Cultivation and morphology of blood cells of the olm *Proteus anguinus*

Gojenje in morfologija krvnih celic močerila *Proteus anguinus*

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Sex identification in *Proteus anguinus* using external morphological characteristics is not possible because of the lack of sexual dimorphism. We can identify their sex only during the reproductive season when mature eggs are visible through the non-pigmented skin of females and the cloaca is swollen in males. Accurate sex identification is crucial for demographic studies in natural populations, and would also be important for the establishment of a successful captive breeding program that could be used for evolutionary developmental studies as well as conservation measures.

We have optimized a non-destructive method, derived from human cytogenetics, to visualize chromosomes in order to search for potential dimorphism in sex chromosomes (Gredar 2016). We initially assumed that *P. anguinus* has similarly heteromorphic X and Y sex chromosomes as its closest living relative the North American mudpuppy, *Necturus maculosus* (Sessions 1980). Our cell culture approach was based on a previously described method used in *N. maculosus* (Seto et al. 1964) involving *in vitro* cultivation of blood cells in primary cell culture, but extensive modifications of *in vitro* cell culturing conditions are required. The first step is optimization of growth conditions, including the correct choice of culture medium and its osmolality, and the optimal incubation temperature. It was also necessary to determine the optimal concentration and time course of treatment with mitogen (phytohemagglutinin or. PHA-M) and antimitotic (colchicine) for the adequate stimulation of cell divisions of lymphocytes in cell culture and subsequent arrest in metaphase. We determined that the concentrations of mitogen and antimitotic in *P. anguinus* must be at least 3 or 4 times higher, with much longer treatment times, than are routinely used in human cytogenetics. One of our most surprising results is that the cultivation of cells was successful at an incubation temperature of 25°C indicating that *P. anguinus* cells can tolerate *in vitro* higher temperatures than previously believed. We successfully established a viable blood cell culture and were able to visualize the chromosomes. However, a recent study in our laboratory showed that *P. anguinus* is characterized by a translocation between the X and Y sex chromosomes so that both males and females have identical-looking chimeric sex chromosomes (Sessions et al. 2016). Consequently it is not possible to identify their sex chromosomally. Nevertheless, the establishment of successful cell culture methodologies potentially enables other kinds of studies including cell biology, cell physiology, genetics, biochemistry, toxicology and others in a way that does not require sacrificing the animal. This is especially important for protected and vulnerable species like *P. anguinus*.

Very little is known about the morphology of blood cells and hematological parameters in *P. anguinus*. Therefore we used blood smears for a detailed morphological description of proteus blood cells as well as for differential counts. The relative abundance of different types of white blood cells (WBC) can provide important information about the physiological condition of animals and, by extension, problems with the surrounding environment (Allender & Fry 2008).

In almost all Urodeles the erythrocytes (red blood cells or RBC) mature in the peripheral blood because of lack of bone marrow (Turner 1988). The same is true for *P. anguinus*, and the number of erythroblasts, that are immature RBC, is quite high (3.9–10.5% of all cells; N = 3) in comparison with axolotl (0.1–0.35%; N = 3) (Gredar 2016). The RBCs of *P. anguinus* are relatively large (52.7 ± 0.48 µm × 29 ± 0.24 µm; N = 300) and are among the largest in amphibians due to the large genome size, which is about 15 times that of the human genome. RBC sizes in vertebrates show a positive correlation with genome size (Gregory 2001) and a negative correlation with metabolism in animals, thus it is not surprising that metabolic rate in *P. anguinus* is considerably lower than that of the most surface dwelling amphibians (Hervant et al. 2001). The differential count of

leukocytes (WBC) in *P. anguinus* shows that lymphocytes and neutrophils are the predominant types (53.5–90.1% and 6.9–37.2%, respectively (N = 3)), while the eosinophils and monocytes are less numerous (0.0–7.0% and 1.7–3.0%, respectively (N = 3)) and basophils are almost absent. The low ratio between neutrophils and lymphocytes (0.20) indicates that animals were not under stress during captivity (Davis & Maerz 2008). This research was done on a small sample size and further studies are needed to obtain baseline values of WBC in *P. anguinus*. Also, more analyses are needed for accurately interpreting hematological data and for identifying abnormalities. Future research will focus on deviations from baseline values of the WBC profile, which can signal various physiological problems.

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