Impact of desiccation pre-exposure on deltamethrin-induced oxidative stress in *Bombina variegata* juveniles

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ABSTRACT

Global warming represents a severe threat to existing ecosystems, especially for anuran tadpoles who encounter significant fluctuations in their habitats. Decreasing water levels in permanent and temporary water bodies is a significant risk for larval survival or fitness. On the other hand, the natural environment of amphibians is extremely polluted by various xenobiotics. This study evaluated how pre-exposure of *Bombina variegata* tadpoles to chronic environmental stress (desiccation) modulates the biochemical response of juvenile individuals to following acute chemical stressor (pesticide deltamethrin). Our results demonstrated that individually applied pesticide changed the thiol and lipid status of the treated juveniles but animals subjected solely to desiccation pressure were more tolerant to free radicals and showed no induction of lipid peroxidation. Comparison of juveniles exposed to deltamethrin revealed that desiccation pretreatment during the larval stage of development modified cellular protection in the juveniles. Higher activities of CAT, GSH-Px and GR were recorded in the pre-exposed group, as well as a lower degree of lipid peroxidation relative to the group that was not pre-exposed to low water stress. Pre-desiccated groups displayed the greatest range of coordination of investigated antioxidant parameters, supported by Pearson's correlations. Activation of the GSH-redox system is a significant marker in juveniles against stress caused by desiccation and a chemical stressor. The stressful environment experienced during tadpole development produced an adaptive reaction to subsequent exposure to another stressor in juveniles. To develop relevant management and conservation strategies, more studies of the interactive effects of environmental and chemical stressors are necessary.

Keywords: amphibian larvae; desiccation pre-treatment; pesticide; oxidative damage; toad juveniles

1. Introduction

Accelerated industrial and urban development over the last hundred years has intensified the need for the exploitation of natural resources, which has in turn aggravated the worldwide problem of environmental pollution. The natural environment is increasingly contaminated with various hazardous compounds, such as organic contaminants, heavy metals, organometallic mixtures, microplastics, active pharmaceutical ingredients and gaseous pollutants (Walker et al., 2012; Freitas et al., 2020; Pagano et al., 2020). Anthropogenic contamination has to a great extent disordered the natural balance of ecosystems and has triggered harmful consequences on humans, plants and animals (Qyli et al., 2020). Due to their toxicity, persistence and non-degradability, most hazardous substances represent a potential threat to living organisms. Through circulation in the biosphere they accumulate at different trophic levels and can contribute to the degradation of natural ecosystems by reducing species diversity and abundance (Pushkar et al., 2021). When contaminants enter organisms through the water, air or food, they can have fatal consequences (Paital et al., 2019; Paital and Agrawal, 2020).

The toxicity of pollutants and their effects on amphibians have aroused increased concern for over two decades as their populations have declined worldwide (Stuart et al., 2004; Samanta and Paital, 2016). Because of their biphasic development, amphibians are a unique sentinel group among all vertebrates. Throughout their lifecycle they are integral components of the aquatic

habitat as larvae, and as adults, of both aquatic and terrestrial habitat. This makes amphibians vulnerable to numerous environmental stressors arising from anthropogenic activities, such as pollution and climate change, as well as from natural sources (Noyes et al., 2009; Narayan, 2016), and are recognized as one of the most threatened vertebrate groups (Blaustein and Wake 1990; Blaustein, 1994). Climatic factors are also exerting considerable impact on population dynamics and amphibian pervasiveness. Global warming, as a crucial aspect of climate change, is a serious risk to existing ecosystems and is related to a variety of consequences, such as unfavorable meteorological conditions, resource consumption and biodiversity loss (Verplanken et al., 2020). Many amphibian species live and develop in permanent water bodies such as lakes, rivers and swamps, but some of them depend on provisional water bodies that are sporadically filled by rain (Gomez-Mestre et al., 2013). In such an inconstant natural environment, desiccation is a significant environmental risk factor influencing larval survival rate or fitness of following developmental phases.

During a set of modifications necessary for the metamorphosis, amphibians undergo serious transformations of oxidative metabolism (Gomez-Mestre et al., 2013; Burraco et al., 2017) and enhanced production of reactive oxygen species (ROS) (Johnson, et al., 2013). Environmental conditions experienced during the larval stage often affect and influence the development and overall fitness of post metamorphic individuals (Ruthsatz et al., 2020). The consequences of suboptimal developmental conditions can be transferred to the next stage of development and cause serious carry-over effects on juveniles and adults (Pechenik, 2006, Ruthsatz et al., 2020). However, the adaptive reaction to stressful conditions can elevate cellular defenses that can ensure additional protection from a second, more serious stressor (Mattson,

2008). Numerous studies reported the adaptive response of adult amphibians to pesticides, as one of the most ubiquitous anthropogenic disruptors (Isnas et al., 2012; Özkol et al., 2012; Radovanović et al., 2017; Loutfy and Kamel, 2018). Yet, the influence of deltamethrin on post-metamorphic amphibians has not been sufficiently investigated, neither their influence in combination with environmental stressors during previous developmental stages. More attention should be paid to the juvenile phase in amphibian development in terms of oxidative stress because it can be seen as an endpoint of the action/co-action of multiple stressors.

In the context of all the above, the aim of our investigation was to examine whether and how pre-exposure to chronic desiccation stress (in the form of low water conditions) during larval development modulates the adaptive response of *Bombina variegata* juveniles to following acute stress caused by a selected chemical stressor (pesticide). We also assessed whether the supplementary chemical stress exacerbates the consequences of desiccation experienced during metamorphosis. The biological responses in juveniles were evaluated in accordance with individual biomarkers (oxidative stress and neurotoxicity) and the integrated biomarker response (IBR). We predicted that initial environmental stressor influenced the effects of the chemical stressor and that successively subjected stressors will interact across life stages, producing modifications of the examined parameters and the response of the antioxidant system (throughout IBR). Gained results can be crucial to define the magnitude and mutual influence of different stressors on amphibian populations in order to develop appropriate management and conservation strategies.

2. Material and methods

2.1. Experimental setup

The sampling area is located in the National Park Fruška Gora, in northern Serbia, (539 m s.l., 45°0'N-45°15' N, and 16°37'E-18°01'E) where *Bombina variegata* (yellow-bellied toad) uses temporary or ephemeral ponds as breeding sites. On 22nd May 2020, we collected the newly deposited eggs of five clutches from different forest ephemeral ponds. The eggs were brought to the Institute for Biological Research "Siniša Stanković", Belgrade and were maintained in individual 2 L-tanks, filled with dechlorinated tap water, until the organisms reached the free swimming and feeding developmental stage 25 (Gosner, 1960). In the laboratory, constant environmental conditions were provided (a natural photoperiod and 20ºC room temperature).

All eggs were collected under the permit provided by the Ministry of Environmental Protection of the Republic of Serbia (Permit No. 353-01-2876/2019-04). The Veterinary Directorate, Department of Animal Welfare, Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia, approved the study design (Approval No. 323-07-08393/2020-05/4). The experimental procedure was conducted following the European Directive (2010/63/EU) on the protection of animals used for experimental purposes, as well as the ARRIVE guidelines and Code of Practice for the Housing and Care of Animals used in Scientific Procedures.

2.1.1. Desiccation treatment

At Gosner stage 25 (Gosner, 1960), 30 tadpoles per clutch (150 individuals in total) were randomly selected and placed in plastic containers (42 × 32 × 21.5 cm). Tadpoles were raised in cohorts of five individuals per container to avoid density effects on life history traits. In the experimental setup two groups were formed as follows: the group that served as the control was maintained in 10 L of water and a water depth of 9.4 cm; the desiccation group was kept in 5 L, water depth 4.7 cm. The low water level treatment was initiated the when individuals reached stage 36 (Gosner, 1960; the appearance of individual toes).

A total of 30 experimental units (plastic containers) were positioned randomly in the room (with 5 tadpoles per box). The water (dechlorinated tap water) was changed every 4th day following a random repositioning of containers to avoid any effects due to position. On every second day the individuals were fed *ad libitum* with one tablet per tank of commercial fish food (Tetra TabiMin®, Tetra GmbH, Melle, Germany). Containers were checked daily for accurate detection of metamorphosing individuals.

After reaching the stage 42 and the appearance of forelimbs (Gosner, 1960), a wet-terrestrial environment was prepared for the metamorphic individuals until the tail was fully resorbed at stage 46 (Gosner, 1960). In this phase we recorded the body mass (BM), and body length (snout-to-vent length (SVL)) from photos using the geometric morphometric software TpsDig2 (Rohlf, 2015-Rohlf, F.J. TPS Dig 1.31 and TPS relative wards software, 2001, State University of New York, Stony Brook, USA). The condition factor (CF) was calculated according to Bagenal and Tesch (1978) and served as an indicator of the overall health of the animals.

2.1.2.. Pesticide treatment

These post-metamorphic individuals were further used in the part of the experiment which was concerned with acute stress caused by the chemical stressor (pesticide). From the total number of juvenile individuals (75 animals from the control treatment and 75 animals from the desiccation treatment), we randomly selected 20 individuals developed in a constant water level, and 20 individuals developed under desiccation conditions, and formed 4 groups of 10 individuals each (Figure 1). The remaining individuals were returned to their natural habitat on Fruška Gora.

The first group served as the control (individuals that were not exposed to either desiccation or pesticide action are referred to as the control-control group (CC). The second group included individuals that were not subjected to the desiccation process but only to the action of pesticides in the post-metamorphic phase and are referred to as the control-deltamethrin group (CDM). The animals from the third group were exposed to the desiccation process but not to the activity of the pesticide and are referred to as the desiccation-control group (DC). The 4^{th} group was comprised of juveniles that were first exposed to the desiccation conditions during development and then to deltamethrin, and are referred to as the desiccation-deltamethrin group (DDM).

Prior to pesticide exposure, all 40 yellow-bellied toad juveniles were dehydrated for 5 h in 4 unlined, clean plastic 20 L boxes (38.5 × 28 × 19 cm) (Van Meter et al., 2014). This enabled easier uptake through the dermis and rehydration when the exposure step was initiated. After 5 h of dehydration, individuals were transferred to four experimental boxes with soil substrate.

Experimental units were 20 L plastic boxes (38.5 × 28 × 19 cm), lined with 750 g of soil (Van Meter et al., 2014). In two experimental chambers, 250 mL of previously prepared solution (0.1 mL K-Othrine SC 25 dissolved in 250 mL of tap water) was sprayed evenly over the surface of the soil and 10 individuals were immediately added to each chamber for a 24-h of pesticide exposure (CDM and DDM exposure groups). The concentration of deltamethrin was 1 mg/100 mL H_2O. The experimental solution was prepared according to the instructions of the producer (manufacturer). Commercial technical-grade deltamethrin (2.5 %) was chosen as a product widely used in agriculture. It was prepared freshly in water to prevent potential instability.

In the remaining two experimental boxes, the soil was sprayed with 250 mL of tap water and another ten individuals were transferred to each chamber (CC and DC exposure groups). To maintain juveniles in contact with pesticide-sprayed soil, a mosquito net was provided inside each box at a height of 2.5 cm over the soil surface (Van Meter et al., 2014). This prevented juveniles from climbing the plastic walls of the chambers and allowed them to move freely on the ground. At termination of the experiment, after 24-h of exposure, the animals were sacrificed by immersion in liquid nitrogen and stored at 80°C until further biochemical analysis (Underwood et al., 2013; Pinya et al., 2016).

2.2. Tissue processing

The bodies of juveniles were finely cut into pieces and mixed to obtain sufficient homogenous samples. For assessment of thiobarbituric acid-reactive substance (TBARS) concentration, 0.2 g of the tissue was taken. Minced tissue parts were homogenized and

sonicated at pH 7.4 in 10 volumes of ice-cold Tris-HCl buffer. After 10 min centrifugation in 40 % TCA at 10 000×g at 4°C, the obtained supernatants were used for lipid peroxidation (LPO) determination. The remaining tissue was used for the determination of antioxidant and neurotoxicity parameters and determination of the protein carbonylation (PC) level.

The tissue samples were homogenized in 5 volumes of 25 mM sucrose buffer, pH 7.4 (Lionetto et al., 2003) containing 10 mM TrisHCl and 5mM EDTA, (Rossi et al., 1983) with a homogenizer Ultra-Turrax; Janke and Kunkel, IKA-Werk. The homogenates were sonicated with a Sonopuls ultrasonic homogenizer (HD 2070, Bandelin Electronic, Germany) for 30 s at 10 kHz, and sonicate aliquots were immediately taken for total glutathione (GSH) concentration determination. For the determination of additional biochemical parameters, the remaining sonicate was centrifuged for 90 min at 100 000 g at 4°C (Takada et al., 1982) to acquire the supernatant.

2.3. Biochemical analyses

The total protein concentration was estimated at 500 nm using the Lowry method with bovine serum albumin (BSA) as standard (Lowry et al., 1951). The activity of superoxide dismutase (SOD) was measured according to the procedure of Misra and Fridovich (1972), based on the autoxidation of adrenaline to adrenochrome and monitored at 480 nm. The Claiborne assay (1984) for catalase (CAT) analysis was performed at 240 nm; it includes measurement of hydrogen peroxide decomposition. The method for determination of glutathione peroxidase (GSH-Px) activity (Tamura et al., 1982), and the assay for glutathione reductase (GR) activity

(Glatzle et al., 1974) were based on the rate of NADPH oxidation. Glutathione-*S*-transferase (GST) activity was established by interaction of the SH group of GSH with 1- chloro-2,4-dinitrobenzene (CDNB) (Habig et al., 1974). GSH-Px, GR, and GST activities were assessed at 340 nm. The activities of all enzymes were expressed in U/mg protein. The concentration of total GSH was measured by the Griffith method (1980). The reaction took place in the presence of GR and was based on the GSH oxidation (by 5,50 -dithiobis-(2-nitrobenzoic acid) (DTNB)) and NADPH reduction. The concentration was determined at 412 nm and expressed as nmol/g tissue. For measuring oxidative damage of lipids, the TBARS method described by Rehncrona et al. (1980), was used. LPO was evaluated at 532 nm and expressed in nmol TBARS/mg tissue. The level of PC was determined according to the 2,4-dinitrophenylhydrazine (DNPH) alkaline method at 450 nm (Mesquita et al., 2014) and gained values were expressed in nmol/mg protein. The activity of the neurotoxicity parameter cholinesterase (ChE) was monitored by measuring the thiol reaction between DTNB and acetylcholine iodide as a substrate (Ellman et al., 1961). The product detected spectrophotometrically at 412 nm was yellow 5-thio-2-nitrobenzoate, and activity was expressed as μmol/min/g tissue.

All investigated parameters were measured at 25ºC using a UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan). All chemicals used in the experiment were purchase from Sigma (St. Louis, MO, USA).

2.4. Statistical analysis

The Grubbs test preceded other statistical analyses and outlier data were verified and excluded. The data were checked for assumptions of normality with the Kolmogorov-Smirnov one-sample test and the examined components had a normal distribution. We implemented one-way analysis of variance (ANOVA) to test body size differences between the examined groups. Factorial ANOVA was implemented to investigate possible distinctions between two independent variables-desiccation (constant water level for the control and low water level for the desiccation group) and deltamethrin (presence/absence of pesticide) and their relationship with oxidative stress parameters. The post hoc Fisher's least significant difference (LSD) test was used to examine differences among the treated groups. The relationships between investigated biochemical parameters were examined with Pearson's correlations. Analysis was performed independently for each treated group of juveniles. The correlation coefficient was considered significant at $p < 0.05$. The general AOS response was calculated and expressed as the integrated biomarker response (IBR) value based on Devin et al. (2014). All data were analyzed with statistical software STATISTICA 8.0, except the IBR index that was calculated in R 3.4.1.

3. Results

3.1. Biometric parameters

During the experiment, no lethality or visual modification in the activities of post-metamorphic individuals were noted in any of the examined groups. No changes in swimming or eating behavior were detected.

The measured values for the biometric parameters are presented in Table 1. The juveniles from the CC and CDM groups had significantly higher BM in comparison with other two groups. CF of the juveniles from the DC group was statistically lower in respect to CC and CDM groups. Statistical analysis demonstrated that there were no significant distinctions for the SVL parameter among the four investigated groups.

3.2. Oxidative stress and neurotoxicity parameters

The results for factorial ANOVA showed significant differences for the desiccation treatment (decreasing/constant water level) with respect to GSH-Px and GR activities and GSH and LPO concentrations. Significant differences for the deltamethrin treatment (presence/absence of pesticide) were recorded for GSH and LPO concentrations. A significant interaction deltamethrin × desiccation was observed for GSH (Table 2).

The post hoc Fisher's LSD test was performed for each factor and on their significant interaction. The results for antioxidant system parameters are presented in Figure 2. The recorded data show that the activity of SOD was higher in the group of juveniles that were pre-exposed to desiccation and additionally subjected to pesticide stress (DDM) in comparison to the group that was subjected only to desiccation stress (DC) (Fig. 2A). The group of individuals that were exposed to both stressors had significantly higher CAT activity in respect to the CDM

group (Fig. 2B). Comparisons of GSH-Px activity revealed variations among the treated groups. Higher GSH-Px activity was recorded in the DDM group of animals versus the control group and the group subjected only to acute pesticide stress (Fig. 2C). In the group reared in low water conditions (DC) and the group subjected to both stressors (DDM), the activities of GR were statistically higher than in the control. This enzyme was also higher in juveniles exposed to the effects of both stressors (DDM) as compared to the group exposed to the individual pesticide stress (CDM) (Fig. 2D). The GSH level was significantly elevated in all treated groups with respect to the control (Fig. 2F). No significant differences among treated groups were observed for GST activity (Fig. 2E).

The results for oxidative and neurotoxicity stress parameters are presented in Figure 3. The concentration of TBARS, as the marker of LPO, was significantly higher in the CDM group than in the CC and DDM groups. In the group that was exposed to both stressors (DDM) this parameter was elevated with respect to the control and the group of juveniles reared in low water conditions (DC) (Fig. 3A). No significant differences among the treated groups were noted for the PC level and ChE activity (Fig. 3B, 3C).

3.3. Pearson's correlations

Connections between the examined antioxidant parameters were analyzed with Pearson’s correlations (Figure 4). Most significant correlations were detected in groups pre-exposed to low water levels (DC group-seven significant correlations and DDM group-six significant correlations). Only one significant correlation was observed in CDM and CC group.

3.4. Integrated biomarker response (IBR)

The obtained results for IBR showed that the group of toads exposed to desiccation and pesticide stress (DDM) had the highest biomarker response, with an IBR value of 8.53 ± 0.01, followed by DC 6.50 ± 0.02, and CDM 0.56 ± 0.03. On the other hand, according to the evaluation of all biomarker sets, the control group had the lowest value (0.29 ± 0.03). The obtained scores for IBR were specific for every examined group and were used to make the star plot (Figure 5). The lines in each axis are established by the biomarker response to stress exposure and correlate with the relative biomarker response within that particular exposure group. A star plot was created to ensure evaluation among the exposure groups for every set of biomarkers.

4. Discussion

The initial stages in development are life phases in which the level of oxidative stress is significantly elevated because of the correlation between high metabolic activities necessary for growth and consequent ROS formation (Monaghan et al., 2009). In the early developmental phase, environmental factors could additionally enhance ROS formation and provoke serious cell injuries affecting later life stages. Exposure to a stressful environment during the larval stage can extensively disturb performance and fitness through carry-over effects on one hand (Räsänen et al., 2002; Crean et al., 2011), but it can also lead to an adaptive response that can rise up a

cellular reply and protect the organism (Berry and López-Martínez, 2020). In that context, understanding how individuals challenged with chronic stress (desiccation) during early development respond to exposure to acute induced stress (pesticide) during later juvenile life stage can be crucial for understanding the anthropogenic effects on amphibian populations. Pesticides are well known as stress inducers and have been highlighted as one of the main factors leading to amphibian population decline (Hayes et al., 2010). Our study focused on the assessment of individual and interactive effects of the pesticide deltamethrin and desiccation pretreatment on the biometric and biochemical parameters during different developmental phases of *Bombina variegata*.

No significant change in the health status of the animals was observed, except for reduced body mass in the desiccated groups. In our experiment desiccation environment had a negative effect on fitness resulting in a lower BM of *B. variegata* individuals in the DC and DDM groups. The CF was lowest in the DC group. This is consistent with previous results obtained for individuals under metamorphic climax and juveniles of the same species (Sinsch et al., 2020; Petrović et al., 2021). Reduced body mass and size were also observed in *Agalychnis moreletii* juveniles after the desiccation period in comparison to control conditions (Hernández-Herrera et al. 2019).

Our results also indicated that both stressors contributed to alterations in examined oxidative stress parameters. Individually administered DM caused the change in a thiol and lipid status of the treated juveniles. The concentrations of GSH and LPO were higher in CDM group related to control. LPO was proposed as one of the cell mechanism associated with pesticide-induced toxicity (Khrer, 1993). The acquired results indicate that ROS overproduction could be

related to the metabolism of DM, which caused the peroxidation of lipid membranes. Elevation of the GSH content should be considered as a compensatory adaptive response to extreme free radical accumulation. GSH has been marked as an actor in the defensive mechanism that aquatic organisms activate during the initial phases of xenobiotics subjection (Stephensen et al., 2002). Previous investigations have reported the induction of LPO and modulation of AOS parameters by deltamethrin (Radovanović et al., 2017; Nasia et al., 2018) or other pesticides (Isnas et al., 2012; Loutfy and Kamel, 2018) in adult individuals.

Post-metamorphic individuals subjected only to desiccation stress (DC) exhibited higher GR activity and increased GSH concentration in comparison to individuals reared under constant water. The estimated components of the antioxidant defense were modulated in response to increased ROS production, which was insufficient to cause significant lipid damage. Animals subjected to desiccation stress were more tolerant to higher ROS concentrations in the war against free radicals than animals individually exposed to DM. Our results, together with the study of Burraco et al. (2017) on juvenile individuals pre-exposed to drying stress, suggest the capability of the AOS (primarily the GSH system) to cope with ROS production and lipid peroxidation.

To the best of our knowledge, there have been no reports on how joint (successive) desiccation-pesticide exposure affect the investigated biochemical parameters of amphibians. Such integrated actions can damage the antioxidant response, stimulating or suppressing enzyme activity. To neutralize the influence of ROS, several authors reported enhanced enzyme action as a general response to environmental stressors in aquatic animals such as amphibians and fish (Yin et al., 2014; Radovanović et al., 2017; Loutfy and Kamel, 2018; Gavrilović et al., 2021).

The interactive effect of desiccation conditions and pesticide action (DDM) in the present report was observed as increased GSH-Px and GR activities, and GSH and LPO concentrations with respect to non-stressed individuals (CC). GSH and GSH-coupled antioxidant parameters have an important position in the AOS. GSH is a low molecular weight sulfhydryl antioxidant that in response to toxicants behaves as a defensive, cellular-reducing component (Zhang et al., 2004). It acts directly by scavenging ROS, or indirectly as an antioxidant enzyme substrate (Li et al., 2010). The function of GSH-Px is complementary to CAT since both enzymes detoxify H_2O_2, but they differ in their affinities for H_2O_2, as well as in their subcellular localization (peroxisomal and cytosolic, respectively) (Halliwell and Gutteridge, 2015). Besides eliminating H_2O_2, alkyl peroxide, and fatty acid hydroperoxides, GSH-Px is involved in the detoxification of lipid peroxides by neutralizing the organic hydroperoxides found in lipoproteins and complex lipids. In the recovery process the enzyme GSH-Px uses GSH as a hydrogen donor. As the final result of this reaction, GSSG is formed and reduced by GR, and thereby the concentration of GSH in the cell is restored (Halliwell and Gutteridge, 2015). An attempt by the cell to defend itself was unsuccessful herein under the given conditions and oxidative damage occurred; the increases in AOS parameters can be considered as an adaptive reaction that was insufficient to prevent cell injury.

Other authors support the concept that environmental factors modulate the physiological stress response in organisms. The activities of CAT, SOD and glucose-6-phosphate dehydrogenase were enhanced by the herbicide clomazone in *P. nattereri* tadpoles treated at higher temperatures (Freitas et al., 2017). A higher temperature in combination with phenylurea herbicide induced increased activities of GST and GSH-Px in American bullfrog tadpoles (Grott

et al., 2021). In contrast, the level of LPO and GST activity were not affected by the combined effect of temperature, salinity and deltamethrin in black tiger shrimp *Penaeus monodon* (Tu et al., 2012). Taking into consideration all variations of the studied components, we can confirm that pre-exposure to chronic desiccation stress was a significant environmental factor that affected the activities of several biochemical parameters. This disturbance in homeostasis can have serious physiological consequences on individuals and their capacity to adjust their defensive response to stress.

Juveniles pre-exposed to desiccation followed by pesticide application (DDM) exhibited increased SOD activity and LPO concentration as compared to the DC group of toads. As we have seen, deltamethrin itself also causes an increase in LPO concentration in juveniles not previously exposed to desiccation (CDM). In both cases, the pesticide induced perceptible lipid damage, regardless of whether the individuals lived in constant water or in the desiccated environment.

Previous studies on anurans have described different responses in protein carbonyls and TBARS after exposure to different stressors (Falfushinska et al., 2008; Gavrilović et al., 2020). After comparing treated groups of juveniles in the present study, no changes were observed in PC contents. The absence of significant changes in the level of PC, together with the increase in TBARS concentration in individuals exposed to deltamethrin, point to lipid oxidation as a more sensitive indicator of oxidative damage caused by pesticide toxicity. Further, our results reveal an increased tendency to oxidative injury via LPO in juvenile individuals of this species. According to some authors, the absence of marked variations in PC level suggest that there was a rapid degradation of oxidized proteins which reinstated an oxidation equilibrium since it is

known that oxidative stress triggers multifunctional proteases, that degrade oxidized proteins (Starke et al., 1987, Bagnyukova et al., 2007).

Cholinesterases are universal enzymes located in the central nervous system and are implemented as parameters of neurotoxicity (Amiard-Triquet et al., 2012). A reduction in ChE activity was observed as a general pattern after exposure to sublethal concentrations of different neurotoxic pesticides (Booth et al., 1998; Sandoval-Herrera et al., 2019; Berntssen et al., 2021). Contrary to expectation, in the present study no change in ChE activity was detected. The absence of an enzyme response after deltamethrin exposure can be explained as the consequence of short-term exposure through the skin of juveniles. The environmentally realistic concentration applied via the dermal route was not sufficient to cause a neurotoxic effect after 24 h of exposure. The absence of deltamethrin influence on ChE activity has also been reported in the skin and muscle of toad *Bufotes viridis* (Radovanović et al., 2017) and muscle of *Ancistrus multispinis* (Assis et al., 2009).

Comparison of juveniles exposed to deltamethrin (CDM and DDM) revealed that the desiccation pretreatment modified cellular protection. This was observed through higher activities of CAT, GSH-Px and GR in the DDM group, which resulted in a lower degree of lipid peroxidation related to the CDM group. Maintenance of redox homeostasis depends on the joint activities of antioxidants, and effective protection is the result of the cooperation of all parts of the antioxidant system (Costantini et al., 2013). Desiccation exposure also induces a more integrated AOS response (higher values of IBR) in DC and DDM groups. Higher levels of integration can allow the AOS to more efficiently deal with increased ROS concentrations. In this study individuals that were exposed to desiccation during development (DDM) displayed a

better response to pesticide-induced stress, e.g. had lower oxidative damage in comparison to ones that were treated only with deltamethrin (CDM). These results can be interpreted within the framework of hormesis (Mattson, 2008), whereas stressful conditions experienced during the larval period can induce an adaptive response to the subsequent stressor (pesticide exposure). The hormetic response resembles the preparation to oxidative stress (POS), an adaptive physiological response, which was identified in 83 species (Giraud-Billoud et al., 2019). POS was examined in various stressful environmental situations such as dehydration and freezing, but it has been most investigated in hypoxic conditions.

This assumption of elevated adaptive response to the subsequent stressor in the present study is reinforced by the results of Pearson's correlations, where a high degree of mutuality between the examined parameters in the pre-desiccated groups (DC and DDM group, 7 and 6, respectively) was obtained. These groups demonstrated the coordination of examined antioxidant components, which was to be expected, given that the animals were pre-exposed to chronic stress. Over time, a mutual relationship between parameters was built to better protect the organism from deleterious effects. On the other hand, only one correlation was recorded in the control group and the group that experienced just acute-pesticide induced stress (CDM group). Of all the investigated parameters, GSH was the component with the largest number of correlations in the treated groups. Most correlations of GSH were with components of the GSH system, GSH-Px, GST and GR. This implies that GSH has an important role in the juvenile AOS, and that the GSH-redox cycle provides major protection in post-metamorphic individuals against desiccation/deltamethrin mediated stress.

IBR revealed the details of the biochemical responses obtained for each group. The presented data show that the group of juveniles exposed to desiccation and pesticide stress exhibited the highest biomarker response, and that the greatest response of the AOS was provoked by the greatest stress.

In summary, we indicated that desiccation stress is an important factor inducing changes in the ensuing biochemical responses in *B. variegata* juveniles exposed to the chemical stressor. Pre-exposure to environmental stress can enhance the biochemical stress response in amphibians. Changes in one environmental variable (water level) during early developmental phases had a substantial effect on the toxicity of the chemical stressor in later formative stages. Based on the investigated biomarkers, it can be concluded that pre-stress exposure can be beneficial and that *B. variegata* juveniles showed better adaptability after longer subjection to the chronic environmental stressor. Our investigation provides insight into the adaptation of amphibian populations in natural surroundings by highlighting the protective function of GSH and GSH-dependent enzymes.

Declaration of competing interest

The authors have declared no conflict of interest.

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Table 1

The total snout-vent length (SVL), body mass (BM) and condition factor (CF) of *Bombina variegata* juveniles in four experimental groups (CC – control-control, CDM – control-deltamethrin, DC – desiccation-control, DDM – desiccation-deltamethrin), ten animals per group. The data are expressed as the mean ± SE. Different letters indicate significant differences between groups (Fisher LSD, $p < 0.05$).

	CC	CDM	DC	DDM
SVL (cm)	1.64 ± 0.19^{a}	1.59 ± 0.22^{a}	1.62 ± 0.19^{a}	1.60 ± 0.023^{a}
BM (g)	0.47 ± 0.01^{a}	0.45 ± 0.01^{a}	0.39 ± 0.01^{b}	0.40 ± 0.01^{b}
CF	10.81 ± 0.35^{a}	10.71 ± 0.38^{a}	9.22 ± 0.40^{b}	10.04 ± 0.41^{ab}

Table 2

Results of factorial ANOVA for the comparison between the deltamethrin treatment (presence/absence of pesticide) and desiccation treatment (decreasing/constant water level) and the interaction between the treatments for the examined biochemical parameters.

Parameter	Effects	df	F	p
SOD	deltamethrin	1	2.487	0.123559
-	desiccation	1	0.215	0.645929
-	deltamethrin × desiccation	1	2.994	0.092140
CAT	deltamethrin	1	1.890	0.177692
-	desiccation	1	3.157	0.084031
-	deltamethrin × desiccation	1	1.533	0.223713
GSH-Px	deltamethrin	1	0.024	0.876743
-	**desiccation**	**1**	**9.816**	**0.003430**
-	deltamethrin × desiccation	1	0.485	0.49080
GR	deltamethrin	1	0.140	0.710052
-	**desiccation**	**1**	**11.309**	**0.001841**
-	deltamethrin × desiccation	1	0.114	0.737980
GST	deltamethrin	1	0.2171	0.644069
-	desiccation	1	2.1873	0.147849
-	deltamethrin × desiccation	1	0.9876	0.326953

GSH	**deltamethrin**	**1**	**19.681**	**0.000083**
-	**desiccation**	**1**	**8.128**	**0.007173**
-	**deltamethrin × desiccation**	**1**	**13.424**	**0.000794**
LPO	**deltamethrin**	**1**	**59.9881**	**≥0.000001**
-	**desiccation**	**1**	**4.4268**	**0.042427**
-	deltamethrin × desiccation	1	1.5163	0.226164
PC	deltamethrin	1	0.053	0.819119
-	desiccation	1	3.299	0.077664
-	deltamethrin × desiccation	1	0.034	0.854279
ChE	deltamethrin	1	1.419	0.241376
-	desiccation	1	2.853	0.099843
-	deltamethrin × desiccation	1	0.041	0.840279

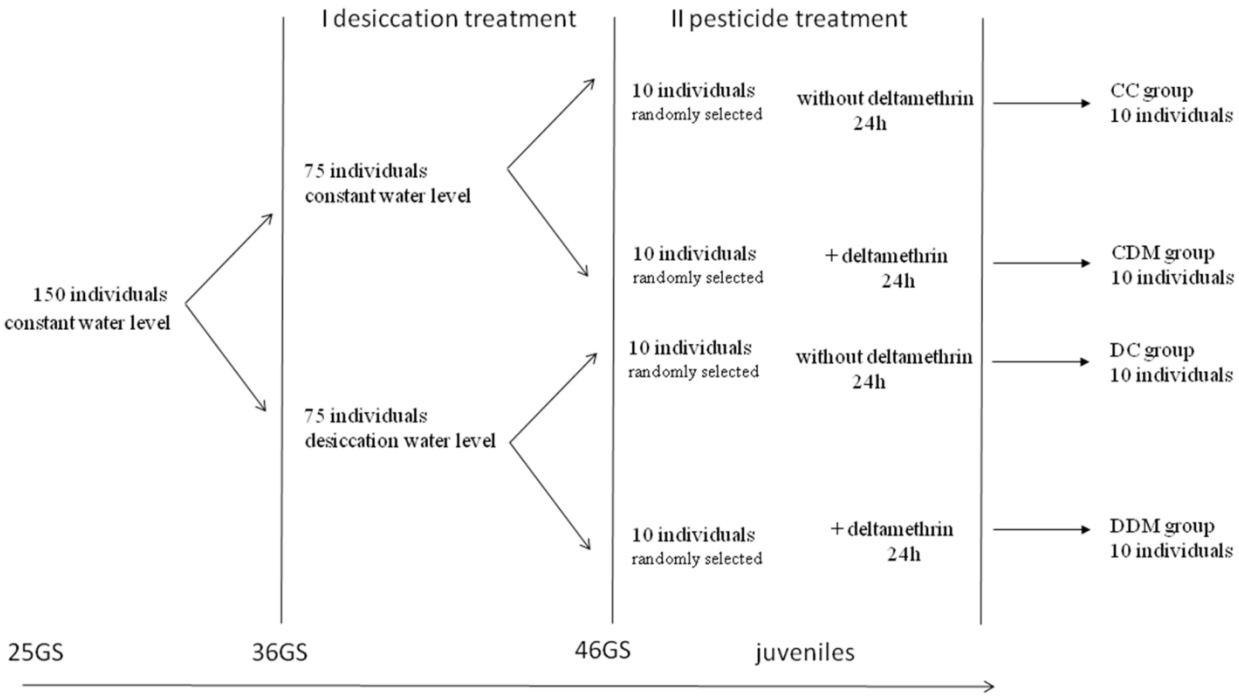

Fig. 1. Experimental setup diagram**.**

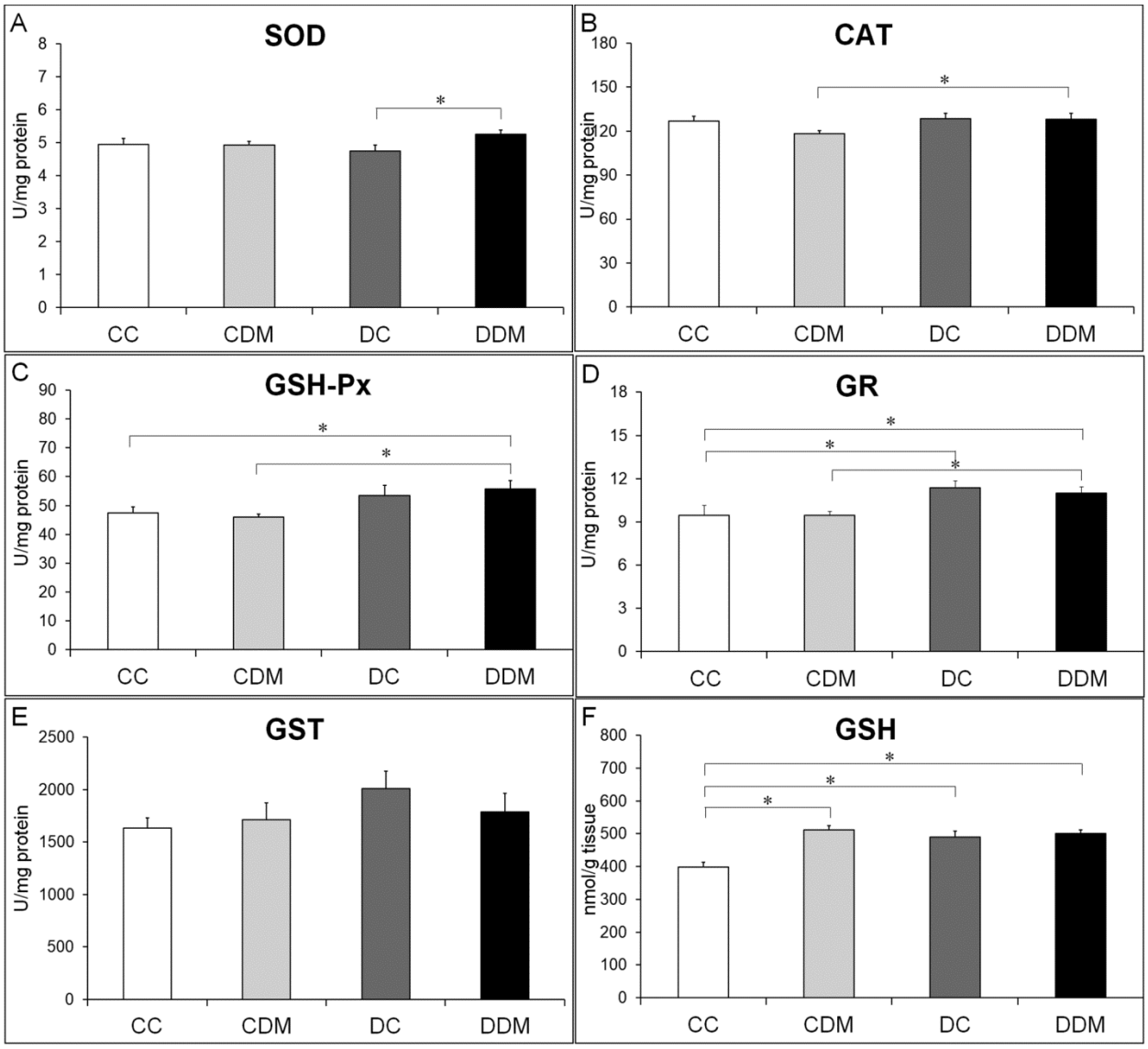

Fig. 2. Parameters of antioxidant system (AOS) (A – superoxide dismutase (SOD); B – catalase (CAT); C – glutathione peroxidase (GSH-Px); D – glutathione reductase (GR); E – glutathione-S-transferase (GST); F – glutathione (GSH)) in 4 experimental groups (CC – control-control, CDM – control-deltamethrin, DC – desiccation-control, DDM – desiccation-deltamethrin,) of

Bombina variegata juveniles. * significant differences between groups (n=10 per group; Fisher's LSD, $p < 0$ .05).

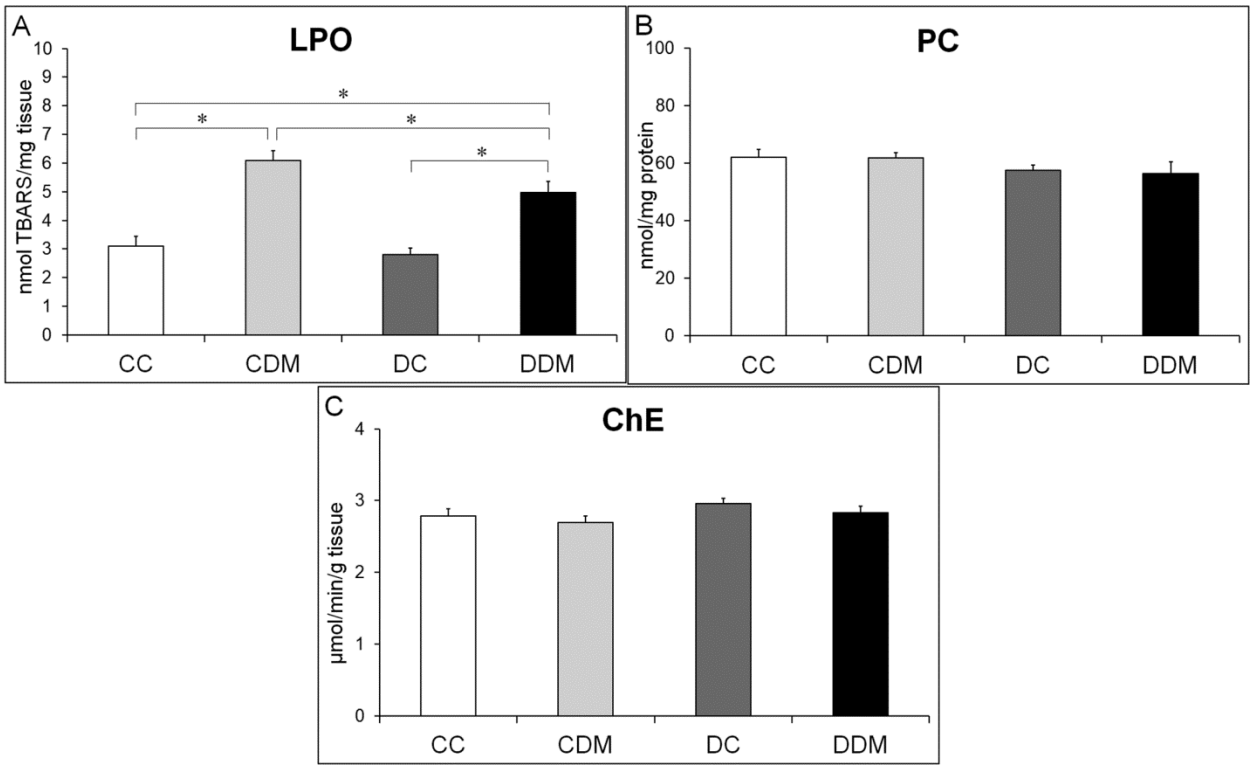

Fig. 3. Oxidative and neurotoxicity stress parameters (A – lipid peroxidation (LPO); B – protein carbonylation (PC); C – Cholinesterase (ChE)) in 4 experimental groups (CC – control-control, CDM – control-deltamethrin, DC – desiccation-control, DDM – desiccation-deltamethrin) of *Bombina variegata* juveniles. * significant differences between groups (n=10 per group; Fisher's LSD, $p < 0\ .05$).

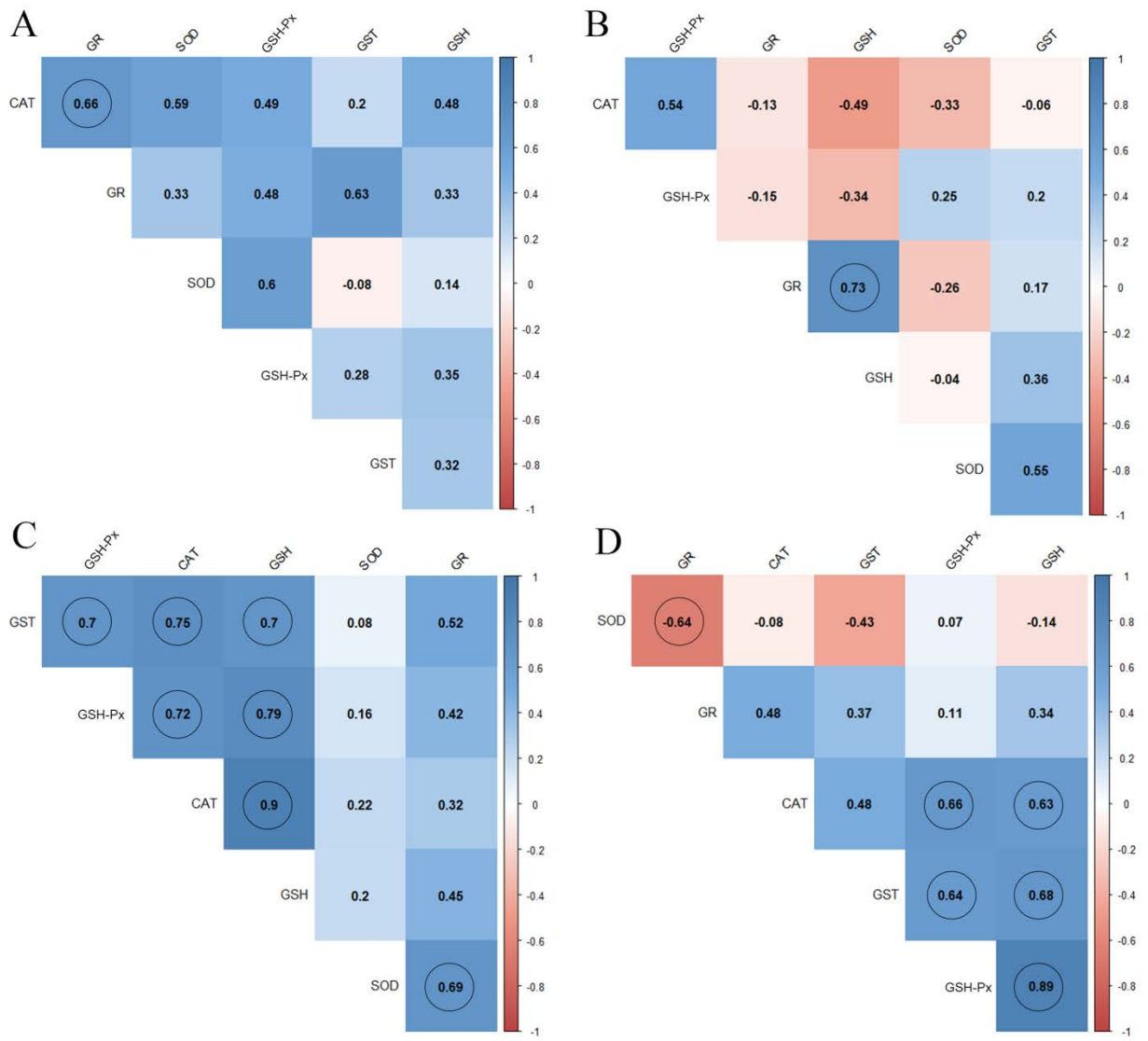

Fig. 4. Pearson correlations of examined parameters of A) CC – control-control group, B) CDM – control-deltamethrin group, C) DC – desiccation-control group and D) DDM – desiccation-deltamethrin group of *B.variegata* juveniles. SOD - superoxide dismutase, CAT - catalase, GSH-Px - glutathione peroxidase, GST - glutathione-S-transferase, GR - glutathione reductase, GSH - glutathione. Statistically significant correlations are marked with a black circle.

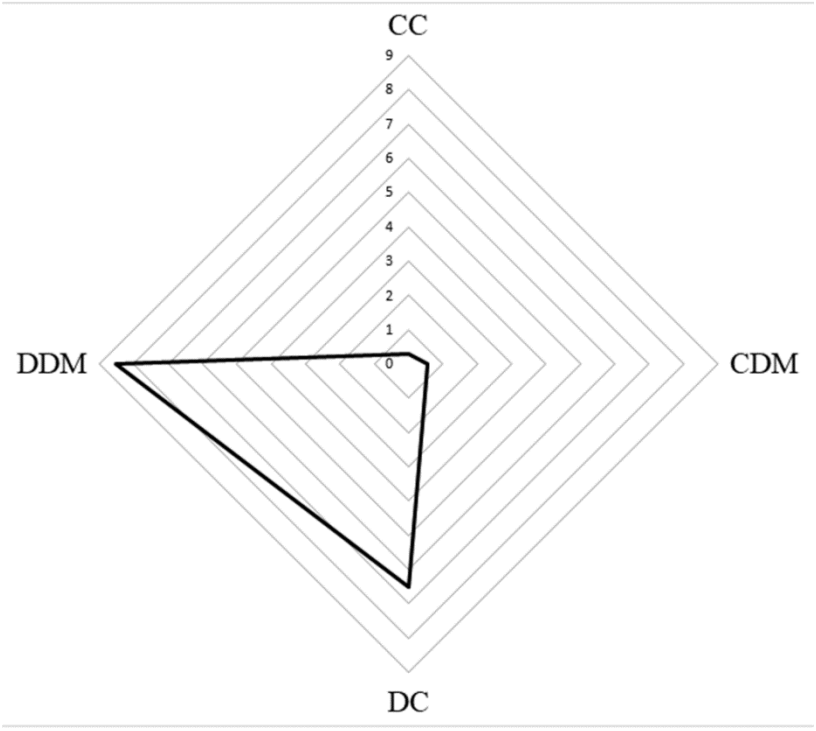

Fig. 5. IBR analysis of the investigated groups of *Bombina variegata* juveniles (CC – control-control, CDM – control-deltamethrin, DC – desiccation-control, DDM – desiccation-deltamethrin).