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Comparative analysis of hematological parameters in wild and captive *Proteus anguinus*

Primerjalna analiza hematoloških parametrov pri močerilu iz narave in v ujetništvu

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Proteus anguinus lives in porous limestone subterranean habitat, which is susceptible to environmental pollution. According to this, and to its specific reproductive biology such as slow and low reproductive rate, longevity and consequently pollutants accumulation as well as its thin non-keratinized skin, proteus is extremely vulnerable to environmental stressors, such as pollution and pathogens, which are of global concern for amphibians. Therefore, assessing health and stress in both wild and captive individuals of proteus is an important and necessary issue in the efforts for protection and conservation of this vulnerable species. Hematological parameters obtained from blood smears, such as white blood cell (WBCs) counts, are an efficient, inexpensive and non-destructive method to assess the health and stress levels of vertebrates, including amphibians (Davis et al. 2008).

In Gredar & Bizjak Mali (2017), we presented the results of a detailed study of blood cell types and their morphology in proteus, which was conducted by Gredar (2016). This study enabled the first preliminary WBCs counts and neutrophils to lymphocytes ratios (N/L ratio) estimations, but the study was carried out on a small number (N=3) of captive animals. Recently, the immunological response of proteus infected with opportunistic black yeast *Exophiala salmonis* was recorded (Bizjak Mali et al. 2018). However, some progression on proteus WBC counts was made up to date (Prša 2018) with efforts to obtain more accurate baseline values of WBCs, especially in wild population of proteus as well as in animals held in captivity. The purpose of this report is to summarize the procedure for safe blood sampling in proteus and to complement previous results on WBC counts and N/L ratio.

Like in other amphibians, the most appropriate site for blood sampling is the heart ventricle. In proteus, the heart is visible through its non-pigmented skin, which makes blood sampling safer (Fig. 1). The blood vessels in the tail, a common site for blood taking in larger urodeles, are too small in proteus. Correct handling in all steps, from anesthesia to recovery, is crucial, including further optimal artificial conditions for rearing these animals in captivity. The common anaesthetic for aquatic vertebrates is tricaine methanesulfonate or MS-222, applied as aqueous solution (Ross & Ross 2008). Both an appropriate concentration of the anaesthetic and the length of anesthesia must be applied and we optimized these two parameters for proteus to be 0.03% and 10–15 min, the latter depending on the animal's weight. Also, the solution must be adjusted to pH 7 with sodium bicarbonate, otherwise it is too acidic and harmful to animal. Before blood sampling, the safe blood volume should be calculated (Heatley & Johnson 2009) and it can be doubled for healthy animals. The safe blood volume is especially important if larger volume of blood is needed, e.g. if blood is needed for several different purposes, such as blood smears, blood culturing or plasma biochemistry. During the procedure, moistening of the skin is necessary and immediately after blood sampling (this takes a few minutes) animals have to be returned to the UV-filtered, aerated and dechlorinated tap water with appropriate temperature for monitored recovering. The awakening from anesthesia is variable but usually takes from few minutes to half an hour. Blood sampling can be repeated in the same animal without any harmful effects on it. However, the recovery time between two consecutive samplings should be long enough. The maximum number of repeated blood samplings in the same animal in our study was 7 times over the course of 3 years. All proteus individuals used for blood sampling have survived. For blood sampling in amphibians, lithium heparin is the recommended anticoagulant because it has the lowest risk for causing artefacts and haemolysis (Wright 2001). Blood smears must be prepared quickly to minimize artefacts and to ensure data quality, and when the blood on the slide is dried, the smears have to be immediately fixed in methanol for 2–3 minutes followed by

Giemsa staining. For optimal differential staining of blood cells, the smears must be stained as soon as possible or at least on the next day.

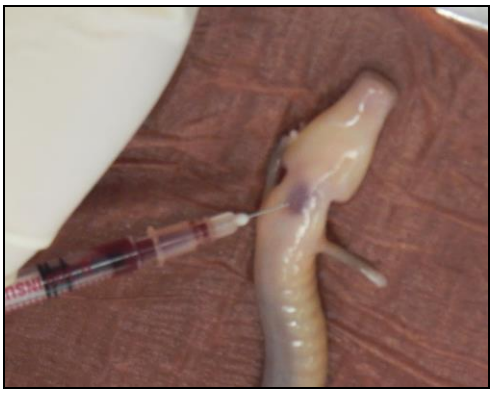

Figure 1. Blood sampling in proteus (photo: Bizjak Mali L.).
Slika 1. Odvzem krvi močerilu (foto: Bizjak Mali L).

Blood cell counts were made for recently captured individuals of proteus (N=9, Planina Cave, SW Slovenia, body length from 210 to 280 mm), beginning with initial blood sampling within 2–12 days after capture and while they were in captivity over a period of up to 3 years. Counts of WBCs from the initial blood sampling showed extensive variation between animals in every blood cell type. Nevertheless, proteus has a typical urodele pattern of WBCs with the majority of WBCs to be lymphocytes (73.0% ± 12.0) followed by neutrophils (15.9% ± 8.9), monocytes (7.7% ± 4.8) and eosinophils (3.2% ± 2.8), with the exception of basophils that were not found. These WBCs values are similar to the results of previous preliminary research on proteus blood cells from a smaller numerus of animals (Gredar 2016). In all captured animals, the neutrophils to lymphocytes ratio (N/L ratio) was quite variable (between 0.01 and 0.43) but below 1.0 and indicates that animals were not stressed (N/L ratio near 1 is an indicator for stress in amphibians (Davis & Maerz 2008)). In addition, N/L ratios of captured proteuses were within the reference range (between 0.01 and 0.6) that had been reported for other amphibian species (Davis 2009). Surprisingly, in five of the nine individuals amoebas were observed in blood during the initial and subsequent blood sampling (Fig. 2), but this was not reflected in their WBC counts and N/L ratio. We found that WBC counts did not change significantly in animals that had been kept in captivity for varying lengths of time. Although their N/L ratios (ranging from 0.02 to 0.86) generally appear to increase with time in captivity, these differences were not statistically significant ($p = 0.63$). An extremely high N/L ratio (7.8) was found only in one of the nine captive individuals after six months of captivity. However, this animal did not show any obvious symptoms of disease except amoebas in the blood, and is in fact still in good condition at the time of writing this report.

In conclusion, our results showed no statistically significant effect of long-term captivity on the physiological condition of proteuses as revealed by hematological parameters evaluated. The presence of amoebas in proteuses blood is remarkable and further studies are required to clarify the phenomenon of a weak immune response of proteuses to protozoan parasites in their blood.

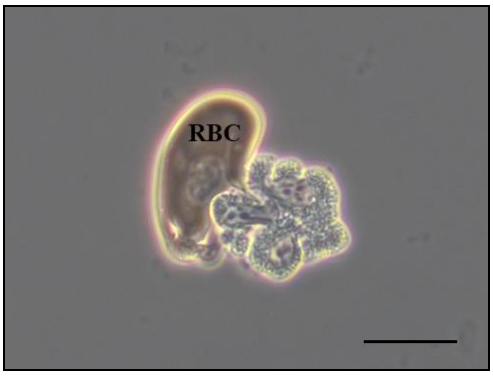

Figure 2. Amoeba in the blood of proteus. RBC – red blood cell, phase contrast, scale bar: 25 µm (photo: P. Prša).
Slika 2. Ameba v krvi močerila. RBC – rdeča krvnička, fazni kontrast, merilo: 25 µm (foto: P. Prša).

All the animals were collected with the approval of the Slovenian Ministry of the Environment and Spatial Planning, permit no. 35601-8/2016-4, and are kept in the Speleobiology Laboratory at the Chair of Zoology, Department of Biology, Biotechnical Faculty, University of Ljubljana, in accordance with the Slovenian animal protection act (Ur.l. RS 37/13).

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