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Biomarkers in Caddisfly Larvae of the Species *Hydropsyche pellucidula* (Curtis, 1834) (Trichoptera: Hydropsychidae) Measured in Natural Populations and after Short Term Exposure to Fenitrothion

E. Berra,[1] M. Forcella,[1] R. Giacchini,[1] B. Rossaro,[2] P. Parenti[1]

[1] Department of Environmental Sciences, University of Milano-Bicocca, Piazza della Scienza 1, 20126 Milano, Italy
[2] Department of Biology, University of Milano, via Celoria 26, 20133 Milano, Italy

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Several biochemical biomarkers are currently used as a tool to identify changes at the subcellular level induced by pollutants before major effects are evident at a higher level of biological organization (McCarthy and Shugart 1990). In freshwater ecosystems, macroinvertebrates represent good biological indicators for toxicant contamination since they are strictly associated with sediments. Changes in enzyme activities in freshwater invertebrates after exposure to xenobiotics have been reported by several authors (for a review see Hyne and Maher, 2003). Several pesticides lack target specificity and cause long lasting population effects on aquatic invertebrates. At the subcellular level, their effects have been investigated by measuring, in most instances, enzyme biomarkers such as acetylcholinesterase and glutathione-S-transferase. A number of species have been selected for these studies, including *Chironomus riparius, Gammarus pulex,* and *Daphnia magna* (Callaghan et al. 2001; Crane et al. 2003; Kheir et al 2001; McLoughlin et al. 2000; Barata et al. 2004).

Recently, caddisflies (Trichoptera) have been added as valuable candidate for evaluating water quality of European rivers (Stuijfzand et al. 1999). The larvae of caddisflies represent one of the largest and most ecologically diverse groups of aquatic insects (Mackay and Wiggins 1979). They are important decomposers of organic matter and are a food source for fish and birds. Several laboratory studies have been undertaken to study sublethal effects of trace metals and organic contaminants (Balch et al. 2000; Schultz and Liess 2000). However, the potential use of enzyme biomarkers to monitor pesticide contamination has been scarcely exploited in Trichoptera. The aim of this work is to present data on a series of enzymes and metabolites measured in caddisfly larvae exposed to sublethal doses of the organophosphate (OP) fenitrothion and to compare them with activities displayed in naturally-occurring individuals.

MATERIALS AND METHODS

Caddisfly larvae (4th instar) belonging to the species *Hydropsyche pellucidula* (Curtis 1834) were collected during a campaign in January and February 2004 from three sites of the Ticino River, a left tributary of the Po River, northern Italy. The sites are close to its outlet from Maggiore Lake close to the localities of

Correspondence to: P. Parenti

Turbigo (station 1; 50°41'N;14°77'E), Oleggio (station 2; 50°05'N;14°73'E), and Castelletto-Cuggiono (station 3; 50°37'N;14°74'E). The Ticino River has a length of 248 km and a drainage basin of 6599 km^2. Caddisfly larvae were collected with a net from overturned rocks and placed in a portable freezer, maintained at 5°C, for transportation to the laboratory. There was no mortality of larvae due to sampling and transport.

Enzyme assays were performed as follows. For each set of analyses, 0.5-3.0 g of larvae (10-30 individuals) of similar size were gathered, suspended and homogenized with 9 volumes of Hepes-Tris 10 mM, pH 7.5, containing 50 mM mannitol and 1 mM dithiothreitol. The suspension was then filtered through two layers of surgical gauze. Crude homogenate was centrifuged at 15,000*g* at 4 °C for 30 min. The resulting supernatant was used to measure enzyme activities. For the larval acetyl-cholinesterase (AChE) assay, the homogenization buffer contained 1% Triton X-100. Lactate dehydrogenase (LDH, EC 1.1.1.27), glutamate-oxalacetate transaminase (GOT, EC 2.6.1.1), glutamate-pyruvate transaminase (GPT, EC 2.6.1.2), catalase (CAT, EC 1.11.1.6), glutathione-S-transferase (GST, EC 2.5.1.18) and AChE (EC 3.1.1.7) were assayed using well-established protocols as reported by Berra et al. (2004). *p*-nitrophenylacetate esterase (PNPAE, EC 3.1.1.2) was assayed according to Karoly et al. (1996); α-naphtylacetate esterase (NAE) (EC 3.1.1.1) according to Zhu and He (2000), phosphotriesterase (PTE) (E.C. 3.1.8.1) according to Guedes et al. (1997), using 1 mM paraoxon as the substrate, trehalase according to Wegener et al (2003) and cellulase (EC 3.2.1.4) was assayed at pH 8.8, with 5.4 mM NaCl, 10.8 mM $CaCl_2$ and 1 mM 4-nitrophenyl-β-cellobioside as the substrate. Assays were performed with 3 replicates at 30°C using 10-40 µl of sample in 1-ml cuvette test and a Cary3 Spectrophotometer. Enzyme activities were analyzed by Cary Win UV application software for Windows 2000, expressed as international units and referred to protein concentration as determined by the method of Bradford (1976). For metabolite analyses 2-6 larvae were used. They were immediately removed from the medium and dropped in liquid nitrogen. Frozen animals were homogenized in the presence of 6% perchloric acid (0.3 ml/mg larvae), and then centrifuged at 3,000*g* for 15 min. Supernatant was neutralized with 2.5 M K_2CO_3 (about 60 µl/ml), the pH checked with 1 µl drop on litmus paper, and then centrifuged again to remove the potassium perchlorate. The resulting supernatant (about 500 µl/mg larvae) was used for determination of metabolites. Concentrations of pyruvic acid, L-lactic acid, malic acid, L-alanine, glucose, ATP, and glucose-6-phosphate (G6P) were assayed spectrophotometrically using standard enzymatic tests based on NADH oxidation or formation. In each assay 20-50 µl of supernatant were used.

Exposure was performed by incubating 15-20 larvae in 500 ml of dechlorinated water containing 0, 0.1, and 1 mg/L fenitrothion for 24h at 18°C. Fenitrothion was added from a concentrated stock solution prepared in acetone.

Regression analyses with fenitrothion concentration as the independent variable and enzyme activity as the dependent variable were performed using Matlab 7 ® statistical toolbox. The standard error of the regression coefficient b was computed according to Davies (1971, p. 92); the ratio of the slope b to its standard error is

distributed as Student's t with n-2 degrees of freedom, were n is the number of observations.

RESULTS AND DISCUSSION

Table 1 shows the activity of the enzymes which have been measured as potential biomarkers in *H. pellucidula* larvae collected at three stations along the Ticino River. Results evidenced a degree of variability among stations, which are here regarded as uncontaminated sites as judged by low levels of phosphates and nitrates (<36μg/L and <1.7 mg/L, respectively). Major variation concerned GST, AChE, LDH, transaminases and cellulase activities. Some of them might be related to different feeding conditions and energy source utilization, such as LDH, transaminases and cellulase. The activity of AChE can be inhibited by several kinds of substances, including OPs, carbamates, and other xenobiotics, even though it has been evidenced that fluctuation in the activity values should be treated with caution because natural variability can occur even in the absence of toxicants (Olsen et al. 2001). We decided to repeat enzyme activity measurements on a group of larvae from station 3, which were maintained in the laboratory in a starved condition for 24 h at 18°C in well aerated vessels. Data reported in Table 1 (column "laboratory") show a significant decrease of transaminases, cellulase, and LDH activities when compared with specimens collected at all three stations. This dissimilarity could be related to differences in metabolic status of the larvae possibly related to different food availability between those in the laboratory and the freshly collected larvae. By contrast the decrease in catalase activity could be associated to a decrease of environmental factors inducing oxidative stresses. However, as catalase is localized in peroxisomes and is involved in fatty acid metabolism, changes in its activity might be difficult to interpret (Stegeman et al. 1992). Larvae reared in the laboratory displayed higher GST activity when compared with specimens collected at station 3, but the value fell in the range of variability observed among sites. AChE was significantly higher in laboratory larvae than in those from all three stations.

Next caddisflies collected from station 3 were exposed for 24 h at 18°C to two sublethal dosages of the OP insecticide fenitrothion, namely 0.1 and 1 mg/L. The *H. pellucidula* larvae remained active all along the treatment and no gross differences were observed in any of the groups. The survival rates were 100% in all treatments. A series of enzyme activities and metabolite concentrations were determined. Enzyme measurements are reported in Table 2 and in Figure 1. As shown, the enzyme pattern was significantly altered between control and exposed larvae and both target and non-target enzymes were affected by the insecticide. The most striking effect was the strong inhibition of several esterase activities, including AChE, PNPAE and NAE. These last two activities are commonly referred to in the literature as carboxylesterases (CbE). The inhibition of AChE by OPs is well known in many invertebrates and vertebrates. Less information is available for the role of CbE in freshwater species. Both PNPAE and NAE belong to the group of B-esterases, which are readily inhibited by OPs. CbE, however, have a limited ability to hydrolyze the insecticide esters and it has been proposed that they are involved in insecticide resistance primarily by insecticide

sequestration through its binding (Zhu and He 2000). As a matter of fact CbE have higher affinity for OPs than AChE and hence could afford substantial protection from the oxons as they are generated within the organism (Barata et al., 2004). By contrast PTE is a type A-esterase and it is not inhibited by OP compounds (Aldridge 1993), as confirmed in the present study. Cellulase is present in caddisfly larvae (Martin 1981) and it has been proposed as a potential biomarker (Hyne and Maher 2003). However, no effect was observed on this enzyme.

Table 1. Enzyme activities in larvae of *H. pellucidula* from the Ticino River

Enzyme	Station 1	Station 2	Station 3	Laboratory
CAT	20.8 ± 0.3	21.9 ± 1.4	22.2 ± 1.0	13.7 ± 1.1*
GST	117 ± 1	169 ± 15	90 ± 8	144 ± 13*
AChE	43.0 ± 2.5	69.5 ± 4.0	85.5 ± 3.0	106 ± 5*
PNPAE	82.0 ± 6.0	85.0 ± 8.0	81.5 ± 3.5	75 ± 2
PTE	0.80 ± 0.01	0.75 ± 0.01	0.80 ± 0.05	1.0 ± 0.1
GOT	330 ± 5	420 ± 11	331 ± 5	242 ± 5*
GPT	27.0 ± 0.5	33.5 ± 0.5	27.0 ± 0.9	15.0 ± 0.5*
LDH	15.6 ± 0.8	29.5 ± 0.2	20.0 ± 0.1	8.2 ± 0.6*
Cellulase	3.55 ± 0.04	1.85 ± 0.01	2.90 ± 0.01	1.70 ± 0.01*

Data are expressed in mU/mg protein, except catalase which is in $min^{-1}\mu g^{-1}$;
* Significantly different ($p < 0.05$, *t* test) from control (Station 3)

Table 2. Effect of fenitrothion on larvae of *H. pellucidula* from Ticino River.

Enzyme	Control	Fenitrothion 0.1 mg/ml	Fenitrothion 1 mg/ml
CAT	17.1 ± 1.1	20.1 ± 2.2	16.5 ± 1.31
GST	151 ± 3	212 ± 19**	210 ± 9***
AChE	70.0 ± 1.0	5.0 ± 0.3***	2.0 ± 0.2***
PNPAE	75 ± 2	28 ± 1***	17 ± 1***
NAE	92 ± 1	23 ± 0.5***	16 ± 0.1***
PTE	1.0 ± 0.1	0.8 ± 0.1	1.1 ± 0.1
GOT	359 ± 12	422 ± 9***	347 ± 9
GPT	24.0 ± 1.5	23.0 ± 0.3	25.0 ± 0.8
LDH	13.0 ± 0.5	14.0 ± 0.2**	15.0 ± 0.2***
Trehalase	0.28 ± 0.01	0.14 ± 0.08*	0.13 ± 0.01**
Cellulase	2.5 ± 0.1	2.1 ± 0.2	2.9 ± 0.3

Data are expressed as in Table 1. *p<0.05; **p<0.02; ***p<0.01, where p are the probabilities that treatment means differ from control

The decrease in esterase activities was accompanied by a significant increase in GST activity. GSTs catalyze the conjugation of reduced glutathione with a number of molecules having electrophylic sites. They are known to be important in the detoxification of OPs and can be induced by them (Callaghan et al., 2001). AChE, PNPAE, NAE and GST activities were significantly affected by an increase in concentration of fenitrothion up to 1 mgl^{-1} in caddisfly larvae (Figure 1).

Moreover, these enzyme activities in exposed larvae appear outside the range of variability measured in natural populations (Table 1).

Table 3. Metabolite concentrations in larvae of *H. pellucidula* from Ticino River.

Metabolite	Station 2	Station 3	Control	Fenitrothion 0.1 mg/l	Fenitrothion 1 mg/l
Lactate	1.48 ± 0.07	1.70 ± 0.04	1.62 ± 0.02	1.20 ± 0.07**	1.38 ± 0.10*
Malate	0.30 ± 0.03	0.23 ± 0.02	0.53 ± 0.01	0.91 ± 0.04**	0.78 ± 0.01**
Pyruvate	0.20 ± 0.01	0.25 ± 0.02	0.29 ± 0.01	0.29 ± 0.02	0.28 ± 0.04
Alanine	1.3 ± 0.1	0.97 ± 0.01	0.97 ± 0.02	2.16 ± 0.17**	4.72 ± 0.11**
Glucose	0.27 ± 0.01	0.37 ± 0.02	0.30 ± 0.02	0.09 ± 0.01**	0.20 ± 0.01**
G6P	0.24 ± 0.03	0.32 ± 0.02	0.30 ± 0.03	0.08 ± 0.01**	0.13 ± 0.01**
ATP	0.23 ± 0.04	0.29 ± 0.02	0.23 ± 0.02	0.23 ± 0.02	0.68 ± 0.02**

Data are in μmol/g wet weight. *$p<0.05$; **$p<0.02$, where p are the probabilities that treatment means differ from control. Larvae were collected at stations 2 and 3 or exposed to fenitrothion after one day of acclimation in laboratory.

Although the primary site of an OP is the nervous system, many biochemical changes have been reported to occur outside this system (Nath et al. 1997; Nath 2000). Our data show some metabolic disorder induced by OP in caddisflies that could be related to an increase of glucose metabolism induced by oxidative stress after fenitrothion exposure. This situation has been described in silkworm larvae (Nath 2000). In particular, GOT was increased in larvae exposed to 0.1 mg/L fenitrothion, an effect interpreted as due to protein mobilization (Nath et al. 1997). GPT, however, was unaffected, and both transaminases display values within the range of natural variability. Therefore their involvement needs further study. A slight but significant increase in LDH activity suggests an increase in anaerobic metabolism of carbohydrate sources. The significant decrease of trehalase activity seems to contradict this conclusion. However, trehalose has been recently claimed to protect protein integrity against a variety of environmental stresses (Chen and Haddad 2004). Reduction of trehalase activity would lead to reduction of trehalose utilization as a protein stabilizer during OP exposure.

Metabolite analysis confirmed changes in metabolic pathways. The concentration of malate and alanine dramatically increased in exposed larvae. This could be due, in part, to an increase in protein mobilization, in agreement with an increase in transaminase activity. Contemporarily glucose metabolism increased, as determined by a decrease in glucose concentration and increase in ATP concentration, and the pyruvate produced by glycolysis was primarily converted to alanine instead of lactate. However, in order to reoxidize NADH of glycolytic origin pyruvate need to be transformed in lactate. Malate increased its concentration via Krebs cycle due to the formation of α-ketoglutarate as a consequence of GPT activity. Malate may serve as a source of reducing power (NADPH) through the activity of malic enzyme, which is relatively high in Hydropsychidae compared to other freshwater larvae (Berra et al. 2004).

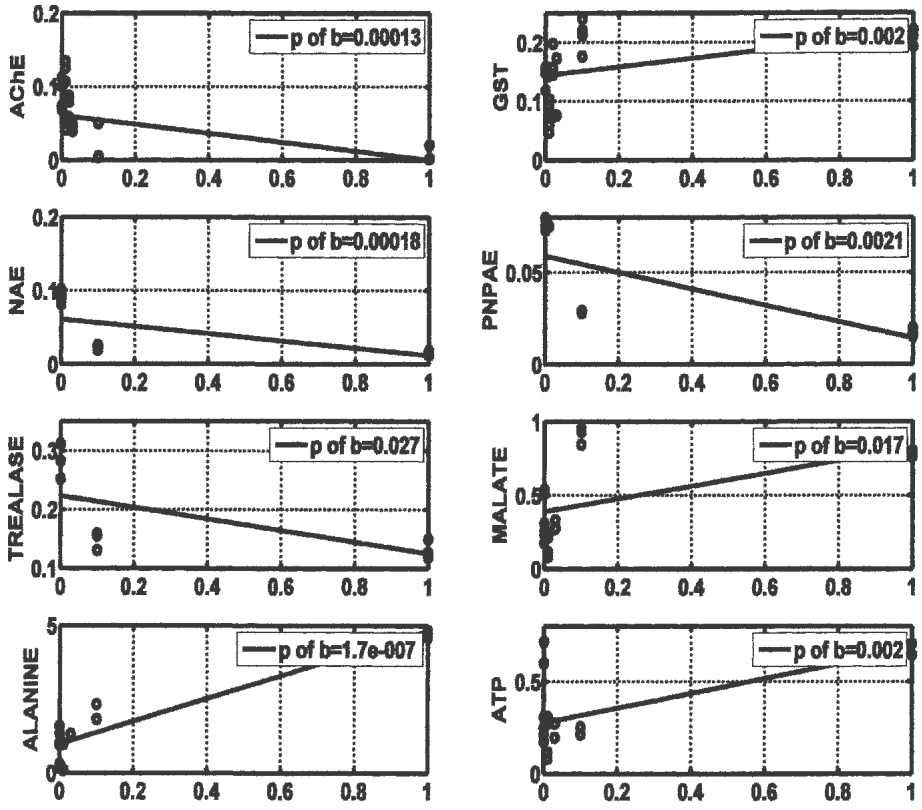

Figure 1. Response of different enzymes and substrates to fenitrothion concentration; p of b: probability that the true regression coefficient estimated by b (slope) is 0. Among the biomarkers tested only those having a significant correlation are reported.

Caddisflies represent a good candidate to monitor OP exposure, because they are widespread and abundant in freshwaters, have a relatively large body size (1-3 cm body length, dry tissue weight 30-100 mg) and significantly respond to OP. The results demonstrate the interplay of specific and nonspecific metabolic effects by OP in a freshwater species and support the importance of using a suite of biomarker responses to describe a particular pollution scenario. In particular three biomarkers appear to be most valuable for testing OP exposure: AChE and NAE activities and alanine concentration. AChE represents the target enzyme, NAE an enzyme involved in OP detoxification, and alanine increases in the presence of stress agents. For routine testing these biomarkers can be easily determined using 2-4 larvae of medium size. Preliminary data on larvae exposed to carbamates evidence a different inhibition/activation pattern. Studies are in progress to produce a comprehensive picture of the effects of insecticides and other pollutants on freshwater macroinvertebrates.

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