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Effects of alpha-cypermethrin and difenoconazole on survival, growth and biomarkers in European green toad tadpoles (*Bufotes viridis*, Laurenti 1768)

Bruno Bekić[1], Anamarija Žagar[2,3,4], Tatjana Simčič[2], Branimir Hackenberger Kutuzović[1], Olga Jovanović Glavaš[1,*]

[1] Department of Biology, Josip Juraj Strossmayer University of Osijek, Cara Hadrijana 8/A, 31000 Osijek, Croatia.
[2] National Institute of Biology (NIB), Department of Organisms and Ecosystems Research, Večna pot 121, 1000 Ljubljana, Slovenia.
[3] CIBIO Research Centre in Biodiversity and Genetic Resources, InBIO, University of Porto, Rua Padre Armando Quintas, No 7, 4485-661 Vairao, Portugal.
[4] Biotechnical faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia.

* Correspondence: ojovanovic@biologija.unios.hr

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In recent decades, the decline of amphibian populations has become more pronounced and accelerated, with one of the contributing factors being the excessive use of pesticides. Alpha-cypermethrin, an insecticide, and difenoconazole, a fungicide, are widely employed pesticides globally. To gain a comprehensive understanding of the acute impact of these two pesticides on amphibians, we used European green toad (*Bufotes viridis*) tadpoles as our model organisms. We assessed mortality and mass as apical endpoints, and examined physiological biomarkers including electron transport system (ETS) activity, catalase activity, and carbonyl protein content. Tadpoles were exposed to varying concentrations of the two pesticides for 48 hours (0.2-10 μg/L for alpha-cypermethrin; 10-100 μg/L for difenoconazole). Our results indicate that the exposure of tadpoles to these pesticides significantly affected their physiology. Mortality was observed exclusively in tadpoles treated with difenoconazole (LC_{50} = 100.832 μg/L), while a decrease in mass occurred in tadpoles exposed to both pesticides. No significant differences across treatments were found for total ETS activity, catalase activity, or the presence of protein carbonyls. In conclusion, our findings suggest that alpha-cypermethrin at environmentally relevant concentrations poses a risk to European green toad tadpoles, while the effects of difenoconazole are become patent at higher concentrations than those recorded in natural aquatic environments.

Key words: Amphibia; Bufonidae; electron transport system; pesticide exposure; toxicology.

The first major decline of amphibians was observed in the 1980s, and species numbers have continued to decline ever since (Hayes *et al*., 2010). The class of Amphibia is considered one of the most endangered groups of animals (Stuart *et al*., 2004), with 40.7% of amphibian species being threatened with extinction (Luedtke *et al*., 2023). Major reasons for their decline include climate change, driving 39% of the declines, followed by habitat loss with 37%. Apart from that, various pathogens

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and diseases, UV-B radiation, the presence of invasive species, and pesticide use are the other major causes (HAYES *et al.*, 2010). Amphibians interact with both aquatic and terrestrial ecosystems during their life cycle, and many environmental stressors contribute significantly to reducing their numbers (BLAUSTEIN & WAKE, 1990). Aquatic forms of amphibians are highly sensitive to water pollution (e.g. pesticides) due to their highly permeable skin (FRYDAY & THOMPSON, 2012). Adverse effects of pesticides on tadpoles, among others, include reduced growth and development, endocrine disruption, impaired metamorphosis, the occurrence of malformations, and increased mortality at environmentally relevant concentrations (e.g. GREULICH & PFLUGMACHER, 2003; HAYES *et al.*, 2010).

Alpha-cypermethrin ($C_{22}H_{19}Cl_2NO_3$) belongs to the group of pyrethroid insecticides that are used to control insect pests in agriculture (GÜRKAN *et al.*, 2016). It is a widely used pesticide due to its low toxicity levels to birds and mammals. However, it is highly toxic to aquatic organisms, both invertebrates and vertebrates, with median lethal concentration (LC_{50}) values for zebra fish (*Danio rerio*) of 1.84 and 2.20 μg/L after 96 or 24 hours of exposure, respectively (WHO, 2021). Considering the concentrations found in the environment (e.g. concentrations of 0.2 to 150 μg/L were found in streams near agricultural areas in Argentina; AGOSTINI *et al.*, 2010) it is highly relevant to study as many different aspects of this pesticide as possible on other aquatic organisms, such as tadpoles.

Alpha-cypermethrin acts as a neurotoxin and several studies have confirmed various negative effects on different amphibian species. It was shown to affect growth in *Hypsiboas pulchellus* tadpoles after 96 h exposure at concentrations of 0.83 μg/L when used as commercial products and 3.44 μg/L as technical grade substance (AGOSTINI *et al.*, 2010). Also, it caused morphological changes and deformities in mouth and tail, visceral edema, and behavioral changes at low concentrations (0.34-4.18 μg/L), while a 100 % malformation rate was observed at 34.4 μg/L (AGOSTINI *et al.*, 2010). Increased oxidative stress, expressed as increased catalase activity and lipid peroxidation, as well as increased activity of the biotransformation enzyme glutathione S-transferase, were recorded after 96 h exposure to 0.3 μg/L of technical grade substance in *Duttaphrynus melanostictus* tadpoles (DAVID *et al.*, 2012). Similarly, increased activity of antioxidant (i.e. superoxide dismutase and catalase) and glutathione S-transferase enzymes, together with increased malondialdehyde (a biomarker of lipid peroxidation) content, shortened swimming distance and even complete immobility, were recorded after 14 days of exposure of *Pelophylax nigromaculatus* tadpoles to a sublethal concentration (0.5 μg/L) of technical grade alpha-cypermethrin (XU & HUANG, 2017). Although most of the aforementioned studies recorded sublethal effects at concentrations below 10 μg/L, concentrations causing mortality are very variable (from as low as 1.73 μg/L; XU & HUANG, 2017; up to 479.7 μg/L; AGOSTINI *et al.*, 2010), which stresses the importance of new data on other amphibian species. In addition, examining other sublethal effects is important as these data can assist in better understanding of the threat posed by this

pesticide to amphibians in the environment.

Difenoconazole ($C_{19}H_{17}Cl_2N_3O_3$) is a widely used triazole fungicide applied to control various fungal diseases of fruits, vegetables and cereals. As such, it is commonly detected in various types of surface waters, with concentrations ranging from negligible 0.09 μg/L to 16 μg/L detected in Canada (HEALTH CANADA, 2021), while soil lixiviate concentrations can reach up to 320 μg/L (ZHAO *et al*., 2018). Fungicides generally cause amphibian malformation and weight loss, reduction in tadpole growth and survival, delay in metamorphosis, reduced mobility, weakening of the immune system, and oxidative stress (BERNABÒ *et al*., 2016). Despite the wide use of difenoconazole, very few data are available on its effects on amphibians.

Nowadays, studies focusing on sublethal effects that impair physiological processes are becoming more important, as this type of data can provide better insight into long-term effects that may not be evident in general toxicity studies that focus only on mortality as a single endpoint (e.g. PHAM *et al*., 2017; BARRIGA-VALLEJO *et al*., 2017). Pesticide exposure, as a common environmental stressor, increases oxidative stress in cells. Oxidative stress occurs when more reactive oxygen species (ROS) are present in cells than the capacity of cells to detoxify them. This results in ROS accumulation that can affect different molecules in the cell. For that reason, exposure of organisms to stressors (e.g. pesticides) can result in a higher energy demand, that translates into increased metabolic activity, including an increase in electron flow within the electron transport system (ETS; ŽAGAR *et al*., 2015). In this context, studying the electron flow in the transport system, which results in production of ATP molecules in the mitochondria, is a good example of such mechanism (THOMSON, 2019). The ETS, which is located in the inner membrane of the mitochondria, acts as a bridge between oxidizing organic matter and oxygen (O_2).

Increased oxidative stress can also be assessed by two widely used biomarkers, catalase activity and the presence of protein carbonyls (RAVI KIRAN & ARUNA, 2010). Catalase (1.11.1.6) is an antioxidant enzyme that catalyzes the degradation reaction of extremely harmful hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) (ZHANG *et al*., 2018). Protein carbonyls are reactive aldehydes and ketones formed by oxidation of the side chains of certain amino acids (DALLE-DONNE *et al*., 2003). They are very stable and can be formed in several ways, the most important being the oxidation of proteins by hydroxyl radicals (-OH), which results from the accumulation of free radicals (IRAZUSTA *et al*., 2011). This leads to changes in amino acid sequence, cleavage of peptide bonds and formation of carbonyls (Dalle-Donne *et al*., 2003).

The European green toad *Bufotes viridis* (Laurenti 1768), of the family Bufonidae, is an example of a species that frequently occurs in urban areas, including suburban agricultural landscapes (e.g. Donaufeld near Vienna; SISTANI, *et al*., 2021) that serve as refugia for this species. Apart from these anthropogenic habitats, it inhabits steppes and wild floodplains (LANDLER *et al*., 2023). In these habitats, it is highly likely to be exposed to various pollutants,

such as pesticides (BĂNCILĂ *et al.*, 2023). Geographically, it is an extremely widespread species, occurring throughout much of Europe (KARAKOUSIS & KYRIAKOPOULOU-SKLAVOUNOU, 1995; AGHASYAN *et al.*, 2017). According to the IUCN Red List of Threatened Species, it is classified as Least Concern (LC), although significant population declines have been observed in some parts of its range (e.g. AGHASYAN *et al.*, 2017).

In our study, we used *B. viridis* tadpoles as a model organism to evaluate the effects of two widely used pesticides (alpha-cypermethrin and difenoconazole) on general endpoints and physiological biomarkers. We hypothesize that *B. viridis* tadpoles are susceptible to the adverse effects of the aforementioned pesticides at environmentally relevant concentrations. We investigated this by measuring tadpole mass and survival, as well as catalase activity, protein carbonyl content, and ETS activity. The outcome of our study will provide valuable data on this widespread species and will assist in better understanding of the potential effects of these pesticides. As well, these results could be applied to other amphibian species with similar ecology and physiology.

MATERIALS AND METHODS

Test organisms and experimental setup

The experiments were conducted according to the EU legislation for animal experimentation. This research was approved by Ministry of Economy and Sustainable Development of Republic of Croatia (Institute for Environment and Nature UP/I-612-07/20-48/205).

We collected *B. viridis* tadpoles from a small private artificial fishpond (2 m in diameter) near Osijek, Croatia (Semeljci; GPS: 45°21'36"N 18°32'24"E), where chances of exposure to agricultural runoff are minimal, and transferred them to the laboratory. After 24 hours of acclimatization, groups of seven tadpoles that were similar in size were randomly selected (Gosner-stage 25-26 determined by inspection of all individuals under a loupe; GOSNER, 1960) and placed in 250 ml glass flasks filled with 100 ml of standardized FETAX solution (625 mg NaCl, 96 mg $NaHCO_3$, 75 mg $MgSO_4$, 75 mg $CaSO_4 \cdot 2H_20$, 30 mg KCl, 15 mg $CaCl_2$ per liter of deionized water; pH = 7.34; conductivity 1322 μS/cm), to which a pesticide at a certain concentration was added (see next paragraph). The sample size was chosen as the lowest possible number of animals which provided enough material for subsequent analysis, so the number of used animals was minimized. The housing volume was selected because tadpoles at Gosner-stage 25 are relatively small and do not need much space, especially since they were not fed during the experiment. Apart from pH and conductivity we did not measure other physical and chemical parameters of the test solutions because previous studies conducted under similar conditions revealed a stability in such conditions (e.g. HENRY *et al.*, 2013; YU *et al.*, 2013).

All used reagents were of analytical grade. Tadpoles were kept at constant temperature (21 ± 1°C) under natural light conditions and were not fed during the experiment. Mortality was checked after 24 and 48 hours of exposure, and all dead tadpoles were removed from the flasks.

After 48 hours, live tadpoles were weighed and stored at -80°C until further analysis

Pesticides

For experimental treatments, two pesticides were used: difenoconazole (commercial preparation DIFCOR, Agro-Chem MAKS) with an active ingredient (a.i.) concentration of 250 g/l, at concentrations of 10, 20, 40, 60, 80 and 100 μg/l a.i., and alpha-cypermethrin (commercial preparation FASTAC® 10 EC, BASF) with an active ingredient concentration of 100 g/l, at the following concentrations: 0.2, 1, 2.5, 5, 7.5 and 10 μg/l a.i.; for the control treatment, FETAX solution was used. Concentrations for both pesticides were chosen based on the existing data to have a range covering from environmentally relevant concentrations to concentrations known to affect tadpoles or other organisms. All concentrations and control treatment were set in three replicates.

Sample preparation and biochemical analysis

For all analyses, whole tadpoles were used for preparation of homogenates. For homogenization of tissue for carbonyl proteins, 50 mM phosphate buffer, 0.5 M EDTA and 100 mM phenylmethanesulfonyl fluoride were used, while for determining the activities of the ETS and catalase, homogenization buffer consisted of 75 μM $MgSO_4$, Polyvinylpyrrolidone 0.15% w/v, Triton-x-100 0.2% (v/v) and phosphate buffer (pH 8.4).

Catalase activity was measured according to the method proposed by AEBI (1984) using UV/Vis spectrophotometer Lambda 25 (Perkin-Elmer, USA) at 240 nm every 30 sec for 2 min at 25°C.

Activity of the ETS was determined following the methodology described in ŽAGAR *et al.* (2015). Homogenates were centrifuged for 4 min at 0°C and 10 000 g, and supernatant was transferred to three microtiter plates together with reagent solution (substrate, 0.25 mM NADPH and 1.7 mM NADH, and iodonitrotetrazolium–INT). Microtiter plates were incubated for 15 min at three different temperatures (20° C, 24°C and 28°C). These temperatures were selected based on the known range of body temperatures of *B. viridis* when active (KATZ & GIL, 1997), to measure the actual expression of metabolism. After incubation, formaldehyde and H_3PO_4 were added to the microtiter plates to stop the reaction. Finally, the absorbance was measured at 490 nm using a microplate reader (Synergy MX BioTek, USA). From the measured values, concentration of consumed oxygen was calculated, which corresponds to the amount of formazan produced by the reduction of INT.

Carbonyl content was measured using a MAK094 (Sigma-Aldrich) commercial carbonyl group assay kit. Protein carbonyl groups are derivatized with 2,4-dinitrophenylhydrazine, which leads to the creation of stable dinitrophenylhydrazone adducts. The quantity of these adducts is directly proportional to the amount of carbonyls in the sample, which are detected spectrophotometrically at 375 nm. In addition, Pierce™ BCA protein analysis kit (Thermo Scientific, USA) was used to determine protein concentration of each sample.

Table 1: Recorded mean values ± standard deviation (SD) for body mass (BM), catalase activity (CAT) and carbonyl proteins (CARB) for different concentrations of alpha-cypermethrin and difenoconazole treatments. Values in bold are different from those at the control group ($P < 0.05$).

Substance	Concentration (μg/L)	BM (g)	CAT (U/g protein)	CARB (nmol carbonyl/mg protein)
α-cypermethrin	0	0.035 ± 0.003	48.94 ± 17.26	7.89 ± 2.88
	0.2	**0.026 ± 0.000**	52.05 ± 8.55	8.66 ± 3.80
	1	**0.026 ± 0.001**	52.78 ± 9.01	7.32 ± 1.32
	2.5	**0.026 ± 0.002**	35.94*	9.81 ± 2.20
	5	**0.024 ± 0.001**	33.87 ± 4.20	9.88 ± 6.03
	7.5	**0.025 ± 0.000**	35.94 ± 8.69	12.31 ± 9.41
	10	**0.024 ± 0.001**	36.80 ± 0.15	6.32 ± 0.37
Difenoconazole	0	0.033 ± 0.003	45.19 ± 19.85	5.90 ± 1.49
	10	0.032 ± 0.003	45.98 ± 2.67	8.24 ± 0.80
	20	**0.025 ± 0.001**	25.15 ± 8.09	9.62 ± 4.06
	40	**0.026 ± 0.000**	37.78 ± 5.29	8.37 ± 2.06
	60	**0.025 ± 0.001**	33.13 ± 9.75	14.61 ± 7.04
	80	**0.025 ± 0.001**	36.90 ± 16.17	7.23 ± 4.04
	100	**0.014 ± 0.010**	64.32 ± 25.30	6.97 ± 1.06

*For two of the three replicates, it was not possible to determine protein content and calculate CAT activity

Statistical analysis

We performed one-way analysis of variance (ANOVA) to determine the differences between control and each pesticide treatment for all variables. For samples that did not follow normal distribution, the nonparametric equivalent, the Kruskal-Wallis test, was used. We then used Tukey's LSD post hoc test. All tests were performed at a significance level of $\alpha = 0.05$. All statistical analyses were performed using R statistical program v.3.6.1. (R Development Core Team, 2019). For difenoconazole after 48 hours of exposure, LC_{50} value was calculated using Probit analysis (Finney, 1971).

Results

Tadpole survival

After 24 hours of exposure of tadpoles to various concentrations of difenoconazole, we observed mortality only at the highest concentration (100 μg/l), with an average mortality rate (±SD) of 42.9% (± 20.2) whereas after 48 hours of pesticide exposure, we observed an average mortality rate of 4.7% (± 6.7) at 80 μg/l and 47.6% (± 17.8) at 100 μg/l. At this exposure time, the calculated LC_{50} of difenoconazole as the tested formulation (DIFCOR) was 100.832 μg/l (95% CI: 91.875-110.662). Alpha-cypermethrin caused no mortality to tadpoles at any tested concentration during the 48 hours of exposure.

Tadpole mass

After 48 hours of exposure to different concentrations of difenoconazole, the mass of tadpoles exposed to 20, 40, 60 and 80 μg/l was lower than that in the control group, whereas there was no significant difference between the control and 10 μg/l groups (Table 1). The mass of tadpoles was lower than in the control group after exposure to alpha-cypermethrin at all tested concentrations (Table 1).

Biomarker analysis

Catalase activity after 48 h of exposure to different concentrations of either difenoconazole or alpha-cypermethrin did not differ significantly between the control and the different pesticide concentrations (Table 1).

The activity of ETS after the exposure of tadpoles to difenoconazole at all temperatures did not show significant differences between control and treatment groups. However, there were significant differences between various concentrations of difenoconazole (Fig. 1); at 20°C there was a significant difference in ETS activity of tadpoles exposed to 20 and 100 μg/l (Fig. 1A). At 28°C, the results were similar to those at 20 °C, with a decrease in

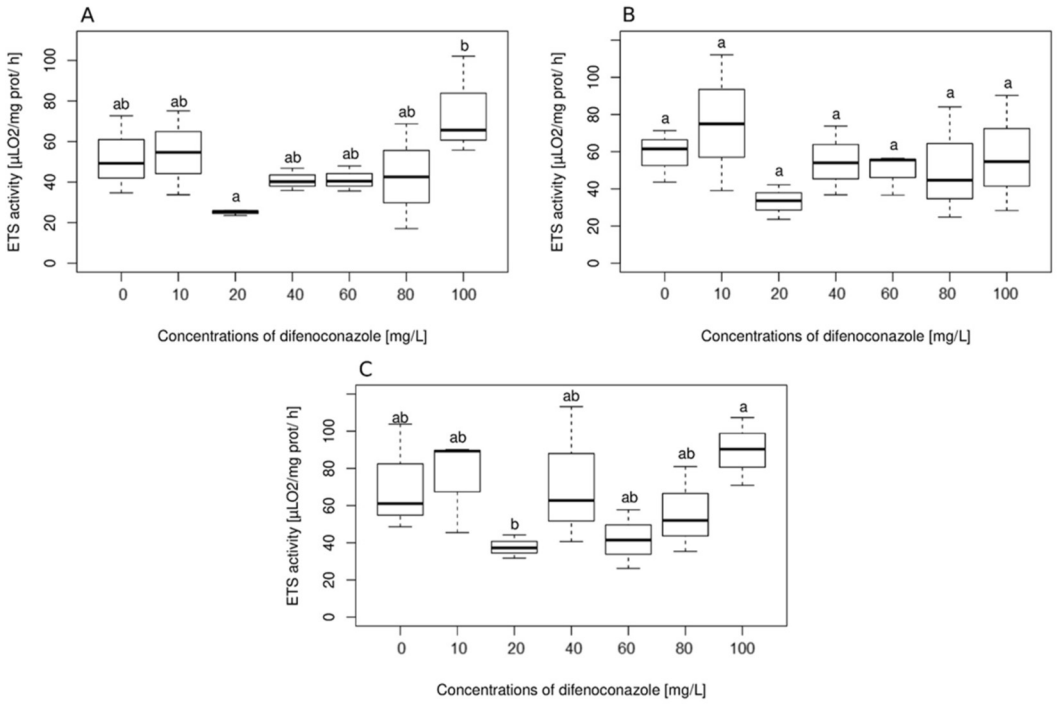

Figure 1: ETS activity [μl O_2/mg prot/h] after exposure of European green toad tadpoles (*Bufotes viridis*) to different concentrations of difenoconazole at (A) 20°C, (B) 24°C and (C) 28°C. Dark black line represent the median, boxes the interquartile range, and whiskers the total range. Statistically significant differences of Tukey's LSD post hoc tests are shown with different letters ($P < 0.05$).

ETS activity at 20 μg/l and an increase at 100 μg/l concentration (Fig. 1C).

ETS activity after the exposure of tadpoles to alpha-cypermethrin at all temperatures did not show significant differences between control and the different pesticide concentrations. However, there were significant differences between various concentrations of alpha-cypermethrin treatment (Fig. 2); at 24°C there was an increase in ETS activity at 1 μg/l in relation to 2.5 μg/l) (Fig. 2B), and at 28°C there was an increase in ETS activity at 1 μg/l in relation to 7.5 μg/l (Fig. 2C).

The presence of carbonyl groups after exposure of tadpoles to difenoconazole or alpha-cypermethrin showed no significant differences between the control and the different pesticide concentrations (Table 1).

Discussion

In this study, we investigated the effects of two widely used pesticides, alpha-cypermethrin (an insecticide) and difenoconazole (a fungicide), on *B. viridis* tadpoles. Specifically, we examined their impact on mortality and mass, as well as effects expressed through oxidative stress and metabolic activity. This was achieved by analyzing catalase activity, carbonyl

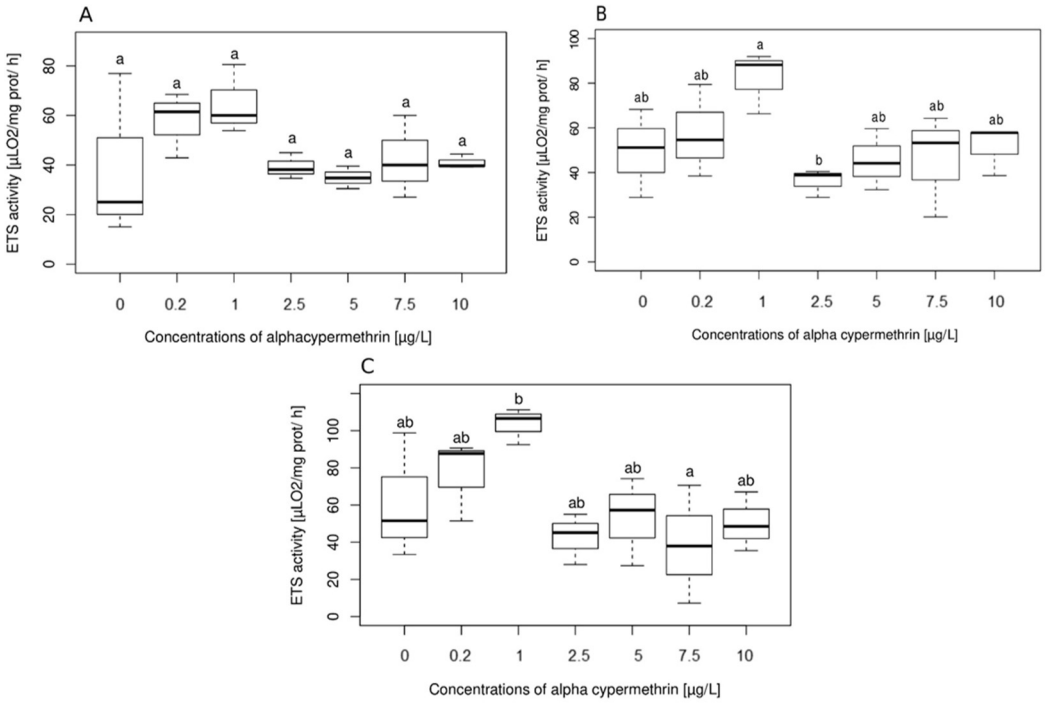

Figure 2: ETS activity [μl O_2/mg prot/h] after exposure of European green toad tadpoles (*Bufotes viridis*) to different concentrations of alpha-cypermethrin at (A) 20°C, (B) 24°C and (C) 28°C. Dark black line represent the median, boxes the interquartile range, and whiskers the total range. Statistically significant differences of Tukey's LSD post hoc tests are shown with different letters ($P < 0.05$).

content, and ETS activity. The obtained results showed that both pesticides had negative effects on *B. viridis* tadpoles. Exposure to difenoconazole caused mortality at concentrations of 80 and 100 μg/l, and both pesticides caused a significant reduction in tadpole mass. In addition, exposure to these pesticides impaired the physiological processes of the animals.

Mortality is a critical indicator of the harmfulness of certain pesticides because it directly affects the number of individuals and thus can cause a reduction in populations during exposure (McMahon *et al*., 2011). After a 24-hour exposure to difenoconazole, average mortality was found to be 42.9% (± 20.2) only at the highest concentration of 100 μg/l. After a 48-hour exposure, average mortality was 47.6% (± 17.8) at 100 μg/l and 4.7% (± 6.7) at 80 μg/l. Mortality data for difenoconazole are not available from amphibian tadpoles, but available data from fish show that amphibian larvae from our study would be more sensitive than fish to this fungicide. Zebra fish (*Danio rerio*) tolerated high concentrations of difenoconazole, with a 48h-LC_{50} of 1.4 mg/l (Sanches *et al*., 2017) and a 96h-LC_{50} of 1.17 mg/l (Mu *et al*., 2013). However, considering environmentally relevant concentrations, our data suggest that difenoconazole does not pose a direct threat to the survival of *B. viridis* tadpoles.

After exposure to alpha-cypermethrin no mortality was observed after either 24 or 48 hours. Gürkan *et al*. (2016) conducted a study on the effect of alpha-cypermethrin on the mortality of *Bufotes variabilis* tadpoles, in which tadpoles were treated for 96 hours with similar concentrations of the pesticide (0.1, 5, and 10 μg/l) as used in the present study. They found the highest mortality at a concentration of 10 μg/l, where 40% of the individuals died. For the South American hylid species *Boana pulchella*, the LC_{50} of 479.7 μg/l showed this species to be tolerant to alpha-cypermethrin regarding mortality, but low concentrations (0.34-4.18 μg/l) caused malformations, confirming adverse effects of the exposure to this pesticide to amphibians (Agostini *et al*., 2010). These data show differential sensitivity to alpha-cypermethrin of different amphibian species, with *B. viridis* expressing high resistance to mortality. A possible explanation for the high tolerance to lethal effects shown by *B. viridis* tadpoles tested in the present study could be the differences in sensitivity of tadpoles depending on their developmental stage. For example, more developed tadpoles of the species *Lithobates clamitans*, appeared to be more sensitive than younger individuals of the same species. This may be attributed to the mode of action of pyrethroid pesticides, which primarily act on the nervous system, i.e. sodium channels (Berrill *et al*., 1993), so that tadpoles with a more developed nervous system are at greater risk of exposure to these pesticides than younger individuals.

In our study, a difference in tadpole mass was observed between the control and all concentrations of the two pesticide treatments (except at 10 μg/l of difenoconazole). Similar to our results, another study on *B. variabilis* tadpoles using alpha-cypermethrin exposure for 96 hours also showed a significant reduction in mass of exposed tadpoles at concentrations from 0.1 to 10 μg/l compared to controls

(GÜRKAN *et al.*, 2016).

Some studies indicate that both pesticides, difenoconazole and alpha-cypermethrin, can increase oxidative stress in aquatic species by generating large amounts of ROS (BAGNYUKOVA *et al.*, 2005; MOREIRA *et al.*, 2020). For example, when alpha-cypermethrin enters the body, it is degraded to a highly toxic and unstable cyanide hybrid molecule, which can be further degraded to cyanides and aldehydes, leading to increased free radical formation and ultimately increased oxidative stress (XU & HUANG, 2017). Catalase is an antioxidant enzyme found in the cytosol, peroxisomes, and mitochondria and is responsible for the degradation of hydrogen peroxide due to oxidative stress (ZHANG *et al.*, 2018). Its activity can be enhanced or suppressed depending on the degree of exposure to harmful chemicals, such as pesticides (RUTKOSKI *et al.*, 2021). Measurements of catalase activity after difenoconazole and alpha-cypermethrin treatments showed no significant differences between the control and the different pesticide concentrations. A study on the effects of difenoconazole on catalase activity in water fleas (*Daphnia magna*) showed that sublethal concentrations of 50 μg/l did not result in a significant increase in catalase activity (MOREIRA *et al.*, 2020). Another study in common carp (*Cyprinus carpio*) following exposure to another triazole fungicide, tebuconazole, showed a decrease in catalase activity at a concentration of 1190 μg/l, which might be due to inhibition of the enzyme by excessive free radical production (TONI *et al.*, 2011). Similarly, a study in walking catfish (*Clarias batrachus*) found a decrease in catalase activity after exposure to pyrethroid insecticides due to an overload of the enzyme defense system against free radicals (TRIPATHI & BANDOONI, 2011).

ETS activity is a commonly used biomarker to determine stress levels due to chemical exposure and to determine metabolic energy requirements (MOREIRA *et al.*, 2020). Measurements of ETS activity in tadpoles showed no significant differences in activity between control and individuals treated with alpha-cypermethrin, which may indicate that the metabolic apparatus did not respond or was not enough damaged to detect the differences. Measurements of ETS activity in tadpoles treated with difenoconazole showed that there were no significant differences between the control and exposed animals at all tested concentrations, but there was a significant difference between 1 μg/l and 10 μg/l concentrations at 24°C and 28°C. It is well established that free radicals, including hydrogen peroxide, can control mitochondrial ATP production by disabling or reducing electron flow in the transport system (THOMSON, 2019). In general, higher ETS values for difenoconazole than for alpha-cypermethrin at the same temperatures suggest that tadpoles were more stressed during difenoconazole treatment and that metabolic activity was higher because they likely had higher energy demands (ŽAGAR *et al.*, 2015).

Carbonyl groups are another indicator of oxidative stress. They are known to be produced by oxidative stress due to increased accumulation of reactive oxygen particles such as hydrogen peroxide, which then damage cellular proteins and lipids (RUTKOSKI *et al.*, 2021). Measure-

ments of both pesticides revealed no significant difference in carbonyl groups compared to the control; this is in line with the results of a study that examined the effect of the triazole fungicide tebuconazole at concentrations of 590, 1190, 1780, and 2370 μg/l on common carp (*C. carpio*), which found no significant difference in the amount of carbonyl groups between exposed and control groups (Toni *et al*., 2011). However, it should be noted that our results showed a slight trend of increasing carbonyl groups with increasing pesticide concentrations, after which the levels decreased at the highest concentrations. Therefore, a particular increase in the amount of carbonyl groups in tadpoles treated with difenoconazole and alpha-cypermethrin might be attributed to an increase in oxidative stress due to damage and disruption of protein metabolism in the cell (Rutkoski *et al*., 2021).

The results of this study suggest that both difenoconazole and alpha-cypermethrin have significant adverse effects on tadpoles of *B. viridis* after acute exposure. Difenoconazole induces tadpole mortality at concentrations of 80 μg/l and 100 μg/l, while no mortality was observed following exposure to environmentally relevant concentrations of alpha-cypermethrin. Both pesticides resulted in a significant reduction in tadpole mass after 48 hours of exposure. Although there were no significant differences in biomarker activities, it is crucial to emphasize that these effects could become more pronounced with prolonged exposure. Furthermore, the reduction in tadpole size may have long-term implications at the population level, as smaller tadpoles are more susceptible to various environmental impacts, such as predation.

Considering environmental relevance of our study, at concentrations of both pesticides measured in the environment, alpha-cypermethrin seemed to pose a higher risk than difeconazole to *B. viridis* tadpoles. Moreover, the large discrepancies between the effects of alpha-cypermethrin among different species of amphibians raise the importance of our study. Further studies are necessary to gain better insight into the sensitivity of *B. viridis* tadpoles to pesticides, given that this species is commonly found near agricultural lands and is at high risk of exposure. As this species is widespread, the results obtained are relevant on a broad geographical scale, and other species may similarly be affected by these two pesticides.

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References

Aebi, H. (1984). Catalase *in vitro*. *Methods in Enzymology* 105: 121-126.

Aghasyan, A.; Avci, A.; Tuniyev, B.; Crnobrnja-Isailovic, J.; Lymberakis, P.; Andrén, C.; Cogalniceanu, D.; Wilkinson, J.; Ananjeva, N.B.; Üzüm, N. *et al.* (2017). *Bufotes viridis*. *The IUCN Red List of Threatened Spe-*

cies 2015. International Union for Nature Conservation and Natural Resources, Gland, Switzerland. Available at http://www.iucnredlist.org/. Retrieved on 31 October 2023.

AGOSTINI, M.G.; NATALE, G.S. & RONCO, A.E. (2010). Lethal and sublethal effects of cypermethrin to *Hypsiboas pulchellus* tadpoles. *Ecotoxicology* 19: 1545-1550.

BAGNYUKOVA, T.V.; VASYLKIV, O.Y.; STOREY, K.B. & LUSHCHAK, V.I. (2005). Catalase inhibition by amino triazole induces oxidative stress in goldfish brain. *Brain Research* 1052: 180-186.

BĂNCILĂ, R.I., LATTUADA, M. & SILLERO, N. (2023). Distribution of amphibians and reptiles in agricultural landscape across Europe. *Landscape Ecology* 38: 1-14.

BARRIGA-VALLEJO, C.; AGUILERA, C.; CRUZ, J.; BANDA-LEAL, J.; LAZCANO, D. & MENDOZA, R. (2017). Ecotoxicological biomarkers in multiple tissues of the neotenic *Ambystoma* spp. for a non-lethal monitoring of contaminant exposure in wildlife and captive populations. *Water, Air and Soil Pollution* 228: 415.

BERNABÒ, I.; GUARDIA, A.; MACIRELLA, R.; SESTI, S.; CRESCENTE, A. & BRUNELLI, E. (2016). Effects of long-term exposure to two fungicides, pyrimethanil and tebuconazole, on survival and life history traits of Italian tree frog (*Hyla intermedia*). *Aquatic Toxicology* 172: 56-66.

BERRILL, M.; BERTRAM, S.; WILSON, A.; LOUIS, S.; BRIGHAM, D. & STROMBERG, C. (1993). Lethal and sublethal impacts of pyrethroid insecticides on amphibian embryos and tadpoles. *Environmental Toxicology and Chemistry* 12: 525-539.

BLAUSTEIN, A.R. & WAKE, D.B. (1990). Declining amphibian populations: a global phenomenon? *Trends in Ecology and Evolution* 5: 203-204.

DALLE-DONNE, I.; ROSSI, R.; GIUSTARINI, D.; MILZANI, A. & COLOMBO, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clinica Chimica Acta* 329: 23-38.

DAVID, M.; MARIGOUDAR, S.R.; PATIL, V.K. & HALAPPA, R. (2012). Behavioral, morphological deformities and biomarkers of oxidative damage as indicators of sublethal cypermethrin intoxication on the tadpoles of *D. melanostictus* (Schneider, 1799). *Pesticide Biochemistry and Physiology* 103: 127-134.

FINNEY, D.J. (1971). *Probit Analysis*. Cambridge University Press, Cambridge, England.

FRYDAY, S. & THOMPSON, H. (2012). Toxicity of pesticides to aquatic and terrestrial life stages of amphibians and occurrence, habitat use and exposure of amphibian species in agricultural environments. *EFSA Supporting Publications* 2012: EN-343.

GOSNER, K.L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16: 183-190.

GREULICH, K. & PFLUGMACHER, S. (2003). Differences in susceptibility of various life stages of amphibians to pesticide exposure. *Aquatic Toxicology* 65: 329-336.

GÜRKAN, M.; SERBEST, S. & HAYRETDAĞ, S. (2016). Acute toxicity of the synthetic pyrethroid alpha-cypermethrin on the tadpoles of variable green toad, *Bufotes variabilis* (Amphibia:Anura). *Ege Journal of Fisheries and Aquatic Sciences* 33: 367-371.

HAYES, T.B.; FALSO, P.; GALLIPEAU, S. & STICE, M. (2010). The cause of global amphibian declines: A developmental endocrinologist's perspective. *Journal of Experimental Biology* 213: 921-933.

HEALTH CANADA (2021). *Difenoconazole and its Associated End-Use Products*. Pest Management Regulatory Agency, Ottawa, Ontario, Canada.

HENRY, M.; BRENNER, F.J.; JONES, A.M; BROCKHAGE, R. & SMELTZER, D.A. (2013). Impact of chemical fertilizer and pesticides on aquatic microcosms. *Journal of the Pennsylvania Academy of Science* 87: 42-49.

IRAZUSTA, V.; MORENO-CERMENO, A.; CABISCOL, E.; TAMARIT, J. & ROS, J. (2011). Proteomic strategies for the analysis of carbonyl groups on proteins. *Current Protein and Pep-*

tide Science 11: 652-658.

KARAKOUSIS, Y. & KYRIAKOPOULOU-SKLAVOUNOU, P. (1995). Genetic and morphological differentiation among populations of the green toad *Bufo viridis* from Northern Greece. *Biochemical Systematics and Ecology* 23: 39-45.

KATZ, U. & GIL, N. (1997). Different temperature relations of two species of toads that coexist at the border of their geographical distributions. *Amphibia-Reptilia* 18: 259-268.

LANDLER, L.; BURGSTALLER, S. & SCHWEIGER, S. (2023). Land-use preferences of the European green toad (*Bufotes viridis*) in the city of Vienna (Austria): the importance of open land in urban environments. *Frontiers in Zoology* 20: 3.

LUEDTKE, J.A.; CHANSON, J.; NEAM, K.; HOBIN, L.; MACIEL, A.O.; CATENAZZI, A.; BORZÉE, A.; HAMIDY, A.; AOWPHOL, A.; JEAN, A. *et al.* (2023). Ongoing declines for the world's amphibians in the face of emerging threats. *Nature* 622: 308-314.

MCMAHON, T.A.; HALSTEAD, N.T.; JOHNSON, S.; RAFFEL, T.R.; ROMANSIC, J.M.; CRUMRINE, P.W.; BOUGHTON, R.K.; MARTIN, L.B. & ROHR, J.R. 82011). The fungicide chlorothalonil is nonlinearly associated with corticosterone levels, immunity, and mortality in amphibians. *Environmental Health Perspectives* 119: 1098-1103.

MOREIRA, R.A.; DE ARAUJO, G.S.; SILVA, A.R.R.G.; DAAM, M.A.; ROCHA, O.; SOARES, A.M. & LOUREIRO, S. (2020). Effects of abamectin-based and difenoconazole-based formulations and their mixtures in *Daphnia magna*: a multiple endpoint approach. *Ecotoxicology* 29: 1486-1499.

MU, X.; PANG, S.; SUN, X.; GAO, J.; CHEN, J.; CHEN, X.; LI, X. & WANG, C. (2013). Evaluation of acute and developmental effects of difenoconazole via multiple stage zebrafish assays. *Environmental Pollution* 175: 147-157.

PHAM, B.; MIRANDA, A.; ALLINSON, G. & NUGEGODA, D. (2017). Evaluating the non-lethal effects of organophosphorous and carbamate insecticides on the yabby (*Cherax destructor*) using cholinesterase (AChE, BChE), Glutathione S-Transferase and ATPase as biomarkers. *Ecotoxicology and Environmental Safety* 143: 283-288.

QUARANTA, A.; BELLANTUONO, V.; CASSANO, G. & LIPPE, C. (2009). Why amphibians are more sensitive than mammals to xenobiotics. PLOS ONE 4: e7699.

RAVI KIRAN, T. & ARUNA, H.K. (2010). Antioxidant enzyme activities and markers of oxidative stress in the life cycle of earthworm, *Eudrilus eugeniae*. *Italian Journal of Zoology* 77: 144-148.

R DEVELOPMENT CORE TEAM (2019). *R: A language and environment for statistical computing.* R Foundation for Statistical Computing, Vienna, Austria. Available at https://www.R-project.org. Retrieved on 10 December 2022.

RUTKOSKI, C.F.; MACAGNAN, N.; FOLADOR, A.; SKOVRONSKI, V.J.; DO AMARAL, A.M.B.; LEITEMPERGER, J.W.; COSTA, M.D.; HARTMANN, P.A.; MÜLLER, C.; LORO, V.L. & HARTMANN, M.T. (2021). Cypermethrin- and fipronil-based insecticides cause biochemical changes in *Physalaemus gracilis* tadpoles. *Environmental Science and Pollution Research* 28: 4377 -4387.

SANCHES, A.L.M.; VIEIRA, B.H.; REGHINI, M.V.; MOREIRA, R.A.; FREITAS, E.C.; ESPÍNDOLA, E.L.G. & DAAM, M.A. (2017). Single and mixture toxicity of abamectin and difenoconazole to adult zebrafish (*Danio rerio*). *Chemosphere* 188: 582-587.

SISTANI, A.; BURGSTALLER, S.; GOLLMANN, G. & LANDER, L. (2021). The European green toad, *Bufotes viridis*, in Donaufeld (Vienna, Austria): status and size of the population. *Herpetozoa* 34: 259-264.

STUART, S.N.; CHANSON, J.S.; COX, N.A.; YOUNG, B.E.; RODRIGUES, A.S.L.; FISCHMAN, D.L. & WALLER, R.W. (2004). Status and trends of amphibian. *Science* 306: 1783-1786.

THOMSON, C. (2019). *Metabolic Patterning and Regulation during Early Embryo Development and Appendage Regeneration*. Ph.D. Disserta-

tion, The University of Manchester, Manchester, UK.

TONI, C.; FERREIRA, D.; KREUTZ, L.C.; LORO, V.L. & BARCELLOS, L.J.G. (2011). Assessment of oxidative stress and metabolic changes in common carp (*Cyprinus carpio*) acutely exposed to different concentrations of the fungicide tebuconazole. *Chemosphere* 83: 579-584.

TRIPATHI, G. & BANDOONI, N. (2011). Impact of alphamethrin on antioxidant defense (catalase) and protein profile of a catfish. *Environmentalist* 31: 54-58.

WHO (2021). *WHO Specifications and Evaluations for Public Health Pesticides.* World Health Organization, Geneva, Switzerland.

XU, P. & HUANG, L. (2017). Effects of α-cypermethrin enantiomers on the growth, biochemical parameters and bioaccumulation in *Rana nigromaculata* tadpoles of the anuran amphibians. *Ecotoxicology and Environmental Safety* 139: 431-438.

YU, S.; WAGES, M.R.; CAI, Q.; MAUL, J.D. & COBB, G.P. (2013). Lethal and sublethal effects of three insecticides on two developmental stages of *Xenopus laevis* and comparison with other amphibians. *Environmental Toxicology and Chemistry* 32: 2056-2064.

ŽAGAR, A.; SIMČIČ, T.; CARRETERO, M.A. & VREZEC, A. (2015). The role of metabolism in understanding the altitudinal segregation pattern of two potentially interacting lizards. *Comparative Biochemistry and Physiology A* 179: 1-6.

ZHANG, W.; LU, Y.; HUANG, L.; CHENG, C.; DI, S.; CHEN, L.; ZHOU, Z. & DIAO, J. (2018). Comparison of triadimefon and its metabolite on acute toxicity and chronic effects during the early development of *Rana nigromaculata* tadpoles. *Ecotoxicology and Environmental Safety* 156: 247-254.

ZHAO, F.; LIU, K.; XIE, D.; LV, D. & LUO, J. (2018). A novel and actual mode for study of soil degradation and transportation of difenoconazole in a mango field. *Royal Society of Chemistry Advances* 8: 8671-8677.