mg protein, unpublished data). Thus, GST activity in the coral *P. astreoides* is greater in larvae than in adults; and similarly high values have been found for the larvae of *C. gigas* (Quiniou et al. 2007).

The exposure of *P. astreoides* larvae to 0.049 µM of inorganic Hg did not change significantly its thiol content (Fig. 2). Similarly, glutathione (GSH) metabolism, related to the thiol content, was unaltered with the exposure of the mussel *Mytilus galloprovincialis* to 0.2 µM of inorganic Hg (Canesi et al. 1999). However, the exposure of *P. astreoides* larvae to B(a)P increased significantly its content of Free SH (Fig. 2A). The latter is an indirect measure of GSH, which is involved in the regulation of many redox-sensitive processes (Drögue 2002). Under oxidative stress conditions, the concentration of GSH frequently increases

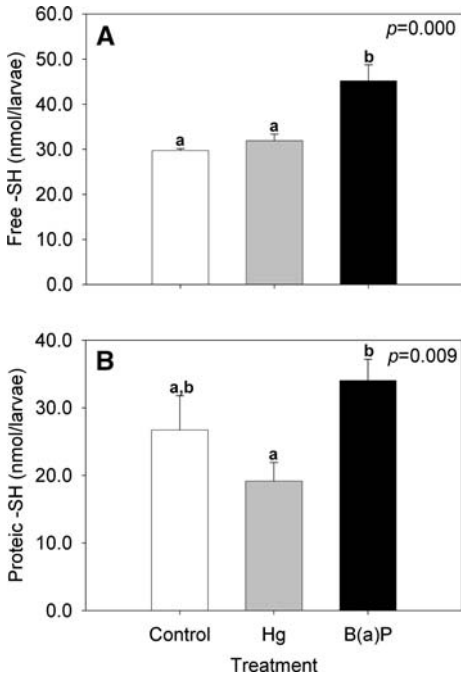

Fig. 2 Thiol concentration per larvae (nmol SH/larvae) after 96 h of exposure to Hg and B(a)P. (**A**) Free thiols (Free SH). (**B**) Proteic thiols (Proteic SH). Bars show mean and error bars represent standard deviation. *p*-value for one way ANOVA. Different letters indicate significant differences (Test LSD, $p < 0.05$)

due to its synthesis *de novo* as demonstrated in the fish *Carassius auratus* exposed to heat (e.g., Lushchak and Bagnyukova 2006). In the bivalve *Perna viridis*, exposure to B(a)P and Aroclor 1254 for 18 days, increased the GSH concentration indicating that this tripeptide is one of the first antioxidant defenses against xenobiotics (Cheung et al. 2004). Thus, the increased thiol content in larvae of *P. astreoides* suggests a response to the oxidative stress caused by exposure to B(a)P.

In this study we report for the first time the presence of biochemical markers in cnidarian larvae exposed to contaminants. Specifically, we showed both enzymatic and nonenzymatic antioxidant responses in larvae of the coral *P. astreoides*. The variability of these responses could be related to the type of contaminant, as *P. astreoides* larvae showed higher survivorship when exposed to mercury than to B(a)P. Also, we observed in these larvae (1) an increased Free SH content in response to B(a)P, and (2) higher CAT and GST activities than in adult corals. The high level of enzymatic activity found in larvae of *P. astreoides* suggests that this early life stage has defense mechanisms against organic and inorganic compounds, as adults do. However, it remains unknown how this enzymatic activity relates to the tolerance of cnidarian larvae and adults against contaminants.

Acknowledgments We would like to thank INTECMAR (Instituto de Tecnología y Ciencias Marinas, USB) for the use of its logistic facilities; M. Barbosa and A. Ostapovitch for laboratory and field assistance; and L. Márquez, C. Lodeiros, H. Guzmán and F. Osborn for critically reviewing this manuscript. This work was supported by Project No. G-2001000599 of FONACIT (Fondo Nacional de Ciencia y Tecnología). Comments from two anonymous reviewers greatly improved a previous version of this manuscript.

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