Histological and Histometric Evaluation of the Liver in *Astyanax Bimaculatus* (Teleostei: Characidae), Exposed to Different Concentrations of an Organochlorine Insecticide

LUCAS MARCON,[1]* NILO BAZZOLI,[1] ANN HONOR MOUNTEER,[2] AND LAÉRCIO DOS ANJOS BENJAMIN[3]

[1]Programa De Pós-Graduação Em Zoologia De Vertebrados Da PUC Minas. Av. Dom José Gaspar, 500, Coração Eucarístico, Belo Horizonte, Minas Gerais, Brasil
[2]Departamento De Engenharia Civil, Universidade Federal De Viçosa. Av. Peter Henry Rolfs S/N, Campus Universitário, Viçosa, Minas Gerais, Brasil
[3]Departamento De Medicina Veterinária, Universidade Federal De Viçosa. Av. Peter Henry Rolfs S/N, Campus Universitário, Viçosa, Minas Gerais, Brasil

ABSTRACT

To investigate possible morphological changes to the liver tissue of lambaris, *Astyanax bimaculatus* (Linnaeus, 1758), females were exposed to treatments of sublethal concentrations of the insecticide Thiodan® for 96 hr. Treatments included three sublethal concentrations of 1.15, 2.3, and 5.6 μg L^{-1} of Thiodan® and a control group without insecticide. The action of Thiodan® at sublethal concentrations did not affect the morphological structure of the liver as a whole, but changes in isolated locations of the hepatic parenchyma were observed. Glycogen depletion, nuclear and cytoplasmic deformation, nuclear and cytoplasmic hypertrophy, hyperemia, and cellular degeneration in liver cells at the different concentrations studied were recorded. These observed changes in the livers were greater in groups exposed to Thiodan® in comparison to the control group. Furthermore, there was a change in the diameter of the nuclei and cytoplasm of hepatocytes in the different treatments. The groups exposed to Thiodan® also exhibited a larger number of hepatocyte nuclei and a reduction in the amount of cytoplasm. We conclude that for the exposure period and concentrations of Thiodan® analyzed, the morphology of hepatic tissue had a cellular adaptive response. Anat Rec, 298:1754–1764, 2015.

Key words: histology; hepatic tissue; hepatocyte; teleost fish

Thiodan® is a commercial organochlorine insecticide comprised of α-endosulfan (70%) and β-endosulfan (30%), the former being the more toxic isomer. Endosulfan sulfate is a metabolic derivative of endosunfan. It is more environmentally stable than endosunfan and, thus, is the most toxicologically significant of its metabolites (Hose et al., 2003). Endosulfan serves as a broad-spectrum insecticide that works through contact and ingestion. This chlorinated hydrocarbon is highly toxic and so its use is banned by many nations (Wan et al., 2005).

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*Correspondence to: Lucas Marcon, Programa de Pós-graduação em Zoologia de Vertebrados da PUC Minas. Av. Dom José Gaspar, 500, Coração Eucarístico, 30.535-610, Belo Horizonte, Minas Gerais, Brasil. E-mail: kalukasss@hotmail.com

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Runoff and infiltration from irrigation brings endosulfan into aquatic ecosystems where its toxicity threatens aquatic organisms (Stegeman and Lech, 1991). Being neurotoxic to fish, even in low concentrations (Pereira et al., 2012), it is not surprising that studies have found fishes to be sensitive to endosulfan (Carriger et al., 2011). The sexes can exhibit differences in sensitivity to endosulfan because of the amount of lipid stored in their tissues (Ballesteros et al., 2007). Furthermore, the insecticide causes behavioral changes such as convulsions and erratic swimming (Capkin et al., 2006), as well as being a contaminant that accumulates in the tissues, as observed in tilapia, *Oreochromis niloticus* (Henry and Kishimba, 2006).

Acute or chronic exposure of fish to endosulfan can cause structural modifications to the gills and, with changes to the osmotic equilibrium, impair gas exchange, although it depends on the concentration (Capkin et al., 2006). Morphological changes in the liver may include the appearance of vacuolization of hepatocytes, glycogen depletion, alteration of capillary sinusoids, change in size of hepatocytes, and necrosis (Cengiz et al., 2001; Glover et al., 2007) which, according to some authors, may influence the synthesis of vitellogenin in the liver and the formation of vitellogenic follicles (Al-Ghais, 2013; Barni et al., 2014; Marcon et al., 2014, 2015). Furthermore, hepatic physiology can be compromised differently depending on the period of exposure to pesticides, the concentration of the contaminant, and dietary habits (Ribeiro et al., 2006; Al-Ghais, 2013; Barni et al., 2014; Benze et al., 2014).

The lambari, *Astyanax bimaculatus* (Linnaeus, 1758) (Teleostei: Characidae), occurs in neotropical rivers such as those found in most Brazilian ecosystems. It is an omnivorous forager and has become a vital part of the nutritional base of many human populations along the coast (De Carvalho et al., 2009). Being small and having a high reproductive rate, the lambari has been found to quickly adapt to being kept in the laboratory and thus is an ideal choice for experimental studies. Furthermore, it has been successfully used as a biomarker of contamination in water (dos Santos et al., 2012), and so we chose to use this species to study the effects of the endosulfan-containing insecticide Thiodan® on fish. There have been few studies that used histometric data in assessing the toxicological effects of Thiodan® on the livers of lambaris. Therefore, the objective of this study was to analyze the amount and intensity of cellular changes in the hepatic tissue of female *A. bimaculatus* at different sublethal concentrations of Thiodan®.

MATERIALS AND METHODS

For the present study, female *A. bimaculatus* in advanced stage of maturation were collected during the months of February to May 2012 at Prata's Fish Farm, Eugenópolis, Minas Gerais (21°05′56″S 42°11′13″W). Individual female lambaris were chosen based on aspects of the body, including a bulging belly, a dilated and vascularized genital pore, a weight of 11.52 ± 2.0 g and a total length of 9.12 ± 0.64 cm (values expressed as mean ± SD).

Fish were transported to the Department of Veterinary Medicine (DVT) of the Federal University of Viçosa (UFV) and kept in 50-L glass aquariums at a density of 2.5 g of fish per liter of water for acclimation for 10 days. Water used in acclimation was acquired from the sewage treatment system of the UFV, and is suitable for human consumption (CONAMA, 2005). The aquariums contained dechlorinated (by evaporation) tap water and had a biological filter (250 L h^{-1}) and an aquarium aerator. Water temperature was maintained using a thermometer and thermostat in each aquarium (26.0°C ± 1.0°C). Fish were fed an ad libitum diet containing 35% protein, twice daily. The self-cleaning design of the aquaria and routine siphoning after feeding removed fecal waste quickly. Fifty percent of the water was replaced at 48-hr intervals and no mortality was recorded during the acclimation period.

Test of Acute Toxicity in a Static System—LC_{50}

We conducted a test of acute toxicity for a static system using a lethal concentration of Thiodan® (endosulfan 350 g L^{-1}) to determine the 50% mortality (LC_{50}) of the animals after 96 hr. One hundred twenty females were subjected to food restriction for 24 hr before the start of the experiment in order to avoid the accumulation of organic matter that could influence the action of the pesticide and the quality of the water, in accordance with NBR 15088 (ABNT, 2007).

Five fish were placed in each of 24 1.5-L plastic aquariums. Three replicates of five fish were established for each concentration of endosulfan and an insecticide-free control group (Fig. 1a,b): 0 μg L^{-1} (control), 1.5 μg L^{-1}, 3.0 μg L^{-1}, 6.0 μg L^{-1}, 12.0 μg L^{-1}, 24.0 μg L^{-1}, 48.0 μg L^{-1}, and 96.0 μg L^{-1}. The different concentrations were obtained by diluting Thiodan® first to 1:100 (Intermediate Standard Solution, containing 3.500 mg L^{-1} of endosulfan) and second to 1:100 (Standard Final Solution, containing 35.000 μg L^{-1} of endosulfan), thus enabling the observation of the total toxicity of the pesticide. Mortality checks were performed every 6 hr for 96 hr, removing dead animals when found in order to minimize any interference by decomposition (Table 1). Water used in the LC_{50} test was acquired from the sewage treatment system of the UFV, and is suitable for human consumption (CONAMA, 2005). The temperature was measured with a thermometer in an aquarium without fish, so that no additional stress was caused to any group. The program used to calculate the LC_{50} was TOXCALC, which determined the value and confidence interval (CI) using the PROBITUS ($P < 0.05$) method.

Test of Sublethal Toxicity in a Static System—Fish Contamination

After determining the LC_{50}−96 hr, we tested effects at sublethal concentrations of 1.15, 2.3, and 5.6 μg L^{-1} of endosulfan under varying acclimation and feeding conditions. All concentrations selected were below the laboratory predetermined LC_{50} as recommended by the Brazilian test standard NBR 15088 (ABNT, 2007). We tested the following conditions: 1) no acclimation, without feeding during the 96 hr of exposure; 2) no acclimation, with feeding during the 96 hr of exposure; 3) 10 days acclimation, without feeding during the 96 hr of exposure; and 4) 10 days acclimation, with feeding during the 96 hr of exposure. Three replicates containing ten fish were established for each concentration of

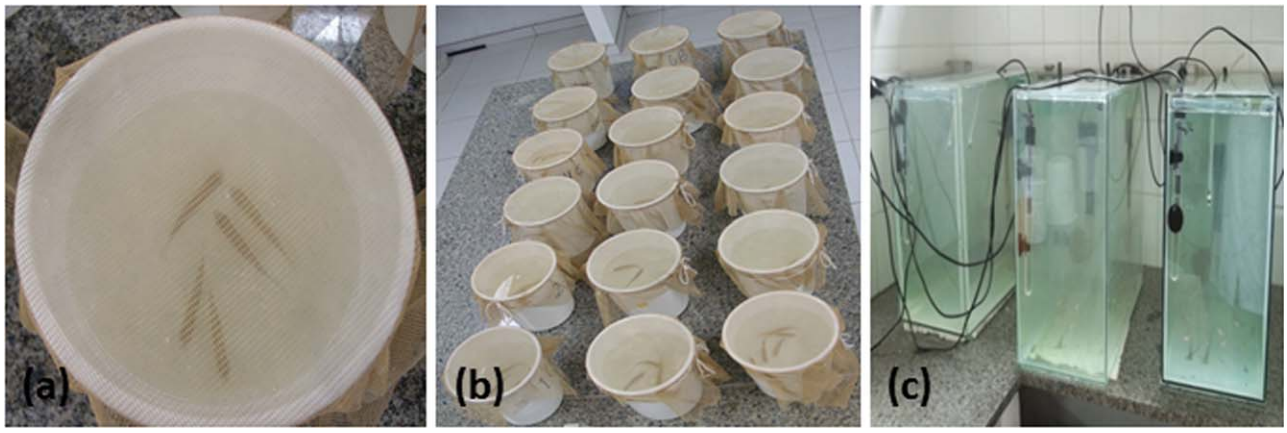


Fig. 1. Distribution of five fish in plastic aquariums (**a**) with three replicates for each concentration (**b**) in the acute toxicity (LC_{50}). Sublethal toxicity exposure test in glass aquaria (**c**).

endosulfan and acclimation/feeding condition, as well as for the endosulfan-free control treatments, for a total of 480 fish. Fish were distributed among 12 50-L glass aquaria daily (Fig. 1c). Temperature was controlled (25.5°C ± 1.0°C) using thermostats in each aquarium and recorded.

Fish were exposed to a 12-hr photoperiod. Water quality was monitored at the beginning and end of the exposure period using procedures described in Standard Methods (American Public Health et al., 2005). Values of the water quality parameters (averages ± standard deviations) were: pH (phmeter model DN21, Digimed, São Paulo) = 7.2 ± 0.3, apparent color (Nessler visual comparison) = 4.2 ± 3.5 mg L^{-1} color units, turbidity (turbidimeter model 2100AN, Hach, São Paulo) = 1.8 ± 1.3 NTU, dissolved oxygen (iodometric titration) = 6.9 ± 0.6 mg $L^{-1,}$ 5-day biochemical oxygen demand = 4 ± 0.6 mg L^{-1}, free residual chlorine (DPD colorimetric kit, Hach, São Paulo) was not detected, nitrate (nitrate kit, Hach, São Paulo) = 0.24 ± 0.28 mg L^{-1} and total hardness (titration) = 28.2 ± 5.9 mg L^{-1} $CaCO_3$. Water quality parameters remained within the range recommended for the NBR 15088 static sublethal test system (ABNT, 2007) as well as for fish breeding and farming (CONAMA, 2005) during the exposure period.

Histological Analysis

After 96 hr of exposure to the insecticide, six fish from each group were collected from the different experiments. Liver fragments were collected after the fish were euthanized with benzocaine (1:10,000) and by transverse section of the spinal cord, in accordance with the ethical principles established by the Brazilian College of Animal Experimentation (COBEA, http://www.cobea.org.br). The research was approved by the Committee of Ethics of the Federal University of Viçosa (Protocol 24/2009).

TABLE 1. Values recorded for mortality in lambaris *A.bimaculatus* exposed to Thiodan® for determination of LC_{50}−96 hr

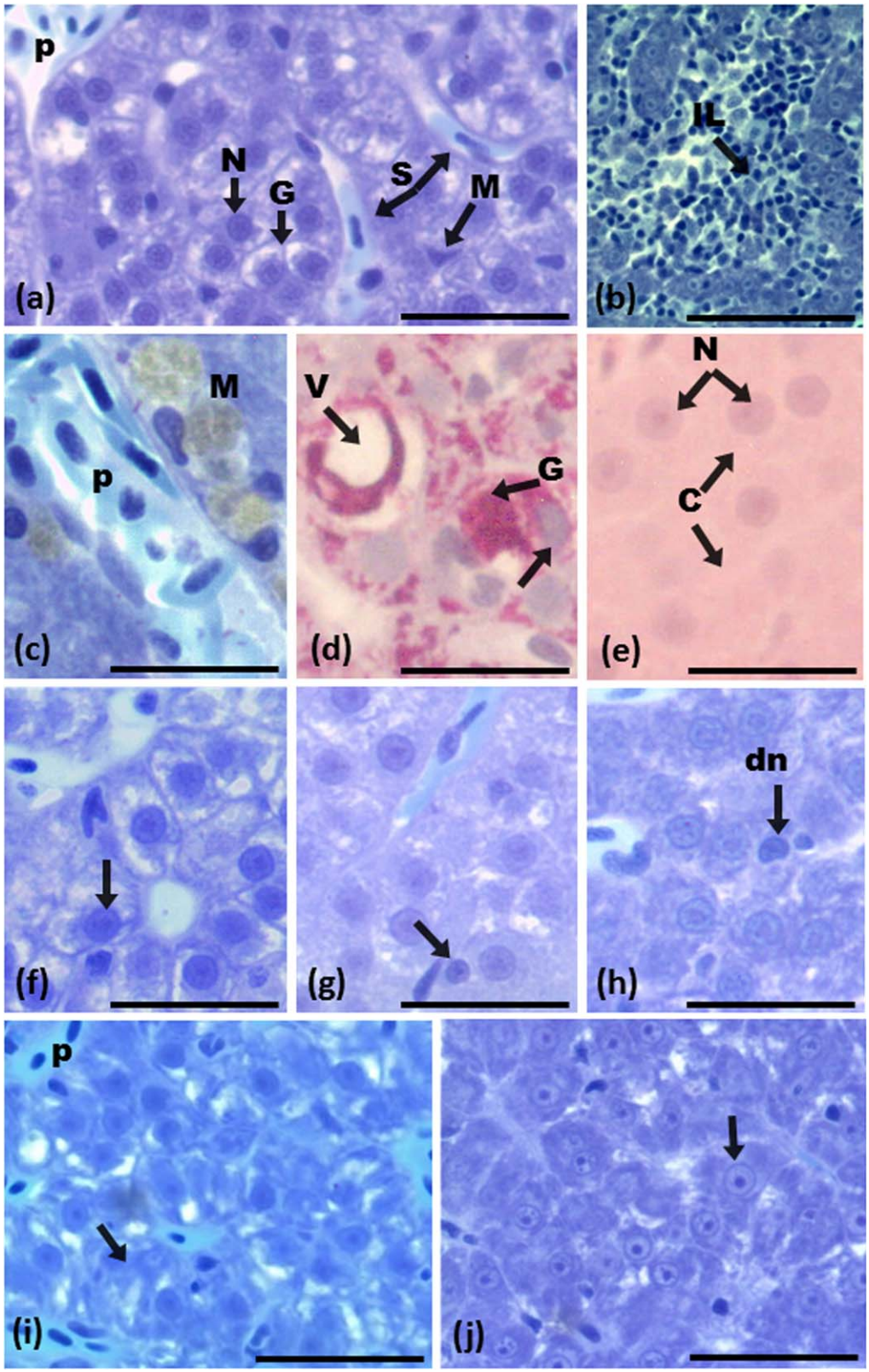
Thiodan® (μg L^{-1})	Expost (*n*)	Dead (*n*)
0	15	0
1.5	15	0
3	15	1
6	15	3
12	15	4
24	15	11
48	15	15
96	15	15

For histology, all six samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 24 hr, and embedded in glycolmethacrylate (Leica, Historesin). Sections with a thickness of 3 μm were stained with toluidine blue for histologic and histometric analyses. Slides were mounted with Entellan and analyzed on an Olympus CX31 light microscope. Images were obtained using a SC 020 digital camera through GETIT Analysis, Olympus software. Histological slides were analyzed throughout their length and 10 fields were photographed, at random by selecting areas with normal tissue and without pathological alteration, with a 40× magnification for morphological and histometric analyses that were representative of the action of Thiodan® in the hepatic tissue. These analyses were compared to the pattern of organization of the livers of the control group.

Histopathological changes were evaluated semiquantitatively by the Degree of Tissue Change (DTC), which is based on the severity of each lesion for each organ, according to Schwaiger et al. (1997) and Benze et al. (2014). The DTCs were classified as progressive stages of impairment to organ function adapted from Poleksic and Mitrovic-Tutundzic (1994): Stage I, changes which do not compromise the organ's function; Stage II, more severe and impairment to the organ's function; and Stage III, very severe and irreversible damage. The value of DTC was calculated for each animal using the formula: $DTC = (10^{\circ} \times \sum I) + (10^{1} \times \sum II) + (10^{2} \times \sum III)$, with I, II, and III corresponding to number of lesions in Stage I, II, and III in 10 photographed fields for each animal, respectively. The DTC value obtained for each fish ($n = 6$) was used to calculate the mean index for each Thiodan® exposed group and their respective controls. Mean DTCs were placed into one of five categories: 0–10 = normal tissue; 11–20 = mild to

moderate tissue damage; 21–50 = moderate to severe tissue damage; 51–100 = severe tissue damage; and greater than 100 = irreparable damage to the tissue.

The amount of glycogen in hepatocytes was evaluated semiquantitatively, and visualized through the histochemical reaction of periodic acid Schiff (PAS) counter-



stained with hematoxylin. To better understand the results, as described by Camargo and Martinez (2007), levels were determined using the following score: 0 = no glycogen; 1 = little glycogen; 2 = moderate amount of glycogen; and 3 = much glycogen. After this analysis the average glycogen content ($n = 6$) of the hepatocytes was calculated for the control groups and for of the groups exposed to different toxic concentrations.

Histometric Analysis

For histometric analysis, measurements of the diameters (μm) of ten nuclei and cytoplasm of hepatocytes were performed for each animal. Sixty measurements were made for each group using the Image-Pro Plus program by drawing two perpendicular lines at the limits of the nucleus and the cytoplasm of the hepatocytes. For calculating liver tissue formation, the number of nuclei and cytoplasm hepatocytes, sinusoids, and macrophages with elongated or irregular shape next to the sinusoids were counted in 10 photographic fields per fish liver using a grid with 441 intersection points per photo in an area of 10,000 μm^2, for a total of 4,410 points/animal. The mean and standard deviation for the values of measurements of the diameters and liver tissue formation were calculated for each treatment group.

Hepatosomatic Index and Gonadosomatic Index

To calculate the Hepatosomatic Index (HSI) and Gonadosomatic Index (GSI) of each specimen, the biometric data of body mass (BW, g), liver mass (LW, g) and ovary mass (OW, g) were recorded. The mean and standard deviation for the values of HSI (FWx100/BW) and GSI (OWx100/BW) were calculated for each treatment group.

Statistical Analysis

Quantitative variables were submitted to homoscedasticity (Levenes) and normality (Shapiro-Wilk) tests. Subsequently, analysis of variance was performed to test for significant differences in the average values of the indices, calculated at the time of collection (96 hr), between the control groups and the treated groups. The coefficient of variation to prevent Type I and Type II statistical errors were determined, and the parametric test of Duncan (DTC, GSI, and HSI) or Tukey (diameter μm of hepatocyte nuclei and cytoplasm) were performed to compare mean values. When the assumptions of normality and homogeneity were not met, even after appropriate transformations, the data were subjected to the nonparametric Kruskal–Wallis test. For the qualitative variables (values for glycogen), the Kruskal–Wallis test was used and $P < 0.05$ was the significance level employed

RESULTS

Test of Acute and Sublethal Toxicity in a Static System

For female *A. bimaculatus*, $LC_{50}-96$ hr = 13.67 μg L^{-1}, (CI of 10.1–18.4 μg L^{-1} $P < 0.05$) at an average water temperature of 23.3°C ± 0.72°C. In the static test system, Thiodan® exposure for 96 hr at sublethal levels resulted in abnormal behavior including erratic swimming, attempts to aggression, escape, surface gasping, and rapid respiration. These behaviors were observed up to 24 hr regardless of the exposure concentration but especially at 5.6 μg L^{-1} in the experiment without acclimation/without food, and in which average temperature was higher (26.9°C ± 0.9°C) than the other experiments (24.9°C ± 0.5°C).

Histological Analysis

Morphologically the liver of lambaris showed polygonal hepatocytes with vacuolated granular cytoplasm, central rounded nuclei, evident nucleoli, and the presence of a hepatopancreas. Hepatocytes were arranged in strands bordering the sinusoids (Fig. 2a) and there was no presence of bile stagnation. Leukocyte infiltrates were observed in all groups (Fig. 2b). In the treatment with acclimation/without food at 1.15 μg L^{-1}, macrophages with brown pigments were observed next to blood vessels and in liver parenchyma (Fig. 2c).

Vacuolation was evident in the hepatocytes of the analyzed females (Fig. 2a). For the treatment without acclimation/with food at 5.6 μg L^{-1}, the females' livers showed lipid accumulation in the hepatocytes through the presence of large vesicles surrounded by positively labeled glycogen, as shown by the PAS reaction (Fig. 2c). Furthermore, displaced nuclei were observed at periphery of the hepatocytes. In the experiment without acclimation/without food at the concentration of 5.6 μg L^{-1}, there was a negative reaction to PAS in the cytoplasm of the hepatocytes (Fig. 2d).

DTC Stage I alterations were observed in the control group and those exposed to Thiodan®, such as nuclear contour deformation, nuclear displacement to the periphery of the cell, nuclear atrophy, disorganization of hepatic cords, and cytoplasmic and nuclear hypertrophy. The displacement of the nucleus to the periphery of the hepatocyte was observed only in females of the treatment of without acclimation/with food at the concentration of 5.6 μg L^{-1}. For DTC changes at Stage II, nuclear degeneration (Fig. 3a) was rarely present in the treatments exposed to Thiodan®. Cytoplasmic degeneration (Fig. 3b) and hyperemia (Fig. 3c) were uncommon in all experiments. The presence of a pyknotic nucleus (Fig. 3d) was more frequent in the treatments of without acclimation/with food and with acclimation/with food. DTC changes at Stage III (necrosis) were absent in all

Fig. 2. (**a**) Normal histological organization of liver tissue of *A. bimaculatus*. (**b**) leucocyte infiltration (IL) in liver tissue of *A. bimaculatus*. (**c**) macrophages (M) next to a blood vessel. (**d**) Hepatocyte with clear vesicles (Vc) surrounded by glycogen (G) positively marked by PAS reaction and nucleus (arrow) displaced to the cell periphery. (**e**) Hepatocyte, with absence of glycogen shown by negative PAS reaction. Changes in DTC, Stage I—(**f**) Hepatocyte with nuclei (arrow) in the periphery. (**g**) Nuclear decrease (arrow). (**h**) Nuclear deformation (dn). (**i**) Hypertrophic cell (arrow). (**j**) Nuclear enlargement (arrow). Nucleus (N) and cytoplasm (C) of hepatocytes, sinusoids (S), and capillaries (P) with erythrocytes and glycogen accumulation (G) in the cytoplasm, and macrophages (M). Bars: a (30 μm); b (65 μm); c, d, and e (20 μm); f, g, and h (28 μm); i and j (30 μm). Staining: Toluidine blue in (a)–(c) and (f)–(j). Periodic acid Schiff (PAS) in (d) and (e).

treatments. DTC Stage I (Fig. 2e–i) and Stage II (Fig. 3a–d) alterations were observed in the control group and those exposed to Thiodan® ($P < 0.05$), but were more frequent in the treated groups, indicating moderate damage (Table 2) and possible impairment of hepatic function.

In all experiments, the control and treated groups had mean values for glycogen ranging from 1.00 to 3.00, except in the experiment without acclimation/without food at of 5.6 μg L^{-1}, which registered the lowest average. However, significant differences in liver glycogen ($P < 0.05$) were only found between the treatment at 5.6 μg L^{-1} (0.10 ± 0.10) and the treatments at 1.15 μg L^{-1} (2.66 ± 0.81) and 2.3 μg L^{-1} (3.00 ± 0.00).

Histometric Analysis

For fish exposed to Thiodan® at concentrations of 1.15 and 2.3 μg L^{-1}, nuclei diameter of hepatocytes was greater ($P < 0.05$) than the control in the experiment without acclimation/without food. For the experiment with acclimation/with food at 2.3 μg L^{-1}, we recorded a smaller nuclei diameter the hepatocytes ($P < 0.05$) than the control (Table 3). The diameter of the cytoplasm of the hepatocytes was greater in the experiments without acclimation/without food and the experiment with acclimation/without food in fish exposed to pesticides than the control ($P < 0.05$) (Table 3). For the experiments without acclimation/with food and with acclimation/with food, there was less cytoplasm of the hepatocytes in fish exposed to pesticides than the control fish ($P < 0.05$) (Table 3).

A higher number of hepatocyte nuclei were observed in fish exposed to pesticides than fish in the control groups ($p < 0.05$) (Table 4). When quantifying the cytoplasm of hepatocytes, a lower number ($P < 0.05$) were recorded in fish exposed to pesticides than the control fish (Table 4). For the number of macrophages, there was no significant difference ($P > 0.05$) between fish exposed to pesticides and the control fish (Table 4). The number of sinusoids was similar in most fish exposed to pesticides when compared to the controls (Table 4).

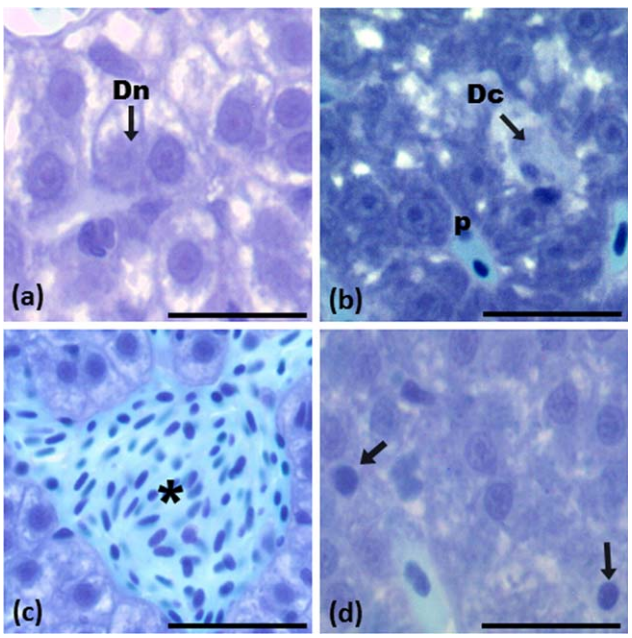


Fig. 3. Changes in DTC, Stage II—(**a**) Nuclear degeneration (Dn). (**b**) cytoplasmic degeneration (Dc). (**c**) hyperemia (*). (**d**) Presence of pyknotic nuclei (arrows). Capillaries (P) with erythrocytes. Bars: a, b and d (25 μm); c (30 μm). Staining: Toluidine blue.

Hepatosomatic Index and Gonadosomatic Index

HIS and GSI did not differ significantly ($P > 0.05$) between exposed and control fish. For HIS, however, differences between the concentration of 5.6 μg L^{-1} (0.38 ± 0.31) and 1.15 μg L^{-1} (0.88 ± 0.3) and 2.3 μg L^{-1} (0.84 ± 0.38) under the conditions of without acclimation/without food did differ significantly ($P < 0.05$).

DISCUSSION

In the present study, female *A. bimaculatus*, despite being small, showed a LC_{50}−96 hr of 13.67 μg L^{-1} endosulfan, which is above the concentration allowed by Brazilian legislation according to the National Environmental Council (CONAMA, 2005). Organochlorines, such as Thiodan®, act on axon sodium channels, increasing the period that these channels are open, which leads to an increase in flow of sodium into the membrane and causing a prolongation of the depolarization phase after the peak of the action potential. Because of this, fish die by hyperexcitation (Pereira et al., 2012). Consequently, the continued propagation of the nerve impulse causes tremors, convulsions, prostration, and subsequently death, as observed in insects (Sharma et al., 2012). Moreover, it can affect any type of crustacean, fish and mammal, even at low concentrations (Eto, 1990; Pereira et al., 2012). The CONAMA resolution 357 of 17 March 2005 established the allowable maximum value of 0.22 μg L^{-1} of endosulfan in fresh water to be used for animal farming (CONAMA, 2005). Gurure (1987) determined LC_{50}s' of 1.42, 3.80 and 10.3 μg L^{-1} for 96, 48, and 24 hr of exposure to endosulfan, respectively, for

TABLE 2. Degree of tissue change in the livers of female *A. bimaculatus* at different concentrations of Thiodan® after 96 hr

Experiment	Control	1.15 μg L^{-1}	2.3 μg L^{-1}	5.6 μg L^{-1}
1	8.83 ± 10.34^{a}	18.50 ± 10.09^{ab}	28.83 ± 8.32^{b}	25.66 ± 5.50^{ab}
2	4.33 ± 5.57^{a}	28.83 ± 8.10^{b}	27.80 ± 13.88^{b}	27.66 ± 5.71^{b}
3	5.66 ± 5.85^{a}	26.00 ± 7.73^{b}	25.50 ± 10.78^{b}	27.33 ± 9.20^{b}
4	7.50 ± 5.12^{a}	22.17 ± 7.80^{b}	20.33 ± 8.75^{b}	23.66 ± 8.80^{b}

1: Without acclimation and without food. 2: Without acclimation and with food. 3: With acclimation and without food. 4: With acclimation and with food. Values are expressed as mean ± standard deviation ($n = 6$). Different lowercase letters (a and b) indicate differences ($P < 0.05$) between groups in the same experiment. Parametric Duncan test.

TABLE 3. Average diameter (μm) of the hepatocytes' nuclei and cytoplasm in the livers of female *A. bimaculatus* at different concentrations of Thiodan® after 96 hr

Experiment	Control	1.15 μg L^{-1}	2.3 μg L^{-1}	5.6 μg L^{-1}
Nuclei				
1	4.61 ± 0.40^{ac}	4.98 ± 0.20^{b}	4.87 ± 0.28^{b}	4.39 ± 0.27^{c}
2	5.16 ± 0.71^{a}	5.01 ± 0.40^{a}	5.02 ± 0.20^{a}	5.15 ± 0.25^{a}
3	4.94 ± 0.19^{a}	4.96 ± 0.36^{a}	4.92 ± 0.82^{a}	5.08 ± 0.33^{a}
4	5.61 ± 0.44^{ac}	5.24 ± 0.40^{bc}	5.09 ± 0.28^{b}	5.33 ± 0.19^{c}
Cytoplasm				
1	8.85 ± 0.99^{a}	10.22 ± 1.09^{b}	9.74 ± 0.80^{b}	10.05 ± 1.29^{b}
2	10.84 ± 1.36^{a}	9.87 ± 1.53^{b}	9.75 ± 0.81^{b}	9.31 ± 0.80^{b}
3	9.60 ± 0.95^{a}	10.22 ± 0.94^{b}	10.03 ± 1.20^{ab}	10.33 ± 1.08^{b}
4	10.86 ± 1.30^{ab}	10.4 ± 1.35^{bc}	10.22 ± 1.38^{c}	10.22 ± 1.06^{c}

1: Without acclimation and without food. 2: Without acclimation and with food. 3: With acclimation and without food. 4: With acclimation and with food. Values are expressed as mean ± standard deviation ($n = 60$). Different lowercase letters (a,b and c) indicate differences ($P < 0.05$) between groups in the same experiment. Parametric Tukey test.

TABLE 4. Average number of hepatocytes' nuclei, cytoplasm, macrophage, and sinusoides in a grid with 441 intersection points in the liver tissue of female *A. bimaculatus* at different concentrations of Thiodan® after 96 hr

Experiment	Control	1.15 μg L^{-1}	2.3 μg L^{-1}	5.6 μg L^{-1}
Nuclei				
1	25.53 ± 4.94^{a}	29.8 ± 4.64^{b}	28.4 ± 4.59^{b}	29.1 ± 3.74^{b}
2	23.71 ± 4.90^{a}	28.9 ± 5.41^{b}	28.9 ± 5.45^{b}	29.0 ± 5.14^{b}
3	25.60 ± 5.38^{a}	29.4 ± 4.80^{b}	28.6 ± 5.55^{b}	29.3 ± 5.52^{b}
4	27.70 ± 5.94^{a}	31.4 ± 10.28^{b}	34.7 ± 6.64^{bc}	32.2 ± 6.54^{c}
Cytoplasm				
1	395.51 ± 10.56^{a}	396.66 ± 8.77^{a}	393.60 ± 15.26^{a}	400.80 ± 7.27^{a}
2	399.96 ± 13.07^{a}	398.30 ± 7.22^{a}	396.18 ± 11.43^{ab}	388.65 ± 19.54^{b}
3	396.03 ± 17.55^{a}	390.11 ± 16.20^{b}	394.45 ± 13.88^{ab}	392.05 ± 22.12^{ab}
4	393.81 ± 30.25^{a}	385.55 ± 27.23^{b}	390.36 ± 22.75^{ab}	391.36 ± 18.92^{b}
Macrophage				
1	1.20 ± 1.13^{a}	1.25 ± 0.89^{a}	1.01 ± 1.00^{a}	1.06 ± 0.94^{a}
2	1.01 ± 1.01^{a}	1.24 ± 0.79^{a}	1.02 ± 0.84^{a}	1.31 ± 0.92^{a}
3	0.98 ± 0.89^{a}	1.28 ± 0.97^{a}	1.15 ± 0.95^{a}	0.96 ± 0.90^{a}
4	1.01 ± 0.94^{a}	1.28 ± 1.09^{a}	0.86 ± 0.79^{a}	1.01 ± 0.79^{a}
Sinusoids				
1	18.75 ± 2.39^{a}	13.3 ± 3.46^{a}	17.96 ± 12.10^{a}	11.03 ± 4.65^{a}
2	16.03 ± 7.84^{ab}	12.60 ± 5.32^{a}	14.09 ± 6.31^{ab}	22.03 ± 6.85^{b}
3	18.30 ± 5.79^{a}	20.36 ± 7.73^{a}	16.81 ± 5.60^{a}	18.65 ± 9.64^{a}
4	18.50 ± 14.00^{a}	18.31 ± 13.26^{a}	$15{,}10 \pm 7.74^{a}$	16.45 ± 7.39^{a}

1: Without acclimation and without food. 2: Without acclimation and with food. 3: With acclimation and without food. 4: With acclimation and with food. Values are expressed as mean ± standard deviation ($n = 60$). Different lowercase letters (a,b and c) indicate differences ($P < 0.05$) between groups in the same experiment. Nonparametric Kruskal–Wallis test.

adult tilapia. It is evident that lambari is more resistant than adult Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758), provided that the experimental conditions were the same.

Altered behaviors of female *A. bimaculatus* were observed within 24 hr in all test concentrations, as reported by Pereira et al. (2012), who also found a decrease in acetylcholinesterase (AChE) activity. Da Cuna et al. (2011) observed behavior suggestive of neurotoxicity but, unlike the observations made by Pereira et al. (2012), there were no differences in AChE activity. Together these investigations demonstrate the deleterious effects to the nervous system caused by exposure to endosulfan. The present study also reported behavioral alterations that may have been due to neurotoxicity, but did not analyze AChE activity as it was not part of the objective.

Hepatic histological descriptions of the females in the present study were similar to those found for other fish (Wolf and Wolfe, 2005; Chehade et al., 2014), where hepatocytes are organized into rows or double strands of plates. Histology and physiology of fish livers are similar to that of mammals, however, Wolf and Wolfe (2005) report that there are some marked structural differences in the liver parenchyma of fish, in particular the absence of lobulation, which allows slow blood flow in the sinusoids with reduced chemical penetration into hepatocytes.

One of the functions of hepatocytes distributed in the liver is the production of the detoxification enzyme cytochrome P450 (Omiecinski et al., 2011), which is present in many tissues of mammals (Stegeman and Lech, 1991). The presence of pancreatic cells distributed in the

liver of *Astyanax altiparanae* (Chehade et al., 2014), as in *A. bimaculatus*, suggests that these cells have a role in detoxification (Lorenzana et al., 1989), but future studies are needed to prove this in these species. Another important structure for detoxification is the capillary endothelium, where the presence of cytochrome P450 has been identified and confirmed by immunoblot techniques (Lorenzana et al., 1989). Furthermore, endothelial cells of sinusoids can synthesize the cytokeratin filaments required for the regulation of blood flow in capillaries in *A. altiparanae* (Chehade et al., 2014). The structural integrity observed in endothelial and hepatopancreatic cells of *A. bimaculatus* indicated that these cells were not affected morphologically by Thiodan® in any of the tested concentrations.

The absence of bile stagnation in the livers of female *A. bimaculatus* is important in that it shows that Thiodan® did not interfere with the physiology of biliary excretion in the different experiments. However, the study by Nowak and Kingsford (2003), confirmed ultrastructural changes in hepatocytes of catfish, *Tandanus tandanus* (Mitchell, 1838), caused by residues of endosulfan in the bile and hepatic tissue after 28 days of exposure to the insecticide. Histopathologically, this type of change is observed in the cytoplasm of hepatocytes by the presence of yellowish-brown granules where bile would be synthesized. This change would demonstrate a pathophysiological condition caused by lack of biliary excretion and metabolism (Pacheco and Santos, 2002) through the lack of release of the bile into the digestive tract (Simonato et al., 2008; Benze et al., 2014). This type of change can be found in fish exposed to toxins (Pacheco and Santos, 2002; Benze et al., 2014) and also in fish that have some nutritional problem when kept in captivity (Langiano Vdo and Martinez, 2008). The present study found that the different concentrations of Thiodan® in the different 96-hr treatments were insufficient to develop histopathological evidence of bile stagnation.

The present study found macrophages to be present in the hepatic tissue of *A. bimaculatus*. Macrophages are related to the breakdown of erythrocytes (Herraez and Zapata, 1986), antigen presentation (Ellis, 1980), iron storage, and detoxification (Herraez and Zapata, 1986). Hartley et al. (1996) proposed using macrophages as a biomarker of aquatic pollution through quantifying these structures. There are some comparative studies of different species which show that variation in the number, size, and pigmentation of these structures relate to age, nutrition, and health (Agius and Roberts, 2003). Toxins can also cause an increase of macrophages in the liver and other organs (Pascoli et al., 2011; Boran et al., 2012), that may have various types of pigments (Agius and Roberts, 2003; Wolf and Wolfe, 2005), which contradicts the work by Sarma et al. (2012). Thus, macrophages provide evidence of absorption of exogenous substances, thus suggesting that the presence of these cells is indicative of toxicity at the Thiodan® concentrations tested.

The characteristic presence of lipid accumulation in hepatocytes was found in two fish that were without acclimation/with food at the concentration of 5.6 $\mu g\ L^{-1}$. Several Studies reported accumulation of lipids in hepatocytes associated with toxic exposure (Thiyagarajah and Grizzle, 1985; Braunbeck et al., 1990; Biagiantirisbourg and Bastide, 1995). Glover et al. (2007) subjected *Salmo salar* (Linnaeus, 1758) to a diet containing 4 $\mu g\ kg^{-1}$ endosulfan for 14 and 35 days and Sarma et al. (2012) exposed *Channa punctatus* (Bloch, 1793) to a concentration of 8.1 $\mu g\ L^{-1}$ endosulfan for 96 hr. Both of these studies observed changes in the appearance of liver tissue including large spherical vesicles in hepatocytes and characteristics of lipid deposition. The appearance of these structures also in in the hepatic tissue of *A. bimaculatus* suggests that the insecticide may have affected lipid deposition, as was also observed by Braunbeck et al. (1990) and Krovel et al. (2010).

In this work, the action of Thiodan® at sublethal concentrations was found not to affect the liver as a whole, but did manifest itself in the form of the occurrence of specific lesions in the liver parenchyma in the different treatments. DTC in the livers of lambaris were not severe, as they showed no necrosis and exhibited only Stage I and II morphological changes (these stages being classified as repairable) as reported in other studies (Langiano Vdo and Martinez, 2008; Simonato et al., 2008; Benze et al., 2014; Wolf et al., 2014). Moreover, an increased frequency of histological alterations can indicate dysfunctions induced by toxic agents, since metabolically active areas of the liver are reduced, leading to a possible reduction in the overall functions performed by this organ (Oliveira Ribeiro et al., 2002; Pacheco and Santos, 2002; Benze et al., 2014). Our result suggests that Thiodan® in the different treatments may impair liver function even at very low concentrations, regardless of whether the fish is fed or not.

The effects of Thiodan® may influence the increased action of the depolarization of the axon channels in the nervous system at higher temperatures (Pereira et al., 2012), such as those observed in our experiment without acclimation/without food. Capkin et al. (2006) reported that the effects of cyclodiene are directly related to water temperature. The depletion of glycogen observed in the present study, as indicated by the decreased hepatosomatic index (Prado et al., 2011), may be due to biochemical toxicity interfering with normal liver glycogen deposition (Glover et al., 2007) and increase in energy metabolism (Narain and Singh, 1991) due to the altered behavior of the fish during exposure to endosulfan (Pereira et al., 2012).

Change in the diameters of the nuclei of hepatocytes can be considered a response to a stressor, and is indicative of activation of liver function as a result of increased metabolic activity against adverse conditions (Langiano Vdo and Martinez, 2008; Sarma et al., 2012; Benze et al., 2014). On the other hand, a decrease in the size of the nuclei can be related to the possible multiplication of hepatocytes during the replacement of damaged cells (Braunbeck et al., 1990; Gul et al., 2004). In the work of Oliveira Ribeiro et al. (2002), a decrease in the diameter of the nuclei of hepatocytes of *A. bimaculatus* was found after exposure to the toxin tributyltin. This type of change is indicative of the cell degeneration process, since it starts with the condensation and reduction of the diameter of nuclei (pyknosis), suggesting an early apoptosis event (Nowak and Kingsford, 2003; Rabitto et al., 2005; Ribeiro et al., 2005). Apparently organochlorine insecticides can induce an increase in the cell death (Salas and Burchiel, 1998; Duchiron et al., 2002). The hypertrophy of hepatocytes in our experiments without acclimation/without food and with acclimation/

without food can also be associated with contaminants (Arnold et al., 1995). Furthermore, other studies report that cell change may be due to structural changes related to hepatocyte cell function (Oliveira Ribeiro et al., 2002; Nowak and Kingsford, 2003), physiological conditions related to reproduction (Wester, 2003; Wolf et al., 2014), and the increased metabolic activity due to pollution (Takashima and Hibiya, 1995; Nowak and Kingsford, 2003; Sarma et al., 2012). In the present study, the observed changes in cell diameters show that there were physiological effects on the adaptive response of cells to the new environment in which the fish were exposed to Thiodan®, as shown by the impact on liver function in exposed females compared to the control groups.

Quantification of the nuclei and cytoplasm of hepatocytes in the different experiments provides evidence of hepatocyte proliferation indicative of the toxic action of Thiodan® on the hepatocytes during detoxification, as observed in the Gul et al. (2004). The percentage of liver tissue occupied by hepatocyte type cells ranged from 87.42% to 90.88% in the hepatic parenchyma of *A. bimaculatus*. A few studies mention the cellular constitution of fish livers, for example, hepatocytes occupied 84.5% to 87.3% of the hepatic parenchyma in trout (Hampton et al., 1989) and 89.9% in the Golden Fish (Rocha et al., 1997). These values showed that most of the hepatocytes of female *A. bimaculatus* are playing an important role in the hepatic structural integrity.

In some studies, macrophages were observed in the hepatic parenchyma, for example between 0.2% and 0.7% of *Oncorhynchus mykiss* and *Salmo trutta fario*, respectively (Hampton et al., 1989; Rocha et al., 1997). In the present study, values ranged from 0.21% to 0.29% in *A. bimaculatus*. Meseguer et al. (1994) observed abundant free macrophages in the liver parenchyma, suggesting that they move freely within the parenchyma, forming macrophage aggregates when there is an increase in phagocytic activity such as an immune response to damage in individuals exposed to contaminants. Moreover, an increase in the number of granulocytes can be related to the occurrence of necrosis (Rabitto et al., 2005; Sarma et al., 2012), besides increasing oxidative activity in phagocytic cells in fish (Sarma et al., 2012). In our study, necrosis in the liver was not observed among the fish subjected to Thiodan®, indicating that hepatic structures remained compact and homogeneous, contrary to what has been observed in *Anguilla anguilla* (Linnaeus, 1758) exposed to bleached kraft pulp mill effluent (Pacheco and Santos, 2002). An increased number of macrophages and sinusoids can be signs of stress in fish, which may be caused by the presence of chemical agents (Pacheco and Santos, 2002; Rabitto et al., 2005; Wolf et al., 2014). However, in the present study, exposure to the concentration levels of Thiodan® used in the different 96-hr experiments was not sufficient to activate an immune response in *A. bimaculatus*.

Physiological processes determine the accumulation and distribution of aquatic contaminants within fish after entry through the gills or with food. One such process is lipid mobilization in the maturation of ovaries (Gundersen et al., 2000). This lipid mobilization would transport lipophilic organochlorine pesticides that had been ingested or were in lipid energy reserves to the ovarian follicles (Menone et al., 2000; Marcon et al., 2015). The higher levels of lipids of female fish may have influenced the endosulfan resistance (Ballesteros et al., 2007) of our study fish, which were in an advanced stage of maturation. Only additional research can determine if lipid levels are a factor in the resitance of *A. bimaculatus* to Thiodan®.

CONCLUSION

This study demonstrated that for the concentrations of Thiodan® used and the amount of cellular change in the liver tissue of lambari, *A. bimacutaus*, did not affect the liver in a severe and irreversible way for this hardy fish. The combination of histological biomarkers proved to be a useful tool for investigating sublethal effects of environmental contaminants. Under the conditions that the study was conducted, at the concentrations of Thiodan® used, and after the exposure time of 96 hr, we conclude that females of *A. bimaculatus* are in the early process of intoxication, a pathological process that is still reversible. Thus, this study contributes to the understanding of the hepatic structure and toxicity responses of female *A. bimaculatus* in maturation stage. The adaptive response of *A. bimaculatus* to the different levels of exposure to Thiodan® is more clearly understood in light of these results.

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