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Oxidative stress parameters in two *Pelophylax esculentus* complex frogs during pre- and post-hibernation: Arousal *vs* heavy metals

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ABSTRACT

In spring, frogs from temperate regions are faced with arousal-induced oxidative stress and exposure to various xenobiotics from the environment. The question is whether pollutants can significantly modify the antioxidative defense system (AOS) response of hibernators during recovery from hibernation. If this assumption is true, we would then expect different patterns of seasonal variations in the AOS between individuals exposed to different levels of pollution. To examine this assumption, we determined the relationship between seasonal variations of accumulated metals and AOS parameters in the skin and muscle of two frog species from the *Pelophylax esculentus* complex (*P. ridibundus* and *P. esculentus*) inhabiting two localities (the Danube-Tisza-Danube canal and the Ponjavica River) with different levels of pollution during pre- and post-hibernation periods, respectively autumn and spring. Our results showed that even though there were differences in the concentrations of accumulated metals and AOS parameters between localities and species, the frogs displayed almost the same patterns of AOS variations during seasons, with a higher AOS response observed in spring. The parameters SH groups, GSH, GR and SOD had been contributed most rather than others. Our findings indicate that oxidative stress during the post-hibernation period was mainly caused by the organisms' recovery from hibernation, as the result of natural selection acting on the AOS, and that the accumulated metals did not significantly modify the AOS response. The present study provides new insight into the biological and physiological cellular responses of frogs to arousal stress.

1. Introduction

Frogs as ectotherms are greatly influenced by environmental changes (Duellman and Trueb, 1994), especially species from temperate regions where the seasons are clearly defined. During the winter, due to low temperatures and lack of food frogs hibernate, which is accompanied by a significant depression in aerobic metabolic rate (Storey and Storey, 1986). Aside from hibernation, the pre- (autumn) and post-hibernation (spring) periods are characterized by different changes that prepare hibernators for survival (Chainy et al., 2016). The antioxidative defense system (AOS) plays an important role in this response, especially in spring, when the increased metabolic rate during arousal is accompanied by high oxygen consumption and the generation of reactive oxygen species (ROS) (Bagnyukova et al., 2003; Orr et al., 2009; Faggio et al., 2016; Burgos-Aceves and Faggio, 2017). Oxidative stress (OS) in natural populations of frogs in spring can be induced, besides arousal, by various xenobiotics from the environment. This raises the question whether their presence in tissues affects the AOS of hibernators. Heavy metals are a potentially good xenobiotic choice to test this assumption. They are persistent, accumulate and are well known oxidative stress inducers (Espín et al., 2014; Koivula et al., 2011). Depending on the redox state, heavy metals can induce oxidative stress directly through the Fenton and Haber-Weiss reactions in the case of redox active metals, or indirectly through changes in the AOS, which is characteristic of redox inactive metals (Valko et al., 2005; Aliko et al., 2015; Pagano et al., 2015, 2017).

As a model organism we choose *P. ridibundus* (*Rana ridibunda*) and *P. esculentus* (*R. esculenta*) frogs, which are genetically very close semiaquatic, hibernating, widespread European frog species considered to be good bioindicators of metal exposure (Sura et al., 2006; Stolyar

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et al., 2008; Falfushynska et al., 2016; Borković-Mitić et al., 2016; Prokić et al., 2016a). Differences in the metabolism, feeding and wintering habits of these species lead to interspecific differences in metal bioaccumulation and AOS parameters (Prokić et al., 2016b, 2017), which could be interesting for this study. Seasonal fluctuations in the response of the AOS were studied in different tissues of *P. ridibundus*, while *P. esculentus* has been studied to a lesser extent (Bagnyukova et al., 2003; Falfushynska et al., 2008; Feidantsis et al., 2013). But only a few studies dealt with the possible effects of heavy metals on the AOS in hibernators during arousal. For some hibernators (bats and snakes) it has been noted that accumulated heavy metals can significantly modify the AOS during arousal (Zukal et al., 2015; Gavrić et al., 2017), as confirmed by the increased correlations between concentrations of heavy metals and the biomarkers of AOS in spring. In frogs, the opposite was suggested. Namely, due to the high sensitivity of AOS parameters to environmental conditions (anoxia, freezing, thermal acclimation and recovery from hibernation) pollution cannot influence these biomarkers in frogs (Sura et al., 2006). To test this assumption, we determined and compared the seasonal variations in metal accumulation and AOS parameters in the skin and muscle of *P. ridibundus* and *P. esculentus* from two localities with different levels of heavy metal pollution during pre- (autumn) and post-hibernation (spring). We assumed that heavy metals would not affect the AOS response during arousal because of the importance of the AOS to frog survival. If this proved to be true, we would then expect very similar patterns of AOS variation in individuals from the different localities between the two examined species.

2. Materials and methods

2.1. Sampling site

Our previous studies marked the canal Danube-Tisza-Danube (DTD) (45°36′11.95″ N and 20°3′7.42″ E) as a locality burdened with heavy metal pollution, while the River Ponjavica (44°44′56.03″ N and 20°44′22.44″ E) has moderate heavy metal pollution (Borković-Mitić et al., 2016; Prokić et al., 2016a). The DTD canal is mainly polluted by anthropogenic activity (the meat industry, pig farms, sugar refineries, communal wastewaters, a vegetable cooking-oil factory and metal and leather processing factories) (Krčmar et al., 2017).

In contrast to the DTD canal, the River Ponjavica receives negligible wastewaters from small neighboring settlements and agricultural fields, and there are no major pollutants from industry. It is marked as a nature park composed of a variety of natural and semi-natural habitats, formed as a diluvial area of the river Danube. The River Ponjavica's flow is minimal or even absent, and it represents a good habitat for frogs of the *P. esculentus* complex (Radulović et al., 2007).

2.2. Sampling design

Hibernation and arousal depend on air and water temperature. In Greece, which also has a temperate but warmer climate than Serbia, hibernation of *P. ridibundus* frogs begins in the second half of November and lasts about 100 days, while arousal starts at the end of February (when the water temperature is 9–11 °C), and lasts until the end of April (Feidantsis et al., 2013). In Serbia, due to colder winters, frog arousal begins a little bit later. In this experiment, frogs were caught in the spring (April) and autumn (late October) with hand nets. Capture was always conducted at the same time of day and at the same position (in a stretch of 20 m). All individuals were checked and healthy ones were transported in native water to the laboratory for analyses. We measured *in situ* the physicochemical parameters of the water (temperature, pH, dissolved oxygen and conductivity) using mobile analytical equipment (WTW Multi 340i). Measurements were taken at three different points and in triplicate. For heavy metal analyses, water was taken from three points in 0.5-l plastic bottles that were previously acidified with 65% nitric acid (Merck) to pH < 2 in order to prevent metal adsorption.

2.3. Animals and tissue processing

Samples were counted for each locality in every season. Species with average biometric parameters (the SVL – the length from the tip of the snout to the posterior end of the cloaca and BM – body mass) are presented in the Supplementary Table (S1). The absence of sex-related differences in the AOS allowed us to merge both sexes in order to obtain larger sizes of samples. Both sexes were also used in our previous studies (Borković-Mitić et al., 2016; Prokić et al., 2016a, 2016b, 2017). Animals were killed by decapitation, and the skin from the ventral part of the body and hind-leg muscles were removed and divided into two halves for chemical and biochemical analyses. Chosen tissues were proven in our early studies as potentially good ones for examining heavy metals accumulation and AOS in frogs, due to their function and importance (Borković-Mitić et al., 2016; Prokić et al., 2016a, 2016b). All samples were stored at − 80 °C according to the standard procedure (Jansen et al., 2013).

Frog capture was approved by the Serbian Ministry for Energy, Development and Environmental Protection and Institute for Nature Conservation of Vojvodina Province (Permissions No. 353-01-364/2014-08 and No. 03-299/2). 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.

2.4. Determination of metal concentrations

The tissues were oven-dried at 105 °C and transferred to polytetrafluoroethylene (PTFE) cuvettes to which 7 ml of 65% HNO_3 and 1 ml 30% H_2O_2 were added. The microwave oven used for digestion has a rotor that can hold 10 PTFE cuvettes (Ethos 1, Advanced Microwave Digestion System, Milestone, Italy). The digestion procedure was as follows: 10 min warm-up to 200 °C, which was maintained for 15 min. Cooled samples were quantitatively transferred into volumetric flasks (25 ml) and diluted with distilled water. The concentrations of the elements: Cr, Fe, Ni, Cu, Zn, As, Cd, Pb, Co and Hg were measured in the water, muscle and skin of the frogs by inductively coupled plasma atomic emission spectrometry (ICP-OES; Thermo Scientific, United Kingdom). iTEVA software controlled the system. The selected wavelengths (nm) for the metals were as follows: Fe 259.9; Cr 267.7; Ni 231.6; Cu 324.7, Zn 213.8; As 193.7, Cd 214.4; Pb 220.3 and Hg 194.2. Certified reference material TORT-2 (lobster hepatopancreas reference material for trace metals; NRC Canada) was used to check the accuracy and precision of the instruments (Table S2, Supplementary material).

2.5. Biochemical analysis

Skin and muscle tissues 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 IKA-Werk Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany). The homogenates were further sonicated for 30 s at 40 kHz on ice, and then centrifuged in a Beckman ultracentrifuge at 100,000 × *g* for 90 min at 4 °C. The supernatants were taken for biochemical analyses.

The biomarkers of AOS were measured using a Shimadzu UV-160 spectrophotometer. Antioxidant enzyme activities were expressed as U mg protein^{-1}, and the concentrations of the nonenzymatic components (GSH and SH groups) as nmol/g tissue. The total protein content was measured according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

SOD activity was measured at 480 nm according to the procedure of Misra and Fridovich (1972), which is based on the autoxidation of adrenaline to adrenochrome. CAT activity was determined by the rate of hydrogen peroxide (H_2O_2) decomposition at 240 nm (Claiborne,