Functional morphology and environmental studies on *Proteus*

Funkcionalno-morfološke in okoljske raziskave na proteju

Lilijana BIZJAK MALI & Boris BULOG, Department of Biology, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia; Email: lilijana.bizjak-mali@bf.uni-lj.si

The purpose of this paper is to summarize some recent studies of the research group, Functional Morphological Studies of Vertebrates, of Prof. Dr. Boris Bulog (recently retired). We focused our investigations on the biological adaptations of the blind cave salamander (*Proteus anguinus*) to its habitat in the context of vulnerability to toxic chemical and organic pollution. Our long-term goal was to understand factors that can affect its survival in its natural environment. The following sections highlight some of the main areas of our recent studies.

Reproductive biology. Information on reproductive biology is important for the long-term goal of establishing a successful captive breeding program of this endangered cave amphibian. We have focused on the development of non-destructive methods for identifying sex, since *Proteus* males and females are indistinguishable by external morphological criteria. We have also examined variation in gonad morphology and the dynamics of gametogenesis. Our initial prediction was that we could use chromosomes from cultured blood to identify sex since the closest relative of *Proteus*, *Necturus,* has heteromorphic sex chromosomes. However, our results showed that the sex chromosomes of *Proteus* are homomorphic and therefore not useful for sex identification. On the other hand, we found interesting banding patterns indicating an X-Y translocation (Sessions et al. 2016). The significance of this finding is the basis for future research. The optimization of the non-destructive in vitro method using the cultivation of blood cells has been successfully achieved. We anticipate that we can utilize the culturing methods we have developed to answer several important questions about the cell biology and possibly even developmental genetics of *Proteus*. We are also currently trying to develop molecular markers for identifying sex.

The morphology and histology of gonads, as well as the ultrastructural characteristics of maturation stages of gametes, have been described in detail (Bizjak Mali & Bulog 2010, Kovačič 2013). Our research on gonads shows that reproductive characteristics reflect the fact that *Proteus* lives in a relatively stable habitat with environmental conditions that are conducive to non-seasonal reproductive cycles, however, some peculiarities have been revealed. We found that 1) there is no correlation between the size of the adult animal and condition of the gonads, 2) testis morphology is highly variable and at least three testis morphologies were found, 3) gametogenesis is mainly non-seasonal but with a tendency for maturation of gametes during the autumn for ♂ and winter for ♀, and 4) spermatogenesis and oogenesis may not be synchronous; courtship and insemination via spermatophore can probably occur weeks or months before oviposition as is common for some other salamanders. Surprisingly, numerous atretic follicles were revealed in the ovaries and were more frequent in ovaries of food-deprived specimens (Bizjak et al. 2011, 2013). The affected oocytes were mainly in vitellogenic stages, underlining the fact that yolk resorption by phagocytic cells can be an important energy-conservation process essential for normal development of the remaining oocytes, ensuring the reproductive potential of the species. The most remarkable discovery is that we found testis-ova in testes regardless of the morphology or meiotic condition of the testes (Bizjak et al. 2015, Bizjak & Bulog 2015). Testis-ova in other species of amphibians are usually associated with hormonal dysfunction or possible exposure to endocrine disruptors. In the case of *Proteus* we think they might be related to current evidence that *Proteus* has undergone a sex-chromosome turnover involving X-Y translocation (Sessions et al. 2016).

Conservation biology and monitoring pollution. From the early 1990's to the present, we have conducted environmental investigations of contamination of the cave habitats of *Proteus* and the accumulation of toxic compounds in its tissues. We paid special attention to the underground waters in the SE region of Slovenia, the locality of the black subspecies *P. a. parkelj*, which is an area smaller than 15–20 square kilometres. This is potentially one of the most threatened populations of *Proteus*. The water resources in karst underground are extremely vulnerable to all kinds of pollution and contamination with hazardous

organic and inorganic compounds originating from agricultural and industrial development, and require long-term monitoring. Among the most serious chemical pollutants are pesticides, polychlorinated biphenyls (PCBs), and heavy metals which persist in the environment, being slowly, if at all, degraded by natural processes. Our studies revealed the accumulation of zinc and arsenic as well as PCBs in both tissues and habitat of *Proteus*, and we also found a very high level of nitrates in the underground water (Bulog et al. 2002). The elevated concentrations of zinc in the tissues of *P. a. parkelj* were connected with the Zn-rich sludge deposit from an adjacent factory. After our intervention, the deposits were removed and the situation was improved. In the nearby vicinity of Jelševnik there are many vineyards and fields that use arsenic-containing pesticides in agricultural processes that can lead to arsenic accumulation in sediments and consequently to its bio-accumulation in living organisms. The concentration of arsenic found in the tissues of the black *Proteus* was more than 42 times higher than in the underground water of its habitat and about 65 times higher than in the tissues of white species of *Proteus* from unpolluted areas (Bulog et al. 2002). A recent study also revealed the accumulation of high levels of PCBs in the tissues of *Proteus* from the Krupa River, which is at least 28 times higher than those from unpolluted sites (Pezdirc et al. 2011). The results also showed that the Krupa River and its hinterland are still burdened with PCBs. The ability of *Proteus* to survive such a high PCB loading in its tissues is remarkable and deserves further study. The results of the monitoring of physical-chemical parameters in the period from 2000 to 2009 at the localities of the black subspecies of *Proteus* showed high levels of nitrates ranging between 1–9.7 mg/L (in unpolluted waters it is usually not higher than 1 mg/L), and the level increased in 2010 and 2011 when a biogas plant at Lokve in Črnomelj began to operate (Bulog 2012). The values were significantly increased in the spring and reached values higher than 15 mg/L, probably as a consequence of intensive manuring and pouring of slurry onto agricultural surfaces, and the consequent leaching of nitrates into underground water. Therefore, further long-term regular monitoring of potentially harmful pollutants in the underground water, in conjunction with continued studies of the reproductive biology of *Proteus*, is crucial.

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