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PII: S1095-6433(19)30090-X
DOI: https://doi.org/10.1016/j.cbpa.2019.05.004
Reference: CBA 10494

To appear in: *Comparative Biochemistry and Physiology, Part A*

Received date: 20 February 2019
Revised date: 6 May 2019
Accepted date: 6 May 2019

Please cite this article as: M.D. Prokić, J.P. Gavrić, T.G. Petrović, et al., Oxidative stress in Pelophylax esculentus complex frogs in the wild during transition from aquatic to terrestrial life, Comparative Biochemistry and Physiology, Part A, https://doi.org/10.1016/j.cbpa.2019.05.004

Oxidative stress in *Pelophylax esculentus* complex frogs in the wild during transition from aquatic to terrestrial life

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Abstract

During life, anuran individuals undergo drastic changes in the course of transition from aquatic to terrestrial habitat, when they are faced with metabolically demanding processes (growth, responses to developmental pressures), which result in increased production of reactive oxygen species (ROS), signaling molecules involved in development that can induce oxidative damage and stress. This situation can be further complicated by environmental influences. The aim of this study was to investigate oxidative stress parameters in naturally developing *Pelophylax esculentus* complex frogs during four developmental periods: premetamorphosis, prometamorphosis, metamorphic climax and juvenile stage, in order to examine changes in the response of the antioxidative system (AOS) and oxidative damage during the transition from aquatic to terrestrial life. Results show that ontogenetic shifts in anurans are accompanied by different levels of damage and AOS responses, which vary from the increased first-line enzymatic activities during the early period of development (premetamorphosis), through increased changes in the non-enzymatic complement during the metamorphic climax, to changes in both the enzymatic and non-enzymatic components observed in juvenile individuals. Premetamorphic individuals and individuals in metamorphosis displayed higher levels of lipid peroxidation, indicating that direct exposure to the environment for the first time and the modulation of organs are the most susceptible stages for oxidative damage. On the other hand, lower oxidative damage in juveniles points to the ability of their AOS to efficiently respond to challenges of the terrestrial environment. This study highlights the importance of ROS and the AOS of anurans in response to different developmental and/or environmental pressures that individuals face.

Keywords: anurans; antioxidative system; development; oxidative stress; tadpoles; juvenile

1. Introduction

Managing oxidative stress (OS) is considered as one of the major determinants of an individual's life history as it can have direct effects by way of increased oxidative damage of macromolecules (Costantini, 2014), and/or indirect costs due to higher investment of energy in the antioxidative system (AOS) and repair mechanisms (Janssens and Stoks, 2018; Eikenaar et al., 2018; Prokić et al., 2018a). Boosted antioxidant defenses in the damselfly (*Lestes viridis*) and blackbirds (*Turdus merula*) lead to a trade-off in immune functioning (Janssens and Stoks, 2018; Eikenaar et al., 2018), also higher need for investment in antioxidant defense in the crested newts hybrid (*Triturus* spp) could have diverted the limited resources away from other functions and affected hybrid fitness (growth, survival, reproduction and longevity) (Prokić et al., 2018). OS in ecological and evolutionary studies is mostly examined in the context of reproduction and much less in the context of development/growth (Metcalfe and Alonso-Alvarez, 2010); however, interest in the effect of ontogenetic changes on OS is on the rise (Romero-Haro et al., 2016; Salmón et al., 2018). There is evidence that the relationship between growth and OS depends on the developmental stage of the organism, and that at certain points during development an individual is more vulnerable to oxidative damage (Smith et al., 2016). This is particularly important at birth/hatching because of changes in partial oxygen pressure and metabolic rate (Nussey et al., 2009). OS during development is often seen as a constraint and the price of increased growth (Smith et al., 2016), with possible long-term effects on fitness (Metcalfe and Monaghan, 2001). The requirements of increased cellular activity during metabolic processes can lead to the elevated production of reactive oxygen species (ROS) as a byproduct of metabolism. When the concentrations of ROS (such as $O_2^{\cdot -}$ and H_2O_2) are tightly controlled, they can act as intracellular signaling molecules and perform critical functions in organisms (regulating circulation, energy metabolism, reproduction and remodulation of cells, tissues and organs) (Allen and Tresini, 2000). On the other hand, increased ROS can react with biomolecules (lipids, proteins and DNA), affecting their normal function, and as a result of oxidative damage and stress they can produce malformations or death (Halliwell and Gutteridge, 2015; Faggio et al., 2016; Aliko et al., 2018; Burgos-Aceves et al., 2018; Gobi et al., 2018; Hodkovicova et al., 2019; Stara et al., 2019). It was hypothesized that since animals are faced during life with different growth intensities and metabolic rates, they have evolved mechanisms to counteract the effects of ROS, and that different antioxidative components are used to

counteract the ROS produced at different stages of development (Metcalfe and Alonso-Alvarez, 2010). The antioxidative machinery that deals with potentially destructive ROS is equipped with both enzymatic and nonenzymatic components that are in complex interrelationships (Costantini, 2014). The first line of this defense consists of rapidly acting neutralizing enzymes (superoxide dismutase – SOD, catalase – CAT and glutathione peroxide – GSH-Px) that suppress or prevent the formation of free radicals. The second line of defense, which is comprised of scavenging antioxidants (glutathione – GSH, uric acid, vitamins C and E), removes radicals, inhibiting the initiation and propagation reactions (Halliwell and Gutteridge, 2015).

Amphibians represent an interesting model organism for examining the physiological mechanisms that mediate life-stage transitions, as they possess a unique life cycle among tetrapods (involving egg, tadpole, juvenile, subadult and adult stages) which is related to their inhabiting terrestrial and aquatic environments (Burraco et al., 2018; Prokić et al., 2018a; Prokić et al., 2018b; Turani et al., 2018). The anuran individual undergoes a biphasic developmental process, from a free-living tadpole that metamorphoses into the adult. This change is regulated by thyroid hormones and requires a series of dynamic and systemic adaptations from a low-oxygen (aquatic) environment and herbivorous diet, to an oxygen-requiring terrestrial and carnivorous life (Kulkarni et al., 2017; Ruthsatz et al., 2018). It is followed by increased growth, together with absorption of larva-specific organs, the appearance of adult-type organs, numerous physiological and biochemical changes and oxidative metabolism in particular (increased OS, shortening of telomere lengths, changes in corticosterone levels) (Gomez-Mestre et al., 2013; Burraco et al., 2017a, 2017b). It was assumed that the AOS plays an important role in managing the developmental processes and responses to environmental changes that tadpoles are faced with (Melvin, 2016; Pinya et al., 2016; Prokić et al., 2017). Besides the AOS, during anuran development external environmental inputs (desiccation, pollution, temperature changes and predator exposure) can activate the HPA-axis (and cause increased corticosterone production), which together with thyroid hormones can affect growth and development (Boorse and Denver, 2003; Burraco et al., 2017b). This activation leads to enhanced ROS production (Costantini et al., 2011). Rizzo et al. (2007) showed that during the earliest stages of anuran development the produced ROS was removed by the activity of SOD and CAT, while the GSH system is formed when the animal is about to be exposed to environmental stressors for the first time. The period of transformation and formation of the adult intestine and reduction of the tail is followed by

increased production of ROS and OS (Menon and Rozman, 2007; Johnson et al., 2013). Later life stages, such as the subadult and adult, differ also in the overall correlation of the AOS and the way it deals with ROS (Samanta and Paital, 2016; Prokić et al., 2018b). Even though there are studies examining OS during different stages of anuran development, to our knowledge there is no comprehensive study of the development from tadpole to juvenile.

Natural populations in particular, are challenged with more variable and harsher environments than laboratory-reared individuals, and can show different relationships between development-induced OS. Although studies conducted on organisms from the wild do not readily allow experimentation or repeated sampling, they can provide an essential insight into the evolutionary and ecological causes and consequence of variations in OS (Nussey et al., 2009).

In the present study, we followed oxidative stress parameters in developing *Pelophylax esculentus* complex frogs in the wild during four periods of development (premetamorphosis, prometamorphosis, metamorphic climax and juvenile). Premetamorphosis is characterized by individuals that begin free-swimming and are for the first time directly exposed to the environment. As prometamorphic individuals, they also display intense growth, feeding and accumulation of energy (lipids) necessary for further life stages. In contrast to them, individuals in metamorphic climax stop growing, their body mass declines and feeding is reduced, and their tissues undergo remodeling. Completely metamorphosed juveniles are prepared for the terrestrial life style (jumping, carnivorous diet, exposure to UV light and oxygen). Our aim was to determine the changes in the AOS and their role in oxidative damage during transition from the aquatic to the terrestrial environment. We hypothesized that that the levels of oxidative stress will be proportional to the levels of change that individuals undergo during development (e.g. greater oxidative stress in premetamorphic and metamorphic individuals due to more drastic changes).

2. Materials and methods

All frogs were caught at the locality “Stevanove ravnice” within the Special Nature Reserve “Deliblatska peščara” (44˚49'47.8" N and 21˚18'23.6" E) Serbia (Supplementary Fig. S1), from May to July 2017, which covered the entire period of frog development. The locality is in Vojvodina, the northern part of Serbia, and is marked as the largest European continental sandy locality. The richness in flora and fauna make it one of the most important centers of biodiversity in Serbia and Europe. It is protected by the Government of the Republic of Serbia

and included in the Ramsar List of Wetlands of International Importance (Stupar et al., 2017). The Reserve is removed from large sources of pollution. At the sampling locality vernal ponds are formed due to sand depression and elevated groundwater. Ponds are located near the Danube River and are covered with hydrophilic or mixed grasses, making this site desirable for anuran reproduction. Even though several species reproduce in these ponds, we did not observe any interspecific competition. *Pelophylax esculentus* complex frogs from all stages of development were caught in the same pool in order to avoid possible environmental differences between pools. Sampling was always performed from 9-13 AM. The great number of males and females at the beginning of the reproductive period provided a variety of tadpoles belonging to different families. All individuals finished their development before complete desiccation of the pool (end of August). Frogs were caught with a hand net and Gosner stages (GS) were determined in the field, according to which individuals were assigned to one of four groups: premetamorphic (covering 25-35 GS), prometamorphic (35-41 GS), metamorphic (42-46 GS) and juvenile frogs. Before placing the individuals in liquid nitrogen and transfer to the laboratory for further analyses, we photographed the individuals in order to obtain their body lengths (Underwood et al., 2013; Lillywhite et al., 2016; Pinya et al., 2016). The images were made using a Sony DSC-F828 digital camera (24-bit color and 3,264 x 2,448 pixel resolution, MP; Sony Corp., Tokyo, Japan). Landmarks were digitized using TpsDig software (Rohlf, 2005) and calculated as Euclidian distances between landmarks using Tmorphgen6 (Sheets, 2001).

At the sampling locality some of the physicochemical parameters of the water (temperature, pH, dissolved oxygen and conductivity) were measured using mobile analytical equipment (YSI Multiparameter Water Professional Plus Water Quality Meter). Measurements were taken in triplicate.

Animal capture was approved by the Ministry of Agriculture and Environmental Protection of the Republic of Serbia (permit no. 353-01-515/2016-17) and the Institute for Nature Conservation of Vojvodina Province (permit no.03-456/2). All animal procedures complied with the EC Directive and 86/609/EEC European Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes.

2.1. Sample processing

Whole bodies were finely chopped and mixed to obtain as much homogenous material as possible. Individuals at earlier stages were pooled in order to obtain the required amount of

sample (0.6-0.7 g). About 0.2 g was taken for determination of the concentration of thiobarbituric acid reactive substances (TBARS), while the rest of the sample was used for determination of AOS parameters. The samples for the latter were homogenized (using an Ultra-Turrax homogenizer, Janke and Kunkel, IKA-Werk, Staufen, Germany) in 5 volumes of 25 mM sucrose containing 10 mM Tris-HCl, at pH 7.5, according to Lionetto et al. (2003). The homogenates were sonicated using an ultra-homogenizer Sonopuls (Bandelin, Germany) at 40 kHz for 3 x 10 s each. A part of the sonicate was taken for total GSH concentration determination, while the rest was centrifuged in a L7-55 ultracentrifuge (Beckman, USA) at 100,000 x *g* at 4°C for 90 min (Takada et al., 1982). The supernatants were used for measuring AOS parameters.

2.2. Biochemical analyses

SOD activity was measured based on the autoxidation of adrenaline to adrenochrome at 480 nm by the method of Misra and Fridovich (1972). CAT activity was determined according to the rate of hydrogen peroxide (H_2O_2) decomposition at 240 nm (Claiborne, 1984). Glutathione peroxidase (GSH-Px) activity was based on the reduction of t-butyl hydroperoxide with nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm (Tamura et al., 1982). To determine glutathione reductase (GR) activity, we recorded the reduction of glutathione disulfide (GSSG) to reduced GSH using NADPH as a substrate at 340 nm (Glatzle et al., 1974). The method described by Habig et al. (1974) was used for determination of glutathione-S-transferase (GST) activity, based on the reaction of the SH group of GSH with 1-chloro-2, 4-dinitrobenzene (CDNB) at 340 nm. The activities of all enzymes were expressed in U/mg protein. Protein concentrations in samples were measured according to the method of Lowry et al. (1951).

The concentration of total GSH was determined after oxidation of GSH using 5,5´-dithiobis-(2-nitrobenzoic acid) (DTNB) and reduction by NADPH in the presence of GR at 412 nm according to Griffith (1980). The method of Ellman (1959) was used to measure the concentrations of SH groups. TBARS was estimated according to the method of Rehncrona et al. (1980). The concentration of TBARS was measured after treating the samples with cold thiobarbituric acid reagent (10% trichloroacetic acid, 0.6% thiobarbituric acid) and heating at 100°C, at 532 nm.

Measurements were performed at 25°C using a Shimadzu UV 1800 UV-VIS spectrophotometer with a temperature-controlled cuvette holder.

2.3. Statistical analyses

To check for possible outliers, the normality of data and homogeneity of variance, we applied the Grubb, Kolmogorov-Smirnov and Levine tests, respectively. All criteria were respected and data were expressed as the means±standard error. We examined differences between the different groups (premetamorphic, prometamorphic, metamorphic and juvenile) with respect to OS parameters through one-way ANOVA ($p<0.05$). To determine the differences between each group, we performed post hoc analyses (Tukey's HSD for unequal N with $p<0.05$ as the criterion for significance). The overall response of the AOS was calculated as the value of Integrative Biomarker Response (IBR) based on the procedure given by Devin et al. (2014). To investigate the variation in AOS parameters within and between examined groups, we used Principal Component Analysis (PCA). All statistical analyses were performed using STATISTICA 8.0 (StatSoft, Inc., 2007) with the exception for the IBR value and Pearson's correlations, which were calculated in R 3.4.4. (R Development Core Team, 2018) using the script provided by courtesy of Devin S for IBR and package 'corrplot' for correlogram.

3. Results

The physicochemical parameters of water at the sampling pool are presented in the Supplementary material, Table S1.Values for total body length of individuals were as follows: premetamorphic individuals: 27.80 ± 4.01 mm; prometamorphic: 42.39 ± 5.15 mm; metamorphic: 25.28 ± 8.96 mm; juvenile: 21.45 ± 1.28 mm. The results for OS parameters are given in Table 1 and Figure 1. SOD activity differed significantly between almost all examined groups. The highest was in premetamorphic and juvenile individuals, followed by prometamorphic individuals, and the lowest were in individuals under metamorphic climax. CAT was statistically higher in juvenile individuals as compared to the other groups. Premetamorphic individuals had higher GSH-Px and GST activities than the other groups. GST was also higher in prometamorphic individuals in comparison to juveniles. The activity of GR was higher in the premetamorphic and juvenile groups than in the other two. Concentrations of nonenzymatic components (GSH and SH) were higher in juveniles and individuals under metamorphic climax as compared to earlier stages. GSH was also higher in prometamorphic individuals in comparison to premetamorphic ones. The concentration of TBARS as the marker of the lipid peroxidation process (LPO) was higher in premetamorphic individuals than in individuals under metamorphic climax, followed by prometamorphic and juvenile frogs. The

IBR for AOS parameters was highest in premetamorphic individuals (3.91 ± 0.04), followed by juvenile (3.40 ± 0.02), metamorphic (0.62 ± 0.02) and prometamorphic frogs (0.30 ± 0.00).

PCA was applied to explore the relationships between the examined AOS parameters (Table 2 and Figure 2). The first PC axis separated premetamorphic individuals from individuals in metamorphic climax and juveniles, with GST and SOD as the parameters that contributed most to the observed separation (Table 2). Prometamorphic individuals were consigned in between. The second PC axis distinguished prometamorphic individuals from juveniles, with the separation mainly the result of CAT activity and GSH concentrations. Pearson's correlations between AOS parameters are given in Figure 3.

4. Discussion

The path of transition from aquatic, swimming, herbivorous tadpole to terrestrial, carnivorous adult in anurans is accompanied by different development/growth, feeding (e.g. overfeeding and fasting) rates and different cell processes (proliferation and apoptosis). All of these processes can directly or indirectly affect metabolic rates and result in increased ROS production and oxidative damage (Smith et al., 2016). Low levels of produced ROS can affect cell signal transduction, while excessive ROS production damages macromolecules and leads to apoptosis (Scandalios, 2005). Individuals from natural populations can also challenge various environmental factors that can affect their oxidative balance (Nussey et al., 2009).

Variations in OS at different stages can be the result of the different pressures that challenge individuals at each stage, and are followed by different levels of oxidative damage and antioxidative responses. The highest levels of lipid peroxidation in this study were observed in premetamorphic individuals and those that were undergoing metamorphosis. Higher levels of LPO during the early stage (hindlimb bud) and metamorphic climax of *Polypedates maculatus* anurans was also reported by Mahapatra et al. (2001). By definition oxidative damage is raised because of exceptional ROS concentrations. It is interesting that the response to ROS differs among stages. These variations are influenced by complex processes during the metamorphosis of tadpoles, including the response of the hypothalamic-pituitary-intrarenal axis to both internal and external stressors (Glennemeier and Denver, 2002).

Larvae of amphibians tend to obtain sufficient energy necessary for growth and lipid stores, and it has been suggested that stored lipid are vital for survival of individuals during later stages (Scott et al., 2007). So, the role of the AOS in early stage (premetamorphic) tadpoles

would be to cope with ROS induced primarily by growth and external environmental factors (such as light, oxygen, pollution) (Gomez-Mestre et al., 2013; Burraco et al., 2017a, 2017b). Premetamorphic individuals start to swim freely and eat intensively, and are for the first time directly exposed to the environment (Rizzo et al., 2007). Their body and metabolic rate increase (Kirschman et al., 2017). Rapid growth, development and adjustment to independent feeding increased plasma malondialdehyde (MDA) concentrations in the lamb of Soay sheep (*Ovis aries*) (Nussey et al., 2009). During the premetamorphic stage, frogs displayed the highest AOS response (e.g. IBR values), with higher values for the enzymatic components of the AOS (SOD, GSH-Px, GR and GST). Increased activities of SOD and GSH-Px, as enzymes directly involved in removing superoxide ($O_2^{\cdot-}$) and H_2O_2, could provide a compensatory mechanism against increased ROS generation in the mitochondria due to an increase in overall physical activity and growth. It was shown that the accelerated growth of damselflies, *Chalcolestes viridis* (*Lestes viridis*), was accompanied by higher SOD and CAT activities (De Block and Stoks, 2008). GST of tadpoles would help protect them against compounds from the environment. The importance of GSH-dependent enzymes (GR and GST) in protection from environmental stressors has already been highlighted in *Xenopus laevis* tadpoles after hatching (Amicarelli et al., 2004; Rizzo et al., 2007). Even though the AOS was stimulated, it was incapable of stopping ROS production and the process of lipid peroxidation in individuals at this stage.

Individuals under metamorphic climax face developmental pressures (intensive changes of the intestine, skin, tail and limbs) in preparation for a terrestrial lifestyle. During metamorphic climax, oxygen consumption decreases, individuals enter an obligatory fast, loose body mass and are generally immobile unless disturbed (Hourdry et al., 1996; Schreiber et al., 2005). Food deprivation in animals can alter normal mitochondrial functioning and increase ROS production (H_2O_2 and $O_2^{\cdot-}$) (Sorensen et al., 2006; Salin et al., 2018). It was suggested that mitochondria-derived ROS play an important regulatory role in the modulation of organs in anurans (Menon and Rozman, 2007; Johnson et al., 2013). They activate the apoptosis-related pathway that underlies the mechanism of thyroxin-induced shortening of the tadpole tail and remodeling of the intestine (Hanada et al., 1997; Kashiwagi et al., 1999). The observed relatively lower activities of enzymes (SOD, CAT and GSH-Px) involved in removing mitochondrial ROS ($O_2^{\cdot-}$ and H_2O_2) during metamorphic climax can be result of ROS production/accumulation. Similar results to ours were reported in the intestine and tail of *X. laevis* frogs during metamorphosis (Menon and

Rozman, 2007; Johnson et al., 2013). The authors pointed out the requirement for ROS in this process. Johnson et al. (2013) also showed increased concentrations of NO and reactive nitrogen species (RNS) during metamorphic climax. An increased GSH concentration, the component of the AOS that is capable of scavenging a variety of ROS, would protect the cells of individuals under metamorphosis from even greater oxidative damage. GSH is a component of the AOS that modulates cell processes by interacting with ROS and preventing damage to proteins or DNA, and also by participating in DNA repair (Huang et al., 2001). The cytoprotective role of nonenzymatic antioxidants, such as ascorbic acid, against oxidative attack during metamorphosis was also reported by Menon and Rozman (2007). Burraco et al. (2017a) observed an increase in total GSH concentration which was associated with elevated growth and developmental rates of tadpoles exposed to predators. Similar to our results, tadpoles were exposed to oxidative stress, with the inactivation of antioxidant enzymes probably due to increased ROS production, as the authors suggested. Protein synthesis is crucial for the formation of muscle and new adult structures, and ROS interaction with SH groups during the process of metamorphic change can produce conformational changes that allow for better protein-protein or protein-DNA interactions (Magder, 2006). Higher levels of LPO in metamorphic individuals can be due to fasting and elevated ROS concentrations that affect the fatty acid composition of cell membranes as a result of an increased number of double bonds and the peroxidizability index, making the membranes of fasted animals more sensitive to lipid peroxidation (Sorensen et al., 2006). Gomez-Mestre et al. (2013) detected elevated lipid peroxidation in tadpoles at metamorphic climax in comparison to prometamorphic individuals, regardless of the changes in environmental factors (e.g. water regime levels) under which they were reared. An incremental rise in MDA concentrations in tadpoles during metamorphosis was also reported for *X. laevis* frogs (Menon and Rozman, 2007). The process of metamorphosis is also fueled by triglycerides (an important primary energy storage for amphibians) (Sawant and Varute, 1973; Sheridan and Kao, 1998; Melvin, 2016), which according to Perez-Rodriguez et al. (2015) correlates with MDA concentrations and can interfere with the obtained results.

The rapid transition from aquatic to terrestrial respiration and the substantial increase in oxygen consumption during this transition was accompanied by different physiological changes (Burggren and Doyle, 1986; Crowder et al., 1998). The significant increase in oxygen fueled by higher oxygen consumption relative to the environment is reflected in the higher metabolic

activity level in juvenile frogs (Kirschman et al., 2017). In juvenile frogs, the response of the AOS included both first (SOD, CAT, GR) and second line defense (GSH and SH groups) components. A boosted first-line of defense enzymes, together with the second (GSH system), would be beneficial in protection from the harsher, high-oxygen terrestrial environment, which can produce an increase in endogenous production of ROS and an increase in the potential risk of oxidative injuries. This response was successfully accompanied by lower oxidative damage, observed as a lowering TBARS concentration in juvenile frogs, revealing the organism's ability to efficiently adapt to a new lifestyle (terrestrial locomotion, carnivorous diet, high oxygen levels, exposure to UV light, etc.).

H_2O_2 is a major ROS produced by cells. Besides its prooxidative effect, it also acts in signaling pathways as a second messenger and can have important roles at the cellular level during development and ageing (Rampon et al., 2018). In anurans, H_2O_2 participates in cell cycle progression at the beginning of development (Han et al., 2018), in organ remodeling during metamorphosis (Kashiwagi et al., 1999; Mahapatra et al., 2001; Menon and Rozman, 2007; Johnson et al., 2013), in ageing and lifespan (Kashiwagi et al., 2005). The regulation of its concentration is delayed during the life cycle of anurans (Prokić et al., 2018b). Differences in peroxisomal enzyme activity during development were reported for *Rana japonica* and *Rana nigromaculata* (Kashiwagi et al., 1999).We observed an interesting pattern in processes that control the levels of H_2O_2 in premetamorphic and juvenile individuals. Both stages were characterized by elevated SOD activity, but unlike the high GSH-Px activity observed during premetamorphosis, in juvenile frogs CAT was the main enzyme involved in the removal of H_2O_2. The roles of both enzymes are complementary, but differ in their affinities for H_2O_2, as well as in their subcellular localizations (peroxisomal and cytosolic, respectively) (Halliwell and Gutteridge, 2015). GSH-Px is also involved in the regulation of mitochondrial ROS production and in the reduction of hydroperoxides of polyunsaturated fatty acids to their corresponding alcohols, thereby indirectly limiting the products of hydroperoxides (i.e. malondialdehyde and hydroxynonenal) and counteracting the toxic effects of lipid peroxidation (Halliwell and Gutteridge, 2015). In the present study, enhanced GSH-Px activity could have been the result of higher LPO concentrations and increased mitochondrial ROS production in premetamorphic individuals due to intensive growth and physical activity. Burraco et al. (2017a) showed a strong positive association of GSH-Px activity and plasticity in the developmental and growth rates of

anuran tadpoles in response to environmental factors (pond desiccation and predator presence), which were related to enhanced mitochondrial respiration. In contrast to GSH-Px, the higher CAT activity could be associated with air-breathing of juvenile frogs. Several authors reported a rapid increase in CAT activity at the end of metamorphosis and in juvenile individuals (Kashiwagi and Kashiwagi, 1996; Mahapatra et al., 2001; Starrs et al., 2001).

The level of oxidative stress in frogs developing in the wild clearly differed between the examined ontogenetic stages, and was reflected in the different responses of the AOS and oxidative damage, probably as a reaction to the developmental and/or environmental pressures that follow every stage. In order to obtain a more complete picture about OS in anurans during development, these data should be compared with findings obtained in a controlled experimental setting.

Acknowledgements

The authors are grateful to Dr. Goran Poznanović for proofreading the manuscript and Dr. Zorica Saičić for her support.

Funding

This study was supported by the Ministry of Education, Science and Technological Development of Republic of Serbia, Grant No. 173041.

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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Table content

Table 1. Results of One-way Anova of the comparison between different developmental stages (premetamorphosis, prometamorphosis, metamorphic climax and juvenile); N- number of individuals.

Variable	F	*p*	S.E. of estimate	N
SOD	51.89	0.0000	0.48	83
CAT	62.59	0.0000	10.77	83
GSH-Px	7.91	0.0001	1.26	83
GST	51.33	0.0000	14.68	83
GR	17.89	0.0000	1.35	83
GSH	173.86	0.0000	0.03	81
SH	79.93	0.0000	11.94	83
LPO	25.67	0.0000	0.29	83

Table 2. Loadings of oxidative stress variables onto the principal components (PC).

	PC loadings			
	PC1	PC2	PC3	PC4
SOD	-0.793	-0.246	0.074	0.499
CAT	0.278	-0.885	-0.049	0.129
GSH-Px	-0.646	-0.208	-0.716	-0.148
GST	-0.894	0.0408	0.193	-0.268
GR	-0.750	-0.459	0.335	-0.223
GSH	0.699	-0.560	0.050	-0.205
% of each axis	49.60	23.56	11.21	7.52

Figure captions

Fig. 1. Differences in oxidative stress parameters (SOD, CAT, GSH-Px, GST, GR, GSH, SH groups and TBARS) between developmental stages (premetamorphosis, prometamorphosis, metamorphic climax and juvenile). All data are presented as mean ± standard error. Significant differences ($p<0.05$) are marked with the letters a, b, c which indicate differences among the examined groups; "a" in respect to premetamorphic individuals, "b" in respect to prometamorphic and "c" in respect to metamorphic.

Fig. 2. Principal component analyses of the antioxidative parameters (SOD, CAT, GSH-Px, GST, GR, and GSH) of premetamorpohic, prometamorphosic, metamorphic and juvenile individuals.

Fig. 3. Pearson correlations of examined parameters of A) premetamorphic, B) prometamorphic, C) metamorphic and D) juvenile individuals. SOD- superoxide dismutase, CAT- catalase, GSH-Px- glutathione peroxidase, GST- glutathione s-transferase, GR- glutathione reductase, GSH- glutathione. Statistically significant correlations are black rounded.

Highlights

- Ontogenetic shifts in anurans are accompanied by different levels of oxidative stress
- Higher enzymatic antioxidative activities characterized premetamorphosis
- Higher levels in the non-enzymatic components characterized metamorphic climax
- Both the enzymatic and non-enzymatic components were higher in juveniles
- Higher levels of lipid peroxidation were in premetamorphosis and metamorphic climax

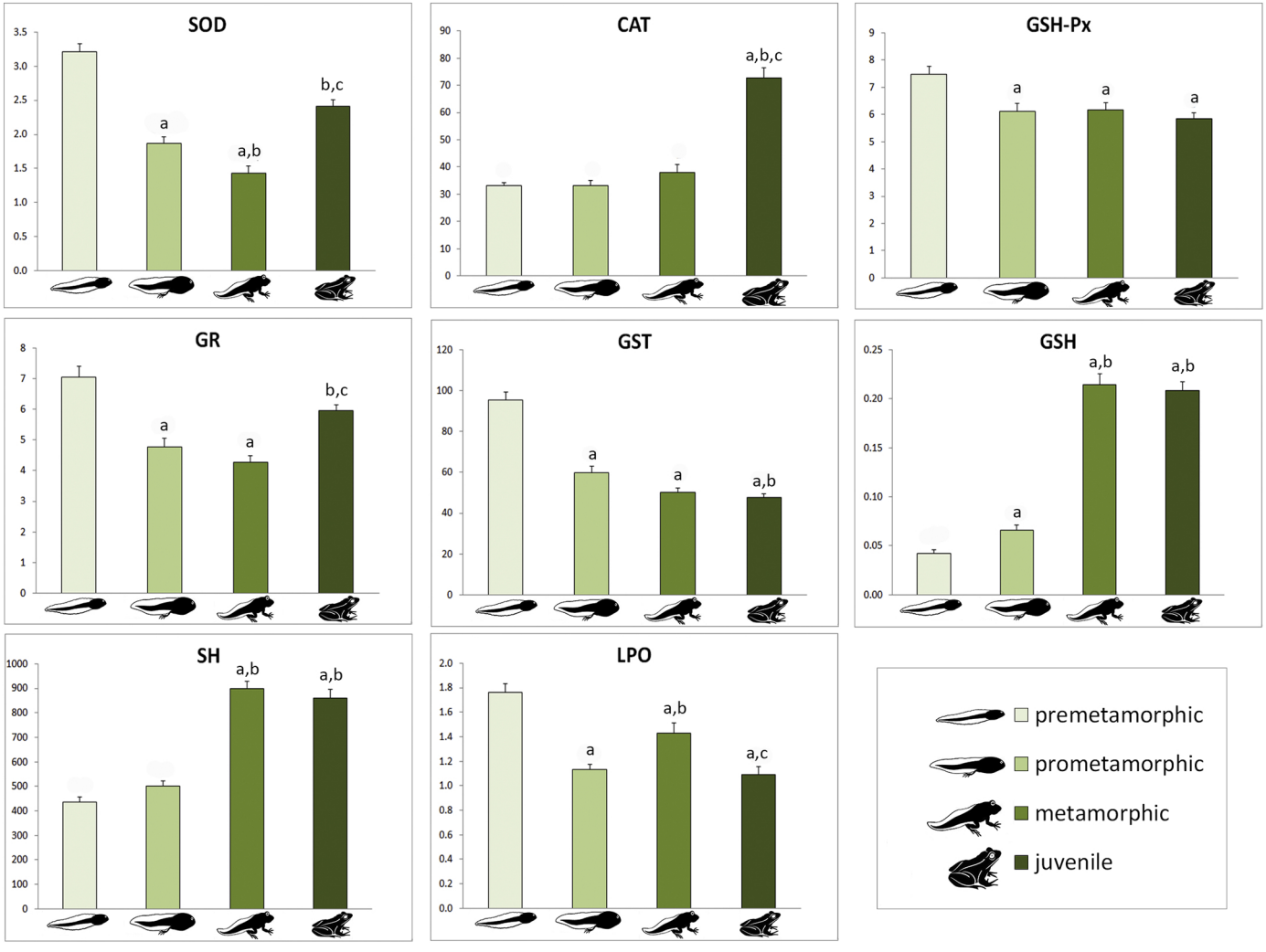
SOD
a
a,b
b,c
CAT
a,b,c
GSH-Px
a
a
a
GR
a
a
b,c
GST
a
a
a,b
GSH
a
a,b
a,b
SH
a,b
a,b
LPO
a
a,b
a,c
premetamorphic
prometamorphic
metamorphic
juvenile

Figure 1

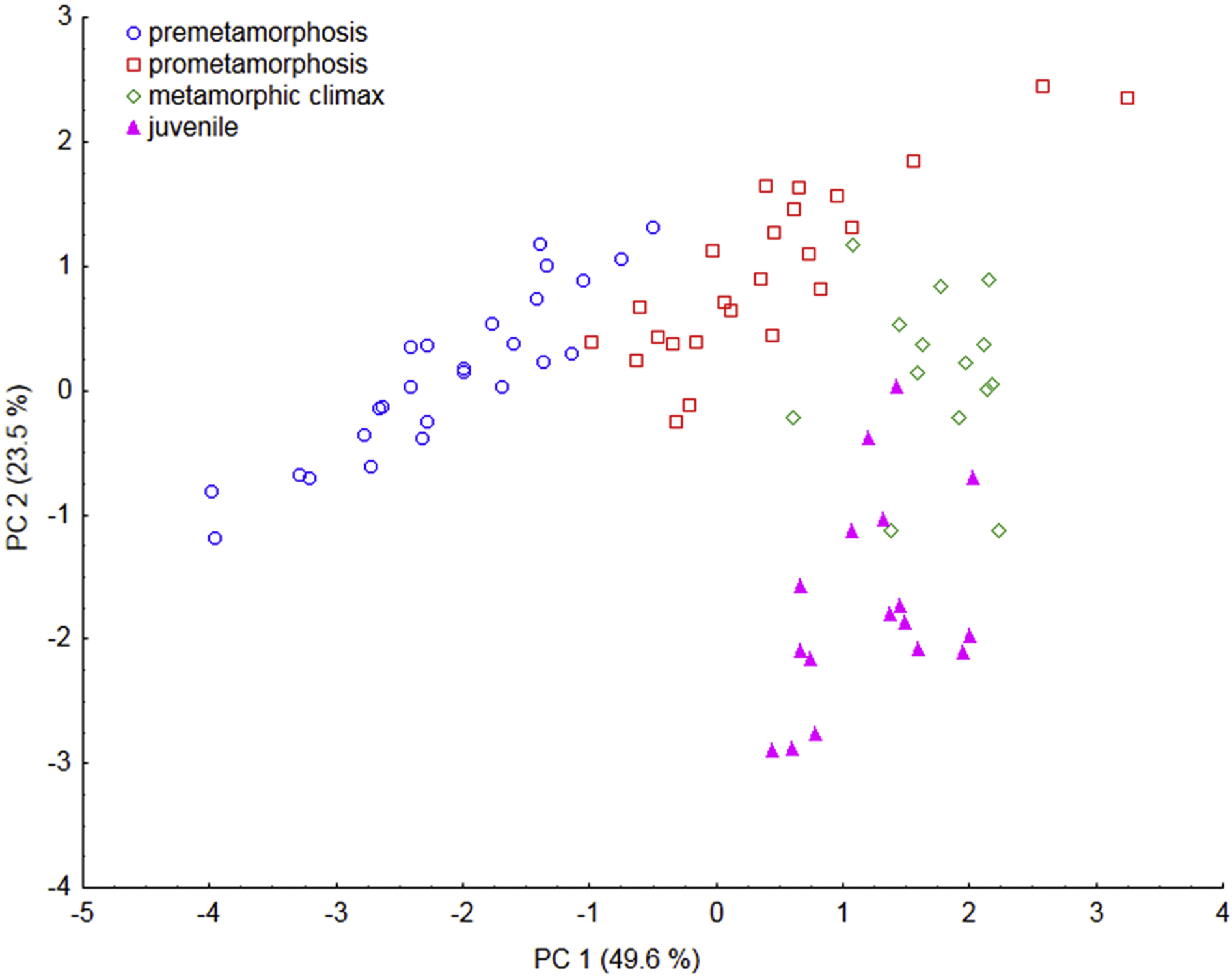
premetamorphosis
prometamorphosis
metamorphic climax
juvenile
PC 2 (23.5 %)
PC 1 (49.6 %)
3
2
1
0
-1
-2
-3
-4
-5
-4
-3
-2
-1
0
1
2
3
4

Figure 2

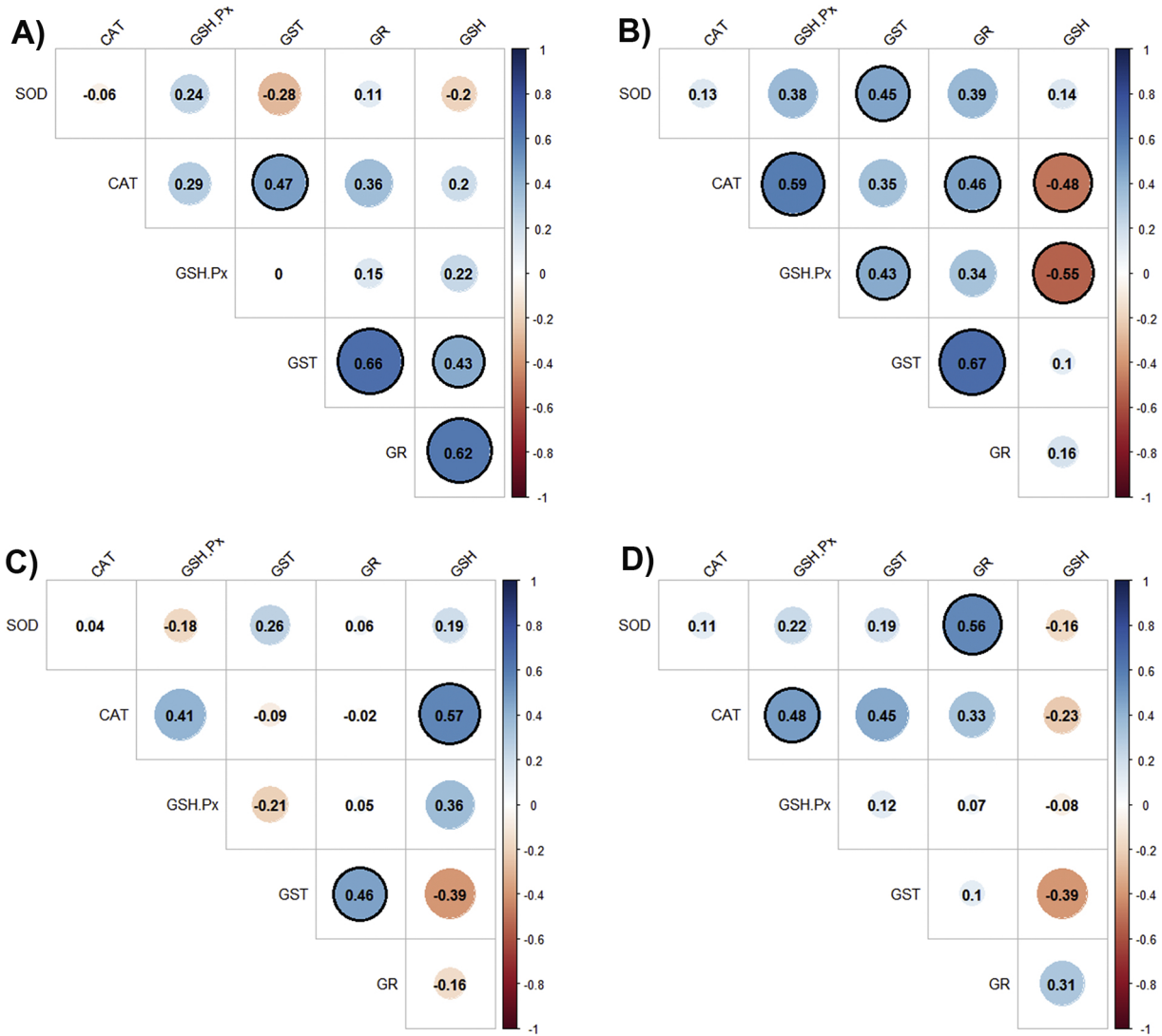
A)
CAT
GSH.Px
GST
GR
GSH
SOD
-0.06
0.24
-0.28
0.11
-0.2
CAT
0.29
0.47
0.36
0.2
GSH.Px
0
0.15
0.22
GST
0.66
0.43
GR
0.62
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
B)
CAT
GSH.Px
GST
GR
GSH
SOD
0.13
0.38
0.45
0.39
0.14
CAT
0.59
0.35
0.46
-0.48
GSH.Px
0.43
0.34
-0.55
GST
0.67
0.1
GR
0.16
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
C)
CAT
GSH.Px
GST
GR
GSH
SOD
0.04
-0.18
0.26
0.06
0.19
CAT
0.41
-0.09
-0.02
0.57
GSH.Px
-0.21
0.05
0.36
GST
0.46
-0.39
GR
-0.16
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
D)
CAT
GSH.Px
GST
GR
GSH
SOD
0.11
0.22
0.19
0.56
-0.16
CAT
0.48
0.45
0.33
-0.23
GSH.Px
0.12
0.07
-0.08
GST
0.1
-0.39
GR
0.31
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

Figure 3