BIOMARKERS, VOL. 8, NO. 2 (MARCH–APRIL 2003), 128–141

Taylor & Francis
healthsciences

Metallothionein induction related to hepatic structural perturbations and antioxidative defences in roach (*Rutilus rutilus*) exposed to the fungicide procymidone

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Received 8 July 2002, revised form accepted 2 October 2002

A variety of stresses, hormones, glucocorticoids and cytokines are known to induce metallothioneins (MTs) in animals. The aim of this study was to investigate the effects of chemical stress induced by the dicarboximide fungicide procymidone on hepatic structure, MT content and antioxidative defences (catalase and glutathione reductase activities and glutathione content) in the common fish *Rutilus rutilus*. Catalase and glutathione reductase activities remained stable throughout the experiment. Four days of exposure to 0.2 or 0.4 mg l^{-1} of procymidone induced an obvious increase in liver MT content, perturbation of metal MT contents, and an increase in hepatic glutathione content. After 14 days' exposure, obvious and large structural alterations of the hepatic parenchyma occurred simultaneously with a decrease in MT and glutathione content. These events were interpreted as degeneration of the liver. Fish exposed for 14 days to procymidone and then placed for 14 days in clean water showed nearly complete decontamination of the liver, but MT concentrations remained high. The toxicological significance of these events is discussed.

Keywords: liver, metallothioneins, oxidative stress, procymidone, *Rutilus rutilus*.

Introduction

Metallothioneins (MTs) are a class of inducible metal-binding proteins characterized by a low molecular weight (6000–10 000 Da), a high content of cysteine (about 30% in vertebrates), a lack of aromatic amino acid residues and heat stability (Viarengo 1985). These proteins have been detected in a wide range of prokaryotes and eukaryotes, including plants, invertebrates and vertebrates (Kägi and Kojima 1987). MTs play a major role in the detoxification of heavy metal ions such as cadmium or mercury, and in the homeostasis of essential metals such as zinc and copper (Petering *et al.* 1990, Zhang and Schlenk 1995). It has been established that metals (copper, cadmium and zinc) are particularly powerful inducers of MTs (Hogstrand 1991). Thus considerable effort has been focused on the use of MTs in the biomonitoring of metal contamination in fish (Petering *et al.* 1990, Zhang and Schlenk 1995, Cosson and Amiard 1998, Paris-Palacios *et al.* 2000a,b). Detection of high MT levels in feral aquatic organisms is usually understood as an indication of metal contamination (Roch *et al.* 1982, George and

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Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2003 Taylor & Francis Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/1354750021000050511

Olsson 1994, Linde *et al.* 2001). However, the specificity of MT induction by metals has recently been questioned by increasing knowledge of the functions and regulation pathways of MTs (Davis and Cousins 2000). A variety of stresses (physical trauma, high oxygen pressure, starvation, inflammation, ultraviolet irradiation, etc.), hormones (glucocorticoids, oestrogens, glucagon, insulin, etc.), vitamins, cytotoxic agents, antibiotics and cytokines act via intracellular signalling mechanisms to induce MTs in animals (George and Langston 1994, Davis and Cousins 2000). Moreover, evidence has been produced that suggests that MTs could act in a number of biochemical processes such as growth regulation or the scavenging of free radicals and reactive oxygen metabolites (Samson and Gedamu 1998, Andrews 2000). An antioxidant response element (ARE) has been identified and shown to activate MT transcription *in vitro* (Samson and Gedamu 1998, Andrews 2000). It is now well known that pesticides can induce an increase in the concentration of reactive oxygen species in cells and, consequently, an increase in antioxidative defences such as catalase or glutathione reductase (GRd) (Davies *et al.* 1994, Cossu *et al*. 1997). If MTs participate in these cellular defences, they may also be induced by pesticide contamination. Aquatic ecosystems are frequently contaminated by a mix of metal and pesticides. However, there is a lack of information about the capacity of pesticides to induce MTs. Thus it is important to assess whether MTs can be used as specific biomarkers of metal contamination in the presence of pesticides, or whether pesticides can have a notable influence on their level. The aim of this study was to investigate the effect of chemical stress produced by a fungicide (procymidone) on the hepatic MT content of the roach *Rutilus rutilus*. Quantitative and qualitative (metal content) analysis of MTs in roach liver exposed to procymidone for 4, 7 or 14 days was conducted. Simultaneously, hepatic histopathology and total protein content were studied to determine the toxic impact of the pesticide. Evaluation of some antioxidative defences (catalase and GRd activities and glutathione content) was performed in order to study the relationship between MT induction and the development of oxidative stress.

Roach is a relevant potential biomonitor of aquatic pollution because this member of the Cyprinidae is an abundant sedentary fish present in most European rivers, lakes and ponds. Procymidone [*N*-(3,5 dichlorophenyl)-1,2 dimethylcyclopropane-1,2 dicarboximide] is a moderately systemic fungicide with a rather selective action. It is especially effective in the control of *Botrytis* and *Sclerotinia* species in various vegetables, fruits, flower crops and ornamentals. Procymidone is applied at a dose of 0.5–1 kg/hectare (Tomlin 1994), often repeatedly, on the aerial parts of fruits and vegetables, sometimes until shortly before harvest to avoid fruit- and vegetable-rot during transport. It is also used on young plants and the top layer of soil against soil-borne diseases. It is stable to light, temperature and moisture and persists for several weeks in soil (Tomlin 1994). Thus, procymidone may pollute aquatic ecosystems and organisms after run-off. Few studies have examined the toxicity of procymidone. *The Pesticide Manual* (Tomlin 1994) indicates a 96 h-CL50 of 22.9 mg l^{-1} for *Lepomis macrochirus* and 3.6 mg l^{-1} for *Oncorhynchus mykiss*. Paris-Palacios *et al.* (1998) and Paris-Palacios (1999) observed no lethality in *Brachydanio rerio* exposed to 0.5, 1 or 3 mg l^{-1} of procymidone (procymidone

solubility in water 4 mg l^{-1}). However, the large histocytological perturbations induced by the pesticide clearly revealed the hepatotoxicity of these concentrations. In order to induce adaptive responses to chemical stress in liver while limiting degenerative events, in this study *Rutilus rutilus* were exposed to lower procymidone concentrations (0.2 and 0.4 mg l^{-1}). Roach were also replaced in clean water after 14 days of exposure to procymidone in order to study their capacity for decontamination and recuperation.

Materials and methods

Fish maintenance

Roach (90 fish aged 16 months) were purchased from a pisciculture centre in Reims, France, and acclimatized for 14 days to laboratory conditions at a fish density of 1.5/l of bottled spring water (eau Cristaline, source des Grands Bois, Fismes, France) in the following conditions: pH 8 ± 0.5, temperature 9 ± 1°C, photoperiod 12 h/12 h, $CaCO_3$ concentration 300 mg l^{-1}, Ca^{2+} concentration 124 mg l^{-1}, Mg^{2+} concentration 25 mg l^{-1}, Na^{2+} concentration 11 mg l^{-1}, HCO^{3-} concentration 399 mg l^{-1}, and Cl^{-} concentration 16 mg l^{-1}. After acclimatization, roach were separated into three groups of 30 roach. One group of controls were kept in clean water, two groups were exposed for 14 days to procymidone concentrations of 0.23 ± 0.02 mg l^{-1} and 0.44 ± 0.03 mg l^{-1}, respectively. Fungicide concentrations were measured by high performance liquid chromatography (HPLC) every 2 days and maintained at a stable level; the water was renewed every 4 days (semi-static conditions). After 14 days' exposure, 12 roach from each group were placed for 14 days in clean water (depuration). Six fish from each group were killed by decapitation after 4, 7 or 14 days of exposure and after 14 days of exposure followed by 7 or 14 days of depuration.

Hepatic content of procymidone

Three pooled sets of specimens were obtained for each treatment by homogenizing liver samples from three roach in acetonitrile. After 72 h of extraction at 4°C, specimens were centrifuged (2500 *g* for 10 min at 4°C). Supernatants were filtered (Millipore filter, 0.22 μm pores) and injected on a column (Spherisorb S5ODS2, length 25 cm, internal diameter 4.6 mm) eluted with 1 ml min^{-1} of acetonitrile:water (60:40) containing 0.1% trifluoroacetic acid. Analysis of the absorbency spectra (200–400 nm) was performed using a UVD34DS Gynkotek detector. Calibration was done with procymidone (99.7% purity, Dr Ehrenstorfer GmbH). Analysis of each of the three pools was performed in triplicate ($n = 3$).

Histology

Liver samples from four roach from each group (control or treated with 0.2 or 0.4 mg l^{-1} procymidone) were immediately immersed in Bouin's fluid for 24 h, rinsed in distilled water, dehydrated by ethanol and embedded in paraffin. Sections of 6 μm were stained with nuclear fast red and picro-indigo carmine. Samples of liver from the same fishes were also fixed by immersion in 3% glutaraldehyde buffered to pH 7.2 with 0.1 M sodium cacodylate. Specimens were washed three times in buffer, dehydrated with acetone and embedded in Spurr resin. Semi-thin sections were stained with methylene blue-azure II (Richardson *et al*. 1993). The percentage of lysed hepatocytes in liver was measured with an precision of 5% using a picture analysis program (Cyberview 2.5) on at least 20 slides from four roach in each group ($n = 20$).

Hepatic glutathione content and catalase and GRd activities

Pools of three livers were obtained in triplicate for each group of nine roach and homogenized in sodium phosphate buffer (0.1 M, pH 7, 4°C). Homogenates were centrifuged (2300 *g* for 15 min at 4°C) and the total protein content of the supernatants was determined using the method of Bradford (1976), with bovine albumin (Sigma) for calibration.

Catalase activity in the supernatants was determined using the method of Teisseire *et al*. (1998) and expressed as millimoles of hydrogen peroxide consumed per milligram of protein per minute. Analysis of each of the three pooled livers was carried out in triplicate.

A volume of 40 μl of supernatant was used for the determination of total glutathione content. Tests were performed in triplicate for each of the three pools in phosphate buffer (50 mM, pH 7.5) with 2.5 mM of ethylene diamine tetra-acetic acid (EDTA), 0.5 mM of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), 0.1 mM of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 0.5 units of

GRd, with a final volume of 1 ml, according to the method of Paris-Palacios *et al*. (2001). The change in absorbency at 412 nm was followed during incubation for 3 min, and the total glutathione content was obtained using a reduced glutathione (GSH) calibration procedure (ICN).

GRd activity measurements were performed in triplicate for each of the three pools. First, 300 µl of each supernatant was added to 2.4 ml of phosphate buffer (50 mM, pH 7.6) and 150 µl of $NaHCO_3$ containing 2 mM NADPH (Carlberg and Mannervik 1985). After incubation (5 min at 25°C) to reduce the native oxidized glutathione (GSSG), the reaction was started by the addition of GSSG (150 µl, 20 mM) in phosphate buffer (50 mM, pH 7.6). The decrease in absorbency at 340 nm due to NADPH consumption caused by GSSG reduction was followed over 4 min, and GRd activity was calibrated using commercial GRd (Sigma G-47851). GRd activity was expressed as micromoles of GSSG reduced per minute per milligram of protein.

MT analysis

Analysis of total MT and of MT fractions was performed in triplicate using 0.4 g of pooled liver tissue from three roach. Liver tissue was homogenized in 3 ml of 20 mM Tris HCl buffer (pH 8) containing 20% glycerol, 2 mM mercaptoethanol, 0.006 mM leupeptin and 0.5 mM phenylmethylsulphonyl fluoride (PMSF). The homogenate was centrifuged at 30 000 *g* for 20 min at 4°C. The supernatant was loaded onto an equilibrated Sephadex G 75 column (3 × 60 cm) previously calibrated for molecular weight determination using a kit from Sigma (6500–66 000 Da). Columns were eluted with 1 mM phosphate buffer (pH 8) at 0.6 ml min^{-1} and analysis of the absorbency spectra (200–300 nm) was performed using a UVD3405 Gynkotek detector. MT fractions were pooled and the total MT content was determined spectrofluorimetrically in triplicate for each pool using the method of Paris-Palacios *et al*. (2000a). Then 1 ml of the MT fraction was saturated with copper (by the addition of increasing amounts of $CuSO_4$ in aqueous solution). The emission of the Cu-MTs formed was measured at 540 nm (excitation at 268 nm) with a fluorometer (Perkin Elmer LS50B) equipped with a filter (Oriel 59154, absorbency 400–700 nm) to cut the second-order harmonic of the lamp (Paris-Palacios *et al*. 2000a). Cu-MT was quantified using a calibration curve obtained by copper saturation of commercial rabbit liver MTs (Sigma).

The copper, cadmium and zinc contents of the MT fractions were determined using atomic absorption spectrometry. Measurements were performed in triplicate for each of the three pools. In fish, zinc, copper and cadmium are concurrently bound to MTs as $(Zn,Cu)_7$-MTs or $(Cd,Cu)_7$-MTs (Kägi and Kojima 1987, Richardson *et al*., 1993). Thus the metal-MT quantities in liver were estimated as ([Cu] + [Cd] + [Zn])/7.

Statistical analysis

The Student's *t*-test was used to determine the statistical significance of the results. Differences were considered to be statistically significant when $p < 0.01$ (two experimental groups were compared against two control groups).

Results

No fish lethality was observed during the 14 days' exposure to 0.2 or 0.4 mg l^{-1} of procymidone or the 14 days' of depuration.

Hepatic content of procymidone

No procymidone was detected in the livers of the control fish. Concentrations of procymidone in exposed fish increased progressively during the exposure period. The procymidone accumulation was time and concentration dependent (Table 1). Bioaccumulation factors reached 265 and 332 in the livers of roach exposed for 14 days to 0.2 and 0.4 mg l^{-1} of procymidone, respectively (Table 1). When roach were replaced in clean water, the procymidone concentrations in the liver decreased rapidly; only traces of procymidone were detected after 7 days depuration and none after 14 days.

Table 1. Procymidone concentrations in livers of roach exposed to procymidone.

Procymidone exposure (mg l^{-1})	Duration of exposure (days)	Hepatic procymidone concentration (μg g^{-1} fw)[a]	Bioaccumulation factor[b]
0	14	ND	—
0.4	4	6±3	15
	7	52±8	129
	14	133±9	332
	14 (+7 dupuration)	<5	—
	14 (+14 dupuration)	<5	—
0.2	4	<5	12
	7	9±3	44
	14	53±8	265
	14 (+7 dupuration)	<5	—
	14 (+14 dupuration)	ND	—

[a] Values are the mean of three determinations ± SD.
[b] Bioaccumulation factor = [procymidone] in liver (mg kg^{-1})/[procymidone] in water (mg l^{-1}).
fw, fresh weight; ND, not detected.

Histopathological events

The homogenous hepatic parenchyma of *R. rutilus* was composed of hepatocytes (225 ± 23 μm^2) arranged in typical tubular architecture around the bile system (Figure 1). The hepatic structure of the control roach was characteristic of fish in good health (Biagianti-Risbourg 1997, Braunbeck 1998, Paris-Palacios *et al*. 2000b).

After 7 and 14 days of exposure to procymidone, liver perturbations were obvious. Numerous areas, mostly located near hepatic veins, were completely lysed (Figures 2 and 3). These areas contained elements of broken hepatocytes (free nuclei, mitochondria, etc.). The percentage of lysed hepatocytes increased with the exposure time, but was similar in roach exposed to 0.2 or 0.4 mg l^{-1} of procymidone (Figure 3). After 14 days' exposure, 40 ± 10% of the liver was

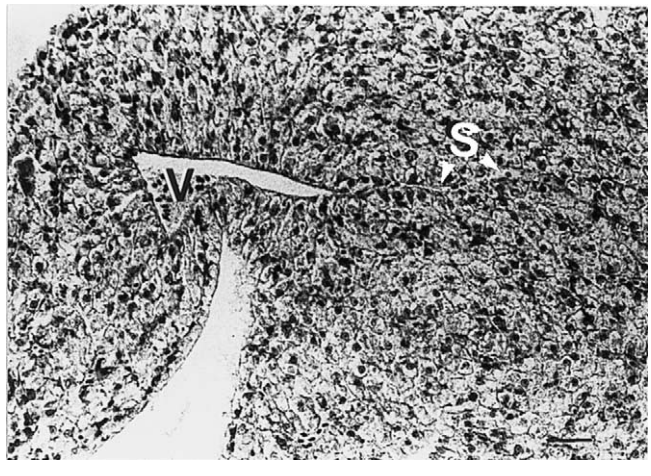

Figure 1. Hepatic parenchyma of control roach. The parenchyma was homogeneous and showed a regular tubular organization of hepatocytes. Semi-thin section stained with Richardson blue. S, sinusoid; V, vein. Bar = 50 μm.

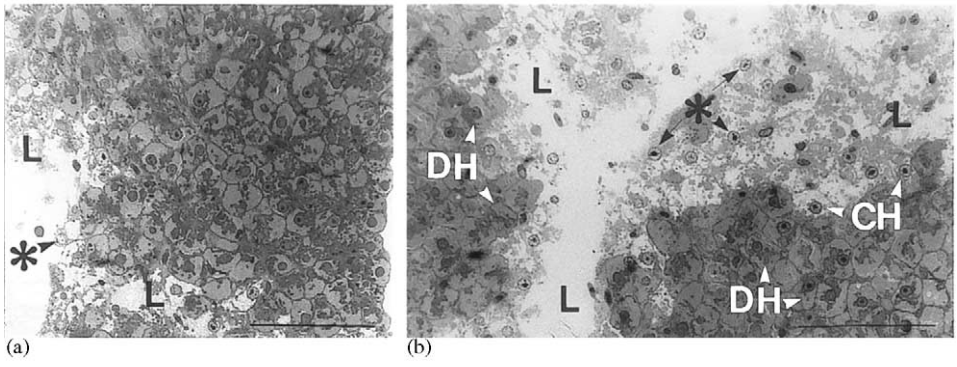

Figure 2. Hepatic parenchyma of roach exposed for 7 days to 0.2 mg l^{-1} (a) or 14 days to 0.4 mg l^{-1} (b) of procymidone. The parenchyma was composed of clear hepatocytes (CH) and dark (highly basophilic) hepatocytes (DH). There was a large lysed area (L). Semi-thin section stained with Richardson blue. *, free nucleus in lysed area. Bar = 50 μm.

composed of lysed hepatocytes (Figures 2 and 3). Numerous clear and large hepatocytes (373 ± 91 μm^2) were seen around the lysed areas (Figure 2). These had a poorly basophilic cytoplasm and nucleus. They constituted $30 \pm 10\%$ of the parenchyma in roach exposed for 14 days to 0.2 or 0.4 mg l^{-1} of procymidone (Figure 3). The remainder of the parenchyma was composed of smaller basophilic hepatocytes (192 ± 30 μm^2) (Figure 2).

Liver of roach exposed for 14 days to 0.2 or 0.4 mg l^{-1} of procymidone and replaced for 14 days in clean water showed a structure comparable to that observed during exposure (Figure 4). However, a slight decrease in the percentage of lysed hepatocytes was seen (Figure 3).

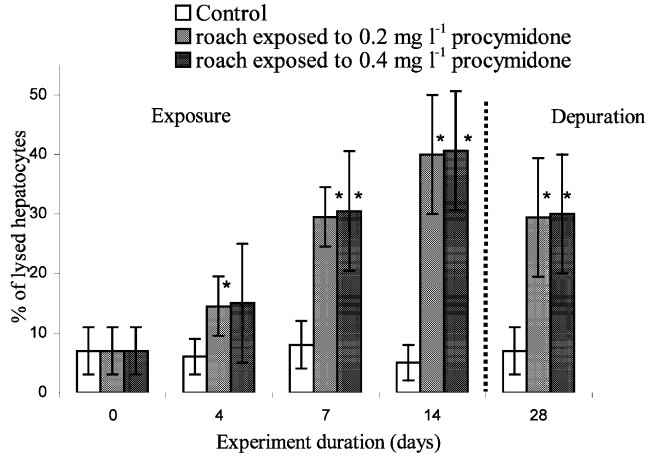

Figure 3. Development of lysis in roach exposed to procymidone (measurement performed with a precision of 5% on five slides for four roach by group; $n = 20$). Values are mean $\pm$ SD. *, significantly different from control (Student's t-test, $p < 0.01$).

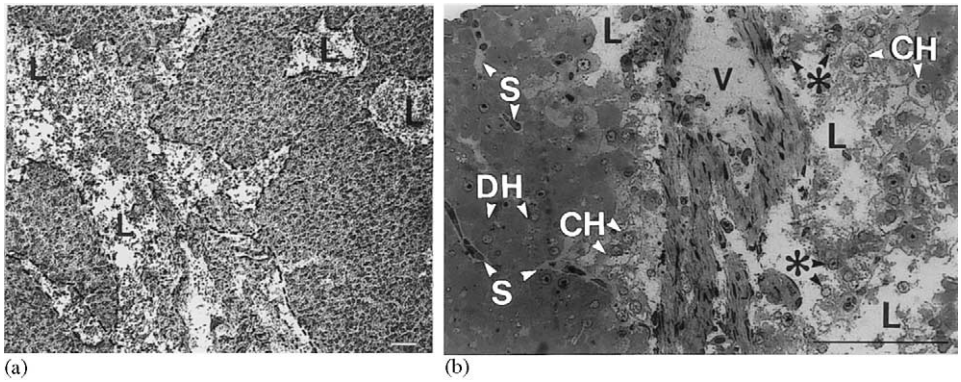

Figure 4. Hepatic parenchyma of roach exposed for 14 days to 0.4 mg l^{-1} procymidone and replaced for 14 days in clear water, showing large lysed areas. Section stained with picro-indigo carmine and nuclear red (a) or Richardson blue (b). CH, clear hepatocyte; DH, dark hepatocyte; L, lysed area; S, sinusoid; V, vein; *, free nucleus in lysed area. Bar = 50 μm.

Antioxidative defences

Control roach showed no variation in glutathione content or catalase and GRd activities in all the experiments. Procymidone exposure induced no perturbation of catalase or GRd activity (Table 2). However, the hepatic level of total glutathione content (GSH+GSSG) varied greatly (Figure 5). After 4 days of exposure to procymidone a clear increase in GSH+GSSG of +100% was observable in the livers of roach exposed to 0.2 or 0.4 mg l^{-1} of procymidone. For longer exposures the hepatic glutathione concentrations decreased progressively, but remained significantly higher than control values. This evolution of GSH+GSSG concentrations in liver was time dependent, but was similar for both procymidone

Table 2. Catalase and GRd activities and protein content in livers of roach exposed to procymidone.

		Procymidone concentration		
Parameter measured	Exposure duration (days)	Control	0.2 mg l^{-1}	0.4 mg l^{-1}
Catalase activity (μmol min^{-1} mg^{-1} protein)	4	3.8±0.6	3.6±0.7	3.1±0.4
	7	3.4±0.7	3.5±0.5	3.1±0.7
	14	3.2±0.5	3.2±0.8	3.3±0.6
	14 (+14 depuration)	3.2±0.5	3.4±0.6	3.1±0.7
GRd activity (μmol min^{-1} mg^{-1} protein)	4	1.45±0.11	1.69±0.21	1.65±0.24
	7	1.27±0.07	1.26±0.25	1.21±0.21
	14	1.32±0.18	1.21±0.18	1.22±0.16
	14 (+14 depuration)	1.62±0.17	1.34±0.26	1.43±0.17
Protein content (mg g^{-1} fw)	4	2.6±0.5	2.8±0.5	3.1±0.5
	7	3.1±0.5	2.8±0.5	3.1±0.5
	14	2.9±0.5	2.9±0.5	3.1±0.5
	14 (+14 depuration)	3±0.5	2.9±0.5	3.2±0.5

Values are the mean of three determinations±SD. fw, fresh weight.

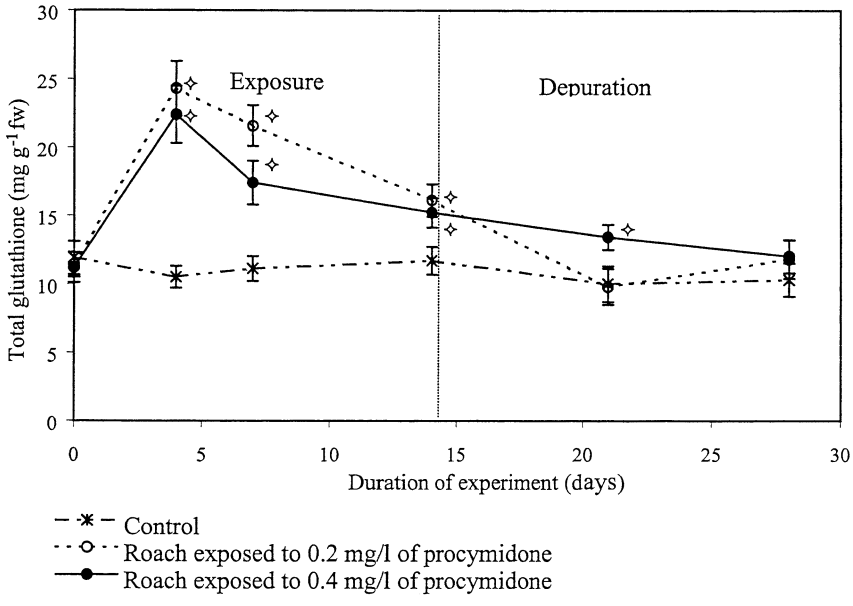

Figure 5. Hepatic changes in total glutathione content in roach exposed to procymidone. Values are means of three determinations ± SD. ◇, significantly different from control (Student's *t*-test, $p < 0.01$).

concentrations. When roach were replaced in clean water the glutathione content continued to decrease and reached control values (Figure 5).

MT content

The hepatic protein content of roach exposed to both concentrations of procymidone was stable during all the exposure and depuration periods (Table 2). The two methods of quantification of MT used (Table 3) revealed a marked increase in MT concentrations in the liver of roach exposed for 4 days to procymidone (10-fold higher than controls). Longer exposure to procymidone (7 and 14 days) induced a decrease in the hepatic MT content, but this remained clearly higher than control values. The changing pattern of MT concentrations was dependent on the duration of exposure but was similar for both procymidone concentrations used, despite the difference in hepatic procymidone accumulation. When roach were replaced in clear water, the hepatic MT content was stable in roach exposed to 0.4 mg l^{-1}, but showed a further increase in roach exposed to 0.2 mg l^{-1} of procymidone (Table 3).

In the control roach, 75 ± 5% of the hepatic MTs were conjugated to metals. In fish exposed to procymidone, the concentration of metal-MTs clearly increased. However, the total MT content of the liver increased faster than metal-MTs concentration, producing a decrease in the percentage of metal-MTs to 55 ± 5%. Additionally, a marked perturbation of MT nature occurred. In the control roach, metal-MTs were mostly conjugated to zinc (74%). However, in roach exposed to procymidone (independently of the concentration and duration of exposure), about 40% of the metal-MTs were Cu-MTs and Zn-MTs decreased to about 40% (Table

Table 3. MTs in livers of roach exposed to procymidone.

Procymidone exposure (mg l^{-1})	Duration of exposure (days)	Total MTs (nmol MT g^{-1} fw)	Metal MTs (nmol MT g^{-1} fw)	Cu MTs (% of total metal MT)	Zn MTs (% of total metal MT)	Cd MTs (% of total metal MT)
Control		17±3	12±3	3.4±0.9	74±2	22±3
0.2 mg l^{-1}	4	177±19*	93±19*	38±10*	43±18*	19±7
	7	130±11*	71±13*	41±4*	37±9*	22±5
	14	107±8*	59±10*	38±3*	39±7*	23±8
	14 (+14 depuration)	90±7*	51±11*	39±5*	39±12*	21±6
0.4 mg l^{-1}	4	298±15*	153±18*	47±14*	34±17*	21±8
	14	92±9*	63±10*	41±15*	36±16*	23±7
	14 (+14 depuration)	147±21*	74±19*	44±6*	35±10*	23±14

Values are the mean of three determinations ±SD. fw, fresh weight.
* Significant difference from control ($p < 0.01$, Student's t-test).

3). These proportions (40% Cu-MTs, 40% Zn-MTs) also persisted during the depuration period (Table 3). The percentage of Cd-MTs was constant and was similar to control values in all the experiments (Table 3).

Discussion

The hepatic MT concentrations in control roach were similar to those found in previous studies on roach or other Cyprinid such as *Brachydanio rerio* (Paris-Palacios 1999, Paris-Palacios *et al*. 2000a). In normal liver, zinc is the predominant metal bound to MTs (Kägi and Vallée 1960, Bukler and Kagï 1975). According to previous studies, MTs in control roach liver contained 74% zinc, 22% cadmium and 3% copper (Samson and Gedamu 1998). As cadmium is a non-essential metal, the presence of Cd-MTs may indicate previous cadmium contamination. This is not surprising as cadmium is a ubiquitous environmental pollutant (Goyer 1997). Moreover, the detection of cadmium bound to MTs was a constant feature of numerous studies (Kägi and Vallée 1960, Riordan and Vallée 1991, Wagmann *et al*. 1994).

Roach exposed to procymidone showed significant hepatic accumulation of this pesticide. The dicarboximide produced a clear induction of MTs in liver. Several regulation pathways of MT transcription have been correlated with the presence of different binding transcription factors (Davis and Cousins 2000). The ARE activates transcription in response to the increase in reactive oxygen species concentrations in cells (Samson and Gedamu 1998, Andrews 2000). No perturbation of catalase or GRd activities occurred in the liver of roach exposed to procymidone. These findings argue against the development of oxidative stress during procymidone exposure. However, significant perturbations of total glutathione (GSH+GSSG) content did occur. Glutathione is involved in several cellular functions, including phase II metabolism through glutathione-S-transferase activity (Sies 1988, Narbonne and Michel 1997) and antioxidative defences in relation to its redox capacity through GRd and glutathione peroxidase activities (Cossu *et al*. 1997, Lackner 1998). Additionally, glutathione acts as a source of amino acids for protein synthesis (Meister and Anderson 1983). It is involved in the regulation of metal homeostasis and distribution and acts as an intermediate in the transfer of metals from MTs (Viarengo 1989, Davis and Cousins 2000). In oxidative stress, low GSH/GSSG ratios enhance zinc release from MTs to apoproteins (Jiang *et al*. 1998). In procymidone-exposed roach liver, the correlation between changes in the hepatic contents of glutathione and MT may suggest that both are involved in the same function (antioxidative defences, metal homeostasis, cell repartition, or other unknown functions).

Davis and Cousins (2000) reported that cytokines and various hormones act through intracellular signalling mechanisms and signal transducers and activators of transcription (STAT) to induce MT expression. Two glucocorticoid response elements (GREs) induce MT transcription in response to glucocorticoids during stress such as physical trauma or microbial infection (Kelly *et al*. 1997). The development of a large percentage of lysed hepatocytes during roach exposure to procymidone revealed the high hepatotoxicity of this fungicide. Small basophilic

hepatocytes and large clear hepatocytes were also seen. These changes may reflect alterations in cellular osmotic regulation capacity. Treated roach were subjected to a chemical stress that may induce hepatic MT synthesis through hormonal response and GRE activation.

Metal-MT complexes constituted 75±5% of the total MT present in control fish (Paris-Palacios *et al*. 2000a), but decreased to 55±5% in treated roach. We hypothesize that procymidone exposition induced an increase in the concentrations of apoMTs or MTs not saturated by metals (ratio of metal/mol MTs of lower than 7). The large new pool of MTs produced in treated roach liver (10-fold higher than in control fish) bound significant quantities of copper and zinc. It is likely that there was displacement of these metals to MTs from other sites in the cell or organism. In this case, some MT metal-binding sites would be unsaturated. The functions of MTs include intracellular metal metabolism and storage; MTs serve as a reservoir of zinc, allowing rapid exchange with apometalloproteins to rescue their enzymatic activities (Davis and Cousins 2000). It is possible that, in procymidone-contaminated liver, high levels of MTs can capture metals from other metalloproteins and so lead to their inactivation. This type of metal exchange between MTs and other proteins is facilitated by the presence of GSSG (Jiang *et al*. 1998). The increase in hepatic GSH+GSSG content in treated roach may be related to changes in the MT metal pool. The metal response element (MRE) sequences of DNA induce MT transcription in response to binding of a multiple zinc finger protein named MTF-1. The degree of metal occupancy of MTF-1 depends on the cellular zinc pool (but not that of other transition metals) and this determines its binding to MREs and thus MT transcription (Samson and Gedamu 1998, Andrews 2000). Moreover, perturbation of cellular levels of Zn-MTs related to MT induction by metals, oxidants or electrophiles could regulate gene expression, cell proliferation or apoptosis (Davis and Cousins 2000). Thus in roach exposed to procymidone, MT induction and perturbations of metal-MT content may be of multiple origins and may cause important modifications of cellular status. This could play a major role in the development of the degenerative processes (lysis) observed in livers of procymidone-exposed roach.

With increasing time of exposure to procymidone (7–14 days), degenerative alterations in the liver became obvious, particularly at the end of the exposure period (45±5% of liver lysed). Simultaneously, a decrease in GSH+GSSG content and MT levels occurred and significant inhibition of phase I metabolism is seen (Paris-Palacios *et al*. 2001). It is clear that the roach liver entered the exhaustion phase of stress (Monod 1997, Segner and Braunbeck 1998, Biagianti-Risbourg 1990, Paris-Palacios *et al*. 2000b, 2001). However, when fish were replaced in clean water, no mortality was observed and hepatic decontamination was rapid and complete. At the same time roach livers showed a new increase in MT concentration and high induction of phase I metabolism (Paris-Palacios *et al*. 2001). On one hand these results indicated a certain recuperation of hepatic function during depuration but, on the other hand, at the end of the depuration period the livers have not been restored to their normal status. These observations lead to the conclusion that MTs (and also glutathione) may participate in the adaptive response of liver to the stress induced by procymidone.

Conclusions

Livers of roach exposed to procymidone strongly accumulated this pesticide. The hepatotoxicity of procymidone is clearly revealed by the structural alterations that occur. However, it is important to notice that the concentrations of procymidone used (0.2 and 0.4 mg l^{-1}) were higher than concentrations used in the field. GERBE (1998) measured procymidone concentrations varying from 0–20 μg l^{-1} in a basin of run-off water recuperation from the vineyards of Champagne, Reuil, France, between 1996 and 1999. Procymidone exposure induced a clear increase in MT levels and perturbations of metal pools bound to MTs in roach liver. It is noteworthy that MT is induced even though no metal was administered, showing that this biomarker response may reflect an 'effect' and not the exposure *per se*. Results indicated the possible participation of MTs in the adaptive response to stress. The magnitude of MT induction observed in liver of roach exposed to procymidone (10-fold) must change the way in which MTs are used as a biomarker. On the one hand, it shows that MTs cannot be used as a specific biomarker of metal exposure and that pesticide contamination can be responsible for high MT levels in feral organisms. On the other hand, MTs could be a useful non-specific biomarker to study global environmental perturbations, as most aquatic ecosystems are contaminated by a mixture of pollutants.

Acknowledgements

We gratefully acknowledge Europôl'Agro for financial support of this work within the 'Toxicologie et Ecotoxicologie des Pesticides et des Métaux lourds' research programme.

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