EFFECT OF POTASSIUM DICHROMATE AND FENITROTHION ON HEMOGLOBINS OF *CHIRONOMUS RIPARIUS* MG. (DIPTERA, CHIRONOMIDAE) LARVAE: POTENTIAL BIOMARKER OF ENVIRONMENTAL MONITORING

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Abstract. In an attempt to identify *Chironomus* hemoglobins as biomarkers for environmental monitoring, alterations in the hemoglobins in *Chironomus riparius Mg.* (Diptera: Chironomidae) larvae, exposed to potassium dichromate and fenitrothion, were investigated under laboratory conditions. The hemoglobins were evaluated in terms of their total contents by a cyanomethemoglobin procedure, individual components by electrophoresis of isoelectric focusing, and their oxidation by multi wavelength rapid-scanning spectrophotometry. The total hemoglobin contents increased at the high level of fenitrothion exposure. No variations in the individual hemoglobin component levels were found, by exposure to either fenitrothion or potassium dichromate. Whereas, the absorption spectra of the hemoglobins showed decreases in the peaks corresponding to the oxyhemoglobins by exposure to both compounds, but more sensitively by the chromium, which probably reflects the increase of the autoxidation of the oxyhemoglobins to methemoglobins by these compounds. These results suggest that autoxidation of the hemoglobins in *Chironomus riparius* seems to be a sensitive parameter in response to redox-active chemical exposure, and this biochemical parameter could be developed as a biomarker in environmental monitoring.

Keywords: biochemical parameter, biomarker, *Chironomus riparius*, environmental monitoring, fenitrothion, hemoglobin, potassium dichromate

1. Introduction

The aquatic larvae of non-biting midges (Chironomidae, Diptera) are widely used in fresh water environmental monitoring. They are ubiquitously distributed, sensitive to many pollutants, easy to culture and have a short life cycle (Ingersoll and Nelson, 1991), which make them suitable species for biomonitoring.

One of the main particularities of *Chironomus*, however, is the possession of hemoglobins (Hbs) during their larval stage. *Chironomus* Hbs show many interesting features, such as a high degree of polymorphism, high affinity for oxygen and an extracellular localization (Osmulski and Leyko, 1986). *Chironomus thummi thummi* larvae are reported to secret up to 16 Hbs and 12 globin polypeptides, and more than 30 cloned globin genes have been sequenced (Weber *et al.*, 1985; Green

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et al., 1998; Bergtrom, 1999; Gruhl *et al*., 2000). From an evolutionary point of view, it is generally admitted that the presence of Hbs in invertebrates reveals the adaptation of these organisms to unfavorable environmental conditions, since these pigments help to sustain aerobic metabolism under low-oxygen conditions (Weber and Vinogradov, 2001). *Chironomus* Hbs appear to fulfill clear physiological roles in transporting and storing oxygen in the larvae that burrow in polluted and hypoxic muds (Osmulski and Leyko, 1986). According to Weber (1980) and Lindegaard (1995), the extracellular Hbs enhance the good exploitation of hypoxic oxygen. Moreover, a possible, but as yet undefined, role has been proposed for *Chironomus* Hbs in the metabolism of xenobiotics in frequently polluted environments, where *Chironomus* flourish (Osmulski and Leyko, 1986; Weber and Vinogradov, 2001).

Considering the potential of *Chironomus* larvae as biomonitoring species, and the above-mentioned physiological particularities of *Chironomus* Hbs, the invertebrate respiratory pigment has a considerable potential as a sensitive biomarker in environmental monitoring. The measurement of Hbs has been employed as a biomarker of xenobiotic pollution in isolated marine fish erythrocytes (Boge and Roche, 1996; Roche and Boge, 2000), but rarely applied in invertebrates. In this study, to identify *Chironomus* Hbs as a potential biomarker of environmental monitoring, we evaluated the changes of this respiratory protein in 4th instar *Chironomus riparius Mg.* (Diptera: Chironomidae) larvae, by exposure of two chemicals with different modes of action, namely, potassium dichromate and fenitrothion. Alterations to the *Chironomus* Hbs were estimated in terms of their total contents by a cyanometHb procedure, the individual components by electrophoresis of isoelectric focusing (IEF), and their oxidation by multi wavelength rapid-scanning spectrophotometry in the 4th instar *C. riparius* larvae.

2. Materials and Methods

2.1. Organisms

The *C. riparius* larvae were obtained from adults reared in our laboratory. The original strain was given by the French institute in charge of the Industrial Risks and Protection of Environment and Health (INERIS; Institut National de l'Environnement Industriel et des Risques, 60550 Verneuil en Halatte, France). Rearing was performed in aerated 25 L glass aquaria, filled with aerated tap water. A 5cm thick artificial sediment layer, consisting of washed siliceous sand and cellulose (Sigma-Aldrich, Saint Quentin Fallavier, France), was introduced to the bottom of the aquaria. The adult midges were retained from escape using wooden cages covered with a 1mm mesh size metal net. Aquaria were placed under a constant temperature (20 °C) and photoperiod (14 h day 10 h^{-1} night). In order to obtain homogenous samples (size and age), egg masses were transferred from rearing aquaria into 2 L glass experimental tanks, and left for 24 h, with the non-hatched eggs then being removed.

2.2. EXPERIMENT CONDITION

Glass tanks (20 × 15 × 20 cm), containing 2 L of dechlorinated tap water and 1 cm of 'sediment' layer, were used for the experiments. Trials were carried out using groups of 4th instar larvae collected inform the rearing aquaria. The larval instar was determined using the head capsule size. Larvae were then randomly introduced into the experimental aquaria. The cellular and extracellular proteins were measured in the larvae during the middle of the 4th instar development, the last larval stage prior to pupation (i.e. 32 days after the eggs had hatched). Analysis of the total Hb contents during the end period of 4th instar development was conducted 32 to 38 days after the eggs had hatched. All the toxicity experiments were conducted on larvae 32 days after hatching.

The toxicity of potassium dichromate ($K_2Cr_2O_7$) is due to the presence of hexavalent chromium (Cr(VI)), which is a common environmental contaminant, with a high oxidizing potential (Eisler, 1986), and is used as a reference compound in many aquatic toxicity testing procedures (Pawlisz *et al*., 1997). Fenitrothion is an organophosphate insecticide, which acts by irreversibly inhibiting the activity of Acetylcholinesterase (Eto, 1974). Sublethal concentrations, such as $LC_{50,24h}/1000$ and $LC_{50,24h}/100$, were determined as the exposure levels, which corresponded 0.5 and 5 mg l^{-1} for potassium dichromate and 2 and 20 μg l^{-1} for fenitrothion. The selection of the experimental concentrations was based on the results of preliminary acute toxicity tests, and on the effective concentrations of the Hbs metabolism related parameters, such as antioxidant enzyme activities (Choi *et al*., 2000; 2001). Water was used as the solvent for the potassium dichromate and acetone for the fenitrothion. For each experiment, 2 ml of the test solutions were added to the experimental tanks prior to the introduction of the larvae. The exposures were carried out under a constant temperature (20 ± 1 °C) and photoperiod (14 h day 10 h^{-1} night). The larvae were collected 24 h after exposure from the control and experimental tanks, and the chemicals effects evaluated.

2.3. BIOCHEMICAL MEASUREMENT

Ten larvae from the control and experimental tanks were pooled, and hemolymphs were withdrawn by opening the body wall, and the body fluids were transferred into eppendorf cups containing ice-chilled physiological solution (NaCl 10.3 mM).

The protein contents were separately assessed, as described previously (Choi *et al*., 2001), on the hemolymphs and larval bodies for extracellular and cellular measurement. The total Hb contents of the hemolymphs were estimated by the cyanometHb procedure, with Drabkin's solution (Kampen and Zijlstra, 1961; Tentori and Salvati, 1981), using a plasma Hb kit (Sigma-Aldrich, Saint Quentin Fallavier, France).

Electrophoreses of isoelectric focusing (IEF), in nondenaturing systems, were used to characterize the *Chironomus* hemoglobin, which allowed the separation of native proteins due to their isoelectric point (pI). The IEF was performed as de-

TABLE I

The cellular and extracellular proteins, and hemoglobin contents, measured in 4th instar *Chironomus riparius* larvae (mean ± SEM; n = 10)

Fraction	Proteins (mg g^{-1} dry tissue)	Hemoglobins (mg g^{-1} dry tissue)
Cellular	116.1 ± 0.7	–
Hemolymph	178.2 ± 1.5	164.7 ± 0.8

– : not detected.

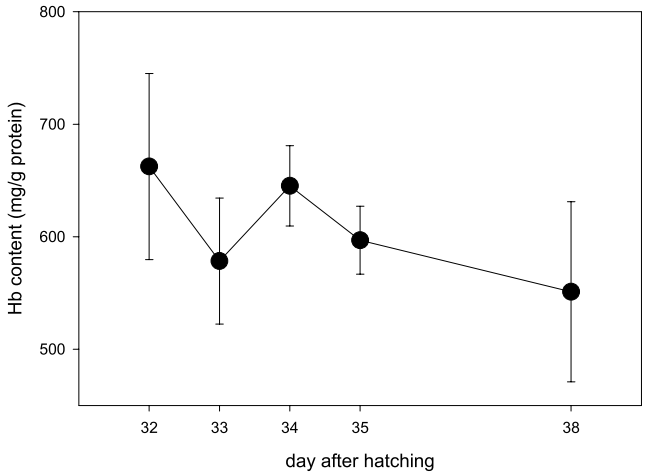

Figure 1. Hemoglobin content in the hemolymph of 4th instar *Chironomus riparius* larvae measured 32 to 38 days after the eggs had hatched (mean ± SEM; n = 5).

scribed previously (Choi, 1998). Briefly, 6% acrylamide gel was prepared, with ampholyte mix (range of pH 3.5–10; Sigma-Aldrich, Saint Quentin Fallavier, France), and a pH gradient was formed on the gel by pre migration of the gel, with ampholyte, for 15 min at 500V. The electrophoresis was performed on nondenatured hemolymph protein samples, for 2 h at 500V. After the electrophoresis, the gels were stained with silver nitrate (Sigma-Aldrich, Saint Quentin Fallavier, France), and the pI was determined from a standard curve prepared from marker proteins (range of pI 3.6–9.3; Sigma-Aldrich, Saint Quentin Fallavier, France).

100 μl of the hemolymph samples from the control and treated larvae were diluted with 500 μl of ultra pure water, and multi wavelength rapid-scanning spectrophotometry performed immediately, between 500 and 650 nm using a spectrophotometer (Uvikon 930, Kontron Instruments, Montigny-le-Bretonneux, France) to measure oxyHb.

3. Results and Discussion

3.1. TOTAL HEMOGLOBIN CONTENTS

In our laboratory experimental conditions, 4th instar development of *C. riparius* larvae persisted 22 to 38 days after the eggs had hatched (Choi, 1998), and the measurements of the cellular and extracellular proteins were conducted on the larvae in the middle of the 4th instar development (i.e. 32 days after the eggs had hatched). Table I presents, the 4th instar *C. riparius* larvae, were hemolymph protein constitutes 60% of the total protein, and the main property was proven to be Hb (about 92%). The variance of the total Hb content was investigated during the end period of the 4th instar development (i.e. 32nd to 38th days of development) in the *C. riparius* larvae (Figure 1). It has previously been reported that the Hb concentration decreases at the end of the 4th instar stage, due to a decrease in the synthesis, and the enzymatic degradation due to hormonal regulation (Vafopoulou-Mandalos and Laufer, 1984; Lindegaard, 1995; Schin *et al*., 1998). Although a slight fluctuation was observed during the measurements, the Hb contents tended to decrease by the end of the 4th instar development in our experiment (Figure 1).

The effects of the potassium dichromate and fenitrothion, on the total Hb contents, were investigated in the 4th instar *C. riparius* larvae (Figure 2). A significant increase was observed at high level of fenitrothion exposure, which seems to confirm the reports that elevated concentration of respiratory proteins confers a high tolerance to some pollutants in *Chironomus* (Lindegaard, 1995), as no severe population disturbance was observed at similar levels of exposure in our previous study (Choi, 1998). It is also known that Hb possessing fresh water macroinvertebrates (i.e. *C. thummi thummi, C. riparius, C. plumosus, Tubifex tubifex*) are usually highly tolerant to environmental pollution (Osmulski and Leyko, 1986). A good supply of oxygen may help in the active and rapid removal of toxic compounds, by accelerating metabolic reactions. Our results for the increased Hb contents, using an organic pollutant (fenitrothion), not a metallic compound (potassium dichromate), could contribute to support this hypothesis, as fenitrothion exerts its toxicity through metabolic activation (i.e. *via* Cytochrome P450).

3.2. HEMOGLOBIN MULTIPLICITY

Although, Hbs multiplicity is a well-known phenomenon in Chironomid species (Weber *et al*., 1985; Green *et al*., 1998, Bergtrom, 1999, Gruhl *et al*., 2000), few have been reported in *C. riparius*. In this study, before identifying the pollutants effects, the Hb multiplicity in 4th instar *C. riparius* larvae and preliminarily characterization of 7 different isotypes were examined by their pI (Figure 3). The electrophoresis of IEF revealed the existence of 7 hemolymph proteins, and their properties were presumed to be those of Hb, as the red color was visible on the bands of the gel prior to the staining procedure (data not shown). Six bands were

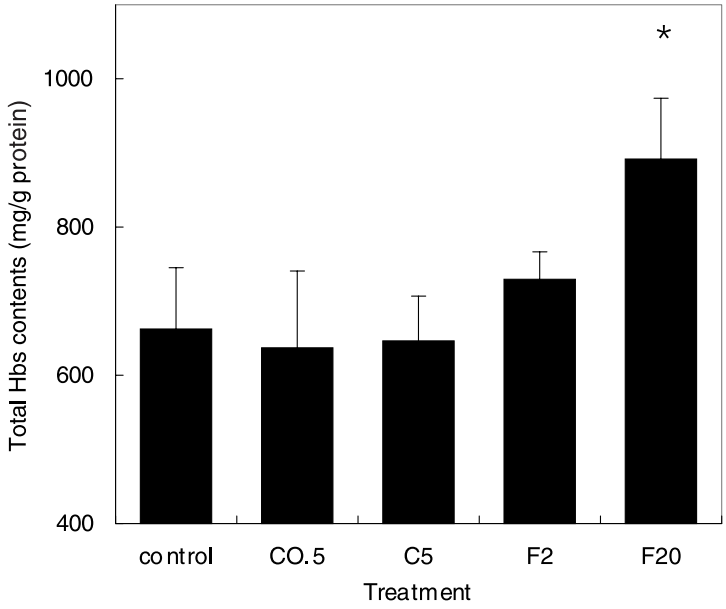

Figure 2. Hemoglobin contents in the hemolymph of 4th instar *Chironomus riparius* larvae measured 24 h after exposure to 0.5 and 5 mg l^{-1} of potassium dichromate and 2 and 20 μg l^{-1} of fenitrothion (mean $\pm$ SEM; n = 5). C0.5 = potassium dichromate 0.5 mg l^{-1}; C5 = potassium dichromate 5 mg l^{-1}; F2 = fenitrothion 2 μg l^{-1}; F20 = fenitrothion 20 μg l^{-1}. * = significantly different from control (t-test: $p < 0.05$).

found in the pI range between 5.7 and 7.1, and one protein band of pI 8.6 was also found.

It has been reported that Hb multiplicity might contribute to their flexibility toward various intrinsic or extrinsic environmental conditions (Osmulski and Leyko, 1986, 1991; Gruhl *et al*., 1997), however, in this study no toxicant induced variations were found on the individual Hb components. More sophisticated molecular techniques (i.e. western blot analysis) may be needed to elucidate the changes in the Hbs multiplicity in response to toxicants (i.e. appearance or disappearance of specific Hbs bands), and is ongoing work in our laboratory.

3.3. Hemoglobin oxidation

The absorption spectra indicated that both chemicals tested induced a decrease in the peaks corresponding to oxyHb (i.e. 542 and on 576 nm) in 4th instar *C. riparius* larvae (Figure 4). These decreases were observed in a concentration-dependant manner for potassium dichromate exposure, whereas they were only observed with high concentration of fenitrothion. The decrease in the oxyHb probably reflects the increased autoxidation of the oxyHb to metHb caused by these compounds.

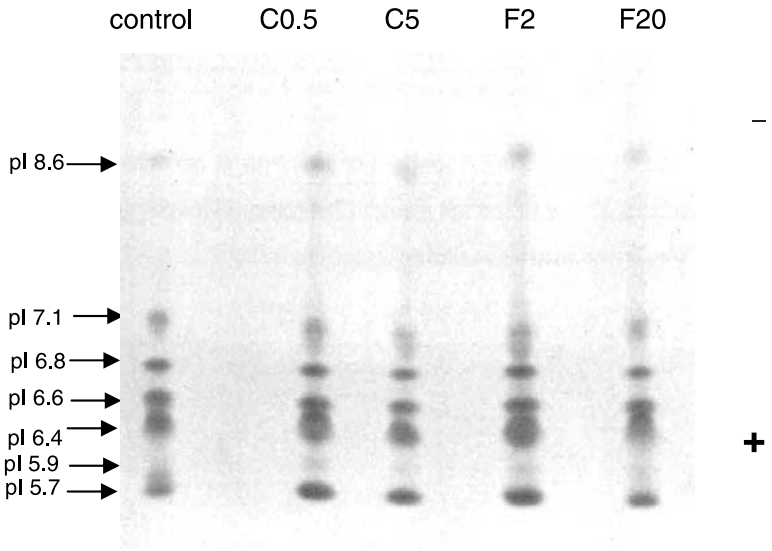

Figure 3. IEF electrophoresis of hemoglobin in 4th instar *Chironomus riparius* larvae measured 24 h after exposure to 0.5 and 5 mg l^{-1} of potassium dichromate and 2 and 20 μg l^{-1} of fenitrothion. C0.5 = potassium dichromate 0.5 mg l^{-1}; C5 = potassium dichromate 5 mg l^{-1}; F2 = fenitrothion 2 μg l^{-1}; F20 = fenitrothion 20 μg l^{-1}.

Hemoglobin reacts with a wide variety of redox active compounds, and numerous studies have suggested that environmental pollutants can give rise to Hb oxidation (Medeiros, 1983; Winterbourn, 1985). Autoxidation of oxyHb to metHb is considered one of the major sources of oxyradicals (Misra and Fridovich, 1972, Weber *et al.*, 1973, Abele-Oeschger and Oeschger, 1995). Hb, from lower animals, is especially known to be auto-oxidized more rapidly than the corresponding mammalian proteins (Abele-Oeschger and Oeschger, 1995).

The presence of highly active respiratory pigments implies that these organisms possess efficient antioxidant enzymatic systems. Our previous study showed that *C. riparius* larvae survived in severely stressed conditions, with little disturbance to the population level (Choi *et al.*, 1998; 2002). Such a tolerance suggests these animals possess efficient protection equipment at biochemical or physiological levels, which was elucidated in our previous studies (Choi *et al.*, 2000, 2001). The rapid decrease of oxyHb, observed in Figure 4, could be related to our previous result of the superoxide dismutase (SOD) activation caused by chromium and fenitrothion (Choi *et al.*, 2000), as the increased SOD activity, following exposure to these chemicals, might be partially due to the extra production of superoxide anion during the autoxidation of Hb (Abele-Oeschger and Oeschger, 1995). Hb autoxidation seems to reflect chemically induced oxidative stress in *C. riparius*. Therefore, along with the other oxidative stress markers, identified previously (i.e.

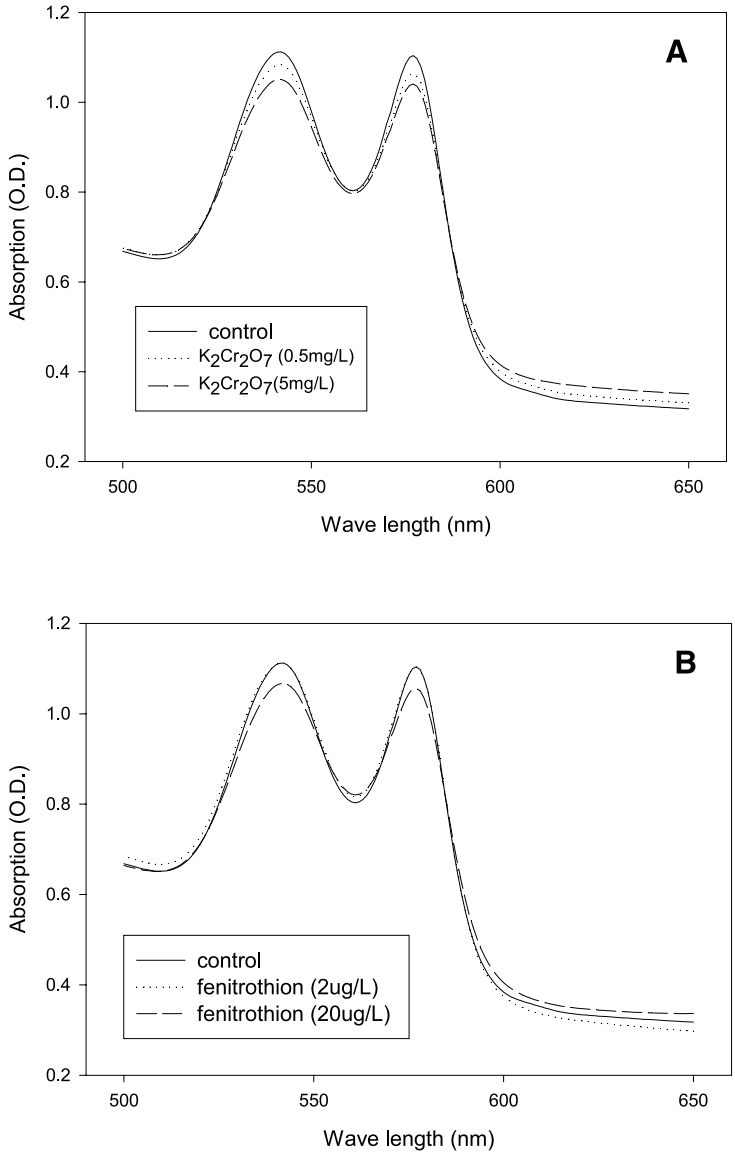

Figure 4. Absorption spectrum of oxyhemoglobin of 4th instar *Chironomus riparius* larvae measured 24 h after exposure to 0.5 and 5 mg l^{-1} of potassium dichromate (A) and 2 and 20 μg l^{-1} of fenitrothion (B).

antioxidant enzymes), oxyHb could be developed as a biomarker for the exposure to chemicals that exert their toxicity through oxidative stress, such as chromium.

4. Conclusion

The total Hb contents, the individual Hb components and oxyHb were assessed in *C. riparius* larvae exposed to low levels of potassium dichromate and fenitrothion. The present study suggests that among the Hb parameters tested in *C. riparius*, autoxidation could be a more sensitive biomarker of chromium exposure than the total contents or the individual components. Whereas, the total contents could be identified as a biomarker of fenitrothion exposure, however, for this type of contamination, the Hb parameters do not seem to be as sensitive as the other biomarkers previously identified (i.e. SOD, AChE, GSTs). In conclusion, the Hb parameters in *C. riparius* have proved to be potential biomarker in environmental monitoring, especially for contamination by redox-active chemicals.

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