RESEARCH PAPER
www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences
DOI: 10.4194/1303-2712-v18_10_04

Effect of Prometryn-Containing Herbicide Gesagard on Hematological Profiles and Biochemical Parameters in Goldfish Liver and Plasma

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Abstract

The impact of goldfish exposure for 96 h to herbicide Gesagard 500 FW at concentrations 0.2, 1, or 5 mg L^{-1} (corresponding to 0.1, 0.5 or 2.5 mg L^{-1} of effective compound prometryn) on the hematological profile of blood and biochemical parameters of plasma and liver was studied. Fish exposure to low concentration of the herbicide (0.2 mg L^{-1}) slightly decreased liver glycogen and plasma lactate levels. Plasma glucose levels rose by 27% in goldfish exposed to 1 mg L^{-1} Gesagard. The activity of lactate dehydrogenase decreased by 63% and 36% in plasma of fish exposed to herbicide at concentrations 1 and 5 mg L^{-1}, respectively, but was not affected in liver.

Goldfish exposure to the highest concentration of Gesagard (5 mg L^{-1}) decreased hematocrit by 23% and increased monocyte count by 57%, and elevated triacylglycerol level by 91% in plasma. Overall, the results indicate that acute exposure to Gesagard induced minor changes in the hematological and biochemical parameters of goldfish, suggesting that disruptions of these parameters may provide early warning signs that could be useful for assessing acute or sublethal toxic effects of pesticides on aquatic species.

Keywords**:** blood parameters, glycogen, lactate dehydrogenase, transaminases, triacylglycerol.

Introduction

Triazine herbicides are widely used to control broadleaf and some grassy weeds in agriculture and industrial areas (Hostovsky, Blahova, Plhalova, Kopriva, & Svobodova,, 2014). Prometryn (2,4-bis(isopropylamino)-6-methylthio-s-triazine) belongs to thiomethyl triazines and is frequently used alone or as the commercial preparations Gesagard and Caparol for the control of weeds at plantations of cotton, celery, pigeon peas, and dill (Dikić, 2014). Also, prometryn applies in aquaculture to remove filamentous algae, aquatic weeds, and other harmful algae in the fish, shrimp, crab, shellfish, and sea cucumber aquaculture industries (Zhao & Zhu, 2016). The herbicide activity of prometryn arises from the inhibition of photosynthesis by binding to a plastoquinone-binding cite on the D1 protein of photosystem II complex in thylakoid membranes of chloroplasts (Das, McElroy, & Cooper, 2000; DeLorenzo, Scott, & Ross, 2001).

The excessive use of prometryn in agriculture as well as its relatively high stability in water (half-life reach 70 days) and soil (half-life reach 316 days) has led to the pollution of ecosystems, especially aquatic (Dikić,

2014). It was found in surface water of Greece (Vryzas, Alexoudis, Vassiliou, Galanis, & Papadopoulou-Mourkidou, 2011), France and the Czech Republic at levels ranging from 0.021 μg L^{-1} to 4.40 μg L^{-1} (Caquet et al., 2013; Stará, Kouba, & Velíšek, 2014).

Fish is one of the major targets of toxicants in aquatic environment. Several studies have been conducted using fish as a model for assessing of promentryn toxicity in aquatic organisms. For example, Velisek, Stara, Zuskova and Svobodova (2013) observed changes in hematological and biochemical parameters in plasma of common carp after chronic exposure to prometryn. Other authors reported oxidative stress induction in red swamp crayfish (*Procambarus clarkii*) and common carp (*Cyprinus carpio* L.) after acute or chronic exposure to prometryn (Stará, Kristan, Zuskova, & Velisek, 2013: Stará et al., 2014). To date, however, there is no information on the effects on fish of the commercial preparation of the prometryn-containing herbicide Gesagard that is widely used in agriculture. Gesagard contains 50% prometryn, but the remainder of its composition is not provided by the producers. Hence, it is important to test not only the impact of the pure active ingredient of this industrial pesticide, but also the effects of the commercially used preparations. For example, the herbicide Roundup produced by Monsanto is used as isopropylamine salt of the active component glyphosate [N-(phosphonomethyl)glycine] and also contains a surfactant (polyethoxylated tallow amine) which enhances the effectiveness of glyphosate. Importantly, deleterious effects of the surfactant on non-target organisms may exceed those of the active ingredient (Lushchak, Kubrak, Storey, Storey, & Lushchak, 2009). Previously, we studied the effects of Gesagard on free radical processes in goldfish tissues (Mosiichuk et al., 2015). Therefore, the present work was designed to investigate the impact of 96 h acute exposure to the prometryn-containing herbicide Gesagard on blood and liver parameters of goldfish. Hematological parameters together with investigation of leukocyte profile and some biochemical indices in fish blood can be valuable tools for nonlethal diagnostics of fish intoxication. Furthermore, the evaluation of hematological and biochemical characteristics in fish blood has become an important means of understanding the mechanisms of herbicide toxicity.

Materials and Methods

Reagents

Phenylmethylsulfonyl fluoride (PMSF), β-nicotinamide adenine dinucleotide (NAD), β-nicotinamide adenine dinucleotide reduced (NADH), ethylenediamine-tetraacetic acid (EDTA), KH_2PO_4, NaCl, lactic acid, pyruvic acid, lactate dehydrogenase from bovine heart were purchased from Sigma–Aldrich Corporation (USA). Detection kits for estimation of triacylglycerol (Liquick Cor-TG) and glucose (Liquick Cor-GLUCOSE) levels were purchased from PZ CORMAY (Poland). Detection kits for estimation of activities of alanine aminotransferase and aspartate aminotransferase were received from "Phyllis-Diagnosis" (Ukraine). Gesagard 500 FW was purchased from Syngenta AG (Switzerland). All other reagents were of analytical grade.

Animals and Experimental Conditions

Specimens of goldfish (*Carassius auratus* L.) weighing 80-100 g were obtained commercially from a local fish farm (Halych district, Ivano-Frankivsk region, Ukraine) in September 2013. Fish were acclimated in

the laboratory for four weeks in a 1000 L tank under natural photoperiod in aerated and dechlorinated tap water at 19.0-20.0°C, pH 6.9-7.1, 8.1-8.6 mg L^{-1} O_2 and hardness (determined as Ca^{2+} concentration) 38-40 mg L^{-1}. Fish were fed with commercial pellets of CarpCo Excellent for Cyprinids (Koi Grower, The Netherlands), containing 36% protein, 7% fat, 3.6% cellulose, 8.7% ash, 1% phosphorus and vitamins C, A, D_3 and E. Fish were fed during the acclimation period (four weeks), but were fasted for 1 day prior to and during experimentation.

Experiments were carried out in 120 L glass aquaria (containing 100 L of water), in a static mode with or without the addition of the commercial herbicide Gesagard 500 FW (Syngenta AG,Switzerland) which contains prometryn [6-methylsulfanyl-2-N,4-N-di(propan-2-yl)-1,3,5-triazine-2,4-diamine] at a concentration of 500 g L^{-1}. Groups of seven fish were placed in aquaria with different nominal concentrations of Gesagard: 0.2, 1 and 5 mg L^{-1}, which corresponds to 0.1, 0.5 and 2.5 mg L^{-1} of prometryn, respectively. Gesagard concentrations used in this work were selected based on LC_{50}^{96} value (half-lethal concentration after 96 h exposure) for goldfish determined to be 4 mg L^{-1} (Erickson & Turner, 2002). Animals were exposed to these conditions for 96 h (no mortality occurred during exposures). Fish in the control group were maintained in the same manner, but Gesagard was omitted. Aquarium water was continuously aerated with air pumps and was not changed over the 96 h course to avoid stressing the animals. Levels of dissolved oxygen, temperature and pH were monitored every 24 h. The experiments were carried out in two independent experimental replicates with a total number of at least five biological replicates for every measured parameter.

After fish exposure, blood was quickly taken from caudal vessels using a syringe rinsed with 50 mM Na_2EDTA as an anticoagulant. Blood samples were centrifuged for 15 min at 3,000 g and 4°C. Plasma was collected and used for biochemical analyses. Fish were then quickly sacrificed by transspinal transsection without anesthesia and livers were dissected, rinsed in ice-cold 0.9% NaCl, dried by blotting on filter paper, frozen, and stored in liquid nitrogen until use.

All experiments were conducted in a strict accordance with the Ethics Committee of Precarpathian National University.

Evaluation of Hematological Parameters and Leukocyte Formula in Blood

Total hemoglobin concentration was determined after erythrocyte hemolysis in Drabkin's solution using a commercial kit (Genesis Co, Ltd., Ukraine) following the manufacture instructions.

Hematocrit was determined following the procedure of Ptashynski, Pedlar, Evans, Baron and Klaverkamp (2002). Immediately after blood sampling, small amounts of whole blood were transferred to microcapillary tubes, which were then carefully sealed on both ends and centrifuged (2000 g, 20 min, 4°C) using an OPN-8 centrifuge (USSR). Hematocrit values were calculated as the percentage of red blood cell pellet in the total blood column.

For microscopic examination of leukocyte content, small drops of whole blood were directly smeared on slides (n=2 per fish) and air-dried. Smears were fixed and stained with azure-eosine water solution as described previously (Vasylkiv, Kubrak, Storey, & Lushchak, 2010). Cytological analysis was conducted by scoring at a 1600 x magnification using a Leitz microscope (Leitz Wetzbar GmbH, Germany). Different

types of leukocytes were identified according to the Fish Blood Cell Atlas (Ivanova, 1983). A total of 200 leukocyte cells were counted per smear and assigned to different leucocyte categories. Data are shown as the percentage of different leucocytes per 200 cells counted.

Determination of Metabolic Stress Indices in Liver and Plasma

For measurement of glucose and glycogen concentrations aliquots of frozen liver samples were homogenized 1:10 (w:v) with 50 mM potassium phosphate buffer containing 0.09% NaN_3. Fresh prepared plasma was mixed 2:1 (v:v) with 50 mM potassium phosphate buffer containing 0.09% NaN_3. Then samples were incubated in a water bath (70°C) for 5 min in tightly covered centrifuge tubes, followed by chilling on ice. After centrifugation (16,100 g, 15 min, 21°C) of samples the thermally denatured proteins were removed and supernatants used for determination of glucose concentration by the Liquick Cor-GLUCOSE commercial kit (Cormay, Poland) with spectrophotometric detection at 540 nm. Glucose concentrations in samples (mg L^{-1} or mg gwm^{-1} for plasma or liver, respectively) were estimated using a linear regression of data from a standard curve. For determination of glycogen concentration the samples were first incubated with 0.5 U amyloglucosidase and then glucose concentration was assayed in the same manner. Glycogen concentration in the samples was expressed in milligrams of glucose equivalents per gram wet mass of tissue (mg gwm^{-1}).

Protein concentration was measured by the Coomassie brilliant blue G250 method (Bradford, 1976) using bovine serum albumin as a standard. Data are expressed as milligrams of total protein per milliliter of blood or liver homogenate (mg mL^{-1} or mg gwm^{-1}).

For measurement of triacylglycerol content aliquots of liver samples were homogenized (1:10, w:v) with phosphate-buffered saline (PBS) containing 0.05% Triton X100. Fresh prepared plasma was mixed 1:1 (v:v) with the same medium. Samples were incubated for 15 min in a boiling water bath. Thereafter, homogenates were cooled and centrifuged 7 min at 5,000 g and 21°C. The resulting supernatants were used for determination of triacylglycerol (TAG) content using commercial kit Liquick Cor-TG (Cormay, Poland) according to the manufacturer's protocol. Data are expressed as microgram triacylglycerol (TAG) per milliliter of plasma (mg mL^{-1}) or per gram wet mass of tissue (mg gwm^{-1}) for liver.

The concentration of lactate was measured via oxidation of lactate to pyruvate by L-lactate dehydrogenase (LDH) (Cuddihee & Fonda, 1982). The amount of NADH formed was measured spectrophotometrically at 340 nm. For quantitative conversion of lactate to pyruvate, the medium was supplied with hydrazine, which reacts with pyruvate to produce a pyruvate hydrazone. Samples of tissue were homogenized 1:10 w:v (for liver) or mixed 1:1 v:v (for plasma) with ice 0.5 M perchloric acid (PCA) and then centrifuged (15 min, 16,100 g, 4°C). Supernatants were neutralized with 2 M KOH. The reaction mixture contained 0.5 M glycine-hydrazine buffer (pH 9.0), 2 U/mL LDH, 2 mM NAD and 50 µl of preparation. Lactate concentration in samples (µmol) was calculated using a linear regression of data from a standard curve made with 10-240 µM lactate. The concentration of lactate was expressed as micromoles of lactate per milliliter of plasma (µmol mL^{-1}) or per gram wet mass of tissue (µmol gwm^{-1}) for liver.

Activities of Enzymes

www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences DOI: 10.4194/1303-2712-v18_10_04

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured according to the method of Reitman and Frankel (1957) using commercial kits (Phyllis-Diagnosis, Ukraine) following the manufacturer's guidelines. The activities of these enzymes were expressed as international units (U) per milligram soluble protein.

The activity of lactate dehydrogenase (LDH) was assayed spectrophotometrically using a Specol 211 spectrophotometer (Germany) by monitoring the change in NADH absorbance at 340 nm (Lushchak, Bagnyukova, Storey, & Storey, 2001). One unit of enzyme activity is defined as the amount of enzyme consuming 1 μmol of substrate per minute. The activity is expressed as international units per milligram soluble protein.

Statistical Analysis

Statistical analysis was performed by Mynova software (version 1.3) using ANOVA followed by the Dunnett's test to compare multiple experimental treatments to the single control value. Prior to statistical analysis, all data were tested for normality of distribution using the Shapiro-Wilk test. All data were normally distributed. Data are presented as means ± S.E.M. The probability value of $P < 0.05$ was considered to be statistically significant.

Results and Discussion

Hematological Parameters and Leucocyte Count in Gesagard Exposed Fish

Change in haematological variables are frequently evaluated in clinical diagnoses of fish physiology to determine the effects of external stressors (Fazio et al., 2014). In our experiments, no significant changes in total hemoglobin levels were observed in goldfish exposed to Gesagard at any of the three concentrations used (Table 1). Similarly, other authors found no changes of total hemoglobin levels in common carp after chronic exposure to simazine or terbutryn (Oropesa, Garcia-Cambero, Gymez, Roncero, & Soler, 2009; Velisek, Stará, Kolarova, & Svobodova, 2011; Velisek, Stara, Machova, & Svobodova, 2012). Short-term exposure to the triazine herbicide Sencor also had no effect on the total hemoglobin levels in goldfish (Husak et al., 2014). Short term exposure to commercially formulated insecticide Cyperdicot had no effect on hemoglobin levels in African catfish (Odo et al., 2017). In contrast, chronic (60 days) exposure of common carp to prometryn at concentrations of 8 and 80 μg L^{-1} enhanced hemoglobin levels (Velisek et al., 2013).

The hematocrit measures the volume percentage of packed red blood cells in a volume of blood. In our experiments, hematocrit was lower by 23% in fish exposed to the highest concentration of Gesagard (5 mg L^{-1}) as compared to the control group, whereas at rest concentrations it did not influence the parameter (Table 1). A decrease in the number or size of red blood cells reduces the amount of space that they occupy which can result in a lower hematocrit (Banaee, Mirvagefei, Rafei, & Amiri, 2008). Also, a reduced hematocrit value could suggest either a disruption of hemopoietic processes, or enhanced elimination of erythrocytes from the blood stream (Ural, 2013). Significant decreases in the hematocrit have been reported in common carp exposed to 45 μg L^{-1} simazine for 90 days (Oropesa et al., 2009). However, neither simazine at concentrations of 0.06-4 μg L^{-1}, nor terbutryn at concentrations 0.02-2 μg L^{-1} changed

hematocrit in common carp after a 90 day treatment (Velisek et al., 2011; 2012). In contrast, goldfish exposure to the metribuzin-containing herbicide Sencor resulted in enhanced hematocrit (Husak et al., 2014).

Leucocytes are critical elements of immune system and changes in their counts after exposure to pesticides indicate disturbances in the system. This may result in non-adequate responses to environmental challenges. There are five main types of white blood cells (lymphocytes, monocytes, neutrophiles, eosinophiles and basophiles), each of which play different roles in response to the presence of foreign organisms in the body (Banaee et al., 2008; Saravanan, Prabhu Kumar, & Ramesh, 2011). In this study, we found only one significant change in the relative amount of a single leukocyte type (monocytes) in goldfish exposed to Gesagard: relative counts of monocytes were enhanced by 57% in fish exposed to 5 mg L^{-1} Gesagard (Table 1). In the previous study, we found increased relative counts of stab neutrophils and monocytes in fish exposed to Sencor (Maksymiv et al., 2015). Similarly, other authors reported increased counts of monocytes, and stab and segmented neutrophils in common carp after chronic exposure to atrazine, as well as acute poisoning with metribuzin (Svobodova & Pecena, 1988; Velisek, Svobodova, Piackova, & Sudova, 2009). Odo et al. (2017) reported increased monocytes count in African catfish exposed to insecticide Cyperdicot. Overall, the change in monocyte count in goldfish indicates the potential development of inflammation resulting from fish exposure to the prometryn-containing herbicide Gesagard at its highest concentration used in this study (5 mg L^{-1}).

Blood of fish has been shown to be sensitive to pesticide-induced stress and, hence, evaluation of hematological characteristics is an important way to understand normal and pathological processes and toxin impacts (Fazio et al., 2014). Chronic oral exposure of mice to prometryn was found to disrupt operation of the immune system, markedly affecting lymph nodes and thymus, as well as altering blood biochemical parameters (Dikić et al., 2009a;b). Other methylthio-s-triazines, such as terbutryn and simetryn, disturbed blood parameters in common carp under chronic exposure (Oropesa et al., 2009; Velisek et al., 2011; 2012).

Plasma Metabolite Levels

Carbohydrates serve as energy source for most cellular processes including responses to environmental challenges (Martinez-Porchas, Martinez-Cordova, & Ramos-Enriquez, 2009). In our experiments, the concentration of glucose in the plasma of control fish was 2.42 ± 0.05 mg mL^{-1} which was enhanced by 27% only in the fish exposed to Gesagard at concentration 1 mg L^{-1} of Gesagard (Figure 1). Elevation of blood glucose levels is widely used as a secondary marker of a stress response (Fırat et al., 2011). It has been reported that the increased blood glucose is usually observed in fish under undesirable conditions and it helps the animal by providing energy substrates to vital organs to cope with the increased energy demand (Banaee, Sureda, Mirvaghefi, & Ahmadi, 2011). Velisek and colleagues (2011) also reported an increase in glucose levels in plasma of common carp after long-term exposure to terbutryn, as a response to metabolic stress. Similar effects were observed in common carp (Velisek et al., 2009) and goldfish (Maksymiv et al., 2015) after acute poisoning with the herbicide Sencor. However, chronic exposure to

prometryn and simazine did not influence plasma glucose levels in common carp (Velisek et al., 2012; 2013).

Triacylglycerols (TAG) are the main lipid depot and important energy source in fish. In this experiment, the concentration of TAG in plasma of control goldfish was 0.41 ± 0.05 μg mL^{-1} and it was increased by 91% only in fish treated with the highest 5 mg L^{-1} level of herbicide (Figure 2). Higher TAG levels in plasma may suggest goldfish acclimation to the stress conditions and provide a compensatory response of lipids to maintain energy balance. Previously, we did not observe changes of TAG content in plasma of goldfish after short-term exposure to the triazine herbicide Sencor (Maksymiv et al., 2015). Similarly, plasma TAG levels were unchanged in common carp exposed to simazine (Velisek et al., 2012). Velisek and colleagues (2009) reported diminished TAG levels in plasma of common carp after acute treatment with Sencor (Velisek et al., 2009).

Blood plasma contains a complex mixture of various proteins. Determination of total protein, albumin, and globulin in plasma is used to monitor the course of immune disorders, liver dysfunction and impaired kidney activity (Banaee *et al.* 2011; Chen et al., 2013). In our experiments, no significant change in plasma total protein concentrations was observed in goldfish exposed to Gesagard (Table 2).

Plasma lactate concentrations represent a balance between lactate production and catabolism. Many experimental and clinical studies have shown that blood lactate levels may increase at tissue hypoxia (Bakker & de Lima, 2004; Phypers & Pierce, 2006). However, we found decreased by 22% plasma lactate level in fish exposed to Gesagard at concentration 0.2 mg L^{-1}, whereas at higher concentrations the herbicide had no effect on the lactate level (Table 2). The significant drop in the plasma lactate concentration could indicate a decrease in the glycolytic process due to the lower metabolic rate in fish exposed to Gesagard. Similarly, the triazine herbicide metribuzin decreased lactate concentration in blood plasma of common carp after acute exposure (Velisek et al., 2009). On the contrary, lactate concentration was increased in plasma of common carp exposed to terbutryn or prometryn for a long time (Velisek et al., 2011; 2013). Also, these authors did not find changes in plasma lactate level of common carp after chronic exposure to simazine (Velisek et al., 2012).

Enzyme Activity in Plasma

The activities of several enzymes in blood plasma have been considered as relevant stress indicators. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities are commonly used in the diagnosis of fish diseases as well as in the detection of specific tissue damage caused by environmental pollution (Chen et al., 2013; Fırat et al., 2011; Kumar et al., 2016). ALT is located mainly in the cytoplasm of hepatocytes, whereas AST exists in liver cell cytoplasm and mitochondria. Under normal conditions, the activities of ALT and AST in plasma are low. However, when liver cells are damaged, ALT and AST are released into the bloodstream. The release of ALT into the blood results from a loss of integrity of hepatocyte membranes, whereas elevated plasma AST activities indicates also mitochondrial disruption (Chen et al., 2013; Fırat et al., 2011; Loteste, Scagnetti, Simoniello, Campana, & Parma, 2013). In our experiments, no statistically significant differences in plasma ALT and AST activities were found in any of the Gesagard-treated fish (Table 2). In contrast to our results, Velisek and colleagues (2013) observed

enhanced AST activity and diminished ALT activity in plasma of common carp exposed to prometryn for 30 and 60 days. Chronic exposure of common carp to simazine caused a significant decrease of ALT activity, whereas no change in AST activity in plasma was determined (Velisek et al., 2012). The triazine herbicide terbutryn increased AST activity, but had no effect on ALT activity in plasma of common carp after long term treatment (Velisek et al., 2011).

Lactate dehydrogenase is an enzyme involved in carbohydrate metabolism and has been used as indicative criteria of exposure to chemical stress and to diagnose cell, tissue and organ damage (Kumar et al., 2016). The activity of LDH was 7.64 ± 0.93 mU mg protein^{-1} in plasma of control goldfish, and it decreased by 63% and 36% in fish exposed to 0.2 and 5 mg L^{-1} Gesagard, respectively (Figure 3). The decreased plasma LDH activity could indicate a decrease in the glycolytic process resulted from herbicide toxicity. Similarly, acute exposure of common carp to the triazine herbicide metribuzin decreased LDH activity in plasma (Velisek et al., 2009). Previously, Velisek et al. (2013) reported no changes of plasma LDH activity in common carp after treatment with prometryn for 30 or 60 days. Long term exposure of common carp to simazine did not affect plasma LDH activity and lactate concentration, whereas under the same conditions terbutryn enhanced these parameters (Velisek et al., 2011; 2012).

Liver Metabolic Parameters

Liver is regarded as a major site of storage, biotransformation, and excretion of pesticides. The liver has dual blood sources from the hepatic vein and hepatic artery. The hepatic vein supplies blood from the gastrointestinal tract. Through first pass elimination, the liver processes xenobiotics absorbed from dietary sources. The liver also receives blood flow from the hepatic artery, which allows for biotransformation of xenobiotics systemically available in the bloodstream (Parkinson & Ogilvie, 2007). Therefore, biochemical parameters of plasma are tightly related with liver functional status. However, in our experiments virtually none of the altered plasma parameters was changed in liver of goldfish: Gesagard exposure had no effect on total protein, glucose, triacylglycerol and lactate levels in liver and only in the liver of fish exposed to Gesagard at concentration 0.2 mg L^{-1} glycogen level was 18% lower than that in the control group (Table 2). Glycogen, the main reserve carbohydrate and fuel source in animals, is known to be depleted rapidly when organisms are under stress due to direct utilization for energy production (Oruç & Üner, 1999; Suneetha, 2012). Previously, we did not observe significant changes of TAG content in liver of goldfish after short-term exposure to the triazine herbicide Sencor (Maksymiv et al., 2015). Also we did not find significant changes in AST, ALT and LDH activities in liver of goldfish exposed to any of the Gesagard concentrations used (Table 2).

The results of the present study indicate that acute exposure to Gesagard at concentrations 0.2, 1, or 5 mg L^{-1} induced only minor changes in the hematological, morphological and biochemical parameters of goldfish, *C. auratus*. Treatment of fish with Gesagard at high concentrations, the pesticide reduces hematocrit, induces inflammation and impairs metabolic rate. These changes perturb energetic status and enhance the need for energy which is met by an increase in blood levels of TAGs, the most efficient energy source. At lower concentrations Gesagard slows glycolytic processes via decreasing lactate level and LDH activity. But fish organism compensates it via enhanced level of glucose and utilization of glycogen for

energy production. Alterations of these parameters may provide an early warning signal for the determination of acute and sublethal toxic levels of pesticides and their impact on fish. The findings of the present study also provide a better understanding of the toxicological endpoints of Gesagard that can be used to ascertain a safer level of this pesticide in the aquatic environment in order to protect its habitants.

Acknowledgments

The research received partial support from a discovery grant from the Natural Sciences and Engineering Research Council of Canada (6793) to KBS.

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ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences DOI: 10.4194/1303-2712-v18_10_04

Table 1. The levels of total hemoglobin, hematocrit value and relative content of different leukocytes in blood of goldfish, exposed to control conditions or 0.2, 1 or 5 mg L^{-1} of Gezagard for 96 h

Parameter		Fish group			
		Control	0.2 mg L^{-1}	1 mg L^{-1}	5 mg L^{-1}
Hematological parameter	Total hemoglobin (g L^{-1})	89.2±4.6	82.0±4.9	79.2±6.3	81.1±7.2
	Hematocrit (%)	37.7±1.1	33.2±1.8	32.6±2.1	29.0±1.8*
Leukocytes type	Lymphocytes (%)	62.9±9.2	66.0±1.2	64.8±2.3	60.1±1.9
	Stab neutrophils (%)	12.7±2.1	12.7±0.9	11.3±1.2	13.2±1.5
	Segmented neutrophils (%)	5.83±1.52	5.78±1.48	8.01±2.15	8.24±1.58
	Monocytes (%)	9.30±1.83	9.85±1.54	10.2±0.6	14.6±1.5*
	Metamyelocytes (%)	3.50±0.53	2.35±0.48	1.91±0.37	2.83±0.54
	Myelocytes (%)	3.00±0.64	2.70±0.23	3.24±0.70	2.33±0.37

Data are amount of different leucocytes, evaluated per 200 leucocytes counted. Some blast leucocytes (hemocytoblasts, myeloblasts and promyelocytes) and granulocytes (eosinophils and basophils) were either not found or present only in very low amounts (0-1 per total 200 leucocytes counted) and were omitted from the table. Data are presented as means ± S.E.M, n=6-7. *Significantly different from the respective control value with $P<0.05$ according ANOVA followed by Dunnett's test.

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ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences DOI: 10.4194/1303-2712-v18_10_04

Table 2. Biochemical parameters in plasma and liver of goldfish, exposed to control conditions or 0.2, 1 and 5 mg L^{-1} of Gesagard for 96 h. Parameters abbreviated are: triacylglycerol (TAG) concentration and the activities of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

Parameters	Fish group			
	Control	0.2 mg L^{-1}	1 mg L^{-1}	5 mg L^{-1}
Plasma				
Total protein (mg mL^{-1})	20.2 ± 2.2	18.4 ± 2.0	22.0 ± 3.2	17.9 ± 2.0
Lactate concentration (µmol mL^{-1})	2.47 ± 0.20	1.94 ± 0.17*	2.84 ± 0.30	2.71 ± 0.21
ALT activity (U mg $protein^{-1}$)	0.80 ± 0.11	0.77 ± 0.12	0.66 ± 0.08	0.73 ± 0.08
AST activity (U mg $protein^{-1}$)	4.39 ± 0.52	4.22 ± 0.47	3.44 ± 0.44	3.97 ± 0.26
Liver				
Total protein (mg gwm^{-1})	27.5 ± 0.9	27.5 ± 0.9	29.9 ± 0.7	29.8 ± 1.9
Glucose concentration (mg gwm^{-1})	8.04 ± 1.13	6.67 ± 1.24	7.52 ± 1.14	9.94 ± 1.08
Glycogen concentration (mg gwm^{-1})	160 ± 7	131 ± 8*	170 ± 8	154 ± 10
TAG concentration (mg gwm^{-1})	2.78 ± 0.30	2.75 ± 0.44	3.22 ± 0.45	3.35 ± 0.38
Lactate concentration (µmol gwm^{-1})	9.73 ± 0.95	9.10 ± 1.50	9.06 ± 1.35	7.65 ± 0.86
LDH activity (U mg $protein^{-1}$)	7.31 ± 0.49	7.95 ± 0.49	7.35 ± 0.34	7.77 ± 0.34
ALT activity (U mg $protein^{-1}$)	89.5 ± 3.5	90.0 ± 2.7	85.8 ± 3.3	89.8 ± 4.0
AST activity (U mg $protein^{-1}$)	103 ± 10	96.5 ± 6.8	96.5 ± 7.5	106 ± 10

Data are presented as means ± S.E.M, n = 5–7. *Significantly different from the control group with $P < 0.05$ according ANOVA followed by Dunnett's test.

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Turkish Journal of Fisheries and Aquatic Sciences
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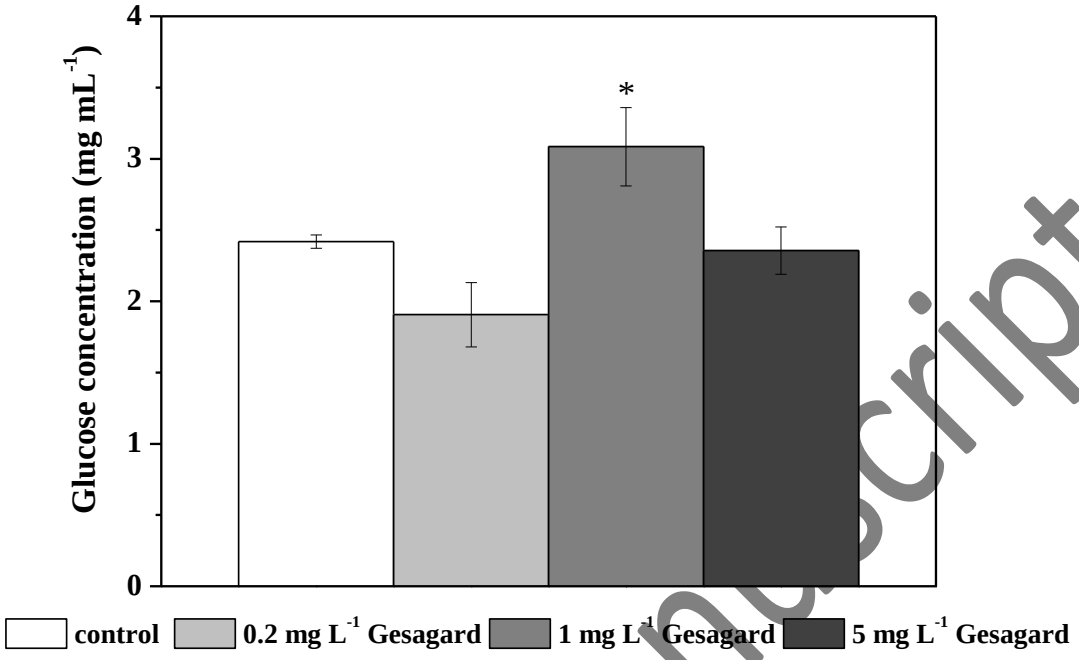

Figure 1. Glucose concentration in plasma of goldfish exposed to control conditions or 0.2, 1 or 5 mg L^{-1} of Gesagard for 96 h. Data are presented as means ± S.E.M, $n = 5–7$. *Significantly different from the control group with $P < 0.05$.

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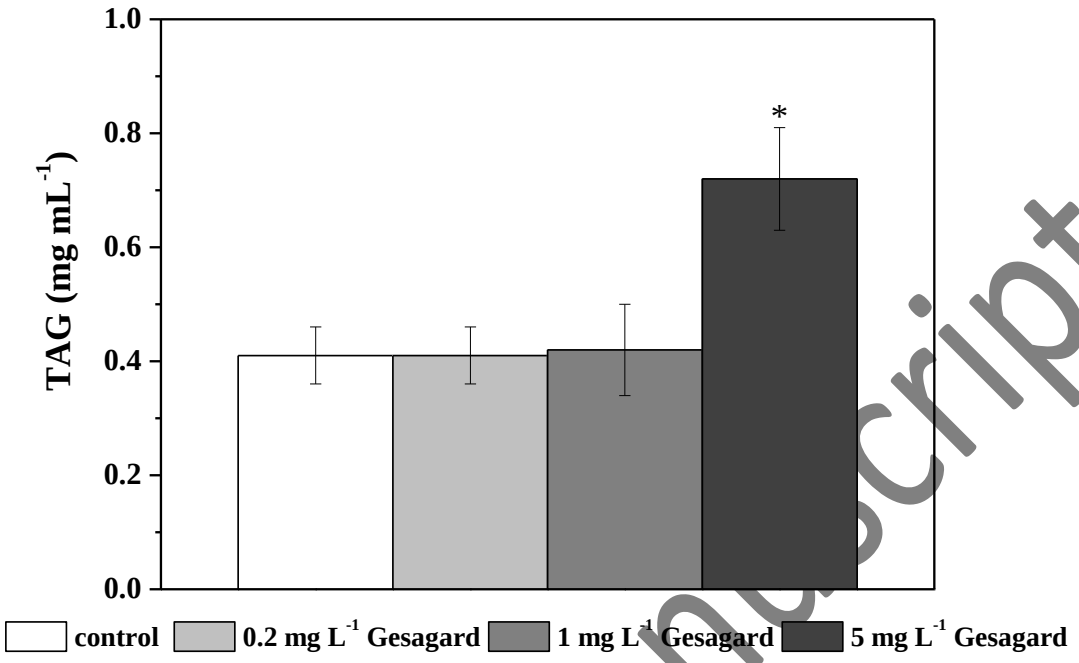

Figure 2. Triacylglyceride concentration (TAG) in plasma of goldfish exposed to control conditions or 0.2, 1 or 5 mg L^{-1} of Gesagard for 96 h. Data are presented as means ± S.E.M, n = 5–7. *Significantly different from the control group with $P < 0.05$.

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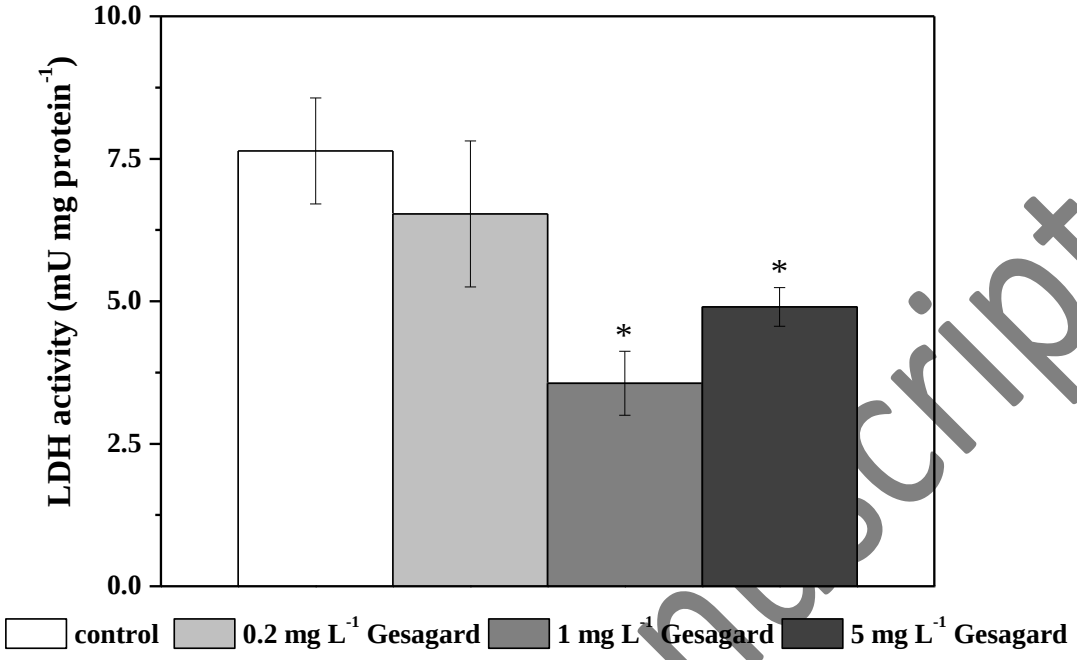

Figure 3. Lactate dehydrogenase (LDH) activity in plasma of goldfish exposed to control conditions or 0.2, 1 or 5 mg L^{-1} of Gesagard for 96 h. Data are presented as means ± S.E.M, $n = 5–7$. *Significantly different from the control group with $P < 0.05$.