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ORIGINAL ARTICLE

Comparative study of oxidative stress parameters and acetylcholinesterase activity in the liver of *Pelophylax esculentus* complex frogs

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Abstract Comparative activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), the phase II biotransformation enzyme glutathione-S-transferase (GST), the concentrations of total glutathione (GSH), sulfhydryl groups (–SH) and the activity of the neurotoxicity biomarker acetylcholinesterase (AChE) were investigated in the livers of species belonging to the *Pelophylax esculentus* "complex" (parental species *Pelophylax ridibundus*, *Pelophylax lessonae*, and their hybrid *Pelophylax* kl. *esculentus*) from the wetland, Obedska bara in Serbia. The condition factor (CF) and hepato somatic index (HSI) were also calculated. All three species were caught at same locality and were exposed to the same environmental conditions. Liver SOD activity was lower in *P. ridibundus* than in *P.* kl. *esculentus* and *P. lessonae*; higher activities of CAT, GR and GST were observed in *P.* kl. *esculentus* frogs as compared to their parental species. The activity of GSH-Px was significantly lower in *P.* kl. *esculentus*. The activity of AChE was increased in *P. lessonae* as compared to *P.* kl. *esculentus* and *P. ridibundus*. Similar concentrations of GSH and ─SH groups were observed in all investigated species. *P.* kl. *esculentus* had a higher CF, while the HSI was lower when compared to the parental species. Our findings suggest that the parental species (*P. ridibundus* and *P. lessonae*) possess more similar antioxidative responses

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to environmental conditions than the hybrid species *P.* kl. *esculentus*. The obtained results improve our understanding of the biology and physiology of these three closely related species.

1. Introduction

In the past decades, the ecology and ecophysiology of amphibians has received increasing attention (Sparling et al., 2000) because of the global decline in amphibian populations (Houlahan et al., 2000). Changes in the environment (temperature and oxygen levels) and human borne pollutants (metal ions, pesticides, oil and related pollutants) act as stressors via induction of oxidative stress (Lushchak, 2011). The most susceptible targets for oxidative stress are proteins, membrane lipids and DNA (Davies, 1995). All oxygen-utilizing organisms possess a constitutive antioxidant defense system (AOS), comprised of enzymatic and non-enzymatic components that render harmless toxic reactive oxygen species (ROS) that are continuously produced as by-products of aerobic metabolism. Enzymatic components include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), the phase II biotransformation enzyme glutathione S-transferase (GST) and non-enzymatic components, include total glutathione (GSH) and sulfhydryl groups (-SH) all of which serve as markers of oxidative stress. Acetylcholine esterase (AChE) serves as a biomarker of neurotoxicity (Halliwell and Gutteridge, 1999).

Water frogs complex (*Pelophylax esculentus* complex former name *Rana esculenta* complex) is genetically and ecologically well-investigated because of its hemiclonal mode of reproduction that characterizes natural interspecies hybrid lineages. The *P. esculentus* complex includes the parental species, *Pelophylax ridibundus* Pallas 1971 and *Pelophylax lessonae* Camerano 1882 and the hybridogen *Pelophylax* kl. *esculentus* Linnaeus 1758 which are found in Europe (Graf and Polls, 1989). Hybrids can reproduce by hybridogenesis whereby the set of chromosomes derived from one parental species is completely discarded, while the set from the other parental species undergoes compensatory duplication. *P.* kl. *esculentus* gametes thus contain an unrecombined genome derived from one parental species (Ragghianti et al., 2007). The taxa coexist in the same environment but differ in their ecological niches. Several studies have indicated that *P.* kl. *esculentus* has a broader environmental tolerance relative to its parental species (Semlitsch et al., 1996, 1997). *P.* kl. *esculentus* hybrids are superior to their hosts in several fitness-related viability and fecundity traits (Hotz et al., 1999); they are less sensitive to environmental stressors, such as pond drying, interspecies larval competition, food limitation (Semlitsch et al., 1997), hypoxic conditions during hibernation (Tunner and Nopp, 1979) and the common agricultural fungicide triphenyltin (Fioramonti et al., 1997). The ecological success of *P.* kl *esculentus* has been suspected to be due to heterotic effects. Heterosis would allow hybridogens to tolerate and to exploit a broader range of ecological conditions, supporting a more widespread distribution than their parental species (general purpose genotype hypothesis-heterozygote superiority) (Van Doninck et al., 2002; Voituron et al., 2005). Based on this hypothesis we expect that *P.* kl. *esculentus* should be more tolerant to oxidative stress induced by environmental factors than the parental species (*P. ridibundus* and *P. lessonae*).

To improve our understanding of the physiology of water frog species (*P. esculentus* complex) and to test general purpose genotype hypothesis, we determined the differences in levels of response of antioxidant defense and neurotoxicity parameters in the livers of parental species, *P. ridibundus* and *P. lessonae*, and their hybrid species, *P.* kl. *esculentus* from the wetland Obedska bara in Serbia.

2. Materials and methods

2.1. Animals collection

The nature reserve "Obedska bara" is a vast swamp-forest area stretching along the Sava River in southern Srem (northern Serbia) and is the oldest protected area in Serbia (since 1874). It has acquired the status of a Special Nature Reserve and is on UNESCO's list of internationally important aquatic habitats (Ramsar area). It is characterized by a complex assembly of aquatic, wetland, meadow and forest phytocoenoses.

Physico-chemical parameters of water (temperature, pH and dissolved oxygen) were measured *in situ* using mobile analytical equipment (WTW Multi 340i). Water analysis was carried out in triplicate.

Animal capture was approved by the Serbian Ministry for Energy, Development and Environmental Protection (Permissions No 353-01-364/2014-08 and 03-299/2).

The following specimens of adult frogs of the *P. esculentus* complex were collected: 10 specimens of *P. ridibundus*, 10 specimens of *P.* kl. *esculentus* and 6 specimens of *P. lessonae*. Frogs of both sexes were collected in the middle of May 2014 at Obedska Bara in Serbia (44°44′8.89″ N, 19°59′15.67″ E) (Fig. 1). Individuals were caught with a net and transported to the laboratory in cages with native water. Body mass was measured with a BJ 610C Precisa scale and snout-vent-length with a Vernier caliper.

The condition factor (CF) was calculated according to Bagenal and Tesch (1978) by multiplying the body mass (g) by 100 and dividing by the cube of the snout-vent-length (mm). The liver was removed, dried with filter paper and weighed. The hepato somatic index (HIS) was calculated by dividing the liver mass (g) by the body mass (g) and multiplying by 100 (Jelodar and Fazli, 2012).

2.2. Liver tissue processing

All samples were stored under the same conditions to avoid differences caused by storage. Liver tissues were kept at −80 °C until analysis. Livers were minced and homogenized in 5 volumes of 25 mmol/L sucrose containing 10 mmol/L Tris–HCl, pH 7.5 at 4 °C (Lionetto et al., 2003), using an

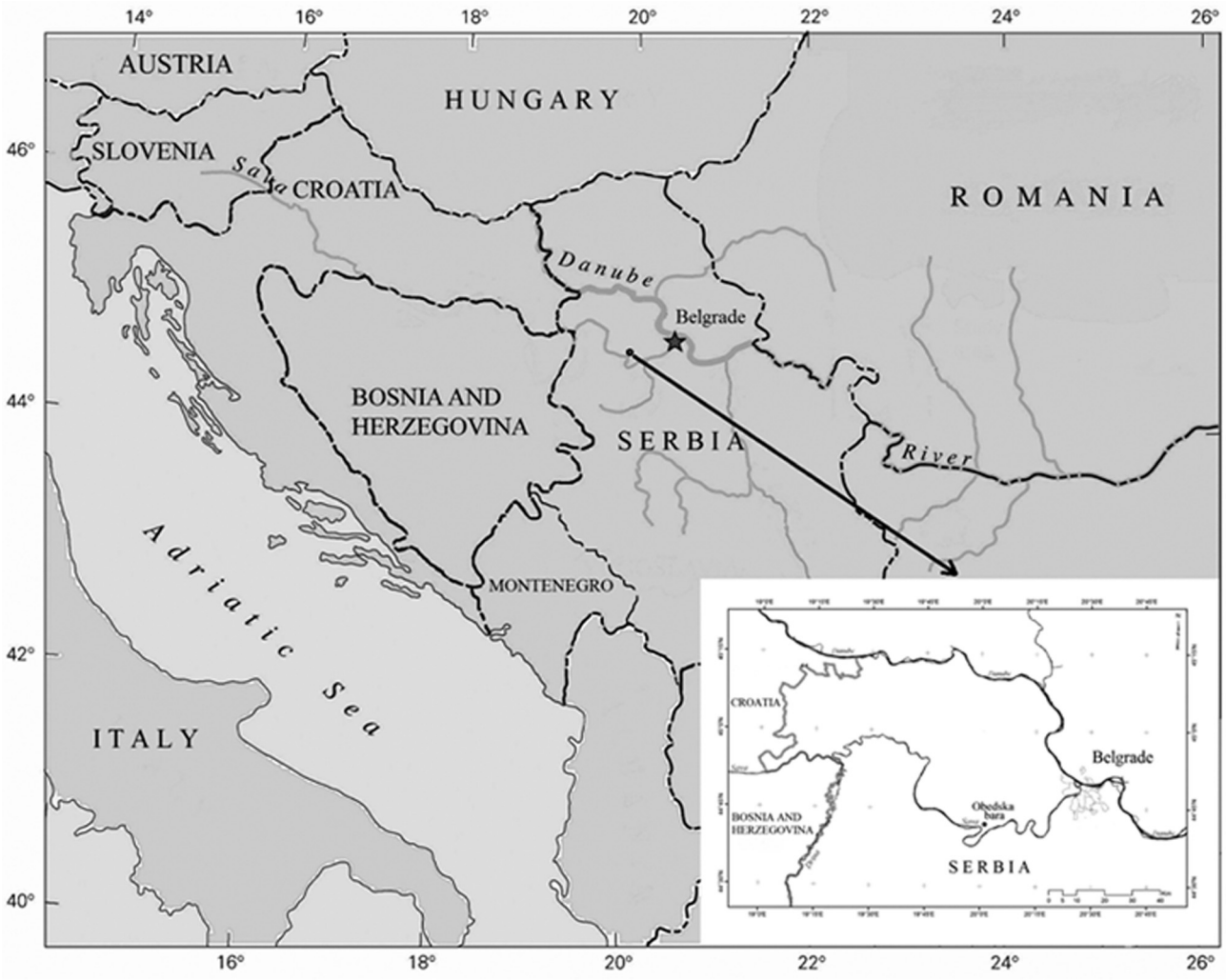

Figure 1 Location of the sampling site Obedska Bara in Serbia.

IKA-Werk Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany). The homogenates were sonicated for 30 s at 10 kHz on ice to release enzymes, followed by centrifugation in a Beckman ultracentrifuge at 85,000×*g* for 90 min at 4 °C. The resulting supernatants were used for biochemical analyses.

2.2.1. Biochemical analyses

Protein concentrations in the supernatants were determined according to the method of Lowry et al. (1951), using bovine serum albumin as a standard. The activities of the antioxidant enzymes were measured simultaneously in triplicate for each sample, using a Shimadzu UV-160 spectrophotometer with a temperature-controlled cuvette holder. Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured by the procedure of Misra and Fridovich (1972) which is based on the autoxidation of adrenaline to adrenochrome. The change in absorbance was monitored at 480 nm. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of autoxidation of adrenaline. The activity of catalase (CAT; EC 1.11.1.6) was quantified by the decomposition of H_2O_2 at 240 nm according to the method of Claiborne (1984). One unit of CAT activity was defined as the amount of enzyme that catalyzed the dismutation of 1 μmol of H_2O_2 per min. The concentration of total GSH was determined by the method of Griffith (1980) and expressed in nmol/g tissue. Glutathione peroxidase (GSH-Px; EC 1.11.1.9) activity was estimated in a coupled enzyme system (Tamura et al., 1982), where GSH-Px reduced t-butyl hydroperoxide and NADPH was consumed by GR to convert the formed oxidized glutathione (GSSG) to its reduced form (GSH). One unit of GSH-Px activity was defined as the amount of enzyme that oxidized 1 nmol of NADPH per min. The activity of glutathione reductase (GR; EC 1.6.4.2) was detected from the oxidation of NADPH during the reduction of GSSG (Glatzle et al., 1974). One unit of GR activity was defined as the amount of enzyme that oxidized 1 nmol of NADPH per min. Glutathione S-transferase (GST; EC 2.5.1.18) activity was estimated as described by Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene (CDNB) and GSH as substrates. One unit of GST activity was defined as the amount of enzyme that produced 1 nmol of CDNB-GSH conjugate per min. The assays of GSH-Px, GR and GST were measured at 340 nm. All antioxidant enzyme activities were expressed as specific activities in U mg protein^{-1}. The concentration of sulfhydryl groups was determined using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) according to the Ellman

Table 1 Average body mass, snout-vent length, liver mass, condition factor and hepatic somatic index in species of the *Pelophilax esculentus* complex. Significantly different values ($p < 0.05$) are marked with * which indicates differences between *Pelophylax ridibundus* and *Pelophylax* kl. *esculentus*; #Indicates differences between *Pelophylax lessonae* and *Pelophylax* kl. *esculentus*.

	Average body mass (g)	Average snout-vent length (mm)	Average liver mass (g)	Condition factor (CF)	Hepatic somatic index (HSI)
Pelophylax ridibundus	63.20 ± 5.84	90.1 ± 2.92	2.76 ± 0.37	8.56 ± 0.51	4.37 ± 0.27
Pelophylax kl. *esculentus*	47.94 ± 5.06	75.9 ± 3.08	1.65 ± 0.35	9.89 ± 0.29*#	3.44 ± 0.15*#
Pelophylax lessonae	19.34 ± 3.25	60.83 ± 3.27	0.89 ± 0.14	8.26 ± 0.42	4.65 ± 0.67

(1959) method, and expressed in nmol/g tissue. Acetylcholinesterase (AChE; EC 3.1.1.7) activity was determined according to Ellman et al. (1961). The assay consisted of measuring the reaction of the thiocholine with 5,5′-dithiobis-(2-nitrobenzoic acid) DTNB. The yellow anion of 5-thio-2-nitrobenzoic acid formed in the reaction was detected spectrophotometrically at 412 nm.

2.3. Native electrophoresis

Electrophoresis was carried out at 4 °C according to a modified procedure of Gabriel (1971) in a 1.5 mm 10% polyacrylamide gel in standard Tris–glycine buffer (pH 8.3) for SOD, and in 8% polyacrylamide gel for CAT. After electrophoresis, in-gel activities of SOD were observed by the NBT method of Mavelli et al. (1984) as follows: the gel was soaked in 25 ml of NBT for 15 min, briefly washed, then soaked in the dark in 30 ml of potassium phosphate buffer (pH 7.0) containing TEMED and 2.8×10^{-2} mM riboflavin for another 15 min. The gel was briefly washed again and illuminated with visible light. CAT activity was visualized after soaking the gel in 3.27 mM H_2O_2 for 30 min, washing in double distilled water and staining with a mixture of 1% (w/v) potassium ferricyanide and 1% (w/v) ferric chloride (Woodburry et al., 1971).

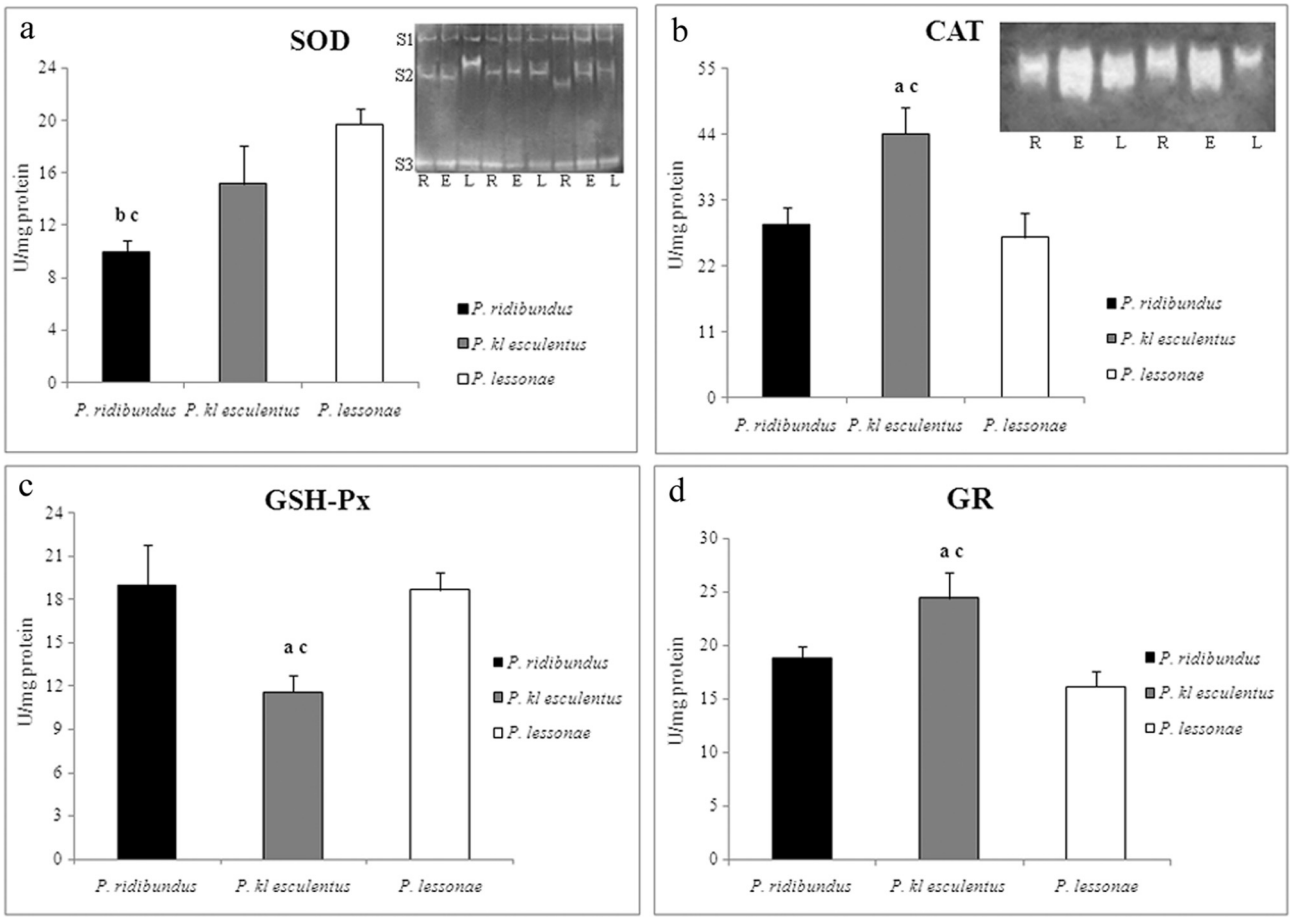

Figure 2 (a) SOD, (b) CAT, (c) GSH-Px and (d) GR activities in the livers of species of the *Pelophylax esculentus* complex. Data are expressed as mean ± S.E. The parametric one-way ANOVA test was used to establish significant differences between samples. A minimum significance level of $p < 0.05$ was accepted. Significantly different values are marked with letters indicating which values are different from each other; a, b and c indicate differences among liver enzyme activities in the *Pelophylax esculentus* complex. "a" is *P. ridibundus*, "b" is *P.* kl. *esculentus*; "c" is *P. lessonae*. *Abbreviations:* SOD – superoxide dismutase (S1, S2, S3 – SOD isoforms), CAT – catalase, GSH-Px – glutathione peroxidase, GR – glutathione reductase; R – *P. ridibundus*, E – *P.* kl. *esculentus*, L – *P. lessonae*.

All chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.4. Data analysis

All data were expressed as mean ± standard error of the means (SE). Data were normally distributed (Lilliefors' test and Kolmogorov–Smirnov test) (Zar, 1999) and a parametric one-way ANOVA test with $p < 0.05$ as the criterion for significance was performed. Canonical discriminant analysis was used to evaluate the differences among the frogs based on the measured activities of antioxidant enzymes (SOD, CAT, GR, GSH-Px and GST), concentrations of nonenzymatic components (GSH and SH group) and AChE activity (Darlington et al., 1973). Statistical analyses were performed with STATISTICA 8.0.

3. Results

At the time of sampling, the water temperature was 16.77 ± 0.15 °C. The concentrations of dissolved oxygen was 6.94 ± 0.23 (mg O_2/L) and pH 7.69 ± 0.10.

A significantly higher CF value was obtained in *P.* kl. *esculentus*, while the HSI was significantly lower than in parental species ($p < 0.05$) (Table 1).

The antioxidative parameters in the livers of water frogs (*P. esculentus* complex) are presented in Figs. 2 and 3. The activity of SOD in *P. ridibundus* was significantly lower ($p < 0.05$) than the activities in *P.* kl. *esculentus* and *P. lessonae* (Fig. 2a). CAT, GR and GST activities were significantly higher ($p < 0.05$) in *P.* kl. *esculentus* as compared to *P. ridibundus* and *P. lessonae* (Figs. 2b, d and 3a). The opposite trend in activity was observed for GSH-Px activity (Fig. 2c). Namely, GSH-Px activity was significantly lower in the liver of *P.* kl. *esculentus* when compared to the parental species ($p < 0.05$). *P.* kl. *esculentus* had a lower GSH concentration in the liver compared to the other two species, however, the change was not statistically significant (Fig. 3c). The activity of AChE was markedly lower in the liver of *P. ridibundus* when compared to *P. lessonae* ($p < 0.05$) (Fig. 3b). No significant differences in —SH group concentrations were detected between the investigated species (Fig. 3d).

The results of SOD and CAT activities are presented in Fig. 2a and b. By electrophoresis we identified three bands

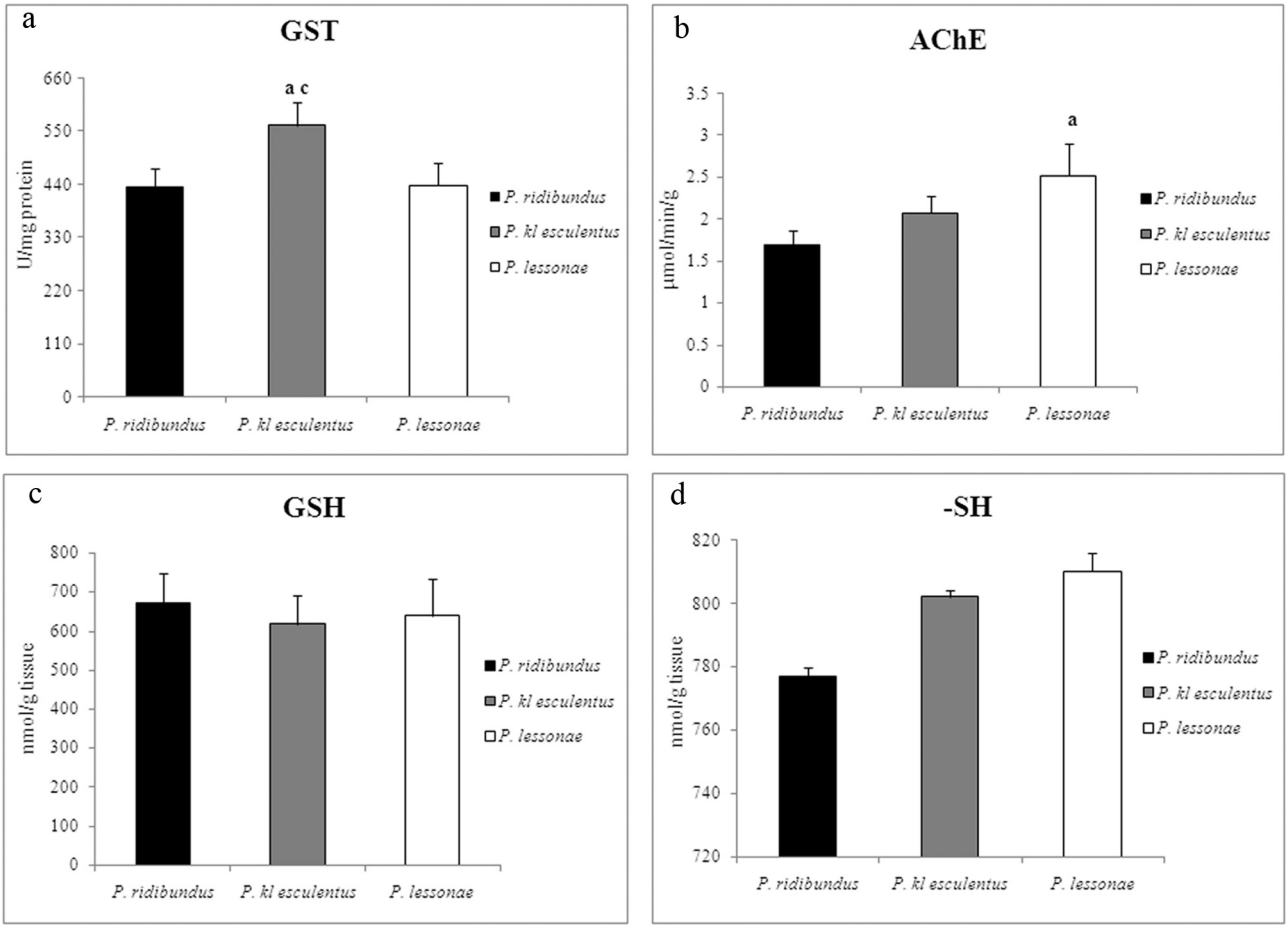

Figure 3 (a) GST and (b) AChE activities and concentrations of (c) GSH and (d) —SH groups in the livers of species of the *Pelophylax esculentus* complex. Data are expressed as mean ± S.E. The parametric one-way ANOVA test was used to establish significant differences between samples. The minimum significance level was $p < 0.05$. Significantly different values are marked with letters indicating which values are different from each other; a, b and c indicate differences among liver enzyme activities in the *Pelophylax esculentus* complex. "a" is *P. ridibundus*, "b" is *P.* kl. *esculentus*; "c" is *P. lessonae*). *Abbreviations:* AChE – acetylcholinesterase, GST – glutathione-S-transferase, GSH – total glutathione, SH – sulfhydryl groups.

corresponding to SOD-1, SOD-2 and SOD-3 isoforms in the livers of the *P. esculentus* species complex. We observed only one CAT isoform in all three species. CAT activity was highest in *P.* kl. *esculentus* compared to other two species.

Canonical discriminant analysis differentiated between *P. esculentus* complex species (Fig. 4). The first canonical function (Root 1) in the analysis accounted for 75.9% of total heterogeneity. Root 1 indicates that *P.* kl. *esculentus* was separated from the other two species. The second canonical function (Root 2) in the analysis accounted for 24.1% of the total heterogeneity. Root 2 showed separation between parental species *P. ridibundus* and *P. lessonae*.

4. Discussion

Recent studies have often focused on terrestrial or aquatic organisms while amphibians in the wild have been examined in only a few studies (Hayes et al., 2006). Amphibians were mostly studied in the laboratory, while the effects of natural conditions remain unknown. Only a few studies have been devoted to examinations of the *P. esculentus* complex. The present work is the first to examine the comparative antioxidative defense system in the *P. esculentus* complex exposed to the same environment, i.e. that inhabited the same locality.

Plenet et al. (2000) examined the different oxygen requirements of species belonging to the *P. esculentus* complex. The authors reported that *P. ridibundus* was more sensitive to changes in oxygen levels than *P. lessonae* and *P.* kl. *esculentus*. The reason for this is probably due to two mechanisms: storage of fermentable fuels (mainly glycogen) and different metabolic rates in normoxia. *P.* kl. *esculentus* and *P. lessonae* have higher glycogen levels and lower metabolic rates compared to *P. ridibundus* (Hochachka, 1980). Different metabolic rates can affects the generation of reactive oxygen species (ROS) and the level of antioxidative defense system (Sohal and Weindruch, 1996). Lushchak (2011) assumed that oxidative stress may be responsible for adaptation of organisms to a broad range of environmental stressors.

CF has been used as an indicator of general health of frogs. Frogs in contaminated ecosystems generally have low CF values (Thammachoti et al., 2012). The liver is the most metabolically active organ, it plays an essential role in drug and xenobiotic metabolism (Maton et al., 1993). HSI is associated with liver energy reserves and metabolic activity (Pyle et al., 2005). The results of CF and HSI indicated that *P.* kl. *esculentus* was generally in a better state than the parental species.

SOD, CAT and GST activities were lower in *P. ridibundus*. At the same time, the concentration of GSH in the liver of *P. ridibundus* was higher. GSH is an important radical scavenger if the reaction with SOD is prevented (Munday and Winterbourn, 1989). Lower SOD, CAT and GR activities was accompanied by higher GSH-Px activity in *P. ridibundus*, suggesting that coordinated action toward hydrogen peroxide induced oxidative attack.

Results of our investigation revealed similar antioxidative response in *P. lessonae* compared to *P. ridibundus*. The higher activity of liver SOD which generates H_2O_2 is counter balanced by lower CAT and GR activities. The produced H_2O_2 can be detoxified by CAT and GSH-Px. In *P. lessonae*, H_2O_2 is eliminated by higher GSH-Px activity, thus compensating for lower CAT activity.

In the liver of *P.* kl. *esculentus* we observed opposite trends in CAT and GSH-Px activities. Liver CAT activity was higher while the activity of GSH-Px was lower. Similar trends in GR and GST activities were observed. Higher GR activity can be linked with a lower GSH concentration in the liver of *P.* kl.

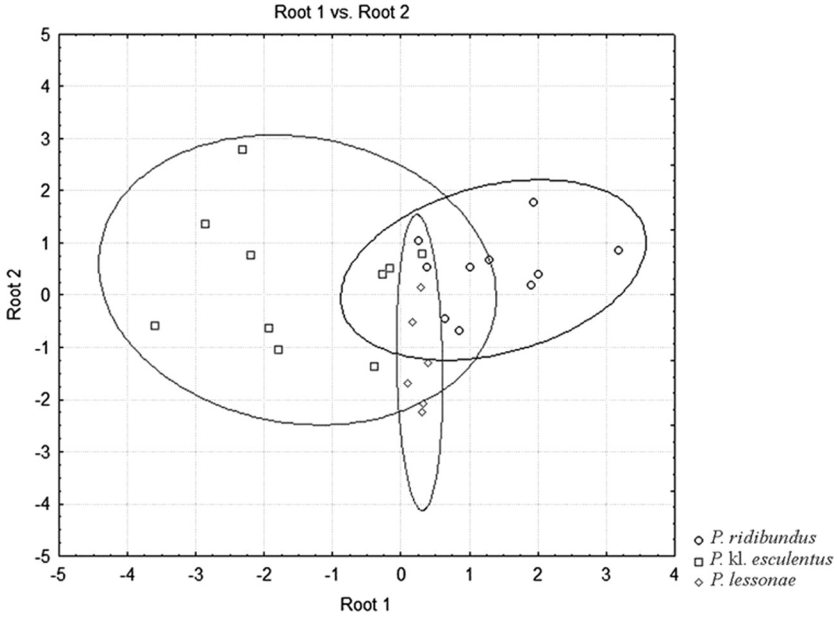

Figure 4 Canonical discriminant analysis of liver antioxidant enzymes (SOD, CAT, GSH-Px, GR and GST) and AChE activities and concentrations of GSH and —SH groups in species of the *Pelophylax esculentus* species complex. Groups were formed by Root 1 (the first canonical function) and Root 2 (the second canonical function). Differences between the species are visualized by ellipses generated by STATISTICA 8.0.

esculentus and with attempts of the liver to maintain physiological GSH concentration. GR has a crucial role in preserving normal functioning of GSH-dependent enzymes (Deponte, 2013).

GST and AChE activities can be used as biomarkers of pollution with xenobiotics, especially organophosphorus and carbamate pesticides (Hayes and McLellan, 1999; Hobbiger, 1961). Booth et al. (1998) reported that two organophosphorus pesticides, chlorpyrifos and diazinon, affect GST and AChE activities in earthworm *Apporectodea caliginosa*. The pesticides induced opposite trends, with GST increasing and AChE decreasing (Booth et al., 1998). A similar trend was reported by Gungordu (2013) who studied different frog species exposed to pesticides. In our study, GST activity was significantly higher in the liver of *P.* kl. *esculentus* than in parental species. Lower AChE activity was reported in the liver of *P. ridibundus* and *P.* kl. *esculentus* when compared to *P. lessonae*. The results of examinations of GST and AChE activities suggest that *P.* kl. *esculentus* react to the presence of some organic xenobiotics. Fagotti et al. (2005) reported that the degree of organochlorine pesticide bioaccumulation was higher in the hybrid *P.* kl. *esculentus* than in the host species *P. lessonae*.

Non-denaturing electrophoresis detected CAT and SOD isoforms. Results of CAT activity revealed the presence of one isoform in all species. The band was more intense in *P.* kl. *esculentus* compared to parental species. These results are in agreement with the activities of CAT that were assessed spectrophotometrically. De Quiroga et al. (1985) also reported the presence of one CAT isoform in the liver of *R. ridibunda*. SOD isoforms S1 and S3 detected in all three species exhibited similar patterns, whereas isoform S2 displayed considerable variation.

Canonical discriminant analysis revealed that liver CAT, GR, GST and GSH-Px activities significantly contributed to differentiation between hybrid *P.* kl. *esculentus* and parental species *P. ridibundus* and *P. lessonae*. CAT, GR and GST activities were significantly higher in *P.* kl. *esculentus,* while GSH-Px was significantly lower when compared to the parental species. The main contributors to differentiation between parental species were SOD and AChE activities. SOD and AChE activities were significantly higher in *P. lessonae*.

5. Conclusion

The parental species, *P. ridibundus* and *P. lessonae*, display more similar antioxidative responses to environmental-induced oxidative stress than the hybrid *P.* kl. *esculentus*. Our results suggest that the preferred pathway of H_2O_2 detoxification in *P.* kl. *esculentus* utilizes CAT and GSH-Px in *P. ridibundus* and *P. lessonae*. The antioxidative strategy of *P.* kl. *esculentus*, suggests that the hybrid could be better equipped for fighting against environmental-induced oxidative stress, and the one of the reasons was it has broader environmental tolerance as compared to the parental type. The overall results supports hypothesis of heterozygote superiority. The obtained results point the way for comparative physiological studies of wildlife species. These findings are a potentially useful tool for understanding the physiology and long-term conservation of the *P. esculentus* complex frogs.

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.

Conflict of interest

The contents of the manuscript have not been published previously, or been submitted elsewhere for consideration, nor are they in press. All of the authors have seen and approved the manuscript. There is no conflict of interest, including any financial, personal or other relationships with other people or organizations.

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