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Do Low Doses of Imidacloprid Cause Oxidative Stress in Adult Marsh Frogs?

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Abstract. Studying the effects of neonicotinoids on non-target organisms is still an urgent task in today's world, where they are used in substantial quantities. Initially, it is interesting to find out the change in easy-to-detect parameters under insecticide exposure conditions that may indicate some exposure. Therefore, we investigated the parameters of oxidative changes (SOD, CAT, MDA, and total protein) in sexually mature marsh frogs caused by exposure to imidacloprid. Thirty frogs were selected for the experiment and divided into five groups of six individuals each. The exposure concentrations of imidacloprid were 10 μg/L and 100 μg/L. The duration of insecticide exposure was 7 and 21 days. After the finish of the experiment, the liver, kidney, and blood were taken from the animals under anesthesia. It was found that after 7 days of exposure, the group exposed to 100 μg/L imidacloprid solution had lower serum total protein than the control and 10 μg/L exposure groups ($p<0.05$). Also, after 21 days of imidacloprid exposure at a concentration of 100 μg/L, there was an increase in the SOD value in the frog liver compared to the control group ($p<0.05$). No significant differences were found in other parameters ($p>0.05$). The findings cast doubt on the unconditional use of common oxidative stress indices in adult marsh frogs as biomarkers of low-dose imidacloprid exposure. The obtained results for the adult marsh frog may support the possibility of using the liver SOD level only as a biomarker of imidacloprid exposure at environmentally significant concentrations.

Key words: antioxidant system, amphibians, biomarkers, neonicotinoid insecticides, imidacloprid, *Pelophylax ridibundus*.

Introduction

Various crop pests in the context of global climate change are causing significant and increasing economic damage. Pests destroy up to 40% of global crops and cost $220 billion in losses (Kolombar et al., 2020; IPPC Secretariat, 2021). Synthetic insecticides (SI) are increasingly used worldwide to protect plants against pests (Martynov et al., 2019; Sharma et al., 2019). However, these substances have toxic effects on non-target organisms, including humans (Vial et al., 1996; Kozak et al., 2020). Furthermore, both invertebrate and vertebrate animals are non-

target targets of SI. Therefore, agricultural pest control measures should consider the likely adverse environmental effects (Martynov et al., 2019).

Insecticides can disrupt cells' reactive oxygen species (ROS) metabolism (Nedzvetsky et al., 2021). This disruption leads to changes in enzymatic defenses and an increase in free radicals (Duzguner et al., 2012). As a result, this process can lead to apoptosis (Ge et al., 2015). Antioxidant enzymes, such as catalase (CAT) and superoxide dismutase (SOD), are known to be a part of the antioxidant system in living organisms (Aslanturk et al., 2011). As these enzymes are involved in the biochemical mechanisms of cellular detoxification, their values can be biomarkers of pollutant contamination of aquatic ecosystems (Monteiro et al., 2006). Malonic dialdehyde (MDA) is one of the leading products of lipid peroxidation. Therefore, this aldehyde can be used as an indicator of animal oxidative stress (Veedu et al., 2022).

Imidacloprid belongs to the group of neonicotinoid insecticides. It is used worldwide as a plant protection agent and has a global market share of 25% of neonicotinoids (Thompson et al., 2020). At the same time, imidacloprid can be carried by surface runoff and enter open water bodies (Yadav & Watanabe, 2018). This insecticide is susceptible to photolytic degradation, but the natural pH of the environment prevents its hydrolysis (Tišleret et al., 2009). Smit et al. (2015) concluded that the approximate quality standard for imidacloprid in water bodies should not exceed 8.3 ng/L for long-term exposure and 0.2 μg/L for short-term exposure.

Consequently, imidacloprid can have toxic effects on aquatic organisms and be transported through trophic chains (Van Dijket et al., 2013). In addition, imidacloprid can damage body systems responsible for transport and detoxification (Qadir et al., 2014). Several papers have also reported that imidacloprid can cause oxidative stress in cells of various organs of hydrobionts (Vieira et al., 2018; Hong et al., 2020; Huang et al., 2021). It is also known that imidacloprid can reduce the survival rates of amphibians, which may lead to a reduction in their populations (Ade et al., 2010).

Due to habitat confinement, high cutaneous permeability, and the aquatic larval stage of development, amphibians, opposite terrestrial vertebrates, are particularly susceptible to environmental influences. Therefore, amphibians can be convenient sentinels for assessing and bioindicating ecosystem pollution by pollutants (Şişman et al., 2021). Furthermore, as amphibians may cross agricultural land during migration, they are affected by pesticides and their half-life products (Leeb et al., 2020). It is also worth noting that migrations may coincide with the period of pesticide application (Lenhardt et al., 2014).

The marsh frog *Pelophylax ridibundus* (Pallas 1771) is a common species in many parts of Europe and Asia. It is a part of the green frog complex (Pysanets & Kukushkin, 2016; Suriadna et al., 2020; Baranovski, 2021). This hygrophilous amphibian occupies permanent water bodies, where it goes into anabiosis for wintering (Pysanets, 2007). In developed agrarian areas, *P. ridibundus* biotopes often locate adjacent to agricultural fields. As a result, insecticides enter coastal and aquatic ecosystems, which have toxic effects on the species (Kryvoltsevych et al., 2022). Also, invertebrates exposed to insecticides may enter the diet of the marsh frog (Pafilis et al., 2019; Alnoaimi et al., 2021).

Data on the effects of low doses of imidacloprid on adult amphibians are incredibly scarce (Campbell et al., 2022). Existing data on the toxic effects of neonicotinoids have been obtained mainly on tadpoles (Gasso et al., 2020b).

Our work aimed to investigate the experimental effects of imidacloprid in low doses on oxidative stress in marsh frogs.

Materials and Methods

Thirty individuals of *P. ridibundus* were selected for the study. The amphibians were caught at night in the Orel River coastal ecosystem (Dnipro region, Ukraine) during the post-reproductive period (late August – early September). The animals were trans-

ported to the laboratory in ventilated containers (Saad et al., 2022). The weight of the frogs was 50.89 ± 4.21 g, and the length was 82.62 ± 2.06 mm.

Before starting the experiment, each individual underwent three-day acclimatization in the laboratory. Each individual was kept in a 3-litre plastic container with a light/dark cycle (12/12 hours) at an average temperature of 27°C. The containers were filled with dechlori-nated tap water, which was changed every two days. The water had the following parameters: pH 7.2, dissolved oxygen 9.0 ppm, total ammonia 0.05 mg/L, total chlorine 0.05 mg/L, total dissolved solids 269.0 ppm, salinity 207.0 ppm, Ca^{2+} 60.0 mg/L, Fe^{2+} 0.05 mg/L. The water volume in the containers was 1 L.

The frogs were divided into five groups. Each group consisted of six individuals of *P. ridibundus*.

We used the "resource equation" method (Arifin & Zahiruddin, 2017) to calculate the sample size for analysis. We measured a value "E", which should lie within the limits of 10–20: E = total number of animals − total number of groups. In our case, E = (6 × 5) – 5 = 30 − 5 = 25, which is more than 20, hence sample size in this experiment is more than necessary.

The first and second frog groups were exposed to ecologically relevant concentrations of 10 and 100 µg (Van Dijk et al., 2013; Anderson et al., 2015) respectively for 7 days. The third and fourth groups were exposed to the same concentrations respectively for 21 days. The last group of frogs was kept in dechlorinated water and used as a control.

Frogs were not fed during the experiment due to possible interference with the absorption of the compound (Iturburu et al., 2018). Blood sampling, autopsy, and organ extraction (liver and kidney) were performed after the finish of the experiment under anesthesia (Stepchenko, et al., 2021).

Blood was collected in 3 ml tubes (Vacutainer® EDTA tubes). Part of the whole blood was used for catalase analysis. The rest of the blood was centrifuged at 1000-1200 rpm for 10-15 minutes. The resulting serum was used for MDA and SOD analyses.

The liver and kidney samples were homogenated in 0.1 M phosphate buffer (1:10) and centrifuged at 20 000 g for 15 min to obtain the extracts for further study. The total protein concentrations were determined according to the Bradford protocol (Bradford, 1976). The activity of catalase (CT, EC 1.11.1.6) was measured by evaluating the ability of hydrogen peroxide to form a stable-colored complex with molybdenum salts and expressed in ncat/mg of protein (Koroliuk, et al. 1988). The activity of superoxide dismutase (SOD, EC 1.15.1.1) was measured by assessing the ability of the enzyme to inhibit quercetin oxidation and expressed in arbitrary units per mg of protein (AU/mg protein) (Kostyuk et al., 1990). Malonic dialdehyde (MDA) content was determined as described previously (Ohkawa et al., 1979), based on the reaction of MDA with thiobarbituric acid (TBA) and expressed in nmoles of TBA-reactive substances (TRAS) per mg of protein.

The study was carried out following the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986) and Law of Ukraine No. 3447-IV "On the Protection of Animals from Cruelty" (Revision on August 8, 2021).

Results are presented by calculating the mean and standard error (SE) values. A non-parametric Kruskal-Wallis (K-W) test, followed by Dunn's test, was performed. The level of statistical significance was $p<0.05$. Statistical processing was performed with Origin Software, version 9.8 (Origin Lab Corp, 2021).

Results

The total protein content in the liver and kidneys of *P. ridibundus* did not differ between the control groups and the groups exposed to imidacloprid concentrations (K-W test, $p>0.05$) after 7 and 21 days. However, in the serum of amphibians that were exposed to an imidacloprid solution of 100 µg/L for seven days, there was a tendency for this index to decrease compared with the control group and the group which was exposed to 10 µg/L (Dunn's test, $p<0.05$). It is worth noting that

after 21 days of exposure, there was no difference in serum total protein content between the imidacloprid and control groups (K-W test, $p>0.05$) (Table 1).

The study of CAT activity in marsh frog serum, liver, and kidney showed no significant difference after 7 and 21 days of imidacloprid exposure compared to the amphibian control group (K-W test, $p>0.05$) (Fig. 1). There is a tendency for catalase activity to increase in the liver and kidneys at 100 μg/L imidacloprid concentration.

In the group of 21 days of imidacloprid exposure at a concentration of 100 μg/L in the frog liver, an increased level of SOD was observed when compared to the control (Dunn's test, $p<0.05$). However, 21-day exposure to imidacloprid at a concentration of 10 μg/L caused no significant abnormalities (Dunn's test, $p>0.05$). There were also no significant differences between the groups of frogs under seven days' exposure to the insecticide (K-W test, $p>0.05$).

Table 1. Effect of imidacloprid on total protein values in liver, kidney, and serum of *P. ridibundus*. Significant differences: * – to control; # – to 10 μg/L exposure.

Days of exposure	Concentration of imidacloprid, μg/L	Liver	Kidney	Serum
Control		20.47 ± 1.45	7.42 ± 0.70	115.44 ± 15.45
7	10	17.64 ± 1.77	8.34 ± 1.05	130.26 ± 23.77
	100	20.06 ± 1.88	7.48 ± 0.88	61.32 ± 7.91*#
21	10	19.12 ± 0.66	7.16 ± 2.33	106.31 ± 18.85
	100	16.30 ± 2.60	5.91 ± 1.26	108.74 ± 28.29

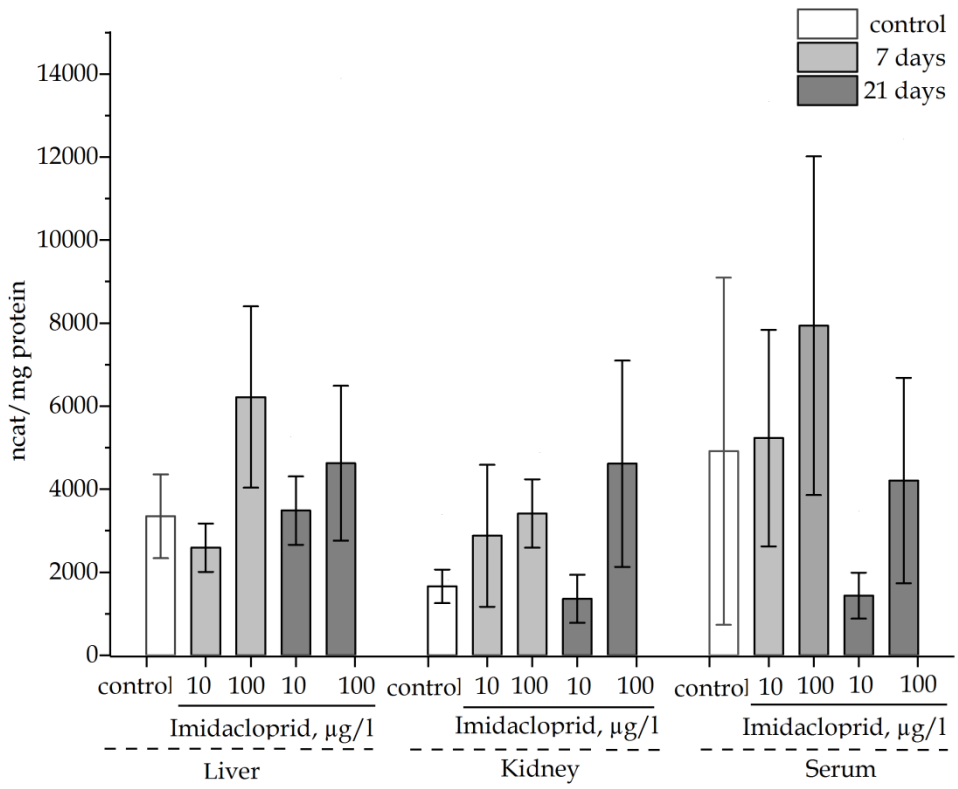

Fig. 1. Effect of imidacloprid on catalase activity values in liver, kidney, and serum of *P. ridibundus*. Data are presented as mean ± SE.

In the group of 21 days of imidacloprid exposure at a concentration of 100 μg/L in the frog liver, increased activity of SOD was observed when compared to the control (Dunn's test, $p<0.05$). However, 21-day exposure to imidacloprid at a concentration of 10 μg/L caused no significant abnormalities (Dunn's test, $p>0.05$). This activity was also no differences between the groups of frogs under seven days' exposure to the insecticide (K-W test, $p>0.05$).

When the SOD content of the kidneys and serum of the frogs was examined, imidacloprid was found to cause no change in this index (K-W test, $p>0.05$). Although there is no statistically significant difference in renal SOD activation, a trend is observed. After 7 days of exposure to both concentrations, there is a non-significant decrease. However, at day 21 there is a reversal of the trend towards an increase relative to control. Also, when exposed to concentrations of 10 μg/L imidacloprid for 7 and 21 days, an increasing trend in serum SOD activity can be observed (Table 2).

In the liver, kidneys, and serum of *P. ridibundus*, which were exposed to 10 μg/L and 100 μg/L imidacloprid solution, MDA had no significant differences (K-W test, $p>0.05$) (Fig. 2). After 21 days of imidacloprid exposure, serum levels of MDA tended to increase at concentrations of 10 μg/L. However, at 100 μg/L this trend is not observed.

Table 2. Effect of imidacloprid on the activity of superoxide dismutase in liver, kidney, and serum of *P. ridibundus*. * – significant differences to control

Days of exposure	Concentration of imidacloprid, μg/L	Liver	Kidney	Serum
Control		0.18 ± 0.03	1.30 ±0.32	0.06 ± 0.02
7	10	0.13 ± 0.05	0.56 ± 0.24	0.19 ± 0.14
	100	0.17 ± 0.06	1.07 ± 0.55	0.09 ± 0.02
21	10	0.23 ± 0.02	1.91 ± 0.56	0.17 ± 0.10
	100	0.32 ± 0.03*	2.69 ± 1.37	0.10 ± 0.06

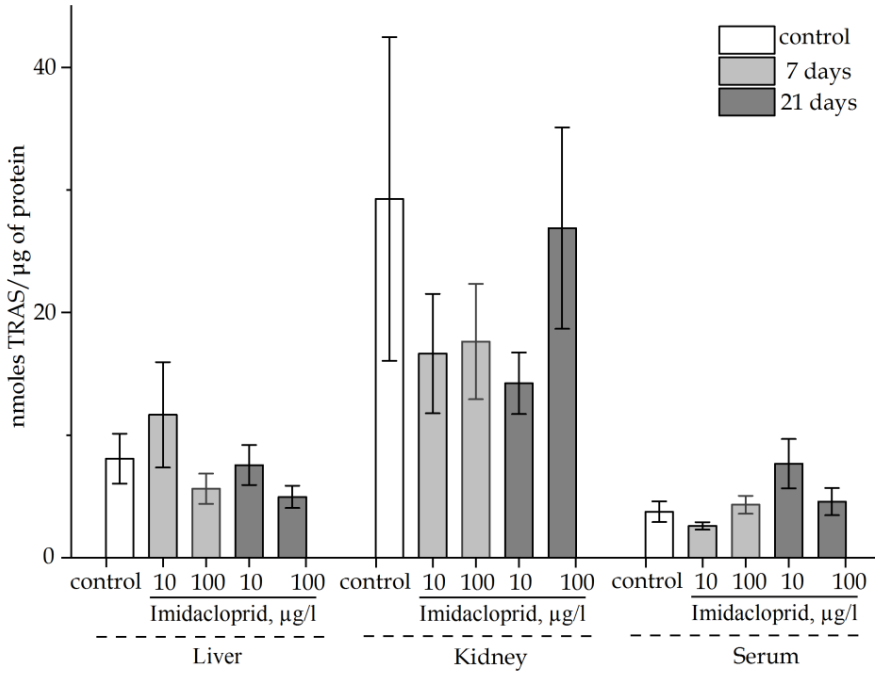

Fig. 2. Effect of imidacloprid on malonic dialdehyde content in the liver, kidney, and serum of *P. ridibundus*. Data are presented as mean ± SE.

Discussion

Neonicotinoids could provoke changes in antioxidant enzyme activity, leading to a deterioration of cells' antioxidant protection (Yang et al., 2020). However, existing studies have been conducted at higher concentrations of these insecticides. Loutfy & Kamel (2018) found that levels of total protein, albumin, GSH, and SOD declined, and MDA raised in the serum of the African common toad *Sclerophrys regularis* (Reuss, 1833) after the 12-day impact of Actara 20% (0.03 g/L) and Acetamore 25% (0.05 g/L) solutions. Thiamethoxam (30 mg/L) and acetamiprid (40 mg/L) have similar toxic effects on *S. regularis* (Saad et al., 2022). Guo et al. (2022) observed changes in concentrations of SOD, CAT, and MDA at tadpoles of the dark-spotted frog *Pelophylax nigromaculatus* (Hallowell, 1861) by acetamiprid (0.18 mg/L and 1.85 mg/L) during 28 days. Camlica et al. (2017) found that acetamiprid at a concentration of 1×10^{-6} M decreased the acetylcholinesterase and CAT activities but increased the MDA level in the sciatic nerve of *P. ridibundus* after 120 minutes of exposure. These findings support an idea of peripheral nervous toxicity of imidacloprid.

It is known that the effects of pesticides can decrease plasma protein content (Rivarola et al., 1991). Reduced total protein level may indicate degradation or deterioration of protein synthesis (Shakoori et al., 1990). As serum proteins are predominantly synthesized in the liver, this may indicate a deterioration of this organ (Thapa & Walia, 2007). It is worth mentioning that although no significant difference was observed, this was accompanied by a tendency for catalase activity to increase. All of this may indicate a possible strain on the liver (Tuzmen et al., 2007). Most toxicity studies on neonicotinoids have been carried out on tadpoles of tailless amphibians (Lee-Jenkins et al., 2018; Holtswarth et al., 2019; Gavel et al., 2021; Saka & Tada, 2021). Low concentrations of these insecticides cause significant oxidative stress in amphibian larvae (Robinson et al., 2021; Guo et al., 2022). However, data on the response of adult amphibians are very limited (Loutfy & Kamel, 2018; Saad et al., 2022).

Luo et al. (2021) found that after 21 days of exposure to imidacloprid (100 and 1000 μg/L) SOD and CAT levels were increased in the intestines of zebrafish *Danio rerio* (Hamilton, 1822). Vieira et al. (2018) reported that SOD concentrations rose in the liver of the streaked prochilod *Prochilodus lineatus* (Valenciennes, 1837) by the impact of low imidacloprid doses (12.5 μg/L, 125 μg/L, and 1250 μg/L) during 120 hours. Our data on increased SOD activities in the liver of studied *P. ridibundus* could be connected with ROS production and indicated oxidative stress. El-Garawani et al. (2021) also described grows of this indicator in the liver of the Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) after 21 days of imidacloprid exposure. However, SOD activity decreased with an increase in the concentration of imidacloprid. This effect is probably because ROS production exceeds the cells' antioxidant defense capacity (Saddick et al., 2017).

Qadir and coauthors (2014) likewise noted that total protein decreased after two days of imidacloprid impact on the rohu *Labeo rohita* (F. Hamilton, 1822). However, this parameter levelled off concerning the control group on the following days of the experiment. We found that imidacloprid at a concentration of 100 μg/L declined serum total protein after seven days of exposure. Nevertheless, this response was absent after twenty-one days of the experiment. As so, serum total protein in adult marsh frogs is not a good biomarker of low doses of imidacloprid exposure.

The data on slight changes in oxidative stress indices obtained in our study may indicate a greater resistance of adult frogs to low concentrations of imidacloprid in comparison to tadpoles. In this case, the commonly used CAT and MDA indices may not show the presence of that insecticide's low concentration in the environment.

The authors are aware of the fact that if the sample size were increased, the observed trends in changes in oxidative stress indicators would probably prove to be statistically significant differences. On the other hand, given the problem of the conservation of natural populations, it is necessary to use the minimum possible number of animals for such experiments. Biochemical research regularly uses 5-7 animals per experimental group (Serdar, 2021). Often the same principles of

sufficient sample size are also applied to wildlife populations under study. In this case, we often see trends not supported by statistical validity. Data obtained demonstrate that amphibians taken from natural populations could show an ambiguous response to exposure to low doses of imidacloprid-type synthetic neonicotinoids. However, the observed changes, both statistically significant and trending, indicate some adverse effects of imidacloprid exposure on non-target amphibian species. At the same time, the findings suggest the need for more sensitive exposure biomarkers in the case of low concentrations of synthetic neonicotinoids affected adult frogs weighing about 50 g or more should be sought.

Campbell et al. (2022) found that imidacloprid at ecologically relevant concentrations can cross the blood-brain barrier and bioaccumulate in the brain of the northern leopard frog *Lithobates pipiens* (Schreber, 1782). Thus, if penetrated the central nervous system, imidacloprid can induce changes at the molecular level. Neuromolecular markers such as glial fibrillary acidic protein (Tikhomirov et al., 2016; Shiyntum et al., 2017; Sukharenko et al., 2017) or replicative protein A (Nedzvetsky et al., 2020) provide an opportunity to detect the effects of toxicity factors (Gasso et al., 2020a).

The validity of these parameters was confirmed in experiments on invertebrates (worm *Eisenia fetida* (Savigny, 1826) (Huslystyi et al., 2021)), fish (rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792)) (Nedzvetsky et al., 2020)), and frog *P. ridibundus* (Yemolenko et al., 2022). Therefore, the use of these biomarkers, as opposed to standard oxidative stress indices, may be promising parameters for further research into the effects of low imidacloprid concentrations on adult amphibians.

Conclusions

As a result of 7-day and 21-day exposures of *P. ridibundus* to imidacloprid in low doses known in the environment, only several biochemical parameters changed among the studied ones. Seven days of exposure to imidacloprid at a concentration of 100 µg/L reduced the total protein level in the frogs' serum. In addition, after 21 days of exposure to the appropriate imidacloprid concentration, the amphibians showed an increase in liver SOD activities. However, no significant changes in CAT and MDA activities were detected. The findings cast doubt on the unconditional use of common oxidative stress indices in adult marsh frogs as biomarkers of low-dose imidacloprid exposure. The obtained results for the adult marsh frog may support the possibility of using the liver SOD level only as a biomarker of imidacloprid exposure at environmentally significant concentrations. Slight changes in oxidative stress indices obtained in our study may indicate a certain resistance of adult frogs to imidacloprid in low concentrations. Our data suggest that more sensitive exposure biomarkers should be used for adult frogs weighing about 50 g or more to low concentrations of synthetic neonicotinoids.

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