The effect of short-term fasting on the oxidative status of larvae of crested newt species and their hybrids

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Abstract

In nature, animals often face periods without food caused by seasonal fluctuations and/or prey scarcity. An organism's physiological response to imposed energetic limitations is followed by changes in mitochondrial functioning (adjustment of energy metabolism) and a reduction of non-essential processes. However, this energy-saving strategy can have its costs. In this study, we examined oxidative stress as one of the possible physiological costs of short-term, two-week-long food deprivation on developing amphibian larvae of the crested newts *Triturus macedonicus* and *Triturus ivanbureschi* and their hybrids. We investigated whether this exogenous factor additionally affected the oxidative status (fitness-related trait) of hybrid individuals. The fasting treatment led to lower growth and a lower body mass and body condition index of individuals. The results revealed that the antioxidant system (AOS) of food-deprived larvae could not cope in a proper manner with reactive oxygen species production under limited energy availability, leading to higher lipid oxidative damage. The lowest AOS response was observed for H_2O_2 scavenging parameters (catalase, glutathione peroxidase, and total glutathione), which together with the elevated activity of superoxide dismutase suggested increased H_2O_2 concentrations. Comparison between parental species and their hybrids showed that hybrid individuals suffered greater oxidative damage (as demonstrated by higher concentrations of lipid peroxides), indicating that they were more susceptible to fasting-induced oxidative stress. Overall, this study illustrates that: (i) an oxidative event is one of the costs amphibian larvae face during short-term periods of fasting, (ii) hybrids are less capable of dealing with this stressful condition, which can lower their chances of survival in a changing environment.

Keywords: antioxidant system; oxidative stress; hybridization; fitness; food deprivation; amphibians; larvae

1. Introduction

All processes of life, even the most basic ones, have an energy cost (Porter and Gates, 1969). An animal's energy and the constitutive compounds required for normal functioning are obtained through food ingestion (McCue, 2010). Although food is essential for development, growth, self-maintenance and reproduction, individuals are often challenged by the absence of food in nature (McCue, 2012). For most amphibian species, periods of intermittent food supply are the result of bouts of foraging in stochastic environments during seasonal fluctuations (Milanovich and Maerz, 2013). Most of the data on this topic in amphibians are focused on prolonged food deprivation during hibernation and/or estivation in adults (Storey, 2002; Secor, 2005; Moreira et al., 2020). However, short-term fasting during the larval period has been studied to a lesser extent than in adults, even though it is commonly seen in the wild (Crespi and Denver, 2005). In larvae, food deprivation can have a negative effect on the duration of the developmental period and individual survival (Denoël and Poncin, 2001). Harsh conditions at early stages might have lasting consequences on the life history of individuals during later life (Scott et al., 2007).

To overcome the energetic limitations imposed by fasting, organisms have developed mechanisms to mobilize internal energy stores, adjust energy metabolism and downsize non-essential processes (Secor and Carey, 2016). Maintaining the biological system under fasting conditions carries hidden costs. The acquisition of sufficient energy in larvae (necessary for growth and maintenance of homeostasis on one hand and the establishment of lipid stores on the other, during periods of food absence) can be followed by molecular damage (Kirschman et al., 2017; Burraco et al., 2020). One of the potential costs of fasting is oxidative stress, mainly because of the metabolic changes caused by modifications to the mitochondria, the major site of reactive oxygen species (ROS) production (Salin et al., 2018), but also because of the activation of the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis (Crespi and Denver, 2005), and alterations in prooxidant/antioxidant balance (Sorensen et al., 2006; Vázquez-Medina et al., 2010). An increase in ROS levels (especially H_2O_2) as the result of the energy-saving strategy for fasting has been reported for different groups of organisms (Sorensen et al., 2006; Sharma et al., 2011; Zhang et al., 2013; Salin et al., 2018). Increased ROS levels are usually accompanied by the response of the antioxidant system (AOS), which is equipped with enzymatic and non-enzymatic components (Costantini, 2014). The first line of this defense consists of enzymes (superoxide dismutase – SOD, catalase – CAT, and glutathione peroxidase – GSH-Px) that neutralize ROS (O_2^-, H_2O_2). The second line of the defense comprises the scavenging non-enzymatic antioxidants (glutathione – GSH, sulfhydryl

(SH) groups, uric acid, vitamins C and E), that remove free radicals, inhibiting the initiation and propagation reactions. Beside GSH, the GSH system includes glutathione reductase (GR) that reduces the oxidized form of glutathione, and the biotransformation enzyme glutathione S-transferase (GST) that participates in the conjugation of GSH with various substances (Halliwell and Gutteridge, 2015). Studies showed that fasting state can increase (Vázquez-Medina et al., 2011; Schull et al., 2016) or decrease (Morales et al., 2004; Sorensen et al., 2006; Rocha et al., 2008) activities/concentrations of AOS components, depending on various factors (species, tissue, duration of food deprivation). However, the state of oxidative stress occurs only in cases when the produced ROS overwhelms the capacities of the AOS (Halliwell and Gutteridge, 2015).

For the examination of possible oxidative costs of fasting on amphibian larvae we used crested newts, *Triturus macedonicus* and *T. ivanbureschi*, and their hybrids as a model organism. We monitored the parameters of the AOS (SOD, CAT, GSH-Px, GSH, GR, GST, and SH groups), oxidative damage (lipid peroxidation (LPO)), and body size (snout-vent length (SVL) and body mass (BM)) after two weeks of food deprivation. The ability of *T. macedonicus* and *T. ivanbureschi* to hybridize in nature and produce fertile hybrids (Wielstra and Arntzen, 2012; Wielstra et al., 2017; Arntzen et al., 2018), allowed us to also examine the possible effects of food deprivation as an exogenous factor on hybrid fitness. Hybrid individuals often have lower fitness in comparison to parental species due to the effects of endogenous and/or exogenous factors (Burke and Arnold, 2001). In hybrid individuals of crested newts, mitonuclear mismatch (endogenous factor) was marked as one of the main reasons for the increased metabolic rate as compared to parental species (Gvoždík, 2012). Our previous study showed that F_1 hybrid individuals of crested newts (of *T. macedonicus* and *T. ivanbureschi*) had higher values of antioxidant parameters and exhibited a less integrated AOS in comparison to parental species under non-stressful conditions, indicating the trend of decreased fitness due to endogenous factors (Prokić et al., 2018a). The degree of effects of parental incompatibilities on hybrid fitness can also depend on the environmental (exogenous) factors in which hybrids occur (Stolzenberg et al., 2009; Havird et al., 2019). We believe that following oxidative stress parameters in hybrid larvae that develop under a challenging environment would provide insight into the hybridization phenomenon in natural populations.

Based on the findings that fasting can alter mitochondrial function, induce a significant increase in ROS levels, and activate the HPA/I axis, we assumed that the energy-limited state will: (1) alter the AOS, especially the H_2O_2-scavenging part of the system (CAT, GSH-Px

and GSH) due to the increase in mitochondrial H_2O_2 levels; (2) cause oxidative stress in crested newt larvae in both parental species and hybrids. If a higher investment in the maintenance of oxidative balance due to parental incompatibilities (mitonuclear mismatch) occurs in hybrids, we expect that (3) periods of fasting will lead to higher levels of oxidative damage in hybrids in relation to the parental species.

2. Materials and Methods

2.1. Experimental design

Triturus ivanbureschi and *T. macedonicus* adults (the parental generation) were collected from natural populations in 2014 and 2015, respectively. The capture of animals for the experiment 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). For the present experiment, the animals were crossed at the beginning of March 2018, after hibernation in the cold chamber at a constant temperature (4°C). Crossings between parental species were performed in semi-natural conditions (large, 500-L containers were filled with water, closed with a protective net, containing plastic strips as underwater vegetation for egg deposition, bricks for shelter and plastic floating islets). Four different crossings were made to obtain larvae of the species and their reciprocal F_1 hybrids, as follows: 1) *T. ivanbureschi* (*T. ivanbureschi* 6♀ × 3♂), 2) *T. macedonicus* (*T. macedonicus* 3♀ × 3♂), 3) *T. ivanbureschi*-mothered hybrids (3 *T. ivanbureschi* ♀ × 3 *T. macedonicus* ♂), and 4) *T. macedonicus*-mothered hybrids (2 *T. macedonicus* ♀ × 2 *T. ivanbureschi* ♂).

When females started depositing eggs, they were transferred to separate 10-L aquaria, half-filled with dechlorinated tap water. Plastic strips were set for egg deposition. Eggs were collected daily and raised in Petri dishes. After hatching, the larvae were raised in 100-mL plastic cups. When larvae reached stage 50 (beginning of the formation of hind limbs; Glücksohn, 1932), they were transferred to 2-L plastic containers (one larva per container) half-filled with dechlorinated tap water. The water in the Petri dishes, cups and containers was changed every other day. To minimize potential environmental effects, all individuals were reared in the same controlled laboratory conditions under an ambient photoperiod. The temperature was maintained at 19°C. During the earlier stages, the larvae were fed *ad libitum*

with *Artemia* sp., and at later stages with *Tubifex* sp. At stage 62 (fully developed digits, limbs and tail; Glücksohn, 1932) the larvae only increased in size until the end of metamorphosis. At this stage, the larvae were randomly divided into fasting and control groups. The number of individuals in the fasting group was as follows: for *T. ivanbureschi* = 13, *T. macedonicus* = 15, *T. ivanbureschi*-mothered hybrid = 13 and *T. macedonicus*-mothered hybrid = 15. The number of individuals in the control group was as follows: for *T. ivanbureschi* = 8, *T. macedonicus* = 10, *T. ivanbureschi*-mothered hybrid = 9 and *T. macedonicus*-mothered hybrid = 15. Larvae were kept individually in 2-L plastic containers (one larva per container), to avoid intraspecific aggression and cannibalism, and for comparison of biometric parameters of individuals at the beginning and end of the experiment. Individuals in the control group were maintained at the food ratio they were exposed to earlier. Twice a day, feces were removed from the containers to minimize possible feces ingestion and to keep the water clean. Water in containers was changed every other day. Individuals in the fasting group were maintained on the fasting treatment for 14 days, which is a credible period of short-term food deprivation that might be experienced by newts in nature (Irwin et al., 1999; Milanovich and Maerz, 2013). All individuals survived until the end of the experiment. The larvae were killed by immersion in liquid nitrogen and were kept at -80 °C until further analyses (Underwood et al., 2013).

The body mass and SVL of individuals were measured at the start and end of the experiment. For determination of SVL, we used photographs of the dorsal view (from the tip of the snout to the level of the posterior edge of hind legs) of the larvae with a Sony DSC-F828 digital camera (24-bit color and 3264 × 2448 pixel resolution, MP; Sony Corp., Tokyo, Japan), and ImageJ software (Rasband, 2019). The body condition index (CI) was calculated on log-transformed data for BM and SVL according to Labocha et al. (2014).

2.2. Sample processing and biochemical analyses

The whole body of larvae was minced and mixed to obtain material as homogenous as possible. About 0.2 g of mixed tissues were taken for LPO determination while the rest was used for other biochemical analyses. Analysis of the level of LPO included homogenization and sonication in 10 volumes of an ice-cold Tris-HCl buffer (pH 7.4), followed by centrifugation at 10,000 × g for 10 min at 4°C in 40% trichloroacetic acid (TCA) to obtain the supernatant. For measurement of antioxidant parameters, the tissue was homogenized for 45 s in 5 volumes of an ice-cold 25 mM sucrose buffer (pH 7.4) containing 10 mM Tris-HCl and 5 mM EDTA, with an Ultra Turrax homogenizer (T-18, IKA-Werk, Germany). The

homogenates were then sonicated at 10 kHz for 30 s on ice (Takada et al., 1982) using a Sonopuls ultrasonic homogenizer (HD 2070, Bandelin Electronic, Germany). For the determination of GSH concentration, the sonicates were centrifuged at 5,000 × g for 10 min in 10% sulfosalicylic acid and the protein precipitate was discarded. For measurement of the other AOS parameters, the sonicates were centrifuged at 100,000 × g for 90 min at 4 °C to obtain the supernatant.

The protein content was determined at 500 nm using the Lowry method (Lowry et al., 1951) with bovine serum albumin (BSA) as standard. SOD activity was measured at 480 nm according to the assay described by Misra and Fridovich (1972) that is based on the ability of SOD to inhibit the autoxidation of epinephrine. The Claiborne (1984) method for CAT analysis, which includes measurement of hydrogen peroxide degradation at 240 nm, was performed. GSH-Px activity was measured according to the protocol outlined by Tamura et al. (1982); to estimate GR activity we used the assay of Glatzle et al. (1974). According to both methods, the activity is based on the rate of NADPH oxidation. GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was determined using the protocol of Habig et al. (1974). GSH-dependent enzymes (GSH-Px, GR, and GST) were measured at 340 nm. The activities of all antioxidant enzymes were expressed in U/mg protein. GSH concentration was determined according to the 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) enzymatic recycling method (Griffith, 1980) and was expressed in nmol/g tissue. The method of Ellman (1959) was used for the estimation of protein sulfhydryl (-SH) group concentrations, expressed as μmol/mg protein. Non-enzymatic parameters (GSH and protein -SH groups) were measured at 412 nm. For the measurement of LPO, we used the thiobarbituric acid-reactive substance (TBARS) assay of Rehncrona et al. (1980). Lipid peroxide concentrations were evaluated at 532 nm and expressed in nmol/mg tissues. All biochemical measurements were performed at 19°C with a Shimadzu UV 1800 UV-VIS, (Shimadzu, Japan) spectrophotometer with a temperature-controlled cuvette holder. More details for the biochemical measurements are presented in the Supplementary Material.

2.3. Statistical analyses

Possible outliers were checked by Grubb's test. Assumptions of normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levine's test) were respected. Possible differences between two independent variables: "species" (parental species and hybrids), treatments (fasting and control) and their interaction for biometric and oxidative stress parameters, were analyzed using factorial ANOVA. On parameters for which a

significant interaction between factors (treatment x species) was reported, we performed pairwise multiple comparisons – Tukey's test. For parameters with significant differences for each factor, we applied the post hoc Tukey HSD test to determine further differences between the parameters. In the analysis of biometric parameters recorded at the end of the experiment, we included the initial values as covariate in the ANCOVA test. The criterion for significance was set as $p \leq 0.05$. Statistical analyses were performed using STATISTICA 8.0 (StatSoft, Inc., 2007), with the exception of pairwise multiple comparisons, which were calculated in XLSTAT, Ver. 2014.5.03 (Addinsoft, 2015).

3. Results

3.1. Biometric parameters

Biometric parameters (SVL, BM and CI) for all examined groups are given in Table 1. At the beginning of the experiment, we observed significant differences only between species (Table 2). At the end of the experiment, we observed differences after the treatments for all parameters and for the BM between species (Table 2). No significant interactions between factors were detected (Table 2). Two-week fasting individuals of all groups had significantly lower values for BM, SVL and CI in comparison to control individuals at the end of the treatment (*T. macedonicus* SVL– $F=24.8$, $p<0.0001$, $df=1$, BM– $F=52.0$, $p<0.0001$, $df=1$, CI– $F=41.4$, $p<0.0001$, $df=1$; *T. ivanbureschi* SVL– $F=47.6$, $p<0.0001$, $df=1$, BM– $F=59.1$, $p<0.0001$, $df=1$, CI– $F=37.2$, $p<0.0001$; *T. macedonicus* mothered hybrids: SVL– $F=39.0$, $p<0.0001$, $df=1$, BM– $F=39.2$, $p<0.0001$, $df=1$, CI– $F=34.6$, $p<0.0001$, $df=1$; *T. ivanbureschi* mothered hybrid SVL– $F=40.9$, $p<0.0001$, $df=1$, BM– $F=70.7$, $p<0.0001$, $df=1$, CI– $F=53.9$, $p<0.0001$, $df=1$).

3.2. Oxidative stress parameters

The main aim of this study was to determine the effects of fasting on crested newt larvae (control vs fasting), and possible differences between the examined species in response to fasting; thus, we will present only these results. The rest of the results (such as differences between species in the control group) can be found in the Supplementary Material (Tables S1 and S2). The results for factorial ANOVA showed significant differences for all examined parameters for the factors species and treatment, the exception being GR, where a significant difference was detected only between the species (Table 3). Significant interactions for treatment x species were observed for SOD, GSH-Px, SH groups and LPO (Table 3). All possible combinations of the post hoc test on parameters with significant interactions are shown in Table S2. Post hoc analysis of the interaction between species and treatment for

SOD showed that fasting individuals of both parental species (*T. macedonicus* and *T. ivanbureschi*) had higher values than those of the control; also, SOD activity was higher in the parental species in comparison to the hybrids (*T. macedonicus*-mothered and *T. ivanbureschi*-mothered hybrid) under fasting treatment (Fig. 1 and Table S2). The GSH-Px value was lower in the fasting group of *T. macedonicus*, *T. macedonicus*-mothered and *T. ivanbureschi*-mothered hybrid in comparison to matching controls (Fig. 1 and Table S2). Comparison under fasting treatments revealed that *T. ivanbureschi* and *T. macedonicus*-mothered hybrid had higher activities of GSH-Px than *T. macedonicus* and the *T. ivanbureschi*-mothered hybrid (Fig. 1 and Table S2). For the interactions of SH groups, we observed a lower concentration in fasting individuals of *T. macedonicus* and *T. ivanbureschi*-mothered hybrid in comparison to matching controls, and as regards differences between species under the fasting regime, SH groups were higher in *T. ivanbureschi* and the *T. macedonicus*-mothered hybrid than in the other two species (Fig. 2 and Table S2). Comparison of LPO values showed that fasting larvae of all examined groups had higher values than the control (Fig. 2 and Table S4). Fasting hybrid individuals had a higher concentration of LPO than individuals of fasting parental species, *T. ivanbureschi*-mothered hybrid than both parents, while the *T. macedonicus*-mothered hybrid had a higher concentration of LPO than only *T. macedonicus* (Fig. 2 and Table S2). For parameters with significant differences for the factors species and treatment (CAT, GSH and GST), post hoc tests were performed for the treatments (control vs fasting) and species (all species under fasting treatment); interspecies differences under non-stressful (control) conditions were of interest in our previously published study (Prokić et al., 2018a). CAT activity was lower in fasting individuals of *T. macedonicus*, *T. ivanbureschi* and *T. ivanbureschi*-mothered hybrid as compared to the control (Fig. 1). Comparison between species in fasting groups showed that *T. ivanbureschi* individuals had the highest value for CAT activity. Catalase activity was also higher in *T. macedonicus* and the *T. macedonicus*-mothered hybrid in comparison to the *T. ivanbureschi*-mothered hybrid (Fig. 1). Fasting lowered GSH concentration in all examined species, while comparison between species revealed that GSH was lowest in *T. macedonicus* (Fig. 1). Significant differences in GST activity between fasting and control individuals were reported for *T. ivanbureschi* and *T. macedonicus*-mothered hybrid. The fasting larvae of *T. ivanbureschi* also had higher GST than all other groups under the fasting treatment (Fig. 1). GR was the only parameter with significant differences only for the factor species, however, the post hoc test showed significant differences only between control individuals (Table S1).

4. Discussion

When the balance between energy consumption and expenditure is disrupted and the energetic conditions decline, organisms are forced to induce shifts in energy resource allocation, diverting them away from different functions such as growth, reproduction, immune and antioxidant defenses (De Block and Stoks, 2008; Monaghan et al., 2009; Isaksson et al., 2011; Schull et al., 2016; Prokić et al., 2018b; Prokić et al., 2019). Two weeks of fasting led to body-mass loss and lower growth (body length), affecting the body condition of crested newt larvae. The observed lower CI indicates a lower fat content that together with non-optimal growth conditions in larvae can further alter the development of new structures and metamorphosis (Metcalfe and Monaghan, 2001). Even though we did not follow post fasting effects, in the literature a smaller body size of amphibian larvae either results in a smaller size of metamorphs or it can lead to an increase in 'catch-up' growth to compensate for the adverse effects of fasting. Both possible scenarios can have negative effects on different fitness traits of juveniles (locomotor abilities, metabolic rates, endurance, resistance to desiccation and feeding success, immune function) (Semlitsch et al., 1988; Beck and Congdon, 2000; Gervasi and Foufopoulos, 2008; Burraco et al., 2017, 2020; Székely et al., 2020).

The newt larvae are very sensitive to different perturbations of environmental conditions (different temperatures, permanency of the ponds, etc.)(Denoël and Poncin, 2001). Short-term fasting is among the most common negative factors, caused by different environmental perturbations. In this study, we assumed that short-term fasting affects the oxidative status of the developing larvae of crested newts. The prediction was based on the finding that a two-week fasting period in ectotherms (brown trout *Salmo trutta*) induced a significant increase in hepatic mitochondrial H_2O_2 levels (Salin et al., 2018), and the activation of the HPI axis in amphibian larvae (an increase in corticosterone and corticotropin-releasing factor) in response to food unavailability (Crespi and Denver, 2005). Herein, we observed a lower antioxidant defense and higher oxidative damage (LPO concentration) in fasting larvae of crested newts in comparison to controls. The only AOS parameter that was higher was SOD (result seen in both parental species). The corresponding increase in SOD is expected due to an increased mitochondrial superoxide radical leak (Schull et al., 2016). Activation of SOD causes increased formation of intracellular H_2O_2 (Halliwell and Gutteridge, 2015). However, the two subsequently-activated components in this biochemical cascade, the H_2O_2-scavenging enzymes, failed. The absence of an adequate

response from CAT and GSH-Px in removing H_2O_2 is in agreement with the significant increases in H_2O_2 concentrations that are observed during fasting in the mitochondria of different animals (Sorensen et al., 2006; Salin et al., 2018; Roussel et al., 2019). Aside from direct effects, such as oxidative damage of biomolecules, it was suggested that increased H_2O_2 concentration as a secondary messenger could play a role in the re-feeding signal (Sylvie et al., 2012) by modulating cellular hormesis (Costantini, 2014; Schull et al., 2016). A decrease was also observed in the concentration of GSH, an important component of the second line of defense. GSH is crucial for the adaptive response against oxidative stress and a variety of ROS (Halliwell and Gutteridge, 2015). It is a potent reductant and cofactor for GSH-Px and GST, and is involved in the removal of hydrogen peroxide and lipid hydroperoxides, the main targets for the GSH redox system (Forman et al., 2009). Lower concentrations of GSH in fasting larvae can further contribute to increased concentrations of H_2O_2 and the accumulation of oxidative damage. Lower thiol concentrations were also reported in *T. macedonicus* and *T. ivanbureschi*-mothered hybrid under limited food availability, suggesting further alterations in the redox status. Higher GST activity in *T. ivanbureschi* and *T. macedonicus*-mothered hybrids could be the result of the activation of the detoxification system that prevents lipid hydroperoxide formation. Increased generation of lipid peroxides was reported during both short- and long-term fasting and in ectho- and endotherms (Ogasawara et al., 1989; Pascual et al., 2003; Morales et al., 2004; Sorensen et al., 2006; Furné et al., 2009; Sinha et al., 2015; Varju et al., 2018). Fasting in animals, aside from increasing mitochondrial ROS production, also affects lipid metabolism, leading to high fatty acid unsaturation (higher content of unsaturated fatty acids rich in double bonds) in the cell membranes making them more prone to oxidative damage (Sorensen et al., 2006). Moreover, changes in the lipid composition of the mitochondrial membrane can further modify mitochondrial H_2O_2 generation (Ramsey et al., 2005).

The oxidative stress during food deprivation could be interpreted using resource- or energy-based models (Costantini, 2019). Limited-energy conditions can result in the inability of an organism to increase its antioxidant defense towards increased ROS production. Maintaining the AOS and repair systems requires the consumption of energy (Monaghan et al., 2009). It was shown that in the absence of food (energy), the animals were incapable of maintaining high levels of AOS activity (Costantini and Møller, 2008; De Block and Stoks, 2008; Isaksson et al., 2011). Studies on poikilothermic organisms suggest that food deprivation can cause an impairment of the glutathione redox system (Furné et al., 2009), and significantly lower the concentration of GSH (Pascual et al., 2003; Sinha et al., 2015; Varju

et al., 2018) There is evidence that nutritional restriction negatively affects GSH metabolism, due to a deficiency of the cysteine-precursor amino acids used for GSH synthesis, which are obtained directly from food or by the metabolism of dietary methionine (Shimizu and Morita, 1992; Paterson et al., 2001). This may also produce a resource-allocation conflict between cellular GSH levels and the synthesis of proteins containing cysteine (de Magalhães and Church, 2006). Moreover, the enzymatic activity of GSH-Px depends on the presence of the essential dietary micronutrient selenium (Kumar et al., 2009; Isaksson et al., 2011). Newt larvae during the period of fasting continue to grow, but at a lower rate in comparison to controls. However, the energy used for somatic growth can additionally deviate energy from the AOS (Monaghan et al., 2009).

Two weeks of fasting revealed that hybrid individuals suffered greater oxidative damage than the parental species (evidenced by the increase in LPO concentration) – *T. ivanbureschi*-mothered hybrid in comparison to both parents, and the *T. macedonicus*-mothered hybrid in comparison to *T. macedonicus*. Hybrid individuals also displayed lower SOD activity. The more pronounced effects of fasting in hybrid individuals can be the result of the greater amount of energy they need to invest in the maintenance of physiological processes (standard metabolic rates and the AOS) (Gvoždík, 2012; Prokić et al., 2018a; Petrović et al., 2020). Previous observations on hybrid individuals of crested newts showed that they displayed an elevated AOS, were more active and aggressive, and were larger in body size in comparison to parental species under non-stressed conditions (Prokić et al., 2018a; Petrović et al., 2020). The availability of food resources was found to be crucial in limiting *Triturus* newt hybrids' endurance in the environment because of the greater expenditure of energy in the maintenance of high metabolic rates caused by mitonuclear mismatch (Gvoždík, 2012). A higher oxidative stress can be observed as a physiological constraint affecting fitness-related traits and mediating the life-history of hybrids through the progressive deterioration of tissues. The limited capacity to cope with exogenous factors and stress in a period of food deprivation could affect the survival of hybrid individuals in natural populations and could contribute to relatively narrow geographical hybrid zones in *Triturus* newts.

5. Conclusion

Even short-term food deprivation in crested newt larvae can lead to an oxidative event that is followed by a lower response of the AOS. The effects of the observed oxidative costs on the short- and long-term life histories (metamorphosis, growth patterns, reproductive

performance, aging and survival) of newts still require investigation. Comparisons between parental species and hybrids revealed more pronounced oxidative damage in hybrid individuals. The results from this study should be useful in our efforts to gain a better understanding of the possible role of oxidative stress in animals facing limited energy conditions, as well as in ecologically-mediated hybrid inferiority.

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Tables

Table 1. Biometric parameters: snout-vent length (SVL in mm), body mass (BM in g), and condition index (CI) at the beginning and end of experiment for each group. SVL and BM are given as mean ± standard error of raw data, while the CI was calculated on log-transformed data. F – fasting; C – control; N – number of individuals.

		SVL		BM		CI		N
		beginning	end	beginning	end	beginning	end	
T. macedonicus	F	24.49±0.29	25.59±0.32	1.22±0.05	1.08±0.04	0.245±0.006	0.221±0.005	15
	C	24.70±0.48	27.18±0.58	1.09±0.05	1.29±0.06	0.223±0.010	0.247±0.006	10
T. ivanbureschi	F	25.66±0.54	26.64±0.43	1.28±0.06	1.17±0.06	0.249±0.006	0.231±0.007	13
	C	25.89±0.51	28.19±0.50	1.22±0.10	1.44±0.07	0.240±0.011	0.263±0.007	8
T. macedonicus hybrid	F	27.82±0.33	28.87±0.29	1.59±0.05	1.43±0.05	0.282±0.005	0.260±0.005	15
	C	27.85±0.23	30.19±0.24	1.59±0.06	1.72±0.05	0.281±0.005	0.290±0.005	15
T. ivanbureschi hybrid	F	23.41±0.45	24.08±0.51	0.95±0.06	0.86±0.06	0.206±0.008	0.190±0.008	13
	C	24.69±0.43	27.06±0.5	1.01±0.06	1.18±0.06	0.214±0.008	0.233±0.007	9

Table 2. Results of the comparison between the treatments (fasting and control), "species" (*T. macedonicus*, *T. ivanbureschi* and hybrids) and for the interaction between the treatment x "species" for biometric parameters (snout-vent length – SVL, body mass – BM and condition index – CI). Data in **bold** indicate statistical differences.

Parameter	Effect	df	F	p
SVL-at the beginning	Treatment	1	2.04	0.1565
	Species	3	**35.89**	**<0.0001**
	treatment x species	3	0.76	0.5189
BM-at the beginning	Treatment	1	0.57	0.4517
	Species	3	**31.38**	**<0.0001**
	treatment x species	3	0.89	0.4508
CI-at the beginning	Treatment	1	1.09	0.2990
	Species	3	**30.84**	**<0.0001**
	treatment x species	3	1.32	0.2745
SVL-at the end	**Treatment**	1	**87.70**	**<0.0001**
	Species	3	1.62	0.1912
	treatment x species	3	1.37	0.9371
BM-at the end	**Treatment**	1	**142.9**	**<0.0001**
	Species	3	**3.82**	**0.0130**
	treatment x species	3	0.74	0.5321
CI-at the end	**Treatment**	1	**42.65**	**<0.0001**
	Species	3	1.57	0.2016
	treatment x species	3	2.50	0.0654

Table 3. Results of factorial ANOVA for the comparison between the treatment (fasting and control), "species" (*T. macedonicus*, *T. ivanbureschi* and hybrids), and the interaction between the treatment x "species" for oxidative stress parameters. Data in **bold** indicate statistical differences.

Parameter	Effect	df	F	p
	treatment	1	**13.16**	**0.0004**
SOD	**Species**	3	**18.34**	**<0.0001**
	treatment x species	3	**6.22**	**0.0006**
	treatment	1	**27.46**	**<0.0001**
CAT	**Species**	3	**10.01**	**<0.0001**
	treatment x species	3	2.38	0.0749
	treatment	1	**35.06**	**<0.0001**
GSH-Px	**Species**	3	**13.40**	**<0.0001**
	treatment x species	3	**3.83**	**0.0124**
	treatment	1	**58.43**	**<0.0001**
GSH	**Species**	3	**3.24**	**0.0257**
	treatment x species	3	1.35	0.2649
	treatment	1	2.03	0.1576
GR	**Species**	3	**3.00**	**0.0344**
	treatment x species	3	1.30	0.2809
	treatment	1	**6.75**	**0.0109**
GST	**Species**	3	**3.55**	**0.0175**
	treatment x species	3	2.16	0.0984
	treatment	1	**6.63**	**0.0116**
SH	**Species**	3	**25.74**	**<0.0001**
	treatment x species	3	**3.06**	**0.0324**
	treatment	1	**38.76**	**<0.0001**
LPO	**Species**	3	**5.61**	**0.0014**
	treatment x species	3	**2.76**	**0.0466**

Fig.1. Parameters of the AOS (SOD, CAT, GSH-Px and GSH) in fasting and control individuals of parental species (*T. macedonicus* and *T. ivanbureschi*) and hybrids (*T. macedonicus*-mothered and *T. ivanbureschi*-mothered). "*****" indicates significant differences between treatments (fasting vs control); different letters indicate significant differences between species under fasting treatment. Dot – mean value; box – standard error; bars – minimal and maximal value.

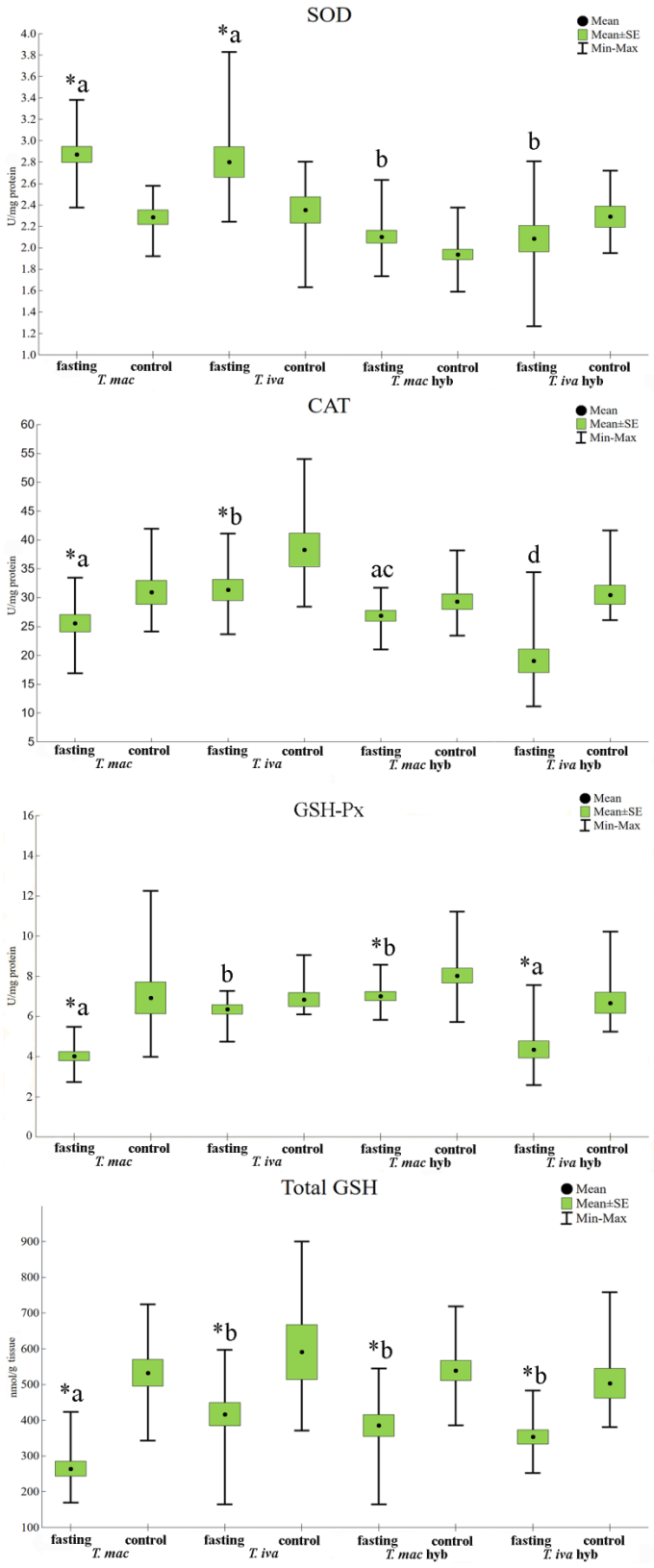
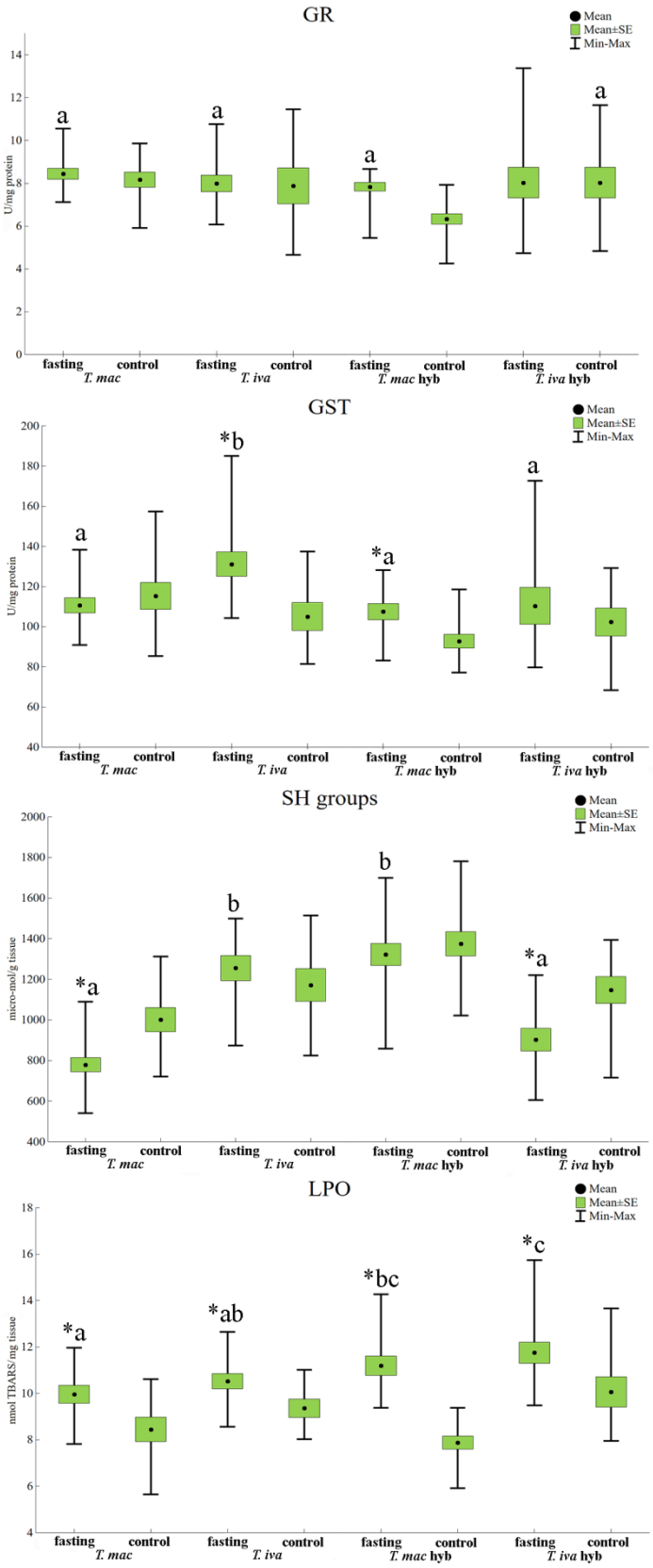


Fig.2. Oxidative stress parameters (GR, GST, SH groups and LPO) in fasting and control individuals of parental species (*T. macedonicus* and *T. ivanbureschi*) and hybrids (*T. macedonicus*-mothered and *T. ivanbureschi*-mothered). "*****" indicates significant differences between treatments (fasting vs control); different letters indicate significant differences between species under fasting treatment. Dot –mean value; box – standard error; bars – minimal and maximal value.


GR
Mean
Mean±SE
Min-Max
U/mg protein
a
a
a
a
fasting
control
T. mac
T. iva
T. mac hyb
T. iva hyb
GST
*b
*a
a
SH groups
micro-mol/g tissue
*a
b
b
*a
LPO
nmol TBARS/ mg tissue
*a
*ab
*bc
*c