Oxidative cost of interspecific hybridization: a case study of two *Triturus* species and their hybrids

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SUMMARY

Hybrid individuals of crested newts had higher values of antioxidant parameters and a less integrated antioxidant defense system in comparison to parental species, indicating higher investment in maintaining oxidative balance.

ABSTRACT

Oxidative stress has most recently been suggested as one of possible mechanisms responsible for reduced fitness of hybrids. To explore possible oxidative cost of hybridization, we examined antioxidant defense system parameters (superoxide dismutase, catalase, glutathione peroxidase, glutathione s-transferase, glutathione reductase, glutathione, SH groups), their interconnectedness (index of integration), and levels of oxidative damage (concentrations of lipid peroxides-TBARS) in lab-reared newt species, *Triturus macedonicus* and *T. ivanbureschi*, and their hybrid. Our results showed that parental species differed in antioxidant defense system parameters, but not in the levels of integration of the whole system and oxidative damage. Individuals of *T. ivanbureschi* had higher activities of superoxide dismutase, glutathione s-transferase and concentrations of glutathione. Hybrid individuals of crested newts displayed higher levels of the antioxidant defense system (higher superoxide dismutase, catalase, glutathione peroxidase activities and concentrations of SH groups), and a lower overall correlation of antioxidant system (lower index of integration) in comparison to both parental species, suggesting that they may possess a less efficient antioxidant defense system and a higher investment in maintaining oxidative balance. The higher investment in the antioxidant system could divert limited resources away from other functions and affect further hybrid fitness. The presented findings contribute to a better understanding of the antioxidant defense system of crested newts and their interspecies differences, and support the hypothesis that oxidative stress is one of the costs of interspecific hybridization.

Keywords: oxidative stress; crested newts; *Triturus*; interspecific hybridization; antioxidative defense system

INTRODUCTION

Interspecific hybridization is followed by different intrinsic and/or extrinsic costs that can affect hybrid fitness (Wolf et al., 2010). In natural hybrid populations at species contact zones, a small loss of fitness can reflect on hybridization (Barton and Hewitt, 1985). Extrinsic costs are the result of a mismatch between phenotype and environment due to variations in physical or social factors (Schluter, 2000). On the other hand, intrinsic costs are independent of the environment and arise from incompatibilities that result from the recombination of co-adapted genomes (ploidy levels, chromosomal rearrangements, genic and mitonuclear interactions). These incompatibilities can influence different biochemical and physiological processes and lead to a disturbance in the sophisticated cell homeostatic system (Ellison and Burton, 2008; Olson et al., 2010). Studies conducted on hybrids of different species (chickadee *Poecile*, Olson et al., 2010; copepod *Tigriopus,* Barreto and Burton, 2013; flycatchers *Ficedula,* McFarlane et al., 2016; sunfish *Lepomis,* Borowiec et al., 2016) including two *Triturus* newt species (Gvoždík, 2012) showed that hybrid individuals experience metabolic cost, seen as significantly increased standard/basal metabolic rates relative to parental species. The authors suggested that this cost was the result of mitonuclear mismatch that leads to a disturbance in mitochondria and aerobic respiration (ATP, the oxidative phosphorylation (OXPHOS) pathway), and to increased oxygen consumption. Even though aerobic respiration and metabolism are closely related to the rate of production of reactive oxygen species (ROS), oxidative stress in hybrids has received limited attention despite the fact it is a fitness-related trait.

Oxidative stress is considered as a likely physiological cost of increased metabolic investment (development, growth, reproduction, parental care). It is an important mediator of the life history trade-off that could have detrimental consequences on an individual's fitness (health, longevity and reproductive output) (Costantini, 2008; Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010, Costantini, 2014). Oxidative stress is often defined as the imbalance between ROS production and neutralization processes, as the result of increased ROS production or a disturbed antioxidant defense system (AOS) (Halliwell and Gutteridge, 2015). A major consequence of oxidative stress is the loss of function and structural integrity of modified biomolecules (DNA, lipids, proteins), which has been implicated as a mechanism responsible for cell senescence and death (Pamplona, 2008). Management of oxidative stress and protection against it is pivotal for organism function (Pamplona and Costantini, 2011). To protect themselves from oxidative stress, animals have

evolved an integrated cellular antioxidant system. The efficiency of the AOS depends on the antioxidant components of the system and their functional interdependency achieved through direct or indirect biochemical reactions (Halliwell, 1999). Maintaining this highly complex system with a large number of components and pathways is an energetically demanding processe that shares the same limited resources of with other functions. Knowledge of the AOSs' response is important from evolutionary and ecological standpoints as it provides us with ability to identify and quantify underlying costs of free radical production and species ability to respond to their environment (Cohen et al. 2012).

The monophyletic group of nine *Triturus* newt species and their hybrid zones provide an excellent natural model system to understand hybridization and its consequences (Crnobrnja-Isailović et al., 1997; Arntzen et al., 2009, 2014; Wielstra et al., 2013, 2017). Newts are widely distributed across western Eurasia with a range of different hybrid zones and hybridization outcomes, from sterile hybrids without introgression, to a relatively broad contact zone between *T. macedonicus* and *T. ivanbureschi*, with generation of Fn hybrids and introgression (Arntzen et al., 2009; Arntzen et al., 2014). The hybridization phenomenon in newts has mostly been studied with regard to its morphological and genetic traits (Crnobrnja-Isailović et al., 1997; Arntzen et al., 2009; Slijepčević et al., 2015; Vučić et al., 2018), while data about possible biochemical and physiological differences are scarce. Herein we studied *T. macedonicus, T. ivanbureschi*, two genetically distinct species that have overlapping niches and naturally hybridize in the central and eastern part of Serbia, and their viable and fertile hybrid (Arntzen and Wallis, 1999; Arntzen et al., 2014). Both species displayed differences in some aspects of development, morphology and ecological preferences (Vukov et al., 2011, 2014; Džukić et al., 2016).

Interspecific hybridization in newts is often followed by mitonuclear mismatch because of differences in nuclear and mitochondrial DNA in parental species (Maletzky et al., 2008; Arntzen et al., 2009; Gvoždík, 2012). Mitonuclear mismatch can directly affect normal mitochondrial function ((OXPHOS) pathway) leading to increased electron leak and increased ROS levels (Du et al., 2017), and indirectly to upregulation of the AOS in response to increased ROS production. This upregulation is an energetically costly process, but if ROS production overwhelms antioxidant defences, this can disrupt further the oxidative balance in the hybrid, resulting in higher levels of oxidative damage in comparison to the parental species.

The aim of this study was to compare the levels of oxidative stress of *T. macedonicus* and *T. ivanbureschi* and their F1 hybrid (*T. macedonicus* ♀ × *T. ivanbureschi* ♂) under

laboratory conditions in order to determine interspecific differences in the AOS and to examine the possible oxidative cost of interspecific hybridization.

MATERIALS AND METHODS

Animal housing

Parental individuals used in experimental crossing to obtain larvae used in this study originated from natural populations with known genetics (Wielstra et al., 2013), *Triturus macedonicus* (Karaman, 1922) (location: Ceklin, Montenegro- 42°21´N; 18°59´E) and *T. ivanbureschi* (Arntzen and Wielstra, 2013) (location: Zli Do, Serbia- 42°25´N; 22°27´E). After hibernation in a cold chamber at constant temperature (4°C), experimental crossings were performed in March of 2017: 1) *T. macedonicus* ♀ × *T. macedonicus* ♂ (*T. macedonicus* larvae), 2) *T. ivanbureschi* ♀ × *T. ivanbureschi* ♂ (*T. ivanbureschi* larvae), and 3) *T. macedonicus* ♀ × *T. ivanbureschi* ♂ (hybrid larvae). Females were transferred to separate aquaria to lay eggs (*T. macedonicus* individuals belong from breeding of 3 females and 4 males; *T. ivanbureschi* from 3 females and 5 males; while hybrid individuals from breeding of 3 females of *T. macedonicus* and 5 males of *T. ivanbureschi*). Eggs were collected daily and kept in plastic Petri dishes immersed in dechlorinated tap water. After hatching, each group was separately transferred to small tanks until they could find food on their own. Afterwards, they were transferred to 12 L aquaria half filled with dechlorinated water. Stones and bricks were provided for shelters. All larvae were raised under the same laboratory conditions. The temperature was kept between 18 and 19°C. Water was changed twice a week. Larvae were fed every other day with *Artemia* sp. at early stages, and with *Tubifex* sp. at later stages. All animals were killed at stage 62 (Glücksohn, 1932), which is characterized by fully developed limbs and tail. The number of individuals per group was as follows: 13 for *T. macedonicus*, 16 for *T. ivanbureschi* and 16 for the hybrid.

Animal capture was approved by the Ministry of Energy, Development and Environmental Protection of the Republic of Serbia (permit no. 353-01-75/2014-08), and the Environmental Protection Agency of Montenegro (permit no. UPI-328/4). The experimental procedure was approved by the Animal Ethical Committee of the Institute for Biological Research “Siniša Stanković”, University of Belgrade (decision no. 03-03/16).

Sample processing

Whole bodies were finely chopped and mixed to obtain as much homogenous material as possible. About 0.1 g was taken for TBARS while the rest was used for other biochemical analyses (approximately 0.3-0.4 grams). The remaining sample was homogenized with an Ultra-Turrax (Janke and Kunkel, IKA-Werk, Staufen, Germany) homogenizer in 5 volumes of 25 mM sucrose containing 10 mM Tris-HCl, pH 7.5 (Lioneto et al., 2003). The homogenates were then sonicated at 40 kHz with three bursts for 10 s each. A part of the sonicate was taken for measuring the total GSH concentration, while the rest was centrifuged at 100,000 x g at 4°C for 90 min (Takada et al., 1982). The obtained supernatants were used for measuring the parameters of the AOS.

Biochemical analyses

SOD activity was measured according to Misra and Fridovich (1972). This method is based on the autoxidation of adrenaline to adrenochrome. The Claiborne (1984) method was used to determine CAT activity, while GSH-Px activity was assessed according to Tamura et al. (1982). This method is based on the reduction of t-butyl hydroperoxide with nicotinamide adenine dinucleotide phosphate (NADPH). To determine glutathione reductase (GR) activity we used the method of Glatzle et al. (1974), which includes reduction of glutathione disulfide (GSSG) to reduced glutathione (GSH) using NADPH as a substrate. GST activity was measured based on the reaction of the SH group of GSH with 1-chloro-2, 4-dinitrobenzene (CDNB) (Habig et al., 1974). All enzyme activities were expressed in U/mg protein. Protein concentrations were measured according to Lowry et al. (1951).

The concentration of GSH was estimated in a process in which GSH is oxidized by 5,5´-dithiobis-(2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of GR (Griffith, 1980). The concentrations of SH groups were measured using DTNB according to the method of Ellman (1959). The concentrations of thiobarbituric acid reactive substances (TBARS) as markers of the lipid peroxidation process (LPO) and potential oxidative damage were estimated according to the method of Rehncrona et al. (1980). The content of TBARS formed spontaneously was measured upon treating the samples with cold thiobarbituric acid reagent (10% trichloroacetic acid, 0.6% thiobarbituric acid) and subsequent heating at 100°C.

All measurements were performed in triplicate at 19 Cº using a Shimadzu UV 1800 UV-VIS spectrophotometer with a temperature-controlled cuvette holder (Gvoždík et al., 2007; Abele et al., 2011). Wavelengths for biochemical methods were: for SOD 480 nm; CAT – 240 nm; GSH-Px, GR and GST – 340 nm; GSH and SH groups – 412 nm; TBARS/LPO – 532 nm. Average coefficient of variation between replicates was: 4.39% for SOD, 2.55% – CAT, 3.35% – GSH-Px, 3.28% – GST, 3.69% – GR, 3.32% – GSH, 3.49% – SH and 4.02% for LPO 5.74%; the intraclass correlation coefficients (ICC) for each method were as follows: 0.982 – SOD, 0.978 – CAT, 0.988 – GSH-Px, 0.992 – GST, 0.975 – GR, 0.983 – GSH, 0.987 – SH, and 0.786 – LPO. All chemicals were obtained from Sigma–Aldrich (St Louis, MO, USA).

Statistical analyses

Assumptions of normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levine's test) were respected. All analyses were performed on log-transformed data expressed as the means ± standard deviation. Individuals did not differ in age (stage 62) and size. To investigate possible differences between groups (parental species and hybrids) with respect to the oxidative stress parameters, we performed first one-way ANOVA. We further applied post hoc analyses (Tukey HSD for unequal N with $p<0.05$ as the criterion for significance) to determine differences between each group. Principal component analysis (PCA) and cluster analysis were performed to explore the variation in AOS within and between parental species and hybrids. The effect sizes (Cohen's d) were calculated based on the means, standard deviations and number of subjects.

The level of overall correlation between components of the AOS was estimated by the index of integration, which was calculated as the variance of eigenvalues (VE) of correlation matrices calculated for each experimental group separately for all examined groups (Wagner, 1984; Costantini et al., 2011, 2013). Higher correlations among components correspond to higher values of VE because most of the variance can be explained by one or several eigenvalues. Lower correlations among components that correspond to lower VE indicate a more even distribution of variance (Pavlicev et al., 2009). To reduce possible errors, a small sample size correction was used according to Cheverud (1996). The significance of the differences between parental species and hybrid VE were calculated by resampling the data with replacement and recomputing the VE. We used the ratio of the VE of the two compared groups as a test statistic. The p-value was obtained as the number of times the randomized ratio exceeded the original one (based on 1000 permutations) (Manly, 1992).

All statistical analyses, except the index of integration, intraclass correlation coefficients and Cohen d effect size, were performed using STATISTICA 8.0 (StatSoft, Inc., 2007). The index of integration was calculated using PopTools 3.2.5 (Hood, 2011), while ICC (package 'ICC' 2.3.0), correlogram (package 'corrplot') and Cohen d effect size (package 'effsize' 0.7.1) were conducted in R 3.4.4, (R Development Core Team).

RESULTS

Oxidative stress parameters in parental species and hybrids are presented in Figure 1 (A-I), while in Tables 1 and 2 we provide the statistics (one-way Anova, F, p values and S.E of estimate; raw data, post hoc Tukey HSD for unequal N with p, and Cohen's d values respectively). Comparison between parental species revealed that individuals of *T. ivanbureschi* had significantly higher SOD and GST activities and increased concentrations of GSH. Hybrid in comparison to both parental species (*T. macedonicus* and *T. ivanbureschi*) had higher the activities of SOD, CAT, GSH-Px and the concentration of SH groups. The index of integration, as a measure of integration between all AOS components, showed that the hybrid had a significantly lower value than both parent species. In the hybrid, we also observed higher activities of GST, GR and an increased concentration of GSH in comparison to the maternal species *T. macedonicus*. The concentrations of TBARS did not differ significantly among the examined groups. Pearson's correlations between oxidative stress parameters of parental species and hybrids are given in the supplementary material (Fig. S1-3). Individuals did not differ in snout-vent length (SVL for *T. macedonicus* was 19.41 ± 1.59, for *T. ivanbureschi* was 18.48 ± 1.92, and hybrid individuals were 18.58 ± 1.07; p = 0.21).

PCA was applied to explore the relationships between parameters of AOS (Table 3 and Figure 2). The first PC axis clearly distinguished hybrid individuals from individuals belonging to *T. macedonicus*, with GR and GST as the parameters that contributed most to the separation (Table 3). Individuals of the paternal species (*T. ivanbureschi*) were consigned in between. Cluster analyses showed that parental species displayed more similar AOS in comparison to the hybrid (Figure 2).

DISCUSSION

Comparison of the AOS of *T. macedonicus, T. ivanbureschi* and their hybrid

In this study we provide some basic information about the AOS in two crested newt species (*T. macedonicus* and *T. ivanbureschi*) and their hybrid. Different ecological preferences and life history are often followed by different metabolic demands and/or levels of oxidative stress (Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010; Costantini, 2010). Studies conducted on birds and mammals confirm a strong bond between oxidative stress and ecology and the life history of species (development, survival rate, body size, clutch size, metabolic rate, longevity, locomotion) (Cohen et al. 2008; Nussey et al., 2009; Costantini, 2010). Even though *T. macedonicus* and *T. ivanbureschi* reproduce in similar aquatic habitats and are exposed to extremely variable environmental conditions, they differ in certain aspects (Džukić et al., 2016). Larvae of *T. ivanbureschi* tend to develop faster. This is a more active species that becomes sexual mature earlier and lives shorter in comparison to *T. macedonicus* (Furtula et al., 2009; Vukov et al., 2011; 2014; Džukić et al., 2016). All mentioned traits, especially the differences in developmental rates, can be linked with an increased metabolic activity and higher ROS production; the differences in basal antioxidant capacity in the examined species. *T. ivanbureschi* were characterized by higher AOS values (SOD, GST and GSH) in comparison to *T. macedonicus*. SOD activity plays an important role during accelerated and early development in amphibians, while the GSH system assumed greater importance later when the animals were exposed to environmental stressors (Salin et al., 2012; Gomez-Mestre et al., 2013). Beside the observed differences in the AOS, both species exhibited very similar levels of integration of the whole system and oxidative damage. Although comparisons between two species are insufficient to form a general picture about the AOS in newts, the obtained results can provide a well-founded basis for a future multispecies comparative study.

Phenotypically, hybrids can be similar to the paternal or maternal species due to dominance or the maternal effect, intermediate between the parental species, or it can exceed the qualities of both parental phenotypes (Wolf et al., 2010). Maternal matching was reported for basal metabolic rates of the grasshopper mouse hybrid (genus *Onychomys*), suggesting a strong effect of the mitochondrial genotype on the metabolism in hybrids (Shipley et al., 2016). Despite the fact that mitochondrial DNA is inherited from the maternal species and that mitochondria are the main source of ROS production, in this study we report that the hybrid has an AOS which is more similar to the paternal species (*T. ivanbureschi*). Hybrid

individuals differed in all examined AOS parameters from *T. macedonicus* individuals, and the differences were most pronounced for SOD, GST and CAT activity. The obtained results point to a mitonuclear mismatch in hybrid individuals. A study of some morphological traits conducted on the same species newt species (*T. macedonicus* and *T. ivanbureschi*) and their hybrids showed that even hybrid individuals differed from the parents, the hybrids were more similar to *T. ivanbureschi* (Vučić et al., 2018).

Oxidative stress as the cost of hybridization

Several studies dealing with hybridization and the energy cost of this process have suggested a possible link between oxidative stress and increased metabolism observed in some hybrids (Gvoždík, 2012; Borowiec et al., 2016). Hybrids of the marine copepod (*Tigriopus californicus*) had elevated levels of DNA oxidative damage (higher levels of 8-OH-dG) than parental lines as a consequence either of increased basal ROS leakage due to a dysfunctional OXPHOS system, or from a reduced antioxidant capacity (Barreto and Burton, 2013).

We observed that the hybrid of *T. macedonicus* x *T. ivanbureschi* differed significantly from the parental species with respect to the parameters of the AOS and the level of its integration, but not in the level of oxidative damage (lipid peroxidation). The hybrid displayed an increased response of the AOS that was most pronounced in the first line of defense enzymes (SOD, CAT and GSH-Px) which are directly involved in ROS scavenging, and concentration of SH groups. SOD eliminates the superoxide anion radical which is mainly generated in the mitochondria, by converting it to H_2O_2. The concentration of produced H_2O_2 is further lowered by the coordinate action of CAT and GSH-Px. These enzymes catalyze the reduction of H_2O_2 to non-harmful products. Higher GSH-Px activity can be accompanied by increased concentrations of thiols, which work as enzymatic cofactors of GSH-Px and help in removing hydroperoxides (Costantini et al., 2011; Halliwell and Gutteridge, 2015). Our results suggest that hybrid individuals are faced with increased ROS production. This was also observed in isolated mitochondria of sunfish hybrids when these were compared to the parents (Du et al., 2017). Regulation of the AOS can be considered as an indirect cost of oxidative stress (Costantini, 2014). The upregulation of the AOS is energetically costly, and it was shown that animals reared at low food levels were incapable of maintaining high activity levels of the system (Monaghan et al., 2009; Isaksson et al., 2011; De Block and Stoks, 2008). The higher need for investment in antioxidant defense in the hybrid of crested newts could divert limited resources away from other functions and affect hybrid fitness (growth, survive, reproduction and longevity). Boosted

antioxidant defenses in the damselfly (*Lestes viridis*) and blackbirds (*Turdus merula*) lead to a trade-off in immune function (Janssens and Stoks, 2018; Eikenaar et al., 2018), while in hybrids of marine copepod, reduced fecundity was associated with disrupted oxidative balance (Barreto and Burton, 2013). Du et al. (2017) suggested that increases in ROS production and oxidative stress contribute to the relatively poor competitive ability of sperm in sunfish F1 hybrids in comparison to that of parental species.

The strength of correlations (and hence integration) between parameters of the AOS under baseline conditions can also be important for the assessment of the response of the AOS to stressful conditions (Dotan et al., 2004). The complete AOS of the hybrid displayed lower values for the index of integration than in parental species. This implied decreased communication between the functional units in the hybrid, rendering the system less efficient in protection from oxidative damage (Pamplona and Costantini, 2011; Costantini, 2014). Lower integration levels and higher AOS response in the newt hybrid indicated that the hybrid invested more energy in maintaining oxidative balance under laboratory conditions in comparison to parental species.

This study provides some basic knowledge about the antioxidant defenses of crested newts. The indirect cost of higher ROS production supports the assumption that oxidative stress is the cost of interspecific hybridization. As the study was conducted under laboratory conditions, it remains to be clarified how the hybrid will manage in harsher and more dynamic natural conditions. Thus, in future studies we will monitor hybrid fitness under both laboratory and natural setting and will examine more oxidative stress parameters with the aim of determining any long-term or delayed adverse effects of increased investment in the AOS.

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Ethical standards

All animal procedures complied with the European Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes.

Competing interests

The contents of the manuscript have not been published previously, they have not been submitted elsewhere for consideration, nor are they in press. All of the authors have seen and approved the manuscript. There are no competing interests, neither financial, personal or other relationships with other persons or organizations.

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Tables

Table 1. Results of One- way Anova of the comparison between experimental groups (*T. macedonicus, T. ivanbureschi* and hybrid); N- number of individuals.

Variable	F	*p*	S.E. of estimate	N
SOD	37.16	0.0000	0.057	45
CAT	17.52	0.0000	0.059	45
GSH-Px	18.87	0.0000	0.086	45
GST	23.59	0.0000	0.073	45
GR	5.03	0.0107	0.086	45
GSH	4.73	0.0140	0.020	45
SH	20.18	0.0000	0.082	45
LPO	0.72	0.4919	0.028	43

Table 2. Mean and standard deviation of raw data for examined parameter and *p* and d values from comparison between *T. macedonicus*, *T. ivanbureschi* and hybrid. ANOVA post hoc Tukey HSD for unequal N, with $p<0.05$ as level of significance; d- Cohen's d.

	T. mac	*T. ivan*	*hyb*	*T.mac* vs *T.ivan*	*T.mac* vs *hyb*	*T.ivan* vs *hyb*
SOD	3.10 ± 0.10	3.96 ± 0.23	5.26 ± 0.15	p=0.003 d=1.25	p=0.000 d=4.32	p=0.000 d=1.67
CAT	29.5 ± 0.9	33.5 ± 1.4	40.06 ± 1.24	p=0.089 d=0.92	p=0.000 d=2.62	p=0.001 d=1.39
GSH-Px	6.68 ± 0.28	6.94 ± 0.43	10.15 ± 0.59	p=0.964 d=0.11	p=0.000 d=1.96	p=0.000 d=1.54
GST	62.0 ± 3.4	86.5 ± 3.0	94.22 ± 4.09	p=0.000 d=2.19	p=0.000 d=2.70	p=0.403 d=0.64
GR	7.20 ± 0.21	8.58 ± 0.58	9.53 ± 0.51	p=0.219 d=0.69	p=0.011 d=1.50	p=0.310 d=0.46
GSH	0.45 ± 0.01	0.52 ± 0.01	0.53 ± 0.02	p=0.035 d=1.13	p=0.035 d=1.04	p=0.986 d=0.001
SH	497.3 ± 18.1	478.7 ± 26.9	727.7 ± 39.88	p=0.776 d=0.30	p=0.000 d=2.12	p= 0.000 d=2.04
LPO	1.51 ± 0.06	1.58 ± 0.04	1.56 ± 0.03	p=0.527 d=1.03	p=0.694 d=0.79	p=0.946 d=0.39

Table 3. Loadings of variables onto the principal components (PCA).

	PCA loadings					
	PCA1	PCA2	PCA3	PCA4	PCA5	PCA6
SOD	-0.749	0.195	0.440	0.401	0.127	0.061
CAT	-0.744	0.359	0.088	-0.433	0.035	-0.051
GSH-Px	-0.749	-0.070	-0.518	0.181	0.030	-0.355
GST	-0.865	-0.027	0.169	-0.110	0.284	-0.063
GR	-0.784	-0.367	0.286	-0.028	-0.467	-0.084
GSH	-0.578	-0.712	-0.192	-0.056	0.150	0.284
SH	-0.687	0.484	-0.374	0.077	-0.190	0.312
% of each axis	54.94	14.98	10.83	5.77	5.38	4.60

Figures

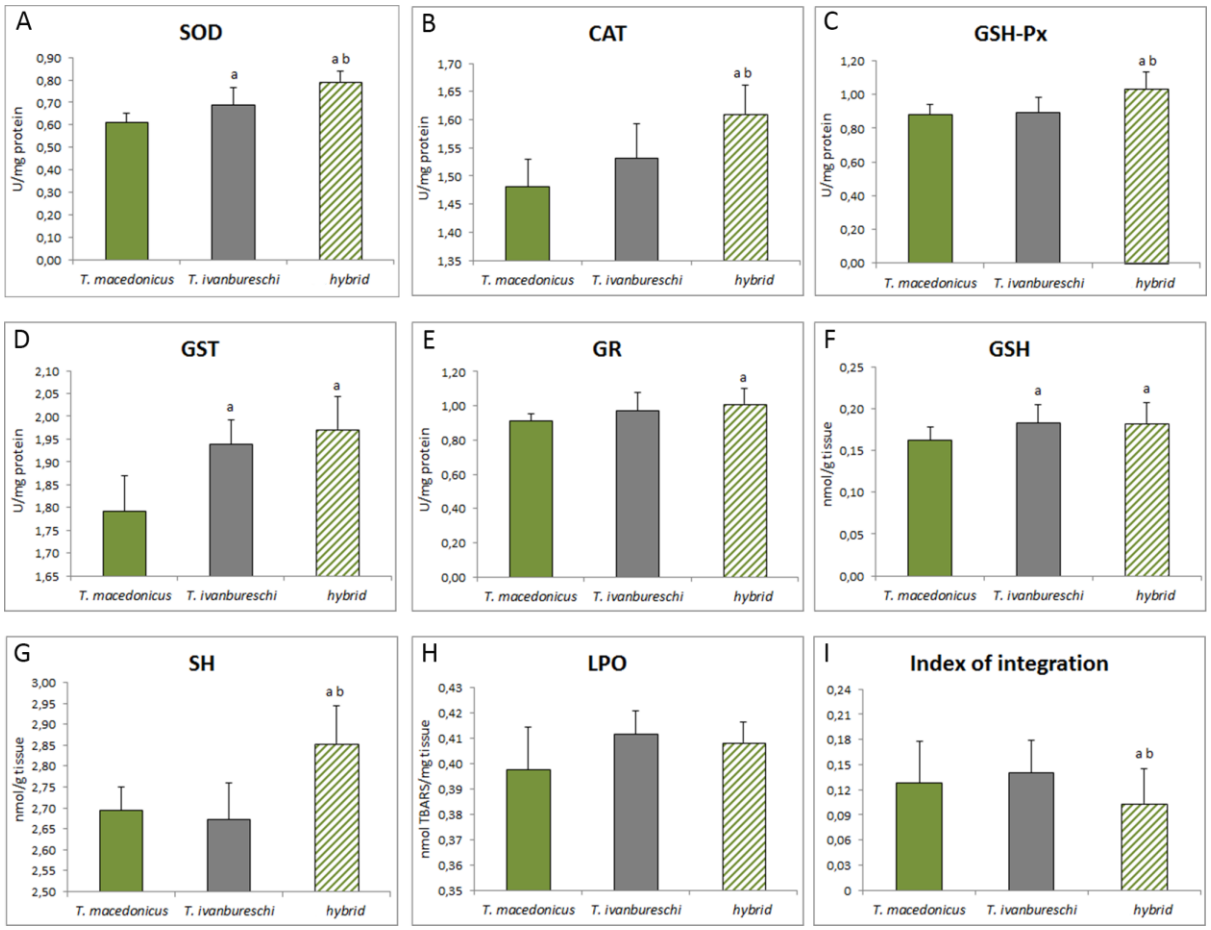

Fig. 1. Oxidative stress parameters and indices of integration in *Triturus macedonicus*, *T. ivanbureschi* and their hybrid. All data are presented as mean ± standard deviation. Significant differences ($p<0.05$) are marked with the letters a and b which indicate differences among the examined groups; "a" from *T. macedonicus*, "b" from *T. ivanbureschi*. A) SOD; B) CAT; C) GSH-Px; D) GST; E) GR; F) GSH; G) SH groups; H) TBARS; I) Index of integration.

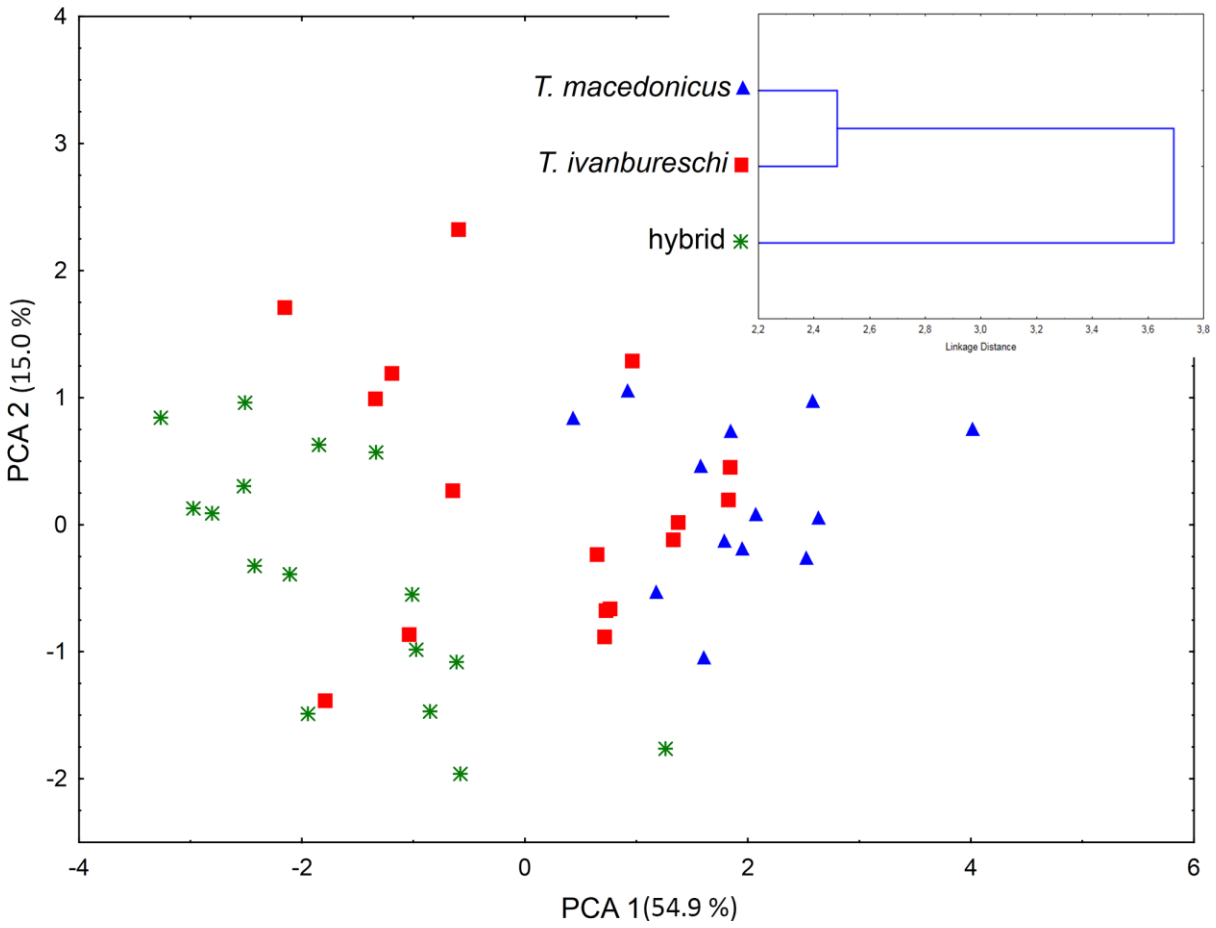

Fig. 2. Principal component and cluster analyses of parameters of the AOS of *Triturus macedonicus*, *T. ivanbureschi* and their hybrid.

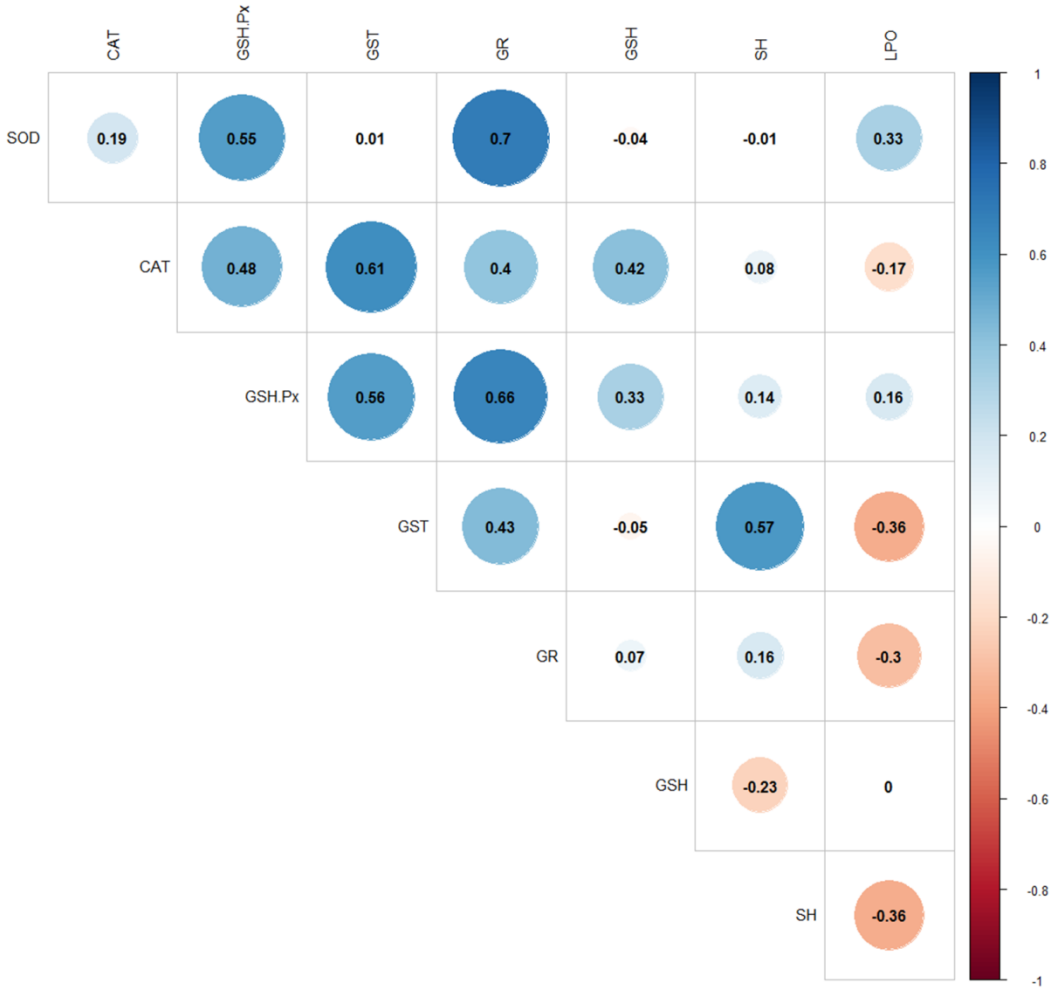

Figure S1. Pearson correlations of examined parameters of *T. macedonicus* individuals. SOD- superoxide dismutase, CAT- catalase, GSH-Px- glutathione peroxidase, GST- glutathione s-transferase, GR- glutathione reductase, GSH- glutathione, SH groups, LPO- lipid peroxidation, e.g. TBARS.

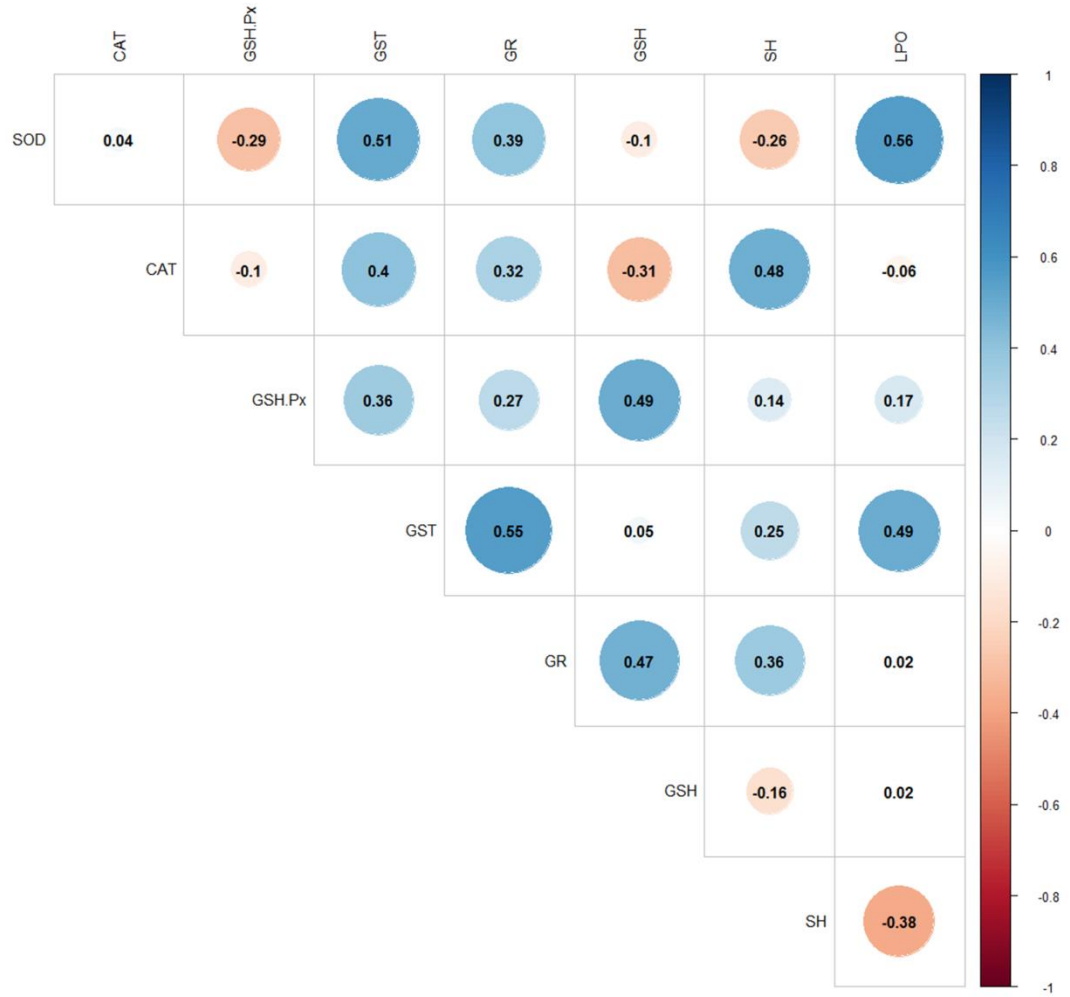

Figure S2. Pearson correlations of examined parameters of *T. ivanbureschi* individuals. SOD- superoxide dismutase, CAT- catalase, GSH-Px- glutathione peroxidase, GST- glutathione s-transferase, GR- glutathione reductase, GSH- glutathione, SH groups, LPO- lipid peroxidation, e.g. TBARS.

Figure S3. Pearson correlations of examined parameters of hybrid individuals. SOD- superoxide dismutase, CAT- catalase, GSH-Px- glutathione peroxidase, GST- glutathione s-transferase, GR- glutathione reductase, GSH- glutathione, SH groups, LPO- lipid peroxidation, e.g. TBARS.