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Hypoxia, hyperoxia and exposure to potassium dichromate or fenitrothion alter the energy metabolism in *Chironomus riparius* Mg. (Diptera: Chironomidae) larvae

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Abstract

Short-term (24 h) effects of four stressors (hypoxia, hyperoxia, potassium dichromate, fenitrothion) on the activity of the electron transport system (ETS) and total lipid, glycogen and protein contents were assessed in 4th instar larvae of *Chironomus riparius.* Hypoxia and hyperoxia caused an increase in ETS activity and protein content. Glycogen content decreased when larvae were placed under hypoxic conditions. ETS activity increased following exposure to 2 $\mu g\ l^{-1}$ of fenitrothion. It decreased in larvae exposed to 20 $\mu g\ l^{-1}$ of this insecticide. A decrease in lipid and glycogen contents was observed in larvae exposed to potassium dichromate or fenitrothion. Changes in ETS activity and lipid and glycogen contents may be related to the activation of the respiratory chain due to an increase in energy cost associated with homeostatic phenomena, such as detoxification processes. These results suggest that some parameters related to energy metabolism, such as ETS activity and lipid and glycogen contents, may be used as biomarkers of environmental disturbance in *Chironomus riparius* larvae.

Keywords: Biomarkers; *Chironomus riparius*; Electron transport system; Energy-yielding substrates; Fenitrothion; Hyperoxia; Hypoxia; Potassium dichromate; Short-term response

1. Introduction

Various stressors may initiate compensatory adjustments in the energy metabolism of an organism to maintain physiological or biochemical functions at a normal (i.e. homeostatic) level. As a consequence, consumption of energy-yielding substrates (e.g. glycogen and lipids) is frequently increased in stressed individuals (Hervant et al., 1996). Therefore, measurement of the amounts of these substrates in organisms has sometimes been proposed as a tool for environmental monitoring (Mayer et al., 1992; Peakall, 1992). The balance between respiratory energy cost and energy available for metabolism reflects the functional metabolic state of the organism. The activity of enzymes associated with the respiratory electron transport system (ETS), which is closely linked

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with oxidative phosphorylation, may be considered as a good indicator of energy consumption (Packard, 1971, 1985). The amounts of energy-yielding substrates available for metabolism may be simultaneously assessed through the measurement of tissue content of glycogen, lipids or proteins.

Midge larvae of the genus *Chironomus* are highly tolerant to many environmental stressors and are frequently able to survive and develop in polluted sediment and/or under hypoxic conditions. Considering the worldwide distribution of *Chironomus* larvae and their potential as sentinel organisms in environmental monitoring (Choi et al., 1998), information about changes in their physiological condition and energy metabolism by natural stressors (i.e. physical stress) or toxicants seems to be relevant.

This paper describes the effects of 24-h exposure to hypoxia, hyperoxia, potassium dichromate and fenitrothion on ETS activity and on the amounts of glycogen, lipids and proteins in 4th instar larvae of *Chironomus riparius*. Effects of 96-h hypoxia and fenitrothion exposure are also presented.

2. Materials and methods

2.1. Organisms

C. riparius strain was provided by INERIS (Institut National de l'Environnement Industriel et des Risques, F-60550 Verneuil-en-Halatte, France). Larvae were reared in aerated 25-l glass aquaria filled with dechlorinated tap water at constant temperature (20 ± 1°C) and light conditions (14 h day/10 h night). A 5-cm-thick layer of washed siliceous sand and cellulose (Sigma S-3504) was used as 'sediment'. This mixture was also used for exposure experiments. Adult chironomids were retained using wood cages covered with steel wire mesh (1 mm mesh size) and reproduced continuously.

2.2. Experimental conditions

Glass tanks ($20 \times 15 \times 20$ cm) containing 2 l of dechlorinated tap water and 1 cm of 'sediment' layer were used. The experiment was performed at a constant temperature (20 ± 1°C) and light conditions (14 h day/10 h night). The effects of physical and chemical stressors were assessed using groups of 4th instar larvae collected in rearing aquaria. Larval instar stage was determined using head capsule size. Larvae were then randomly introduced into control or treated aquaria.

2.3. Physical stress

Control tanks were left uncovered during the duration of the experiment to ensure normal dissolution of atmospheric oxygen in water. For hypoxia treatment, the tanks were sealed with Parafilm®. To increase the oxygen content of water for hyperoxia treatment, atmospheric air was introduced in the test tanks using an electrically powered air pump 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 conditions for 24–96 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 GmbH, Weilheim, Germany). Mean values of dissolved oxygen concentration are reported in Table 1.

2.4. Chemical stress

Potassium dichromate ($K_2Cr_2O_7$) and fenitrothion (*O*,*O*-dimethyl-*O*-nitro-*m*-tolyl phosphoro thioate) were selected as representative metal and pesticide stressors, respectively. Potassium dichromate is frequently used as a reference compound in aquatic toxicity tests (Pawlisz et al., 1997). Its toxicity is due to the presence of hexavalent chromium (CrVI) which is a common

Table 1
Mean (±S.E.) values of oxygen concentration in each treatment

Treatment	Concentration (mg O_2 l^{-1})	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

Values are expressed as mg O_2 l^{-1} and % saturation ($n = 5$ for each treatment).

environmental contaminant presenting a high oxidizing potential. The organophosphate insecticide fenitrothion acts through irreversible inhibition of acetylcholinesterase (AChE).

Intoxication levels used to evaluate the effects of sublethal concentrations of potassium dichromate and fenitrothion were determined using the results of preliminary acute toxicity tests. Water was used as a solvent for potassium dichromate whereas acetone was used for fenitrothion. Groups of 10 larvae were exposed to four concentrations of each compound whereas others were kept as control. Acute toxicity was determined after 24 h of exposure, using death of individuals as an end-point. Log-probit transformation of the data were used in order to estimate $LC_{50,\ 24\ h}$ values and the corresponding 95% C.I. values. Toxicant concentrations corresponding to either 1/1000 or 1/100 of $LC_{50,\ 24\ h}$ were subsequently used to evaluate the effects of both compounds on biochemical parameters. Depending on the experiment, larvae were collected 24–96 h after the beginning of exposure in control and experimental tanks and biochemical measurements were performed as described below.

2.5. Measurements of electron transport system (ETS) activity and lipid, protein and glycogen content

The ETS activity was estimated by the measurement of the maximum enzymatic activity involved in the respiratory processes under saturating substrate condition (Packard, 1985). For each measurement, 10 4th instar larvae were pooled. Larvae were frozen immediately in liquid nitrogen and homogenized in glycine-NaOH buffer (pH 9.4) containing 75 μM $MgSO_4$, 0.2% (v/v) Triton X-100 and 0.15% (w/v) polyvinyl pyrrolidone using a Potter–Elvehjem homogenizer. Crude homogenate was centrifuged at $500 \times g$ for 1 min (4°C) to obtain a clarified homogenate. All samples were kept at −80°C until further analysis. ETS assessment is based on the measurement of formazan production by the electron transport enzymes, with succinate, NADH and NADPH as electron donors, and the tetrazolium salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) as electron acceptor. The rate of tetrazolium reduction was determined spectrophotometrically at 490 nm, in accordance with the technique of Packard (1971) modified by Kenner and Ahmed (1975).

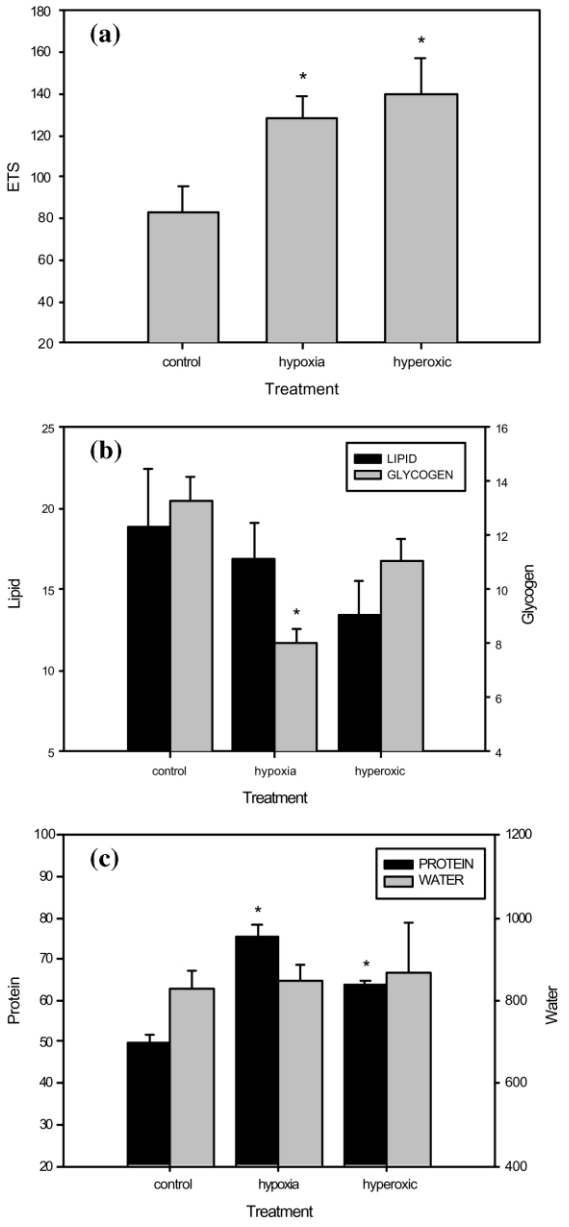

Fig. 1. Effect of 24 h hypoxia and hyperoxia on (a) ETS activity, (b) lipid and glycogen contents and (c) protein and water contents (mean ± S.E., expressed in (a) O_2 ml/h per mg protein, $n = 5$ and (b,c) mg/g fresh weight, $n = 4$) measured in 4th instar larvae of *C. riparius* (significantly different from control, $^*P < 0.05$).

For lipid, protein and glycogen content measurements, 30 4th instar larvae were pooled. Larvae were homogenized in chloroform/methanol (2:1, v/v). Remaining tissues were washed using KCl and total lipids were weighed (Folch et al., 1956). Lipid-free remains were filtered and digested in NaOH (1 mol l^{-1}) for protein and

Table 2
Effects of 96 h hypoxia on ETS activity, lipid, glycogen, protein and water contents (mean ± S.E., expressed as percent of the corresponding control, $n = 4$, except for ETS where $n = 5$) measured in 4th instar larvae of *C. riparius*

Parameters	Exposure time (h)			
	24	48	72	96
ETS	155.2 ± 13.1*	44.1 ± 4.8*	90.0 ± 14.1	74.1 ± 4.9
Lipid	81.1 ± 9.2	63.2 ± 17.4	61.2 ± 16.7	91.7 ± 10.2
Glycogen	60.1 ± 8.0*	60.7 ± 11.5	38.1 ± 5.2*	58.7 ± 2.5*
Protein	151.0 ± 3.5*	74.8 ± 1.4	62.7 ± 3.4*	89.3 ± 6.3
Water	101.8 ± 9.2	104.1 ± 10.9	94.1 ± 7.6	113.8 ± 15.6

Significantly different from control, $^*P < 0.05$.

glycogen measurements. Protein was assessed by the method of Lowry et al. (1951). Glycogen, precipitated by ethanol, was hydrolyzed by amyloglucamylase and corresponding glucose concentration was measured by the glucose oxidase technique adapted from Huggett and Nixon (1957) using ABTS as chromogen. Dry weight was evaluated after drying of larvae at 105°C for 24 h.

2.6. Chemicals

Fenitrothion and potassium dichromate were obtained from Cluzeau Info Labo (Sainte Foy la Grande, France) and Prolabo (Fontenay-sous-bois, France) respectively. Biochemicals were purchased from Sigma Chemical Company Europe (Saint Quentin Fallavier, France).

2.7. Data analysis

Statistical differences between control and stressed larvae were checked using parametric *t*-test. Correlations were calculated using Pearson's coefficient. All analyses were performed using Statview 4.02 for Macintosh computers (Abacus Concepts Inc., USA).

3. Results

3.1. Physical stress

Both hypoxia and hyperoxia caused a significant increase in ETS activity (55 and 69% as compared with the control, respectively) and protein content (51 and 28% of control value, respectively) (Fig. 1). Glycogen content in 4th instar of *C. riparius* decreased under hypoxia (40% of control value). Oxygen concentration did not have any effect on lipid and water contents.

The effects of 96-h hypoxia exposure are presented in Table 2. ETS activity increased during the first 24 h, but dropped dramatically after 48 h of exposure. Protein content showed important fluctuations with an initial increase (151.0% of control value after 24 h exposure) followed by a decrease (62.7% of control value after 72 h of exposure). Glycogen content was always lower in exposed than in control animals. No significant changes were noted in lipid content due to the high dispersion of values. No effects were demonstrated on water content.

3.2. Chemical stress

Fenitrothion was much more acutely toxic than potassium dichromate (Table 3). Concentrations for the assessment of sublethal effects were 0.5 and 5 mg l^{-1} and 2 and 20 μg l^{-1} for potassium dichromate and fenitrothion, respectively.

Acetone did not have any effect on the studied parameters. The effects of potassium dichromate and fenitrothion exposure on ETS activity and lipid, glycogen, protein and water contents are presented in Fig. 2. ETS activity increased when larvae were exposed to the lower concentration of fenitrothion (37% relative to control). Exposure to the highest concentration of fenitrothion had an opposite effect with a 20% decrease of this parameter compared to control. A decrease in lipid (52 and 35% of control, respectively) and

Table 3
Results of 24 h acute toxicity test performed on 4th instar larvae of *C. riparius*

Compound	$LC_{50,\ 24h}$ (mg l^{-1})	95% CI
Potassium dichromate	528.0	[403.0–748.0]
Fenitrothion	2.86	[1.98–3.76]

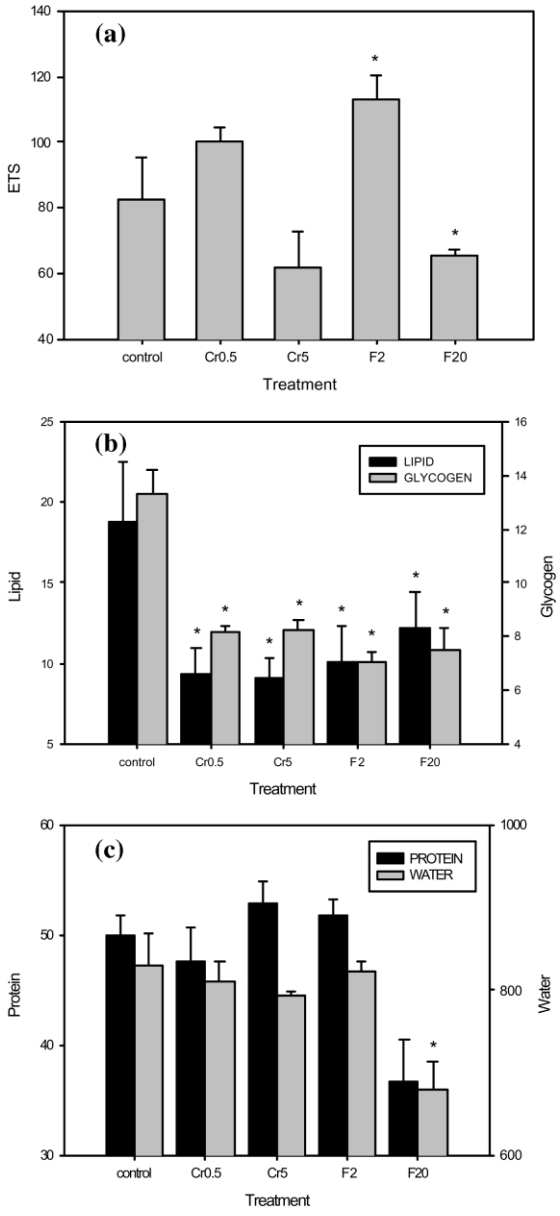

Fig. 2. Effect of 24 h potassium dichromate and fenitrothion exposure on (a) ETS activity, (b) lipid and glycogen contents and (c) protein and water contents (mean ± S.E., expressed in (a) O_2 ml/h per mg protein, $n = 5$ and (b,c) mg/g fresh weight, $n = 4$) measured in 4th instar larvae of *C. riparius* (Cr 0.5: 0.5 mg l^{-1} potassium dichromate, Cr 5: 5 mg l^{-1} potassium dichromate, F 2: 2 μg l^{-1} fenitrothion, F 20: 20 μg l^{-1} fenitrothion; significantly different from control, $^*P < 0.05$).

glycogen (38 and 47% of control, respectively) content was observed with both compounds. Water content also decreased in larvae exposed to 20 μg l^{-1} of fenitrothion (22% relative to control).

Effects of 96 h exposure to 2 μg l^{-1} of fenitrothion are presented in Table 4. ETS activity remained higher in exposed than in control larvae for 48 h and then decreased to control values. The initial (24 h) decrease in glycogen content was followed by a rapid recovery. Protein and water contents did not change during the observation period.

4. Discussion

The ability of *C. riparius* larvae to live in hypoxic or polluted environments is related to the presence of high amounts of hemoglobin in their hemolymph (Weber, 1980) and their highly efficient defense system against oxidative stress (Choi et al., 1999, 2000). Under hypoxic conditions, hemoglobin is required to support aerobic metabolism and hemoglobin synthesis is stimulated by hypoxia (Choi et al., 2000). Since hemoglobin is the most abundant protein in this animal (approx. 60% of the total protein content; Choi, 1998), the increase in protein content observed during this study for larvae placed under low oxygen conditions was probably due to hemoglobin synthesis.

However, hemoglobin is not the unique factor ensuring survival of midge larvae during severe hypoxia. It has been frequently reported that *Chironomus* larvae may switch-on partial anaerobic metabolism under low-oxygen concentration conditions (Hamburger et al., 1994, 1995, 1996; Penttinen and Holopanen, 1995). Hamburger et al. (1994) reported that anaerobic metabolism represented 3 (at 3 mg O_2 l^{-1}) to 40% (at 0.5 mg O_2 l^{-1}) of total energy production in *Chironomus* larvae. In this study, oxygen concentration in water was very low for animals placed under hypoxic conditions (1.6 ± 0.2 mg O_2 l^{-1}). The dramatic decrease of ETS activity after 48 h of exposure to hypoxia may be due to a switch from aerobic to partial anaerobic metabolism. A severe decrease in glycogen content, and no change in lipid content support this hypothesis, since glycogen is the only energy source in anaerobic metabolism. A decrease in glycogen content has already been reported in *Chironomus* larvae undergoing anaerobic metabolism under severe oxygen depletion conditions (Hamburger et al., 1994).

The increase of ETS activity after exposure to low concentrations of fenitrothion may be related to an activation of respiratory chains due to an

Table 4
Effects of 96 h fenitrothion exposure (2 μg l^{-1}) on ETS activity, lipid, glycogen, protein and water contents (mean ± S.E., expressed as percent of the corresponding control, $n = 4$, except for ETS where $n = 5$) measured in 4th instar larvae of *C. riparius*

Parameters	Exposure time (h)			
	24	48	72	96
ETS	137.2 ± 8.6*	146.7 ± 18.8*	93.5 ± 17.7	125.4 ± 40.4
Lipid	38.7 ± 0.5*	78.1 ± 4.3	56.1 ± 6.5	66.1 ± 10.8
Glycogen	52.7 ± 3.0*	112.1 ± 22.0	76.1 ± 8.3	123.3 ± 15.7
Protein	104.0 ± 2.8	103.3 ± 9.6	106.3 ± 9.0	101.9 ± 8.9
Water	99.2 ± 1.4	98.3 ± 7.8	102.3 ± 12.9	103.2 ± 8.3

Significantly different from control, $^*P < 0.05$.

increase of energy needs associated with chemical detoxification. The decrease of this parameter in individuals exposed to high concentration of fenitrothion may be the consequence of various processes, such as oxidative stress. Previous experiments have shown that glutathione peroxidase activity is significantly decreased in *C. riparius* larvae following both potassium dichromate and fenitrothion exposures (Choi et al., 2000). Since this enzyme is involved in the reduction of lipid hydroperoxide, a decrease of its activity may enhance the peroxidation of cell and organite membranes (Chiu et al., 1989; Roche and Bogé, 1993). Therefore, partial damage to the inner mitochondria membrane by lipid peroxidation may impair the function of electron transport system and reduce the value of ETS activity. Stolze and Nohl (1994) observed that xenobiotic-induced inhibition of the respiratory activity in mitochondria isolated from the heart and liver of rat was correlated with lipid peroxidation in the mitochondria, but few studies have been carried out in invertebrates.

Decrease in glycogen content after exposure to chemicals is obviously related to its consumption by processes involved in the reaction to stress. Approximately 75% of lipids in *Chironomus* larvae are non-structural components, which constitute reserves for metabolism (e.g. triglycerides; Choi, 1998). Therefore, the decrease in lipid content following exposure to chemicals may be the consequence of the use of reserve lipids as energy sources. The resulting energy may be used to meet the supplementary requirements induced by the detoxification of toxic compounds. Use of lipid and glycogen reserves during the larval stage may have consequences on further development of larvae (pupation, metamorphosis) and on adult life-history traits. For example, glycogen is considered the main source of energy during metamorphosis and non-feeding adult life stage in *Chironomus* (Hamburger et al., 1996). Changes in lipid and glycogen utilization may be derived from perturbations of the homeostatic control of metabolic and mineral constituents in stressed individuals.

ETS activity and lipid and glycogen content seem to be sensitive parameters accurately reflecting physiological state of *Chironomus* larvae and they may be useful to assess the effects of physical or chemical stress. However, this study shows that, although significant and rapid, the responses of ETS activity and glycogen content were not stressor specific whereas changes in lipid content were more specific of chemical stress. The use of such biochemical parameters as biomarkers in situ requires a better knowledge of the physiological significance of their variations following exposure to chemicals and additional information on their variations in natural environment (e.g. hypoxia) as well as on their intrinsic variability.

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