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Acute effects of diflubenzuron on the freshwater fish *Prochilodus lineatus*

Lindalva Pereira Maduenho, Claudia B.R. Martinez *

Departamento de Ciências Fisiológicas, Universidade Estadual de Londrina, Londrina, Paraná, C. P. 6001, CEP 86051-990, Brazil

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ABSTRACT

The effects of diflubenzuron (DFB), an insecticide to control ectoparasites in fish farms, on muscle acetylcholinesterase (AChE), detoxifying and antioxidant enzymes, hematological and physiological parameters, and liver histopathology were evaluated in *Prochilodus lineatus* after 6, 24 and 96 h of exposure to 25 mg L^{-1} of DFB. The insecticide caused a reduction in the number of erythrocytes and hemoglobin content after 96 h exposure, probably due to hemolysis. Hyperglycemic response indicated energy mobilization, and may have contributed to the increase in osmolarity after 96 h exposure to DFB. The induction of glutathione-*S*-transferase (GST) and catalase activities in liver pointed to the activation of xenobiotic metabolic pathways and antioxidant defenses. The decrease in muscle AChE at all experimental times showed that DFB is an AChE inhibitor. In addition, DFB induced hepatic alterations that might impair normal liver functions. These results show that DFB can cause health disorders in fish and further studies are required to better define its safe use in aquaculture.

1. Introduction

Parasite infections in fish represent a serious problem currently affecting commercial aquaculture, and chemical products are extensively used for the control of these parasites (Guimarães et al., 2007). However, the lack of products specific for fish farming leads to the indiscriminate use of pesticides, which have formulations appropriate for agriculture activities (Boyd and Massant, 1999). At present there is a growing concern worldwide over the arbitrary use of such chemicals that results in environmental pollution and toxicity risk to nontarget organisms (Rao, 2006). Fish culture in Brazil is an important economic activity that has been growing fast in the last few years (Toró et al., 2003). Nevertheless the large use of pesticides is associated with the adoption of production systems that maintain high fish densities, and constitutes one of the main environmental problems derived from the intensive fish culture (Lopes et al., 2006). Diflubenzuron (DFB) is one of the most widely used insecticides to control ectoparasites in Brazilian fish farms (Mabilia and Souza, 2006), but there are no specific formulations of this pesticide for fish treatment.

DFB is a benzoylphenylurea derivative (1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea), which acts as a potent broad-spectrum insect growth regulator that interferes with chitin synthesis at the time of molting and is effective in controlling immature stages of insects and crustaceans. This pesticide has been frequently utilized in agricultural areas against insect pests, and also in fish farming due to its efficacy in controlling fish ectoparasites such as the crustaceans *Lernaea cyprinacea* (Martins, 2004) and *Dolops carvalhoi* (Schalch et al., 2005), among others. Studies with different fish species have examined the toxicity of DFB and have indicated that its mean lethal concentration for 96 h exposure (LC_{50} 96 h) is greater than 50 mg L^{-1}, which characterizes this product as having low toxicity to fish (Fisher and Hall, 1992). However, fish can accumulate diflubenzuron from water up to 160 times (Eisler, 1992). Besides, one of the metabolites of DFB, 4-chloroaniline, shows a higher toxicity to fish than the original product (Fisher and Hall, 1992) and was classified by EPA (2006) as a probable human carcinogen.

The majority of studies evaluating diflubenzuron toxicity on fish involve the determination of the LC_{50} of the insecticide to coldwater fishes (Fisher and Hall, 1992) or the efficacy of the product in combating ectoparasites (Costello at al., 2001; Bouboulis et al., 2004; Schalch et al., 2005). Nevertheless, very little is known about sublethal effects of DFB on fish.

Biological changes in fish related to the exposure or to the effects of chemicals are called biomarkers (Van der Oost et al., 2003). The enzyme acetylcholinesterase (AChE) is recognized as one of the oldest biomarkers, which is sensitive to selected organophosphate and carbamate pesticides, but also may be responding to other contaminants in the environment (Payne et al., 1996). Other prominent biomarkers include biochemical variables such as detoxifying and antioxidant enzyme activities; hematological data (hematocrit, hemoglobin content and erythrocyte count); physiological variables, such as plasma levels of cortisol, glucose, ions and osmolarity; and histological alterations in target organs, such as liver. The determination of these

* Corresponding author. Tel.: +55 43 33714650; fax: +55 43 33714207.
E-mail address: cbueno@uel.br (C.B.R. Martinez).

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biomarkers on fish acutely exposed to DFB is important to expand the knowledge concerning the effects of therapeutic compounds used in aquaculture on the health of fish (Winkaler et al., 2007).

Prochilodus lineatus (Order Characiformes) is an important medium size fish for subsistence and commercial fishing in the south of Brazil, and a potential species for fish culture considering its high productivity in fish farms (Winkaler et al., 2007). Due to its economic importance and because of its well-known biology and sensitivity to pollutants, this fish species is appropriate for testing the toxicity of DFB.

Thus, considering the intensive use of DFB in fish farming and its unknown effects on fishes, this study was designed to investigate the short-term effects of a sublethal concentration of DFB on enzyme activities (liver catalase, GST and muscle AChE), physiological parameters (hematological, ionic, metabolic and endocrine) and in the liver histology of *P. lineatus*. To our knowledge the present investigation is the first to be published showing the effects of DFB to a neotropical fish species, and the approach taken, considering several biomarkers, at different biological levels, permits the identification of changes before more deleterious effects of DFB on fish health take place.

2. Materials and methods

2.1. Animal

Juveniles of *P. lineatus* (Valenciennes, 1847), with body mass of 17.35±5.15 g and total length of 11.24±1.27 cm (mean±SD, n=48), obtained from the Universidade Estadual de Londrina Hatchery Station, were acclimated for 7 days in a 600-L tank filled with dechlorinated water (t: 21.1±0.2 °C; pH: 7.4±0.1; hardness: 52±2.8 mg L^{-1} $CaCO_3$), with constant aeration (DO: 6.2±0.2 mg O_2L^{-1}) and a 12/12 h light/dark photoperiod. During the period of acclimation, the animals were fed every 2 days with commercial pellet food containing 36% of protein (Guabi®, BR). Feeding was suspended 24 h before the start of toxicity tests and fish were not fed during the exposure periods.

2.2. Toxicity tests

After acclimation, the fish were submitted to static acute toxicity tests (6, 24 and 96 h). The commercial formulation of DFB, Dimilin® (wettable powder 25% active ingredient, Crompton, Brazil), was used at a concentration of 25 mg L^{-1} of the active ingredient DFB. The product was mixed directly with water, 24 h before the animals were exposed. The tests were carried out in glass aquaria of 100 L, with continuously aerated dechlorinated water and 7 to 8 fish in each aquarium. There were a total of six aquaria; three corresponding to experimental groups (one for each exposure period) and the other three to control groups. Experimental groups were exposed to DFB and terminally sampled after 6 h, 24 h or 96 h. One control group, with animals exposed only to water, without the contaminant, was sampled at each experimental time (6, 24 and 96 h), concomitantly with the groups exposed to DFB.

During the toxicity tests, water from the aquaria was monitored daily for temperature, pH, dissolved oxygen (DO), conductivity and hardness. The physical–chemical characteristics of the water, in all the exposure periods, remained stable for both control and DFB exposed groups, and the mean values (±SD) were, respectively, temperature: 21.3±1.3 °C and 20.9±1.1 °C; pH: 7.3±0.1 and 7.5±0.1; OD: 7.1±1.3 mg O_2L^{-1} and 6.8±1.1 mg O_2L^{-1}; conductivity: 99 µS cm^{-1} and 96 µS cm^{-1}; hardness: 50 mg $CaCO_3$ L^{-1} and 54 mg $CaCO_3$ L^{-1}.

2.3. Sampling

Immediately after removal from the aquaria, the fish were anesthetized with benzocaine (0.1 g L^{-1}), and blood samples were taken from the caudal vessels into heparinized 1 mL plastic syringes. Subsequently, animals were killed by cervical section, measured and weighed. The liver and fragments of white muscle were removed. The muscle and a part of the liver were stored frozen at −80 °C. Another part of the liver was fixed in ALFAC (85% alcohol, 10% formalin and 5% acetic acid) for histological analyses.

2.4. Analysis of blood parameters

Hematocrit (Hct) values were determined by blood centrifugation (5 min, 5000 *g*) in glass capillaries, using a microhematocrit centrifuge. Hemoglobin content (Hb) was measured by the cyanomethemoglobin method (Kit Analisa, Brazil). For total red blood cell (RBC) counting, the blood (5 µL) was diluted in formalin-citrate (1 mL) and RBC was determined manually with an improved Neubauer counting chamber, under light microscope (400× magnification). Mean Corpuscular Volume (MCV) was calculated as (Hct×10)/RBC.

The remaining blood was centrifuged for 5 min at 5000 *g* and plasma was stored frozen at −20 °C. Plasma cortisol was determined using an immunoenzymatic assay (kit Diagnostic Systems Laboratories, USA) and absorbance was measured in a microplate reader at 450 nm. Plasma glucose was determined by the glucose oxidase method (kit Labtest, Brazil) in a spectrophotometer at 505 nm. Plasma concentrations of Na^+ were determined by flame photometry and Cl^- was determined by the mercuric thiocyanate method (commercial kit, Labtest, Brazil), in a spectrophotometer at 470 nm. Osmolarity was measured by freezing point using a micro-osmometer.

2.5. Biochemical assays

Liver samples were homogenized in 10 vol. of ice-cold 0.1 M K K-phosphate buffer, pH 7.0, and centrifuged for 20 min at 4 °C and 15,000 *g*. The supernatant was used for catalase (CAT) and glutathione-*S*-transferase (GST) assays. CAT activity was determined as described by Beutler (1975), by measuring the rate of decomposition of H_2O_2 in a spectrophotometer at 240 nm. GST activity was determined by enzymatic conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) in a spectrophotometer at 340 nm, according to Habig et al. (1974). CAT activity was expressed as µmol min^{-1} mg liver $protein^{-1}$ and GST activity was expressed as nmol min^{-1} mg liver $protein^{-1}$.

Muscle samples were homogenized in 10 volumes of ice-cold 0.1 M K-phosphate buffer, pH 7.5. Homogenates were centrifuged at 10,000 *g* for 20 min at 4 °C and supernatant was used for acetylcholinesterase (AChE) assay. AChE activity was determined by the method of Ellman et al. (1961) and adapted for microplate as described by Alves Costa et al. (2007). Final concentration for the substrate acetylcholine iodide was 9 mM and for the color reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was 0.5 mM. Absorbance was measured in a microplate reader, at 415 nm, and enzyme activity was expressed as nmol min^{-1} mg muscle $protein^{-1}$. The concentration of protein in liver and muscle samples were determined according to Lowry et al. (1951) using bovine albumin as standard.

2.6. Histological analyses

Liver samples were fixed in ALFAC, embedded in paraffin and sectioned (5 µm). The slides were stained with hematoxylin-eosin (HE), examined under light microscope and photographed using a digital camera. At least 10 sections per animal were analyzed.

Liver tissue changes were evaluated semi-quantitatively by the Degree of Tissue Change (DTC), which is based on the severity of the lesions and the possibility of recovery. For DTC calculation (modified from Poleksić and Mitrović-Tutundžić, 1994), liver changes were classified into three progressive stages of impairment of the hepatic function: stage I = changes that do not damage the liver tissue to such

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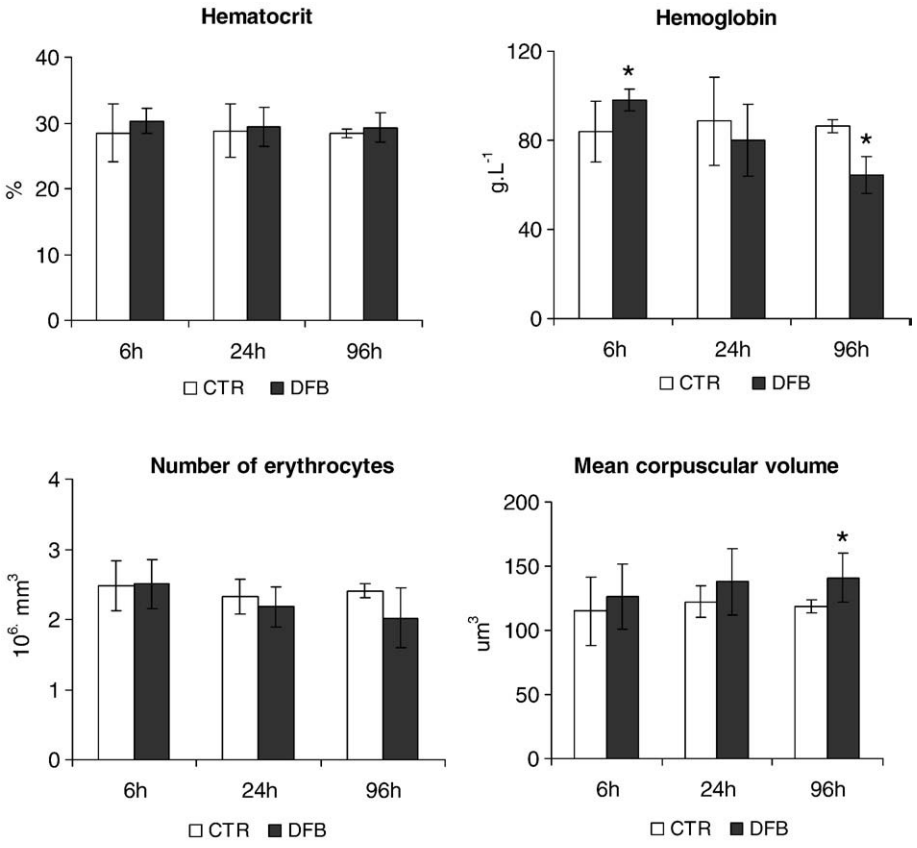

Fig. 1. Hematocrit, hemoglobin content, number of erythrocytes and Mean Corpuscular Volume of *Prochilodus lineatus* exposed to 25 mg L^{-1} of DFB, or only water (CTR), for different experimental periods (6, 24 and 96 h). Bars represent means and vertical lines of SD (number of animals: 6–7). *Different from respective control ($P<0.05$).

an extend that the organ cannot repair itself; stage II = repairable changes that are more severe and affect the associated tissue function; and stage III = changes that preclude the restoration of the structure of the liver, even with an improvement in water quality. The DTC was calculated by the sum of the number of lesion types within each of three stages multiplied by stage index, using the following mathematical equation proposed by Poleksić and Mitrović-Tutundžić (1994): $DTC=(1\times\Sigma I)+(10\times\Sigma II)+(100\times\Sigma III)$; where *I*, *II* and *III* are the number of lesions of stages I, II and III respectively. The DTC value obtained for each fish was used to calculate the median index for each DFB exposed group and their respective controls. The median DTC was divided into 5 categories; 0–10: functionally normal liver, 11–20: slightly to moderately damaged liver, 21–50: moderately to heavily damaged liver, 51–100: severely damaged liver, and >100: irreparably damaged liver. DTC values permitted the comparison of the occurrence and severity of the hepatic lesions between the fish exposed to DFB and the control group at the different experimental times.

2.7. Statistical analyses

For all the parameters analyzed, excluding DTC, differences between DFB exposed group and control group, at each exposure time (6, 24 and 96 h), were analyzed by Student's *t*-test. DTC results were compared by Mann–Whitney test. Values of $P<0.05$ were considered significant. Statistical analyses were performed using SigmaStat 3.5.

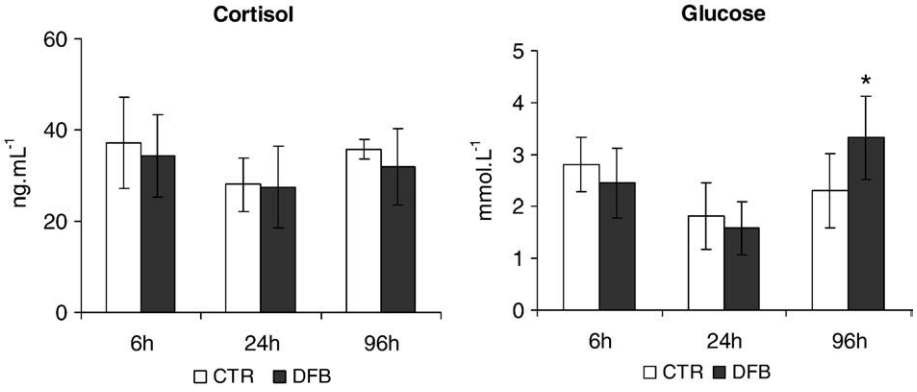

Fig. 2. Plasma cortisol and glucose concentrations of *Prochilodus lineatus* exposed to 25 mg L^{-1} of DFB, or only water (CTR), for different experimental periods (6, 24 and 96 h). Bars represent means and vertical lines of SD (number of animals: 4–7). *Different from respective control ($P<0.05$).

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3. Results

3.1. Hematological parameters

Hb was significantly higher in fish exposed to DFB for 6 h, than the respective control group (t=2.617; P=0.022). After 96 h Hb decreased significantly in comparison to control (t=9.209; P<0.001) and RBC also showed a significant reduction (t=3.299; P=0.004), along with a significant increase in MCV (t=4.035; P<0.001), in relation to the respective controls. Hct remained unaltered (Fig. 1).

3.2. Cortisol and glucose

There was a significant increase in plasma glucose concentration (t=2.930; P=0.009) in fish exposed to DFB only at the experimental time of 96 h, in relation to its control group. No significant alterations were found for plasma cortisol level at any of the experimental times (Fig. 2).

3.3. Osmo-ionic parameters

Plasma chloride concentrations and osmolarity increased significantly (t=2.550; P=0.023 and t=9.308; P<0.001) after 96 h of exposure to DFB in relation to the control group. No variations were observed in plasma sodium concentrations in fish exposed to DFB at any of the experimental times (Fig. 3).

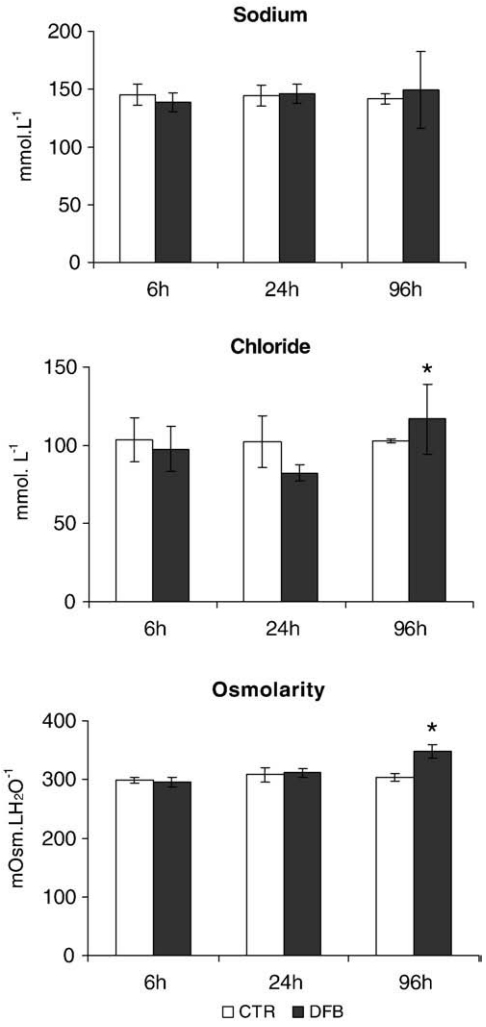

Fig. 3. Plasma concentrations of sodium and chloride and plasma osmolarity of *Prochilodus lineatus* exposed to 25 mg L^{-1} of DFB, or only water (CTR), for different experimental periods (6, 24 and 96 h). Bars represent means and vertical lines of SD (number of animals: 4–14). *Different from respective control (P<0.05).

3.4. Liver catalase and glutathione-S-transferase activities

Fish exposed to DFB showed a significant increase in hepatic activity of GST (t=8.334; P<0.001) and catalase (t=3.516; P=0.002) after 96 h of exposure when compared with the control groups (Fig. 4).

3.5. Acetylcholinesterase activity in muscle

A significant inhibition in muscle AChE activity was observed in *P. lineatus* exposed to DFB, at all the experimental periods (6 h: t=3.537 and P=0.006; 24 h: t=2.760 and P=0.028; 96 h: t=2.273 and P=0.035), when compared to respective control groups (Fig. 5).

3.6. Liver histopathology

The fish exposed to DFB showed several histological changes in the liver (Table 1; Fig. 6). The most frequent alterations were: increase in nuclear and cellular volume, cytoplasmic degeneration, nuclear degeneration, nuclear vacuolation (Fig. 6B and C) and bile stagnation (Fig. 6D). DTC values (Fig. 7) determined for the liver of *P. lineatus* exposed to DFB was significantly greater than the respective controls, at all the experimental times (6 h: t=16 and P=0.016; 24 h: t=36 and P=0.024; 96 h: t=64 and P=0.048).

4. Discussion

In the present work DFB was tested at a concentration of 25 mg L^{-1}, which corresponds to one half of the LC_{50} 96 h determined for the majority of fish species tested (Fisher and Hall, 1992), but exceeds DFB solubility in water, that varies from 0.1 mg L^{-1} at 20 °C to 1.0 mg L^{-1} at 25 °C. This DFB concentration, over its solubility in water, was selected for evaluation considering that DFB can be administered to fish, as an agent against parasites, both by bath methods and by medicated feed. In fact, soon after DFB was added to the water a white precipitate was formed in the bottom of the experimental aquaria and during fish sampling it was observed that all fish exposed to DFB had their digestive tract (stomach and mainly intestine) filled with a white powder. Therefore the procedure here employed allowed fish to be exposed to DFB both through water and food.

Exposure to various types of chemicals, including those related to current aquaculture practices, may induce changes in some of the hematological variables of fish (Heath, 1995) which are frequently used to assess fish health (Martinez and Souza, 2002). In the present work *P. lineatus* showed a significant decrease in Hb content and RBC count after 96 h of exposure to DFB; these reductions were not followed by a decrease in hematocrit, indicating that the volume of the remaining erythrocytes might have increased, as demonstrated by MCV values. These hematological alterations indicate that DFB is causing hemolysis.

The DFB metabolite 4-chloroaniline is highly toxic to fish (Fisher and Hall, 1992). The degradation of 4-chloroaniline, via cytochrome P450, can generate reactive oxygen species (ROS) that are capable of causing hemolytic anemia. ROS can oxidize cellular components, causing damage to the cytoskeleton and membranes, leading to lysis of erythrocytes (Budinsk, 2000). The increased activity of detoxifying enzymes such as GST and antioxidant enzymes such as catalase, showed by *P. lineatus* after 96 h exposure to DFB, corroborates the hypothesis that hemolysis observed in this study might be the result of oxidative stress induced by DFB.

The detoxification process for xenobiotics involves an enzyme apparatus that includes biotransformation and antioxidant enzymes. Glutathione Glutathione-S-transferases (GST) are a group of enzymes catalyzing conjugation of reduced glutathione (GSH) with a variety of

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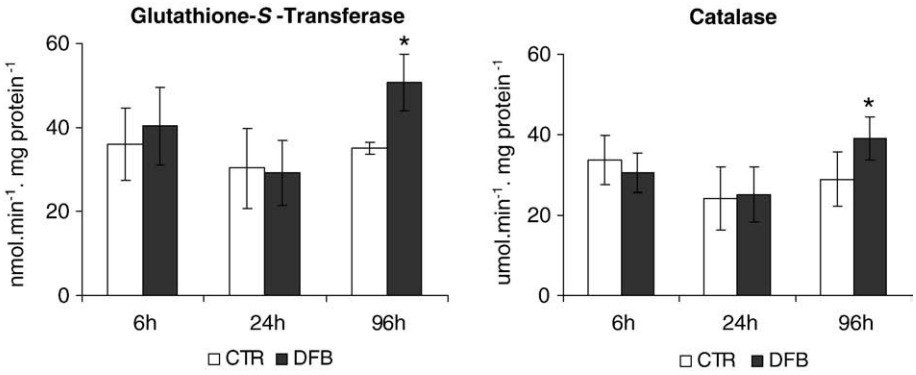

Fig. 4. Hepatic activity of glutathione-*S*-transferase and catalase of *Prochilodus lineatus* exposed to 25 mg L^{-1} of DFB, or only water (CTR), for different experimental periods (6, 24 and 96 h). Bars represent means and vertical lines of SD (number of animals: 5–13). *Different from respective control ($P<0.05$).

electrophilic metabolites, and involving detoxification of both reactive intermediates and oxygen radicals (Di Giulio et al., 1995). Raised GST activity has been associated with defensive adaptation of organisms to the presence of a variety of organic compounds in the environment (Van der Oost et al., 2003). In the present work, fish exposed to DFB for 96 h showed an increase in hepatic GST activity, indicating that the conjugation reactions that occur in phase II of xenobiotic metabolism would be one of the pathways in the metabolism of DFB or its metabolites.

These reactions occurring during the metabolism of xenobiotics can generate ROS, such as $O_2^{-}\cdot$, H_2O_2 and $\cdot OH$, that can cause damage to cellular components. Normally, the cells possess antioxidant defense systems that prevent the formation of ROS (Lackner, 1998). Catalase is an antioxidant enzyme that acts specifically on H_2O_2, forming oxygen and water. The increase in catalase activity in the liver of *P. lineatus* after 96 h of exposure to DFB indicates the induction of this enzyme, probably due to the increased production of hydrogen peroxide during metabolism of the insecticide.

Cortisol is the major corticosteroid hormone in fish and toxicants may have a significant effect on its dynamics (Wendelaar Bonga, 1997; Mommsen et al., 1999). In *P. lineatus* exposed to DFB no changes on cortisol levels were detected and blood cortisol concentration varied from 27 to 37 ng mL^{-1} (Fig. 2). These values are quite low in comparison to the mean plasma cortisol concentration, 165 ng mL^{-1}, determined for *P. lineatus* submitted to confinement stress (Camargo and Martinez, 2006). The absence of a significant elevation in plasma cortisol does not necessarily indicate the lack of a stress response due to the presence of DFB. The increase in cortisol might have occurred before the first blood sampling, which was only 6 h after of the onset of the pollutant exposure (Langiano and Martinez, 2008). Most fish species tested show their highest plasma increase in cortisol within about 0.5–1 h after a stressful disturbance (Barton, 2002). It is also important to consider that exposures to certain types of toxicants are obviously detrimental to fish in that they cause impair fish health, but do not necessarily evoke characteristic increases in cortisol titer, normally associated with the stress response (Barton and Iwama, 1991). Thus, the measurement of plasma cortisol alone may not necessarily reflect the degree of stress experienced by the fish (Barton, 2002) and cortisol levels complemented by other parameters, such as plasma glucose, may give a better indication of the organismal stress response.

When *P. lineatus* was exposed to DFB for 96 h, an increase in plasma glucose was observed. This hyperglycemia is commonly shown by fish under stress and it helps the animal by providing energy substrates to tissues such as brain, gills and muscles, to cope with the increased energy demand (Barton, 2002). In many teleost species the hyperglycemic response is mainly mediated by adrenaline and cortisol, and can occur by glycogenolysis and gluconeogenesis (Wendelaar Bonga, 1997). In the present study, it was not possible to correlate the hyperglycemic response with cortisol circulating levels. However, it is important to take into account that cortisol-induced changes in tissue metabolism occur even in the absence of increased plasma cortisol levels (Mommsen et al., 1999).

Cholinesterase enzymes (acetylcholinesterase and butyrylcholinesterase) hydrolyze the neurotransmitter acetylcholine in cholinergic synapses of vertebrates, but there is evidence that acetylcholinesterase (AChE) is most important in fish muscle (Habig et al., 1988; Alves Costa et al., 2007). Acetylcholine is the primary neurotransmitter in the sensory and neuromuscular systems of fish and AChE is involved in the deactivation of acetylcholine at nerve ends. The activity of this system is vital to normal behavior and muscular function (Ferrari et al., 2007) and it represents a prime target on which some toxicants can realize a detrimental effect (Kirby et al., 2000). AChE is sensitive to

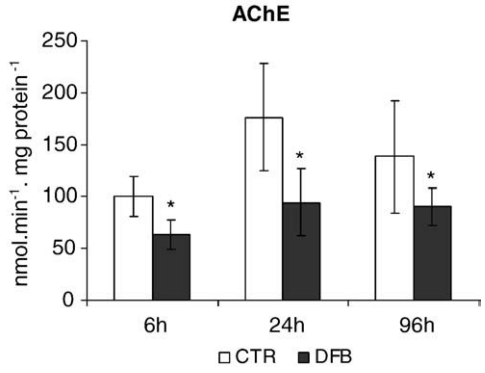

Fig. 5. Acetylcholinesterase activity (AChE) in muscle tissue of *Prochilodus lineatus* exposed to 25 mg L^{-1} of DFB, or only water (CTR), for different experimental periods (6, 24 and 96 h). Bars represent means and vertical lines of SD (number of animals: 4–13). *Different from respective control ($P<0.05$).

Table 1
Histological alterations found in the liver of *P. lineatus* following acute exposures to DFB and their respective stages of damage to the tissue

Stage I
Melanomacrophages aggregates
Cellular hypertrophy
Nuclear hypertrophy
Nuclei in the periphery
Stage II
Nuclear vacuolation
Cytoplasmic degeneration
Nuclear degeneration
Bile stagnation
Pyknotic nuclei

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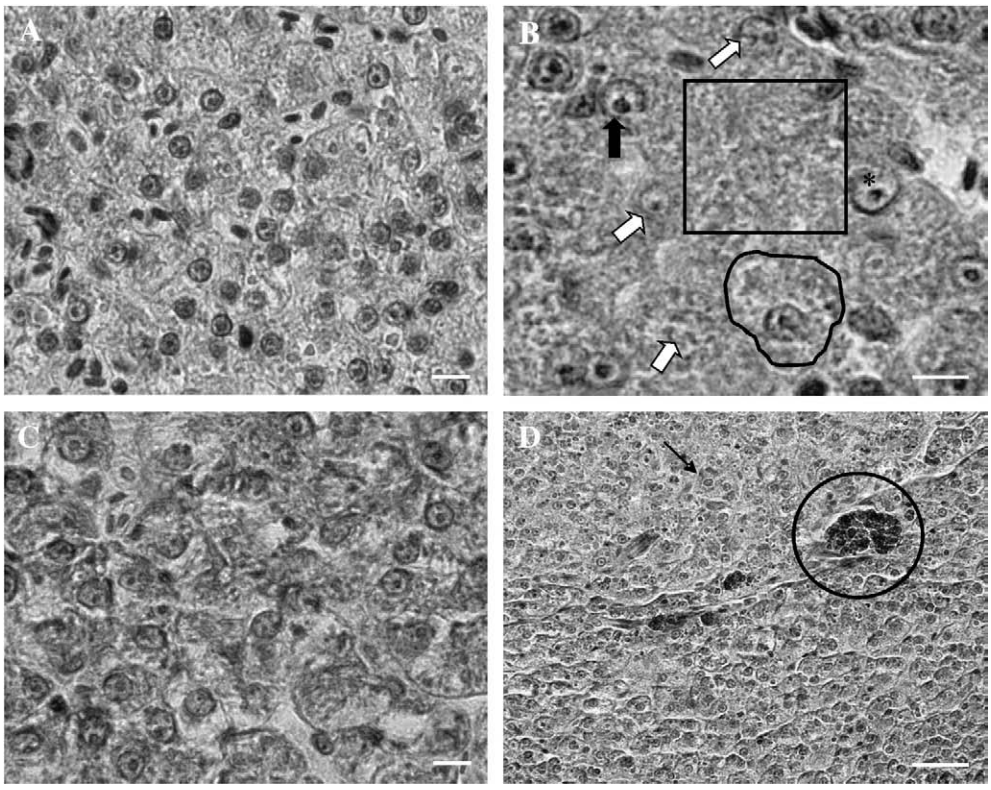

Fig. 6. Photomicrograph of the hepatic tissue of *Prochilodus lineatus* exposed to DFB. (A) Normal hepatic tissue from control fish; (B) Nuclear vacuolation (black arrow), nuclear degeneration (white arrows); cytoplasmic degeneration (square); nuclear hypertrophy (*) and cellular hypertrophy (contour); (C) Area showing cytoplasmic and nuclear degeneration; (D) Melanomacrophages aggregate (circular area) and bile stagnation (arrow). (HE). Scale bar corresponds to 5 μm (A, B and C) and 20 μm (D).

organophosphate and carbamates pesticides that are well known as potent AChE inhibitors (Van der Oost et al., 2003). Besides these two classes of pesticides, AChE has been shown to be sensitive to other types of contaminants, such as other pesticides, metals, detergents and products from the combustion of hydrocarbons, which contain tertiary and quaternary ammonia, among others (Payne et al., 1996). In the present work, *P. lineatus* exposed to DFB showed significant decrease in muscle AChE activity at all the experimental times, indicating an inhibitory effect by this product. The inhibition of muscle AChE by DFB might be a possible explanation for the results found by Ellgaard et al. (1979) (cited by Eisler, 1992). These authors reported that mosquitofish, *Gambusia affinis*, exposed to DFB were 4 times more active than controls. The inhibition of muscular AChE enzyme causes a continuous and excessive stimulation of the nerve/muscle fibers which may result in tetany, paralysis and death (Kirby et al., 2000).

Inhibition of AChE activity has been shown to be associated with a hyperglycemic response (Rahimi and Abdollahi, 2007). This is explained by the fact that with inhibition of muscle AChE, the animal spends more energy due to manifestations such as hyperesthesia, intermittent spasms, muscular tremors and convulsions. This involuntary energy demanding activity triggers the release of glucose by glycogenolysis in the liver to meet the body's energy requirement. Rahimi and Abdollahi (2007) reported that during these conditions, the flow of oxygen is greatly augmented and results in markedly increased rate of reactive oxygen species generation and consequently leads to occurrence of oxidative stress. Therefore, both hyperglycemia and enhanced catalase activity as demonstrated in the present work, may also be related to the inhibition of muscle AChE in *P. lineatus* exposed to DFB.

In freshwater fish, the osmotic water influx and diffusive losses of ions, such as Na^+ and Cl^- are compensated by the excretion of large volumes of dilute urine and the active ion uptake to replace lost ions (Evans et al., 1999). Many pollutants cause ionic dysfunctions by affecting the organs involved in osmoregulation, through changes in their metabolism or mechanisms of active transport of ions (Heath, 1995; Pelgrom et al., 1995). In the present work, fish exposed to DFB showed significant variation in Cl^- concentration along with osmolarity, both parameters increased significantly after 96 h of exposure, but plasma concentrations of Na^+ remained unaltered. The increased plasma osmolarity might be a consequence of the increase in plasma glucose observed after 96 h of exposure to DFB. While the increase in plasma Cl^-, without a concomitant increase in plasma Na^+, could

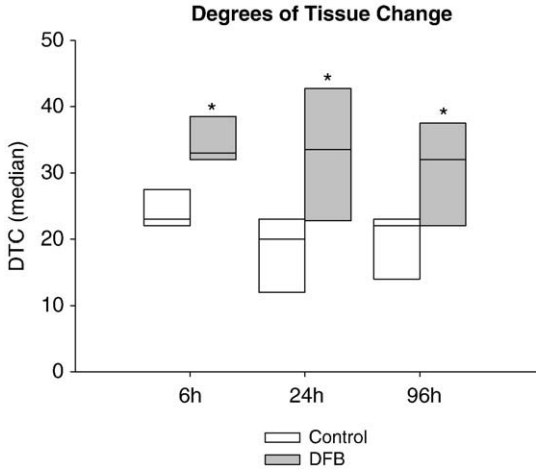

Fig. 7. Degree of Tissue Change (DTC) calculated for the hepatic tissue of *Prochilodus lineatus* exposed to 25 mg L^{-1} of DFB, or only water (CTR), for different experimental periods (6, 24 and 96 h). Vertical line inside the vertical box represents the median and the upper and lower limits of the box represent the percentiles (75 and 25% respectively). Number of animals varied from 5 to 7. *Different from respective control ($P<0.05$).

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represent a minor transitory ionic variation, without a great influence on osmolarity.

The liver is considered to be the main organ responsible for the detoxification of xenobiotics (Hinton et al., 1992), and is thereby subject to alterations, since it normally accumulates high concentrations of toxic substances (Heath, 1995). In the present study, the fish exposed to DFB showed a series of histological alterations in the liver. The most pronounced were: increase in cellular and nuclear volume, cytoplasmic and nuclear degeneration and bile stagnation. These histological alterations were considered not pesticide specific but changes generally associated with the response of hepatocytes to toxicants (Van Dyk et al., 2007). Quantitative analysis of the hepatic alterations showed that *P. lineatus* was affected after exposure to DFB, since DTC values for the liver of fish exposed to the insecticide were significantly greater than the respective controls, at all the experimental times. Exposure period to DFB did not influence DTC values, which ranged from 30 to 35, indicating the occurrence of alterations that might impair normal organ function, but are reversible upon removal of the toxic agent from the water.

The cellular and nuclear hypertrophy observed in the hepatic tissue indicates intensive metabolic activity of the hepatocytes. Given that the size of these cells reflects their functional state, hypertrophy can be considered a response to stress which does not compromise the normal functions of the organ (Takashima and Hibiya, 1995). Increased metabolic activity can be confirmed by the induction of liver GST, as observed in fish exposed to DFB. This insecticide also promoted hepatocellular changes and altered GST liver activity in mice, after its oral administration (Young et al., 1986). In contrast, cytoplasmic and nuclear degeneration represent more serious lesions (stage II alterations) which, although reversible, can impair the functions performed by the liver, since the metabolically active tissue area is diminished. These more severe alterations might be related to oxidative damage in the hepatic tissue caused by oxyradicals generated by the herbicide exposure. This idea is supported by the increased catalase activity in the liver of *P. lineatus* after DFB exposure and also by the fact that liver is the site of multiple oxidative reactions and maximal free radical generation (Gul et al., 2004). Bile stagnation is characterized by the remains of the bile in the form of brownish-yellow granules in the cytoplasm of the hepatocytes and indicates that the bile is not being released from the liver. This accumulation of bile indicates possible damage to hepatic metabolism (Fanta et al., 2003).

The current results show that acute exposure to DFB can cause alterations in several parameters of the neotropical fish *P. lineatus*. The insecticide leads to a reduction in the number of erythrocytes, accompanied by a decrease in hemoglobin content after 96 h exposure, probably due to hemolysis. Hyperglycemia indicates energy mobilization, and may have contributed to the increase in osmolarity after 96 h exposure to DFB. The induction of GST and catalase activities indicates the activation of xenobiotic metabolic pathways and antioxidant defenses. The decrease in muscle AChE at all the experimental times demonstrates that DFB is an effective AChE inhibitor. In addition, the occurrence of hepatic lesions, although moderate, can impair normal liver function. The above results clearly indicate that DFB can cause health disorders in fish and further studies are needed to better define its safe use in fish farming.

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