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EFFECTS OF PHYSICAL (HYPOXIA, HYPEROXIA) AND CHEMICAL (POTASSIUM DICHROMATE, FENITROTHION) STRESS ON ANTIOXIDANT ENZYME ACTIVITIES IN *CHIRONOMUS RIPARIUS* MG. (DIPTERA, CHIRONOMIDAE) LARVAE: POTENTIAL BIOMARKERS

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Abstract—Effects of physical (hypoxia, hyperoxia) and chemical (potassium dichromate, fenitrothion) stress on antioxidant enzyme activities and hemoglobin content in *Chironomus riparius* Mg. (Diptera, Chironomidae) larvae were evaluated under laboratory conditions in order to identify pertinent biomarkers of stress. Hypoxia and hyperoxia caused an increase in Cu,Zn-superoxide dismutase (SOD) and Mn-SOD activities and a simultaneous decrease in total peroxidase (Px) and glutathione peroxidase (GSH-Px) activities. A parallel increase in hemoglobin concentration in the hemolymph was observed. The exposure to sublethal concentrations of both potassium dichromate and fenitrothion led to an increase in Cu,Zn-SOD and Mn-SOD activities and to a decrease in GSH-Px activity. Activation of catalase (CAT) was observed in the larvae exposed to high fenitrothion concentration (20μg/L). The response of SODs was rapid and sensitive to low chemical concentrations, but changes in CAT, Px, and GSH-Px were more specific and less sensitive.

Keywords—*Chironomus riparius* Potassium dichromate Fenitrothion Antioxidant enzymes Biomarkers of stress

INTRODUCTION

Antioxidant enzymes such as superoxide dismutases (SODs), catalase (CAT), and glutathione peroxidase (GSH-Px) are involved in the elimination of reactive oxygen species generated by physical, chemical, or metabolic processes [1,2]. In spite of this protection, various types of intracellular damage such as DNA alteration or lipid peroxidation may occur when the prooxidant–antioxidant balance is disrupted [3]. Many environmental contaminants induce oxidative stress either directly or after bioactivation (phase I reactions) and antioxidant enzyme activities are frequently considered as pertinent biomarkers of such stress, especially in aquatic organisms [4]. Oxidative injuries may also result from a variety of environmental stressor disorders, such as reductive stress generated by xenobiotics, and exposure to O_2 at partial pressure above normoxia [5]. In freshwater ecosystems, chironomid larvae of the subfamily Chironominae are frequently exposed to nearly anoxic conditions because of their sedimentary habits. Among these, *Chironomus riparius* Mg. larvae are well adapted to low oxygen concentration because they contain large amounts of hemoglobin [6,7]. Hemoglobin is required to support aerobic metabolism under such conditions. However, autoxidation of the heme in invertebrate respiratory fluids releases superoxide radicals. These superoxide anions may react with hemoglobin either through oxidation of oxyhemoglobin to methemoglobin or through reduction of methemoglobin to oxyhemoglobin [8]. We have shown recently that enzymatic radical scavengers, including SODs, are abundant in *C. riparius* larvae, and we have made the hypothesis that these enzymes play a compensatory role relative to hemoglobin decrease, in order to maintain radical detoxification [9]. Therefore, *C. riparius* larvae constitute potential sentinel organisms for the monitoring of oxidative stress caused by either physical or chemical stress, provided that the influence of natural (i.e., physical) stressors has been evaluated.

In our study, the effects of changes in dissolved oxygen concentration in water on the antioxidant processes and hemoglobin content of larvae were analyzed. Potassium dichromate ($K_2Cr_2O_7$) and fenitrothion were used as model chemical contaminants. Toxicity of $K_2Cr_2O_7$ is due to the presence of hexavalent chromium (Cr(VI)), which exhibits a high oxidizing potential [10] and acts toxically through the formation of oxygen radicals [11] The organophosphate insecticide fenitrothion (*o*,*o*-dimethyl-*o*-nitro-*m*-tolyl phosphoro thioate) undergoes oxidation by cytochrome P450 monooxygenases [12]. This activation step may generate oxidative stress, as shown in fish for other environmental contaminants [13,14]. The corresponding oxon form (fenitrooxon) is able to irreversibly inhibit acetylcholinesterase (AChE) and may therefore seriously perturb the function of nervous system.

The present paper describes the short-term effects of physical (hypoxia, hyperoxia) and chemical stress ($K_2Cr_2O_7$ and fenitrothion) in fourth instars of *C. riparius*. A multiparametric investigation on detoxification processes involved in oxidative stress and physiologic compensation has been carried out in order to identify putative biological markers suitable for freshwater monitoring using this species, in addition to antioxidative enzymatic activities. Tissue glutathione *S*-transferases (GSTs) and AChE activities were investigated because the former is associated in insects with peroxidase activity and the latter is inhibited by organophosphates. The concentration of hemoglobin in the hemolymph was also measured in *C. riparius* larvae, because of its important role in the oxidative process.

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Table 1. Mean (±SE) values of oxygen concentration in each treatment ($n = 5$ for each treatment)

Treatment	Concentration (mg O_2/L)	Saturation (%)
Control	7.4 ± 0.7	86.7 ± 4.1
Hypoxia	1.6 ± 0.2	18.3 ± 1.7
Hyperoxia	9.3 ± 0.2	104.7 ± 1.9

MATERIALS AND METHODS

Organisms

Fourth instars were used for the present study. The *C. riparius* strain was provided by Institut National de l'Environnement Industriel et des Risques (INERIS, Verneuil-en-Halatte, France). Larvae were reared in our laboratory in aerated 25-L glass aquaria filled with dechlorinated tap water at constant temperature (20 ± 1°C) and photoperiod (14:10 h light:dark). A 5-cm-thick layer of washed siliceous sand and cellulose (Sigma S-3504, Sigma-Aldrich, Saint-Quentin-Fallavier, France) was used as sediment. Adult chironomids were retained using wood cages covered with steel wire mesh (1-mm mesh size) and reproduced continuously.

Experimental conditions

Glass tanks (20 × 15 × 20 cm) containing 2 L of dechlorinated tap water (pH: 7.68; conductivity: 550 μS/cm; total hardness: 50 mg $CaCO_3$/L) and 1 cm of sediment layer were used for all the experiments.

The effects of physical and chemical stress on antioxidant enzyme activities were assessed using groups of fourth instars collected in rearing aquaria. Larval stage was determined using head capsule size. Larvae were then randomly introduced in either control or treated tanks.

Physical stress

Control tanks (normoxic conditions) were left uncovered during the duration of the experiment to ensure natural diffusion of atmospheric oxygen in water. For hypoxia treatment, the tanks were hermetically covered with Parafilm ''M'' (Poly-Labo, Strasbourg, France). In order to increase the oxygen content of water (hyperoxia treatment), atmospheric air was continuously introduced into water using an electrically powered air pump (Rena 101, Rena, Annecy, France) fitted with silicon tubing and a ceramic diffusing device. Larvae were introduced 48 h after the beginning of the experiment and maintained under either normoxic, hypoxic, or hyperoxic condition for 24 to 96 h, depending on the experiment.

The concentration of dissolved oxygen in water was monitored in each tank on a daily basis using a WTW OXI-96 oximeter (WTW, Weilheim, Germany). Mean values of dissolved oxygen concentration are reported in Table 1.

Chemical stress

Two xenobiotics, $K_2Cr_2O_7$ and fenitrothion, were assayed to evaluate antioxidant enzymes responses to chemical stress. Potassium dichromate is used as a reference toxicant in many aquatic toxicity testing procedures [10]. In this study, $K_2Cr_2O_7$ was considered as a model stressor to assess metabolic responses of *C. riparius* larvae to an established oxidative agent (positive control). Fenitrothion generates active oxygen species during its bioactivation in addition to its ability to irreversibly inhibit AChE.

In order to evaluate the short-term (24-h) effects of $K_2Cr_2O_7$ and fenitrothion on antioxidative enzymes, sublethal exposure concentrations were selected from the results of preliminary toxicity tests [15]. For $K_2Cr_2O_7$, two concentrations corresponding to &1frac1,000; and &1frac100; of the 24-h LC50 (0.5 and 5 mg/L, respectively) were chosen. Four fenitrothion concentrations ranging between &1frac1,000; and &1frac100; of the 24-h LC50 (2, 5, 10, and 20 μg/L) were selected. The lower fenitrothion concentration (2 μg/L) was also used to assess the medium-term (96-h) effects of exposure to this compound. Water was used as a solvent for $K_2Cr_2O_7$, whereas acetone was used for fenitrothion. For each experiment, 2 ml of toxic solution was added to the experimental tanks before the introduction of larvae. Exposure was carried out under constant temperature (20 ± 1°C) and photoperiod (14:10 h light:dark). Larvae were collected daily in control and experimental tanks. Enzymatic and biochemical assays were performed as described below.

Sample preparation

Ten larvae were pooled for each set of biochemical analyses. Body fluids (i.e., hemolymph) were collected by placing cut larvae in 2-ml plastic containers filled with ice-chilled 0.6% (w/v) NaCl-distilled water solution for 5 min. Larval remains were then collected and placed immediately in liquid nitrogen. Body fluid samples were kept at −80°C until enzymatic activities and total hemoglobin content measurements. Larval remains were homogenized in 2.5 ml of Tris-EDTA buffer (40 mM, pH 7.8) using a Potter-Elvehjem homogenizer (Biollock-Scientific, Illkvich, France). Crude homogenate was then centrifuged at 500 *g* (4°C) for 15 min to remove tissue debris. The resulting supernatant was then centrifuged at 15,000 *g* (4°C) for 30 min to eliminate mitochondria. The resulting supernatant (i.e., postmitochondrial fraction) was used to measure cytosolic SOD, CAT, GSH-Px, GST, and AChE activities (postmitochondrial enzymes). After repeated washing with homogenizing buffer, the pellet was used to measure mitochondrial SOD activity.

Enzyme assays

The SOD activity was determined after hemoglobin precipitation with ethanol:dichloromethane (2:1, v/v) by the chemiluminescence method of Bensinger and Johnson [16], using an LKB Wallac type 1250 luminometer (EG2G Wallac, Turku, Finland). This method is based on the ability of SODs to inhibit oxidation of luminol after the hypoxathine–xanthine oxidase reaction (25°C, pH 7.8). The different forms of SOD were distinguished using subcellular fractionation and specific inhibitors as previously described [9]. Results were expressed in arbitrary SOD units, with one unit corresponding to the amount of enzyme producing a 50% inhibition of chemiluminescence compared to control.

Total peroxidase (Px) activity was estimated using the guaiacol test (25°C, pH 7) [17]. The CAT activity was quantified by the disappearance rate of H_2O_2 monitored at 240 nm (25°C, pH 7) [18]. The GSH-Px activity was determinated using the consecutive glutathione reductase reaction and oxidation of NADPH, with cumene hydroperoxide as substrate (25°C, pH 7.6) [19]. The GST activity was assessed spectrophotometrically through the measurement of glutathione–1-chloro-2,4-dinitrobenzene conjugate production [20]. The AChE activity was measured by the method of Ellman et al. [21], using acetylthiocholine as a substrate. Enzyme activities were related

Table 2. Effects of short-term (24-h) hypoxia, hyperoxia, and potassium dichromate exposure on various biochemical parameters (mean ± SE; $n = 5$, except for GSH-Px where $n = 3$) measured in fourth instars of *Chironomus riparius*. Enzymatic activities are expressed as unit/mg protein; hemoglobin content is expressed as mg/100 mg hemolymph protein

				Potassium dichromate	
Parameter[a]	Control	Hypoxia	Hyperoxia	0.5 mg/L	5 mg/L
Cu,Zn-SOD (e)	126.7 ± 7.2	127.4 ± 6.3	111.8 ± 1.9	118.4 ± 16.7	136.1 ± 6.8
Cu,Zn-SOD (p)	153.7 ± 8.6	299.1 ± 7.4B	259.9 ± 0.9B	219.6 ± 14.3A	227.6 ± 17.1A
Presumed Fe-SOD (p)	68.5 ± 2.2	59.8 ± 2.5A	65.2 ± 6.8	86.4 ± 5.5A	65.1 ± 10.8
Mn-SOD (m)	52.0 ± 5.3	89.4 ± 3.0B	86.0 ± 3.6B	79.1 ± 5.4A	88.0 ± 4.3A
CAT (p)	285.1 ± 16.8	317.2 ± 7.9	242.6 ± 21.6	269.4 ± 25.7	282.3 ± 48.9
GSH-Px (p)	10.7 ± 1.1	8.8 ± 2.7	7.8 ± 2.2	7.9 ± 2.0	5.2 ± 0.6A
Px (e)	1,550 ± 41	815 ± 22B	1,452 ± 103	1,435 ± 249	1,641 ± 168
GSTs (p)	40.5 ± 0.3	50.6 ± 2.0A	41.3 ± 4.6	38.5 ± 1.1	39.8 ± 11.6
AChE (p)	0.63 ± 0.02	0.64 ± 0.02	0.60 ± 0.07	0.56 ± 0.06	0.63 ± 0.04
Hemoglobin (e)	66.2 ± 8.2	94.3 ± 13A	87.4 ± 3.2A	63.7 ± 10.8	64.6 ± 6.2

[a] SOD = superoxide dismutase; e = extracellular; p = postmitochondrial; m = mitochondrial; CAT = catalase; GSH-Px = glutathione peroxidase; Px = total peroxidase; GSTs = glutathione *S*-transferases; AChE = acetylcholinesterase. A = significantly different from control, $p < 0.05$; B = significantly different from control, $p < 0.01$.

to protein concentration of the extracts measured by the dye-binding method of Bradford [22] using bovine serum albumin as a standard. Hemoglobin of hemolymph was estimated by the cyanomethemoglobin procedure (Sigma diagnostic kit, 525-A, Sigma) [23].

Chemicals

Fenitrothion and $K_2Cr_2O_7$ were obtained from Cluzeau Info Labo (Sainte-Foy-la-Grande, France) and Prolabo (Fontenay-sous-Bois, France), respectively. Biochemicals were purchased from Sigma.

Statistics

Statistical differences were checked using parametric *t* test. Correlations were calculated using Pearson's coefficient. All analysis was performed using Statview 4.02 for Macintosh computer (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Short-term stress

The values obtained for control larvae and larvae exposed to either hypoxia, hyperoxia, or $K_2Cr_2O_7$ for 24 h are indicated in Table 2. In control larvae, activities of SODs and CAT were very high. Previous experiments have shown that three types of SOD may be measured in *C. riparius* larvae: Cu,Zn-SOD in extracellular and postmitochondrial fractions, Mn-SOD in mitochondrial fraction, and putative Fe-SOD of an unclear origin (endosymbiotic or parasitic) [9]. In this study, approximately 70% of total SOD activity was due to Cu,Zn-SOD, whereas presumed Fe-SOD and Mn-SOD activities contributed to 17 and 13% of total SOD activity, respectively. Extracellular SOD activity represented 30% of total SOD activity and consisted exclusively of Cu,Zn-SOD. Selenium-dependent GSH-Px activity was not detected in larvae, suggesting that the measured GSH-Px activity was exclusively due to peroxidase activity of GSTs. More than 90% of total peroxidase activity was located in the hemolymph. Exposure to hypoxia, hyperoxia and both $K_2Cr_2O_7$ concentrations elicited a considerable increase in postmitochondrial Cu,Zn-SOD (+94.7, +69.2, +42.9, and +48.1% of control value, respectively) and Mn-SOD activity (+71.9, +65.4, +52.1, and +69.2%, respectively). Presumed Fe-SOD activity was not significantly affected by hypoxia or hyperoxia, although a slight decrease was observed when larvae were exposed to low oxygen concentrations. This activity significantly increased (+26%) after exposure to the lowest concentration of $K_2Cr_2O_7$ but not to the highest concentration.

Total Px activity was severely altered by hypoxia (−47.0% compared to control), whereas the hemoglobin content of the hemolymph substantially increased under both hypoxia (+42.4%) and under hyperoxia (+32.0%). The other parameters were not significantly modified by either short-term hypoxia or hyperoxia or $K_2Cr_2O_7$ exposure.

In order to determine whether fenitrothion may elicit a response in antioxidant enzyme activities, a more detailed study of the effects of sublethal exposure to this compound was performed (Table 3). The two highest tested concentrations (10 and 20 μg/L) were shown to prevent adult emergence in chronic toxicity tests, whereas the lowest concentrations did not [15].

Fenitrothion caused significant AChE inhibition, in a typically concentration-dependent way (Pearson's $r = -0.903$, $p < 0.05$). The amount of inhibition relative to control ranged from 28.6 to 90.4%. This insecticide also produced a stimulating effect on GST activity, which was not correlated with concentration.

Postmitochondrial Cu,Zn-SOD significantly increased in treated larvae (+86.0, +31.9, +24.4, and +24.7% of control value, for increasing concentrations of fenitrothion, respectively), but this effect was more important for the lowest exposure level (2 μg/L). Simultaneously, a significant increase in mitochondrial Mn-SOD activity (+57.7, +103.8, +67.3, and +22% of control value, respectively) was observed. Presumed Fe-SOD activity increased in exposed larvae, but difference with the activity of this enzyme in control was not always significant. The CAT and GSH-Px activities were only significantly affected by the highest fenitrothion concentration (20 μg/L). The former increased whether the latter decreased. Furthermore, a significant correlation between CAT activity and fenitrothion concentration has been demonstrated (Pearson's $r = 0.97$, $p < 0.01$).

Medium-term stress

Because the short-term effects of hyperoxia exposure were almost comparable to those obtained for hypoxia and because midge larvae undoubtedly more frequently experience hypoxic

Table 3. Effects of short-term (24-h) fenitrothion exposure on various biochemical parameters (mean ± SE; $n = 5$, except for GSH-Px where $n = 3$) measured in fourth instars of *Chironomus riparius*. Enzymatic activities are expressed as unit/mg protein; hemoglobin content is expressed as mg/100 mg hemolymph protein

	Fenitrothion (μg/L)				
Parameters[a]	0 (control)	2	5	10	20
Cu,Zn-SOD (e)	126.7 ± 7.2	149.3 ± 4.1A	122.2 ± 7.0	148.7 ± 12.0	151.9 ± 8.1
Cu,Zn-SOD (p)	153.7 ± 8.6	285.9 ± 12.7A	202.8 ± 5.7A	191.2 ± 11.9A	191.6 ± 11.1A
Presumed Fe-SOD (p)	68.5 ± 2.2	77.3 ± 4.4	94.0 ± 6.3A	78.2 ± 5.5	80.1 ± 2.3A
Mn-SOD (m)	52.0 ± 5.3	82.0 ± 3.7A	106.5 ± 2.7B	87.0 ± 5.1A	67.7 ± 3.8
CAT (p)	285.1 ± 16.8	261.4 ± 31.6	304.6 ± 12.5	338.5 ± 31.9	444 ± 13.6B
GSH-Px (p)	10.7 ± 1.1	8.5 ± 1.7	7.0 ± 0.8	6.9 ± 2.3	5.5 ± 0.7A
Px (e)	1,550 ± 41	1,711 ± 202	1,467 ± 55	1,968 ± 61A	1,468 ± 165
GST (p)	40.5 ± 0.3	56.8 ± 1.8A	57.9 ±1.2A	54.6 ± 3.7A	55.2 ± 1.3A
AChE (p)	0.63 ± 0.02	0.45 ± 0.02B	0.33 ± 0.09B	0.11 ± 0.08B	0.06 ± 0.04B
Hemoglobin (e)	66.2 ± 3.5	72.9 ± 3.7	88.3 ± 4.4A	—	89.2 ± 8.23

[a] SOD = superoxide dismutase; e = extracellular; p = postmitochondrial; m = mitochondrial; CAT = catalase; GSH-Px = glutathione peroxidase; Px = total peroxidases; GSTs = glutathione *S*-transferases; AChE = acetylcholinesterase. A = significantly different from control, $p < 0.05$; B = significantly different from control, $p < 0.01$.

conditions in the natural environment, we focused on the results obtained with larvae exposed to a low dissolved oxygen level. We also assessed the effects of a 96-h exposure to the lowest fenitrothion concentration (2 μg/L). The results of these experiments are presented in Table 4.

Extracellular Cu,Zn-SOD activity was significantly reduced after 72 h of hypoxia (30% of reduction approximately) whereas postmitochondrial Cu,Zn-SOD activity almost doubled in the first 24 h of exposure and then decreased (by approximately 30–35%). The Mn-SOD activity was significantly stimulated during the first 48 h of exposure. Then, values were no longer different from the control. The CAT and GSH-Px activities exhibited the same pattern because both activities were significantly lowered (about 40–60% reduction) as soon as 48 h after the beginning of hypoxia exposure and then remained approximately constant. Extracellular Px activity in larvae exposed to hypoxia was always one half of the value in control larvae.

The exposure to 2 μg/L of fenitrothion did not have any effect on extracellular Cu,Zn-SOD, postmitochondrial CAT, and extracellular Px enzymatic activities. However, postmitochondrial Cu,Zn-SOD and mitochondrial Mn-SOD activities were significantly higher in treated larvae at the beginning of the experiment, whereas GSH-Px presented significantly lower values in treated larvae on various occasions. Presumed Fe-SOD activity was not affected by either hypoxia or fenitrothion exposure. Unlike the activation of GST, the inhibition of AChE by fenitrothion was irreversible during the experiment period.

DISCUSSION

The ability of *C. riparius* larvae to live in a hypoxic environment is related to their high hemoglobin content. Hemoglobin synthesis is stimulated by low oxygen concentration and this phenomenon persists even under prolonged hypoxia [24]. In vertebrate red blood cells, autoxidation of oxyhemoglobin to methemoglobin increases under reduced oxygen pressure [25], and this is considered as one of the major sources of superoxide radicals and, to a lesser extent, of hydrogen peroxide [26]. Hemoglobin autoxidation and hydrogen peroxide production have been investigated in the marine worm *Arenicola marina* exposed to hypoxic conditions [27]. Hemoglobin from lower animals autoxidizes more rapidly than the corresponding mammalian proteins [28], but methemoglobin level is maintained at a low value (less than 1% of total hemoglobin) by CAT and methemoglobin reductase activities (if present) [27]. Many invertebrates possess efficient enzymatic oxygen radical scavengers (SODs, CAT, or Px) that protect them from oxidative tissue damage and SOD activities are generally higher in hemoglobin-containing animals [29]. Analysis of our data suggests that SODs are very active in *C. riparius* larvae and that hypoxia causes an increase in both hemoglobin synthesis and autoxidation, which in turn may generate superoxide anions.

Among the various forms of SOD, postmitochondrial Cu,Zn-SOD and mitochondrial Mn-SOD were significantly activated by both chemical and physical stress. Usually, Mn-SOD and Fe-SOD are not stress-inducible [2]. However, intrinsic conditions may have an effect on the metabolism of mitochondria and act directly on mitochondrial enzymes, as shown in fish red blood cells [30]. In addition, the presence of Mn-SOD in body fluids seems to enable benthic animals to survive extended periods of hypoxia [29].

Selenium-dependent GSH-Px activity is not present in *C. riparius* larvae. Insects exhibit peculiar GST that presents Se-independent GSH-Px activity, which is involved in lipid hydroperoxide reduction [2]. Conversely, CAT activity is elevated in insects, which seems necessary to compensate for peroxidase activity deficiency [31]. Steady-state accumulation of hydrogen peroxide is higher in insects than in other animals. These short-lived animals require high energy levels during embryogenesis, tissue differentiation, and metamorphosis, which causes periodic elevations in hydrogen peroxide contents in mitochondria and cytosol, because of active ATP synthesis [2]. Therefore, high levels of CAT activity are needed to protect larvae against these periodic increases in production of reactive oxygen species.

Peroxidase activity is a well-known property of hemoglobin [32]. Analysis of our data shows that under normoxic conditions, about 90% of total Px activity is located in hemolymph and that hemoglobin content and Px activity are positively correlated [9]. Thus, in these conditions Px activity is essentially hemoglobin-dependent. Surprisingly, total Px and GSH-Px activities decreased under prolonged hypoxia, whereas hemoglobin content increased. Diminution of Px activity was correlated with an inhibitory effect on CAT. Increased SOD activity can be hypothesized to generate an excessive amount

Table 4. Effects of hypoxia and fenitrothion exposure on various biochemical parameters (mean ± SE, $n = 5$) measured in fourth instars of *Chironomus riparius*. Results are expressed as percent of the corresponding control

		Exposure time (h)[c]			
Parameters[a]	Treatment[b]	24	48	72	96
Cu,Zn-SOD (e)	$O_2(-)$	100.5 ± 5.0	102.6 ± 4.9	73.7 ± 4.4A	74.7 ± 3.4A
	F	117.8 ± 3.8	81.5 ± 4.2	97.9 ± 3.2	97.1 ± 4.2
Cu,Zn-SOD (p)	$O_2(-)$	194.7 ± 9.3B	67.4 ± 14.0	65.3 ± 5.7A	73.1 ± 5.3
	F	186.1 ± 15.4A	196.8 ± 7.3A	159.3 ± 12.7	154.8 ± 11.9
Presumed Fe-SOD (p)	$O_2(-)$	87.2 ± 3.1	78.6 ± 12.3	77.8 ± 4.2	87.8 ± 3.2
	F	112.8 ± 7.3	103.6 ± 11.4	106.7 ± 5.1	118.9 ± 10.9
Mn-SOd (m)	$O_2(-)$	171.9 ± 9.8B	202.5 ± 12.6A	100.2 ± 11.1	133.3 ± 21.1
	F	157.7 ± 11.1A	158.6 ± 42.9	125.6 ± 25.8	101.5 ± 25.5
CAT (p)	$O_2(-)$	111.2 ± 3.1	59.0 ± 5.3A	58.6 ± 2.0B	53.1 ± 1.6B
	F	91.7 ± 10.2	96.6 ± 7.4	74.6 ± 14.0	99.3 ± 9.1
GSH-Px (p)	$O_2(-)$	83.0 ± 20.9	41.7 ± 7.8A	49.6 ± 1.9A	59.8 ± 10.8A
	F	79.5 ± 2.7A	53.8 ± 8.6A	97.6 ± 31.8	60.2 ± 4.4A
Px(e)	$O_2(-)$	52.6 ± 3.2B	48.15 ± 1.4B	59.8 ± 4.8A	56.8 ± 2.2A
	F	110.4 ± 13.1	110.1 ± 3.9	113.7 ± 8.4	100.4 ± 3.4
GST (p)	$O_2(-)$	124.8 ± 6.25A	85.23 ± 7.8	90.9 ± 2.8	112.6 ± 8.1
	F	140.1 ± 6.3B	91.5 ± 6.5	90.7 ± 4.2	97.8 ± 11.2
AChE (p)	$O_2(-)$	102.1 ± 4.4	100.8 ± 19.8	135.5 ± 83.8	83.5 ± 17.9
	F	72.3 ± 2.7B	76.4 ± 2.8B	71.3 ± 4.4B	79.9 ± 5.1B
Hemoglobin (e)	$O_2(-)$	142.3 ± 18.4B	155.4 ± 7.4B	151.4 ± 8.7B	149.7 ± 9.5B
	F	110.1 ± 5.6	102.7 ± 9.0	99.4 ± 10.8	94.7 ± 4.0

[a] SOD = superoxide dismutase; e = extracellular; p = postmitochondrial; m = mitochondrial; CAT = catalase; GSH-Px = glutathione peroxidase; Px = total peroxidases; GSTs = glutathione *S*-transferases; AChE = acetylcholinesterase.
[b] $O_2(-)$ = hypoxia; F = fenitrothion (2 μg/L).
[c] A = significantly different from control, $p < 0.05$; B = significantly different from control, $p < 0.01$.

of hydrogen peroxide, thus causing severe cellular damage and an inactivation of other enzymes. Similar results were found in the hemoglobin-containing marine invertebrates *Arenicola marina* and *Astarte borealis* exposed to hypoxia [27]. Xenobiotics may act directly on the redox state of iron-containing proteins such as hemoglobin. Most of the free organic radicals generated in the course of detoxification processes are electrophilic and react with nucleophilic groups of proteins. This causes various deleterious effects such as oxidative denaturation of hemoglobin [33]. Exposure to lethal and sublethal doses of fenitrothion in fifth instars of the silkworm (*Bombyx mori*) has been shown to cause the induction of a severe proteolysis in hemolymph and fat body [34]. Such a phenomenon has not been observed in chironomids. On the contrary, we have observed a slight increase of hemoglobin content in the hemolymph of larvae following intoxication by fenitrothion. This could be due to a good supply of oxygen, suggesting a primary defense system by an increase in hemoglobin synthesis.

Oxyradical formation and oxidative stress after exposure to xenobiotics frequently have been reported [35,36]. Similar phenomena were observed in *C. riparius* larvae intoxicated by either $K_2Cr_2O_7$ or fenitrothion. Hexavalent chromium is a strong oxidizer. Because of ability to induce oxidative DNA damage and of its genotoxicity, chromates and dichromates are considered to be toxic to a large variety of terrestrial and aquatic organisms [37]. The bioreduction of toxic Cr(VI) to the relatively nontoxic Cr(III) occurs by enzymatic processes as soon as it penetrates through biological membranes. Mitochondrial metabolism and membrane-bound sulfhydryl-enzyme activities are severely affected by hexavalent chromium [38]. Moreover, recent studies have suggested that reduction of Cr(VI) plays an important role in carcinogenesis in relation to free-radical production with emphasis on hydroxy-radical generation [39].

Interaction between organophosphate intoxication and oxidative stress has been poorly investigated in insects. In carp (*Cyprinus carpio* L.) and catfish (*Ictalurus nebulosus*), organophosphates produce changes characteristic of oxidative stress other than inhibitory effects on AChE [40]. In *C. riparius* larvae, we observed that exposure to fenitrothion caused an increase of Cu,Zn-SOD and Mn-SOD and, to a lesser extent, of presumed Fe-SOD activities. The CAT activity increased whereas GSH-Px activity decreased in a concentration-dependent way following 24 h of exposure to fenitrothion. The activated form of fenitrothion, fenitrooxon, is formed by oxidative desulfuration catalyzed by the cytochrome P450-dependent system [41]. With the mitochondrial respiratory chain, the microsomal cytochrome P450-dependent system is known as the main oxyradical source in the living cell [42]. Fenitrothion may consequently involve the generation of oxyradicals and thus stimulate antioxidant enzyme systems through biotransformation reactions based on cytochrome P450-dependent enzymes. Other organic pollutants such as polychlorinated biphenyls (PCBs), which are known to cause induction in cytochrome P450-dependent monooxygenase activities, simultaneously produce an alteration in the antioxidant defense of mammals. On the contrary, in aquatic organisms such as fish, hepatic P450-dependent enzyme activities seem to be increased simultaneously with elevation of antioxidant enzymes activities in liver, white muscle, or kidney [43].

In conclusion, our results show that antioxidant enzymatic systems are sensitive parameters in *C. riparius* larvae because of the particular characteristics of this organism (e.g., high

amounts of hemoglobin in the hemolymph). However, the significant and rapid response of Cu,Zn-SOD and Mn-SOD is not stressor-specific, whereas changes in CAT, Px, and GSH-Px are more specific. The in situ calibration and validation of these biochemical markers remain necessary. The use of inducible detoxification enzymes as non-stressor-specific parameters in a useful multilevel and multibiomarker approach for environmental monitoring.

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