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ABSTRACT

Ectotherms are particularly sensitive to global warming due to their limited capacity to thermoregulate, which can impact their performance and fitness. From a physiological standpoint, higher temperatures often enhance biological processes that can induce the production of reactive oxygen species and result in a state of cellular oxidative stress. Temperature alters interspecific interactions, including species hybridization. Hybridization under different thermal conditions could amplify parental (genetic) incompatibilities, thus affecting a hybrid's development and distribution. Understanding the impact of global warming on the physiology of hybrids and particularly their oxidative status could help in predicting future scenarios in ecosystems and in hybrids. In the present study, we investigated the effect of water temperature on the development, growth and oxidative stress of two crested newt species and their reciprocal hybrids. Larvae of *Triturus macedonicus* and *T. ivanbureschi,* and their *T. macedonicus*-mothered and *T. ivanbureschi*-mothered hybrids were exposed for 30 days to temperatures of 19°C and 24°C. Under the higher temperature, the hybrids experienced increases in both growth and developmental rates, while parental species exhibited accelerated growth (*T. macedonicus*) or development (*T. ivanbureschi*). Warm conditions also had different effects on the oxidative status of hybrid and parental species. Parental species had enhanced antioxidant responses (catalase, glutathione peroxidase, glutathione S-transferase and SH groups), which allowed them to alleviate temperature-induced stress (revealed by the absence of oxidative damage). However, warming induced an antioxidant response in the hybrids, including oxidative damage in the form of lipid peroxidation. These findings point to a greater disruption of redox regulation and metabolic machinery in hybrid newts, which can be interpreted as the cost of hybridization that is likely linked to parental incompatibilities expressed under a higher temperature. Our study aims to improve mechanistic understanding of the resilience and distribution of hybrid species that cope with climate-driven changes.

1. Introduction

Human activity and the resulting global change underlie a dramatic worldwide loss of biodiversity (Simide et al., 2016; Román-Palacios and Wiens, 2020; Mi et al., 2022). Habitat fragmentation, diseases, lower resource levels and extreme environmental events are among the main factors threatening wildlife (Blaustein et al., 2001; Strong et al., 2017). Although organisms have evolved mechanisms to detect and respond to

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changes in habitat, the intensity and pace of these changes often exceed the tolerance limits of different organisms and thus negatively affect their performance and fitness (Hoffmann and Sgrò, 2011; Román-Palacios and Wiens, 2020).

Beyond the effects of global change at the individual level, environmental shifts influence changes at the population and species levels, driving ecological and evolutionary processes (Visser and Both, 2005; Williams and Jackson, 2007; Traill et al., 2010). One potential way of coping with environmental variation is species hybridization. Theoretical and empirical studies often indicate that interspecific hybrids differ ecologically from both parents and that hybridization can promote rapid population segregation and the development of novel adaptive traits (Seehausen, 2004). However, hybridization has associated costs such as reductions in an individual's capability to maintain optimal levels of certain physiological pathways (Arnqvist et al., 2010; Koevoets et al., 2012; Gvoždík, 2012; Barreto and Burton, 2013; Du et al., 2017; Prokić et al., 2018). One of the costs is related to perturbations in mitochondrial functioning arising from incompatibilities between maternal mitochondrial and parental nuclear DNA (mitonuclear mismatch) (Burton et al., 2006). This mismatch can lead to inefficiency of oxidative phosphorylation (OXPHOS) (Burton et al., 2006; Koch et al., 2021), disturbances in electron flow (Gusdon et al., 2007; Rand et al., 2018) and redox balance, and Krebs cycle flux, with downstream consequences on the biosynthesis, repair, gene expression and phenotype of individuals (Miller and Matute, 2017; Rodríguez et al., 2021).

Ectotherms have a limited ability to use metabolic heat to maintain their body temperature, and some of their basic physiological functions such as locomotion, growth and reproduction are strongly influenced by ambient temperature (Kingsolver et al., 2013). Many ectotherm species hybridize and the resulting hybrids usually face the same environmental conditions as their parents. The interplay between intrinsic chromosomal rearrangements, genetic and mitonuclear interactions and extrinsic environmental render hybrid individuals more sensitive to environmental (temperature) shifts and can involve maladaptive responses that alter hybrid development and distribution (Koevoets et al., 2012; Chunco, 2014; Canestrelli et al., 2017; Miller and Matute, 2017). Climate change can mediate hybrid zone movement and result in changes in gene flow and alterations in hybrid functioning, leading to either the breakdown or buildup of hybrid boundaries (Ryan et al., 2018). Therefore, studying the mechanisms that underlie the responses of ectothermic hybrids to environmental change and in particular to temperature conditions, will assist us in understanding of their evolutionary potential and limits, as well as provide solutions for conservation efforts and management of natural resources.

Among vertebrates, amphibians are an ideal study system to investigate the impact of global climate change on hybrid species. Amphibians are the most threatened vertebrate group (Blaustein et al., 2010; Catenazzi, 2015), with interspecific hybridization a commonly observed process (Chunco, 2014). It is anticipated that many extant amphibian populations will be increasingly endangered in the following decades, and that most European species will be incapable of adequately coping with the predicted scenarios of global climate change (Catenazzi, 2015). Many amphibians have a biphasic life cycle that includes abrupt transformations from an aquatic larva to a terrestrial juvenile through metamorphosis, with the size at metamorphosis often predictive of survival later in life (Cabrera-Guzmán et al., 2013; Székely et al., 2020; Zhu et al., 2021). Also, amphibian larvae frequently maintain and express plastic responses at the expense of energetically demanding processes (Gervasi and Foufopoulos, 2008; Burraco et al., 2022a), which, together with their highly permeable skin (Yu et al., 2015; Strong et al., 2017; Ruthsatz et al., 2018) and limited vagility (Enriquez-Urzelai et al., 2022) make them highly vulnerable to environmental variations. Increases in ambient temperature are predicted to negatively affect amphibian populations and are likely linked to carry-over effects at a later age, including altered developmental or growth rates (Tejedo et al., 2010; Ruthsatz et al., 2020; Sinai et al., 2022). As in other ectotherms, the temperature exerts pervasive effects on the metabolic rate, locomotor activity, water balance, feeding behavior (Baškiera and Gvoždík, 2019), breeding phenology, gametogenesis (Blaustein et al., 2001, 2010) and susceptibility to infection (Sauer et al., 2018) in amphibians. Most of these processes can lead to increased production of reactive oxygen species (ROS) (Speakman, 2005; Halliwell and Gutteridge, 2015; Koch et al., 2021). Even though ROS have signaling roles, the mismatch between the rate of ROS production and the capacity of the antioxidant defense system (AOS) components disrupt the oxidative balance (ROS steady-state) and lead to oxidative stress (Halliwell and Gutteridge, 2015; Costantini, 2019). Oxidative stress is accompanied by damage to essential biomolecules and the formation of oxidative damage products (carbonyl proteins, lipid peroxides and 8-oxo-7,8-dihydro-2'-deoxyguanosine) that disrupt cell and tissue homeostasis (Halliwell and Gutteridge, 2015). As regards amphibian hybrids, research has shown that their antioxidant machinery often has different dynamics than that of their parents (e.g., higher enzymatic activities and lower levels of overall correlation with the antioxidant system index of integration) (Prokić et al., 2018), in addition to a larger investment in the AOS and higher metabolic rates (Gvoždík, 2012; Prokić et al., 2018, 2021a), which points to the metabolic cost of hybridization. The question is whether changes in habitat conditions such as temperature can potentially exacerbate differences in the oxidative status of amphibian hybrids.

Herein we present the findings of our investigation into the physiological effects of different ambient temperatures on two species: *Triturus macedonicus* and *T. ivanbureschi* and their reciprocal hybrids (*T. macedonicus*-mothered and *T. ivanbureschi*-mothered). These species belong to the crested newt group of the monophyletic genus *Triturus*. *Triturus macedonicus* and *T. ivanbureschi* are phylogenetically well-separated species from two distinct clades of crested newts (Wielstra et al., 2019; Rancilhac et al., 2021) that slightly differ in life history traits and morphology and possess distinct distribution ranges (Arntzen et al., 2018; Vučić et al., 2019, Vučić et al., 2020a,b), with the contact zone positioned on the Balkan Peninsula (the central and eastern parts of Serbia; Fig. 1). Complex interactions between the species include expansion of the range of *T. macedonicus* over *T. ivanbureschi*, followed by asymmetrical introgression of *T. ivanbureschi* mtDNA in *T. macedonicus,* which can be regarded as a genomic footprint of the previous range of *T. ivanbureschi* (Arntzen and Wallis, 1999; Wielstra and Arntzen, 2012; Wielstra et al., 2017). At the contact zone, viable hybrid populations consist of only hybrids with *T. ivanbureschi* mtDNA derived from many generations of mutual hybrid crossing and backcrossing with both parental species (Wielstra and Arntzen, 2012; Wielstra et al., 2017; Arntzen et al., 2018). Most crested newt species develop and reproduce between 18°C and 20°C (Litvinchuk et al., 2007), while the larvae experience water temperatures up to 25°C (Smolinský and Gvoždík, 2014; Smith et al., 2015; Winterová and Gvoždík, 2021).

In this study, we examined the effect of two temperature regimes (19°C and 24°C) on the development, growth and oxidative stress parameters of crested newt parental species and hybrid larvae. To determine the oxidative stress status of individuals, we measured AOS parameters (the activities of superoxide dismutase- SOD, catalase- CAT, glutathione peroxidase- GSH-Px, glutathione reductase- GR and glutathione S-transferase- GST, and concentrations of glutathione- GSH and SH groups) and oxidative damage by quantifying lipid peroxidation (LPO). We hypothesized that a higher temperature should accelerate development and induce oxidative stress in the larvae of both parental species and hybrids. Additionally, as growth and development require synchronized actions of both nuclear and mitochondrial genomes, the potential dysfunction of mitochondria associated with mitonuclear incompatibilities in hybrids can decrease the physiological ability of hybrids to tolerate higher temperatures, and as a consequence, this will result in increased oxidative damage. The absence of hybrid individuals with *T. macedonicus* mtDNA in nature suggests that this genotype faces greater perturbations and intense oxidative stress.

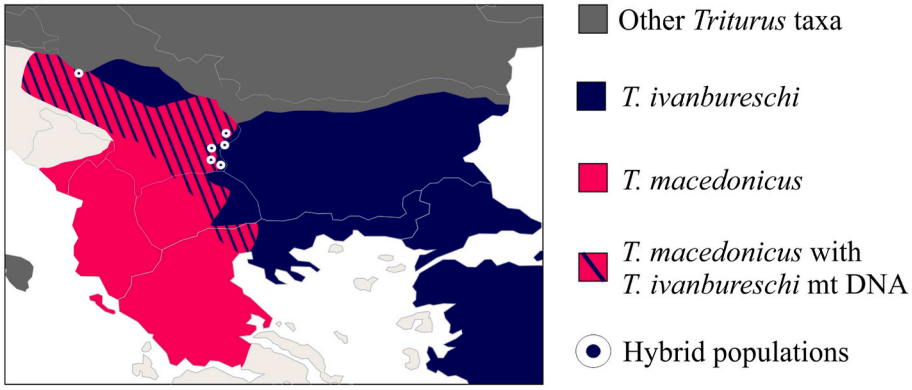

Fig. 1. The contact zone between *Triturus ivanbureschi* (blue) and *T. macedonicus* (pink). The northwestern enclave of *T. ivanbureschi* in central Serbia is separated from the species main range by expanding *T. macedonicus*. Blue lines represent the range of *T. macedonicus* containing *T. ivanbureschi* mitochondrial DNA. The populations with a substantial genetic admixture of the two species' nuclear genome (Wielstra et al., 2017; summarized in Vučić et al., 2020a,b) are highlighted. The hybrid populations consist of individuals derived from many generations of mutual hybrid crossings and backcrossing with both parental species (Arntzen at el., 2018).

2. Materials and methods

2.1. Experimental design

T. macedonicus and *T. ivanbureschi* adult females and males were collected in populations in the wild (*T. macedonicus* in 2015 from Ceklin, Montenegro; 42°21′N, 18°59′E, and *T. ivanbureschi* in 2014 from Zli Dol, Serbia; 42°25′N, 22°27′E). The capture of animals 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). After hibernation, we crossed adult individuals in large mesocosms aiming to simulate their natural environment (i.e., in 500-L tanks filled with dechlorinated water, closed with a protective net and containing plastic strips as underwater 'vegetation' for egg deposition, with bricks for shelter and plastic floating islets). To obtain larvae of the species and their reciprocal F_1 hybrids we made four different crossings as follows: (i) *T. ivanbureschi* (*T. ivanbureschi* 3♀ × 3♂), (ii) *T. macedonicus* (*T. macedonicus* 3♀ × 3♂), (iii) *T. ivanbureschi*-mothered hybrids (*T. ivanbureschi* 3♀ × *T. macedonicus* 3♂), and (iv) *T. macedonicus*-mothered hybrids (*T. macedonicus* 3♀ × *T. ivanbureschi* 3♂). Maternal identity could not be controlled as the combinations of crossings of each species were conducted in a single mesocosm; however, maternal effects were reduced by using males and females originating from the same population (Parichy and Kaplan, 1992; Kaplan, 1998).

Eggs were collected daily and maintained in Petri dishes until hatching. Once hatched, the larvae were raised in 100-mL plastic cups and fed *ad libitum* with *Artemia* sp. until they reached stage 50 according to Glücksohn (1932). At this stage, they were placed individually in 2-L plastic containers half-filled with dechlorinated tap water. From this stage, the larvae were fed with *Tubifex* sp. All individuals were maintained at the same temperature (19°C) and photoperiod. At stage 62 (larvae with fully developed limbs and tail; Glücksohn, 1932), half of the individuals from each genotype were individually and randomly assigned to two temperature treatments – 19°C and 24°C (N = 30 individuals per treatment). The larvae were exposed to the temperatures for 30 days in 2-L plastic containers half-filled with dechlorinated tap water. Individuals were fed with *Tubifex* sp. *ad libitum*. Twice a day feces were removed from the containers to keep the water clean. During the entire experiment, the water was renewed completely every other day. The body size of each individual was recorded at the start and at the end of the experiment by taking photographs of the dorsal view, from the tip of the snout to the level of the posterior edge of hind legs (designated as the SVL), and photographs were analyzed with the help of ImageJ software. All photographs included a metric scale and were taken with a Sony DSCF828 digital camera (24-bit color and 3264 × 2448-pixel resolution (MP, Sony Corp., Tokyo, Japan). The growth rate was calculated as the increase in SVL from the start until the end of the experiment (mm/day). At the end of the experiment, individuals were killed by immersion in liquid nitrogen.

2.2. Sample processing and biochemical analyses

Oxidative stress parameters were measured in whole bodies. To quantify the activity of the antioxidant parameters, each sample was homogenized in 5 vol of 25 mM sucrose buffer, pH 7.4, containing 10 mMTris-HCl and 5 mM EDTA using an Ultra-Turrax, Janke and Kunkel, IKA-Werk (Germany) homogenizer (Lionetto et al., 2003). The homogenate was sonicated for 30 s at 10 kHz with a Sonopuls ultrasonic homogenizer (HD 2070; Bandelin Electronic, Germany).The sonicated samples were immediately separated into aliquots for the measurement of total GSH. GSH sonicates in 10% sulfosalicylic acid were centrifuged at 5,000×*g* for 10 min (Griffith, 1980); for other AOS parameters, samples were centrifuged at 100,000×*g* for 90 min at 4°C using a Beckman ultracentrifuge (Takada et al., 1982; Abele et al., 2011). To determine the activity of SOD, the autoxidation of adrenaline to adrenochrome at 480 nm (Misra and Fridovich, 1972) was followed. CAT activity was assessed by Claiborne's (1984) method, which quantifies the degradation of hydrogen peroxide at 240 nm. To assay GSH-Px activity, the protocol developed by Tamura et al. (1982) was used, and for GR activity, the protocol described by Glatzle et al. (1974). Activities of both GSH-Px and GR are based on the rate of NADPH oxidation. To measure GST activity, the method described by Habig et al. (1974) was used. The activities of three enzymes, GSH-Px, GR and GST, were measured at 340 nm. The activities of all antioxidative enzymes were expressed as U mg^{-1} protein. To measure GSH concentration, the method of Griffith (1980), which is based on 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) enzymatic recycling, was used. GSH concentration was expressed in nmol g^{-1} tissue. After incubation of tissue extracts with DTNB, the contents of protein sulfhydryl (SH) groups were recorded (Ellman, 1959). The concentration of SH groups was expressed as nmol mg^{-1} protein. At 412 nm, the absorbance of both GSH and SH groups was measured. To quantify thiobarbituric acid-reactive substance (TBARS), the assay developed by Rehncrona et al. (1980) was used. Samples were homogenized and sonicated at pH 7.4 in 10 vol of ice-cold Tris-HCl buffer, and centrifuged for 10 min in 40% trichloroacetic acid (TCA) at 10,000×*g* at 4°C. The obtained supernatants were used to measure lipid peroxidation (LPO) at 532 nm and were expressed as nmol $(mg\ tissue)^{-1}$. The total amount of protein contained in samples was quantified according to Lowry et al. (1951) using a UV-VIS spectrophotometer with a temperature-controlled cuvette holder (UV-1800, Shimadzu, Japan); all parameters were measured at 19°C, which is considered the optimal temperature for the studied species (as regards their habitat and body temperature) (Gvoždík et al., 2007; Abele et al., 2011; Prokić et al., 2018).

All reagents were obtained from Sigma (St. Louis, MO, USA). The intraclass correlation coefficients (ICC) were as follows: 0.982, 0.978, 0.988, 0.992, 0.975, 0.983, 0.987 and 0.786 for SOD, CAT, GSH-Px, GST, GR, GSH, SH groups and LPO, respectively.

2.3. Statistical analyses

To check for possible outliers, Grubb's test was used and no outliers were detected. Normal distribution of data and homogeneity of variance were confirmed with the Kolmogorov-Smirnov and Levine tests, respectively. A mixed model for repeated measures was performed to test for differences in SVL within and between genotypes during the experiment, using SVL values at the sampling point (beginning or end of the experiment) as a repeated variable (within-subject), with the genotype and temperature as fixed factors. Differences among the studied factors were checked by posthoc test with Bonferroni's correction for multiple comparisons. The Fisher exact test was used to analyze differences in the percentages of metamorphosed individuals at the end of the experiment. To check for differences between two independent factors, i.e., genotype (parental species and hybrids), temperature (19°C and 24°C), and their interaction with oxidative stress parameters, factorial ANOVA was applied. When a significant interaction between factors (treatment × genotype) was observed, the posthoc test was conducted to check for differences between levels (i.e., pairwise multiple comparisons with Tukey's adjusted P values). In canonical analyses, AOS parameters were included for individuals exposed to a temperature of 24°C to determine the parameters that contributed the most to the differences in stress response among hybrids and parental species. Statistical analyses were performed using STATISTICA 8.0, except for the mixed model, which was performed in IBM SPSS Statistics (Ver. 27.0). Pairwise multiple comparisons were performed in XLSTAT (Ver. 2014.5.03), and intraclass correlation coefficients were calculated in R 3.4.4 (R Development Core Team) with ICC (package 'ICC' 2.3.0).

3. Results

3.1. Growth and development

Differences in growth and developmental rates between hybrids and their parental species were observed. At the beginning of the experiment, the *T. macedonicus*-mothered hybrid had a significantly greater SVL than *T. macedonicus* and the *T. ivanbureschi*-mothered hybrid (Fig. 2). All the examined groups, regardless of the temperature to which they were exposed, at the end of the experiment showed significantly higher SVL values in comparison to the SVL values at the start of the experiment (Fig. 2, Table S1). At the end of the experiment, the larvae of parental species and hybrids reared at 19°C did not differ in SVL. Comparison between groups from the warm environment (24°C) showed that only individuals of *T. ivanbureschi* had significantly lower SVL values than individuals from the other genotypes (Fig. 2, Table S1). *T. ivanbureschi* was the only genotype that at the end experiment did not display significant differences in SVL at either 19°C or 24°C, whereas in all other genotypes, the higher temperature led to a significant increase in SVL (Fig. 2, Table S1). After 30 days of exposure to 19°C, growth rates were similar among species (Fig. 3). In contrast, at 24°C *T. macedonicus* experienced the highest growth rate, followed by both hybrids, while *T. ivanbureschi* exhibited the lowest growth rate (Fig. 3). *T. ivanbureschi* was the only species that did not exhibit a higher growth rate in warm conditions. At the end of the experiment (after 30 days of exposure to temperature treatments), larvae exposed to 19°C did not yet complete metamorphosis. In contrast, 36.7% (11 of 30 individuals) of the *T. macedonicus*-mothered hybrid, 30.0% of the *T. ivanbureschi*-mothered hybrid (9 of 30) and 20.0% of *T. ivanbureschi* (6 of 30) completed metamorphosis after exposure to 24°C. None of *T. macedonicus* larvae completed metamorphosis at either of the temperatures. Comparisons for the number of metamorphosed individuals between genotypes at different temperatures (19°C vs. 24°C) revealed a significantly higher number of metamorphosed individuals at the warm temperature for both hybrids (*T. macedonicus*-mothered hybrid $P = 0.0003$ and *T. ivanbureschi*-mothered hybrid $P = 0.0019$), and *T. ivanbureschi* ($P = 0.0237$). The differences between parental species and their hybrids at 24°C showed that only *T. macedonicus* had a significantly lower number of metamorphosed individuals (none) in comparison to other genotypes (*T. macedonicus* vs *T. ivanbureschi* $P = 0.0237$; *T. macedonicus* vs the *T. macedonicus*-mothered hybrid $P = 0.0003$; *T. macedonicus* vs. the *T. ivanbureschi*-mothered hybrid $P = 0.0019$).

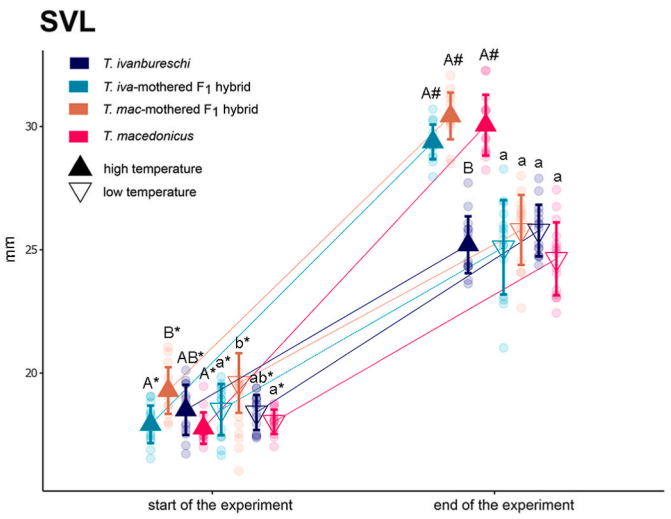

Fig. 2. Snout vent length (SVL-mm) of hybrids and parental species of crested newts larvae (*Triturus*) at the beginning and end of the experiment at temperatures of 19 °C and 24 °C. "*" indicates significant differences between the beginning and end of the experiment for the same genotype/species; "#" indicates significant differences at the end of the experiment between the same species under the 19 °C and 24 °C treatment; lowercase letters indicate differences between species reared under 19 °C water temperature; capital letters indicate differences between species exposed to 24 °C water temperature. Different letters indicate significant differences ($P \leq 0.05$).

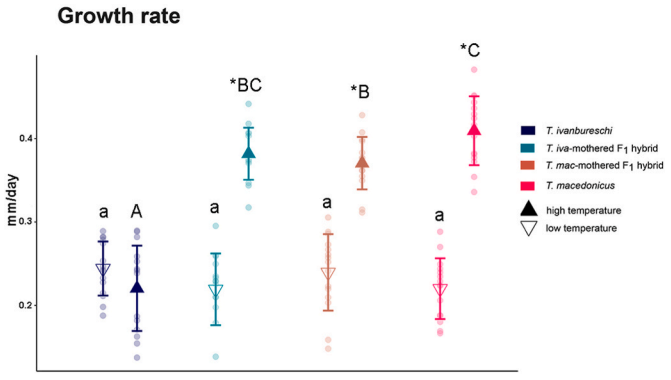

Fig. 3. Growth rates (mean ± standard error) of parental species and hybrid individuals (*Triturus*) reared at 19 °C and 24°C. "*" indicates significant differences between the 19 °C and 24 °C treatment for the same species; lower case letters indicate differences between species reared under 19 °C water temperature; capital letters indicate differences between species exposed to 24 °C water temperature. Different letters indicate significant differences ($P \leq 0.05$).

3.2. Oxidative stress

Factorial ANOVA conducted on oxidative stress parameters showed a significant influence of the genotype × temperature interaction on the activities of SOD and GST, and LPO concentrations (Table 1). Since the

Table 1
Factorial ANOVAs on examined oxidative stress parameters between genotype (*Triturus macedonicus*, *T. ivanbureschi*, *T. macedonicus*-mothered and *T. ivanbureschi*-mothered F1 hybrids), temperature (19 °C and 24 °C), and their interaction (genotype x temperature). Statistical significance ($P \leq 0.05$) is given in **bold**.

Parameter	Effect	Df	*F*	*P*
SOD	temperature	1	0.418	0.5194
	genotype	**3**	**54.190**	**<0.0001**
	genotype x temperature	**3**	**13.826**	**<0.0001**
CAT	**temperature**	**1**	**15.826**	**<0.0001**
	genotype	**3**	**55.030**	**<0.0001**
	genotype x temperature	3	0.298	0.8267
GSH-Px	**temperature**	**1**	**54.641**	**<0.0001**
	genotype	**3**	**17.723**	**<0.0001**
	species x temperature	3	1.245	0.2967
GR	**temperature**	**1**	**4.180**	**0.0432**
	genotype	**3**	**61.345**	**<0.0001**
	genotype x temperature	3	0.959	0.4146
GST	**temperature**	**1**	**173.149**	**<0.0001**
	genotype	**3**	**82.244**	**<0.0001**
	genotype x temperature	**3**	**8.858**	**<0.0001**
GSH	**temperature**	**1**	**13.259**	**0.0004**
	genotype	**3**	**4.442**	**0.0054**
	genotype x temperature	3	0.641	0.5905
SH	**temperature**	**1**	**84.691**	**<0.0001**
	genotype	**3**	**11.582**	**<0.0001**
	genotype x temperature	3	2.028	0.1140
LPO	temperature	1	0.392	0.5323
	genotype	**3**	**3.628**	**0.0153**
	genotype x temperature	**3**	**7.714**	**0.0001**

interaction was significant, the influence of single factors cannot be explained without considering the other factor, hence all post hoc tests on parameters where the interactions were significant are included in Table S2. For the other parameters, CAT, GSH-Px, GR, GSH and SH groups, significant differences for both factors (i.e., genotype and temperature) were recorded but not for their interaction (Table 1).

3.2.1. *Effects of temperature on the oxidative status of hybrids and parental species*

Warm conditions induced increases in SOD, CAT, GSH-Px and GST activities and SH concentrations in *T. macedonicus* individuals (Figs. 4 and 5). In contrast, *T. macedonicus* individuals raised at 19°C had higher GSH and LPO concentrations (Figs. 4 and 5). The higher temperature led to increased activities of CAT, GSH-Px, GST and SH concentration in *T. ivanbureschi*, whereas a lower temperature caused higher SOD activity and GSH concentration. In hybrids exposed to 24°C, an overall increase in the values of oxidative stress parameters compared to those at 19°C was observed. At 24°C, the *T. macedonicus*-mothered hybrid exhibited higher activities of GSH-Px and GST, and the *T. ivanbureschi*-mothered hybrid higher activities of CAT, GSH-Px, GR and GST. Hybrids exposed to the higher temperature had higher concentrations of SH groups and LPO (Figs. 4 and 5).

At the individual level, the growth rate was in significant correlation with GSH-Px ($r = 0.29$, $P = 0.002$), GST ($r = 0.31$, $P = 0.001$) activities and SH ($r = 0.18$, $P = 0.048$) concentration, whereas the correlations between individual growth rate and other oxidative stress parameters were non-significant (Table S3).

3.2.2. *Oxidative stress differences between parental species and hybrids*

Individuals of the *T. macedonicus*-mothered hybrid had significantly higher activities of SOD, CAT, GSH-Px, GR and GST in comparison to the parental species and *T. ivanbureschi*-mothered hybrids at both temperatures (Figs. 4 and 5). At 24°C, the *T. macedonicus*-mothered hybrid also displayed a higher concentration of LPO than both parental species, and of SH groups than *T. macedonicus* larvae (Fig. 5). *Triturus ivanbureschi*-mothered hybrids that were maintained at the lower temperature had lower activities of SOD, CAT and GSH-Px than *T. ivanbureschi*, and the concentration of LPO when compared to *T. macedonicus*. At the warm temperature, the *T. ivanbureschi*-mothered hybrid had lower CAT activity than *T. ivanbureschi,* and lower SOD activity than *T. macedonicus.* Higher LPO values were detected in *T. ivanbureschi*-mothered individuals as compared to *T. macedonicus* and *T. ivanbureschi,* as well as higher concentrations of SH groups than in *T. macedonicus.* Comparisons between parental species revealed that the larvae of *T. ivanbureschi* had higher activities of CAT at both temperatures, of GSH-Px and SOD at 19°C, and SH concentration at 24°C (Figs. 4 and 5). Only the activity of SOD in individuals of *T. macedonicus* at warmer temperature was higher than in *T. ivanbureschi* (Fig. 4).

Canonical discriminant analysis was conducted on AOS parameters at 24°C to obtain possible differences among genotypes in response to thermal stress. *T. macedonicus*-mothered hybrid individuals differed from the other genotypes according to the first canonical function (Root 1–45.74% of the total heterogeneity; Fig. 6). The parameters that contributed most to the observed differences were GST, GR and GSH-Px (Table S4). The second canonical function (Root 2–30.58% of the total heterogeneity) separated parental species; the parameters that contributed the most were SH groups and SOD and CAT activities (Table S4 and Fig. 6).

4. Discussion

In ectotherms, the relationships between mitochondrial activity, ROS production and ambient temperature often show that a higher temperature changes oxidative phosphorylation, increases mitochondrial H_2O_2 production and activates the AOS (Paital and Chainy, 2014; Chung and Schulte, 2015; Wang et al., 2018; Roussel and Voituron, 2020; Jie et al., 2021). Herein we investigated whether ambient temperature alters the life-history traits and oxidative status of larvae of two newt species and their hybrids. A higher water temperature induced overall activation of the AOS response in all four investigated crested newt genotypes. Higher activities of enzymes that remove H_2O_2 (i.e., CAT and GSH-Px) indicated that newt larvae were exposed to increased production of the free radical. The observed higher concentrations of SH groups in animals exposed to warmer temperature can be the result of intense somatic growth, protein reorganization and synthesis. Likewise, increases in GST activity in response to higher temperature match the ongoing process of lipid peroxidation via the formation of lipid hydroperoxide (Pamplona and Costantini, 2011). Even though all genotypes displayed a similar pattern of AOS change in the warm conditions, the AOS of parental species was efficient enough to neutralize ROS production, as suggested by the similar levels of lipid peroxidation observed *at both cold and warm temperatures*. This contrasts with the pattern observed in hybrids since despite the activation of the AOS at the higher temperature, the individuals displayed lipid oxidative damage.

As oxidative stress is a driving force in life-history trade-offs (Costantini, 2008; Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010; Selman et al., 2012; Smith et al., 2016), higher levels of oxidative stress in the hybrids, aside from causing damage to macromolecules and increasing maintenance and repair costs, can lead to mtDNA rearrangements and reduced fidelity of protein translation (Esposito et al., 1999; Du et al., 2017). When these effects are combined, they can result in a detrimental effect on hybrid viability, growth and development rate, but also affect adult reproductive traits (fecundity and sperm swimming speed) and immune status (Alonso-Alvarez et al., 2007; Barreto and Burton, 2013; Hemmer-Brepson et al., 2014; Du et al., 2017; Hill et al., 2018). However, the more pronounced effects of the warm water on the oxidative status of hybrids cannot be explained by the differences in growth and developmental rates nor by thermal tolerance among genotypes. Body growth and developmental acceleration in larvae likely induce overwhelming ROS production partly as the result of increased

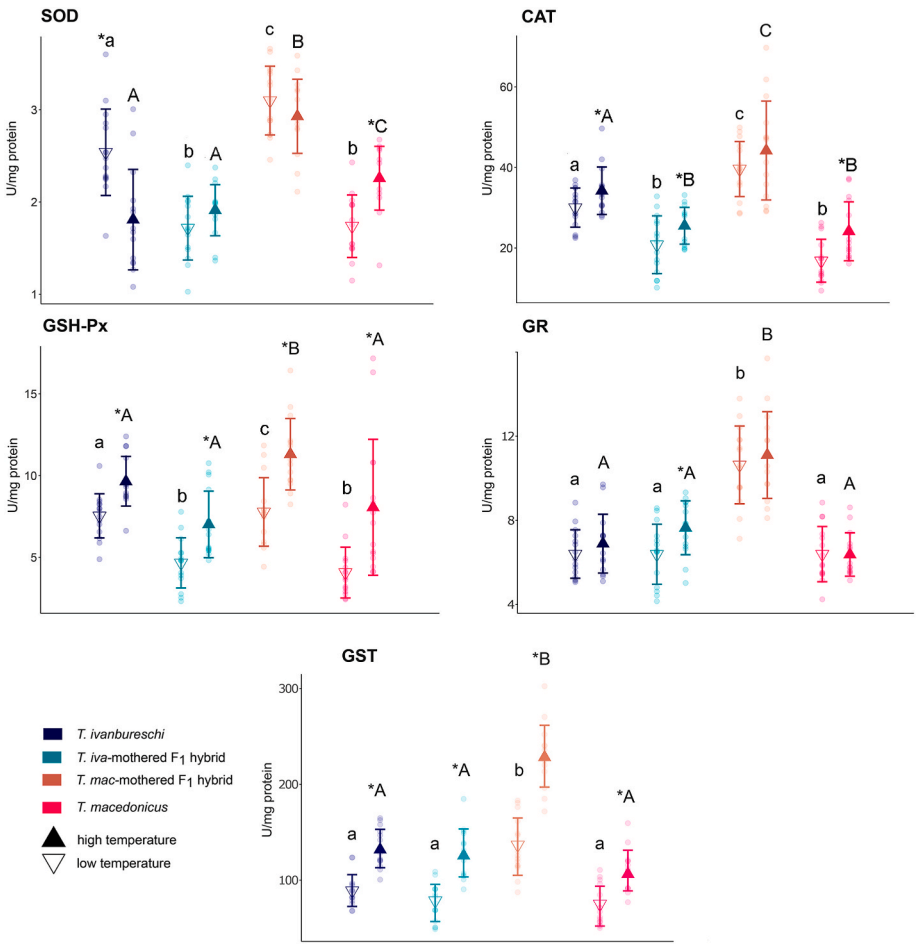

Fig. 4. Enzymatic parameters of the antioxidant system (SOD, CAT, GSH-Px, GR and GST) in individuals of *Triturus macedonicus*, *T. ivanbureschi*, *T. macedonicus* mothered and *T. ivanbureschi* mothered F1 hybrids reared at 19 °C and 24°C. "*" indicates significant differences between the 19 and 24 °C treatment for the same species; lower case letters indicate differences between species reared under 19 °C water temperature; capital letters indicate differences between species exposed to 24 °C water temperature. Different letters indicate significant differences ($P \leq 0.05$). Triangle – mean value; lines – standard deviation; dots –values for each individual. For species and hybrids, abbreviations see Fig. 3.

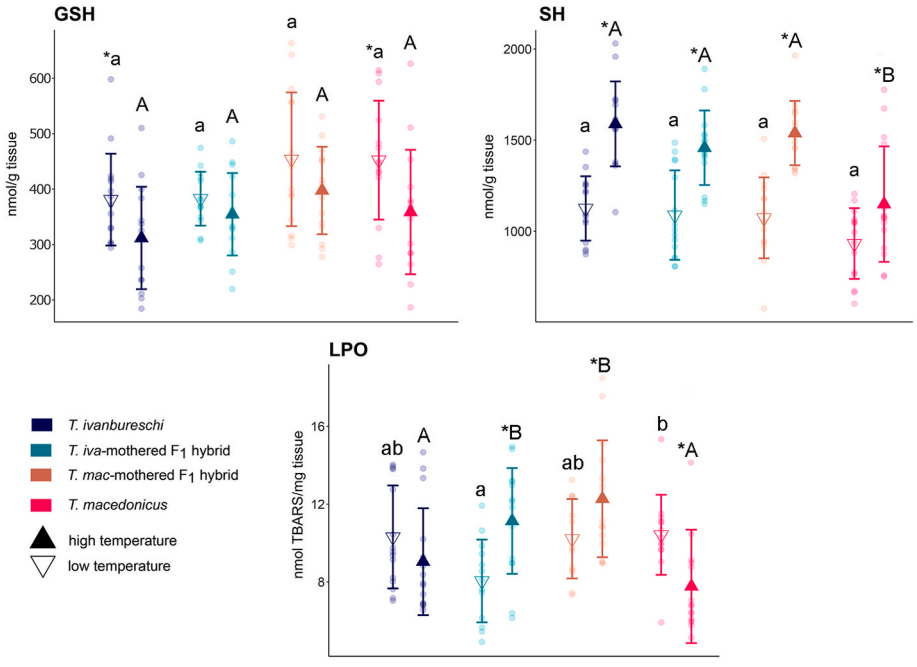

Fig. 5. Non-enzymatic parameters of the antioxidant system (GSH and SH groups) and oxidative damage in lipids (LPO) in individuals of parental species and hybrids of crested newts larvae (*Triturus*) reared at 19 °C and 24 °C. "*" indicates significant differences between 19 and 24 °C treatment under the same species; lower case letters indicate differences between species reared under 19 °C water temperature; capital letters indicate differences between species exposed to 24 °C water temperature. Different letters indicate significant differences ($P \leq 0.05$). Triangle – mean value; lines – standard deviation; dots –values for each individual.

cellular activity that is required to attain body mass and size (Smith et al., 2016; Burraco et al., 2020), and also because corticosterone levels increase during metamorphosis (Costantini et al., 2011; Gomez-Mestre et al., 2013). In this study, we did not observe a significant correlation between body growth and the levels of oxidative damage. In addition, increased growth and accelerated metamorphosis at the higher

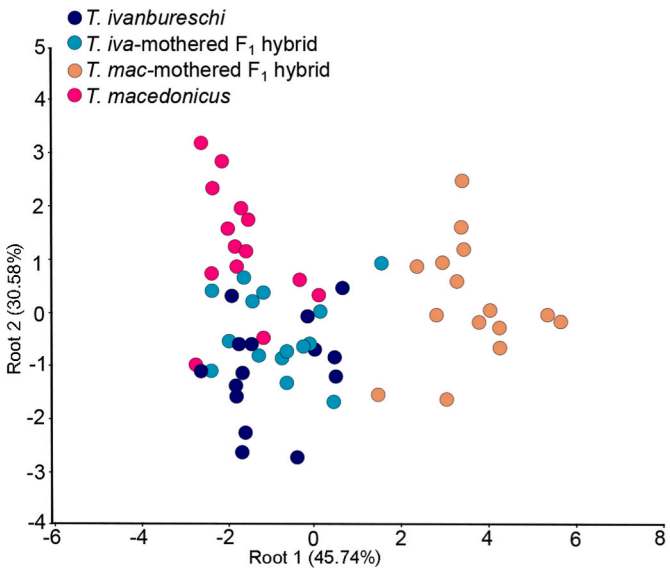

Fig. 6. Canonical discriminant analyses of the antioxidant parameters (SOD, CAT, GSH-Px, GST, GR, GSH and SH groups) of parental species and their hybrids (*Triturus*) reared at 24 °C. See also Supplementary Table 2.

temperature were observed in both hybrids and in the parental species (*T. macedonicus*– significant growth, *T. ivanbureschi* – accelerated metamorphosis). The parental species did not exhibit higher oxidative damage. In natural habitats, *T. macedonicus* and *T. ivanbureschi* are faced with marked temperature gradients across their wide range of distribution. The warm experimental temperature used in our study is above the optimal temperature range for both species and hybrids. Hence, increased oxidative stress in hybrids that had to cope with the warm condition was probably the result of the interaction between the physiological and genetic characteristics of each genotype and the abiotic environment (see Hill et al., 2018).

Several studies in ectothermic animals, insects and lizards, have suggested that hybrids are particularly vulnerable to high temperatures, as exemplified by the negative impacts on fitness-related traits such as survival, fertility and metabolic rates (Wade et al., 1999; Vinšálková and Gvoždík, 2007; Arnqvist et al., 2010; Koevoets et al., 2012; Miller and Matute, 2017; Rodríguez et al., 2021). In crested newt hybrids, the mismatch between mitochondrial and nuclear DNA can include higher metabolic rates and reduced mitochondria efficiency (Gvoždík, 2012). Exposure to thermal stress can amplify this disruption in mitochondrial activity and the metabolic pathways of hybrids showing maladaptive responses. Using the same species and hybrids, we previously revealed that other stressful conditions known to affect mitochondria function, such as fasting, induce greater oxidative damage in hybrids when compared to parental species (Prokić et al., 2021), indicating that hybrids face mitonuclear mismatch to some extent. However, the degree of alteration of mitochondrial functioning and ROS production depends on the type of stress and the mitonuclear background (i.e., the association with maternal nuclear alleles and the mitochondrial genotype), and thus the consequences for the oxidative stress machinery are context-dependent and range from mild to severe (Healy and Burton, 2020; Rodríguez et al., 2021). If hybrid dysfunction is not too severe, a compensatory response may mitigate to some extent fitness loss, but if mitigation fails and the cost of mounting the response exacerbates physiological dysfunction, it can contribute to the absence of hybrids in natural populations (Barreto et al., 2014).

The differences in oxidative stress between the four studied genotypes (i.e., parental species and their reciprocal hybrids) match the occurrence of these genotypes in the natural hybrid zone. Individuals of the *T. macedonicus*-mothered hybrid are not found in natural populations (Wielstra et al., 2017; Wielstra and Arntzen, 2020). This hybrid also had the most disturbed redox balance in our experiment. Besides oxidative damage in response to a higher temperature, this hybrid genotype constitutively displayed high activities of most AOS components (*SOD, CAT, GSH-Px, GST and GR)*, regardless of the temperature and developmental parameters, in comparison to other genotypes, pointing to greater ROS production and intrinsic incompatibilities. Barreto et al. (2014) suggested that aside from oxidative damage, the overexpression of genes involved in the antioxidant response can contribute to the metabolic syndrome and the breakdown of hybrids. The *T. macedonicus*-mothered hybrid also displayed significantly lower integration of the AOS (overall correlation/integration of AOS components) under non-stressful conditions in comparison to parental species, suggesting that hybrids require greater investment than the parents to maintain the same levels of oxidative damage (Prokić et al., 2018). Indeed, the maintenance of the AOS as unregulated is not free of cost, likely because of physiological constraints, and can affect subsequent ontogenetic stages, especially in changed environments (Pamplona and Costantini, 2011; Prokić et al., 2018; Petrović et al., 2020). In this study, we did not observe any cost in body growth, but the trade-off in a more active AOS could be seen in other biological functions (Isaksson et al., 2011; Eikenaar et al., 2018; Janssens and Stoks, 2018). However, *ad libitum* feeding during the experiment could have masked any cost in body size. Our previous results showed that individuals of the *T. macedonicus*-mothered hybrid were significantly more aggressive and active in comparison to *T. macedonicus*, suggesting higher foraging and food intake rates (Petrović et al., 2020). According to the "increased intake hypothesis", higher metabolic rates are linked with increased energy requirements and greater competition (Janča and Gvoždík, 2017). In contrast to the *T. macedonicus*-mothered hybrids, *T. ivanbureschi*-mothered hybrids are present in nature; in our study they exhibited intermediate levels of AOS parameters relative to the parents. In natural populations, *T. macedonicus* individuals with the mtDNA of *T. ivanbureschi* can be found, indicating that mitonuclear mismatch is probably not as marked in this genotype. A similar mitonuclear mismatch was observed in natural populations of horseshoe bats (*Rhinolophus affinis*), whereas individuals with mitochondria introgression exhibited significant upregulation of genes associated with protection against oxidative damage, probably caused by the inefficiency of the OXPHOS pathway (Chen and Mao, 2021).

5. Conclusions

Our results point to a mismatch between developmental growth and oxidative stress responses in amphibian hybrids in warm conditions. Although extrapolating findings from the laboratory to wild populations in the context of climate change is very challenging, the data from this study will serve as a baseline for further research into natural populations where many external factors and their combinations affect the oxidative status and life-history traits. Overall, our study provides some mechanistic insight into species replacement and mitonuclear discordance in hybrids, and can explain the presence of narrow hybrid zones, regardless of a similar reproductive potential and viability of F_1 hybrids and their parents (Bugarčić et al., 2022; Vučić et al., 2022). More pronounced physiological alterations and hybrid breakdown could be expected in F_2 and further generations of hybrids as the mitonuclear incompatibilities are shielded by dominance in the F_1 generation (Burton et al., 2006; for more read Hill et al., 2018). Further data on individuals from wild populations, later life stages and succeeding generations are needed to understand the distribution of hybrid species in nature. Finally, this study aims to encourage further work on the eco-evolutionary consequences of global change on interspecific hybrids, which can include the use of physiological data (such as redox information) in species distribution models (Pallarés et al., 2020; Burraco et al., 2022b) to predict the impact of future environmental scenarios on wild populations.

Credit author statement

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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.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2023.103474.

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