

Antioxidant responses of *Laeonereis acuta* (Polychaeta) after exposure to hydrogen peroxide

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The effects of H₂O₂ were evaluated in the estuarine worm *Laeonereis acuta* (Polychaeta, Nereididae) collected at the Patos Lagoon estuary (Southern Brazil) and maintained in the laboratory under controlled salinity (10 psu diluted seawater) and temperature (20°C). The worms were exposed to H₂O₂ (10 and 50 µM) for 4, 7, and 10 days and the following variables were determined: oxygen consumption, catalase (CAT) and glutathione peroxidase activity in both the supernatant and pellet fractions of whole body homogenates. The concentrations of non-protein sulfhydryl and lipid peroxides (LPO) were also measured. The oxygen consumption response was biphasic, decreasing after 4 days and increasing after 7 and 10 days of exposure to 50 µM H₂O₂ (P < 0.05). At the same H₂O₂ concentration, CAT activity was lower (P < 0.05) in the pellet fraction of worms exposed for 10 days compared to control. Non-protein sulfhydryl concentration and glutathione peroxidase activity were not affected by H₂O₂ exposure. After 10 days, LPO levels were higher (P < 0.05) in worms exposed to 50 µM H₂O₂ compared to control. The reduction in the antioxidant defense was paralleled by oxidative stress as indicated by higher LPO values (441% compared to control). The reduction of CAT activity in the pellet fraction may be related to protein oxidation. These results, taken together with previous findings, suggest that the worms were not able to cope with this H₂O₂ concentration.

Key words: Worms; *Laeonereis acuta*; Hydrogen peroxide; Oxidative stress; Antioxidant enzymes; Oxygen consumption

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Hydrogen peroxide (H₂O₂) is a non-radical reactive oxygen species and the most stable intermediate in the four-electron reduction of O₂ to water. In the aquatic environment, H₂O₂ predominantly derives from UV-driven photoactivation of dissolved organic matter (1). Since H₂O₂ is uncharged, it easily passes through cell membranes by diffusion, and when inside the cells it can react with transition metals liberating hydroxyl radicals (HO•) (2). At high concentrations, these radicals induce peroxidation of lipids and proteins, affecting cell integrity (2,3).

Aquatic organisms have to cope with a wide variety of

environmental pro-oxidants, as well as with those produced by normal aerobic metabolism, leading to the requirement of efficient antioxidant mechanisms. Aerobic cells have acquired a variety of antioxidant mechanisms, including enzymatic (superoxide dismutase, catalase (CAT), glutathione peroxidase (GPx)) and non-enzymatic (glutathione, carotenoids, α-tocopherol, etc.) defenses (4). The existence of antioxidant defenses and oxidative stress generation under exposure to pro-oxidants such as H₂O₂ have been reported in marine organism, with an increase in lipid peroxide (LPO) content (1), induction of antioxidant

enzymes (CAT, superoxide dismutase) (2) and a reduction in oxygen consumption (2,4,5).

Since H_2O_2 is a conspicuous environmental pro-oxidant, the aim of the present study was to evaluate the effects of H_2O_2 exposure on oxygen consumption, on H_2O_2 detoxification enzymes in the pellet and supernatant fractions, non-protein sulfhydryl groups and oxidative damage (LPO) of the estuarine worm *Laeonereis acuta* (Polychaeta, Nereididae). The estuarine worm *L. acuta* has been widely employed in toxicological and environmental studies for analysis of their antioxidant and oxidative damage responses to copper, cadmium and cyanobacterium bloom events and in biomonitoring programs (6-9).

Specimens of *L. acuta* (60-120 mg) were collected in a salt marsh ("Saco do Justino") near Rio Grande city (Southern Brazil, 32° S, 52° W). This site was reported to be unpolluted (6).

The organisms were maintained under laboratory conditions as previously described (10). Briefly, the worms were kept individually in glass dishes (6.0 cm in diameter) containing a thin sand layer and approximately 100 mL of 10 psu diluted seawater at pH 8.0, 20°C. The fixed photoperiod was a 12-h light:12-h dark cycle. During the acclimation period (5 days) the animals were fed frozen *Artemia* sp and 100% of the water was renewed every 2 days.

After the acclimation period the animals were divided into three groups: a control group exposed to diluted seawater (salinity = 10 psu; N = 60), and a second (N = 60) and a third group (N = 60) receiving diluted seawater (salinity = 10 psu) containing 10 and 50 μ M hydrogen peroxide, respectively. The condition was the same as employed in the acclimation period except for the absence of sand in dishes and for water renewal, which was done daily. No mortality was recorded during the experimental period.

At the end of the exposure period of 4, 7, and 10 days, some of the animals (N = 10 per experimental group) were used for the oxygen consumption assay and the remaining ones were frozen at -70°C for later analyses.

For the determination of oxygen consumption (11) the animals were transferred to 10-mL chambers containing 10 psu diluted seawater, pH 8.0, at 20°C. Oxygen consumption was recorded with a manual oximeter (DIGIMED, São Paulo, SP, Brazil). Values are reported as mg O_2 h⁻¹ g wet weight⁻¹.

For the enzymatic determinations (12), whole animals were homogenized with cold buffer (1:3, v/v) containing 0.5 M sucrose and 0.15 M NaCl in 20 mM Tris-HCl, pH 7.6. The homogenate was centrifuged at 500 g for 15 min at 4°C and the resulting supernatant was centrifuged at 12,000 g for 30 min at 4°C. The 12,000-g pellet (peroxisomal and

mitochondrial fraction) was resuspended with the homogenization buffer in the same volume as employed for homogenization. Both extracts (supernatant of 12,000 g and the resuspended pellet) were used for the determination of CAT and GPx activity since CAT activity is expected to occur only in the pellet fraction and GPx activity in the cytosolic fraction.

CAT activity was measured as the rate of enzymatic decomposition of H_2O_2 monitored as a decrease of absorbance at 240 nm (13). Enzyme activity is reported as CAT units, with one unit being the amount of enzyme needed to hydrolyze 1 μ mol H_2O_2 min⁻¹ mg protein⁻¹ at 30°C and pH 8.0. GPx-Se activity (14) was measured as NADPH oxidation measured at 340 nm in the presence of excess glutathione reductase, reduced glutathione, H_2O_2 , and aliquots of the homogenate. The results are reported as GPx units, with one unit being the amount of enzyme necessary to oxidize 1 μ mol NADPH min⁻¹ mg protein⁻¹ at 30°C and pH 7.2.

For the measurement of non-protein sulfhydryl groups (NP-SH) (15), tissues were homogenized (1:20) in 20 mM EDTA. NP-SH content was measured after deproteinization with 50% trichloroacetic acid. NP-SH were detected using 5,5-dithiobis(2-nitrobenzoic acid) and absorbance at 405 nm was determined in a microplate reader. The result was divided by the protein concentration of each sample prior to deproteinization and is reported as specific activity, η mol glutathione/mg protein.

LPO was measured by the Fox method (16) based on Fe^{2+} oxidation by lipid hydroperoxides (FOX reactive substances) at acid pH in the presence of the Fe^{3+} -complexing dye xylenol orange. Samples were homogenized (1:9) in 100% cold (4°C) methanol. The homogenate was centrifuged at 1000 g for 10 min at 4°C and the supernatant was collected and used for LPO determination (580 nm). Cumene hydroperoxide was employed as standard. The results are reported as η mol cumene hydroperoxide/g tissue.

Significant differences between treatments were assessed by ANOVA in combination with the Newman-Keuls *a posteriori* test, with the level of significance set at 5%.

Several studies have demonstrated that when marine invertebrates are exposed to H_2O_2 the main response observed is an increased activity of the enzymes responsible for the detoxification of H_2O_2 , i.e., CAT and/or GPx. This result was observed in shrimp (2), worms (4) and mussels (12). In contrast, *L. acuta* did not present antioxidant responses to H_2O_2 exposure (5). However, the objective of the present study was to observe the activity patterns of both enzymes in the pellet and supernatant fractions, since CAT is a well-known peroxisomal enzyme (3).

The presence of CAT activity in the supernatant fraction would be related to peroxisome damage during homogenization (12). Concerning CAT activity, no statistical difference ($P > 0.05$) was observed in the supernatant fraction (Figure 1A), whereas a lower CAT activity ($P < 0.05$) was observed in the pellet fraction of worms exposed to 50 μM H_2O_2 for 10 days (Figure 1B). The other enzyme that degrades H_2O_2 , GPx, was not affected by exposure to this oxidant either in the supernatant or in the pellet fraction ($P > 0.05$; Figure 1C and D). The other antioxidant mechanism analyzed, NP-SH content, was not affected by H_2O_2 exposure ($P > 0.05$). The mean values (\pm SEM) after 7 days of exposure were 2.5 ± 0.5 , 2.1 ± 0.7 , and 2.5 ± 0.3 nmol/mg protein for the control and 10 and 50 μM H_2O_2 groups, respectively.

The absence of the induction of CAT and GPx activity induction does not mean that the animal is vulnerable to daily variations in H_2O_2 concentration in the natural environment. It has been demonstrated that this species possesses an alternative mechanism to deal with environmental H_2O_2 since it presents a conspicuous mucus secretion that protects its body against environmental H_2O_2 because of high CAT and GPx activities (17). However, during the experimental period the dishes containing the animals were cleaned daily, with the consequent removal of this

protection.

The absence of the induction of activity of the antioxidant enzymes involved in H_2O_2 degradation was paralleled by an increase of almost 441% in LPO levels after 10 days of exposure to the higher H_2O_2 concentration (50 μM ; $P < 0.05$; Figure 2A). This result agrees with the reduction of CAT activity in the pellet fraction during the same period, suggesting oxidative damage at the protein level (18).

The oxygen consumption response was biphasic, decreasing after 4 days and increasing after 7 and 10 days of exposure to 50 μM H_2O_2 ($P < 0.05$; Figure 2B). The behavior of the first phase corroborates reports about *Nereis diversicolor* exposed for 6 h to 5 μM H_2O_2 (4), about the isolated body wall of *Arenicola maritima* (Polychaeta) exposed to 300 μM H_2O_2 (1) and about the shrimp *Crangon crangon* exposed for 5 h to 20 μM H_2O_2 (2). This decrease was related to damage to the membrane transporter mechanisms and to the consequent reduction in intracellular pH (2).

The second phase response, to our knowledge, has not been reported in literature and we consider it to be a stress response that should increase the generation of reactive oxygen species (3). This fact plus exposure to H_2O_2 would lead to the oxidative damage situation observed at this time.

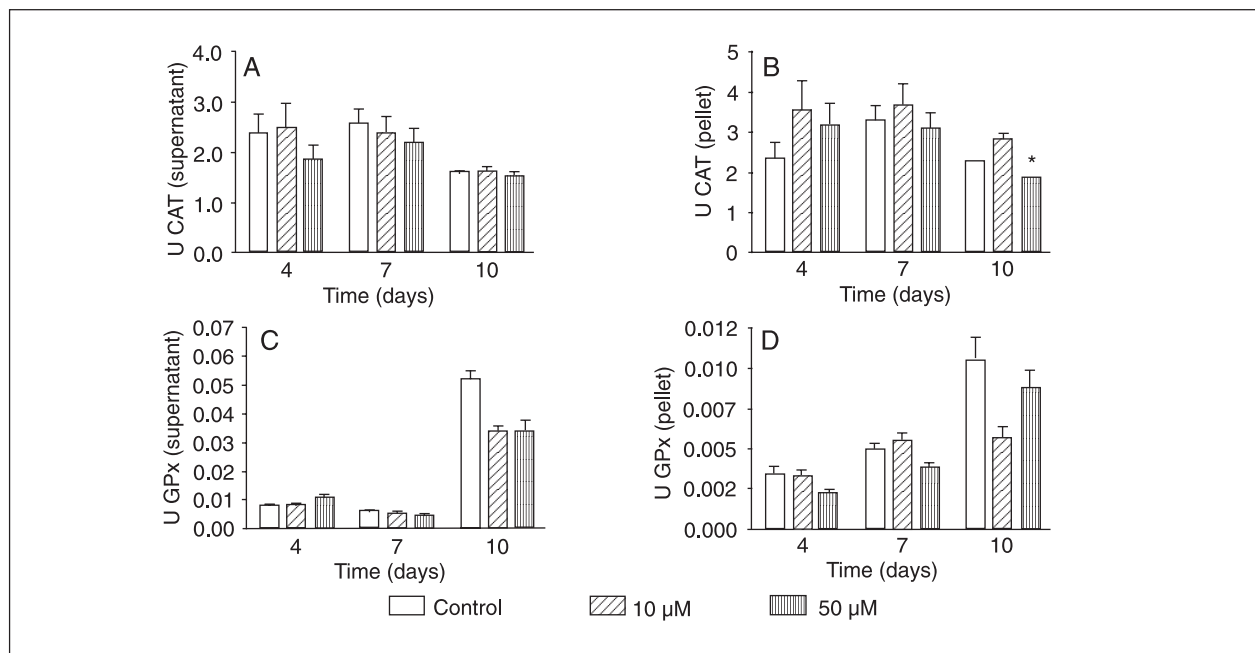


Figure 1. Catalase (CAT) activity (in units, U) in the supernatant (A) and pellet (B) fraction of *Laeonereis acuta* homogenates after exposure to 0 (control), 10, or 50 μM hydrogen peroxide. Glutathione peroxidase (GPx) activity (in units, U) in the supernatant (C) and pellet (D) fraction of *L. acuta* homogenates after exposure to 0 (control), 10, or 50 μM hydrogen peroxide. Data are reported as means \pm SD for $N = 5-9$. * $P < 0.05$ compared to control (ANOVA and Newman-Keuls test).

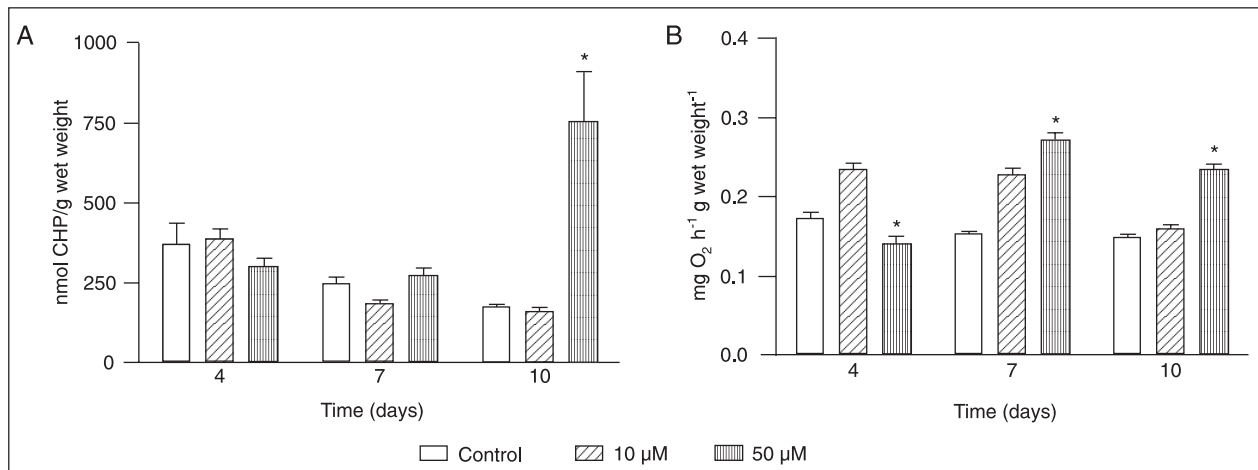


Figure 2. A, Lipid peroxide (LPO) content of *Laeonereis acuta* after exposure to 0 (control), 10, or 50 μM hydrogen peroxide. Units of LPO are in terms of cumene hydroperoxide (CHP) equivalents per gram wet weight (w/w). B, Oxygen consumption of *L. acuta* after exposure to 0 (control), 10, or 50 μM hydrogen peroxide. Units of oxygen consumption are reported as $\text{mg O}_2 \text{ h}^{-1} \text{ g wet weight}^{-1}$ (w/w). Data are reported as means \pm SEM for N = 4-8. *P < 0.05 compared to control (ANOVA and Newman-Keuls test).

After the period of exposure to both concentrations of H₂O₂, morphological alterations were observed, similar to these described in the aquatic oligochaeta *Tubifex tubifex* after 96-h exposure to copper and lead (19) and in *L. acuta* chronically exposed to copper (6). Worms exposed to H₂O₂ showed coiling and necrosis, particularly in the posterior region of their body, where the cuticle is thinner than the other parts (5), with this region being more susceptible to

the effects of H₂O₂.

The present study demonstrates that exposure to higher concentrations of H₂O₂ causes a significant alteration in the metabolism of *L. acuta*, as shown by the oxygen consumption measurements. It was also demonstrated that its antioxidant defense system was not sufficient to deal with H₂O₂ exposure, as evidenced by the oxidative damage and necrosis observed.

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