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RESEARCH ARTICLE

Thiamethoxam causes histochemical changes in the liver of *Aristichthys nobilis* Rich., 1845

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ABSTRACT

In the present study, we aimed to investigate the effects of the neonicotinoid insecticide thiamethoxam on the hepatic glycogen in bighead carp (*Aristichthys. nobilis* Rich.). Fish were exposed to 6.6 mg/L, 10 mg/L and 20 mg/L of the insecticide under laboratory conditions for 96 hours. The PAS-reaction was applied to liver cryostat sections in order to indicate the amount of glycogen. The results showed that the hepatic glycogen amount increased with increasing the insecticide concentrations. On the other hand, we observed glycogen conglomerates in certain hepatocytes. Hence, our results demonstrated an enhanced process of glyconeogenesis in the fish liver under the influence of thiamethoxam.

Key words: histochemistry, PAS-reaction, thiamethoxam, liver, glycogen, bighead carp, *Aristichthys nobilis*

Introduction

The levels of organic compounds found in surface waters have increased in the recent decades as a result of human activities. Thus, pesticides are most commonly detected in flowing waters (Sáenz & Di Marzio, 2009). Insecticides are designed and developed for killing the insects-pests in general and they are not species specific (Cocco, 2002; El-Massad et al., 2012). However, the pesticides and their related chemicals destroy the delicate balance between species that characterize the functioning of the ecosystem. In addition, the extensive use of pesticides in order to protect crops, as well as their toxicity and persistence represents a serious threat to the environment (Tilak et al., 2007).

The neonicotinoids are the newest class of insecticides in the past three decades. They differ from their natural analogues with high persistence in the environment and more pronounced toxic effects (Massaro, 2002).

The fish are particularly sensitive to pollution. They can be exposed to a wide range of toxic substances, such as pesticides during their life cycle. Furthermore, in fish, different pesticides can be absorbed through the gills, skin or alimentary ducts, so they can significantly affect their fundamental physiological processes (Banaee et al., 2013).

The liver is known to be the primary organ for bioaccumulation and therefore, has been extensively studied in regards to the toxic effects of different xenobiotics for few decades (Hinton & Laurén, 1990; Van Dyk et al., 2007; Nunes et al., 2015). In addition, according to Koehler (2004), as the liver is a main detoxification organ, which is essential for both, the metabolism and the excretion of toxic substances, the histological alterations in the liver can be applied as biomarkers for water contamination and the damage that can be produced.

Pesticide-induced hypoxia cause changes in carbohydrate metabolism in fish. These changes include depletion of proteins, glycogen and pyruvate stores in different fish tissues

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such as the liver (Bose et al. 2011). Therefore, according to Pathan et al. (2009) the histochemical tests and in particular, the intensity of staining in hepatic glycogen can be also applied for biomarkers for water pollution, which clearly distinguish the normal liver compared to an impaired one.

Bighead carp (*Aristichthys nobilis* Richardson, 1854) is a common cyprinid species used in recreational fishing. It is also preferred as a protein source through some European countries and particularly in Bulgaria. However, data about the effects of thiamethoxam on the liver of bighead carp, as well as other toxicological data on this fish species are relatively scarce.

In our previous research, we investigated the effects of thiomethoxam on hepatic liver glycogen in common carp, which is another cyprinid fish (Stoyanova et al., 2012), as well as the negative effects of another pesticide with different functions (Georgieva et al., 2013). Thus, in the present study our main goal was to determine the toxic effects of thiamethoxam by observing the amount of glycogen in the liver of bighead carp (*A. nobilis* Rich.) by using histochemical methods. We also aimed to investigate if there is a species-dependent difference based on the obtained results from our previous experiments.

Materials and Methods

Test organisms

We purchased forty fish from the “Institute of Fisheries and Aquaculture” (Plovdiv, Bulgaria). The bighead carps were with a similar size (mean length 19.61±1.2 cm; body mass 51.1±5.1 g) with no external pathological abnormalities. After transportation of the fish to the laboratory at the Faculty of Biology (Plovdiv University), they were placed in 100 l tanks with chlorine-free tap water (by evaporation) in order to acclimatize for one week. The laboratory test included 4 tanks with 10 bighead carps in each one and the fish were not fed prior or during the experiment.

Test chemicals

Thiamethoxam is the active substance of the insecticide “Actara 25 WG”. It is a second generation neonicotinoid insecticide, which belongs to the subclass of thianicotinyl and has unique chemical properties (Fishel, 2005).

The fish were exposed to 3 different thiamethoxam concentrations (6.6 $mg.l^{-1}$, 10 $mg.l^{-1}$ and 20 $mg.l^{-1}$), which represented 30, 20 and 10 times dilution of the stock solution as explained by the manufacturer in the instructions for use. The fourth fish group was placed in clean tap water and served as a control.

All tanks were equipped with air pumps for permanent aeration and the water was kept oxygen saturated. The fish were kept under a natural light/dark cycle (12:12 h). The physico-chemical characteristics of water (pH, temperature, dissolved oxygen, oxygen saturation and conductivity) were measured once per day according to a standard procedure (APHA, 2005) with a combined field meter (WTW, Germany). They were as follows: pH 8.1±0.1; temperature 21.5±0.5°C; dissolved oxygen 9.3±0.15 $mg.l^{-1}$; oxygen saturation 103.43±0.9% and conductivity 310±0.5 S.m.

The present study was performed in accordance with the national and international guidelines of the European Parliament and the Council on the protection of animals used for scientific purposes (Directive 2010/63/EU).

Histochemical analysis

The fish were sacrificed by a sharp hit to the head and the abdomen was opened. The liver was dissected out according to the EMERGE Protocol by Rosseland et al. (2003).

The histochemical analysis was conducted at the laboratory of the Department of Anatomy, Histology and Embryology, Medical University, Plovdiv, Bulgaria. Multiple carp liver sections (6 μm) of each fish were prepared according to a standard methodology using a cryostat (Leica, Jung Frigocut 2800 N). The samples were stained by the PAS method according to McManus (1948) as described by Pearse (1972).

The liver histochemical alterations from the treated with thiamethoxam fish, as well as the control fish livers were appraised individually and semi-quantitatively by using the grading system described by Mishra & Mohanty (2008). We slightly modified the proposed grading system. The positive PAS-reaction was presented in purple-magenta staining in the hepatic cytoplasm.

Results

In general, the PAS-reaction intensity increased in a dose-dependent manner, which means elevated levels of the hepatic glycogen with the increase of the pesticide concentration (Figure 1). We also observed a tendency towards increasing the intensity of PAS-positive grain-like structures, which varied from fine to large grains in all three exposed fish groups, possibly linked with the increase in the

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amount of accumulated glycogen in the liver cells.

The PAS-reaction in the control fish liver was weak and there were no conglomerates of accumulated glycogen in the hepatocytes. The purple-magenta staining demonstrated that the amount of glycogen in the hepatocyte cytoplasm is a result from the liver function as a metabolic organ (Figure 1a). We observed an increased PAS-reaction in the fish group exposed to concentration of 6.6 $mg.l^{-1}$ thiamethoxam. It was moderate, with more intense purple-magenta staining compared to the control group (Figure 1b). This result indicated for increased glycogen at the lowest thiamethoxam concentration compared to the control group.

Likewise, we found similar intensity of the histochemical staining in all cryosections of fish exposed to 10 $mg.l^{-1}$ of the test insecticide. This intensity of PAS-reaction was also moderate. Therefore, we assumed that this particular thiamethoxam concentration did not significantly impact the glyconeogenesis process in the fish liver compared to the lowest concentration.

In contrast of the previous two concentrations, we observed a strong positive PAS-reaction in the fish group exposed to the highest insecticide concentration (Figure 1d). Due to this fact, a more intense PAS-reaction was demonstrated and thus, the highest amount of glycogen in the bighead carp liver. Furthermore, we found conglomerates of accumulated glycogen in certain hepatocytes in the fish exposed to 20 $mg.l^{-1}$ thiamethoxam, which was indicator for the severe impact of the toxicant.

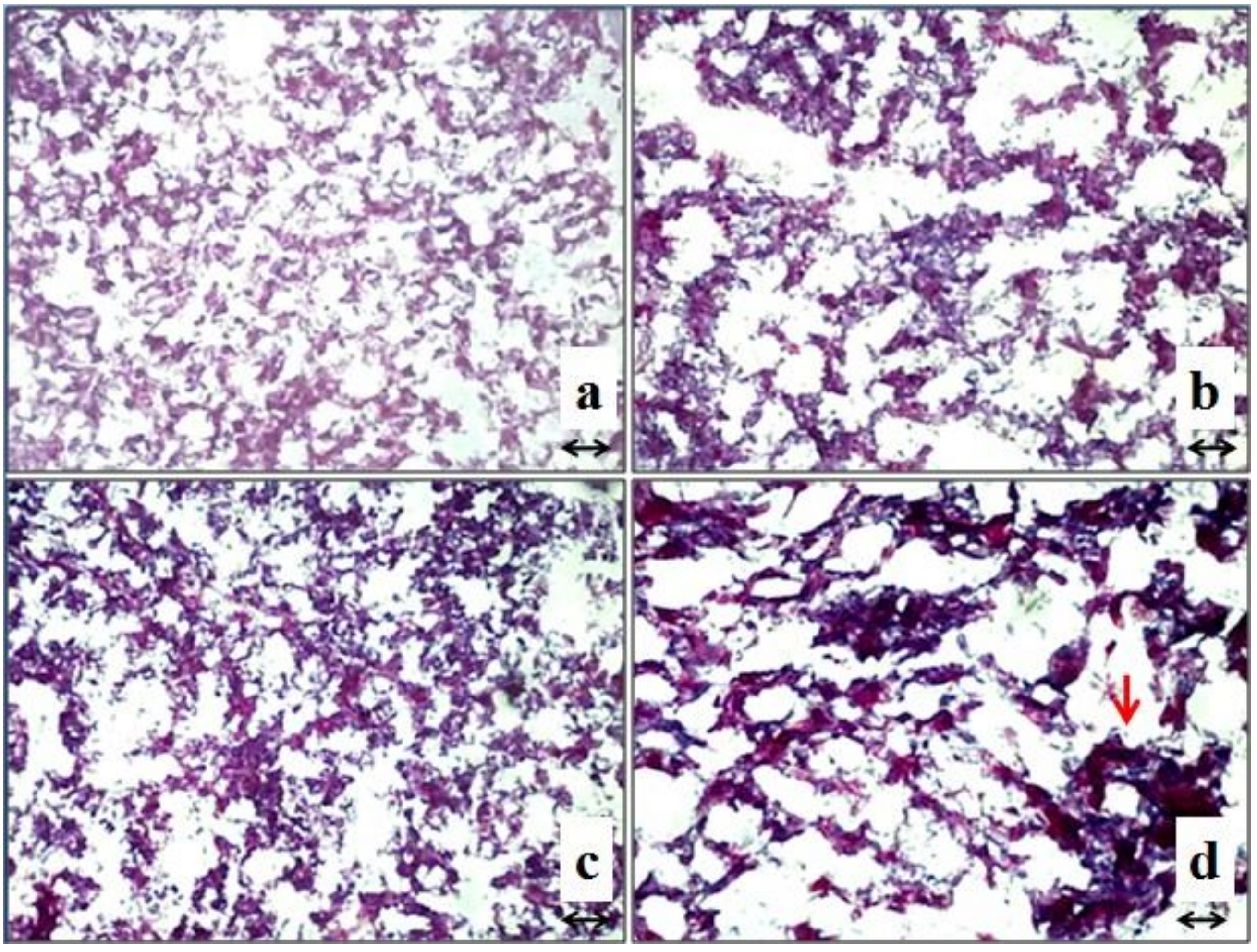

Figure 1. *Intensity of PAS-reaction and conglomerates (red arrow) of accumulated glycogen in bighead carp liver,* ***a*** *– control group, x200;* ***b*** *– 6.6* $mg.l^{-1}$ *insecticide, x400;* ***c*** *– 10* $mg.l^{-1}$ *insecticide, x 400;* ***d*** *– 20* $mg.l^{-1}$ *insecticide, x400.*

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Discussion

The liver is the main carbohydrate store in fish (Ramesh & Saravanan, 2008) and it plays a major role in the blood glucose homeostasis by maintaining a balance between the uptake and storage of glucose (Hoseini et al., 2006). Our data are in agreement with the findings by Rezg et al. (2006, 2007) that hyperglycemia is a temporary process, which is caused by an increase in the fish liver glyconeogenesis under the insecticide effects. Furthermore, the enhanced process of glyconeogenesis caused an increase glycogen deposition in the hepatocytes. Overall, we agree with Samuel & Sasiry (1989) who state that the pesticides may alter the carbohydrate metabolism and particularly the glycogen content. According to Shrivastava (2007), changes in the glycogen amount in the fish liver are the result of changes in the carbohydrate metabolism. The results from this study showed an enhanced process of glycogen accumulation in the liver cells under the increasing concentrations of the test pesticide. These changes may be associated with the inhibition of the enzyme glucose-6-phosphatase and the failure to release glucose into the blood, leading to hypoglycemia. Moreover, the changes in the amount of glycogen in the liver processes can also alter the processes of gluconeogenesis, glycolysis and glycogenolysis. Oruc & Ulner (1998) found that elevated glycogenolysis occured following acute chemical stress and compensatory process developed during chronic exposure in common carp (*Cyprinus carpio* L.). Similarly to the results from our previous study (Stoyanova et al., 2012) we found an increasing intensity of the PAS-reaction in the fish liver under the effects of thiamethoxam. The results from both studies showed that the intensity of PAS-reaction in the liver of bighead carp was more pronounced compared with the intensity in the common carp liver. Therefore, in terms of the toxicity of thiamethoxam we found that bighead carp is the most sensitive cyprinid species.

We can conclude that the observed increase in the PAS-reaction intensity in the fish liver correlated with the increasing thiamethoxam concentrations. In addition, the increase in the glycogen amount also suggested a process of enhanced glyconeogenesis. Hence, we suppose that these results could be acknowledged as a series of compensatory mechanisms in the fish liver metabolism in response to the negative effects of thiamethoxam.

Overall, further investigations in this particular field need to be carried out to better understand the metabolic changes in the liver of different fish species, even if they are from the same family under pesticide influence.

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