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# The effects of pyrethroid insecticide deltamethrin on oxidative stress parameters in different tissues of green toad *(Bufotes viridis)*

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# Efekti piretroidnog insekticida deltametrina na parametre oksidativnog stresa u različitim tkivima zelene krastave žabe (*Bufotes viridis*)

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*Mohammed Melad Lamin Nasia*

# THE EFFECTS OF PYRETHROID INSECTICIDE DELTAMETHRIN ON OXIDATIVE STRESS PARAMETERS IN DIFFERENT TISSUES OF GREEN TOAD (*BUFOTES VIRIDIS*)

## ABSTRACT

One of the main causes of declining frog populations is the increased use of pesticides. The toxicity mechanisms for most pesticides in non-target organisms are associated with oxidative stress. The present doctoral thesis examines acute and chronic *Bufotes viridis* exposure to pyrethroid insecticide deltamethrin (DM). In the part of experiment that examined acute exposure to DM oral application (96 h) was used, while chronic exposure (7, 14 and 21 days) included oral, dermal and combined oral-dermal application. The following oxidative stress, biotransformation and neurotoxicity parameters were analyzed: the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and glutathione-S-transferase (GST); the concentrations of glutathione (GSH), sulfhydryl groups (-SH) and thiobarbituric reactive substances (TBARS); the activity of neurotoxicity biomarker-cholinesterase (ChE) and the expression of phase I biotransformation enzyme cytochrome $P_{450}$1A (CYP1A). The effects of DM were examined on the liver, muscle, gastrointestinal tissue and skin of adult individuals.

The obtained results suggest that DM (although it does not tend to accumulate in tissues) can be hazardous to amphibians and that pesticide exposure modulates the parameters of oxidative stress in selected toad tissues. The data show that the DM-induced systemic toxicity was dose-, route of application- and time-dependent. Toads exposed to this insecticide respond by producing tissue-specific adaptive responses in the course of establishing cell defense to the resulting oxidative stress. The present investigation supports the hypothesis that oxidative stress is one of the steps of pesticide-induced toxicity and that further examination are required to elucidate the entire mechanism.

**Key words**: deltamethrin, oxidative stress, acute and chronic toxicity, biomarkers, *Bufotes viridis*
**Scientific field**: Biology
**Scientific subfield**: Animal physiology
**UDC number**: 597.841:632.951(043.3)

**EFEKTI PIRETROIDNOG INSEKTICIDA DELTAMETRINA NA PARAMETRE OKSIDATIVNOG STRESA U RAZLIČITIM TKIVIMA ZELENE KRASTAVE ŽABE (*BUFOTES VIRIDIS*)**

## SAŽETAK

Jedan od glavnih uzroka opadanja populacije žaba u svetu je povećana upotreba pesticida. Mehanizmi toksičnosti za većinu pesticida u non-target organizmima su povezani sa oksidativnim stresom. U ovoj doktorskoj disertaciji ispitivan je efekat akutne i hronične izloženosti zelene krastave žabe *Bufotes viridis* piretroidnom pesticidu deltametrinu (DM). U delu eksperimenta koji se bavio akutnim izlaganjem DM (96 h), primenjen je oralni način aplikacije, dok je hronično izlaganje (7, 14 i 21 dan) uključivalo oralnu, dermalnu i kombinovanu oralno-dermalnu aplikaciju. Analizirani su sledeći parametri oksidativnog stresa, biotransformacije i neurotoksičnosti: aktivnosti superoksid dismutaze (SOD), katalaze (CAT), glutation-peroksidaze (GSH-Px), glutation-reduktaze (GR) i glutation-S-transferaze (GST); koncentracije glutationa (GSH), sulfhidrilnih grupa (-SH) i tiobarbiturnih reaktivnih supstanci (TBARS); aktivnost biomarkera neurotoksičnosti-holinesteraze (ChE) i ekspresija biotransformacionog enzima faze I-citohroma $P_{450}$1A (CIP1A). Efekti DM ispitivani su na jetri, mišiću, gastrointestinalnom tkivu i koži adultnih jedinki.

Rezultati ove doktorske disertacije sugerišu da deltametrin (iako nema tendenciju akumuliranja u tkivima) može biti opasan za vodozemce i da izloženost pesticidima modulira ispitivane parametre oksidativnog stresa u tkivima zelenih krastavih žaba. Dobijeni podaci pokazuju da je sistemska toksičnost izazvana deltametrinom zavisila od doze, načina i vremena aplikacije pesticida. Jedinke izložene ovom insekticidu su stvorile specifične adaptivne reakcije tkiva u cilju odbrane ćelija od rezultirajućeg oksidativnog stresa. Sadašnje istraživanje podržava hipotezu da je nastanak oksidativnog stresa jedan od razloga toksičnosti pesticida, ali potrebno je dalje ispitivanje da bi se ceo mehanizam u potpunosti rasvetlio.

**Ključne reči**: deltametrin, oksidativni stres, akutna i hronična toksičnost, biomarkeri, *Bufotes viridis*
**Naučna oblast**: Biologija
**Uža naučna oblast**: Fiziologija životinja
**UDK broj**: 597.841:632.951(043.3)

## ABBREVIATIONS

| | |
|---|---|
| AHH | Aryl hydrocarbon hydroxylase |
| AChE | Acetylcholinesterase |
| AHH | Aryl hydrocarbon hydroxylase |
| AOS | Antioxidative defense system |
| ATP | Adenosine triphosphate |
| BChE | Butyrylcholinesterase |
| BSA | Bovine serum albumin |
| CAT | Catalase |
| CDA | Canonical discriminant analysis |
| CDNB | 1-Chloro-2,4-dinitrobenzene |
| ChE | Cholinesterase |
| CuZnSOD | Copper-zinc SOD |
| CYP1A | Cytochrome $P_{450}$1A |
| DDT | Dichlorodiphenyltrichloroethane |
| DM | Deltamethrin |
| DNK | Deoxyribonucleic acid |
| DSPE | Dispersive solid-phase extraction tubes |
| DTNB | 5,5'-dithio-bis-2-nitrobenzoic acid |
| EC-SOD | Extracellular SOD |
| EDTA | Ethylenediaminetetraacetic acid |
| FAD | Flavin adenine dinucleotide |
| FeSOD | Iron SOD |
| GABA | Gamma-aminobutyric acid |
| GIT | Gastrointestinal tissue |
| GLS | Glutathione synthase |
| GR | Glutathione reductase |
| GSH | Glutathione |
| GSH-Px | Glutathione peroxidase |
| GSSG | Glutathione disulfide |
| GST | Glutathione-S-transferase |
| HRP | Horseradish peroxidase |
| HIS | Hepatosomatic index |
| IBR | Integrated biomarker response |
| IUCN | International union for conservation of nature |
| LPO | Lipid peroxidation |
| MDA | Malondialdehyde |
| MnSOD | Manganese SOD |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NiSOD | Nickel SOD |
| OPS | Organophosphate |
| PAGE | Polyacrylamide gel electrophoresis |
| PCA | Principal component analysis |
| PH GSH-Px | Phospholipid hydroperoxide glutathione peroxidase |
| PVDF | Polyvinylidene fluoride |
| RNS | Reactive nitric species |
| ROS | Reactive oxygen species |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SE | Standard error |
| Se GSH-Px | Selenium-dependent glutathione peroxidase |

| | |
|---|---|
| SH | Sulfhydryl group |
| SOD | Superoxide dismutase |
| SVL | Snout-vent length |
| TBA | Thiobarbituric acid |
| TBARS | Thiobarbituric acid reactive substances |
| TEMED | Tetramethylethylenediamine |
| TNB | 2-Nitro-5-thiobenzoic acid |
| Tris | Hydroxymethyl aminomethane |
| UDP | Uridine 5'-diphospho |
| UGP | UDP-glucuronosyltransferase |
| γGLCL | γ-glutamyl-L-cysteine ligase |

**CONTENT**

# 1. INTRODUCTION

### 1.1.Pesticides

The urbanization and industrialization, as a product of advancement, had a negative impact on the environment. Chemical waste resulting from industry represents the harmful pollutants and can pollute water, soil and air. Beside industry, agriculture is one of the main sources of environmental pollution. Pesticides played a great role in the improvement of agriculture worldwide in the twentieth century. However, pesticides used in agriculture are mainly responsible for the contamination of water and soil. Each year, millions of kilograms are applied on agriculture sites, and the extreme use of those chemicals has adversely affected and damaged the fitness of living beings (Pimentel et al., 1992; Pimentel and Greiner, 1997). In the period from 2010 to 2014, mean pesticide use per kg of crop production was 0.645 g, while the mean annual pesticide application was 2.784 kg/ha. The countries with greatest averaged annual pesticide use (kg/ha) for the same period were: Japan (18.94), China (10.45), Mexico (7.87), Brazil (6.16), Germany (5.12), France (4.85), UK (4.03), USA (3.88), and India (0.26) (Zhang, 2018). During 2016, only in Europe approximately 400 000 tonnes of pesticides were used, mainly in agriculture (PAN-Europe, 2016).

Depending on the organism they affect, pesticides are sorted in several groups: insecticides, herbicides, fungicides, miticides, nematicides, molluscicides and rodenticides (Garcia et al., 2012). According to Food and Agriculture Organization of the United Nations data, herbicides and insecticides were the most produced and used pesticides in 2018 (Zhang, 2018). The most commonly used pesticides are organochlorines, organophosphates, pyrethroids and carbamates (Garcia et al., 2012).

Organochlorine insecticides are group of organic substances with chlorine in their chemical structure. They were commonly used pesticides in the past. Organochlorines as lipophilic pesticides display a great affinity for lipids and tend to accumulate in fat tissue of animals. Because of the low bio-degradation rate, organochlorines can persist in the environment and cause serious problems for living organisms (Coats, 1990). This group of pesticides affects neuron membranes through inhibition of enzymes: sodium-potassium and calcium-magnesium adenosine triphosphate-ases (ATP-ases). In this situation sodium ions leaking destabilize membrane potential and normal nerve activity, leading to repeated discharges in the neuron. Some of the well-known organochlorine pesticides are dichlorodiphenyltrichloroethane (DDT), hexachlorocyclohexane (HCH), aldrin and dieldrin chlordane, heptaclor, endosulfan, isodrin, isobenzan, toxaphene (Jayaraj et al., 2016). Many, like DDT, have been removed from the market due to their health and environmental effects.

Organophosphate pesticides (OPS) are esters derived from phosphoric acid. These chemicals are the most used due to weak retention in the nature (Lima and Vega, 2005). Acetylcholinesterase (AChE) represents the main target of this type of insecticides (Costa et al., 2005). Acute exposure to organophosphate causes irreversible inhibition of AChE activity, which is followed by the acetylcholine accumulation and induction of nicotinic and muscarinic receptors. Most known organophosphates are chlorpyrifos, diazinon, fenitrothion, parathion (Savolainen, 2001; Sharma et al., 2005; Aygun et al., 2007).

Pyrethroids originate from pyrethrin, a natural insecticide. The toxic effects of these compounds are achieved through their action on voltage-gated sodium channels. Pyrethroids inhibit the closure of sodium channels keeping them open. In this state the nerves cannot repolarize, leaving the membrane permanently depolarized. In the end, organism is left paralyzed (Soderlund, 2012). Pyrethroids have been classified into two types depending on their structure and toxicity: type I – whithout a cyano group (e.g. allethrin and permethrin), considered to be fewer hazardous than the other class, and type II – which have alpha cyano group (e.g. deltamethrin, cypermethrin and fenvalerate) (Elliott, 1989).

Carbamates represent carbamic acid derivates. Carbamate pesticides are less persistent than organochlorines and organophosphates (Garcia et al., 2012). These chemicals have very similar mechanism of action to one described for OPS. Carbamate generally inhibit a cholinesterase action,

but with shorter duration than that OPS pesticides. Some of the pesticides belonging to this group are carbaryl, carbofuran, oxamyl (Fukuto, 1990).

All kinds of pesticides could be found in the environment so accumulation and biomagnification of these chemicals in the biosphere pose a serious problem. Presence of pesticides in body systems increases the risk of many toxic diseases, such as irreversible developmental and neurological disabilities, physiological and immune system damage. One of physiological damage is the ability of a pesticide to induce reactive oxygen species (ROS) formation and generate oxidative stress and cell death (Sharma et al., 2005).

### 1.1.1. Deltamethrin (DM)

Deltamethrin (DM) was synthesized for the first time in 1974. It represents the synthetic derivative of the natural product pyrethrin which can be found in *Tanacetum cinerariifolium* plant. It is considered as the most toxic pyrethroid for vertebrates (Pham et al., 1984). DM is a member of synthetic pyrethroid pesticides (type II pyrethroid), which contain an alpha cyano group [(S) α -cyano-3-phenoxybenzyl-(1R)-cis-3-(2.2-dibromovinyl)-2, 2-dimethylcyclopropane carboxylate] (Figure 1) (Rehman et al., 2014).

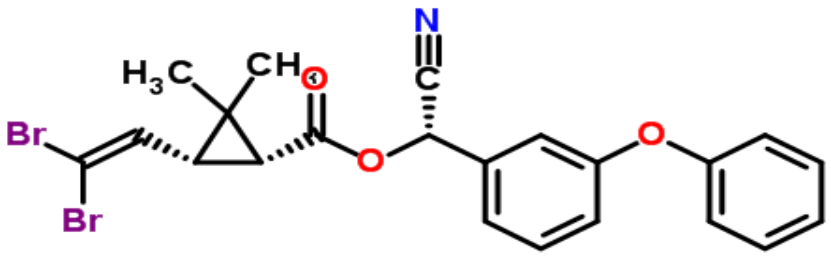


Figure 1. Deltamethrin
(http://www.chemspider.com/Chemical-Structure.37079.html).

Insects can be killed by DM in two ways-through direct contact or ingestion. Mechanism of DM includes activation of sodium channels, which lead to their extension opening, excitation of a membrane (Narahashi et al., 1992) and inhibition of gamma-aminobutyric acid (GABA) receptors. This results in excessive activation of the central nervous system (El-Sayed et al., 2007). In general, DM makes the channels open prolonged time and the membrane potential eventually get depolarized to the moment when action potential initiation is impossible (Narahashi et al., 1992). Several studies showed negative effects of DM on AChE activity and acetylcholine synthesis, thus acting on the cholinergic system. This causes uncontrolled activation of nerve endings and inducing the loss of nerve conduction, which results in convulsions and paralysis (Zhang et al., 2016; Toumi et al., 2015; Worthing and Walker, 1987).

DM changes metabolism of the cell and has genotoxic effects causing DNA damage in human lymphocytes (Scassellati et al., 1994). The elimination period of DM in mammals is approximately 2–4 days. The concentration of DM residuals in the organism is generally very low, only exceptions are lipids where higher residues are observed. The main metabolic reaction that DM undergoes during metabolism in cells is oxidation enabled with the microsomal monooxygenases. The degradation pathways in various animals are almost similar (Casida et al., 1983; Shono et al., 1979).

Due to their lipophilic nature, pyrethroids can be instantly absorbed through oral and dermal paths into organism. After oral administration in rodents, DM is rapidly absorbed from its gastrointestinal system. Investigations on rats indicated that DM, which is ester, is instantly metabolized by esterases and liver microsomal oxidases presented in the gut wall and liver (Rehman et al., 2014).

### 1.2. Oxidative stress

Free radicals are defined as ”atoms, molecules or ions with unpaired electrons” (Halliwell and Gutteridge, 1989). These electrons are very unstable and reactive, leading free radicals to take part in chemical reactions. Radicals can be generated by losing a single electron from a non-radical, leaving behind an unpaired electron and a positive charge; or if a covalent link is disturbed, leaving one electron on each atom. Reactive oxygen species (ROS) and reactive nitric species (RNS) represent two major groups of free radicals. Their production can be a consequence of physiological cell metabolism or disturbed cell homeostasis due to some pathological conditions and presence of xenobiotics (heavy metals, pesticides, X-ray and UV radiation) (Sies, 1986). ROS are mostly produced in aerobic respiration in mitochondria as the result of uncompleted $O_2$ reduction, while RNS are mostly produced during xenobiotic metabolism. Metabolism of xenobiotic could also be followed by the formation of carbonate and sulphur radicals. Other sources of free radicals production are the process of phagocytosis, biotransformation of exogenic and endogenic substrates in endoplasmic reticulum, metabolism of ethanol and during oxido-reduction process in a present of redox active metals. Free radicals are maintained on physiological level by the activity of antioxidant defense system (AOS). However, if free radical production overcomes organism defense, oxidative stress occurs. Oxidative stress is a disbalance between antioxidants and oxidants in favor of the second. Disturbed redox homeostasis leads to a disorder of redox signaling and damage of biomoleculs. Oxidative damage can be a result of increased ROS and RNS production on one hand, and/or failure of the antioxidative system on other (Sies, 2013; Halliwell and Gutteridge, 2015).

Oxidative stress is followed by several processes in living cells, which depends on cell type, intensity and time of exposure. Some of processes are:

1. activation of proliferation,
2. upregulation of AOS,
3. damage of cellular biomolecules (lipids, DNA, protein, carbohydrate),
4. aging,
5. cell death (Halliwell and Gutteridge, 2015).

It should be emphasized that ROS or RNS are not only just hazardous products of metabolism, but, at low to moderate concentrations, essential molecules for cell signaling and regulation. Reactive species are important redox messengers in physiological processes where the signal is transmitted through the redox mechanisms. This process is known as redox signaling (Dröge, 2002; Thannickal and Fanburg, 2000).

### 1.2.1. Reactive oxygen species (ROS)

Reactive oxygen species are products of partial reduction of molecular oxygen during physiological cell metabolism. ROS are classified into two groups: free radicals and non-radicals (Table 1). Free radicals are molecules with one or more unpaired electrons that cause their reactivity. Non-radicals are formed when two free radicals share their unpaired electrons. Superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide ($H_2O_2$) are the main ROS with a physiological importance (Halliwell and Gutteridge, 2015).

Table 1. Radical and non-radical forms of reactive oxygen species (ROS).

| Radicals | Non-radicals |
|---|---|
| Superoxide: $O_2^{\bullet -}$ | Hydrogen peroxide: $H_2O_2$ |
| Hydroxyl: $OH^{\bullet}$ | Hypochlorus acid: HOCL |
| Peroxyl: $RO_2^{\bullet}$ | Hypobrromous acid: HOBr |
| Alkoxyl: $RO^{\bullet}$ | Ozone: $O_3$ |
| Hydroperoxyl: $HO_2^{\bullet}$ | Singlet oxygen: $^1O_2$ |

During aerobic metabolism $O_2$ is gradually reduced with four electrons by mitochondrial electron-transport chain. This process results in water formation. However, the sequential addition of a single electron to $O_2$ gives superoxide anion ($O_2^{\bullet -}$). Superoxide anion radical is not very reactive per se, and is more harmful through the further formation of a more reactive radical species (Bielski and Richter, 1977). However, $O_2^{\bullet -}$ can act as both an oxidant and a reductant, depending on the environmental pH and the substrate with which it reacts (Fridovich, 1986). When two molecules of $O_2^{\bullet -}$ react together, one is oxidised and the other is reduced in a dismutation reaction leading to the formation of hydrogen peroxide, as shown:

$$\mathbf{O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2}$$

This spontaneous dismutation reaction leads to formation of hydroperoxy radicals ($HO_2^{\bullet}$) at pH 4.8 (Lynch and Fridovich, 1978).

At neutral pH, the enzyme-catalysed degradation of $O_2^{\bullet -}$ is more important for the generation of $H_2O_2$. $H_2O_2$ is nonradical, a reactive oxygen metabolite that may contribute further to free radical reactions. One of the first reactions of $H_2O_2$ was the classical Haber-Weiss reaction. This involves the direct reduction of $H_2O_2$ by $O_2^{\bullet -}$ with the generation of the hydroxyl radical ($OH^{\bullet}$), reaction:

$$\mathbf{H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + OH^{\bullet} + OH^-}$$

This reaction proceeds slowly under physiological conditions and is an unlikely source of higher $OH^{\bullet}$ concentrations (Ribiere et al., 1983). $O_2^{\bullet -}$ can also react with metals trough the Haber-Weiss reaction. Reduced metal can further undergo to Fenton reaction with $H_2O_2$ forming $OH^-$ and significant $OH^{\bullet}$ fluxes. The hydroxyl radical is highly reactive with all biomolecules. It possesses large positive redox potential and is the most reactive of all radicals (Anbar and Neta, 1967).

In biological systems several mechanisms of ROS formation are present. ROS are most frequently formed in oxygen metabolism as by-products. 90% of total oxygen, consumed by organisms, is subjected to four-electron reduction in electron-transport chain (Papa and Skulachev, 1997). However, about 10% of molecular oxygen is reduced with less than four electrons which increase ROS concentrations. The central places of electron-transport chain in mitochondria, related

to electrons “leak” and production of $O_2^{\bullet -}$, are complex III and coenzyme Q (Demin et al., 1998). The next most significant place of ROS formation is the electron-transport chain of endoplasmic reticulum (Malhotra and Kaufman, 2007). ROS generation in endoplasmic reticulum is increased with the catabolism of foreign and cellular molecules by cytochromes P450 (CYP). Also, various oxidases, like tryptophan dioxygenase (Li et al., 2007) and xanthine oxidase (Kelley et al., 2010) are responsible for production of certain ROS quantity in cytosol and peroxisomes. Literature data indicate that glucose and amino acids oxidases mostly produce $H_2O_2$, and cytochrome P450 reductase mostly generates $O_2^{\bullet -}$ (Bonnefont-Rousselot, 2002).

### 1.3. Antioxidative defense system (AOS)

The increase of oxygen concentration in the Earth’s atmosphere in the past led to changes in physiological and metabolic systems in living organisms (Embley and Martin, 2006). Those changes included use of molecular oxygen for efficient energy production but also for reduction-oxidation (or redox) processes associated with production of free radicals. These products of oxygen metabolism may have severe damaging effects on an organism, so the protection against them is pivotal for organism normal function (Lane, 2002). Animals have evolved appropriate protective system – antioxidative defense system **(**AOS). The role of this system is to avoid or minimize the production of free radicals, and, if free radicals are produced, to limit their prooxidant activity (Halliwell, 1999). AOS is consisted of various molecules known as antioxidants. Halliwell and Gutteridge (1995) define antioxidant as a “substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate.” Their definition was modified in 2015, new one is that: “Antioxidant is any substance that delays, prevents, or removes oxidative damage to a target molecule” (Halliwell and Gutteridge, 2015).

Antioxidant defense mechanism can include several strategies in protecting cells from free radicals:

- by direct remove of reactive species, trough antioxidative defense enzymes;
- controlling their formation, trough formation of proteins and/or electron carriers complexes (transferrins, albumin, haptoglobins, haemopexin, haem oxygenases, and metallothionein);
- protecting macromolecules using different nonenzymatic components such as chaperones, carotenoids, glutathione, α-tocopherol, ascorbate, and others (Halliwell and Gutteridge, 2015).

Enzymatic and nonenzymatic antioxidants are two main components of AOS (Sies, 2013). The AOS enzymes are localized in the different subcellular compartments and include: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GR) (Halliwell and Gutteridge, 2007). Non-enzymatic antioxidants include low molecular mass antioxidants, such as glutathione (GSH) and other non-protein thiols, the vitamins A, E, C and coenzyme Q, (Halliwell and Gutteridge, 2007). They can protect cell directly from oxidative damage or indirectly as cofactors of different antioxidant enzymes (Halliwell and Gutteridge, 2007).

#### 1.3.1. Superoxide dismutase (SOD)

Superoxide dismutase (SOD; EC 1.15.1.1) is a group of metalloenzymes that represents the first line of defense from ROS. SOD has a pivotal role in cell protection from oxidative stress. It

enables the dismutation of superoxide radical anion into hydrogen peroxide and oxygen (McCord and Fridovich, 1988):

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

Several classes of SOD enzyme appeared in evolution which was related to presents of different metals in biosphere (Alscher et al., 2002). Based on metal ions that occur in active centre and location in cell, SOD can be divided into five isoforms:

- Iron SOD (FeSOD)
- Nickel SOD (NiSOD)
- Copper-zinc SOD (CuZnSOD)
- Manganese SOD (MnSOD)
- Extracellular SOD (EC-SOD).

In animals and fungi, we can find three SOD forms: cytoplasmic CuZnSOD, mitochondrial MnSOD and extracellular CuZnSOD (EC-SOD). All isoforms catalyze the same reaction, but they differ in genes that encode their localization and structure. CuZnSOD is mostly located in the cytoplasm of animal cells but it can be also found in some organelles (peroxisomes, lysosomes and intermembrane space of mitochondria) and nucleus (Crapo et al., 1992; Keller et al., 1991). In mammalian tissues CuZnSOD constitutes approximately 85–90% of total SOD activity, while the rest represents MnSOD with an account of 10–15% (Halliwell and Gutteridge, 2015). Eukaryotic CuZnSODs are dimers built of two identical 16kDa subunits, each containing one catalytically essential $Cu^{2+}$ ion bridged by a His residue to solvent-inaccessible $Zn^{2+}$ ion (Figure 2). The Cu ion is involved in the enzyme reaction (E-enzyme). It is reversibly oxidized and reduced through the following reactions:

$$E\text{-}Cu^{2+} + O_2^{\bullet -} + H^+ \rightarrow E'\text{-}Cu^+ + O_2$$

$$E'\text{-}Cu^+ + O_2^{\bullet -} + H^+ \rightarrow E\text{-}Cu^{2+} + H_2O_2$$

MnSOD is found mainly in the mitochondrial matrix (Fridovich, 1997). In mammals, it is a tetrameric protein encoded in the nuclei, generated in the cytosol, and transmitted after translation into the mitochondria matrix space. The 25 kDa precursor protein has a mitochondrial transit peptide that is cleaved to produce the mature 22 kDa subunit. MnSOD dismutates superoxide radical according to reactions that are based on the activity of manganese ion (Miller, 2012):

$$E\text{-}Mn^{3+} + O_2^{\bullet -} \rightarrow E'\text{-}Mn^{2+} + O_2$$

$$E'\text{-}Mn^{2+} + O_2^{\bullet -} + 2H^+ \rightarrow E\text{-}Mn^{3+} + H_2O_2$$

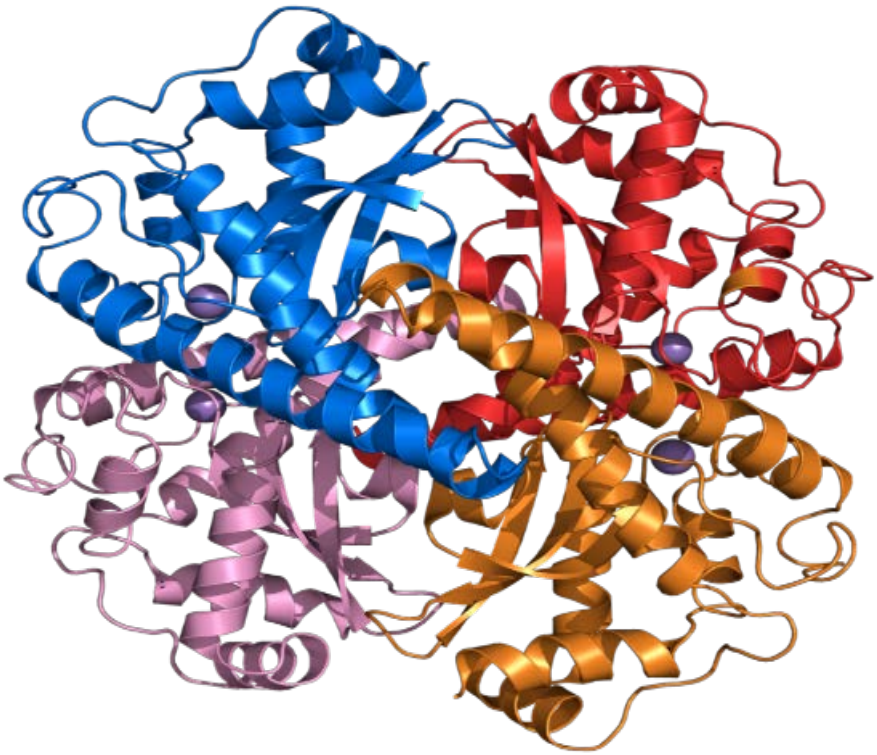

Figure 2. Structure of eukaryotic CuZnSODs
(https://en.wikipedia.org/wiki/superoxide_dismutase).

EC-SOD is commonly located on the cell-surface but outside of blood plasma cells, cerebrospinal fluid and lymph. EC-SODs are usually by structure a tetrameric (dimeric in some species) glycoproteins formed of two pairs of identical subunits, each containing one copper and zinc atom. EC-SOD exists in mammalian cells as a homodimer of molecular weight 135 kDa with a high heparin affinity (Marklund, 1982).

### 1.3.2. Catalase (CAT)

Catalase (CAT; $H_2O_2$ oxidoreductase, EC 1.11.1.6) is found in most aerobic organisms and is considered as one of the most efficient enzymes in living systems. CAT function is to dismutate hydrogen peroxide to water and oxygen (Chelikani et al., 2004). Depending on the substrate concentration CAT can display two types of reactions. At low concentrations of $H_2O_2$ (<1 μM), CAT shows a peroxidase reaction and reduces $H_2O_2$ using different donors of hydrogen (alcohols, ascorbic acid):

$$\mathbf{AH_2 + H_2O_2 \rightarrow A + 2H_2O}$$

At high concentrations of the substrate (> 1 μM), CAT quickly removes $H_2O_2$ through a catalytic reaction, where $H_2O_2$ is a donor and a hydrogen acceptor:

$$\mathbf{2H_2O_2 \rightarrow O_2 + 2H_2O}$$

Based on physical and biochemical properties catalases can be divided into three groups:

- Catalase-peroxidases group with enhanced peroxidatic activity (regarding catalatic activity), extensively distributed in procaryotes and in lower eukaryotes,
- Mn-catalases with manganese in the active center, and
- "True" catalases are widely presented in eukaryotes and many procaryotes (Zámocký and Koller, 1999).

CAT is an intracellular enzyme, molecular mass of 240 kDa, mainly found in cytoplasm and peroxisomes of the animal cells (Radi et al., 1991). It contains four subunits, each of which has Fe (III)-haem at its active site. Every subunit of catalase has a large domain with antiparallel β-pleated sheet and a smaller domain with four α-helices (Figure 3) (Fidaleo, 2010).

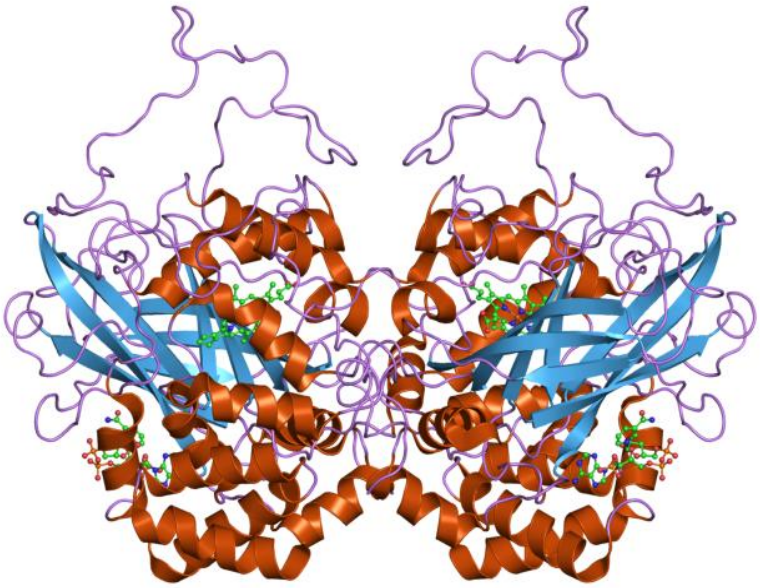

Figure 3. Catalase structure
(https://upload.wikimedia.org/wikipedia/commons/8/86/PDB_7cat_EBI.jpg).

### 1.3.3. Glutathione peroxidase (GSH-Px)

Glutathione peroxidase (GSH-Px, EC 1.11.1.9) has similar role like CAT in $H_2O_2$ cell-detoxification. However, they have dissimilar affinities for $H_2O_2$, and localizations in cellular compartments (peroxisomes and cytosol, respectively) (Halliwell and Gutteridge, 2015). Under normal cellular concentrations, GSH-Px has a more important role in catalyzing the reduction of $H_2O_2$ than CAT due to its significantly higher affinity for $H_2O_2$, while CAT is more active in cases of high $H_2O_2$ production (Kim et al., 1988). Beside $H_2O_2$, GSH-Px can also act on other peroxides (Kruidenier and Verspaget, 2002).

Glutathione peroxidases act using the reduced form of glutathione (GSH) to reduce hydroperoxides and organic peroxides (ROOH) to water and alcohols (ROH) with formation of glutathione disulfide (GSSG):

$$\mathbf{H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O}$$

$$\mathbf{ROOH + 2GSH \rightarrow GSSG + ROH + H_2O_2}$$

Three forms of this enzyme have been described: tetramer selenium-dependent (Se GSH-Px), monomer non-selenium-dependent (non-Se GSH-Px) and monomer phospholipid hydroperoxide glutathione peroxidase (PH GSH-Px) (Figure 4).

In the mammals five isoforms of GSH-Px, that have different tissue distribution, were detected: classical, cellular GSH-Px (cGSH-Px, GSH-Px 1), gastrointestinal GSH-Px (GSH-Px 2), extracellular GSH-Px (GSH-Px 3), phospholipids hydroperoxide GSH-Px (PH GSH-Px, GSH-Px 4) and non-selenium-dependent GSH-Px (GSH-Px 5) (Tabet and Touyz, 2007).

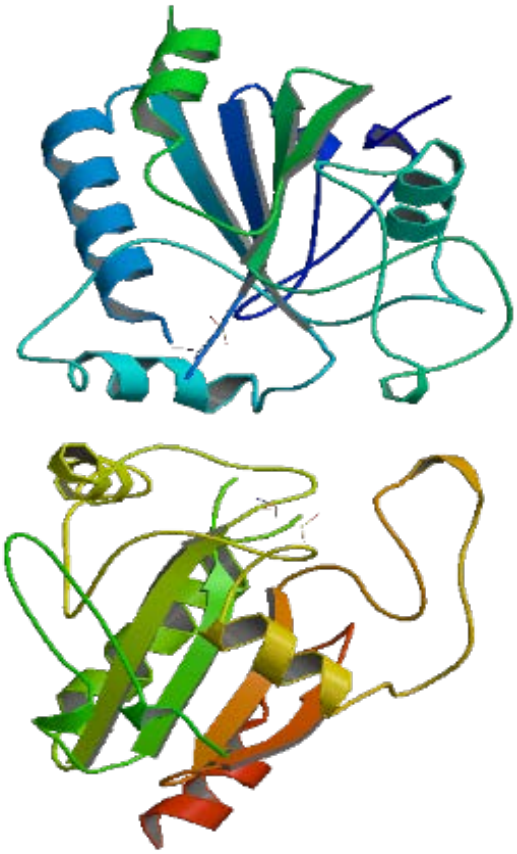

Figure 4. Glutathione peroxidase structure (https://upload.wikimedia.org/wikipedia/commons/8/86/GlutPeroxidase-1GP1.png).

### 1.3.4. Glutathione reductase (GR)

Glutathione reductase (GR, EC 1.6.4.2) (Figure 5) enables the transformation of oxidized glutathione disulfide (GSSG) to the GSH, maintaining cellular GSH/GSSG ratio balanced (Halliwell and Gutteridge, 2015). GR functions as mainly dimeric disulfide oxidoreductase, utilizing flavin adenine dinucleotide (FAD) prosthetic group and NADPH to reduce GSSG into two GSH molecules (Angelucci et al., 2008):

$$\mathbf{GSSG + NADPH + H^{+} \rightarrow 2GSH + NADP^{+}}$$

The GR activity consists of reductive and oxidative reactions. In the reductive reaction, NADPH reduces FAD producing a redox active disulfide. During an oxidative reaction, the GSSG

is reduced to GSH by the formation of an enzyme – glutathione mixed disulfide (Berkholz et al., 2008).

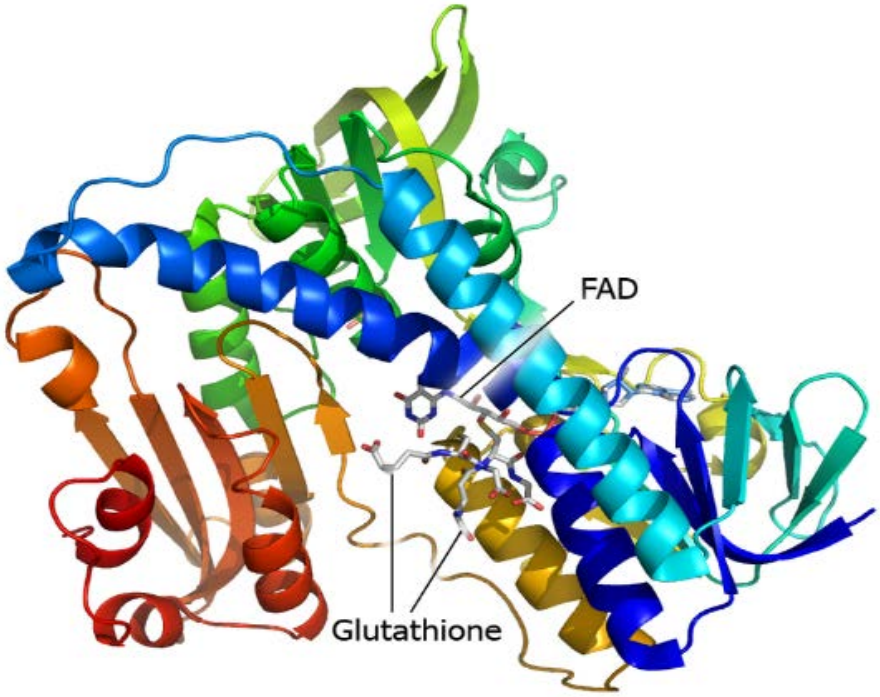


Figure 5. Glutathione reductase structure (https://upload.wikimedia.org/wikipedia/commons/thumb/4/4e/Glutathione_reductase.png/800px-Glutathione_reductase.png).

### 1.3.5. Glutathione (GSH)

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine), and the most abundant non-protein thiol in cells, with multiple functions in cells (Figure 6).

GSH is formed through the two reactions catalyzed by γ-glutamyl-L-cysteine ligase (γGLCL) and glutathione synthase (GLS). In the first step, the enzyme γGLCL forms a peptide link between the γ-carboxy of glutamate and the amino group of cysteine using the energy from the ATP hydrolysis (Lu, 2009):

**L-glutamate + L-cysteine + MgATP → L-γ-glutamyl-L-cysteine + MgADP + Pi**

In the second reaction, glutathione synthetase combines formed dipeptide with glycine, again using the energy providing by ATP (Lu, 2009):

**L-γ-glutamyl-L-cysteine + glycine + MgATP → GSH + MgADP + Pi**

The active thiol group in GSH structure functions as an antioxidant directly through reaction with ROS, RNS, and electrophiles or by operating as a part of GSH dependent system (Sies, 1999). GSH as a direct antioxidant reacts with free radicals. During this direct oxidation thiyl radicals and GSSG are formed.

Both forms of glutathione (reduced and oxidized) act with different redox-active molecules (e.g., NAD(P)H) in maintaining cellular redox balans. In normal conditions, GSSG is maintained at concentrations lower than 5% of total glutathione content in the cell (Sentellas et al., 2014). GSSG produced from the consumption of GSH is potentially toxic and due this it must be excreted from the cell or reduced by GR activity.

GSH is also widely used as a cosubstrate for GSH-Px and, in coupled action with glutathione-S-transferase, can remove xenobiotics (Halliwell and Gutteridge, 2015). GSH can act together with some metal ions, forming metal-GSH complexes and maintain cellular metal homeostasis and/or prevent potential toxic effects of heavy metals. Metal-GSH complexes can have several metabolic functions in the living cells: in the transfer of cations between ligands as well in the metal transport. GSH is an important source of cysteine and a cofactor in redox reactions (Wang and Ballatori, 1998).

GSH also participates in many other aspects of cell normal functioning. It is important for:

- ascorbate, oestrogens, prostaglandins and leukotrienes metabolism,
- the transformation of ribonucleotides to deoxyribonucleotides,
- maintaining communication between cells through gap junctions,
- preventing -SH groups oxidation and cross-linking (the maturation of Fe-S clusters of various proteins),
- stores and transports nitric oxide (Halliwell and Gutteridge, 2015).

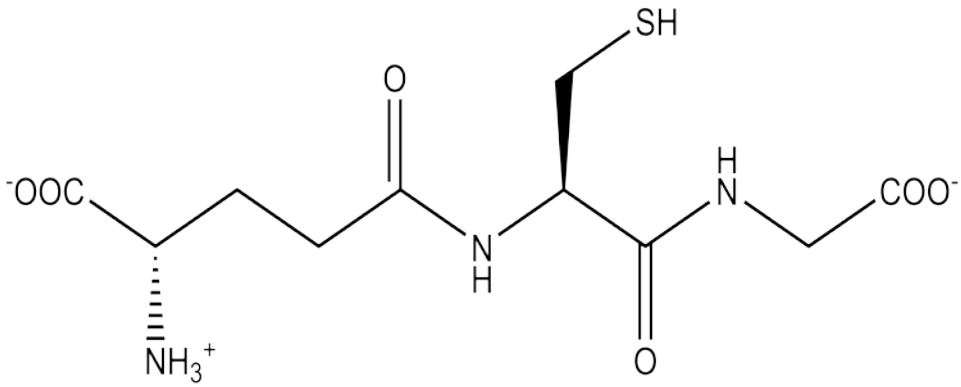


Figure 6. Chemical structure of glutathione (https://upload.wikimedia.org/wikipedia/commons/thumb/8/8c/Glutathione.png/1920px-Glutathione.png).

### 1.3.6. Sulfhydryl (-SH) groups

Thiols are organic compounds that have sulfhydryl or thiol groups (-SH) connected to a carbon atom (Rossi et al., 2009). The intracellular and extracellular concentrations of -SH groups are responsible for preserving the structure and function of proteins (e.g. enzyme activity) and control of transcription factors activity and binding. Thiols compose the main portion of the total cell antioxidants and can be found as oxidized or reduced non-protein thiols, or thiols bound to

proteins (Sen, 2001). -SH groups express their antioxidative potential through a variety of mechanisms (Deneke, 2001).

The concentrations, redox status and form of the thiols are highly variable and are dependent on cellular location and the metabolic activity of the cell. Increased levels of free thiols have been connected with enhanced resistance to oxidative stresses, so during oxidative stress it is beneficial to increase the availability of specific thiols. Cells possess mechanisms that enable the increased intracellular concentration of thiols such as GSH and thioredoxin in response to a variety of stresses (Stocker and Keaney, 2004). Two cysteines residues, in conditions of oxidative stress, can create disulfides. In the reaction S-thiolation, mixed disulfides between low-molecular-mass thiols and protein thiol groups are formed. In the presence of ROS, -SH groups can be reversibly oxidized to sulfenic acids or nitrosothiols (Hansen et al., 2009).

S-glutathionylation of proteins can change protein roll in the cell and has an important function in defense from irreversible oxidation of proteins (Schafer and Buettner, 2001). Enzymes such as thioredoxin, protein disulfide isomerase and mitochondrial glutaredoxin can reverse protein thiols from S-glutathiolated forms (Stocker and Keaney, 2004).

### 1.4. Biotransformation enzymes

Human activities worldwide result with increasing pollution, followed by the production of a great variety of dangerous and foreign chemicals. A great deal of those chemicals constantly enters living organisms through water, air or food. Accumulated xenobiotics can alter cell homeostasis. One of the cell mechanisms to maintain homeostasis and deal with those potentially harmful substances is biotransformation (Sugathan et al., 2017). It is the process through which endogenous enzymes modified the functional groups of organic substances forming chemically different products. These products are hydrophilic and “nature-like” biodegradable compounds, allowing organism to easily eliminate them through the excretory system. The whole process is divided into two phases (Sarasquete and Segner, 2000). During phase I chemical activity of the xenobiotic is modified through the oxidation, reduction or hydrolysis of its functional group with biotransformation enzyme. In phase II a covalent bound between the xenobiotic functional group and endogenous ligand is created and water-soluble conjugate is formed to aid xenobiotic excretion. This way conjugated xenobiotic is more soluble and easily removed (Guengerich, 2001, 2007). The most studied enzymes of phase I are cytochrome P450, cytochrome b5 and NADPH cytochrome P450 reductase, and of phase II are UDP-glucuronosyltransferase (UGT) and glutathione S-transferase (GST).

#### 1.4.1. Phase I enzyme of biotransformation – Cytochrome P450 (CYP)

In environmental and ecotoxicological studies the activity of cytochrome P450 (CYP) enzymes (EC 1.14) is often used as a sensitive and efficient bioindicator of exposure to organic pollution (Rahman and Thomas, 2012). CYP enzymes in the cell are mostly located in the endoplasmic reticulum and mitochondria (Sarasquete and Segner, 2000). The main function of those enzymes is to catalyze a variety of different metabolic reactions, through which xenobiotics (R) are bioactivated and converted to potentially less toxic forms. The great majority of those reactions is oxidations and can be generally presented as (Guengerich, 2001):

$$\mathbf{NADPH + H^+ + O_2 + R \rightarrow NADP^+ + H_2O + RO}$$

The reaction of CYP enzymes is seen as a complex series of radical recombination reactions that is dominated by intermediate and odd electron abstraction/rebound mechanisms of a high-valent $FeO^{3+}$ species (Guengerich, 2007) (Figure 7).

Among CYPs, the CYP1A or AHH (aryl hydrocarbon hydroxylase) subfamily is one of the most important members. Its role in phase I biotransformation is to metabolize endogenous substances such as lipids, steroids, vitamins and foreign compounds like furanes, dioxins, polychlorinated biphenyls (PCBs) and polyaromatic hydro-carbons (PAHs) (Rendic and Carlo, 1997). Metabolical conversion of lipophilic chemicals by CYP1A monooxygenation to more hydrosoluble metabolites determines the further destiny of accumulated chemicals and represents the first step in their excretion and detoxification. Sometimes produced intermediars due to high reactivity can interact and damage biomolecules and thereby being more toxic than the original compound (Guengerich, 2001, 2007; Sugathan et al., 2017).

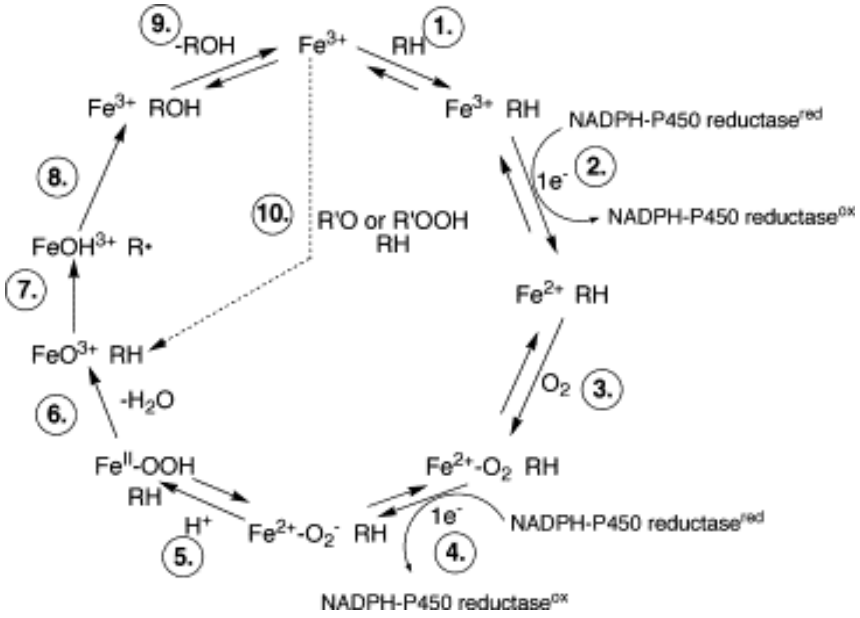


Figure 7. Cycle of CYP enzymes reactions (Guengerich, 2007).

### 1.4.2. Phase II enzyme of biotransformation – Glutathione S-transferase (GST)

The main biological roles of glutathione S-transferase (GST, E.C. 2.5.1.18) are in the detoxification processes of toxic electrophilic endogenous, exogenous molecules and protection against oxidative stress (Ashor et al., 2016). GSTs prevent interaction of xenobiotics with biomolecules by enabling GSH nucleophilic attack on electrophilic atoms (carbon, sulfur and nitrogen) of nonpolar xenobiotics. GST catalyses the conjugation of GSH to target molecules (RX); during conjugation, sulphydryl group reacts with electrophilic sites on xenobiotics (RSG) (Halliwell and Gutteridge, 2015).

**RX + GSH → RSG + HX**

GSTs have a twofold role: to bind substrate and GSH at the enzyme's hydrophobic H-site and hydrophilic G-site, respectively. This enables formation of the enzymes active site. Activation of -SH group of GSH leads to the nucleophilic attack. The resulting conjugat of GSH and toxic

electrophilic substrates becomes highly soluble and fewer reactive. This way xenobiotics cell excretion is easier. Sometimes, the resulting products are also able to damage biomolecules. For example, GSH conjugation with several halogenated hydrocarbons, including dibromo- and dichloroethane, cause kidney damage (Eaton and Bammler, 1999).

This enzyme is present in almost all living systems. It was detected in prokaryotic cells and cytoplasm, mitochondria and microsomes of eukaryotic cells. GST family of enzymes are homodimers or heterodimers, and the molecular weight of each subunit is about 23-28 kDa. Animal cytosolic GST isoenzymes have been grouped in fifteen families-alpha, kappa, mu, pi, omega, sigma, theta and zeta found in mammals, while beta, delta, epsilon, lambda, fi, tau and ro are detected in various non-mammalian species (bacteria, fungi, plants, mollusks, conifers, fish, amphibians, birds). They are distinguished by their molecular masses, isoelectric points, and other properties (Figure 8). Liver, as most important organ in detoxification, is especially rich in GST enzymes and the glutathione conjugates (Masson, 2015).

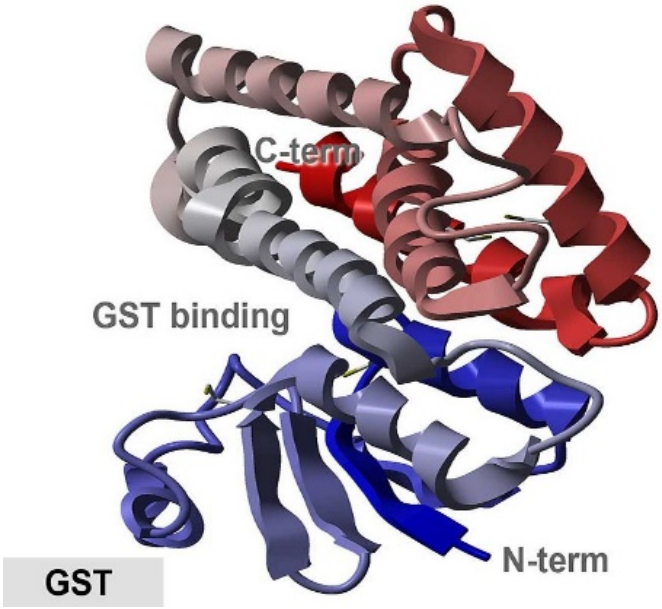


Figure 8. Glutathione S-Transferase structure (https://upload.wikimedia.org/wikipedia/commons/thumb/d/d2/GST-wiki.jpg/800px-GST-wiki.jpg).

Beside the mentioned role of GST, some GST isoforms show a GSH-Px-like activity with organic hydroperoxides, while others may be important protectors against lipid peroxidation metabolizing toxic end-products of lipid peroxidation (e.g. 4-hydroxynonenal and A2/J2 isoprostanes) (Halliwell and Gutteridge, 2015).

### 1.5. Oxidative damage parameters – Lipid peroxidation (LPO)

Lipid peroxidation (LPO) is a multistep process, through which free radicals induce the oxidative damage, affecting cell structure and leading to cell death in both plants and animals. During this process reactive species interact with lipids that contain carbon-carbon double bond(s), mostly with polyunsaturated fatty acids as lipids rich with those bounds (Yin et al., 2011). Oxygen inserted in lipids led to the generation and further propagation of lipid peroxides, rearranging the

double bonds of lipids. This way a normal function of cell membrane is interrupted. By product of this process are ketones, alkanes, alcohols, ethers and aldehydes (Barrera et al., 2008). Cyclooxygenases, CYP and lipoxygenases can also oxidize lipids (Volinsky and Kinnunen, 2013).

Process of lipid peroxidation consists of three phases (Figure 9):

- First-Initiation, during which carbon-centered lipid radical (L˙ ) is formed;
- second phase is propagation, during this phase lipid peroxy radical (LOO˙) and lipid hydroperoxide (LOOH) are formed as a result of free radical chain reactions;
- and the last is phase of lipid hydroperoxide termination during which oxido-modified unsaturated fatty acids or alcohols are cut to nonradical products (Girotti, 1998; Kanner et al., 1991). This process involves induction of various antioxidant enzymes such as glutathione peroxidase and selenium nondependent glutathione-S-transferase.

After initiation of lipid peroxidation, the second phase-propagation will take place as far as termination products are created (Yin et al., 2011).

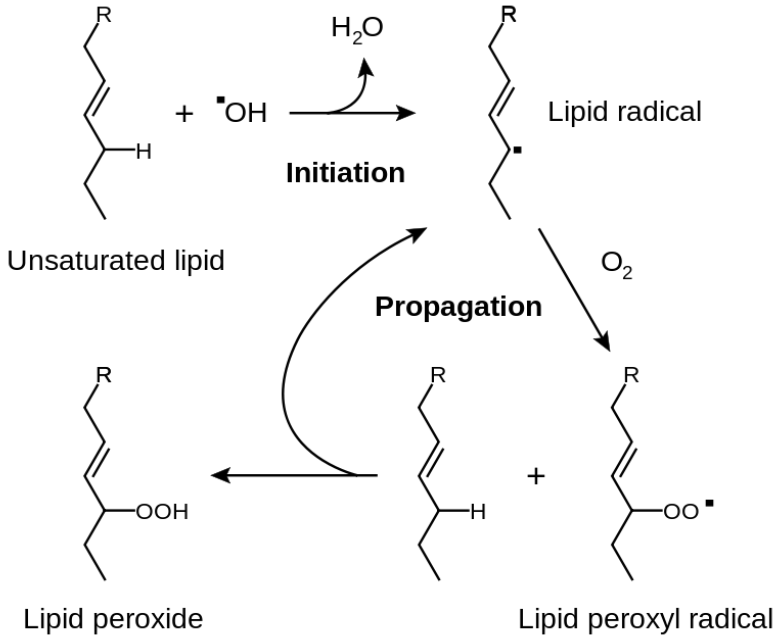


Figure 9. Mechanism of lipid peroxidation (https://en.wikipedia.org/wiki/Lipid_peroxidation).

### 1.6. Neurotoxicity parameters – Cholinesterases (ChE)

Cholinesterase (ChE) represents a family of enzymes – hydrolases that split the ester bond. ChE is present in the central nervous system, and tissues with a higher nervous activity (muscle and red cells). Their main function is to enable the hydrolysis of the acetylcholine to acetic acid and choline, maintaining normal activity of neurons (Sturm et al., 2000). ChE can be divided, based on substrate that prefer, as acetylcholinesterase (AChE) (which enables hydrolysis of acetylcholine) and butyrylcholinesterase (BChE) (which utilizes butyrylcholine as substrate). AChE is located primarily on red blood cell membranes, in neural synapses and in neuromuscular junctions, while BChE in the plasma (serum), pancreas, and liver, where it is synthesized. The rate of hydrolyzing

acetylcholine of those two enzymes differs. AChE decomposes acetylcholine faster, in comparison to BChE which hydrolyzes butyrylcholine (Lionetto et al., 2013). While the main role of AChE is well known, BChE is thought to be involved in some detoxification processes dealing with spectrum of different xenobiotics (Taylor, 1991). Both enzymes are used as parameters of neurotoxicity, especially in studies dealing with toxic effects of pesticides (Triquet-Amiard et al., 2012).

### 1.7. Influence of pesticides on biochemical parameters

During last decades, the toxicological and ecotoxicological studies have been dedicated to examining ability of pesticides to induce oxidative stress, and marked oxidative stress as one of possible mechanisms of their toxicity. Increased free radicals production is important in the toxicity of pesticides and other environmental chemicals. Beside increased generation of free radicals, pesticides are shown to alter antioxidative system, the scavenging enzyme system, and damage molecules (lipids, proteins and DNA). Numerous studies on a various group of animals have confirmed pesticide-induced oxidative stress in various cells and tissues (erythrocytes, liver, kidney and brain) (Gupta et al., 1999; Kale at al., 1999; Giray et al., 2001; Vontas et al, 2001; Gabbianelli et al., 2002; Clasen et al., 2018). Several studies of pesticide-induced oxidative stress have been conducted on amphibians and confirmed negative effect of pesticides exposure to antioxidative system and activity of AChE (Mussi and Calcaterra, 2010; Ezemonye and Tongo, 2010; Pašková et al., 2011; Li et al., 2017; Radovanović et al., 2017; Nasia et al., 2018).

In Asian toad, *Duttaphrynus melanostictus* tadpoles, intoxication with sublethal concentrations of cypermethrin induce higher CAT, GR, GST activity and increased concentrations of lipid peroxides (David et al., 2012). Cypermethrin and permethrin decreased protein concentrations and liver and kidney ChE activities of the frog *Rana tigrina (*Khan et al., 2003). Decrease in total protein concentration was also reported in African toad *Bufo regularis* after chronic exposure to diazinon (Tongo et al., 2012). The same authors reported that diazinon can decrease AChE activity. On the other hand, exposure to another pesticide, endosulfan did not affect brain AChE activity of the African toad *Bufo regularis* (Tongo and Ezemonye, 2013). The study of effects of two pesticides (diazinon and endosulfan) on GST activity showed that *Bufo regularis* after 28 days of treatment had increased GST activity (Ezemonye and Tongo, 2010). *Rana daunchina* tadpoles exposed to atrazine, at low concentrations displayed increased GSH-Px activity, while higher concentrations or longer exposure lead to inhibition of the same enzyme (Yuan et al., 2004).

Most of the studies dealing with DM induced oxidative stress were conducted on aquatic animals, mainly freshwater fishes. Deltamethrin exposure of *Channa punctata* fish induced activity of antioxidant enzymes GSH-Px and GST in liver and kidney but CAT activity decreased in all organs (Sayeed et. al, 2003). Also, Ansari et al. (2009) showed that DM significantly increased LPO concentrations, while activity of antioxidant enzyme SOD was significantly decreased in *Channa punctate*. The study conducted on freshwater fish Nile tilapia, *Oreochromis niloticus* revealed that DM intoxication increased tissues GSH concentration and GSH-Px, SOD and CAT activities (Abdelkhalek et al., 2015). After 48h of exposure to DM it was detected increased lipid peroxidation in the liver, kidney and gills of catfish (*Clarias gariepinus*) while CAT activity was significantly diminished (Amin and Hashem, 2012).

The literature informations regarding to DM impact on amphibians are scarce. It was suggested that DM can cause some neurological effects on frogs, such as spasmodic contractions of *Physalaemus gracilis* embryo, and increased AChE activity of *Xenopus leavis* individuals acutely treated. Reported lethal contraction ($LC_{50}$) for *X. leavis* was 6.26 μg of active ingredient per liter (Aydin-Sinan et al., 2012).

Additional studies on nontarget organisms could give helpful data on the ecotoxicological consequences of DM use. Physiological changes, including oxidative stress parameters, caused by

exposure to DM in animals may have direct consequences on reproduction, longevity and metabolism of these animals (Triquet-Amiard et al., 2012).

## 1.8. Anurans in ecotoxicological studies

Populations of amphibians (including anurans) have been declining worldwide for over a decade (Stuart et al., 2004), and pesticides have long been suspected as being at least partially responsible for observed situations (Cowman and Mazanti, 2000). During the last thirty years the ecology and ecotoxicology of amphibians started to get concerned (Sparling, 2000). Anurans represent a unique group of organisms among all vertebrates due to their biphasic lifestyle. According to Loumbourdis and Wray (1998) anurans are marked as good model system for ecotoxicological studies, because of their characteristics:

- complex life cycle, consisted of egg, larva, juvenile, subadult and adult stage,
- presence in aquatic and terrestrial environment,
- semipermeable skin,
- longevity, population size, broad distribution, and
- limited area of movements.

During lifecycle they are an integral parts of the aquatic environment as tadpole, and both the aquatic and terrestrial as adults. Presence in both habitats makes the anurans susceptible to various stressors and one of the most endangered groups of vertebrates (Blaustein and Wake 1990; Blaustein, 1994). Three main routs of exposure to pesticides in anurans are:

- via water (skin and eggs),
- through diet (gastrointestinal tract), and
- through air (lung and skin)

Pesticides dissolved in water can be absorbed through semipermeable skin during process of respiration and ion exchange. An indirect way of pesticide intake is through the ingestion of treated insects and other invertebrates. Some of these chemicals are present as aerosols and can enter a body through respiration or moisture skin (Sparling, 2000).

### 1.8.1. *Bufotes viridis* toad

Cl. Amphibia
O. Anura
Fam. Bufonidae

*Bufotes viridis* known as Green toad is distributed across Europe mainland (Gasc et al., 1997). When toads reach sexual maturity, their snout-vent length is approximately 4.8-12.0 cm. On dorsal skin we can observe tuberculate with different shades of green spots color and red to red-orange circle points. Their stomach is grey colored (Figure 10). Males poses nuptial pads located on the first finger and are characterised by smaller body size in comparison to female individuals. Tadpoles of green toad eat detritus and algae. Opposite to herbivorous larvae, metamorphosed toads are carnivorous and feed with different invertebrates (Collembola, Coleoptera, Acarina and Diptera). The main food source for adult individuals are different species of crawling invertebrates, including spiders, beetles etc.

Green toad is considered as species with wide distribution. In cold north regions of its distribution individuals are rarely found, but in places where it is found populations are very dense. Natural habitats of *B. viridis* are: woods, steppes, deserts and semi-deserts. They are good adapted

to anthropogenic area and are typically found in cities. In some places their abundance is much higher in anthropogenic environments than in natural habitats. This species is characterised with high resistance to desiccation processes and water lost. It survives well in both wet swampy areas and deserts. In general, this species prefers more open areas and bushlands, in comparison to aquatic environments (Gasc et al., 1997). Urbanization often negatively affects toad's habitats and may cause the decrease of the Green toad abundance on one hand but, forests destruction can favour this species distribution on others.

Figure 10. *Bufotes viridis*- green toad (https://upload.wikimedia.org/wikipedia/commons/thumb/4/43/European_Green_Toad.jpg/800px-European_Green_Toad.jpg).

# 2. OBJECTIVES

Oxidative stress, free radical production and oxidative damage of macromolecules represent the toxicity mechanisms for most pesticides. Due to their life characteristics (complex life cycle, semi-permeable skin), amphibians are susceptible to accumulation of pesticides over water and through a food chain. Experimental studies on pesticide effects on amphibians are almost entirely concentrated on their influence on aquatic species and tadpoles. Accessible results dealing with antioxidant system and oxidative stress in terrestrial adult amphibians subjected to deltamethrin are deficient.

There are certain data of the pesticide toxic effects to amphibians, but it remains unclear how pesticides can act on the body depending on time exposure and the application mode. In order to study the organization and functioning of the antioxidant defense system, for the experiment we have selected the green toad *Bufotes viridis*.

Within the research in this doctoral dissertation, the following parameters were examined:

1. Concentration of deltamethrin in the liver.
2. Oxidative stress parameters:
   superoxide-dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione reductase (GR) activities and total glutathione (GSH) and free sulfhydryl groups (-SH) concentrations,
3. Level of expression of phase I biotransformation protein (CYP1A),
4. Phase II biotransformation enzyme glutathione-S-transferase (GST) activity
5. Lipid peroxidation level (LPO),
6. Cholinesterase (ChE) activity as a parameter of neurotoxicity.

The goals of this doctoral dissertation are:

- ✓ To examine whether and to what extent the pyrethroid insecticide deltamethrin modulates the selected parameters of oxidative stress in the liver, skin, muscle and gastrointestinal tissue of adult species *Bufotes viridis*,
- ✓ Are there differences in the tested parameters depending on the concentration of the pesticide, the length and the way of exposure,
- ✓ To determine the potential tissue differences in the biomarkers response,
- ✓ To determine the concentration of deltamethrin in the liver,
- ✓ To determine which biomarkers would be ecologically significant in ecotoxicological studies of deltamethrin exposure, as well as to improve general knowledge about the impact of this pesticide on adult terrestrial amphibians, that have been very little involved in such research.
- ✓ By simulating similar conditions under which deltamethrin is used, the aim is to determine to what extent the use of this preparation acts on a single endangered and strictly protected species.

# 3. MATERIAL AND METHODS

### 3.1. Site characteristics and sampling methodology

National Park Fruška Gora (Figure 11) is settled in north part of Serbia, or more exactly in the northern part of Srem next to Novi Sad. This is the oldest national park in Serbia and it was proclaimed in 1960. It covers 26.672 ha (Službeni glasnik RS, 84/2015) of protected area placed on an isolated, narrow, island mountain in Pannonia plain. It is rich with forest and crossed with a lot of rivers and streams. Thanks to specific geological history and different microclimatic conditions it is marked as a habitat for many rare and endangered species.


Figure 11. National Park Fruška Gora

The surface higher than 300 meters is covered with thick, deciduous forests. Fruška Gora represents a unique natural phenomenon in geological terms, 78 km long and 15 km wide. Walls of Fruška Gora come from almost all geological periods, beginning with the oldest Paleozoic (a period of 500 million years ago), through Mesozoic, Neogene to the Quaternary. Park is some distance away from any larger pollution sources and can be considered as relative unpolluted area.

All individuals of adult green toads (*Bufotes viridis*) were collected by hand net (Figure 12, 13). Individuals of similar weight were collected to undo potential age and size effect (Canli and Furness, 1993). During experiments the ethical regulations have been followed, according to national and institutional policies for animal well-being. Serbian Ministry of Agriculture and Environmental Protection approved animal capture (permission 353- 01-1492/2015-17).

Figure 12. National Park Fruška Gora – field research
(From T.B. Radovanović archive)

In the laboratory toads were placed into 300-L glass terrariums (120×60×50 cm) for acclimation. According to Goulet and Hontella (2003) acclimation to laboratory conditions lasted for two weeks before starting the experiment. To obtain as much as possible natural conditions in terrariums, a soil substrate containing forest litter and rocks were provided. Twice a week the soil substrate was changed. Once a day terrarium walls were spraying with the dechlorinated tap water. At constant temperature of 20 ± 2°C, the animals were subjected to a natural 12:12 h light/dark photoperiod. Twice a week the toads were fed with earthworms (Ezemonye and Tongo, 2010), and uneaten leftovers were eliminated at each cleaning time.

Deltamethrin used in the experiment was purchased as a commercial product (Bayer K-OTHRINE SC25, 2.5 g/100 mL) and dissolved in dechlorinated tap water (tap water is common solvent in agriculture) in such a way being a more realistic approach (Rendón-von Osten et al. 2005). For the present investigation, commercial technical-grade deltamethrin (2.5%) was chosen as the pesticide widely used in the environment.

Whole experiment was divided in two parts: acute and chronic exposure. For acute treatment 22 individuals were used. Toads were arbitrarily assigned in to four groups: control and three treated groups. Treated animals were orally given three different sublethal concentrations of deltamethrin solution (8, 16 and 32 mg active ingredient/kg body weight) in order to imitate a possible intoxication route in the natural environment of the toads. In control group were 5 individuals, and in treated groups (8, 16 and 32 mg/kg) respectively: 6, 6 and 5 individuals. The acute experiment lasted for 96 h. During this period no mortal outcome was detected.

A chronic exposure involved 84 animals that were arbitrarily assigned to the four experimental groups: control, oral, dermal and combined (oral + dermal) exposure, 21 individual per group. The oral and dermal test groups were subjected to sublethal concentration of pesticide (5 mg/kg body weight/daily) for 7, 14, and 21 days. Combined test group was exposed to combination of oral and dermal exposure to sublethal concentration of pesticide (2.5 mg/kg of oral exposure + 2.5 mg/kg of dermal exposure) also for 7, 14, and 21 days, daily. Seven animals from the control

and test groups each were selected randomly on 7, 14 and 21 days of exposure. No mortality outcome was also reported during this part of experiment.

The average body mass (BM) and snout-vent length (SVL) were measured, before the individuals were euthanized. The toads were euthanized by decapitation. The investigation was permitted by the Animal Ethical Committee of the Institute for Biological Research ”Siniša Stanković”, University of Belgrade. Four different tissues were isolated: the liver, leg muscle, ventral skin and gastrointestinal tissue. After isolation, all tissue samples were washed in saline for poikilotherms (0.65% NaCl) and put into storage at -80°C until further biochemical analysis.

For control and exposed animals hepatosomatic index (HSI) was calculated. HSI represents morphological parameter which is often used in biomonitoring studies. HSI is indicator of the general state of the organism; however its value depends on the influence of other factors such as seasonality, availability of food or disease. HSI is most used in fish studies as an indicator of exposure to environmental toxicants. As the liver is the major detoxification organ, an increase in HSI may indicate an increase in metabolism of xenobiotics. HSI was obtained using the following formula: HSI=liver weight/BM×100, where BM represents body mass (g) of an individual.

Figure 13. Field research (From T.B. Radovanović archive)

### 3.2. Tissue processing

The tissues were minced and approximately 0.2 g of tissue was separated for measuring lipid peroxidation while the rest were homogenized with an Ultra-Turrax homogenizer T-18 (IKA-Werk, Staufen, Germany) in sucrose buffer (5 vol of 25 mM) (Lionetto et al., 2003) containing 10 mM Tris-HCl, pH 7.5 (Rossi et al., 1983). The resulting homogenates were sonicated with Ultrasonicator (Sonopuls HD2070, Bandelin, Germany) at 40 kHz for intervals of 30 s, and portions of the sonicates were used for determination the total concentration of GSH. The remaining sonicated material was centrifuged at 100,000 × *g* at 4°C for 90 min (Ultracentrifuge Beckman L7-55) (Takada et al., 1982) and the formed supernatants were used for determination total protein

concentration, enzyme activities and -SH groups concentration. Following the procedure of Krauss et al. (1983), we prepared the microsomal liver fraction in order to determine CYP1A expression.

The used apparatus:

- analytical balance (Mettler, Switzerland),
- technical balance (Precisa, Switzerland),
- pH meter (WTW, Germany),
- electrovibrator (Heidolf Reax Top, Germany).

All biochemical analyses were performed in triplicate on the Nicolet Evolution 600 UV-Vis spectrophotometer, at 25°C, with a temperature-controlled cuvette holder. All chemicals utilized in present investigation were obtained from Sigma-Aldrich (St Louis, MO, USA).

### 3.3. Deltamethrin determination in the liver

The following reagents were used for the preparation of the samples: acetonitrile, purity for HPLC - chromasolv Sigma-Aldrich (St. Louis, MA, USA); QuEChERS (Quick Easy Cheap Effective Rugged Safe) 50 ml tube with - 4 g of anhydrous magnesium sulphate (MgSO4) and 1.5 g of sodium chloride (NaCl); dispersive solid-phase extraction tubes (DSPE) of 2 ml volume with - 150 mg MgSO4, 25 mg PSA. For gas chromatograph determination, the basic standard solution of deltamethrin in acetonitrile was used (c=10 ng/µl) (Dr. Ehrenstorfer (Augsburg, Germany)).

Preparation of samples:

The chopped, homogeneous sample was soaked in 50 ml of QuEChERS tube with 10 ml of acetonitrile. After shaking and centrifuging, acetonitrile was evaporated to dryness. After reconstitution in 1 ml of acetonitrile, the extract was transferred to a 2 ml dSPE. The contents of the tube is strongly shaken with 1 ml of the extract and then centrifuged. 1µl of aliquot from the supernatant was injected into a gas chromatograph.

Instrumental determination:

The determination of DM in liver samples was carried out on the Varian CP-3380 gas chromatograph (Mulgrave, Australia) with the CP-8400 autosempler and the ECN $^{63}$Ni detector. The injected volume was 1 µl. The separation was carried out on a Zebron ZB-1 Phenomenex (Torrance, USA) column, 30 m long, an inner diameter of 0.25 mm and a film thickness of 0.25 µm. The gas carrier was high purity nitrogen, and the instrument was adjusted to the following operating conditions: injector temperature - 250°C, detector temperature - 300°C, initial temperature of the column 75°C - 1 minute, increase to 255°C with speed of 60°C/min and maintenance for 34 minutes at 255°C. Data was processed by STAR Software Varian (Mulgrave, Australia). Experimentally, in the process of method validation, a lower detection limit of 0.010 mg/kg was determined. The method is linear in the investigated concentration range. The income of the method was from 80% to 96%.

### 3.4. Determination of total protein concentration

The concentration of total proteins was determined by Lowry et al. (1951). For construction of the standard protein curve a series of bovine serum albumin (BSA) dilutions was used (the final concentration ranges between 100-1000 μg/mL). The procedure for protein concentration determination is based on the biuretic reaction of cupric ions ($Cu^{2+}$) with peptide bonds of the protein in the alkaline environment and the reaction of the phosphomolybdenum-phosphoflavolframic reagent (Folin-Ciocalteu reagent) with aromatic amino acids tyrosine and tryptophan, which are constitutive parts of measured proteins. After binding to the peptide bonds, $Cu^{2+}$ ions are reduced in the cuprous ions ($Cu^{+}$) and the $Cu^{+}$-protein complex is formed. This complex further reacts with the added Folin-Ciocalte reagent to form a blue colored complex. The color intensity is proportional to the protein content and is measured spectrophotometrically at 500 nm.

Solutions:

- 0.2 M NaOH,
- Reagent I (2 % $Na_2CO_3$ + 0.2 M NaOH),
- 0.13 M $CuSO_4$ x $5H_2O$,
- 0.13 M K, Na-tartarate
- Reagent II (1 mL 0.13 M CuSO4 x 5H2O + 1 mL of 0.13 M K, Na-tartar, filled to 100 mL with Reagent I) and
- The Folin-Ciocalteu reagent diluted in ratio 1:2 with distilled water.

Experimental procedure:

In a test tube 3 mL of Reagent II, 0.1 mL of 0.2 M NaOH and 0.1 mL of the diluted sample (dilution D = 11) was poured and shaken. For blank, instead of the samples, 0.1 mL of deionized water was added. After 15 minutes of room temperature incubation, 0.6 mL of Folin-Ciocalte reagent was added, shaken and incubated for another 30 minutes at room temperature. The absorbance was measured spectrophotometrically at a wavelength of 500 nm.

### 3.5. Determination of the biochemical parameters

#### 3.5.1. Determination of superoxide-dismutase (SOD) activity

The activity of SOD was determined by the adrenaline method (Misra and Fridovich, 1972), which is based on the ability of SOD to reduce spontaneous autoxidation of adrenaline in the adrenochorme in the alkaline environment. Autoxidation of adrenaline depends on the presence of $O_2^{\cdot-}$. SOD present in the sample removes $O_2^{\cdot-}$ and thus inhibits the autoxidation reaction. Reduction in the rate of adrenaline autoxidation is determined spectrophotometrically at a wavelength of 480 nm. The change in the absorbance is due to the pink-colored adrenaline.

Solutions:

- 3 x $10^{-4}$ M adrenaline in 0.1 M HCl,
- Carbonate buffer (0.05 M $Na_2CO_3$ + $10^{-4}$ M EDTA) pH 10.2 adjusted with 10% HCl and
- 8 mM KCN.

Experimental procedure:

3 ml of carbonate buffer was poured into the glass cuvette, together with an appropriate volume of the pre-adjusted adrenaline and the amount of sample that induces the adrenaline autooxidation inhibition ranging from 16.66% to 66.66%. To calculate the activity of SOD, the value of the sample absorption changes in the blank test (buffer and adjusted adrenaline) was used. The SOD activity unit is defined as the amount of enzyme which leads to 50% inhibition of adrenaline autoxidation in the linear portion of the change in absorbance per minute. The activity of SOD in the samples was expressed in units per milligram of protein (U/mg protein).

### 3.5.2. Determination of catalase (CAT) activity

The activity of CAT was determined by the method described by Claiborne (1984). The method is based on monitoring the decomposition rate of $H_2O_2$ to $H_2O$ and $O_2$ under the action of CAT. Reduction of the absorbance due to the consumption of $H_2O_2$ was detected spectrophotometrically at a wavelength of 240 nm.

Solutions:

- 0.05 M phosphate buffer (pH 7.0) and
- 30% $H_2O_2$.

Experimental procedure:

The $H_2O_2$ solution in phosphate buffer is adjusted so that the blank sample absorption at a wavelength of 240 nm is between 0.525 and 0.550. In a quartz cuvette, 1.5 ml of the adjusted solution of $H_2O_2$ in phosphate buffer was poured, and then the amount of sample which leads to a mean change in the absorbance in the range of 0.03 to 0.06 was added. In the sample starts the process of $H_2O_2$ decomposition reaction due to the presence of CAT. The reduction of the absorbance was monitored spectrophotometrically at 240 nm every 30 seconds for 3 minutes at a temperature of 25 °C. To calculate the CAT activity, a molar extinction coefficient for $H_2O_2$ (43.6 $M^{-1}$ $cm^{-1}$), at a wavelength of 240 nm, was used.

CAT activity unit is determined as the number of millimoles of $H_2O_2$ reduced per minute (mmol $H_2O_2$/min). The activity of enzymes in the tested samples is expressed in units per milligram of protein (U/mg protein).

### 3.5.3. Determination of glutathione peroxidase (GSH-Px) activity

GSH-Px activity was measured by the method developed by Tamura et al. (1982). The principle of the method is based on the coupled activity of GSH-Px (catalyses the oxidation of GSH in GSSG with the reduction of organic hydroperoxides) and GR (enables the reduction of GSSG in GSH with oxidation of NADPH as coenzyme). The organic peroxide tert-butyl hydroperoxide is added to the reaction mixture, with NADPH and GR. The activity of the GSH-Px enzyme was detected by spectrophotometric monitoring of NADPH oxidation into $NADP^+$.

Solutions:

- 1mM GSH,
- 0.2 mM NADPH in 1% $NaHCO_3$,
- 1 mM $NaN_3$,
- 1 mM EDTA,
- 0.03 M tert-butil hydroperoxide,

- 0.5 M phosphate buffer (pH 7,0) and
- GR.

Experimental procedure:

Into quartz cuvette were poured: 1.6 mL of $H_2O$ (or less volume depending on the amount of added sample), 0.3 mL of 1mM GSH, 0.6 mL of 0.2 mM NADPH, 0,1 mL of 1mM $NaN_3$ (which inactivated CAT), 0.1 mL of 1 mM EDTA, 0.3 mL 0,5M phosphate buffer (pH 7.0), 0.1 mL of 0.03 M tert-butyl hydroperoxide, adequte amount of sample and 5μL GR. Decrease of the absorbance was monitored spectrophotometrically at 340 nm every 30 seconds for 3 minutes at a temperature of 25 °C. The activity of GSH-Px was determined according to the blank, using a molar extinction coefficient $6.22 \times 10^3$ $M^{-1}$ $cm^{-1}$ for NADPH at 340 nm.

The GSH-Px enzyme activity unit was defined as the number of oxidized nanomoles of NADPH per minute (nmol NADPH/min), and the activity of GSH-Px in the tested tissues is expressed in units per milligram of protein (U/mg protein).

### 3.5.4. Determination of glutathione reductase (GR) activity

To determine the activity of GR in the tested samples the method according to Glatzle et al. (1974) was used. This method is established on the ability of GR to catalyze the GSSG reduction in to GSH with the oxidation of coenzyme NADPH to $NADP^+$. In the reaction mixture the GSSG and NADPH are added, and GR activity is measured spectrophotometrically by reducing the NADPH concentration.

Solutions:

- 0.5 M phosphate buffer (pH 7.4),
- 0.5 mM EDTA,
- 2 mM GSSG and
- 0.1 mM NADPH in 1% $NaHCO_3$.

Experimental procedure:

In a quartz cuvette 0.6 mL 0.5 M phosphate buffer (pH 7.4), 0.1 mL 2 mM GSSG, 0.1 mL 0.5 mM EDTA, 2 mL $H_2O$ (or a smaller volume depending on the volume of the sample) and 0.1 mL 0.1 mM NADPH were added, and at the end adequte amount of sample. The absorbance was measured at a wavelength of 340 nm every 30 seconds for 3 minutes at a temperature of 25 °C. To calculate the GR activity, a molar extinction coefficient for NADPH at 340 nm of $6.22 \times 10^3$ $M^{-1}$ $cm^{-1}$ was used.

The unit of activity of the enzyme GR is defined as the number of nanomoles oxidized NADPH per minute (nmol NADPH/min). The activity of this enzyme in the tested samples is presented in units per milligram of protein (U/mg protein).

### 3.5.5. Determination of total glutathione (GSH) concentration

The method described by Griffith (1980) was used to measure the concentration of total GSH in the test samples. This method is based on a cyclic enzymatic process: 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) oxidizes GSH, whereby GSSG and 2-nitro-5-thiobenzoic acid (TNB) are formed, and then the GR enzyme reduces GSSG in GSH with NADPH coenzyme oxidation. The formation rate of the yellow coloured TNB compound is monitored and is proportional to the concentration of total GSH in the sample.

Solutions:

- GSH standard,
- 10% sulfosalicylic acid,
- 6 mM DTNB,
- GR,
- 0.3 mM NADPH and
- Buffer (125 mM $NaH_2PO_4$ + 6 mM EDTA, pH 7.5 with NaOH).

Experimental procedure:

0.5 mL tissue sonicates and 0.25 mL of 10% sulfosalicylic acid (for protein precipitation in the sample) were poured in microcentrifuge tubes. After centrifugation for 10 minutes at 5000 rpm, the obtained supernatant was used to determine the concentration of GSH.

0.1 mL 6 mM DTNB, sample, 0.7 mL 0.3 mM NADPH, $H_2O$ to 1 mL of reaction mixture and 5 μL of GR were poured in quartz cuvette. For the standards, instead of the sample, the appropriate volumes of standard solutions with known concentration of GSH. Increase of the absorbance is monitored spectrophotometrically at a wavelength of 412 nm every 30 seconds for 3 minutes at a temperature of 25 °C.

The absorbance is determined spectrophotometrically at 412 nm. The concentration of total GSH in the sample is expressed in nanomoles per gram of tissue (nmol of GSH/g tissue).

### 3.5.6. Determination of the concentration of free sulfhydryl (-SH) groups

The concentration of free -SH groups in the tested samples was measured by the method of Ellman (1959). DTNB oxidizes free -SH groups present in the sample, whereby mixed disulfides and yellow coloured TNB are formed.

Solutions:

- 0.1 M phosphate buffer (pH 7.3) and
- 3 mM DTNB in 0.1 M phosphate buffer (pH 7.3).

Experimental procedure:

In the cuvette 0.5 ml of sample was added, 0.5 mL of 0.1 M phosphate buffer (pH 7.3) and 0.2 mL of DTNB. After incubation for 10 minutes at room temperature, the absorbance at a wavelength of 412 nm was read. To calculate the concentration of free SH groups, a molar extinction coefficient of 14150 $M^{-1}$ $cm^{-1}$ was used. The concentration of free -SH groups in the sample is determined according to the blank and proportion of formed concentration of TNB.

The absorbance is measured spectrophotometrically at 412 nm wavelength. The concentration of free -SH groups in the sample is expressed in micromoles of -SH group per gram of tissue (μmol SH/g tissue).

### 3.5.7. Sodium dodecyl sulfate polyacrylamide gel electrophoretic protein analysis (SDS-PAGE) and CYP1A analysis

Proteins of the examined tissues were separated by molecular weight SDS-PAGE electrophoresis (Laemmly, 1970) in the BioRad Mini-PROTEAN III system. The principle of this method is established on the use of an anion detergent of SDS that denaturates the proteins wrapping around their polypeptide bonds. Negatively charged SDS causes mutual rejection, which leads to the separation of protein chains. Proteins in the electric field travel to a positively charged

half-speed that depends solely on their molecular weight. The structure of the polyacrylamide gel provides resistance to the movement of proteins, wherein the less molecular weight proteins move faster than the protein of a higher molecular weight. The polyacrylamide gel consists of a separation gel and a compaction gel in which the proteins first concentrate before they pass into the separation gel. 12% separation gel (pH 8.8) and 4% compaction gel (pH 6.8) were used.

Polyacrylamide separation gel:

Distilled water 3.35 mL
5 mL of 1.5 M Tris (pH 8.8) + 6.2 mL H2O 2.5 mL
10% SDS 100 μL
30% acrylamide / N'N'-bis-methylene-acrylamide 4 mL
10% APS 50 μ
TEMED 5μ

Polyacrylamide compaction gel:

Distilled water 3.05 mL
4 mL 1.5 M Tris (pH 8.8) + 6 mL H2O 1.25 mL
10% SDS 50 μL
30% acrylamide / N'N'-bis-methylene-acrylamide 665 μL
10% APS 25 μL
TEMED 5μL

After the polymerization of the separation gel between the glass plates (about 30 minutes), a compaction gel was poured into the comb in order to form "wells" in which the samples were later applied. The sample is 1: 1 mixed with sample buffer (3.8 mL of distilled water, 1 mL of 0.5 M Tris-HCl buffer, pH 6.8, 0.8 mL glycerol, 1.6 mL of 10% SDS, 0.4 mL of β-mercaptoethanol and 0.4 mL 10% bromine phenol blue). Then, the volume of the sample containing 20 μg of protein is applied to the gel. The electrophoresis lasted 90 minutes at a voltage of 110 V and a current of 20 mA.

After the electrophoresis, transfer of proteins from gels to PVDF (polyvinylidene fluoride) membrane was performed with apparatus Mini Trans-Blott Cell, Biorad. The membranes were cut to dimensions slightly larger than the gel and activated by flooding in 100% methanol for several seconds, and then washed with distilled water. Membranes and gels were immersed in to transfer buffer (20% methanol, 0.192 M glycine, and 0.025 M Tris pH 8.3). Sandwiches consisting of: sponge, Mini Trans-Blotfilter paper (BioRad), polyacrylamide gel, PVDF membrane, the next filter paper and sponge. The formed sandwich was placed in the transfer apparatus. Transfer of proteins to a membrane was carried out at constant voltage of 120 V for 60 minutes at room temperature. The effectiveness of transfer of proteins from the gel to the membrane was tested by dyeing with 5% Ponceau the color dispersed in acetic acid. PVDF membranes were further blocked in non-fat milk powder dissolved in TBS-T buffer (0.05% Tween20 in 1.5 M NaCl, 0.2 M Tris, pH 7.4), for 2 h at room temperature with mild mixing. Blocking the membrane allows free, non-specific spots on the membrane to be protected so that specific antibodies cannot be tied to them. The membranes were then cut at the level of certain molecular weights based on the Page Ruler Prestained Protein Ladder Marker (10-170kDa, Thermo Scientific) and incubated overnight at 4°C with appropriate primary antibodies for CYP1A (polyclonal rabbit anti-fish CYP1, Biosense, used dilution 1: 500) and β actin (anti-actin antibody, Sigma, 1: 250 dilution) obtained from the rabbit. The antibodies were diluted in 3% milk dissolved in TBS-T buffer. The next day, after washing the excess primary antibody (5 times for 5 minutes each in TBS-T buffer), the membranes were incubated 2h at room temperature in the appropriate dilutions of secondary antibody. For CYP1 we used anti-rabbit (1: 2000 dilution) secondary horseradish peroxidase (HRP) conjugated antibodies (Santa Cruz Biotechnology), and for the β actin anti rabbit IgG peroxidase (Sigma). After incubation, the membranes were washed in TBS-T buffer, and then incubated in a hemiluminous substrate,

luminol. After 2 minutes, the membranes were placed in development cassettes, covered with a plastic film and an X-ray film (Kodak Medical X-ray). After 2 minutes of exposure, the film was developed by immersing in the developer and then into the water and fixer. Signal quantification was done using the Total Lab software package. The CYP1A activity was expressed by an arbitrary unit.

### 3.5.8. Determination of phase II biotransformation enzyme glutathione-S-transferase (GST) activity

The activity of the phase II biotransformation enzyme - GST was measured by the method described by Habig et al. (1974). The principle of the method is based on the ability of the GST to catalyse the reaction of binding 1-chloro-2,4-dinitrobenzene (CDNB) to the sulfhydryl group of cysteine that is a part of the tripeptide GSH, thereby forming a CDNB-GSH conjugate. The rate of absorption due to the formation of CDNB-GSH conjugate is directly comparative to the activity of the GST in the sample.

Solutions:

- 0.5 M phosphate buffer (pH 6.5),
- 20 mM GSH and
- 25 mM CDNB in 95% ethanol

Experimental procedure:

2 ml of $H_2O$ poured into the quartz cuvette (or a smaller volume depending on the amount of sample) were followed by 0.1 mL 25 mM CDNB in 95% ethanol, 0.6 mL of 0.5 M phosphate buffer (pH 6.5), 0.3 mL of 20 mM GSH and adequate amount of sample. The absorbance was monitored spectrophotometrically at a wavelength of 340 nm every 30 seconds for 3 minutes at a temperature of 25 °C. The GST activity was determined according to the blank, using the molar extinction coefficient for the CDNB-GSH conjugate at 340 nm of $9.6 \times 10^3$ $M^{-1}$ $cm^{-1}$.

The absorbance is detected spectrophotometrically at wavelength of 340 nm. The unit of activity of the enzyme phase II biotransformation GST is expressed as the number of nanomoles of the CDNB-GSH conjugate formed per minute (nmol CDNB-GSH/min). GST activity is given in units per milligram of protein (U/mg protein).

### 3.5.9. Determination of lipid peroxides concentration (TBARS)

Concentration of lipid peroxidation (TBARS- thiobarbituric acid reactive substances) in tissues was measured according to method given by Ohkawa et al. (1979). Method is established on reaction of thiobarbituric acid (TBA) with a product of lipid peroxidation- malondialdehyde (MDA), during which bright purple product is formed.

Solutions:

- 1.15% KCl,
- 8.1% sodium dodecyl sulfate (SDS),
- 20% acetic acid (p.H 3.5 ajudsted with NaOH),
- 0.8% thiobarbituric acid (TBA),
- n-butanol and
- pyridine

Experimental procedure:

Samples of tissue were homogenised and sonicated on ice in 1.15% KCl in 1:9 rate. 0.1 mL of gained homogenate was poured in the tube together with 0.2 mL of 8.1% sodium dodecyl sulfate (SDS), 1.5 mL of 20% acetic acid, 1.5 mL of 0.8% TBA and 0.7 mL of deionized water. The mixture was incubated for 60 minutes on 95 ºC, and then left to turn cold on room temperature. When the samples were cooled, from each tube 2 mL was poured in new clean tube togheter with 0.5 mL of distilled water and 2.5 mL of mixture of n-butanol and pyridine (15:1), this proces was followed with constant mixing of solutions. Tubes were centrifuged for 10 min at 4000 rpm, before determining concentration of TBARS. The absorbance was read spectrophotometrically at wavelength of 532 nm. LPO concentration was expressed in nmol/mg tissue.

### 3.5.10. Determination of cholinesterase (ChE) activity

Cholinesterase activity in tissues was determined by the Ellman et al. (1961). The method is based on the hydrolysis of acetyl-thiolcholine wherein the free thiol reacts with DTNB and a yellow coloured TNB is formed.

Solutions:

- 0.05 M phosphate buffer (pH 7.9)
- 0.25 mM DTNB and
- 0.156 M acetyl-thiolcholine iodide.

Experimental procedure:

In a quartz cuvette 3 mL of 0.05 M phosphate buffer (pH 7.9), 0.1 mL of acetylthiocholine iodide and a specific amount of sample were poured. The absorption of the absorbance was read spectrophotometrically at a wavelength of 406 nm every 15 seconds for 1 minute with a delay of 1 minute at a temperature of 25 °C. The activity was determined according to the blank probe and was proportional to the concentration of TNB produced. The activity was expressed in μmol /min/g of tissue.

## 3.6. Statistical analysis of results

In experiment with acute exposure all results were presented as the mean ± standard error (SE). Kolmogorov-Smirnov one-sample test was used for assumptions of normality. The determined parameters had a normal distribution. In order to inspect distinctions between the means of the control and treated groups one-way analysis of variance (ANOVA) and the post hoc Fisher's least significant difference (LSD) test were performed. With Pearson's correlations we tested correlations between the investigated components for studied tissues. For all cases a significance level of $p<0.05$ was established. Subsequently, to identify the most significant variables contributing to tissue specificity and to perceive the variations between the studied tissues, principal component analysis (PCA) was done. STATISTICA Ver 8.0 software was used for all statistical analyses.

In experiment with chronic exposure, in order to upgrade the normality of variables, all data were log transformed and presented as the mean ± standard error (SE). We implemented one-way ANOVA and the post hoc Fisher LSD test in order to examine variations between the means of the control and treated groups. A significance level of $p < 0.05$ was set. We implemented canonical discriminant analysis (CDA) that discovered variation among the treated groups, established on all determined antioxidant parameters for studied tissues (Darlington et al. 1973). STATISTICA 8.0 software was used for all statistical analyses, beside the integrated biomarker response (IBR) value. This parameter was estimated in R 3.4.1. (Devin et al., 2014).

# 4. RESULTS

### 4.1. Concentration of deltamethrin in the liver

Concentration of deltamethrin was determined in the liver after chronic exposure for 21 days. We did not found detectable levels of DM in this tissue.

### 4.2. Results of acute exposure to deltamethrin

#### 4.2.1. Biometric, oxidative stress, biotransformation and neurotoxicity parameters in the green toad *Bufotes viridis* after acute deltamethrin treatment

Regardless of pesticide exposure, no mortality or visual changes in toad behavior were noticed experimental groups. Mean value of the snout-vent length (SVL) was 7.30 ± 0.12 cm, and the average body mass (BM) value was 29.03 ± 1.15 g. The HSI did not disclose any statistical distinction between the control and treated groups of animals. HSI for control group was 3.96 ± 0.28 and for treated groups (8, 16 and 32mg/kg) were respectively: 3.86 ± 0.17, 3.76 ± 0.14 and 4.04 ± 0.23.

Obtained results of the biochemical parameters after 96 h of the acute treatment are presented by tissues.

In the Table 1 and Figure 14 are presented results in the liver of treated toads. Protein concentration, activities of SOD, GST and GSH-Px did not show statistically significant changes in the liver tissue after acute treatment. However, CAT and GR activities were increased in the 32 mg/kg treated group. GSH concentrations were decreased and CYP1A expressions were increased in 8 and 16 mg/kg treated groups in respect to the control. SH group concentrations and ChE activities were decreased, and TBARS concentrations were increased in 16 and 32 mg/kg treated groups after acute treatment in respect to control group.

Examined components in the muscle are showed in the Table 2 and Figure 15. Activities of CAT and ChE as well as -SH concentration did not exhibit significant change in regard to the control. Protein concentration was decrease in the group trated with 8 mg and 16 mg of DM in respect to control. Activities of SOD and GSH-Px were increased in 16 mg/kg treated group and GR and GST concentrations in 16 and 32 mg/kg treated groups. GSH concentration was higher in all exposed groups of toads in respect to control group. TBARS concentration was higher in group of toads that were exposed to 32 mg/kg body mass of deltamethrin.

In the Table 3 and Figure 16 are presented results of gastrointestinal tissue. Protein concentration, activities of CAT, GR and GST did not show statistically significant change after acute treatment. SOD activity was decreased in 16 and 32 mg/kg treated groups. GSH-Px activity and -SH group concentration were higher in all treated groups of toads. GSH concentration and ChE activity were increased and TBARS concentration was decreased in 8 mg/kg treated group.

Examined parameters in the skin are showed in the Table 4 and Figure 17. GSH-Px and ChE activities as well as GSH and TBARS concentrations did not show significant change in respect to the control. Protein concentration was decrease in the group trated 16 mg of DM in respect to control. SOD activity was higher and -SH group concentration was lower in 16 and 32 mg/kg treated groups. CAT and GST activities were increased in 8 and 32 mg/kg treated groups. GR activity was higher in 32 mg/kg treated group.

Table 1: Investigated oxidative stress, biotransformation and neurotoxicity parameters in the liver of *Bufotes viridis* after 96 h deltamethrin (DM) exposure. To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented. Significant differences ($p<0.05$) among control and treated groups of animals are marked with asterisks (*).

| | Control | 8 mg/kg DM | 16 mg/kg DM | 32 mg/kg DM |
|---|---|---|---|---|
| Total proteins (mg/mL) | 19.67 ± 0.65 | 19.51± 0.84 | 20.66 ± 0.63 | 19.53 ± 0.56 |
| SOD (U/mg protein) | 16.46 ± 1.12 | 13.22 ± 0.73 | 14.19 ± 1.34 | 18.04 ± 1.22 |
| CAT (U/mg protein) | 121.64 ± 13.96 | 150.56 ± 4.57 | 157.52 ± 15.48 | 292.69 ± 26.3* |
| GSH-Px (U/mg protein) | 20.83 ± 2.27 | 23.43 ± 1.93 | 23.46 ± 1.95 | 24.69 ± 1.99 |
| GR (U/mg protein) | 3.7 ± 0.33 | 4.00 ± 0.36 | 2.99 ± 0.18 | 4.67 ± 0.4* |
| GST (U/mg protein) | 1290.14 ± 54.86 | 1235.53 ± 83.67 | 1192.24 ± 96.55 | 1228.1 ± 101.99 |
| GSH (nmol/g tissue) | 2364.97 ± 268.99 | 1350.99 ± 95.56* | 1675.14 ± 174.41* | 2154.76 ±.86.86 |
| -SH (µm/g tissue) | 1119.83 ± 0.74 | 1116.01 ± 1.48 | 1114.83 ± 1.29* | 1109.31 ± 1.19* |
| TBARS (nmol/mg tissue) | 1.34 ± 0.12 | 1.63 ± 0.24 | 2.73 ± 0.43* | 2.82 ± 0.79* |
| ChE (µmol/min/g tissue) | 7.3 ± 0.8 | 6.76 ± 0.62 | 5.47 ± 0.48* | 4.77 ± 0.15* |
| CYP1A (arbitrary unit) | 1.01 ± 0.25 | 1.48 ± 0.48* | 1.82 ± 0.24* | 1.06 ± 0.10 |

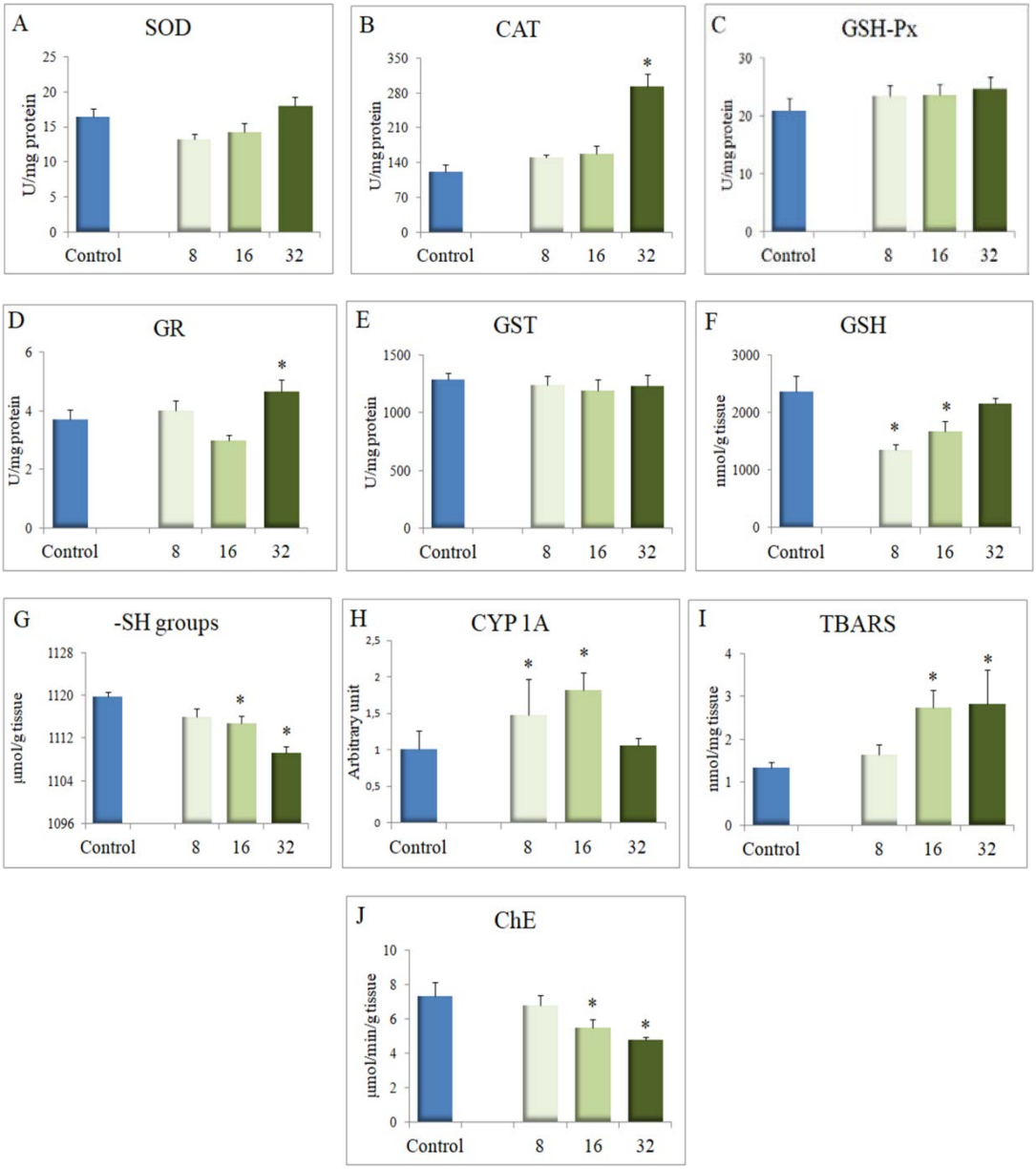


Figure 14. Effects of 96 h deltamethrin (DM) exposure on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups concentration (G), cytochrome P4501A (CYP1A) expression (H), thiobarbituric reactive substances-TBARS concentration (I), and cholinesterase-ChE activity (J) in the liver of *Bufotes viridis*. On the x axis are presented applied concentrations of DM in mg/kg. To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented. Significant differences ($p<0.05$) among control and treated groups of animals are marked with asterisks (*).

Table 2: Investigated oxidative stress, biotransformation and neurotoxicity parameters in the muscle of *Bufotes viridis* after 96 h deltamethrin exposure. To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented. Significant differences ($p<0.05$) among control and treated groups of animals are marked with asterisks (*).

| | Control | 8 mg/kg DM | 16 mg/kg DM | 32 mg/kg DM |
|---|---|---|---|---|
| Total proteins (mg/mL) | 10.77 ± 0.30 | 9.26 ± 0.36* | 8.96 ± 0.27* | 10.13 ± 0.27 |
| SOD (U/mg protein) | 3.97 ± 0.06 | 4.49 ± 0.23 | 5.09 ± 0.18* | 3.7 ± 0.15 |
| CAT (U/mg protein) | 26.4 ± 1.67 | 32.9 ± 3.47 | 22.75 ± 2.45 | 23.38 ± 1.7 |
| GSH-Px(U/mg protein) | 7.94 ± 0.37 | 8.78 ± 0.81 | 11.72 ± 0.89* | 9.23 ± 1.46 |
| GR (U/mg protein) | 2.16 ± 0.1 | 2.86 ± 0.46 | 3.75 ± 0.34* | 3.41 ± 0.12* |
| GST (U/mg protein) | 46.17 ± 2.95 | 51.38 ± 4.48 | 69.71 ± 3.06* | 61.82 ± 3.81* |
| GSH (nmol/g tissue) | 131.28 ± 15.76 | 260.53 ± 50.92* | 312.23 ± 15.62* | 322.17 ± 35.7* |
| -SH (µm/g tissue) | 1083.84 ± 7.48 | 1065.52 ± 10.47 | 1074.63 ± 9.58 | 1075.62 ± 14.79 |
| TBARS (nmol/mg tissue) | 4.21 ± 0.61 | 5.36 ± 0.94 | 3.2 ± 0.4 | 12.09 ± 1.95* |
| ChE (µmol/min/g tissue) | 0.78 ± 0.05 | 0.77 ± 0.05 | 0.88 ± 0.12 | 0.78 ± 0.1 |

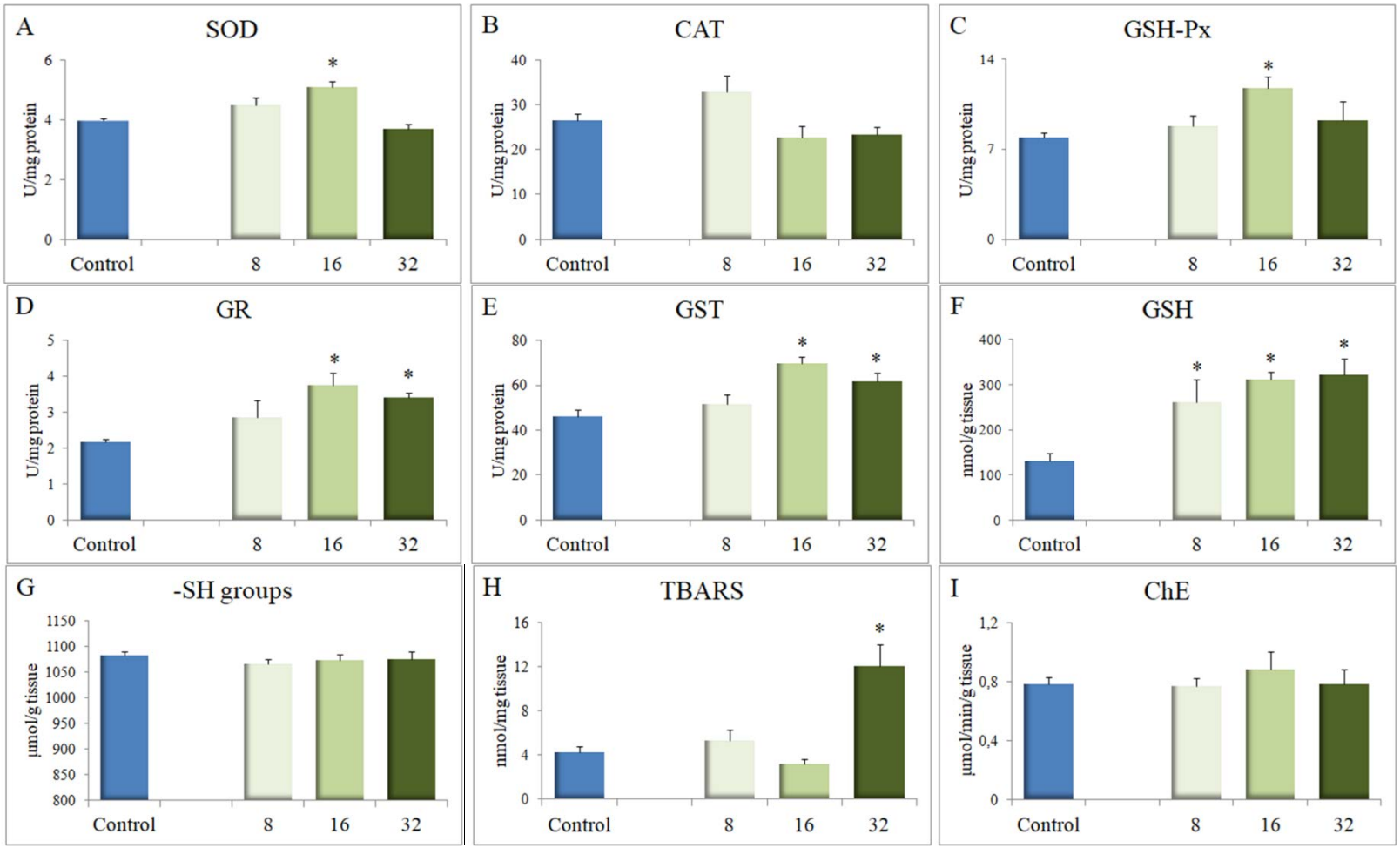


Figure 15. Effects of 96 h deltamethrin (DM) exposure on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G), thiobarbituric reactive substances-TBARS (H) concentrations and cholinesterase-ChE (I) activity in the muscle of *Bufotes viridis*. On the x axis applied DM concentrations are presented in mg/kg. To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented. Significant differences ($p<0.05$) among control and treated groups of animals are marked with asterisks (*).

Table 3: Investigated oxidative stress, biotransformation and neurotoxicity parameters in the gastrointestinal tissue of *Bufotes viridis* after 96 h deltamethrin exposure. To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented. Significant differences ($p<0.05$) among control and treated groups of animals are marked with asterisks (*).

| | Control | 8 mg/kg DM | 16 mg/kg DM | 32 mg/kg DM |
|---|---|---|---|---|
| Total proteins (mg/mL) | 12.11 ± 0.65 | 11.90 ± 0.32 | 12.44 ± 1.31 | 12.12 ± 0.51 |
| SOD (U/mg protein) | 6.57 ± 0.93 | 5.35 ± 0.22 | 4.85 ± 0.36* | 4.04 ± 0.18* |
| CAT (U/mg protein) | 94.76 ± 5.03 | 77.62 ± 7.33 | 102.38 ± 15.15 | 102.69 ± 4.39 |
| GSH-Px (U/mg protein) | 13.9 ± 1.55 | 19.92 ± 2.11* | 21.99 ± 2.57* | 19.7 ± 1.06* |
| GR (U/mg protein) | 13.74 ± 1.28 | 14.05 ± 0.81 | 15.81 ± 1.78 | 15.55 ± 1.95 |
| GST (U/mg protein) | 224.93 ± 18.62 | 242.39 ± 20.24 | 218.45 ± 31.25 | 223.64 ± 17.83 |
| GSH (nmol/g tissue) | 455.34 ± 143.56 | 705.38 ± 60.4* | 506.12 ± 32.66 | 616.98 ± 25.56 |
| -SH (µm/g tissue) | 957.49 ± 34.07 | 1103.34 ± 1.38* | 1100.69 ± 2.46* | 1099.89 ± 1.84* |
| TBARS (nmol/mg tissue) | 5.47 ± 0.66 | 1.23 ± 0.22* | 4.44 ± 1.28 | 5.86 ± 0.97 |
| ChE (µmol/min/g tissue) | 1.12 ± 0.08 | 1.57 ± 0.11* | 1.42 ± 0.1 | 1.4 ± 0.07 |

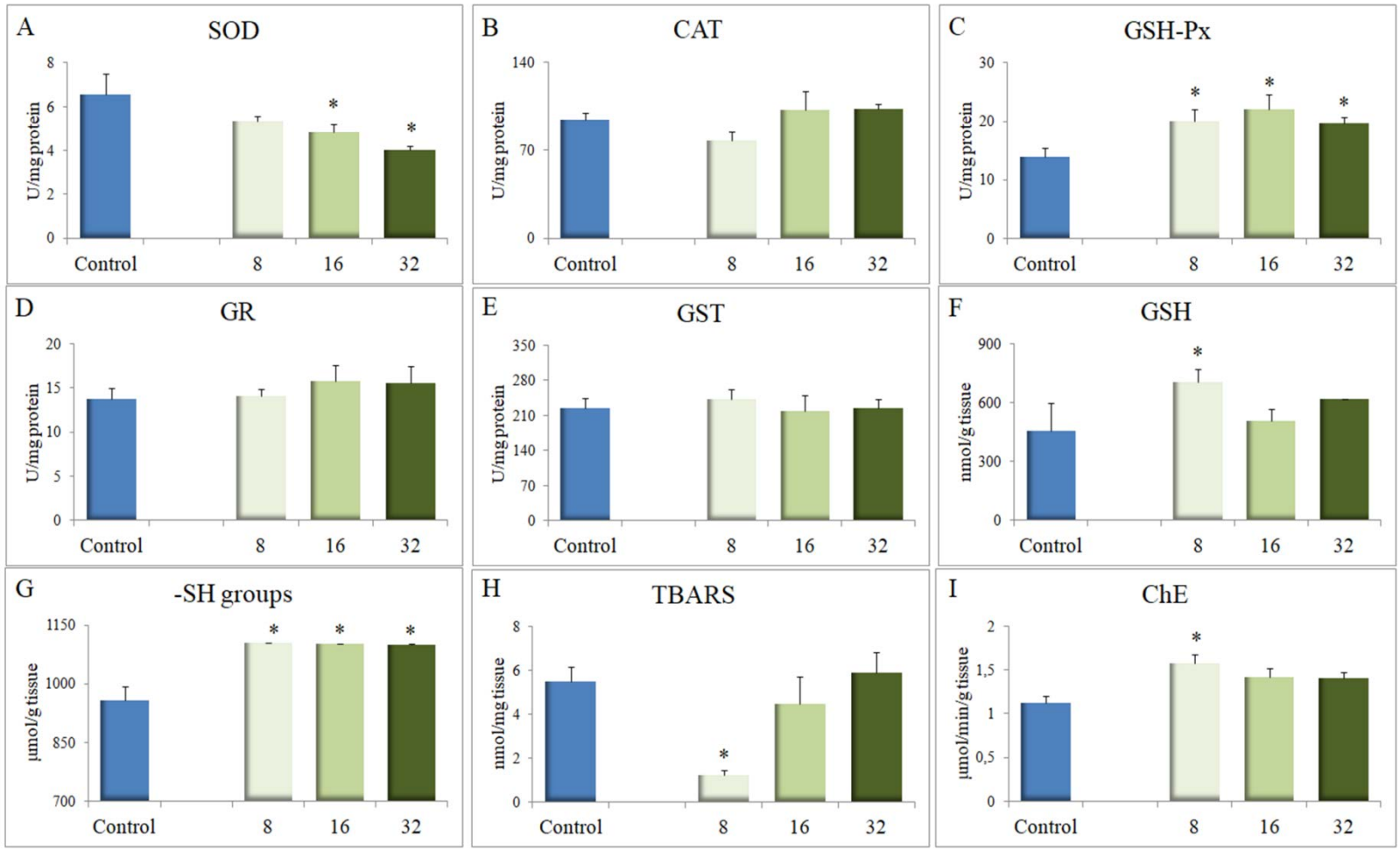


Figure 16. Effects of 96 h deltamethrin (DM) exposure on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G), thiobarbituric reactive substances-TBARS (H) concentrations and cholinesterase-ChE (I) activity in the gastrointestinal tissue of *Bufotes viridis*. On the x axis applied DM concentrations are presented in mg/kg. To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented. Significant differences ($p<0.05$) among control and treated groups of animals are marked with asterisks (*).

Table 4: Investigated oxidative stress, biotransformation and neurotoxicity parameters in the skin of *Bufotes viridis* after 96 h deltamethrin exposure. To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented. Significant differences ($p<0.05$) among control and treated groups of animals are marked with asterisks (*).

| | Control | 8 mg/kg DM | 16 mg/kg DM | 32 mg/kg DM |
|---|---|---|---|---|
| Total proteins (mg/mL) | 10.93 ± 0.54 | 10.69 ± 0.43 | 9.54 ± 0.33* | 10.29 ± 0.23 |
| SOD (U/mg protein) | 6.32 ± 0.64 | 6.13 ± 0.4 | 10.94 ± 0.65* | 18.25 ± 0.97* |
| CAT (U/mg protein) | 27.87 ± 2.44 | 52.49 ± 4.84* | 37.1 ± 4.42 | 54.44 ± 4.71* |
| GSH-Px (U/mg protein) | 6.63 ± 0.37 | 6.28 ± 0.54 | 5.64 ± 0.86 | 6.95 ± 0.46 |
| GR (U/mg protein) | 7.74 ± 0.55 | 8.11 ± 0.42 | 9.42 ± 0.36 | 11.37 ± 1.04* |
| GST (U/mg protein) | 69.91 ± 4.95 | 89.16 ± 4.9* | 75.91 ± 5.06 | 86.54 ± 3.52* |
| GSH (nmol/g tissue) | 1229.39 ± 96.06 | 983.36 ± 51.42 | 1073.16 ± 90.82 | 1052.15 ± 151.58 |
| -SH (µm/g tissue) | 1057.06 ± 23.44 | 972.63 ± 31.12 | 895.69 ± 41.68* | 906.03 ± 51.98* |
| TBARS (nmol/mg tissue) | 0.29 ± 0.07 | 0.19 ± 0.05 | 0.46 ± 0.13 | 0.21 ± 0.02 |
| ChE (µmol/min/g tissue) | 0.8 ± 0.05 | 0.73 ± 0.04 | 0.73 ± 0.03 | 0.87 ± 0.12 |

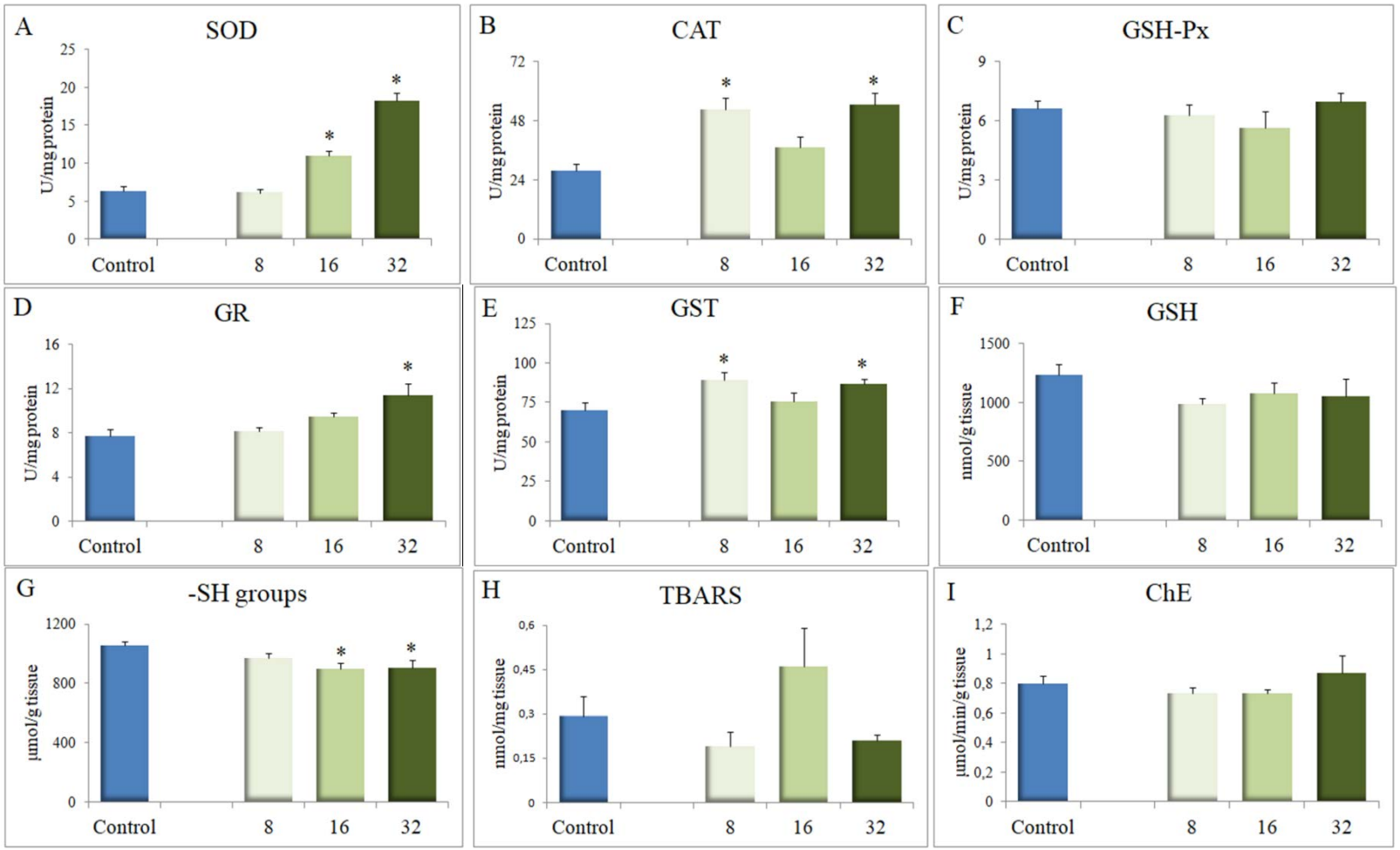


Figure 17. Effects of 96 h deltamethrin (DM) exposure on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G), thiobarbituric reactive substances-TBARS (H) concentrations and cholinesterase-ChE (I) activity in the skin of *Bufotes viridis*. On the x axis applied DM concentrations are presented in mg/kg. To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented. Significant differences ($p<0.05$) among control and treated groups of animals are marked with asterisks (*).

In order to establish the interaction among the examined parameters Pearson's correlation was performed. It showed correlations (positive and negative) between individual AOS parameters (Table 5). CAT, GSH and GR in the liver displayed positive correlations with SOD, while CAT demonstrated negative correlations with -SH groups and ChE. The -SH groups expressed positive correlations with ChE, but with GSH -SH groups expressed negative correlations. In the liver tissue GR and GST showed positive correlations. In the muscle, GSH, GSH-Px, and -SH groups displayed positive correlations with GR just like GST with GSH-Px and GSH with -SH groups. Between TBARS and SOD negative correlation was noticed. On the other hand, in gastrointestinal tissue was obtained a higher number of correlations. Between GSH-Px and GST, CAT, SOD, and GR a positive correlations were obtained, as did GR–CAT, GST–CAT and GR–GST. Negative correlations of the -SH groups with CAT was obtained. In gastrointestinal tissue, TBARS displayed negative correlations with SOD. Only one correlation (between GR and SOD) in the skin was observed.

PCA of examined antioxidant parameters and TBARS in examined tissues was presented in the Figure 18. The analysis discovered a dissociation of the studied tissues (PCA 1:51.69% and PCA 2:18.70%). GST, CAT and ChE were the most important parameters contributing to the liver separation from other tissues along PCA 1. Along PCA 2, TBARS and -SH groups were the major parameters contributing to the differentiation of the skin.

Table 5. Pearson's correlations between the investigated parameters of oxidative stress in the examined tissues of *Bufotes viridis* after 96 h deltamethrin (DM) exposure.

| | SOD | CAT | GST | GR | GSH-Px | GSH | ChE | -SH |
|---|---|---|---|---|---|---|---|---|
| **Liver** | | | | | | | | |
| SOD | | | | | | | | |
| CAT | **0.50** | | | | | | | |
| GST | 0.47 | -0.11 | | | | | | |
| GR | **0.56** | 0.38 | **0.57** | | | | | |
| GSH-Px | 0.41 | 0.36 | 0.21 | 0.08 | | | | |
| GSH | **0.67** | 0.47 | 0.29 | 0.31 | 0.18 | | | |
| ChE | -0.08 | **-0.49** | 0.33 | 0.02 | 0.02 | -0.33 | | |
| -SH | -0.34 | **-0.54** | 0.32 | -0.03 | -0.07 | **-0.51** | **0.57** | |
| TBARS | 0.07 | 0.03 | 0.08 | 0.12 | -0.38 | 0.42 | -0.29 | -0.11 |
| **Muscle** | | | | | | | | |
| SOD | | | | | | | | |
| CAT | 0.17 | | | | | | | |
| GST | 0.21 | -0.39 | | | | | | |
| GR | -0.15 | -0.10 | 0.37 | | | | | |
| GSH-Px | 0.33 | 0.19 | **0.54** | **0.62** | | | | |
| GSH | -0.27 | -0.25 | 0.28 | **0.73** | 0.36 | | | |
| ChE | -0.01 | -0.17 | -0.03 | 0.07 | -0.02 | 0.23 | | |
| -SH | -0.36 | -0.09 | 0.16 | **0.62** | 0.42 | **0.56** | 0.35 | |
| TBARS | **-0.72** | -0.16 | 0.00 | 0.13 | -0.12 | 0.46 | -0.04 | 0.35 |
| **GIT** | | | | | | | | |
| SOD | | | | | | | | |
| CAT | 0.21 | | | | | | | |
| GST | 0.46 | **0.54** | | | | | | |
| GR | 0.36 | **0.71** | **0.70** | | | | | |
| GSH-Px | **0.53** | **0.77** | **0.68** | **0.71** | | | | |
| GSH | 0.03 | -0.19 | 0.30 | -0.14 | -0.11 | | | |
| ChE | 0.24 | -0.27 | 0.10 | 0.00 | 0.01 | -0.08 | | |
| -SH | -0.19 | **-0.62** | -0.22 | -0.40 | -0.35 | 0.32 | 0.14 | |
| TBARS | **-0.56** | 0.08 | -0.09 | 0.09 | -0.08 | -0.32 | -0.14 | 0.06 |
| **Skin** | | | | | | | | |
| SOD | | | | | | | | |
| CAT | 0.23 | | | | | | | |
| GST | -0.10 | 0.36 | | | | | | |
| GR | **0.52** | -0.03 | 0.07 | | | | | |
| GSH-Px | 0.21 | 0.22 | 0.34 | 0.06 | | | | |
| GSH | 0.16 | -0.24 | -0.02 | -0.07 | -0.22 | | | |
| ChE | 0.34 | 0.13 | -0.27 | 0.25 | -0.07 | 0.15 | | |
| -SH | -0.31 | 0.10 | -0.08 | 0.00 | 0.05 | -0.09 | 0.38 | |
| TBARS | -0.05 | -0.39 | 0.02 | 0.01 | 0.19 | -0.03 | -0.13 | 0.09 |

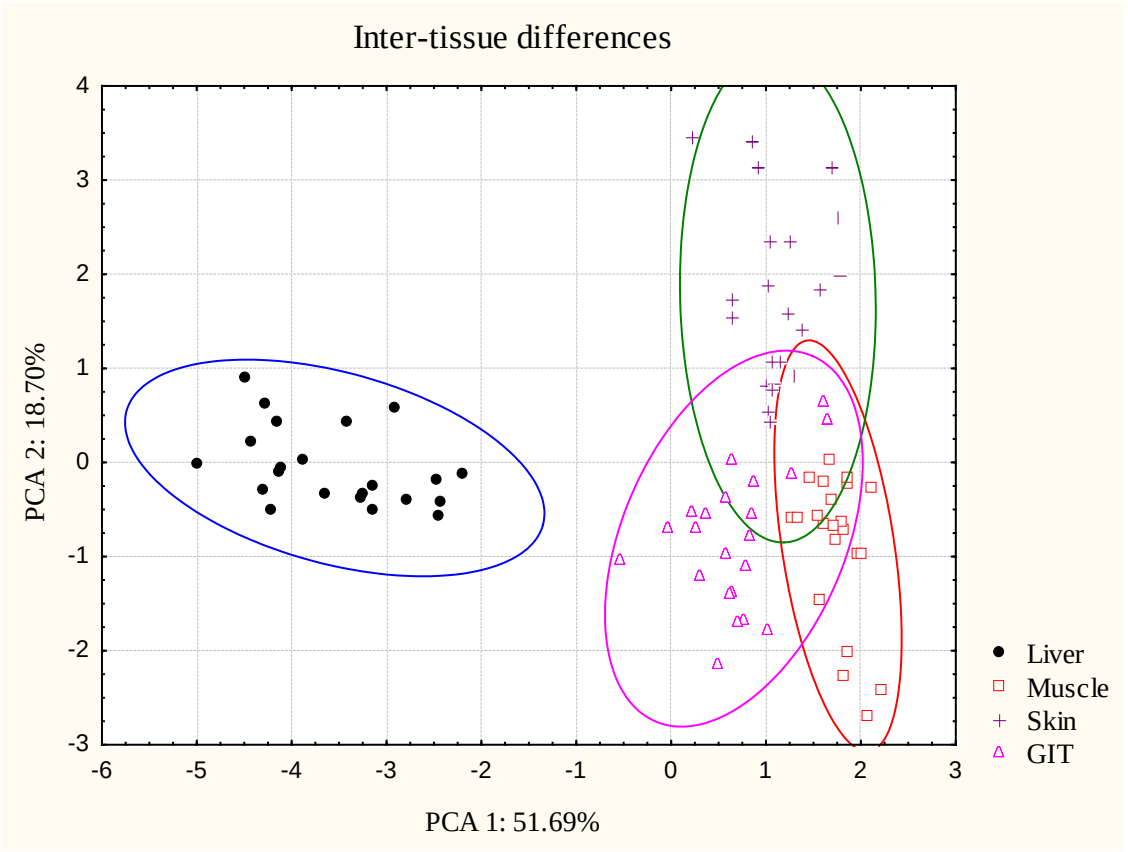


Figure 18. Principal component analysis (PCA) of studied oxidative stress parameters in liver, muscle, skin, and gastrointestinal tissue (GIT) on the factor plan after 96 h deltamethrin (DM) exposure.

### 4.3. Results of chronic exposure to deltamethrin

#### 4.3.1. Biometric, oxidative stress, biotransformation and neurotoxicity parameters in the green toad *Bufotes viridis* after orally applied deltamethrin for 7, 14 and 21 days

During the experiment, no lethality or visual modifications in toad activities were obtained in any of the examined groups. The BM and SVL were in the following order: 7-day control 37.46 ± 2.18 g, 77.86 ± 2.09 mm; 7-day exposure 36.38 ± 4.14 g, 81.43 ± 3.56 mm; 14-day control 28.59 ± 3.39 g, 77.00 ± 2.33 mm; 14-day exposure 31.92 ± 2.26 g, 78.14 ± 1.96 mm; 21-day control 36.41 ± 4.01 g, 80.29 ± 1.91 mm; 21-day exposure 39.17 ± 3.93 g, 81.29 ± 2.05 mm. The HSI (Table 6) did not disclose any statistical variations between the control and treated groups of animals.

Table 6. Hepatosomatic index (HSI) in control and exposure groups.

| HSI | Control groups | Exposure groups |
|---|---|---|
| 7 days | 3.11 ± 0.28 | 3.27 ± 0.32 |
| 14 days | 3.67 ± 0.29 | 3.39 ± 0.20 |
| 21 days | 3.69 ± 0.24 | 3.92 ± 0.13 |

All results of the orally applied deltamethrin after chronic treatment are presented by tissues.

In Table 7 and Figure 19 we presented the results of the study of all analyzed parameters in the liver. Protein concentration was increased on day 21 in the exposed toads as compared to the control group. SOD activity was significantly decreased at days 14 and 21 in respect to the control and day 7. CAT activity and TBARS concentration showed an increasing tendency at day 14 in respect to day 7. This trend was in contrast to GR activity that displayed a decrease on day 14 as compared to day 7 of the tested group. GSH-Px activity showed a decreasing trend on day 21 in respect to the group of animals exposed for 7 and 14 days. The activity of ChE was induced on day 21 in the exposed toads as compared to the control group. A descending trend in the -SH groups concentration was observed in animals exposed for 14 and 21 days when compared to the group exposed for 7 days. GST activity and GSH concentration did not show significant change among investigated groups.

In Table 8 and Figure 20 are presented the results of all investigated parameters in the muscle. Protein concentration was decreased on day 14 in the exposed toads as compared to the groups exposed to 7 and 21 days. Significant inductions in SOD activity in respect to the control were observed on the 21$^{st}$ day of the treatment. The same trend was noticed in activity of GST. GSH-Px activity and the SH groups concentrations reduced on the 14$^{th}$ day of exposure in the comparsion to the control and the group of animals exposed for 7 days. ChE activity was increased on the 21$^{st}$ days of exposure groups in comparison to the control and other treated groups.

Table 9 and Figure 21 show the results of the examination of the parameters in the GIT. Protein concentration was increased on day 21 in the exposed toads as compared to the groups exposed to 7 days. SOD activity exhibited a reduction on the 7$^{th}$ and 14$^{th}$ days of exposure in comparison to the control. The activities of CAT, GSH-Px, GST, ChE and the -SH groups concentration were increased on day 7 in respect to the control group. The concentration of TBARS was increased on the 7$^{th}$ day in comparison to the control group. Activity of GR and GSH concentration did not show significant change among the control and exposed groups.

In Table 10 and Figure 22 are presented the results of all investigated parameters in skin tissue. Protein concentration was decreased on day 14 in the exposed toads as compared to the groups exposed to 7 days. SOD activity showed a declining tendency on the 21$^{st}$ day when match up to the group exposed for 14 days. On day 14 the activity of CAT was increased and GR activity was

reduced in exposed toads as compared to the control group. The concentration of GSH showed a decreasing trend on day 14 of exposure compared to animals treated for 7 and 21 days. ChE activity was increased on the $14^{th}$ and $21^{st}$ days of exposure groups in comparison to the group exposed for 7 days.

Table 7. Effects of orally applied deltamethrin (DM) on investigated oxidative stress, biotransformation and neurotoxicity parameters in the liver of *Bufotes viridis*. Data are log transformed and presented as the means ± SE (standard error). To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented.

| | control 7 | DM 7 days | control 14 | DM 14 days | control 21 | DM 21days |
|---|---|---|---|---|---|---|
| Total proteins (mg/mL) | 1.44 ± 0.01 | 1.44 ± 0.01 | 1.44 ± 0.01 | 1.46 ± 0.01 | 1.35 ± 0.03 | 1.41 ± 0.02$^{*b}$ |
| SOD (U/mg protein) | 1.08 ± 0.03 | 1.07 ± 0.02 | 1.09 ± 0.01 | 0.84 ± 0.02$^{*a}$ | 0.99 ± 0.03 | 0.88 ± 0.02$^{*a}$ |
| CAT (U/mg protein) | 1.82 ± 0.08 | 1.84 ± 0.06 | 2.14 ± 0.06 | 2.18 ± 0.05$^{ac}$ | 2.08 ± 0.05 | 1.98 ± 0.06 |
| GSH-Px (U/mg protein) | 1.31 ± 0.03 | 1.39 ± 0.03 | 1.49 ± 0.02 | 1.41 ± 0.02 | 1.29 ± 0.06 | 1.25 ± 0.04$^{ab}$ |
| GR (U/mg protein) | 0.76 ± 0.05 | 0.79 ± 0.04 | 0.74 ± 0.03 | 0.67 ± 0.02$^{a}$ | 0.74 ± 0.05 | 0.72 ± 0.04 |
| GST (U/mg protein) | 3.10 ± 0.03 | 3.01 ± 0.06 | 3.09 ± 0.03 | 3.00 ± 0.02 | 3.09 ± 0.04 | 3.07 ± 0.03 |
| GSH (nmol/g tissue) | 3.15 ± 0.02 | 3.14 ± 0.06 | 3.21 ± 0.02 | 3.13 ± 0.03 | 3.14 ± 0.05 | 3.04 ± 0.05 |
| -SH (µm/g tissue) | 3.06 ± 0.00 | 3.06 ± 0.00 | 3.06 ± 0.00 | 3.05 ± 0.00$^{a}$ | 3.06 ± 0.00 | 3.06 ± 0.00$^{a}$ |
| TBARS (nmol/mg tissue) | 0.41 ± 0.03 | 0.35 ± 0.01 | 0.40 ± 0.02 | 0.42 ± 0.01$^{a}$ | 0.36 ± 0.01 | 0.39 ± 0.03 |
| ChE (µmol/min/g tissue) | 1.11 ± 0.05 | 1.17 ± 0.04 | 1.12 ± 0.04 | 1.10 ± 0.02 | 0.81 ± 0.03 | 1.10 ± 0.03$^{*}$ |

* - displays significant differences relative to the control; a - displays significant differences relative to the 7$^{th}$ day of exposure; b - displays significant differences relative to the 14$^{th}$ day of exposure; c - displays significant differences relative to the 21$^{st}$ day of exposure;

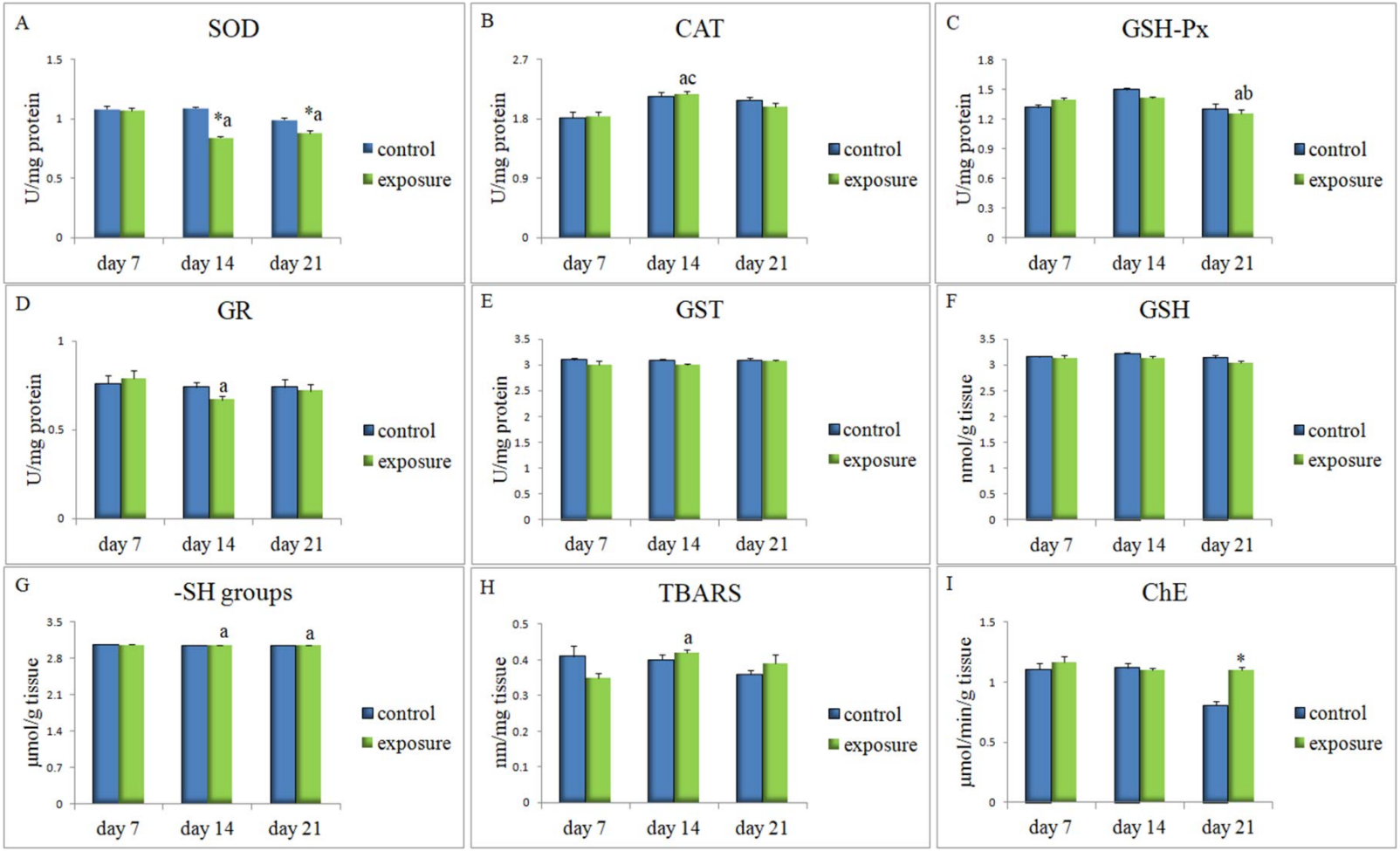


Figure 19. Effects of orally applied deltamethrin (DM) on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G) and thiobarbituric reactive substances-TBARS (H) concentrations and cholinesterase-ChE (I) activity in the liver of *Bufotes viridis*. * - significant differences relative to the control; a - significant differences relative to the 7$^{th}$ day of exposure; b - significant differences relative to the 14$^{th}$ day of exposure; c - significant differences relative to the 21$^{st}$ day of exposure;

Table 8. Effects of orally applied deltamethrin (DM) on investigated oxidative stress, biotransformation and neurotoxicity parameters in the muscle of *Bufotes viridis*. Data are log transformed and presented as the means ± SE (standard error). To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented.

| | control 7 | DM 7 days | control 14 | DM 14 days | control 21 | DM 21days |
|---|---|---|---|---|---|---|
| Total proteins (mg/mL) | 1.03 ± 0.02 | 1.07 ± 0.01 | 1.00 ± 0.02 | 0.98 ± 0.01$^{ac}$ | 1.07 ± 0.02 | 1.04 ± 0.02 |
| SOD (U/mg protein) | 0.65 ± 0.02 | 0.64 ± 0.01 | 0.70 ± 0.02 | 0.69 ± 0.01 | 0.58 ± 0.02 | 0.71± 0.03$^{*a}$ |
| CAT (U/mg protein) | 1.41 ± 0.06 | 1.52 ± 0.04 | 1.39 ± 0.04 | 1.42 ± 0.05 | 1.44 ± 0.05 | 1.54± 0.05 |
| GSH-Px (U/mg protein) | 1.19 ± 0.03 | 1.11 ± 0.04 | 1.25 ± 0.03 | 0.96 ± 0.07$^{*a}$ | 1.02 ± 0.04 | 1.05 ± 0.06 |
| GR (U/mg protein) | 0.45 ± 0.05 | 0.55 ± 0.04 | 0.54 ± 0.03 | 0.46 ± 0.04 | 0.39 ± 0.04 | 0.49 ± 0.03 |
| GST (U/mg protein) | 2.03 ± 0.02 | 1.98 ± 0.03 | 2.07 ± 0.03 | 2.05 ± 0.02 | 1.96 ± 0.04 | 2.06 ± 0.04$^{*}$ |
| GSH (nmol/g tissue) | 2.45 ± 0.02 | 2.43 ± 0.05 | 2.45 ± 0.03 | 2.37 ± 0.03 | 2.46 ± 0.04 | 2.46 ± 0.02 |
| -SH (µm/g tissue) | 3.05 ± 0.00 | 3.05 ± 0.00 | 3.04 ± 0.00 | 3.03 ± 0.01$^{*a}$ | 3.04 ± 0.00 | 3.04 ± 0.00 |
| TBARS (nmol/mg tissue) | 0.66 ± 0.08 | 0.86 ± 0.12 | 0.82 ± 0.05 | 0.81 ± 0.08 | 0.92 ± 0.14 | 1.08 ± 0.09 |
| ChE (µmol/min/g tissue) | 0.32 ± 0.01 | 0.34 ± 0.01 | 0.32 ± 0.01 | 0.31 ± 0.01 | 0.35 ± 0.00 | 0.38 ± 0.01$^{*ab}$ |

* - displays significant differences relative to the control; a - displays significant differences relative to the 7$^{th}$ day of exposure; b - displays significant differences relative to the 14$^{th}$ day of exposure; c - displays significant differences relative to the 21$^{st}$ day of exposure;

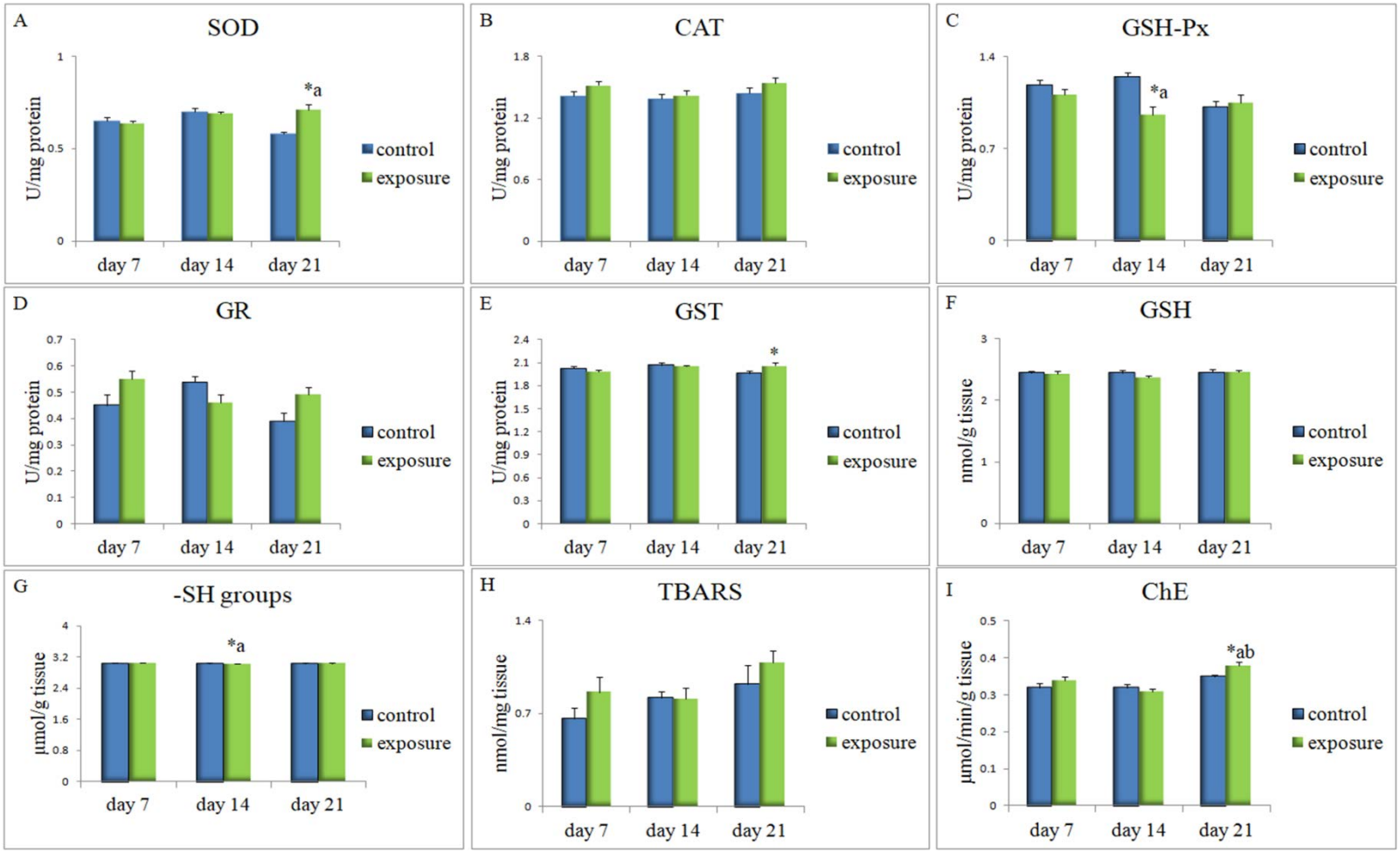


Figure 20. Effects of orally applied deltamethrin (DM) on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G) and thiobarbituric reactive substances-TBARS (H) concentrations and cholinesterase-ChE (I) activity in the muscle of *Bufotes viridis*. * - significant differences relative to the control; a - significant differences relative to the 7$^{th}$ day of exposure; b - significant differences relative to the 14$^{th}$ day of exposure;

Table 9. Effects of orally applied deltamethrin (DM) on investigated oxidative stress, biotransformation and neurotoxicity parameters in the gastrointestinal tissue of *Bufotes viridis*. Data are log transformed and presented as the means ± SE (standard error). To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented.

| | control 7 | DM 7 days | control 14 | DM 14 days | control 21 | DM 21days |
|---|---|---|---|---|---|---|
| Total proteins (mg/mL) | 1.04 ± 0.02 | 1.07 ± 0.01 | 1.12± 0.01 | 1.12 ± 0.02 | 1.18 ± 0.02 | 1.16 ± 0.02[a] |
| SOD (U/mg protein) | 0.74 ± 0.02 | 0.67 ± 0.02[*] | 0.76 ± 0.02 | 0.66± 0.03[*c] | 0.76 ± 0.02 | 0.63 ± 0.02 |
| CAT (U/mg protein) | 1.77 ± 0.07 | 2.19 ± 0.06[*] | 2.01 ± 0.12 | 2.2 ± 0.04 | 2.00 ± 0.05 | 2.08 ± 0.04 |
| GSH-Px (U/mg protein) | 1.22 ± 0.06 | 1.47 ± 0.03[*] | 1.40 ± 0.03 | 1.36 ± 0.01 | 1.30 ± 0.03 | 1.36 ± 0.05 |
| GR (U/mg protein) | 1.05 ± 0.04 | 1.05 ± 0.01 | 1.10 ± 0.04 | 1.05 ± 0.02 | 1.04 ± 0.03 | 1.04 ± 0.02 |
| GST (U/mg protein) | 1.97 ± 0.15 | 2.40 ± 0.04[*] | 2.21 ± 0.08 | 2.31 ± 0.02 | 2.21 ± 0.05 | 2.19± 0.04 |
| GSH (nmol/g tissue) | 1.57± 0.17 | 1.75 ± 0.10 | 1.74 ± 0.07 | 1.58 ± 0.14 | 1.72 ± 0.05 | 1.58 ± 0.06 |
| -SH (µm/g tissue) | 2.97 ± 0.03 | 3.06 ± 0.00[*] | 3.06 ± 0.00 | 3.05 ± 0.00 | 3.04 ± 0.01 | 3.06 ± 0.00 |
| TBARS (nmol/mg tissue) | 0.24 ± 0.03 | 0.35 ± 0.05*[b] | 0.14 ± 0.01 | 0.18 ± 0.02 | 0.22 ± 0.02 | 0.28 ± 0.04[b] |
| ChE (µmol/min/g tissue) | 0.43 ± 0.03 | 0.53 ± 0.02[*] | 0.52 ± 0.01 | 0.50 ± 0.02 | 0.58 ± 0.02 | 0.52 ± 0.01 |

* - displays significant differences relative to the control; a - displays significant differences relative to the 7$^{th}$ day of exposure; b - displays significant differences relative to the 14$^{th}$ day of exposure; c - displays significant differences relative to the 21$^{st}$ day of exposure;

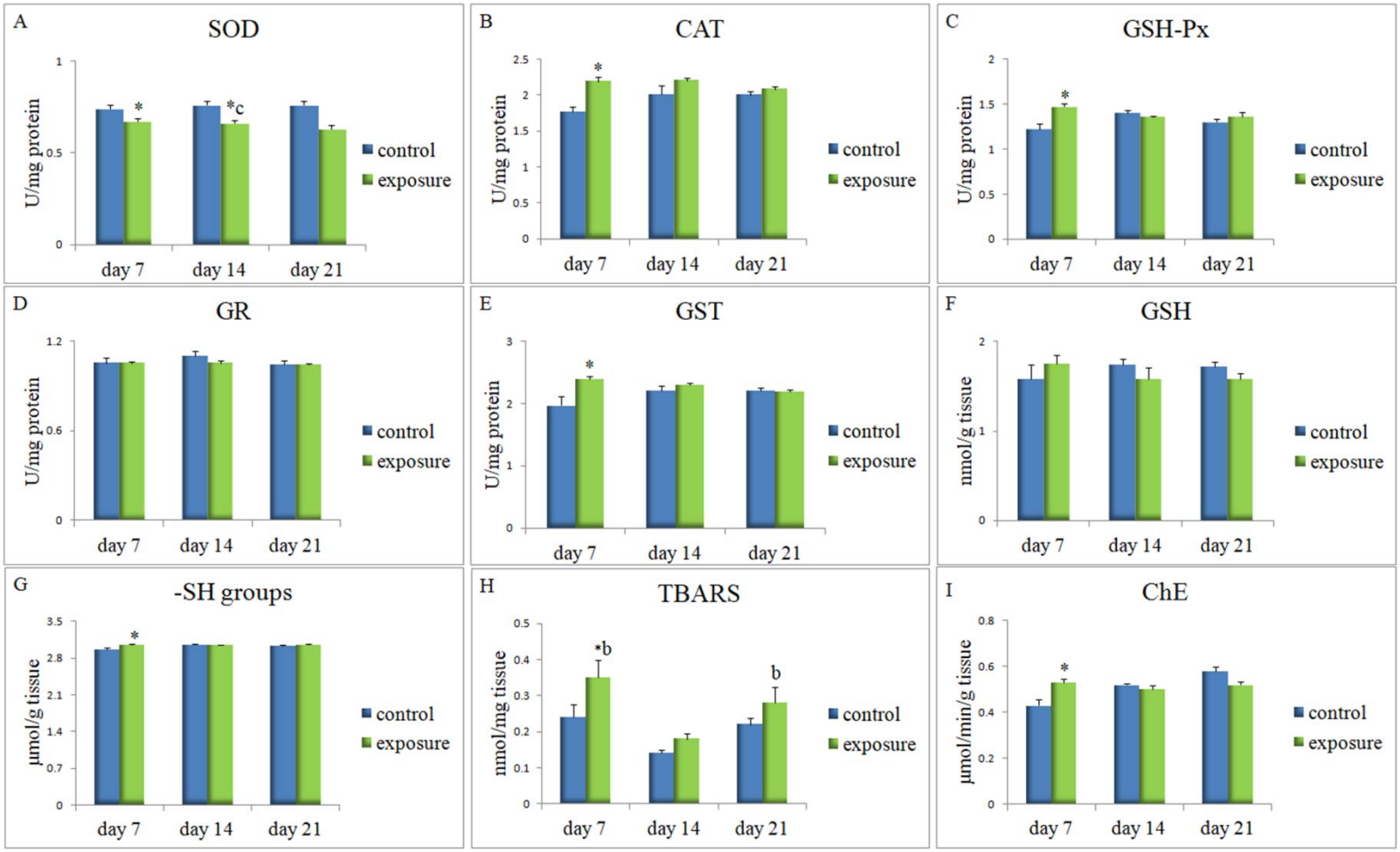


Figure 21. Effects of orally applied deltamethrin (DM) on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G) and thiobarbituric reactive substances-TBARS (H) concentrations and cholinesterase-ChE (I) activity in the gastrointestinal tissue of *Bufotes viridis*. * - significant differences with respect to the control; a - significant differences relative to the 7$^{th}$ day of exposure; b - significant differences relative to the 14$^{th}$ day of exposure; c - significant differences relative to the 21$^{st}$ day of exposure;

Table 10. Effects of orally applied deltamethrin (DM) on investigated oxidative stress, biotransformation and neurotoxicity parameters in the skin of *Bufotes viridis*. Data are log transformed and presented as the means ± SE (standard error). To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented.

| | control 7 | DM 7 days | control 14 | DM 14 days | control 21 | DM 21days |
|---|---|---|---|---|---|---|
| Total proteins (mg/mL) | 1.09 ± 0.01 | 1.08 ± 0.01 | 1.02 ± 0.02 | 1.05 ± 0.02[a] | 1.07 ± 0.01 | 1.04 ± 0.01 |
| SOD (U/mg protein) | 0.69± 0.02 | 0.63 ± 0.03 | 0.64 ± 0.03 | 0.64 ± 0.03[c] | 0.61 ± 0.03 | 0.55 ± 0.03 |
| CAT (U/mg protein) | 1.90 ± 0.03 | 1.91 ± 0.03 | 1.73 ± 0.04 | 1.85 ± 0.03[*] | 1.92 ± 0.03 | 1.93 ± 0.02 |
| GSH-Px (U/mg protein) | 1.00 ± 0.03 | 0.95 ± 0.04 | 1.00 ± 0.03 | 0.95 ± 0.02 | 0.91 ± 0.02 | 0.95 ± 0.02 |
| GR (U/mg protein) | 0.92 ± 0.03 | 0.95 ± 0.01 | 1.04 ± 0.13 | 0.84 ± 0.03[*] | 0.86 ± 0.02 | 0.86 ± 0.03 |
| GST (U/mg protein) | 2.00 ± 0.03 | 1.98 ± 0.03 | 2.03 ± 0.03 | 1.95 ± 0.04 | 2.05 ± 0.03 | 2.05± 0.03 |
| GSH (nmol/g tissue) | 2.73 ± 0.02 | 2.78 ± 0.01 | 2.63 ± 0.07 | 2.67 ± 0.02[ac] | 2.85 ± 0.04 | 2.93 ± 0.03 |
| -SH (µm/g tissue) | 3.03 ± 0.01 | 3.04 ± 0.01 | 3.03 ± 0.01 | 3.02 ± 0.01 | 3.01 ± 0.02 | 3.01 ± 0.02 |
| ChE (µmol/min/g tissue) | 0.25 ± 0.01 | 0.24 ± 0.01 | 0.31 ± 0.01 | 0.31 ± 0.01[a] | 0.27 ± 0.02 | 0.30 ± 0.02[a] |

* - displays significant differences relative to the control; a - displays significant differences relative to the 7$^{th}$ day of exposure; c - displays significant differences relative to the 21$^{st}$ day of exposure;

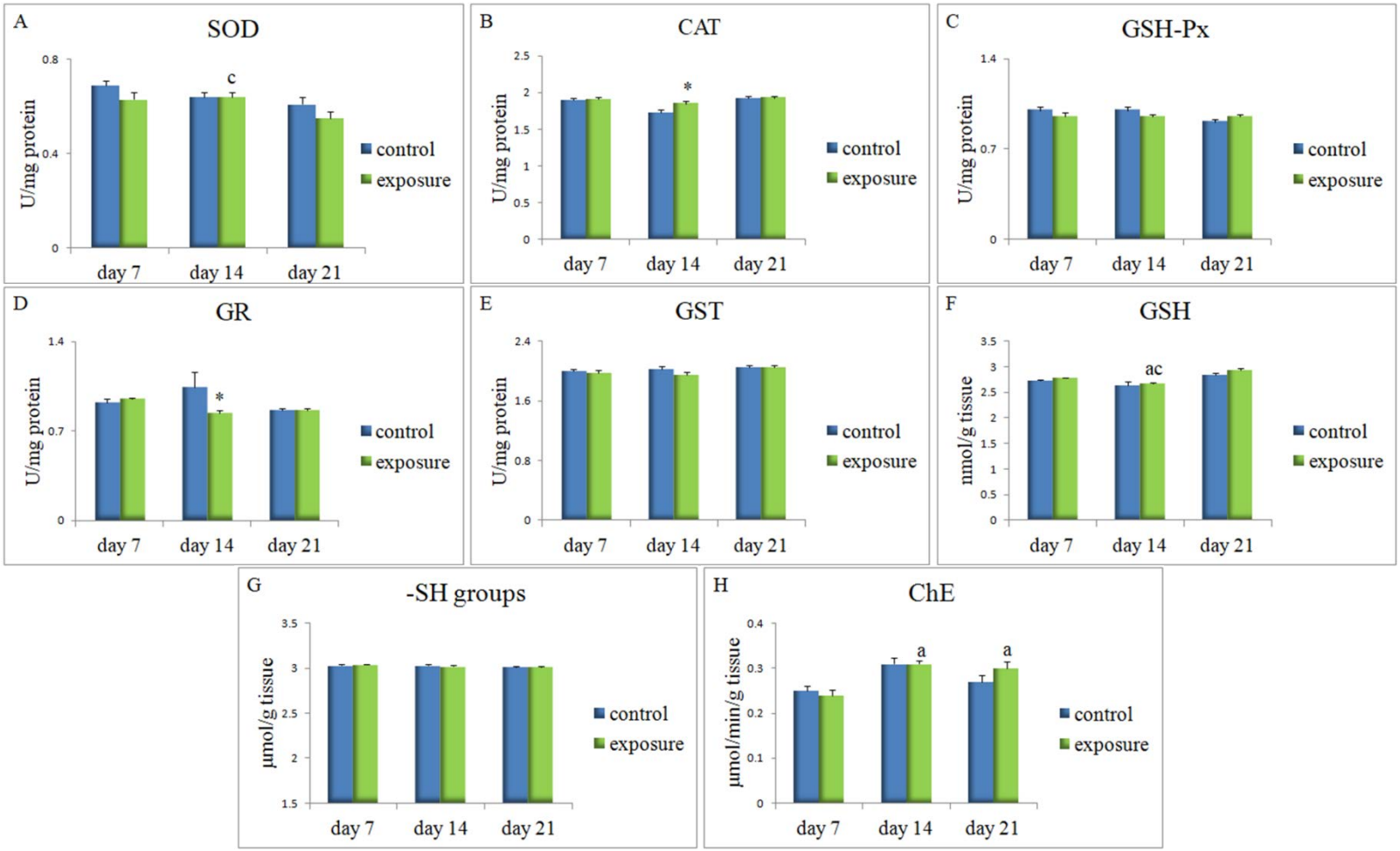


Figure 22. Effects of orally applied deltamethrin (DM) on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G) concentrations and cholinesterase-ChE (H) activity in the skin of *Bufotes viridis*. * - significant differences relative to the control; a - significant differences relative to the 7$^{th}$ day of exposure; c - significant differences relative to the 21$^{st}$ day of exposure;

Based on all determined antioxidant parameters for all studied tissues, Canonical discriminant analysis discovered distinctions among the treated groups, (Table 11, Figure 23). The first canonical function (Root 1) accounted for 80.4% of the total heterogeneity in the liver of the animals. Along the first canonical function 14 and 21 days exposed group were separated from control and 7 days exposed group. They were mainly differentiated regarding to SOD activity and TBARS concentration. Root 2, the second canonical function in the analysis, accounted for 13.0% of the total heterogeneity in toad liver, and GST and GSH-Px activities were the most important parameters that make a contribution to separation of the group of animals exposed for 7 days down the Root 2. The first canonical function accounted for 49.0%, and the second canonical function for 33.0% of the total heterogeneity in the muscle tissue. SOD and GSH-Px activities were the major components in Root 1 that make a contribution to the differentiation among the treated animals. In the Root 2-that were GR and GST activities. The 21 days exposed group was differentiated down the Root 1 from the 7 day exposed group of animals. The Root 1, in the GIT, accounted for 75.90% and the second canonical function for 19.40% of the total heterogeneity. SOD and GSH-Px activities were the main components in Root 1 make a contribution to the distinction of the 7- and 14-day groups of animals from control, and the concentrations of GSH and TBARS in Root 2. In the GIT, along the second canonical function, the 14-day group was separated. In the toad skin Root 1 accounted for 76.2% of the total heterogeneity. Along the first canonical function the 21-day group was separated from 14-day group. It was mainly differentiated by GSH concentration and SOD activity. The second canonical function in the analysis accounted for 13.4% of the total heterogeneity. GR activity and -SH groups concentration the most contributed to the separation.

Table 11. Standardized coefficients used in canonical discriminant analysis of canonical variables in investigated tissues after orally applied deltamethrin (DM).

| | Liver | | Muscle | | GIT | | Skin | |
|---|---|---|---|---|---|---|---|---|
| | Root 1 | Root 2 | Root 1 | Root 2 | Root 1 | Root 2 | Root 1 | Root 2 |
| SOD | **0.938** | 0.207 | **0.832** | -0.234 | **-1.024** | 0.299 | **0.769** | 0.159 |
| CAT | -0.103 | -0.133 | -0.250 | -0.588 | 0.345 | -0.455 | -0.051 | 0.316 |
| GSH-Px | 0.091 | **-0.603** | **-1.079** | -0.029 | **0.606** | 0.325 | 0.180 | -0.315 |
| GR | 0.423 | 0.381 | -0.338 | **-0.904** | 0.079 | 0.101 | -0.466 | **0.807** |
| GST | 0.261 | **0.948** | 0.408 | **0.772** | 0.561 | -0.007 | -0.443 | -0.554 |
| GSH | 0.391 | -0.539 | -0.021 | 0.423 | -0.460 | **0.773** | **-0.784** | 0.260 |
| -SH | 0.316 | -0.583 | 0.396 | -0.217 | -0.133 | -0.174 | -0.051 | **0.712** |
| TBARS | **0.570** | -0.028 | 0.317 | -0.415 | 0.341 | **0.919** | - | - |
| Eigenvalue | 4.513 | 0.729 | 0.794 | 0.536 | 2.636 | 0.674 | 1.018 | 0.179 |
| Cum. Prop | 0.804 | 0.934 | 0.490 | 0.820 | 0.759 | 0.953 | 0.762 | 0.896 |

Statistically significant differences ($p < 0.05$) are in bold.

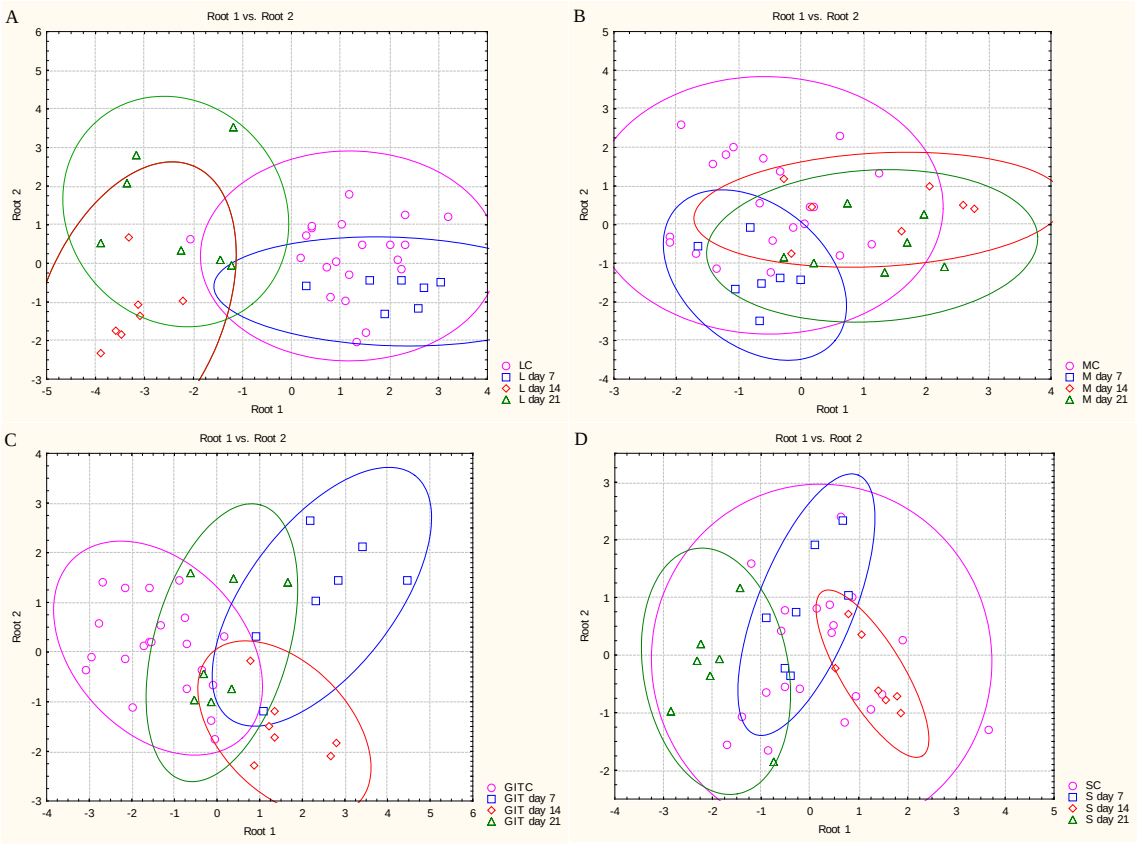


Figure 23. Canonical discriminant analysis of examined oxidative stress parameters in: A – liver (L); B – muscle (M); C – gastrointestinal tissue (GIT) and D – skin (S) on the factor plan after orally applied deltamethrin (DM).

#### 4.3.1.1. Integrated biomarker response (IBR)

Values of IBR for exposed group were, respectively, as follows: control- 0.678; day 7- 0.696; day 14- 3.964; day 21- 7.260. The obtained data demonstrated that the group of animals subjected to the pesticide for 21 days had the maximum response of biomarker (IBR value of 7.260). On the other hand, along with the assessment of all of the biomarker sets, the animals in the control had the lowest IBR value of 0.678. The obtained IBR results were specific for every examined animal group and were used to make the star plot (Figure 24). In each axis the lines are established by the biomarker reply to pesticide application and correspond with the relative biomarker reply within that certain exposed group. Star plot was make to ensure an evaluation among the exposed groups of animals for each set of biomarkers.

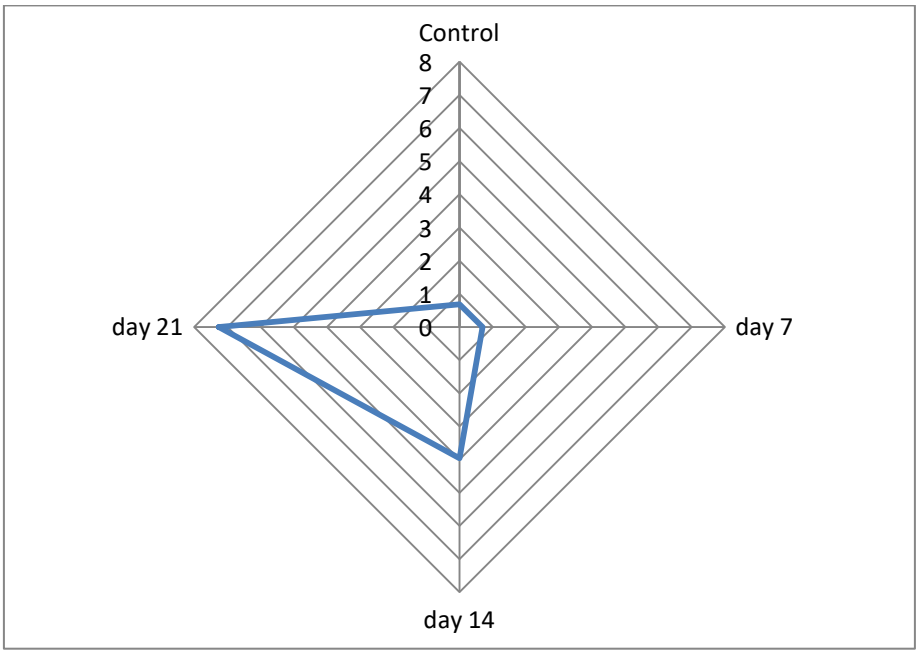


Figure 24. IBR analysis of liver tissue of the dissimilar exposed groups of toads.

### 4.3.2. Biometric, oxidative stress, biotransformation and neurotoxicity parameters in the green toad *Bufotes viridis* after dermally applied deltamethrin for 7, 14 and 21 days

During the experiment, no lethality or visual modifications in animal behavior were obtained in any of the examined groups. The BM and SVL were in the following order: 7-day control 37.46 ± 2.18 g, 77.86 ± 2.09 mm; 7-day exposure 34.56 ± 4.08 g, 79.57 ± 2.77 mm; 14-day control 28.59 ± 3.39 g, 77.00 ± 2.33 mm; 14-day exposure 37.43 ± 5.22 g, 83.86 ± 2.78 mm; 21-day control 36.41 ± 4.01 g, 80.29 ± 1.91 mm; 21-day exposure 38.57 ± 3.76 g, 84.57 ± 2.31 mm. The HSI (Table 12) did not disclose any statistical variations between the control and treated groups of animals.

Table 12. Hepatosomatic index (HSI) in control and exposure groups.

| HSI | Control groups | Exposure groups |
|---|---|---|
| 7 days | 3.11 ± 0.28 | 3.35 ± 0.23 |
| 14 days | 3.67 ± 0.29 | 3.59 ± 0.19 |
| 21 days | 3.69 ± 0.24 | 3.96 ± 0.47 |

All results of the dermally applied deltamethrin after 21 day of chronic treatment to examined parameters are presented by tissues.

In Table 13 and Figure 25 we presented the results of the analysis of all investigated parameters in the liver. SOD activity was significantly decreased on days 14 and 21 as compared to the group exposed to the insecticide for 7 days. Also, after 14 days of exposure a decrease in SOD activity was detected in respect to the control group. CAT activity displayed an increasing trend on day 7 as compared to the control but showed a decreasing trend on day 21 as compared to the group exposed for 14 days. -SH groups concentration was significantly decreased on days 14 and 21 as compared to the group exposed for 7 days. Also, after 14 days of exposure a decrease in SH group concentration was detected in respect to the control group. The activity of ChE was induced on day 21 in the exposed toads as compared to the control group. Protein concentration, GSH-Px, GST and GR activities as well as GSH and TBARS concentrations did not show significant change between the control and exposed groups after different exposure duration.

The results of all investigated parameters in the muscle are presented in Table 14 and Figure 26. Protein concentration was increased in groups exposed to 14 and 21 days in comparison to group exposed 7 days. ChE activity was increased on the 21$^{st}$ day of exposure groups in comparison to the group exposed to the insecticide for 7 days. On the 14$^{th}$ day of exposure ChE activity was increased in comparison to the control. SOD, CAT, GSH-Px, GR and GST activities as well as GSH, TBARS and -SH groups concentrations did not show significant change between the control and exposed groups after different exposure duration.

Table 15 and Figure 27 show results of the examination of the parameters in the GIT. -SH groups concentration was increased on day 7 in exposed toads as compared to the control group. The concentration of TBARS was increased on the 14$^{th}$ day in respect to control and 21 days treated group. Other tested parameters did not show significant change in the gastrointestinal tissue after 21 days of dermal deltamethrin exposure.

The results of all investigated parameters in skin tissue are presented in Table 16 and Figure 28. SOD activity was significantly decreased on day 7 in exposed toads as compared to the control group. CAT activity displayed an increasing trend on day 21 as compared to the group exposed for 14 days. After 14 days of exposure a decrease in GSH-Px activity was detected in respect to the control group. Protein concentration, GR, GST and ChE activities as well as GSH and -SH group concentrations did not show significant change in the skin tissue after chronic dermal deltamethrin exposure.

Table 13. Effects of dermally applied deltamethrin (DM) on investigated oxidative stress, biotransformation and neurotoxicity parameters in the liver of *Bufotes viridis*. Data are log transformed and presented as the means ± SE (standard error). To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented.

| | control 7 | DM 7 days | control 14 | DM 14 days | control 21 | DM 21days |
|---|---|---|---|---|---|---|
| Total proteins (mg/mL) | 1.46 ± 0.01 | 1.48 ± 0.02 | 1.46 ± 0.01 | 1.47 ± 0.02 | 1.37 ± 0.03 | 1.42 ± 0.01 |
| SOD (U/mg protein) | 1.12 ± 0.03 | 1.10 ± 0.03 | 1.13 ± 0.01 | 0.97 ± 0.03$^{*a}$ | 1.03 ± 0.02 | 0.98 ± 0.02$^{a}$ |
| CAT (U/mg protein) | 1.82 ± 0.08 | 2.13 ± 0.05$^{*}$ | 2.14 ± 0.06 | 2.28 ± 0.03 | 2.08 ± 0.05 | 1.99 ± 0.01$^{b}$ |
| GSH-Px (U/mg protein) | 1.33 ± 0.03 | 1.32 ± 0.07 | 1.50 ± 0.02 | 1.38 ± 0.03 | 1.32 ± 0.06 | 1.28 ± 0.06 |
| GR (U/mg protein) | 0.83 ± 0.04 | 0.81 ± 0.03 | 0.81 ± 0.02 | 0.74 ± 0.02 | 0.81 ± 0.04 | 0.77 ± 0.02 |
| GST (U/mg protein) | 3.10 ± 0.03 | 3.09 ± 0.02 | 3.09 ± 0.03 | 3.02 ± 0.04 | 3.09 ± 0.04 | 3.08 ± 0.02 |
| GSH (nmol/g tissue) | 3.15 ± 0.02 | 3.19 ± 0.03 | 3.22 ± 0.02 | 3.23 ± 0.02 | 3.14 ± 0.05 | 3.13 ± 0.05 |
| -SH (µm/g tissue) | 3.06 ± 0.00 | 3.06 ± 0.00 | 3.06 ± 0.00 | 3.04 ± 0.01$^{*a}$ | 3.06 ± 0.00 | 3.05 ± 0.00$^{a}$ |
| TBARS (nmol/mg tissue) | 0.41 ± 0.03 | 0.36 ± 0.03 | 0.40 ± 0.02 | 0.41 ± 0.01 | 0.36 ± 0.01 | 0.39 ± 0.03 |
| ChE (µmol/min/g tissue) | 1.11 ± 0.05 | 1.22 ± 0.03 | 1.12 ± 0.04 | 1.10 ± 0.02 | 0.81 ± 0.03 | 1.14 ± 0.03$^{*}$ |

* - displays significant differences relative to the control; a - displays significant differences relative to the 7$^{th}$ day of exposure; b - displays significant differences relative to the 14$^{th}$ day of exposure; c - displays significant differences relative to the 21$^{st}$ day of exposure;

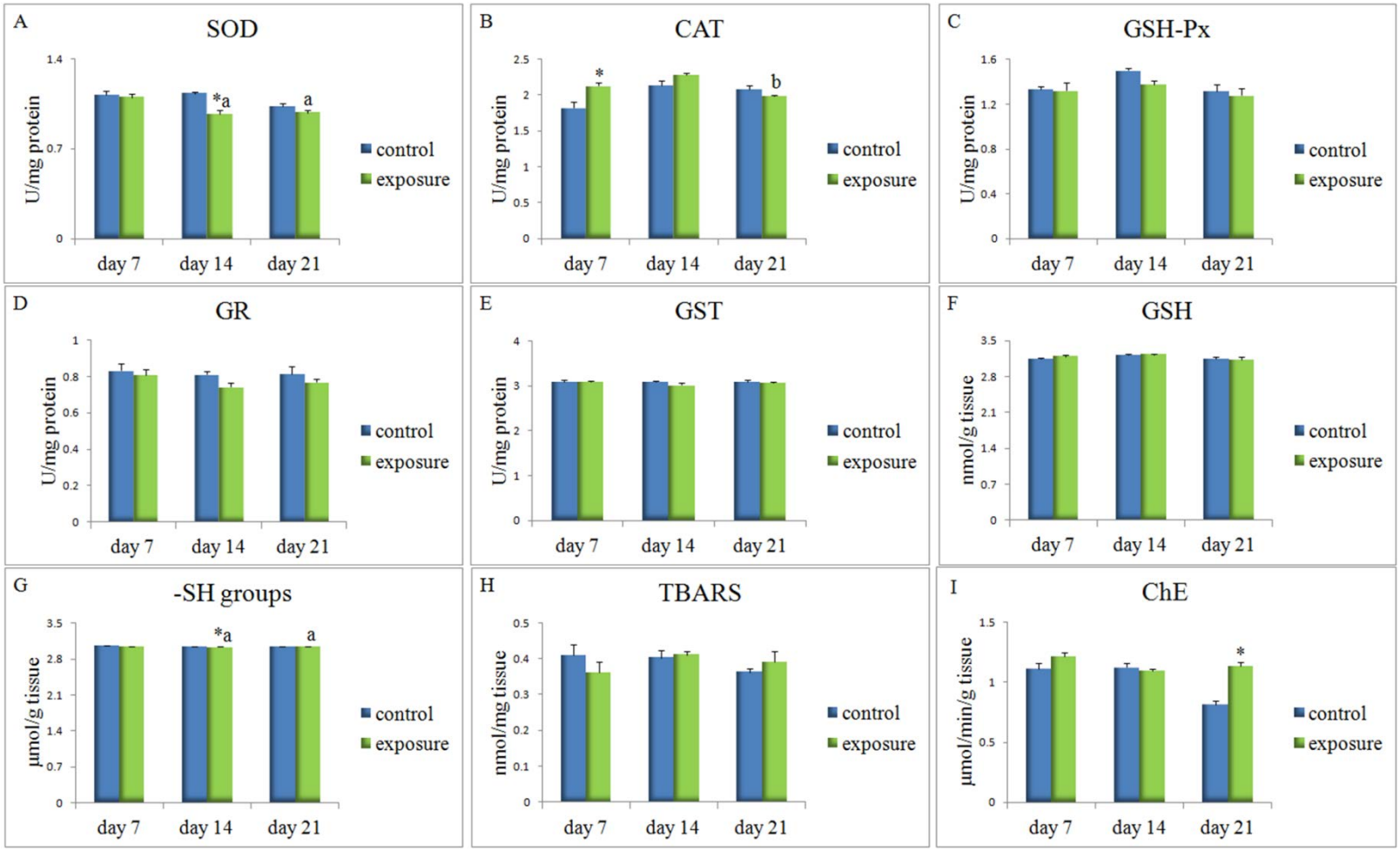


Figure 25. Effects of dermally applied deltamethrin (DM) on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G) and thiobarbituric reactive substances-TBARS (H) concentrations and cholinesterase-ChE (I) activity in the liver of *Bufotes viridis*. * - significant differences relative to the control; a - significant differences relative to the 7$^{th}$ day of exposure; b - significant differences relative to the 14$^{th}$ day of exposure;

Table 14. Effects of dermally applied deltamethrin (DM) on investigated oxidative stress, biotransformation and neurotoxicity parameters in the muscle of *Bufotes viridis*. Data are log transformed and presented as the means ± SE (standard error). To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented.

| | control 7 | DM 7 days | control 14 | DM 14 days | control 21 | DM 21days |
|---|---|---|---|---|---|---|
| Total proteins (mg/mL) | 1.07 ± 0.02 | 1.00 ± 0.01 | 1.04 ± 0.02 | 1.10 ± 0.01[a] | 1.10 ± 0.02 | 1.10 ± 0.02[a] |
| SOD (U/mg protein) | 0.74 ± 0.02 | 0.80 ± 0.01 | 0.78 ± 0.02 | 0.72 ± 0.02 | 0.68 ± 0.01 | 0.76 ± 0.03 |
| CAT (U/mg protein) | 1.43 ± 0.05 | 1.59 ± 0.05 | 1.41 ± 0.04 | 1.58 ± 0.03 | 1.46 ± 0.05 | 1.41 ± 0.03 |
| GSH-Px (U/mg protein) | 1.22± 0.03 | 1.21 ± 0.04 | 1.27 ± 0.03 | 1.13 ± 0.05 | 1.06 ± 0.04 | 1.07 ± 0.06 |
| GR (U/mg protein) | 0.58 ± 0.04 | 0.66 ± 0.03 | 0.65 ± 0.02 | 0.61 ± 0.03 | 0.54 ± 0.03 | 0.59 ± 0.05 |
| GST (U/mg protein) | 2.03 ± 0.02 | 2.02 ± 0.03 | 2.08 ± 0.03 | 2.08 ± 0.02 | 1.97 ± 0.04 | 2.00 ± 0.03 |
| GSH (nmol/g tissue) | 2.45 ± 0.02 | 2.40± 0.03 | 2.45 ± 0.03 | 2.43 ± 0.02 | 2.46 ± 0.04 | 2.47 ± 0.07 |
| -SH (µm/g tissue) | 3.05 ± 0.01 | 3.06 ± 0.01 | 3.04 ± 0.00 | 3.05 ± 0.00 | 3.04 ± 0.01 | 3.05 ± 0.00 |
| TBARS (nmol/mg tissue) | 0.66 ± 0.08 | 0.89± 0.12 | 0.82 ± 0.05 | 1.19 ± 0.05 | 0.92 ± 0.14 | 0.93 ± 0.09 |
| ChE (µmol/min/g tissue) | 0.32 ± 0.01 | 0.34± 0.01 | 0.32 ± 0.01 | 0.36 ± 0.01[*] | 0.35± 0.00 | 0.39 ± 0.01[a] |

* - displays significant differences relative to the control; a - displays significant differences relative to the 7$^{th}$ day of exposure;

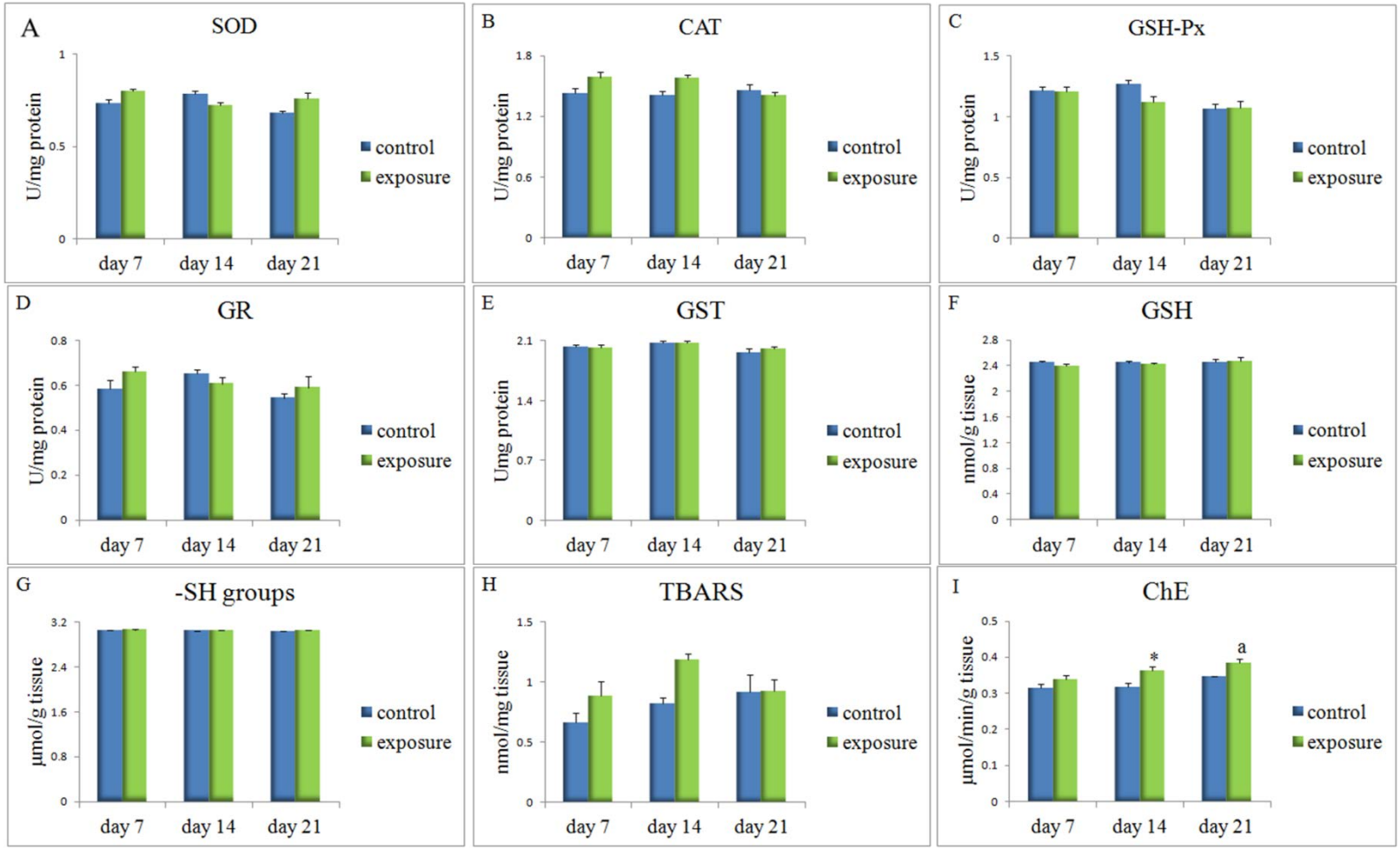


Figure 26. Effects of dermally applied deltamethrin (DM) on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G) and thiobarbituric reactive substances-TBARS (H) concentrations and cholinesterase-ChE (I) activity in the muscle of *Bufotes viridis*. * - significant differences relative to the control; a - significant differences relative to the 7$^{th}$ day of exposure;

Table 15. Effects of dermally applied deltamethrin (DM) on investigated oxidative stress, biotransformation and neurotoxicity parameters in the gastrointestinal tissue of *Bufotes viridis*. Data are log transformed and presented as the means ± SE (standard error). To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented.

| | control 7 | DM 7 days | control 14 | DM 14 days | control 21 | DM 21days |
|---|---|---|---|---|---|---|
| Total proteins (mg/mL) | 1.16 ± 0.02 | 1.21 ± 0.01 | 1.22 ± 0.01 | 1.24 ± 0.01 | 1.25 ± 0.02 | 1.21 ± 0.02 |
| SOD (U/mg protein) | 0.81 ± 0.02 | 0.74 ± 0.02 | 0.83 ± 0.02 | 0.81 ± 0.03 | 0.83 ± 0.02 | 0.83 ± 0.03 |
| CAT (U/mg protein) | 1.78 ± 0.06 | 1.90 ± 0.11 | 2.02 ± 0.12 | 2.10 ± 0.03 | 2.00 ± 0.05 | 1.84 ± 0.08 |
| GSH-Px (U/mg protein) | 1.25 ± 0.06 | 1.37 ± 0.02 | 1.42 ± 0.03 | 1.37 ± 0.01 | 1.32 ± 0.03 | 1.33 ± 0.04 |
| GR (U/mg protein) | 1.09 ± 0.04 | 1.07 ± 0.01 | 1.14 ± 0.03 | 1.09 ± 0.02 | 1.08 ± 0.03 | 1.08 ± 0.01 |
| GST (U/mg protein) | 1.98 ± 0.15 | 2.21 ± 0.07 | 2.21 ± 0.08 | 2.29 ± 0.04 | 2.22 ± 0.05 | 2.17 ± 0.06 |
| GSH (nmol/g tissue) | 1.58 ± 0.16 | 1.76 ± 0.08 | 1.75 ± 0.07 | 1.88 ± 0.11 | 1.72 ± 0.05 | 1.67 ± 0.12 |
| -SH (µm/g tissue) | 2.97 ± 0.03 | 3.06 ± 0.00* | 3.06 ± 0.00 | 3.05 ± 0.00 | 3.04 ± 0.01 | 3.05 ± 0.00 |
| TBARS (nmol/mg tissue) | 0.24 ± 0.03 | 0.28± 0.02 | 0.14 ± 0.01 | 0.37 ± 0.05*c | 0.22 ± 0.02 | 0.22 ± 0.03 |
| ChE (µmol/min/g tissue) | 0.43 ± 0.03 | 0.49± 0.02 | 0.52 ± 0.01 | 0.54 ± 0.02 | 0.58 ± 0.02 | 0.52 ± 0.01 |

* - displays significant differences relative to the control; c - displays significant differences relative to the 21st day of exposure;

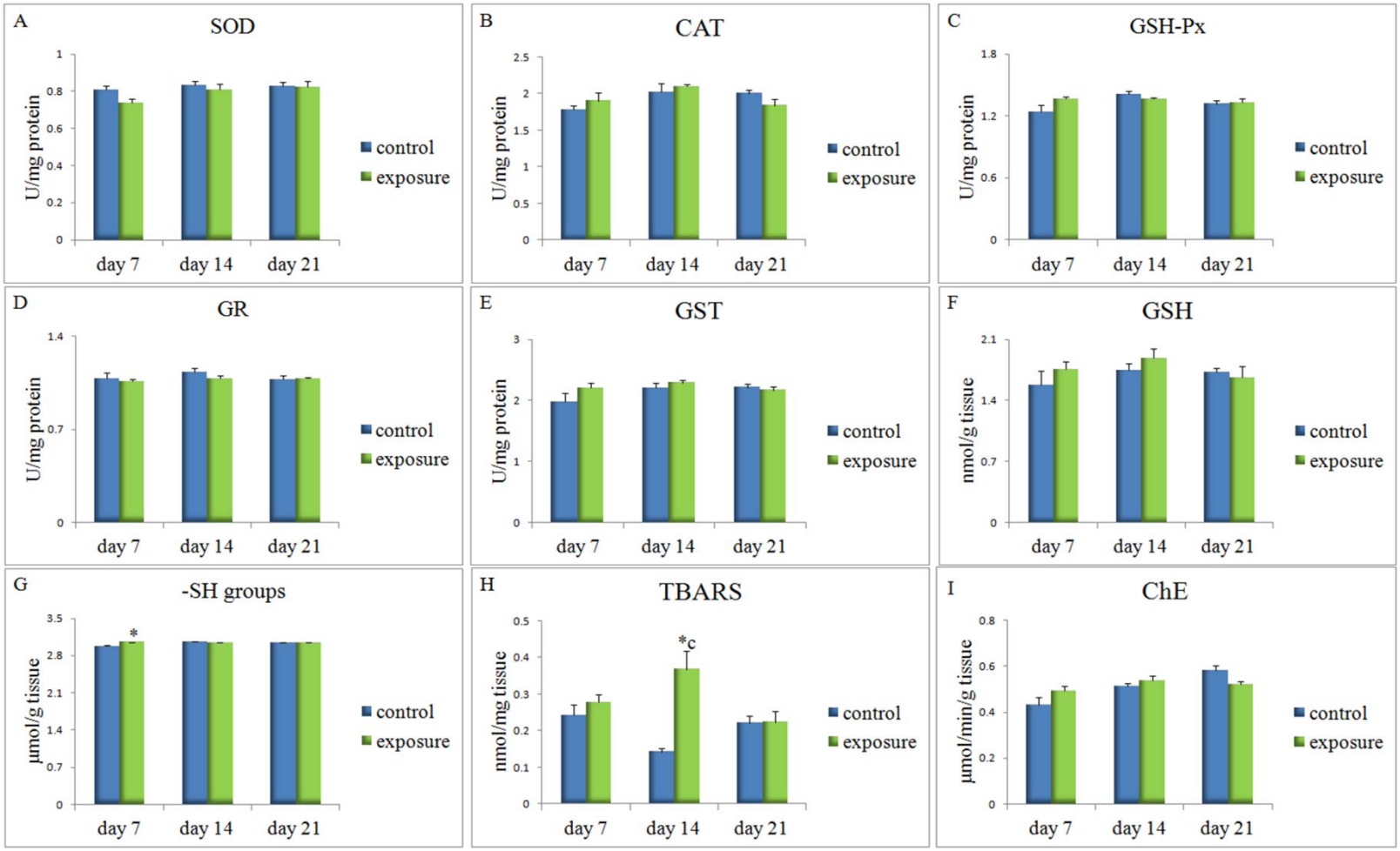


Figure 27. Effects of dermally applied deltamethrin (DM) on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G) and thiobarbituric reactive substances-TBARS (H) concentrations and cholinesterase-ChE (I) activity in the gastrointestinal tissue of *Bufotes viridis*. * - significant differences relative to the control; c - significant differences relative to the 21$^{st}$ day of exposure;

Table 16. Effects of dermally applied deltamethrin (DM) on investigated oxidative stress, biotransformation and neurotoxicity parameters in the skin of *Bufotes viridis*. Data are log transformed and presented as the means ± SE (standard error). To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented.

| | control 7 | DM 7 days | control 14 | DM 14 days | control 21 | DM 21days |
|---|---|---|---|---|---|---|
| Total proteins (mg/mL) | 1.12± 0.01 | 1.14 ± 0.01 | 1.14 ± 0.02 | 1.15 ± 0.02 | 1.15 ± 0.02 | 1.13 ± 0.01 |
| SOD (U/mg protein) | 0.77 ± 0.02 | 0.66 ± 0.01* | 0.73 ± 0.02 | 0.75 ± 0.01 | 0.71 ± 0.03 | 0.74 ± 0.03 |
| CAT (U/mg protein) | 1.91 ± 0.03 | 1.94 ± 0.03 | 1.74 ± 0.04 | 1.97 ± 0.14 | 1.92 ± 0.03 | 2.02 ± 0.03b |
| GSH-Px (U/mg protein) | 1.05 ± 0.03 | 0.96 ± 0.02 | 1.05 ± 0.03 | 0.90 ± 0.01* | 0.96 ± 0.02 | 0.91 ± 0.03 |
| GR (U/mg protein) | 0.97 ± 0.03 | 0.99± 0.02 | 1.08 ± 0.12 | 0.93 ± 0.02 | 0.92 ± 0.02 | 0.97 ± 0.01 |
| GST (U/mg protein) | 2.00 ± 0.03 | 1.97 ± 0.02 | 2.03 ± 0.03 | 1.99 ± 0.02 | 2.06 ± 0.03 | 2.04 ± 0.02 |
| GSH (nmol/g tissue) | 2.73 ± 0.02 | 2.72 ± 0.03 | 2.63 ± 0.07 | 2.67 ± 0.03 | 2.85 ± 0.04 | 2.81 ± 0.05 |
| -SH (µm/g tissue) | 3.03 ± 0.01 | 3.03 ± 0.01 | 3.03 ± 0.01 | 3.02 ± 0.02 | 3.01 ± 0.02 | 3.02 ± 0.01 |
| ChE (µmol/min/g tissue) | 0.25 ± 0.01 | 0.27± 0.01 | 0.31 ± 0.01 | 0.29 ± 0.01 | 0.27 ± 0.02 | 0.29 ± 0.01 |

* - displays significant differences relative to the control; b - displays significant differences relative to the 14$^{th}$ day of exposure;

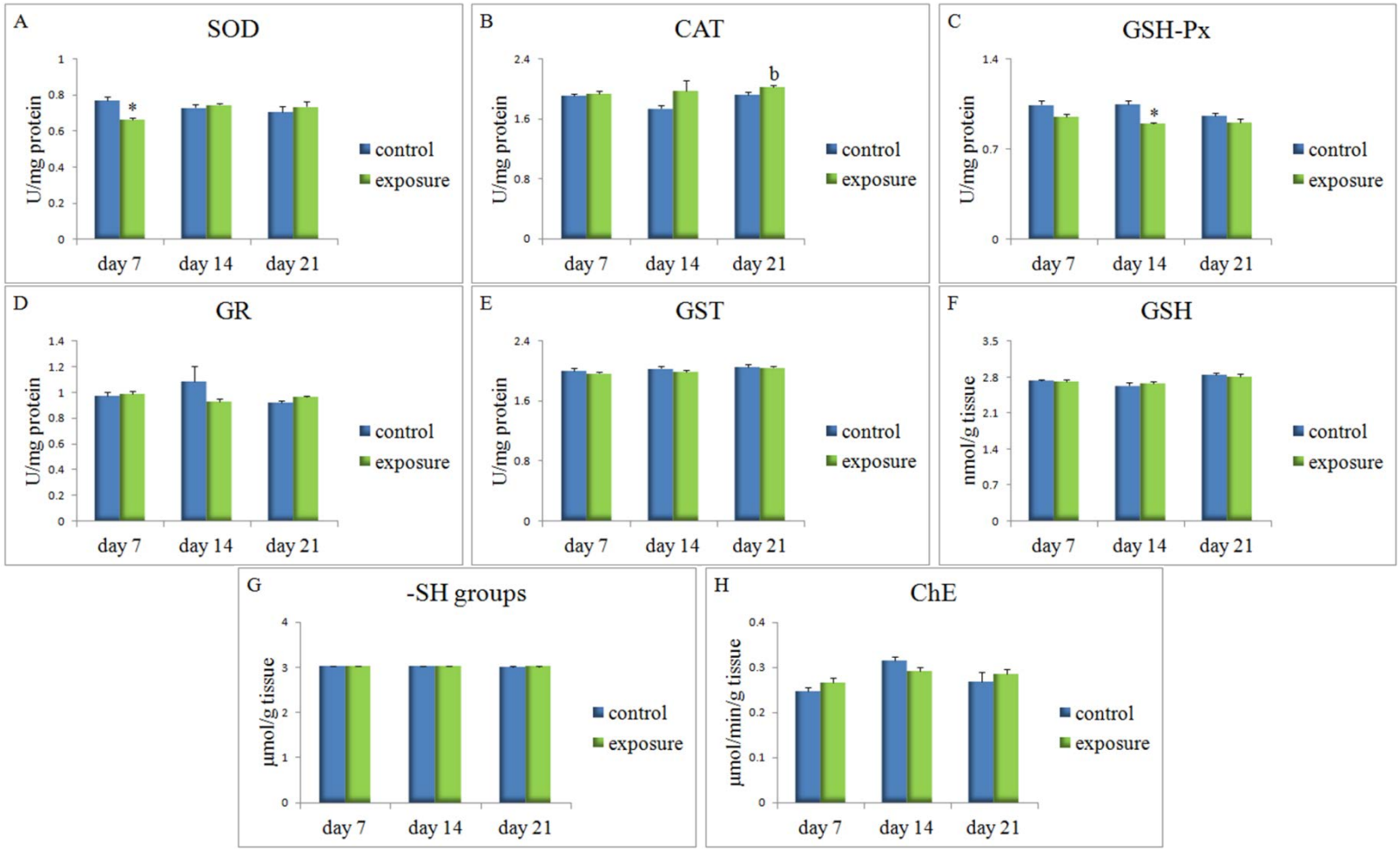


Figure 28. Effects of dermally applied deltamethrin (DM) on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST(E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G) concentrations and cholinesterase-ChE (H) activity in the skin of *Bufotes viridis*. * - significant differences relative to the control; b - significant differences relative to the 14$^{th}$ day of exposure;

Canonical discriminant analysis discovered variations amongst the exposed groups, considering all measured parameters for every investigated tissue (Table 17, Figure 29). Root 1 accounted for 68.5% of the total heterogeneity in animal liver. 7 days exposed group and control group were divided along the Root 1 from 14 and 21 days exposed group and they were mainly differentiated relative to SOD activity and -SH groups concentration. Root 2 accounted for 18.3% of the total heterogeneity in animal liver, and CAT and ChE activities were the most important parameters that provided to the second canonical function differentiation. The first canonical function accounted for 60.5%, and the second canonical function 2 for 24.9% of the total heterogeneity in the muscle tissue. The major factors in the first canonical function, that provided differentiation between the exposed animal groups, were SOD and ChE activities, and in the second canonical function-SOD and CAT activities. The groups subjected for 14 and 21 days were separated along Root 1 from the control, and the group subjected for 14 days from group of toads subjected for 21 days along Root 2. Root 1 accounted for 63.7% and the second canonical function for 27.8% of the total heterogeneity in the GIT. The most important factors in the first canonical function providing separation of the 7- day group of animals from control were SOD activity and TBARS concentration. In the toad skin, the first canonical function accounted for 49.9% of the total heterogeneity. The 21-days animal group was separated along Root 1 from control group, and it was frequently differentiated by activities of CAT and GSH-Px. In toad skin, the second canonical function accounted for 36.7% of the total heterogeneity, and SOD and CAT activities were the major parameters providing separation of the 14-days group from 7 days group along the second canonical function.

Table 17. Standardized coefficients used in canonical discriminant analysis of canonical variables in investigated tissues after dermally applied deltamethrin (DM).

| | Liver | | Muscle | | GIT | | Skin | |
|---|---|---|---|---|---|---|---|---|
| | Root 1 | Root 2 | Root 1 | Root 2 | Root 1 | Root 2 | Root 1 | Root 2 |
| SOD | **0.648** | -0.042 | **1.113** | **-0.682** | **0.504** | **-0.610** | 0.144 | **0.968** |
| CAT | -0.393 | **-0.469** | 0.048 | **0.778** | -0.054 | **-0.616** | **0.643** | **-0.734** |
| GSH-Px | 0.122 | 0.398 | -0.572 | 0.399 | -0.357 | 0.162 | **-0.895** | -0.434 |
| GR | 0.194 | -0.267 | -0.357 | -0.101 | -0.006 | -0.246 | 0.355 | -0.446 |
| GST | 0.488 | 0.102 | 0.197 | 0.603 | -0.132 | 0.581 | -0.064 | 0.307 |
| GSH | -0.333 | 0.078 | -0.209 | -0.300 | -0.095 | -0.607 | 0.123 | 0.324 |
| -SH | **0.559** | -0.253 | 0.200 | -0.021 | -0.112 | **0.641** | 0.043 | 0.396 |
| TBARS | -0.528 | 0.364 | 0.440 | 0.615 | **-0.935** | -0.339 | - | - |
| ChE | -0.264 | **-1.015** | **1.147** | -0.186 | -0.090 | -0.385 | 0.372 | 0.222 |
| Eigenval | 2.589 | 0.692 | 2.462 | 1.012 | 1.067 | 0.465 | 1.014 | 0.745 |
| Cum.Prop | 0.685 | 0.868 | 0.605 | 0.854 | 0.637 | 0.915 | 0.499 | 0.866 |

Statistically significant differences ($p < 0.05$) are in bold.

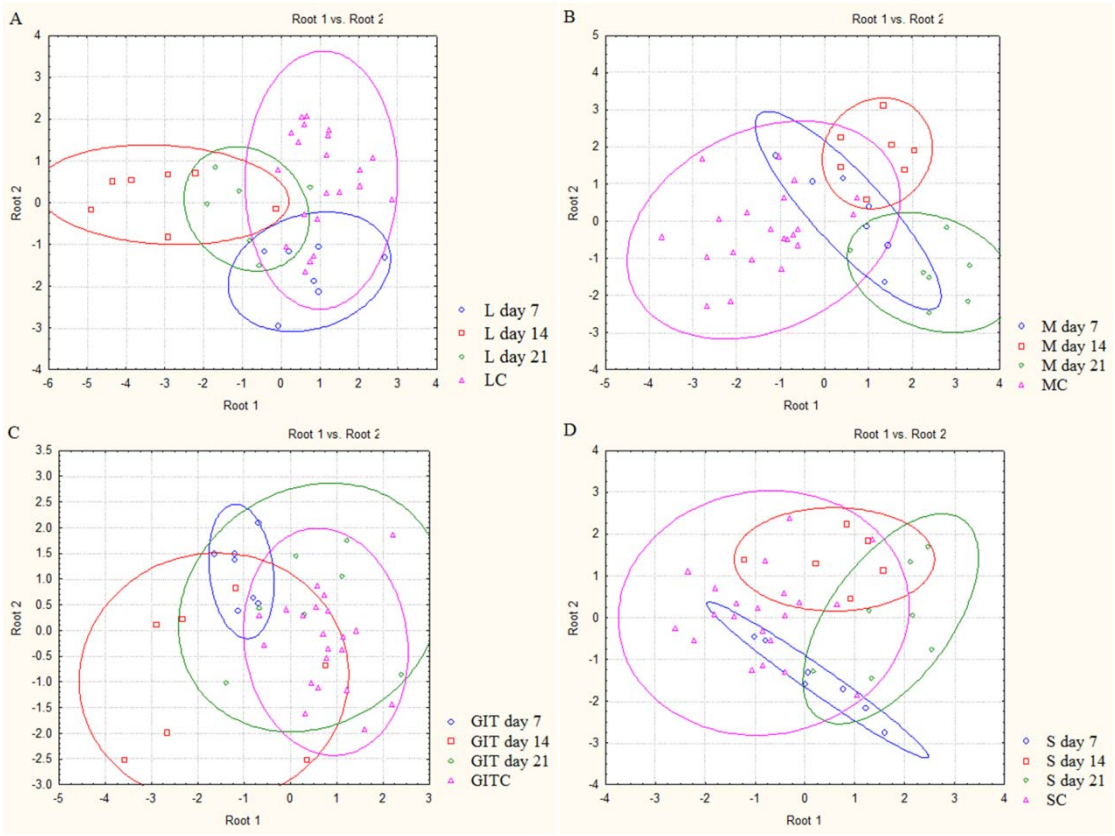


Figure 29. Canonical discriminant analysis of examined oxidative stress parameters in: A – liver (L); B – muscle (M); C – gastrointestinal tissue (GIT) and D – skin (S) on the factor plan after dermally applied deltamethrin (DM).

### 4.3.3. Biometric, oxidative stress, biotransformation and neurotoxicity parameters in the green toad *Bufotes viridis* after combination of orally and dermally (or-der) applied deltamethrin for 7, 14 and 21 days

During the experiment, no lethality or visual modifications in animal behavior were obtained in any of the studied groups. The BM and SVL were in the following order: 7-day control 37.46 ± 2.18 g, 77.86 ± 2.09 mm; 7-day exposure 33.13 ± 3.31 g, 77.71 ± 2.63 mm; 14-day control 28.59 ± 3.39 g, 77.00 ± 2.33 mm; 14-day exposure 37.85 ± 2.85 g, 80.43 ± 1.94 mm; 21-day control 36.41 ± 4.01 g, 80.29 ± 1.91 mm; 21-day exposure 37.80 ± 2.76 g, 78.29 ± 1.08 mm. The HSI (Table 18) did not disclose any statistical variations between the control and treated groups of animals.

Table 18. Hepatosomatic index (HSI) in control and exposure groups.

| HSI | Control groups | Exposure groups |
|---|---|---|
| 7 days | 3.11 ± 0.28 | 3.10 ± 0.34 |
| 14 days | 3.67 ± 0.29 | 3.10 ± 0.22 |
| 21 days | 3.69 ± 0.24 | 3.80 ± 0.24 |

All results after combination of orally and dermally (or-der) applied deltamethrin after 21 day of chronic treatment to examined parameters are presented by tissues.

In Table 19 and Figure 30 we showed the all investigated parameter results in the liver. Protein concentration was increased at day 21 in the exposed toads as compared to the control group. SOD activity was significantly decreased in all treated groups as compared to the control. However, CAT activity displayed an increasing trend on days 7 and 21 as compared to the control. Activity of GSH-Px and GSH concentration were decreased at day 21 in the exposed toads as compared to the group exposed for 14 days. SH group concentration was significantly decreased on days 14 and 21 as compared to the control and group exposed for 7 days. The activity of ChE was increased on day 21 in the exposed toads as compared to the control group. GR, GST activities and TBARS concentration did not show significant change among the control and exposed groups.

The results of all investigated parameters in the muscle are presented in Table 20 and Figure 31. SOD and GST activities were significantly increased on the 21$^{st}$ day of exposure groups in comparison to the control. CAT activity was significantly increased on the 7$^{th}$ day of exposure groups in comparison to the control. GSH-Px activity was decreased on day 14 as compared to the control. GSH concentration was increased on day 21 in the exposed toads as compared to the group exposed for 14 days, and TBARS concentration was higher on day 14 in the exposed toads as compared to the group exposed for 7 days. ChE activity was increased on the 21$^{st}$ days of exposure groups with respect to the control and animals treated for 7 and 14 days. Protein concentration, GR activity and -SH groups concentration did not show significant change among the control and exposed groups.

Table 21 and Figure 32 show the results of the investigated parameters in the GIT. Protein concentration was increased at day 7 in the exposed toads as compared to the control group. SOD activity exhibited a decrease on the 7$^{th}$ and 21$^{st}$ days of exposure relative to the control and group exposed for 14 days. Concentration of -SH groups was induced on 7$^{th}$ day in exposed toads as compared to the control group. ChE activity was increased on the 7$^{th}$ day but decreased on the 21$^{st}$ days of exposure groups with respect to the control. CAT, GSH-Px, GR, GST activities as well as GSH and TBARS concentrations did not show significant change among the control and exposed groups.

The results of all investigated parameters in skin tissue are presented in Table 22 and Figure 33. CAT activity and GSH concentration were significantly increased on the 21$^{st}$ day of exposure groups in comparison to the groups exposed for 7 and 14 days. GSH-Px activity was decreased on day 14 as compared to the control. In other determined parameters was not detected significant change among control and exposed groups.

Table 19. Effects of or-der applied deltamethrin (DM) on investigated oxidative stress, biotransformation and neurotoxicity parameters in the liver of *Bufotes viridis*. Data are log transformed and presented as the means ± SE (standard error). To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented.

| | control 7 | DM 7 days | control 14 | DM 14 days | control 21 | DM 21days |
|---|---|---|---|---|---|---|
| Total proteins (mg/mL) | 1.46 ± 0.01 | 1.51 ± 0.03 | 1.46 ± 0.01 | 1.44 ± 0.02 | 1.37 ± 0.03 | 1.47 ± 0.02* |
| SOD (U/mg protein) | 1.12 ± 0.03 | 0.87 ± 0.03* | 1.13 ± 0.01 | 0.88 ± 0.02* | 1.03 ± 0.02 | 0.88 ± 0.02* |
| CAT (U/mg protein) | 1.82 ± 0.08 | 2.33 ± 0.04* | 2.14 ± 0.06 | 2.27 ± 0.06 | 2.08 ± 0.05 | 2.38 ± 0.05* |
| GSH-Px (U/mg protein) | 1.33 ± 0.03 | 1.34 ± 0.05 | 1.50 ± 0.02 | 1.39 ± 0.04 | 1.32 ± 0.06 | 1.19 ± 0.06b |
| GR (U/mg protein) | 0.83 ± 0.04 | 0.75 ± 0.05 | 0.81 ± 0.02 | 0.73 ± 0.03 | 0.81 ± 0.04 | 0.74 ± 0.04 |
| GST (U/mg protein) | 3.10 ± 0.03 | 2.98 ± 0.06 | 3.09 ± 0.03 | 2.95 ± 0.04 | 3.09 ± 0.04 | 3.11 ± 0.03 |
| GSH (nmol/g tissue) | 3.15 ± 0.02 | 3.16 ± 0.03 | 3.22 ± 0.02 | 3.19 ± 0.01 | 3.14 ± 0.05 | 3.06 ± 0.03b |
| -SH (µm/g tissue) | 3.06 ± 0.00 | 3.06 ± 0.00 | 3.06 ± 0.00 | 3.04 ± 0.00*a | 3.06 ± 0.00 | 3.04 ± 0.00*a |
| TBARS (nmol/mg tissue) | 0.41 ± 0.03 | 0.36 ± 0.02 | 0.40 ± 0.02 | 0.33 ± 0.01 | 0.36 ± 0.01 | 0.39 ± 0.01 |
| ChE (µmol/min/g tissue) | 1.11 ± 0.05 | 1.04 ± 0.03 | 1.12 ± 0.04 | 1.07 ± 0.02 | 0.81 ± 0.03 | 1.18 ± 0.04* |

* - displays significant differences relative to the control; a - displays significant differences relative to the 7$^{th}$ day of exposure; b - displays significant differences relative to the 14$^{th}$ day of exposure;

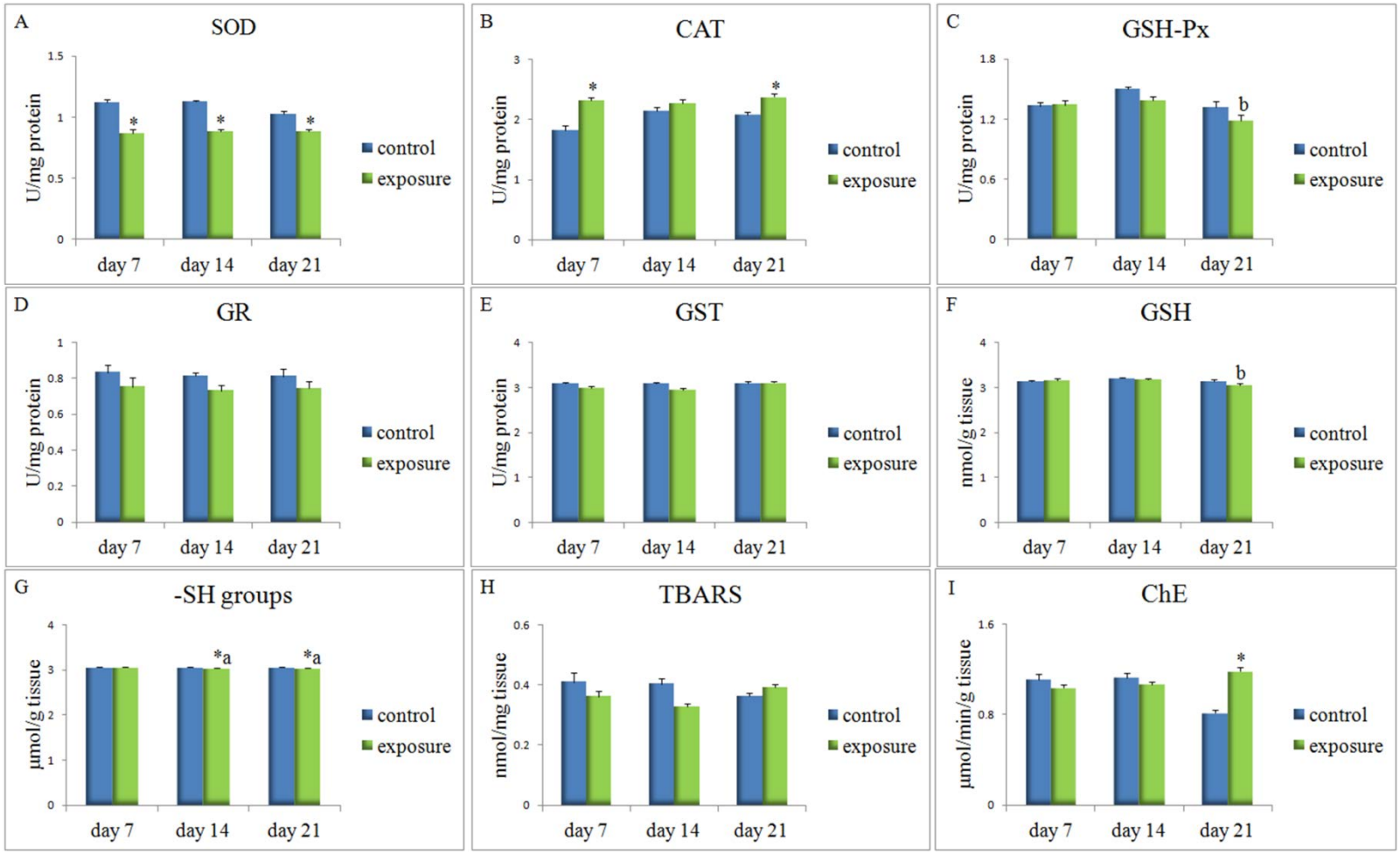


Figure 30. Effects of or-der applied deltamethrin (DM) on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G) and thiobarbituric reactive substances-TBARS (H) concentrations and cholinesterase-ChE (I) activity in the liver of *Bufotes viridis*. * - significant differences relative to the control; a - significant differences relative to the 7$^{th}$ day of exposure; b - significant differences relative to the 14$^{th}$ day of exposure;

Table 20. Effects of or-der applied deltamethrin (DM) on investigated oxidative stress, biotransformation and neurotoxicity parameters in the muscle of *Bufotes viridis*. Data are log transformed and presented as the means ± SE (standard error). To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented.

| | control 7 | DM 7 days | control 14 | DM 14 days | control 21 | DM 21days |
|---|---|---|---|---|---|---|
| Total proteins (mg/mL) | 1.07 ± 0.02 | 1.02 ± 0.02 | 1.04 ± 0.02 | 1.10 ± 0.02 | 1.10 ± 0.02 | 1.04 ± 0.02 |
| SOD (U/mg protein) | 0.74 ± 0.02 | 0.79 ± 0.02 | 0.78 ± 0.02 | 0.72 ± 0.02 | 0.68 ± 0.01 | 0.79 ± 0.02* |
| CAT (U/mg protein) | 1.43 ± 0.05 | 1.68 ± 0.05* | 1.41 ± 0.04 | 1.54 ± 0.04 | 1.46 ± 0.05 | 1.62 ± 0.06 |
| GSH-Px (U/mg protein) | 1.22± 0.03 | 1.21 ± 0.06 | 1.27 ± 0.03 | 1.04 ± 0.06* | 1.06 ± 0.04 | 1.13 ± 0.05 |
| GR (U/mg protein) | 0.58 ± 0.04 | 0.60 ± 0.02 | 0.65 ± 0.02 | 0.62 ± 0.03 | 0.54 ± 0.03 | 0.62 ± 0.03 |
| GST (U/mg protein) | 2.03 ± 0.02 | 2.04 ± 0.02 | 2.08 ± 0.03 | 2.02 ± 0.03 | 1.97 ± 0.04 | 2.15 ± 0.04* |
| GSH (nmol/g tissue) | 2.45 ± 0.02 | 2.45 ± 0.02 | 2.45 ± 0.03 | 2.38 ± 0.04 | 2.46 ± 0.04 | 2.55 ± 0.02b |
| -SH (µm/g tissue) | 3.05 ± 0.01 | 3.05 ± 0.00 | 3.04 ± 0.00 | 3.04 ± 0.01 | 3.04 ± 0.01 | 3.04 ± 0.01 |
| TBARS (nmol/mg tissue) | 0.66 ± 0.08 | 0.79 ± 0.06 | 0.82 ± 0.05 | 1.15 ± 0.05a | 0.92 ± 0.14 | 0.86 ± 0.08 |
| ChE (µmol/min/g tissue) | 0.32 ± 0.01 | 0.34 ± 0.01 | 0.32 ± 0.01 | 0.34 ± 0.01 | 0.35± 0.00 | 0.39 ±0.01*ab |

* - displays significant differences relative to the control; a - displays significant differences relative to the 7th day of exposure; b - displays significant differences relative to the 14th day of exposure;

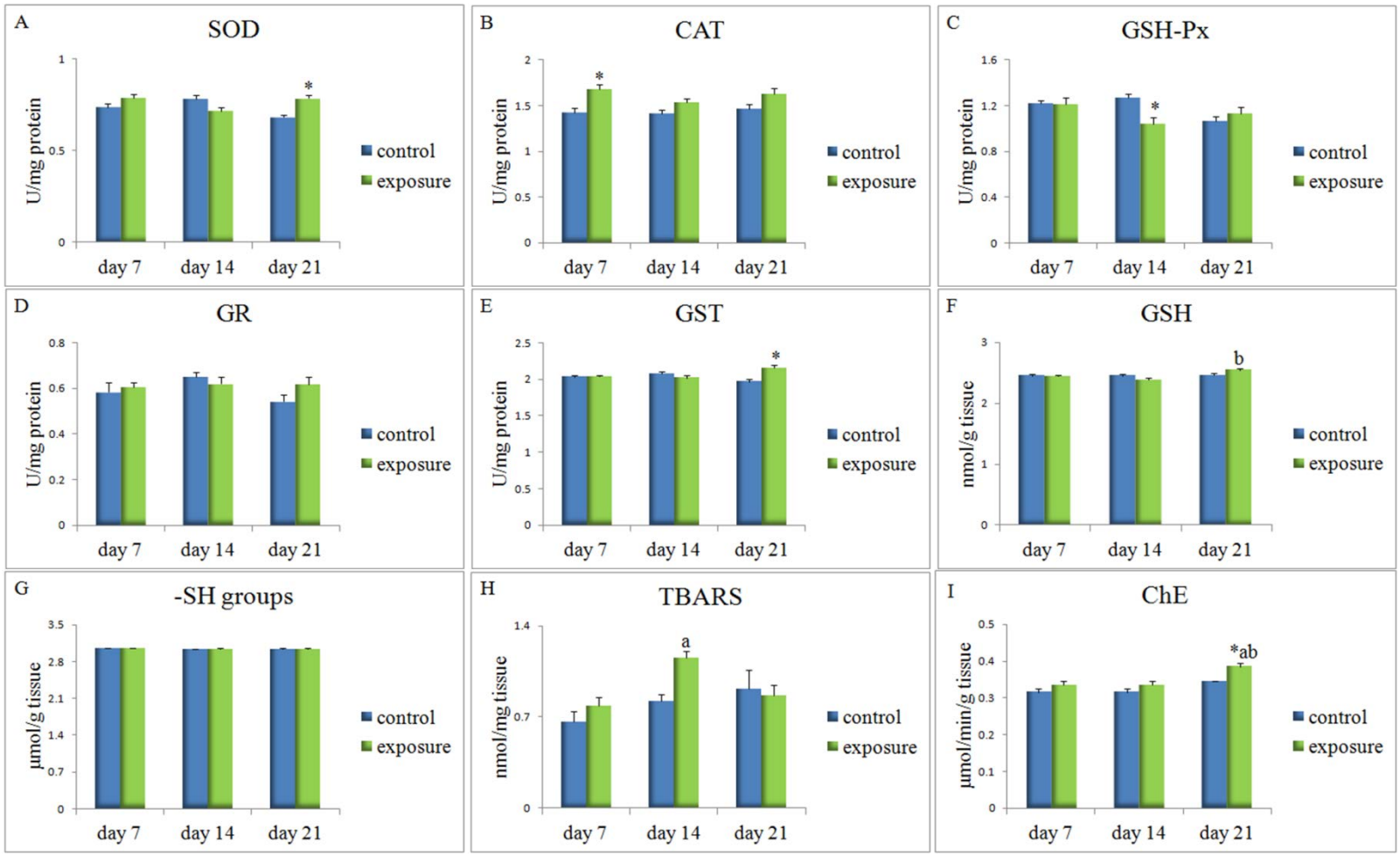


Figure 31. Effects of or-der applied deltamethrin (DM) on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G) and thiobarbituric reactive substances-TBARS (H) concentrations and cholinesterase-ChE (I) activity in the muscle of *Bufotes viridis*. * - significant differences relative to the control; a - significant differences relative to the 7$^{th}$ day of exposure; b - significant differences relative to the 14$^{th}$ day of exposure;

Table 21. Effects of or-der applied deltamethrin (DM) on investigated oxidative stress, biotransformation and neurotoxicity parameters in the gastrointestinal tissue of *Bufotes viridis*. Data are log transformed and presented as the means ± SE (standard error). To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented.

| | control 7 | DM 7 days | control 14 | DM 14 days | control 21 | DM 21days |
|---|---|---|---|---|---|---|
| Total proteins (mg/mL) | 1.16 ± 0.02 | 1.24 ± 0.02* | 1.22 ± 0.01 | 1.18 ± 0.01 | 1.25 ± 0.02 | 1.23 ± 0.01 |
| SOD (U/mg protein) | 0.81 ± 0.02 | 0.70 ± 0.03*b | 0.83 ± 0.02 | 0.84 ± 0.01 | 0.83 ± 0.02 | 0.74 ± 0.01*b |
| CAT (U/mg protein) | 1.78 ± 0.06 | 2.05 ± 0.10 | 2.02 ± 0.12 | 2.05 ± 0.04 | 2.00 ± 0.05 | 1.85 ± 0.05 |
| GSH-Px (U/mg protein) | 1.25 ± 0.06 | 1.40 ± 0.04 | 1.42 ± 0.03 | 1.50 ± 0.03 | 1.32 ± 0.03 | 1.40 ± 0.03 |
| GR (U/mg protein) | 1.09 ± 0.04 | 1.09 ± 0.02 | 1.14 ± 0.03 | 1.12 ± 0.02 | 1.08 ± 0.03 | 1.07 ± 0.02 |
| GST (U/mg protein) | 1.98 ± 0.15 | 2.20 ± 0.06 | 2.21 ± 0.08 | 2.39 ± 0.03 | 2.22 ± 0.05 | 2.26 ± 0.05 |
| GSH (nmol/g tissue) | 1.58 ± 0.16 | 1.70 ± 0.10 | 1.75 ± 0.07 | 1.54 ± 0.06 | 1.72 ± 0.05 | 1.98 ± 0.11 |
| -SH (µm/g tissue) | 2.97 ± 0.03 | 3.06 ± 0.00* | 3.06 ± 0.00 | 3.06 ± 0.00 | 3.04 ± 0.01 | 3.04 ± 0.00 |
| TBARS (nmol/mg tissue) | 0.24 ± 0.03 | 0.21± 0.02 | 0.14 ± 0.01 | 0.24 ± 0.04 | 0.22 ± 0.02 | 0.17 ± 0.01 |
| ChE (µmol/min/g tissue) | 0.43 ± 0.03 | 0.54± 0.01* | 0.52 ± 0.01 | 0.51 ± 0.01 | 0.58 ± 0.02 | 0.50 ± 0.01* |

* - displays significant differences relative to the control; b - displays significant differences relative to the 14$^{th}$ day of exposure;

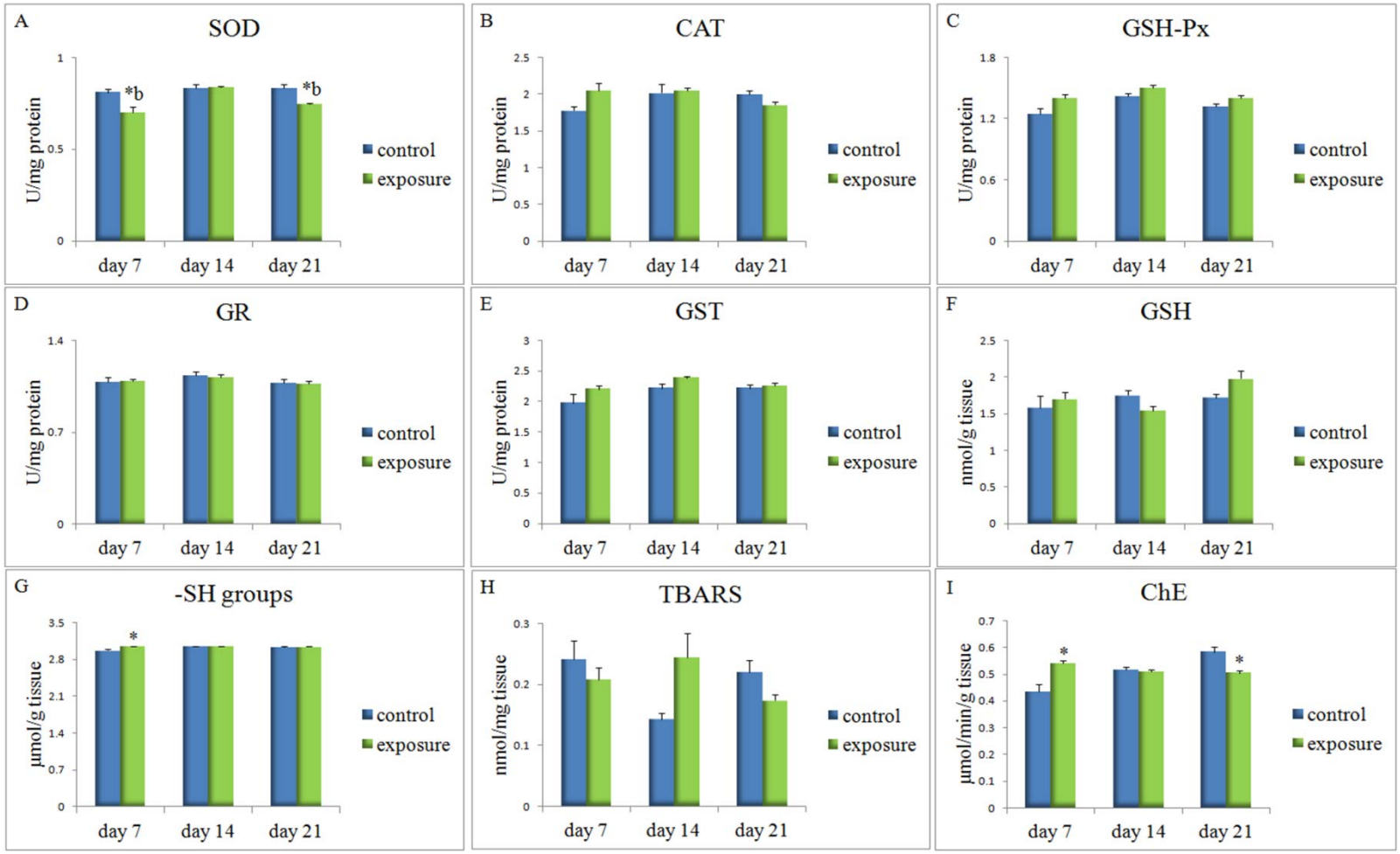


Figure 32. Effects of or-der applied deltamethrin (DM) on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G) and thiobarbituric reactive substances-TBARS (H) concentrations and cholinesterase-ChE (I) activity in the gastrointestinal tissue of *Bufotes viridis*. * - significant differences relative to the control; b - significant differences relative to the 14$^{th}$ day of exposure;

Table 22. Effects of or-der applied deltamethrin (DM) on investigated oxidative stress, biotransformation and neurotoxicity parameters in the skin of *Bufotes viridis*. Data are log transformed and presented as the means ± SE (standard error). To establish significant differences ($p<0.05$) among control and treated groups of animals, the parametric one-way analysis of variance with Fisher's least significant difference post hoc test was implemented.

| | control 7 | DM 7 days | control 14 | DM 14 days | control 21 | DM 21days |
|---|---|---|---|---|---|---|
| Total proteins (mg/mL) | 1.12 ± 0.01 | 1.13 ± 0.01 | 1.14 ± 0.02 | 1.17 ± 0.02 | 1.15 ± 0.02 | 1.13 ± 0.02 |
| SOD (U/mg protein) | 0.77 ± 0.02 | 0.70 ± 0.03 | 0.73 ± 0.02 | 0.72 ± 0.01 | 0.71 ± 0.03 | 0.72 ± 0.02 |
| CAT (U/mg protein) | 1.91 ± 0.03 | 1.84 ± 0.03 | 1.74 ± 0.04 | 1.82 ± 0.04 | 1.92 ± 0.03 | 1.99 ± 0.03$^{ab}$ |
| GSH-Px (U/mg protein) | 1.05 ± 0.03 | 0.96 ± 0.03 | 1.05 ± 0.03 | 0.89 ± 0.02$^{*}$ | 0.96 ± 0.02 | 0.94 ± 0.02 |
| GR (U/mg protein) | 0.97 ± 0.03 | 0.97 ± 0.03 | 1.08 ± 0.12 | 0.93 ± 0.03 | 0.92 ± 0.02 | 0.96 ± 0.03 |
| GST (U/mg protein) | 2.00 ± 0.03 | 1.93 ± 0.04 | 2.03 ± 0.03 | 2.02 ± 0.02 | 2.06 ± 0.03 | 2.05 ± 0.03 |
| GSH (nmol/g tissue) | 2.73 ± 0.02 | 2.66 ± 0.06 | 2.63 ± 0.07 | 2.66 ± 0.03 | 2.85 ± 0.04 | 3.00 ± 0.06$^{ab}$ |
| -SH (µm/g tissue) | 3.03 ± 0.01 | 3.00 ± 0.03 | 3.03 ± 0.01 | 3.00 ± 0.01 | 3.01 ± 0.02 | 2.98 ± 0.02 |
| ChE (µmol/min/g tissue) | 0.25 ± 0.01 | 0.28 ± 0.02 | 0.31 ± 0.01 | 0.30 ± 0.02 | 0.27 ± 0.02 | 0.27 ± 0.02 |

* - displays significant differences relative to the control; a - displays significant differences relative to the 7$^{th}$ day of exposure; b - displays significant differences relative to the 14$^{th}$ day of exposure;

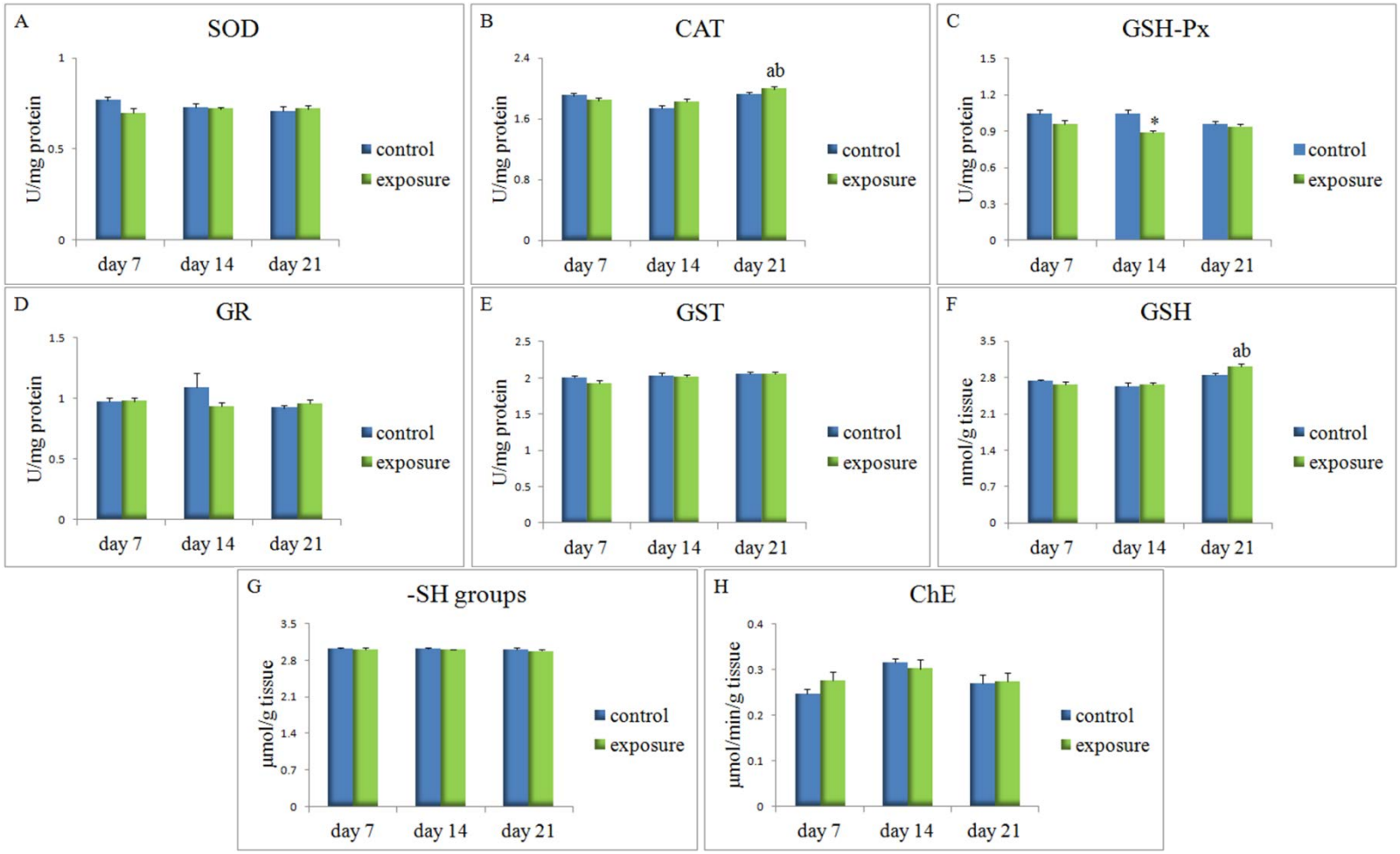


Figure 33. Effects of or-der applied deltamethrin (DM) on superoxide dismutase-SOD (A), catalase-CAT (B), glutathione peroxidase-GSH-Px (C), glutathione reductase-GR (D) and glutathione-S-transferase-GST (E) activities, glutathione-GSH (F), sulfhydryl groups-SH groups (G) concentrations and cholinesterase-ChE (H) activity in the skin of *Bufotes viridis*. * - significant differences relative to the control; a - significant differences relative to the 7$^{th}$ day of exposure; b - significant differences relative to the 14$^{th}$ day of exposure;

Canonical discriminant analysis discovered variations among the exposed groups, considering all measured parameters for every investigated tissue (Table 23, Figure 34). In animal liver, Root 1 accounted for 72.3% of the total heterogeneity. Control group was separated down the Root 1 and it was mainly differentiated relative to activities of SOD and ChE. In animal liver, Root 2 accounted for 17.2% of the total heterogeneity, and GST activity and -SH groups concentration were the most important parameters that provided to differentiation of 7 days from 21 days exposed group down the Root 2. First canonical function, in the muscle, accounted for 68.9%, and Root 2 for 19% of the total heterogeneity. The major parameters in Root 1 that provided to differentiation between the exposed animal groups were activities of GST and ChE, and in Root 2-GSH and TBARS concentrations. The group subjected to pesticide for 21 days was divided downe the Root 1, and there were no clear separation along the Root 2. Root 1 accounted for 52.1% and Root 2 for 31.3% of the total heterogeneity in the GIT. The most important parameters in Root 1 that provided to separation of the 21 days from control group of toads were GSH-Px and GST activities, and in Root 2, the activity of GST and GSH concentration. Along the Root 2, the 14-day group was separated in the GIT tissue. In animal skin, Root 1 accounted for 53.2% of the total heterogeneity. The 21-day group was separated along the Root 1, and it was mainly differentiated by GSH and -SH groups concentrations. In animal skin Root 2 accounted for 31.9% of the total heterogeneity.

Table 23. Standardized coefficients used in canonical discriminant analysis of canonical variables in investigated tissues after combination of oral and dermal exposure to deltamethrin (DM).

| | Liver | | Muscle | | GIT | | Skin | |
|---|---|---|---|---|---|---|---|---|
| | Root 1 | Root 2 | Root 1 | Root 2 | Root 1 | Root 2 | Root 1 | Root 2 |
| SOD | **-0.878** | -0.494 | 0.543 | 0.096 | -1.102 | -0.112 | -0.077 | -0.013 |
| CAT | 0.440 | 0.008 | 0.148 | -0.085 | -0.212 | 0.291 | 0.414 | -0.088 |
| GSH-Px | -0.499 | 0.336 | -0.442 | 0.408 | **0.636** | -0.473 | -0.188 | **0.910** |
| GR | 0.367 | -0.267 | -0.440 | -0.318 | -0.001 | -0.128 | 0.196 | -0.040 |
| GST | 0.015 | **-0.699** | **0.748** | -0.026 | **0.697** | **-1.045** | -0.071 | -0.035 |
| GSH | -0.021 | 0.492 | 0.330 | **0.525** | -0.000 | **1.094** | **0.807** | 0.332 |
| -SH | -0.581 | **0.577** | 0.113 | -0.003 | -0.131 | -0.334 | **-0.499** | 0.168 |
| TBARS | 0.165 | -0.272 | -0.042 | **-0.672** | 0.063 | -0.341 | - | - |
| ChE | **0.699** | 0.110 | **0.926** | -0.181 | -0.188 | 0.063 | 0.116 | **-0.505** |
| Eigenval | 8.687 | 2.074 | 2.798 | 0.773 | 1.945 | 1.167 | 1.056 | 0.634 |
| Cum.Prop | 0.723 | 0.895 | 0.689 | 0.879 | 0.521 | 0.834 | 0.532 | 0.851 |

Statistically significant differences ($p < 0.05$) are in bold.

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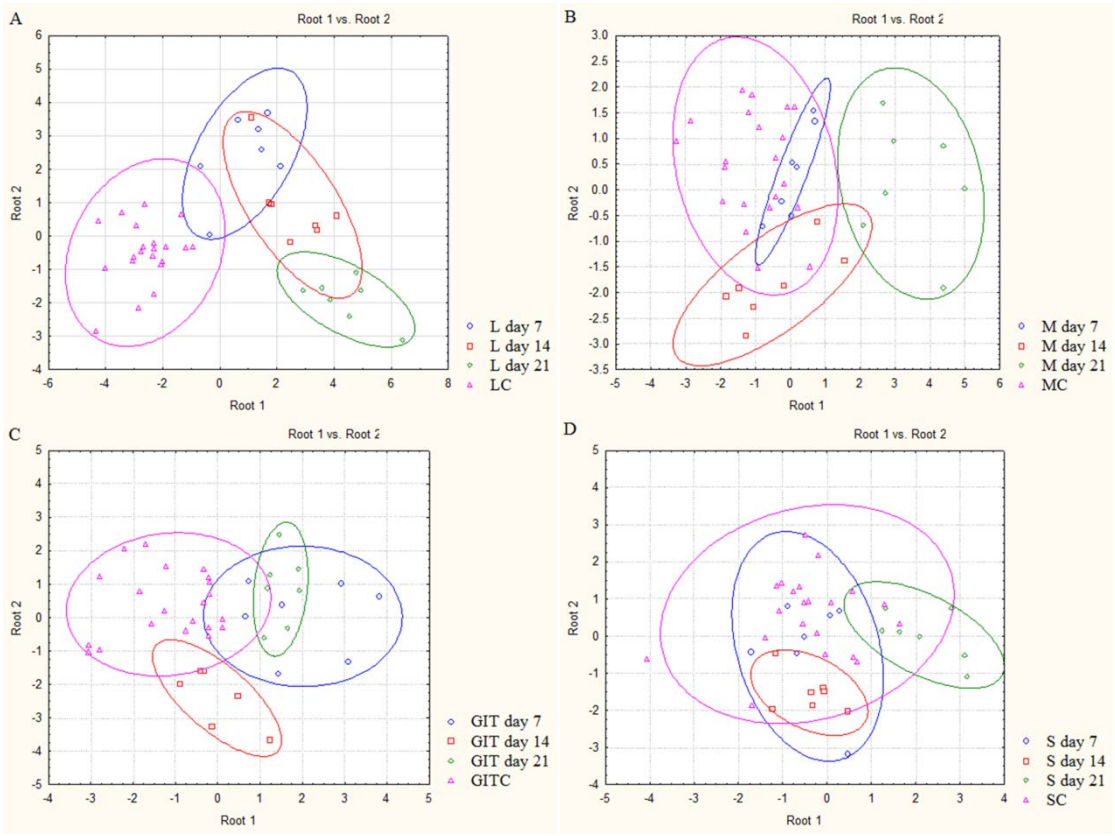


Figure 34. Canonical discriminant analysis of examined oxidative stress parameters in: A – liver (L); B – muscle (M); C – gastrointestinal tissue (GIT) and D – skin (S) on the factor plan after combination of oral and dermal exposure to deltamethrin (DM).

### 4.3.4. Canonical discriminant analysis of all investigated oxidative stress parameters in examined tissues after the 7, 14 and 21 days of the oral, dermal and combined deltamethrin application

In the liver tissue Canonical discriminant analysis discovered variations among the exposed groups, based on examined antioxidant parameters after 7, 14 and 21 days of different application models (Table 24, Figure 35). The first canonical function (Root 1) in the analysis accounted for 78% of the total heterogeneity after 7 days. Group of toads exposed to or-der application was divided down the Root 1 from other treated groups of animals and it was mainly differentiated relating to CAT activity and -SH groups concentration. The second canonical function (Root 2) in the analysis accounted for 14.3% of the total heterogeneity after 7 days. Activities of SOD and ChE were the most important parameters providing to separation of group of animals subjected to dermal application from control and or-der application group, along the second canonical function.

After 14 days of different mode of application, Root 1 accounted for 62.9%, and Root 2 for 26.4% of the total heterogeneity in the liver tissue. The major factor in the first canonical function that provided to the separation between exposed animal groups was SOD activity, and in Root 2- GSH-Px activity and -SH groups concentration. The group of animals exposed to oral application was separated down the Root 1 from control group and along the second canonical function from groups exposed in dermal and combine way (or-der).

The Root 1 accounted for 62.2% and Root 2 for 32.1% of the total heterogeneity after 21 days of different mode of application in the liver tissue. In Root 1 the most important parameters that contributed to the separation of the group subjected to combine way (or-der) of application from control group of toads were activities of CAT and ChE, and in Root 2, the GST and ChE activities,. The group subjected to or-der application was separated down the Root 2 from the group subjected to dermal application of pesticide.

The results for canonical discriminant analysis in muscle of treated group of toads are shown in Table 36 and Figure 25. The Root 1 accounted for 89.3% and Root 2 for 7.5% of the total heterogeneity after 7 days of pesticide exposure. The groups subjected to dermal and or-der applications were separated along the Root 1 from other groups. SOD and ChE activities were the key factors in Root 1 that provided to the separation and in Root 2 it were the SOD activity and GSH concentration. In the GIT, the group subjected to oral exposure was separated along the Root 2.

After 14 days of deltamethrin exposure in the muscle the first canonical function (Root 1) showed 85.2% of total heterogeneity, while another canonical function (Root 2) showed 10.3%. By the first function, control group of animals and group treated in oral way were separated from the groups treated in dermal and combine way, and by the second function there was a separation between control group of animals and group treated in oral way. The factors that provided most to the separation along the first axes were -SH groups and TBARS concentrations and along the second axes were GSH-Px and GR activities.

The first canonical function (Root 1) in the analysis accounted for 74.2% of the total heterogeneity after 21 days in the muscle tissue. Group of toads exposed to or-der application were separated along the Root 1 from control and it was mainly differentiated regarding SOD activity and -SH groups concentration. The second canonical function (Root 2) in the analysis accounted for 19.4% of the total heterogeneity after 21 days. Activities of CAT and GST were the key parameters providing to differentiation along the second canonical function.

Results for canonical discriminant analysis in gastrointestinal tissue revealed differences among the treated group of toads (Table 26, Figure 37). The first canonical function (Root 1) in the analysis accounted for 67.6% of the total heterogeneity after 7 days of exposure. Group of toads exposed to oral application was separated from control along the first canonical function and it was mostly differentiated with regard to GST and GSH-Px activities. The second canonical function (Root 2) in the analysis accounted for 25.6.0% of the total heterogeneity in toad GIT.

Root 1 accounted for 73.4% and Root 2 for 20.9% of the total heterogeneity after 14 days of pesticide exposure. The group subjected to oral exposure was differentiated along the Root 1. The most important parameters in Root 1 that contributed to the separation were activities of CAT and GSH-Px. In Root 2 it was TBARS and GSH concentration. The group subjected to dermal exposure was separated along the Root 2 in the GIT.

After 21 days of different mode of application, Root 1 accounted for 51.3%, and Root 2 for 32.5% of the total heterogeneity in the GIT. The major parameters in Root 1, that provided to the differentiation between exposed animal groups, were SOD and CAT activities, and in Root 2-SOD activity and -SH groups concentration. The group of animals treated in oral way was separated along the first canonical function from dermal group exposure, and the group exposed to combine application along the second canonical function from control group of toads.

Results for canonical discriminant analysis in skin tissue discovered differences among the treated group of animals (Table 27, Figure 38).The first canonical function (Root 1) in the analysis accounted for 49.3% of the total heterogeneity after 7 days of exposure. Difference among the groups along Root 1 was with regard to GR and ChE activities. Root 2 accounted for 45.2% of the total heterogeneity in toad skin. –SH groups concentration and GR activity were the key parameters that contributed to differentiation along the second canonical function.

Root 1 accounted for 77.6% and Root 2 for 16.6% of the total heterogeneity after 14 days of pesticide exposure in the skin tissue. The groups subjected to dermal and combine exposure were separated from control along the first canonical function. The key parameters in the first canonical function, that contributed to the separation were GSH-Px and SOD activities, and in Root 2, the CAT and GST activities. The group subjected to oral exposure was separated along Root 2 in the skin tissue.

After 21 days of different mode of application, Root 1 accounted for 71.5%, and Root 2 for 23.4% of the total heterogeneity in the skin. The key parameters in the first canonical function that provided differentiation between the exposed animal groups were SOD and GST activities, and in Root 2-GSH and -SH groups concentrations. The group of animals treated in combine way was separated along the Root 1 from control group of animals.

Figure 35. Canonical discriminant analysis of examined oxidative stress parameters on the factor plan in the liver of control group (○) of toads and groups treated in oral (□), dermal (◊) and combine (Δ) way of application.

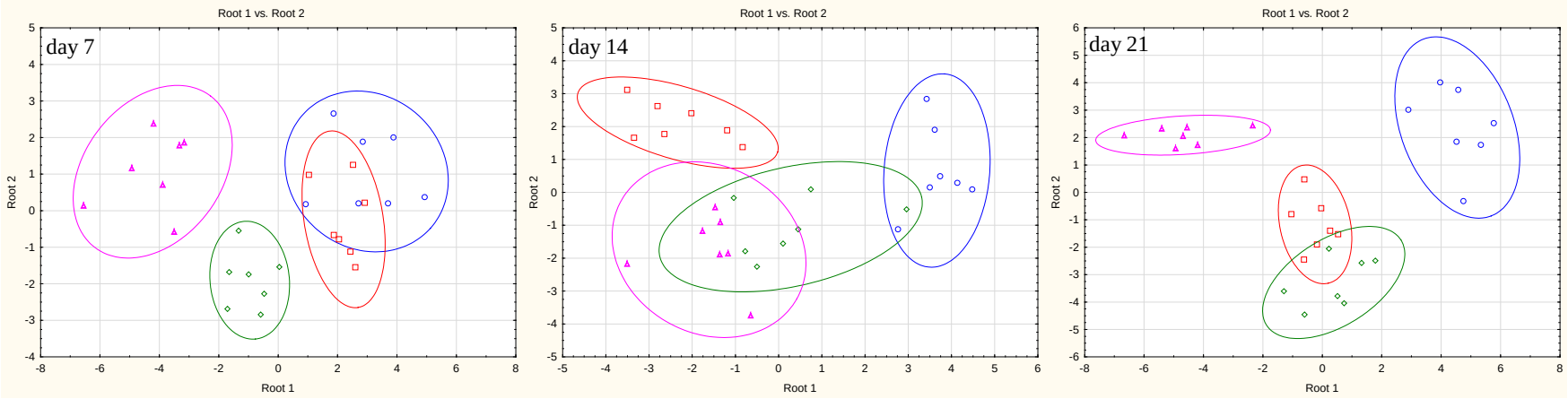


Table 24. Standardized coefficients used in canonical discriminant analysis of canonical variables in the liver of control group of toads and groups treated in oral, dermal and combine way of application. Statistically significant differences ($p < 0.05$) are in bold.

| | Day 7 | | Day 14 | | Day 21 | |
|---|---|---|---|---|---|---|
| | Root 1 | Root 2 | Root 1 | Root 2 | Root 1 | Root 2 |
| SOD | 0,723 | **-0,612** | **0,961** | -0,215 | 0,508 | -1,018 |
| CAT | **-1,404** | -0,493 | -0,272 | -0,121 | **-0,869** | 1,059 |
| GSH-Px | -0,121 | 0,233 | 0,061 | **0,602** | 0,780 | -0,097 |
| GR | -0,108 | -0,473 | 0,022 | -0,286 | 0,087 | 0,261 |
| GST | 0,027 | 0,590 | 0,161 | -0,007 | 0,173 | **1,258** |
| GSH | 0,127 | -0,188 | 0,354 | -0,490 | 0,378 | -0,527 |
| -SH | **-1,153** | -0,218 | 0,270 | **0,585** | 0,279 | -0,215 |
| TBARS | -0,086 | 0,364 | -0,126 | 0,475 | -0,385 | -0,465 |
| ChE | -0,230 | **-0,971** | 0,255 | 0,546 | **-1,027** | **-1,257** |
| Eigenval | 9,474 | 1,737 | 6,286 | 2,642 | 12,488 | 6,450 |
| Cum.Prop | 0,780 | 0,923 | 0,629 | 0,893 | 0,622 | 0,943 |

Figure 36. Canonical discriminant analysis of examined oxidative stress parameters on the factor plan in the muscle of control group (○) of toads and groups treated in oral (□), dermal (◊) and combine (Δ) way of application.

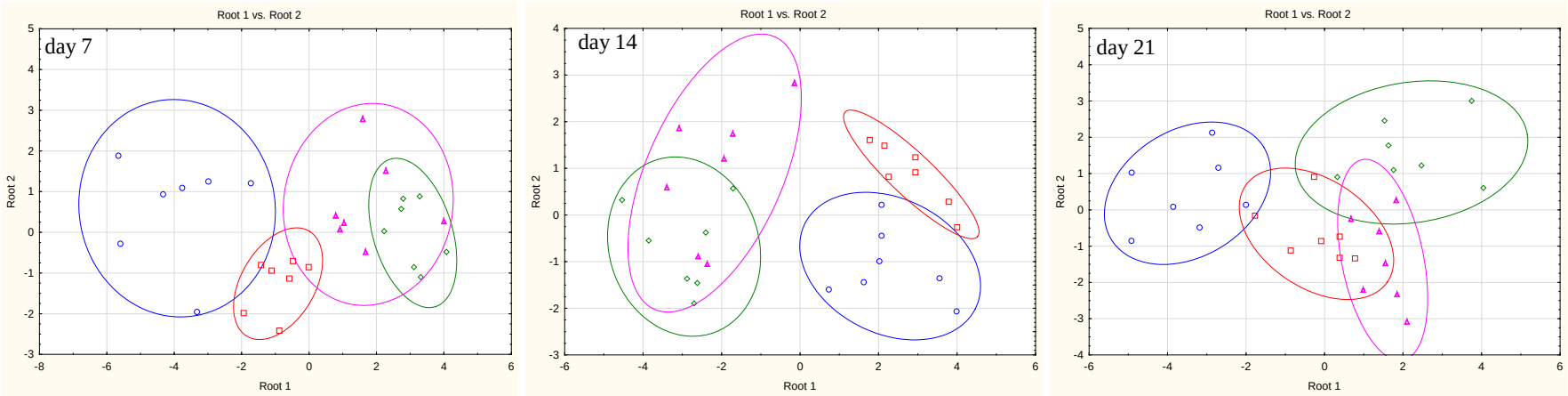


Table 25. Standardized coefficients used in canonical discriminant analysis of canonical variables in the muscle of control group of toads and groups treated in oral, dermal and combine way of application. Statistically significant differences ($p < 0.05$) are in bold.

| | Day 7 | | Day 14 | | Day 21 | |
|---|---|---|---|---|---|---|
| | Root 1 | Root 2 | Root 1 | Root 2 | Root 1 | Root 2 |
| SOD | **1,187** | **0,696** | -0,781 | 0,038 | **1,433** | 0,284 |
| CAT | 0,169 | -0,246 | 0,026 | -0,032 | -0,348 | **-0,827** |
| GSH-Px | -0,334 | 0,208 | 0,573 | **-0,856** | -0,715 | -0,183 |
| GR | 0,519 | -0,561 | -0,174 | **0,765** | 0,277 | 0,567 |
| GST | -0,183 | 0,154 | 0,426 | -0,398 | -0,292 | **-1,086** |
| GSH | -0,708 | **0,749** | 0,513 | -0,423 | 0,517 | -0,196 |
| -SH | -0,187 | -0,241 | **-1,307** | -0,275 | **0,968** | 0,128 |
| TBARS | 0,685 | -0,178 | **-1,115** | -0,274 | -0,115 | -0,002 |
| ChE | **1,197** | -0,426 | -0,924 | -0,110 | 0,461 | -0,263 |
| Eigenval | 8,374 | 0,704 | 7,813 | 0,941 | 5,642 | 1,473 |
| Cum.Prop | 0,893 | 0,968 | 0,852 | 0,955 | 0,742 | 0,936 |

Figure 37. Canonical discriminant analysis of examined oxidative stress parameters on the factor plan in the gastrointestinal tissue of control group (○) of toads and groups treated in oral (□), dermal (◊) and combine (Δ) way of application.

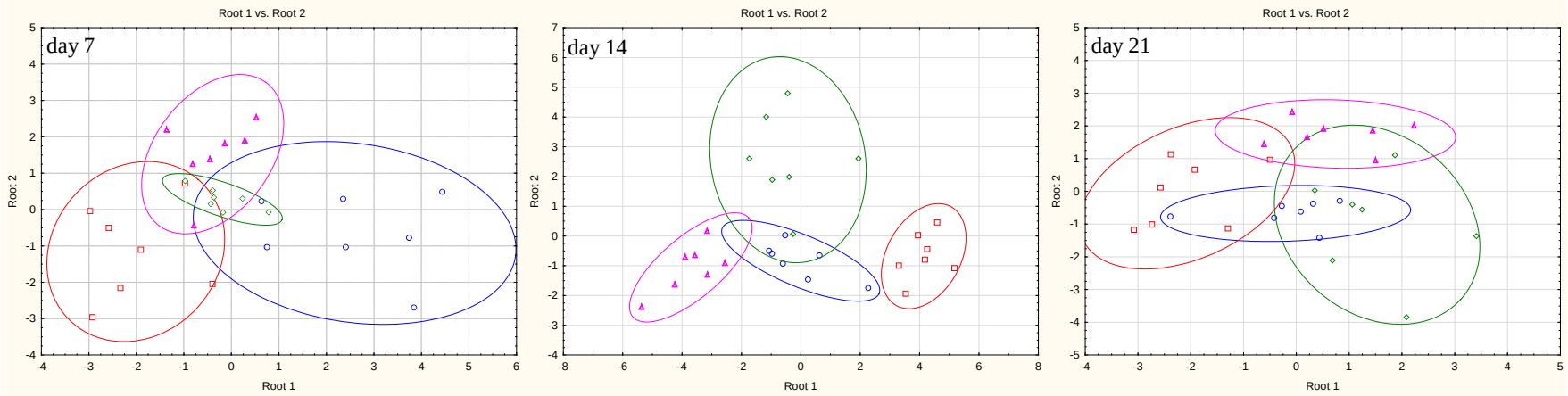


Table 26. Standardized coefficients used in canonical discriminant analysis of canonical variables in the gastrointestinal tissue of control group of toads and groups treated in oral, dermal and combine way of application. Statistically significant differences ($p < 0.05$) are in bold.

| | Day 7 | | Day 14 | | Day 21 | |
|---|---|---|---|---|---|---|
| | Root 1 | Root 2 | Root 1 | Root 2 | Root 1 | Root 2 |
| SOD | 0,366 | 0,680 | -0,819 | 0,279 | **0,744** | **-0,944** |
| CAT | 0,145 | 0,054 | **1,477** | 0,117 | **-0,967** | -0,106 |
| GSH-Px | **-0,802** | -0,171 | **-0,965** | -0,498 | -0,858 | 0,603 |
| GR | 0,251 | 0,015 | -0,708 | -0,117 | 0,417 | 0,432 |
| GST | **-0,809** | **-0,913** | -0,767 | -0,061 | 0,189 | 0,386 |
| GSH | 0,461 | -0,454 | -0,236 | **0,736** | 0,593 | 0,094 |
| -SH | -0,659 | 0,729 | -0,749 | -0,576 | -0,097 | **-0,613** |
| TBARS | -0,224 | -0,759 | -0,431 | **0,760** | -0,066 | -0,305 |
| ChE | -0,013 | **1,058** | -0,259 | 0,089 | -0,028 | -0,391 |
| Eigenval | 3,198 | 1,210 | 9,054 | 2,581 | 2,102 | 1,332 |
| Cum.Prop | 0,676 | 0,932 | 0,734 | 0,943 | 0,513 | 0,838 |

Figure 38. Canonical discriminant analysis of examined oxidative stress parameters on the factor plan in the skin of control group (○) of toads and groups treated in oral (□), dermal (◊) and combine (Δ) way of application.

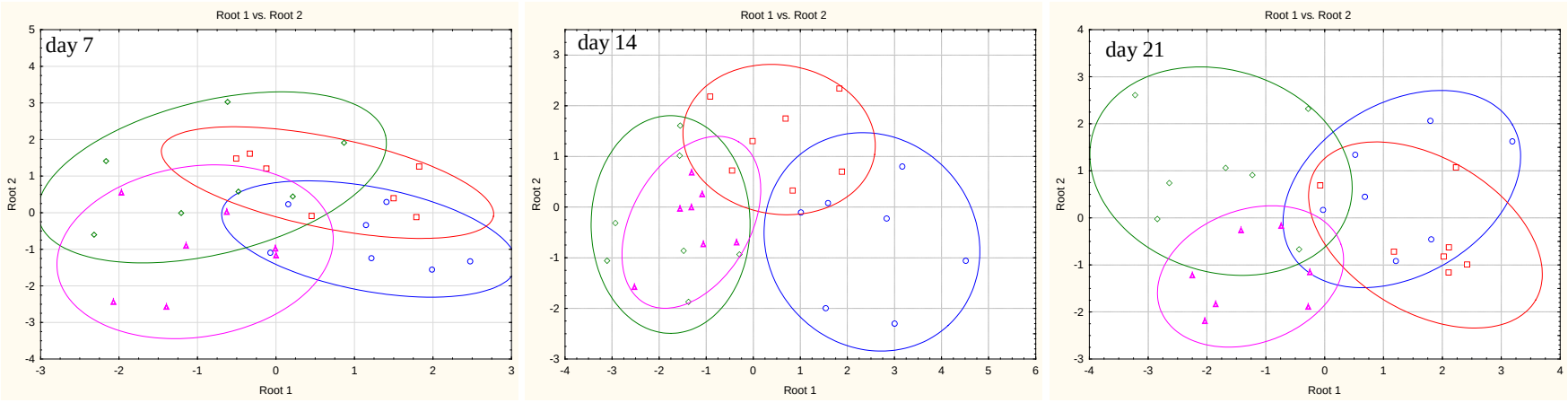


Table 27. Standardized coefficients used in canonical discriminant analysis of canonical variables in the skin of control group of toads and groups treated in oral, dermal and combine way of application. Statistically significant differences ($p < 0.05$) are in bold

| | Day 7 | | Day 14 | | Day 21 | |
|---|---|---|---|---|---|---|
| | Root 1 | Root 2 | Root 1 | Root 2 | Root 1 | Root 2 |
| SOD | 0,218 | -0,757 | **-0,929** | -0,229 | **-0,766** | 0,325 |
| CAT | -0,017 | 0,530 | -0,578 | **0,874** | -0,584 | -0,359 |
| GSH-Px | 0,465 | -1,010 | **0,822** | 0,502 | 0,188 | -0,069 |
| GR | **-0,980** | **1,027** | 0,571 | -0,507 | -0,505 | -0,048 |
| GST | 0,443 | 0,728 | -0,088 | **-0,748** | **0,646** | 0,258 |
| GSH | 0,571 | -0,748 | -0,110 | -0,302 | -0,046 | **-1,080** |
| -SH | -0,143 | **1,071** | -0,018 | -0,576 | 0,177 | **0,590** |
| ChE | **-0,674** | -0,600 | 0,385 | 0,090 | -0,175 | 0,270 |
| Eigenval | 1,041 | 0,952 | 3,346 | 0,716 | 2,724 | 0,888 |
| Cum.Prop | 0,493 | 0,945 | 0,776 | 0,942 | 0,715 | 0,949 |

# 5. DISCUSSION

The metabolism of aerobic organisms implies the ROS generation, so there is a continuing need for their removal. Cells and tissues are in stable condition if prooxidants and antioxidants are in balance. Otherwise, imbalance in favor of prooxidants leads to oxidative stress that can affect all biomolecules (DNA, lipids, proteins and carbohydrates). Thus, oxidative stress can cause different processes - mutagenesis, carcinogenesis, lipid peroxidation, membrane damage, protein oxidation and fragmentation. AOS is a set of protective mechanisms against ROS and, similar to many other biochemical systems, its effects can be changed depending on the degree of alteration of other physiological parameters in the organism (Halliwell and Gutteridge, 2007; Livingstone, 2001). This defense system that protects against oxidative damage includes enzymes (SOD, CAT, GSH-Px, GST and GR) and non-enzymatic components (glutathione, vitamin E, C, A, etc.). In normal conditions, organisms can fight oxidative stress with enzymatic and non-enzymatic components of AOS. However, after exposure to great oxidative challenge, the antioxidant defense may become overwhelmed and disturbed (Halliwell and Gutteridge, 1989).

The toxicity studies used in ecological risk assessments were established on a small number of species. Commonly, toxicity studies for aquatic models have used algae, invertebrates and fish, whereas those for terrestrial models are based on birds and mammals. Amphibians have been underrepresented in ecotoxicology regardless of being one of the most endangered vertebrate taxa (Wake and Vredenburg, 2008) and that hazardous materials negatively impact naturally occurring amphibian populations (Collins and Storfer, 2003). Amphibians represent an important part of ecosystems and relatively complex life cycle makes them important indicators of environmental stress (Sparling et al., 2003). During their life they create relations (as larvae) with the aquatic environment and, (as adults) with both the water and soil. More than 25 years ago global declines in amphibian populations were detected, and obtainable data suggest they are continuing. According to International Union for Conservation of Nature (IUCN) report, of the total number of amphibian species (6285), 1895 are at extinction risk (IUCN, 2016).

Amphibians have a significant position in the ecosystem (in numerous food chains, as secondary consumers) and, consequently, their population destruction has an important influence on other organisms. They are susceptible to very slight changes in the environment and their responses have served as indicators of ecosystem stress, various anthropogenic activities, and the impact of pesticides. Pesticides have a very large impact to the organ systems development and frequently cause lethal/sublethal edema in organisms that have been in contact with pesticides (Pašková et al., 2011). The ecological endpoints influenced by pesticides imply a low degree of egg hatching, reduced growth and big risk of embryonic or larval mortality (Li et al., 2017). The jeopardy connected with pesticide exposure rise primarily during the spring months. This is the time of year in which the frequent use of pesticides is present and coincides with many delicate reproductive processes like spawning, laying of eggs and early maturation of amphibians. Beside first line-skin contact, the toads can be exposed to toxicants via nutrition: insect represents essential food for adult individuals and toads can be subjected to pesticides by ingesting insects which already been subjected to them (Çakıcı, 2015). Exposures are usually seasonal and surge with every applying. Deadly concentrations of active ingredients are frequently found in the environment. Some literature data confirm the fact that commercial formulations of pesticides can be 400 times more lethal than the active ingredient. Mixtures of pesticides are having additional toxic consequences (Relyea, 2004; 2009; Hayes et al., 2006).

The liver of the vertebrata has been widely studied in ecotoxicological studies; it shows a high rate of metabolism and oxygen consumption, and reflects the status of antioxidant defense in the body. It is the major and principal metabolic organ, the central site of different physiological processes, including the foods and xenobiotic compound conversion of, storage of toxic substances, detoxification and excretion (Moreno et al., 2005). This organ represents the main metabolic center of the ROS production of because it is the site of multiple oxidative reactions in the organism.

Otherwise, muscles have a lower metabolic rate and generally are less studied in ecotoxicological studies, but they are assuming to have a key role in the accumulation and transfer of pesticides through the food chain.

Gastrointestinal tissue is important for the analysis of antioxidant potential due to its absorption role. Many toxicants with active transport pass through the wall of the intestine, which can lead to the formation of ROS and the activation of antioxidant protection in this tissue. After DM oral application it is readily absorbed from the GIT in mice and rats (Rehman et al., 2014). For amphibians, such data do not exist.

The skin of amphibians physiologically participates in respiration and the balancing of internal water and ions concentration (Quaranta et al., 2009). Every change in the environment of the frog is first detected through the skin, which represents the primary site of absorption of xenobiotics due to its permeability. Therefore, it can be considered as good candidate for early assessment of the effects of water-soluble substances (Brooks et al., 1998).

Biometric parameters often used in field research are average body mass (BM), snout-vent length (SVL) and hepatosomatic index (HSI). HSI identifies potential changes and liver damage (Ameur et al., 2012). This parameter has been recommended as "exposure index" to environmental pollutants. The presence of herbicides on agricultural surfaces affects the health of the frog, as demonstrated in the study of Thammachoti et al. (2012) by increasing HSI. In this doctoral thesis average body mass, SVL and HSI did not disclose statistical differences between the control group of animals and exposed groups after DM acute or chronic exposure. Also, in the liver it was not found detectable level of DM after chronic exposure for 21 days. Some studies have reveal that DM is fairly rapidly metabolised in rats by esterases and by liver microsomal oxidases to alcoholic moieties and non-toxic acidic (Rehman et al., 2014). Our investigations confirmed that this pesticide does not exibit a tendency to bioaccumulate in toad tissues because of its rapid metabolism. Even though it breaks down quickly and does not tend to bioaccumulate, DM is rapidly absorbed and reaches its principal target-voltage sensitive sodium channels (Anadón et al., 1996).

When the organism is exposed to stress conditions, proteins are utilized as energy source through the amino acid oxidation process (Begum and Vijayaraghavan, 1996). In the present study measured concentrations of total proteins after acute exposure period (96 h) disclose the absence of statistically significant variations in the liver and GIT between treated group of toads. In the muscle was obtained a decrease in the group treated with 8 mg and 16 mg of DM in respect to control and in skin was obtained decrease of total protein concentration in the group treated with 16 mg of DM in respect to control.

Enzyme SOD has the ability to remove $O_2^{\cdot -}$ and thus contribute to maintaining the equilibrium between antioxidants and oxidants. It is one of the principal components of the oxidative defense system and represents a reliable biomarker of oxidative stress (Van der Oost et al., 2003). We did not notice significant variations in SOD activity in the toad liver after acute exposure but in gastrointestinal tissue was obtained reduced activity of this enzyme. Noticed SOD activity decrease could be the outcome of direct SOD injures by the DM or the consequence of its indirect influence on SOD generation, as it was showed that this AOS component has a significant function in the first line of antioxidant defense (Halliwell, 1974). Also, lower SOD activity in GIT could be ascribing to the information that DM was administered in oral and this tissue was the initial line of contact with the pesticide. Some authors indicated that reduced activity of SOD was the outcome of extreme free radical generation and excessive SOD reutilization (Dubey et al., 2012).

After extensive research on American Bullfrog tadpoles (*Lithobates catesbeianus*), which were subjected to different paraquat concentrations for 24 h, it was found that activity of SOD did not enhance beyond the control level after exposure to the highest paraquat concentration (Jones et al., 2010). The conclusion was that the highest given concentration is out of the threshold which can rise an efficient protection from oxidative stress. Alternatively, the nonenzymatic elimination of superoxide could be sufficient to ensure defense against oxidative stress at the elevated paraquat concentration.

Yin et al. (2014) obtained higher SOD activity in all treated animal groups in respect to the control after acute exposure of *Bufo bufo gargarizans* tadpoles to spirotetramat. Only the animals

subjected to the highest concentration showed lower activity of the SOD, so it is obtained that decreased SOD activity point to a down-regulation of the AOS.

In the antioxidant enzymatic system, SOD activity is coupled with activities of CAT or GSH-Px, neutralizing free radical species. According to some authors main reactive oxygen species generated during pesticide biotransformation is hydrogen peroxide (Czarniewska et al., 2003). The most important antioxidant constituents involved in $H_2O_2$ removal are CAT and GSH-Px. CAT posses significantly higher Michaelis-Menten constant (Km) for $H_2O_2$ than GSH-Px (Saint-Denis et al., 1998). Thus GSH-Px is more efficient in the removal of $H_2O_2$ when its concentration is close to the physiological amount (Halliwell, 1974). When generated $H_2O_2$ overcomes the physiological concentration, CAT is activated (Halliwell and Gutteridge, 2007). CAT activity can not be interpreted independently of GSH-Px since these two enzymes have the same substrate- $H_2O_2$.

In the liver and skin of toad, we obtained an increase in CAT activity after acute DM treatment, while GSH-Px activity did not vary comparative to the control in these two tissues. We believe that, after 96 h of exposure, CAT is the main enzyme in charge of $H_2O_2$ degradation. Obtained growth in the activity of CAT is an adaptive response to the $H_2O_2$- induced oxidative in the liver and skin after DM application. David et al. (2012) detected similar results – a higher CAT activity after exposure of Asian toad (*Duttaphrynus melanostictus*) tadpoles to cypermethrin.

On the other hand, Jones et al. (2010) reported that acute paraquat exposure after 24 h did not induce noteworthy variations in liver and muscle CAT activities in tadpoles of American Bullfrog. They concluded that the elevated activities of CAT in examined tissues (particularly the liver) may have been adequate to avoid oxidative disturbance of biomoleculs.

In tongue of frogs *Rana ridibunda* Özkol et al. (2012) registered a decrease in CAT activity after being acutely subjected to omethoate. In some invertebrates, such as black tiger shrimp (*Penaeus monodon*), CAT activity was decreased after exposure to deltamethrin for four days (Tu et al., 2012). DM exposure, joint with the heat stress condition, leads to a significant decrease in the liver, kidney and gills CAT activity, as well as an increase in the liver and kidney GST and GSH-Px activities in the *Channa punctata* (Kaur et al., 2011). These results specify that CAT activity may be modified by DM in various manners in different organisms, and that DM metabolites are capable to modify this enzyme activity, making the animals be more susceptible to harmful consequence caused by ROS.

Besides the $H_2O_2$ elimination role, GSH-Px neutralizes the organic peroxides into alcohols. This enzyme can attack aryl or alkyl halogen groups in the corpus of pyrethroid insecticides (Yu and Nguyen, 1996) which can also provide cellular protection (Kostaropoulos et al., 2001). In all these reactions, GSH represents a hydrogen donor (Halliwell, 1974). As the ultimate result of this reaction mechanism, GSSG is formed and (in the presence of NADPH) reduced by GR; in that way the cell level of GSH is restored (Halliwell and Gutteridge, 2007). In our acute study we did not detected any significant change in the liver GSH-Px activity after acute exposure to DM but in toad muscle and gastrointestinal tissue we registered the increase in GSH-Px activity. This increase in the muscle was joined with an increase in GR activity and GSH level, which is comprehensible by contemplating the entire GSH-Px-GSH-GR machinery. Another study on tadpoles of *Rana daunchina* demonstrated that short-term subjection to low concentrations of herbicide atrazine increased GSH-Px activity, while high concentrations of this pesticide or long-lasting application lead to inhibition of GSH-Px activity (Yuan et al., 2004).

Some previous studies confirmed that concentration of pesticides changed with the time of exposure. Yuan et al. (2004) displayed that in *Rana daunchina* tadpoles short-term subjection to low levels of atrazine contributes to the GSH-Px activity, while higher concentration or longer exposure to this toxin suppresses GSH-Px activity. Some authors remarked that the GSH-Px activity reduced when the toxic substance concentration is very high (Gao et al., 2013).

In the cellular system, many radical and non-radical reactions can lead to oxidation of thiols into disulfides, i.e. GSH in GSSG. The conversion of GSSG into GSH with the activation of GR represents the most important mechanism for maintaining the thiol cell status so GR has important function in antioxidant protection (Halliwell and Gutteridge, 1989). In our investigation on *B.*

*viridis* GR displayed a regular growth in activity after acute application of pesticide. Enzyme activity increased in liver, muscle and skin after the animals were subjected to the highest concentration of pesticide (32mg/kg). In adult frogs *Rana ridibunda* a decrease in tongue GR activity was detected, while it enhanced in lung and varied in muscle and stomach after exposure to organophosphorus insecticide omethoate during 96 h (Özkol et al., 2012). American Bullfrog tadpoles were subjected to growing pesticide concentration for 24 h (Jones et al., 2010). Paraquat treatment led to considerable increases in the liver and muscle GR activity and the authors suggested that this species has a better ability for the GSH regeneration than the other anuran species. After 96h of application of glyphosate and methidathion-based pesticides in the *Bufotes viridis* tadpoles, GR activation was detected (Güngördü et al., 2016). Margarido et al. (2013) determined the activity of biochemical biomarkers in tadpoles of *Scinax fuscovarius* subjected to the fipronil and obtained that the GR activity varied depending to the concentration of pesticide and exposure time. The authors detected GR induction in *Scinax fuscovarius* tadpoles, and that excessive production of ROS may contribute to enzyme inhibition. Ensibi et al. (2013) detected an enhance in the liver GR activity in DM treated fish after 4 days of exposure and correlated this enzymatic adaptation with the rise in CAT activity detected after 4 days of pesticide exposure. This induction of GR has a significant function in preventing the GSH variations at first days of DM exposure.

GST is a multifunctional enzyme included in the detoxification of xenobiotic compounds and has an important function in the protection against oxidative stress. In addition, GSH is the principal low-molecular weight antioxidant in the body (Van der Oost et al., 2003), essential for the functional and structural integrity of cells, tissues and organs. It reacts as a substrate in the conjugation reaction with electrophiles under the catalytic action of GST, but it also has a direct effect with free radicals through oxidation to GSSG. Toxicants spend GSH through a mechanism of reaction catalyzed by GST, and it is likely to stimulate GST activity as a powerful defensive mechanism (Timur et al., 2003). Therefore, the variations in GST activity need to be interpreted together with changes in GSH concentration. After acute exposure to DM in our study, the induction of GST in muscle of *B. viridis* was followed by GSH enhance. The deficiency of variability GST activity in liver was follow by a reduction in GSH concentration, which points to enzyme non activation caused by the pesticide or decline in GSH level (Ensibi et al., 2012). Jayasree et al. (2003) stated that GSH level was negatively influenced and noticed a downfall after DM intoxication of Broiler chicks. A decrease in GSH content was clarified as adaptive reply to lipid peroxidation increase as the outcome of severe oxidative stress (Kavitha and Venkateswara, 2009). In different tissues of frog *Rana ridibunda* Kanter and Celik (2012) investigated the influence of fenthion to oxidative stress biomarkers and detected fluctuation of GSH level during the 96 h exposure to pesticide. They concluded that a substantial decrease in GSH level may be caused by its consumption, dealing with oxidative stress. In our study obtained lack of variation in GST activity in the liver of *B. viridis*, after acute DM application, could be explained with the fact that pesticide was processed by an additional protection mechanism, such is biotransformation with CYP1A.

We detected CYP1A induction in the liver of animals subjected to 8 and 16 mg of DM, in respect to the control group. In general, xenobiotic metabolism in amphibians often displays different patterns than those in mammals. However, there is an insufficiency of information concerning CYP1A activities and expression in amphibians. Xenobiotic metabolism in amphibians frequently shows dissimilar patterns. Bernabò et al. (2014) investigated the influence of two low concentrations of nonylphenol ethoxylate (used mainly as cleaning agents and detergents) on *Lissotriton italicus* liver. They showed an elevated CYP1A expression, subsequent to short-term application of nonylphenol ethoxylate. At higher concentration, the expression increased after 24 h, reaching a peak after 48 h of exposure whereas after 96 h a significant decline in the levels of CYP1A was observed. Diverse CYP1A content was noticed between different groups of amphibians, and it need to be considered; even though CYP expression in amphibians is lower than in mammals, in Urodela was detected an exception (Ertl and Winston, 1998). Some investigators

examined the activity of CYP1A in the liver of *Ancistrus multispinis*, after 4 days of DM exposure. They noticed that obtained induction represented a protective reply to the existence of a toxicant (Silva de Assis et al., 2009). The latest data confirmed that CYP enzymes (together with metabolic pathways of NADPH and carboxylesterase) seem to be the major metabolism pathway for DM (Lu et al., 2019). This explains CYP1A induction and the deficiency of a change in the liver GST activity of toads acutely treated with DM in our experiment.

A large number of proteins contain a functional -SH groups that can be modified through different redox reactions and their reversible modification can initiate modifications in the function of the proteins, which include enzymes activity. All cell structures containing -SH groups have an important function in antioxidant protection and preservation of redox balans (Sen, 2001).

-SH groups and TBARS represent the parameters of macromolecules oxidative disturbance. The acute application of DM significantly modified thiol profile of *B. viridis* in our study, but the concentration of -SH groups exhibited no uniform pattern in investigated tissues (Radovanović et al., 2017). In the liver and skin of treated toads the concentration of -SH groups was lower, but higher in gastrointestinal tissue. Pro-oxidant effect of DM on proteins has been confirmed by the detected reduce in content of -SH group. Pesticide application, followed by the extreme oxidative metabolism, possibly causes -SH group covalent modifications, together with formation of mixed disulfides, S-thiolation and S-nitrosylation (Iciek and Wlodek, 2001). Any of these modifications might be in charge of the reduction of -SH groups content.

Ecotoxicological papers about pesticide influence on amphibians sulfhydryl groups are very scarce and more toxicological effects were tested on fish models. Sayeed et al. (2003) investigated the influence of 48 h DM exposure on antioxidants in the liver, kidneys and gills of *Channa punctata* Bloch. The concentrations of total thiol enhanced significantly in the liver and kidneys, but in the gills was detected decrease in its concentrations. In the same fish thiol profile was also modulated by dual exposure of DM and heat stress in combination or their individual exposure (Kaur et al., 2011). In all examined tissues (except kidney) total thiols content was higher relative to control. Parvez and Raisuddin (2006) investigated the influence of copper pre-exposure on oxidative stress induced by DM in freshwater fish *Channa punctata* Bloch. They recorded reduced level of total thiols in liver of copper-acclimatized DM-subjected animals, in respect to deltamethrin-subjected animals, whereas non-protein thiol concentration was elevated in liver of copper-acclimatized DM-subjected animals in respect to DM-subjected animals.

One of the markers of oxidative stress is lipid peroxidation (Van der Oost et al., 2003). LPO products create a number of free-radical reactions changing the physicochemical characteristic of membrane lipid bilayers, reacting with protein amino-acids, reducing sugars or their oxidation products, thus resulting in serious cellular abnormality (Ensibi et al., 2013). TBARS are a most important oxidation product of peroxidized polyun-saturated fatty acids. Their concentration directly reflects extent of cellular injure induced by lipid peroxidation in the organism.

In the present investigation elevated contents of TBARS after acute oral exposure to DM in the liver and muscle of *B. viridis* were detected. This suggests that free radical production can be associated with DM metabolism, which contributes to peroxidation of lipid membranes.

Other authors detected that 96 h of omethoate application leads to increase of MDA level in the adult frogs *Rana ridibunda* treated with different doses (Isnas et al., 2012). MDA is significant reactive metabolite and marker of lipid peroxidation. In freshwater fish *Channa punctatus* Bloch, after a single 48 h acute exposure to DM, TBARS concentration was considerably increased in liver, gill and kidney. The enhance in the TBARS mean values in liver, gill and kidney were 39%, 182%, and 21%, respectively, with respect to control group of animals (Sayeed et al., 2003).

Some literature data have shown secondary pyrethroid effects on the cholinergic system (Velíšek et al., 2007). In present study after 96 h post-treatment in the liver of treated toads, we observed a decline in the activity of ChE. This result could be the outcome of the direct pesticide influence on the enzyme active site, or a consequence of the ChE synthesis inhibition (Das and Mukherjee, 2000). After subjection to sublethal concentrations of various toxicants, many investigations stated a general reduce in the activity of ChE. Significant decline in ChE activity was

discovered in the fish *Labeo rohita*, after acute and sublethal application of DM (Suvetha et al., 2015). Comparable data were obtained by Velki and Hackenberg (2013) in earthworm *Eisenia andrei*. In the muscle and skin of *B. viridis* we did not detected any changes in ChE activity, which is in compliance with the data published by Assis et al. (2009) who detected an absence of DM effect on the activity of ChE in the *A. multispinis* muscle.

After acute pesticide application in our experiment, we obtained a high degree of mutuality between the examined parameters after using Pearson's correlations. The positive correlation in the muscle and GIT was noticed between GSH-Px and GST. This correlation could be manifestation of the relationship between these two antioxidant components, since GST (similar to GSH-Px), through GSH oxidation, take part in the $H_2O_2$ removal (Koivula et al., 2011). In the liver and gastrointestinal tissue, most of the correlations were detected. This was expected, given that liver represents the major place of storage, biotransformation, and excretion of pesticides and gastrointestinal tissue represents the primary line of contact with the DM. Therefore these tissues showed the upmost extent of coordination of examined antioxidant components. Nevertheless, regardless of the high correlation and reciprocity of the examined components, mainly affected and susceptible tissue was the liver, as indicated by the variations in -SH group levels and TBARS content. In the skin was noticed the lowest number of correlations, which was expected taking into account that it is a marginal (peripheral) tissue.

PCA exhibits the samples dissociation and ensures additional data concerning the variables that generally influence sample distinctions and similarities. In principal component analysis after acute pesticide application Factor 1 demonstrated clear dissociation between the liver and other examined tissues, and factor 2 demonstrated separation of the skin tissue. PCA indicates the function of every component tested in the performance of the entire antioxidant system. In addition, it points to obvious tissue separation, meaning that the entire antioxidative protection system studied in every tissue is differently organized. Dissociation of liver tissue is expected since it has a key function in the biochemical transformations and metabolism of environmental xenobiotics. This tissue represents the central location of the biotransformation process (Kent, 1998); its antioxidant defense arrangement and coordination is unique and distinct.

Despite broad use of pesticide all over the world, ecotoxicological investigations about chronic influence on amphibians are deficient since most of the investigations are based on short-term effects of acute testing (Sanders, 1970; Materna et al., 1995). These studies enable comparison of relative toxicity of chemicals evaluating survival directly. In contrast, low levels of toxicants, to which animals are subjected for a prolonged time, may induce sublethal consequences affecting the existence of the individuals indirectly. Investigations of acute exposure are significant, but data of the chronic exposure effects provides a reliable illustration of the conditions in natural populations. It is easier to extrapolate the potential environment danger of toxicants from laboratory data in the clearly defined laboratory studies with environment similar to that encountered by amphibians in the field. Pesticide chronic toxicity is more hazardous with respect to acute toxicity determine by lab experiments (Webber et al., 2010). Extended exposure can lead to different genetic malformations, defects in birth, tumors generation, nerve infections, endocrine disturbance and effects of reproduction (Islam and Malik, 2018).

After 21 days of oral DM exposure in our study it was obtained enhance in the liver total protein concentration with respect to the control. Nevertheless, when the general trend is observed, in the liver, muscle and skin-the total protein concentration reduction was detected after three weeks of exposure. Jenkins et al. (2003) stated that organism trying to face with prolonged subjection to toxicants with protein immobilization. Extended exposure might reduce the concentration of total proteins as the outcome of augmented proteolysis and possible utilization of the products of metabolism (Ravinder et al., 1988), and as the outcome of higher energy demands for tissue reparation and preservation of cell balance (Neff, 1985). Because of this, some authors suggested variations in the levels of total proteins as oxidative stress markers in toads treated with diazinon (Tongo et al., 2012). Some authors have investigated a total proteins concentration in liver and kidney of *Rana tigrina* after exposure to permethrin and cypermethrin, and noticed decrease in

these tissues (Khan et al., 2003). In African toad *Bufo regularis* Tongo et al. (2012) noticed a fall in total proteins level after diazinon exposure for 28 days. It is hypothesized that prolonged and continued pesticide subjection causes a progressive reduction in the concentration of total proteins.

Dermal chronic exposure to a pesticide in our experiment did not disclose any significant change in liver, skin and GIT total protein content, but in the muscle was evident growth in concentration after 14 and 21 days of exposure in respect to 7 days. Chronic combine way (or-der) revealed an increase in groups subjected to pesticide for 7 and 21 days comparing to control groups in the GIT and liver, respectively.

After oral pesticide application in our investigation, SOD, as first-line defense enzyme, pointed a direct causal connection of activity of the enzyme and time-dependent environmental stress. The activity of this enzyme was decreased with respect to the control on days 14 and 21 in the liver, and on days 7 and 14 in the GIT of treated animals. After dermal application, in the liver tissue SOD activity was as well reduced on days 14 and 21 in comparison to day 7. As animals were subjected to pesticide for an extended period, the enzyme was suppressed. Some authors perceived that pesticide can stimulate mitochondrial $O_2^{-\cdot}$ generation and, if SOD was inhibited, the concentration of $O_2^{-\cdot}$ could get to hazardous amounts (Peixoto et al., 2004). Application of DM resulted in ROS formation and in exclusion of the first line defense provided by SOD in the liver tissue, which was shown in activity reduction. When compared all three application models, we noticed that the combined approach was the most effective; it causes the suppression of liver SOD in all treated groups-after 7, 14 and 21 days with respect to the control group of animals.

Czarniewska et al. (2003) studied the effects of a sublethal dose of paraquat and metoxychlor on the liver AOS of *Rana esculenta* after 6 and 12 days of exposure. They observed no change or even a downfall in SOD activity in frog liver. When the sublethal dose of pesticides was used for 6 days, SOD activity in liver was reduced with respect to the control, but when the frogs were subjected to pesticides for 12 days, SOD activity was very similar as in the control group of animals. They concluded that level of SOD activity can be adequate to defend the liver against superoxide anions generated in the microsomal fraction. Loutfy and Kamel (2018) evaluated the effect of two commercial formulations of neonicotinoid pesticides on Egyptian frog adults (*Bufo regularis*). The authors measured stress parameters after exposing animals to 1/6 of the recommended field concentration. After 12 days of exposure, the study demonstrated a great impairment of SOD activity in the liver of *B. regularis*. Obtained results are attributed to oxidative stress generated by both insecticides. These authors concluded that the inhibition of this antioxidant enzyme could reduce the capacity of frogs to neutralize environmental pollution, making the animals more susceptible to intoxication.

Our acute experiment did not induce a change in the SOD liver activity but chronic exposure did reveal time-dependent decrease in enzyme activity; it can be deduced a connection between DM exposure duration and SOD activity.

Besides SOD, CAT is the enzyme involved in the first-line defense from oxidative stress. After chronic oral exposure in our study, liver CAT activity was increased after 14 days with respect to groups subjected to the pesticide for 7 and 21 days. In the GIT higher CAT activity was observed after 7 days of oral application and in the skin after 14 days of exposure in respect to control. There are contradictory data in the available literature about chronic pesticide influence on CAT activity. Czarniewska et al. (2003) noticed the growth of CAT activity after 12 days of metoxychlor or paraquat injections in *Rana esculenta* liver. Margarido et al. (2013) reported lack of influence of insecticide fipronil on CAT activity in *Scinax fuscovarius* tadpoles. In this experiment tadpoles were exposed to the dissimilar pesticide concentrations. The exposure intervals were 5, 14 and 30 days and authors concluded that deficiency of changes in CAT activity implies that enzyme is not responsive to fipronil in tadpoles under given experimental conditions.

The only way of pesticide exposure generally performed in ecotoxicological studies is direct oral ingestion via gavages or contaminated food. Feeding by contaminated insects is considered the major route of exposure, but in our opinion other routes of uptake have to be investigated to gain realistic assess the hazard of pesticide substances to wildlife. Dermal and non-dietary exposures

considerably contribute to total exposure. After dermal application in our investigation, CAT activity in the liver was increased on days 7 and after combined exposure on days 7 and 21 in respect to control group of animals.

Since proportionally more surface of skin area may get in touch with contaminated soils or plants in amphibians, exposure through the skin may be higher in these species compared to other animals (like birds), at least in terms of the contribution to total exposure. Toxicity experiments demonstrated that through dermal exposure reptiles can take lethal doses of contaminants (Brooks et al. 1998). Reptile skin, like an amphibian, is not impermeable so toxicity via dermal route can be equal or exceed that via the oral exposure. According to Weir et al. (2010), exposure model simulations have further verified the significance of dermal exposure over total exposure in reptiles and, also, indicated important doubts concerning bioavailability and transport across the skin.

Under laboratory conditions, Chang et al. (2018) investigated the influence of insecticide diflubenzuron on the lizard thyroid endocrine system and obtained that both oral and dermal exposures disturbed the thyroid hormone homeostasis and that the treatment via skin could be significant path for the reptiles risk evaluation.

Comparable toxicological studies on amphibians have not been done yet, but we assume that the pattern is similar and that different exposure routes should be considered when assessing the ecotoxicological effects of toxicants in amphibians.

Canonical discriminant analysis revealed that in the liver tissue CAT is the variable that contributed the most (along with -SH groups concentration) to the separation of the group of toads exposed to or-der application after 7 days. After 21 days of exposure CAT was again dominant parameter in group exposed to the combined way of application.

According to our acute experiment we can conclude that liver CAT activity was dose-dependent variable. During the chronic exposure with dermal and or-der application mode, CAT responded very quickly to oxidative stress induced by pesticide. Enhancement in the activity was noticed already after 7 days of exposure.

In the antioxidant enzymatic system, GSH-Px activity is coupled with activities of SOD and CAT in neutralizing generated oxidative stress. By Czarniewska et al. (2003), main ROS produced during pesticide biotransformation is $H_2O_2$. Besides CAT, the most important antioxidant constituents involved in $H_2O_2$ removal of this reactive species is GSH-Px.

In the liver was obtained a reduction in GSH-Px activity on the 21$^{st}$ day of oral exposure compared to other exposed groups. This enzyme is mainly localized to liver mitochondria and cytosol (Duzguner and Erdogan, 2010). Lower activity of GSH-Px in this tissue can be an outcome of $O_2{\cdot}^-$ and $H_2O_2$ hyperproduction for longer exposure period, or final result of enzyme inactivation after pesticide level rise (Bartoskova et al., 2013; El-Tawil and Abdel-Rahman, 2001; Sehonova et al., 2017).

In *Rana daunchina* tadpoles higher concentration or longer exposure to atrazine suppresses activity of GSH-Px (Yuan et al., 2004). Some authors reported downfall in GSH-Px activity when the level of toxicant is higher than the ability of GSH-Px to breakdown that same toxicant (Gao et al., 2013). Yin et al. (2014) studied the effects of spirotetramat after subjection of *Bufo bufo gargarizans* tadpoles. They discovered that GSH-Px was very susceptible investigated parameter which considerably rose on day 4 after exposure. However, this enzyme was reduced after extended exposure for 15 and 30 days, except for the lowest concentration exposed group. GSH-Px activity was stimulated with increased oxygen free radicals formation and it permits the cells to handle oxidative stress and defend membranes from spirotetramat-caused injury throughout the first 4 days of exposure. Yet, the extent of GSH-Px activity was radically reduced during time (day 15). The authors considered the possibility that GSH-Px could not neutralize toxic spirotetramat with a prolongation of exposure time.

Dermal chronic exposure to a pesticide in our investigation did not disclose any significant change in liver, muscle and GIT GSH-Px activity, while chronic combine way (or-der) revealed a decrease in the group exposed to 21 days comparing to group exposed to 14 days in the liver.

If we compare the variations in this enzyme activity in the function of pesticide application time, short-term exposure did not impair the functionality of this enzyme but extended subjection to DM has caused a decline in activity after 21 days of oral and or-der application in liver tissue.

After chronic oral pesticide application GR in the liver of treated animals displayed a reduction in activity on day 14 with regard to day 7 of treatment. In contrast, GR activity in the skin was decline on day 14 in subjected animals with regard to the control. Change in GR activity was not detected in any investigated tissue after dermal and or-der chronic application.

In rats exposed for 28 days to DM, Sharma et al. (2014) obtained liver, kidney and testis depletion in the GR activity as the consequence of a change in the enzyme structure caused by this pesticide. In the study of Li et al. (2010), the chronic effect of fungicide propiconazole was investigated in rainbow trout (*Oncorhynchus mykiss*). Fish were subjected to various sublethal concentrations for 7, 20 and 30 days and GSH concentration and GSH-connected enzyme activities were quantified. After 20 days all enzymes activities in liver and muscle were significantly induced in groups treated with higher concentration, demonstrating the production of ROS and the protective function of GSH-depending enzymes. All investigated enzymes displayed a significant inhibition after 30 days of exposure in muscle, indicating that the growth of ROS has made enzymes poisoned. The authors concluded that even though there was a declining tendency in liver enzyme activities, they were still higher than the control group, which point to the ROS accumulation but not sufficient to make enzymes lose the function. In another fish species, goldfish (*Carassius auratus gibelio*), after DM exposure for 1, 2, 3, 7 and 14 days, Costin et al. (2007) recorded that treated animals produce a significant increase of kidney GR activity in the first two days of exposure. Between 3 and 14 days of intoxication, this activity significantly decreased. The conclusion was that fish kidney overcomes oxidative stress by antioxidant mechanisms and advanced an adaptive response. On the other hand, different concentrations of commercial insecticide fipronil were not altered GR activity after treatment of *Physalaemus nattereri* tadpoles for 2 and 7 days (Freitas et al., 2017).

After acute administration of pesticide in our study we found that the highest pesticide concentration initiated higher response after short-term exposure in the liver, muscle and skin, but after chronic application no regularity in the change of GR activity was noticed.

Antioxidant role of GR cannot be contemplating separately from GST-GSH-GR system. Regardless of the role of GSH-dependent enzymes in toxicity caused by pesticide, in the liver we did not detect significant modifications in the activity of GST and content of GSH, even though GR displayed a descending tendency after 14 days of the oral chronic exposure compared to the group exposed for 7 days. Quite a few studies that investigated the Roundup herbicide effects on GST and GR did not detect any reduction or activity variations, and it was concluded that the toxicant was neutralized by another biotransformation mechanism (Langiano and Martinez, 2008; Lushchak et al., 2009). Loutfy and Kamel (2018) observed decrease in the liver GSH concentration after they subjected adult toads of *Bufo regularis* to two commercial formulations of neonicotinoid pesticides for 12 days. In rainbow trout (*Oncorhynchus mykiss*) chronic effect of fungicide was investigated (Li et al., 2010). The animals were exposed to three different sub-lethal concentrations for 7, 20 and 30 days. In two exposed groups significant reduction in liver GSH content after 20 days was detected, while it resumed to control level after 30 days in group subjected to the highest concentration. A considerable reduction in content of GSH was detected in gill and muscle of animals exposed to the highest concentration for 20 and 30 days. In animals subjected to pesticide for 30 days the highest reduction in GSH concentration was noticed. This investigation demonstrated that application for a long period causes a significant decline in GSH concentration probably as the outcome of low GSH synthesis.

In our study after dermal chronic application change in GSH concentration was not detected in investigated tissues. After chronic combine way (or-der) in liver was obtained decrease and in muscle was detected increase in 21 days treated group with respect to 14 days treated group. In skin was obtained increase in 21 days treated group in respect to 7 and 14 days treated group.

Opposed to the liver, higher GST activity in the GIT and the muscle of the orally chronic subjected animals in our study was connected with reduction of GSH-Px activity in muscle and a raise in activity of GSH-Px in GIT. Detected dissimilar modifications in activity of the enzymes may be the consequence of tissue-specific variations and inconsistent rates of free radical formation. In addition, the toxicant may be represented in different concentrations in the tissues (causing inhibition of varying extent), or the inhibitor could be processed at dissimilar extent (Nasia et al., 2018). The noticed enhancement in the GIT and the muscle GST activities after pesticide application is analogous to the investigation of Ezemonye and Tongo (2010) which examined the diazinon and endosulfan effect on GST activity of *Bufo regularis* adults during 28 days and obtained enhanced activity. In other study the same authors (Tongo and Ezemonye, 2013) detected GST activity growth in *B. regularis*. This increase they explained with detoxification reactions initiated by a pesticide. This defense mechanism is caused with GSH-group addition to xenobiotics, making them additionally hydrosoluble and easy to excrete (Moorhouse and Casida, 1992). From all of the above, it can be concluded that GSH-dependent enzymes have the possibility to restrict the damage produced by the ROS throughout pesticide-detoxification processes (Van der Oost et al., 2003).

The absence of a change in the activity of this enzyme, that takes part in the detoxification mechanism and contributes to phase II of biotransformation, is perhaps surprising but some results confirm our data. In carp *Cyprinus carpio* which was exposed to pesticide for 4 and 15 days, liver GST activity was inhibited after 4 days of exposure, but, on day 15 no significant differences were noticed in the enzyme activity (Hernández-Moreno et al., 2014). It was explained that the unmodified enzyme activity, after pesticide application, may point to enzyme inactivation by GSH consumption, making GST to lose its activity. Ensibi et al. (2013) conducted similar study and specimens of *Cyprinus carpio* were subjected to deltamethrin sublethal exposure for 4, 15 and 30 days. The animals were exposed to pesticide mixed to the aquaria water at different concentrations, which did not influence measured biometric parameters. Only after long exposure period of 30 days liver GST activity was increased in animals subjected to the high concentration of pesticide.

In our study dermal chronic exposure to pesticide did not disclose any important change in GST activity in any of the examined tissues, while chronic combined way (or-der) revealed raise in the muscle of the group exposed to 21 days compared to control group.

Canonical discriminant analysis performed in our study showed that in the gastrointestinal tissue GST and GSH-Px were the variables that contributed the most to the separation of the group of toads exposed to the oral application after 7 days. After 14 days of exposure CAT and GSH-Px were dominant parameters in group exposed to the oral way of application.

Sulfhydryl groups are significant indicators of the protein functional and conformational characteristics, and exposure of protein molecules to free radicals leads to many modifications that can result in structural changes which can bring to loss of enzymatic activity and consequently change physiological function (Headlam and Davies, 2004). After subjection of *B. viridis* specimens to the oral chronic application of DM, it is established that the profiles of thiol groups were significantly altered, even though the -SH groups content did not display any consistencies in examined tissues. The reduced -SH groups level in the liver tissue (after 14 and 21 days of exposure) and in muscle (after 14 days of exposure) validated the pesticide effect on the proteins.

Dermal and combined way (or-der) of application has led to a decrease of -SH groups concentration in liver on days 14 and 21 in respect to the group of toads exposed for 7 days. In the muscle and skin of treated animals no differences in concentration of -SH groups were observed. Nevertheless, in the GIT was detected the increase in concentration after 7 days of treatment with respect to control.

In another study the concentration of total sulfhydryls was determined in the liver of male Wistar rats exposed to deltamethrin 10 days *per os* (Cheshmedjieva et al., 1999). It was found a significant decline in the concentration of low-molecular thiols as a result of the treatments - about 50%, with 1/1000 of $LD_{50}$ of deltamethrin and about 63 %, with 1/100 of $LD_{50}$ of deltamethrin.

In the present study elevated content of TBARS on the 14th day of the research also confirmed liver oxidative damage after oral chronic exposure. The reduced SOD activity in the liver of treated animals in respect to the control, together with the concurrent declining activity of GSH-Px and the enhance in TBARS content, lead us to the assumption that, after prolonged exposure to pesticide, AOS in the liver was extremely endangered by generated free radicals. A lot of papers reported a change in activity of antioxidant enzymes and enhanced TBARS levels in animals after 15 and 30 days exposition to DM (Manna et al., 2005; Rehman et al., 2006).

After dermal application TBARS concentration in GIT was increased on day 14 related to the control group of animals and group treated for 21 days. In the muscle after combined exposure on day 14 was obtained an increase of TBARS concentration in respect to the group of animals subjected to pesticide for 7 days. According to Canonical discriminant analysis, in the muscle TBARS and SH groups were the variables that contributed the most to the separation of the group of toads exposed to the oral application after 14 days of exposure to deltamethrin.

Ensibi et al. (2013) demonstrated an increase in MDA concentration after 30 days of deltamethrin treatment, in the liver of common carp. The rising MDA content pointed at increased production of ROS which is related to deltamethrin metabolism bringing to the membrane lipids peroxidation in the liver. In the study of Yin et al. (2014) the MDA concentration in the treated groups of *Bufo bufo gargarizans* tadpoles decreased significantly compared to the control after 30 days of exposure to insecticide spirotetramat. As they discussed, reduced MDA contents may be a sign of the defensive effects of antioxidant system in *B. gargarizans*. Kaur and Sandhir (2006) investigated the comparative effects of both ways of exposure (acute and chronic) to different oral doses of carbofuran in the liver of Wistar rat. The obtained results demonstrate that rise in lipid peroxidation was higher in chronically exposed rats (60%) than in acute exposed animals (14%). Obtained results they explained with continuous production of free radicals over a long period.

Cholinesterases are widespread enzymes represented in the central nervous system and, in toxicological investigations of the hazardous effects of pesticides, both enzymes (AChE and BChE) are applied as parameters of neurotoxicity (Triquet-Amiard et al., 2012).

After chronic oral exposure to deltamethrin in our experiment, an increase in ChE activity was detected on the 21st day of pesticide application in liver of treated animals with respect to control. An increase in ChE activity was obtained in the GIT after 7 and in the muscle after 21 days of oral exposure concerning the control group. These data are in consentient with the investigation of Dey et al. (2016) who obtained a rise in AChE activity in the muscle of *Heteropneustes fossilis* and *Oreochromis niloticus* after glyphosate intoxication for 30 days. Comparable results reported Moraes et al. (2007) in teleost fish (*Leporinus obtusidens*) as a reply to herbicide exposure.

Our results indicated that toads are able to compensate for the stress, created by long-term DM exposure by increasing the activity of this enzyme. Contrary to our data, Tongo et al. (2012) reported a reduction in AChE activity in the liver and GIT in *B. regularis* in response to 28 days of diazinon application. However, after 28 days of endosulfan application to *B. regularis*, no significant changes in activity were observed (Tongo and Ezemonye, 2013).

After dermal application, we detected increase in ChE activity in the liver on day 21 in respect to control. In the muscle of treated animals we observed rise on day 14 in respect to control, as well as on day 21 with respect to day 7. After combined way (or-der) of application, ChE activity in liver was increased on day 21 in comparison to control. In the muscle was observed a rise in ChE activity after 21 days of treatment with respect to control and other treated groups of animals. Nevertheless, in the GIT was detected the increase in ChE activity after 7 days of treatment and decrease after 21 days of treatment with respect to control groups of animals.

This investigation represents a multi-biomarker study that is often not easy to interpret, and the obtained data are difficult to integrate into the ecotoxicological framework. IBR is mathematical tool that assimilate a variety of biomarkers to a particular value, which can be useful for expression of stress level caused by toxicants (Broeg and Lehtonen, 2006). In our study we define the IBR values for liver tissue since it is the principal metabolic center with powerful antioxidant ability. After chronic orally applied DM, the highest biomarker reply was obtained in the group of animals

subjected to pesticide for 21 days. Evidently that there are differences in the liver tissue between the group of toads subjected longer to the deltamethrin versus groups of animals subjected for brief periods so a causal relationship was observed between time-dependent exposure to the toxicant and examined biomarkers. IBR displayed an adequate accordance with the time of exposure of different animal groups, since the IBR index was the highest in the group of animals exposed to pesticide for 21 days.

When we look all the results together, after chronic orally applied DM we can draw a general conclusion: most of the investigated factors in the GIT displayed major enhance on the $7^{th}$ day of pesticide exposure (CAT, GSH-Px, GST activities and -SH groups and TBARS concentrations). Noticed pattern is associated with the fact that the deltamethrin was orally consumed, so the GIT was the primary contact point with the pesticide.

Canonical discriminant analysis of examined oxidative stress parameters after chronic dermal application revealed dissociation of SOD from other biomarkers in liver, muscle and GIT. In this application mode, like in oral exposure, this enzyme has an important position in antioxidant defense. In the combined mode of exposure, it was not possible to observe any consistencies in examined parameters.

Based on the results of this doctoral dissertation presented so far, it has been established that deltamethrin exposure leads to oxidative stress in the liver, muscle, GIT and skin of *B. viridis*. Detected changes in the values of antioxidant defense parameters display specificity of the response that depends on the applied concentration, time and route of exposure, as well as the type of tissue exposed to pesticides. It was found that different tissues react in different ways and with varying intensities to the presence of toxins.

# 6. CONCLUSIONS

Based on the results presented in present doctoral thesis, after examination of oxidative stress, biotransformation and neurotoxicity parameters in the liver, muscle, gastrointestinal tissue and skin of the green toad *Bufotes viridis*, during acute and chronic exposure to different DM concentrations, the following conclusions can be made:

1. Animals exposed to deltamethrin developed tissue-specific adaptive reactions to protect organism from to the resulting oxidative stress. These reactions do not exhibit a uniform pattern in different tissues and probably are the consequence of either the inconsistent rate of free radical formation in different tissues or of different distributions and deltamethrin concentrations in different tissues.

2. After chronic exposure, a detectable level of DM was not found in liver tissue. Our investigation indicates that this pesticide does not exibit a tendency to bioaccumulate in toad tissues because of its rapid metabolism.

3. The absence of change in the activity of GST in the *B. viridis* liver, after acute deltamethrin application, coincided with CYP1A induction. Our results confirm that CYP enzymes in the liver are involved in the main pathway of DM metabolism.

4. This study confirmed the importance of SOD, CAT and GSH-Px, as the first-line of defense against oxidative damage. SOD and GSH-Px were not activated in the liver during acute DM administration, which was followed by a disturbed oxidative status of sulfhydryl groups and promotion of lipid membrane peroxidation. During chronic administration of the DM, SOD and CAT were activated in the liver. This coordinated action was sufficient to deal with the generated oxidative stress and no increase in TBARS concentration was detected.

5. After acute deltamethrin application, the highest number of correlations between enzymes was revealed in the liver and gastrointestinal tissue. The obtained results indicate that these tissues displayed high degree of coordination of antioxidant parameters: the liver as the main site of storage, biotransformation and excretion of pesticides and gastrointestinal tissue as the first line of contact with the orally taken pesticide. Nevertheless, regardless of the good correlation and reciprocity of the examined parameters, the most affected and most susceptible tissue after acute exposure was the liver, as indicated by variations in -SH group and TBARS content. The lowest number of correlations was obtained for the skin, which was expected, taking into account that it is a peripheral tissue.

6. In our study we defined the IBR index for liver tissue since it is the most important metabolic organ with a powerful antioxidant capability. The highest biomarker response was obtained in the group of toads that were exposed orally to DM for 21 days. A causal relationship was observed between time-dependent exposure to the pesticide and the examined biomarkers.

7. After chronic oral application of DM we conclude that most of the investigated parameters in gastrointestinal tissue displayed significant increases on the $7^{th}$ day of application (CAT, GSH-Px, GST activities and concentrations of -SH groups and TBARS). This pattern could be explained with the fact that the DM was rapidly absorbed after oral administration and that this tissue was the first point of contact with the pesticide.

8. After chronic oral application of DM we observed SOD as the most prominent component among the battery of AOS enzymes. The antioxidative strategy of *Bufotes viridis* was based on the utilization of SOD to maintain the oxidative imbalance at an acceptable level during prolonged DM exposure. After chronic dermal application, discriminant analysis revealed the dissociation of SOD from other biomarkers in the liver, muscle and GIT. As in the oral model of exposure, this enzyme had a significant role in the antioxidant defense against prolonged exposure to the pesticide. After combined exposure, it was not possible to observe any consistencies in the examined physiological parameters.

## GENERAL CONCLUSION

Taking into account its widespread use, the performance of acute and chronic toxicity tests of DM in nontarget species such as toads is necessary. The results of the present doctoral dissertation imply that this pyrethroid is hazardous for amphibians and that exposure to it can have numerous harmful physiological consequences which lead to oxidative stress in different toad tissues. The detected changes in the values of oxidative stress parameters reveal specificity of the response that depends on the applied concentration, time and route of exposure, as well as on the type of tissue exposed to the pesticide. Our results contribute to a better understanding of the mechanisms included in deltamethrin toxicity. The present investigation provides useful data for understanding the effects of DM in future ecotoxicological studies.

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# Изјава о ауторству

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Број индекса Б3048, 2014

**Изјављујем**

да је докторска дисертација под насловом:

The effects of pyrethroid insecticide deltamethrin on oxidative stress parameters in different tissues of green toad (*Bufotes viridis*)

Ефекти пиретроидног инсектицида делтаметрина на параметре оксидативног стреса у различитим ткивима зелене краставе жабе (*Bufotes viridis*)

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- да су резултати коректно наведени и
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**Потпис аутора**

У Београду, _____

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# Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора Mohammed Melad Lamin Nasia

Број индекса Б3048,2014

Студијски програм Анимална и хумана физиологија

Наслов рада:

The effects of pyrethroid insecticide deltamethrin on oxidative stress parameters in different tissues of green toad (*Bufotes viridis*)

Ефекти пиретроидног инсектицида делтаметрина на параметре оксидативног стреса у различитим ткивима зелене краставе жабе (*Bufotes viridis*)

Ментори: Др Тијана Б. Радовановић, виши научни сарадник, Институт за биолошка истраживања „Синиша Станковић" – Институт од националног значаја за Републику Србију, Универзитет у Београду

Др Синиша Ф. Ђурашевић, ванредни професор, Биолошки факултет, Универзитет у Београду

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањена у **Дигиталном репозиторијуму Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

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У Београду, _____

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# Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

The effects of pyrethroid insecticide deltamethrin on oxidative stress parameters in different tissues of green toad (*Bufotes viridis*)

Ефекти пиретроидног инсектицида делтаметрина на параметре оксидативног стреса у различитим ткивима зелене краставе жабе (*Bufotes viridis*)

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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