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Chapter 7

FROGS AND TOADS AS BIOLOGICAL INDICATORS IN ENVIRONMENTAL ASSESSMENT

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ABSTRACT

Frogs and toads are increasingly used as bioindicator organisms in pollution studies in the past decades. However, a majority of frogs and toads species are endangered and protected in most European countries, which makes their study with traditional methods requiring a large amount of tissue samples difficult. We are reporting the development and application of a new analytical technique based on small amount of toe bones to study their elemental contents. Using this method it is not necessary to kill the animals, while the effects of anthropogenic activity on amphibians may well be studied. The first aim of our study was to develop a method using the elemental contents of frog bones that provides good estimates. For this purpose *Rana esculenta* L. individuals were collected from an urban pond in Debrecen (Hungary). Using the developed method our follow up aim was to study the effects of urbanization on the elemental contents of toe bones. *Bufo bufo* individuals were collected also from the urban pond in Debrecen and two rural ponds (Garancsi Pond and Lake Naplás).

In the case of *Rana esculenta* L. individuals P, Ca, Mg, Mn, Na, S and Zn were measured in the skull, spinal, femur, tibia-fibula, tarsal bones, metatarsus, humerus and digits from the front and hind limbs. The elemental contents of toe bones were measured and also estimated from concentrations found in large bones. The measured and estimated elemental contents of toe bones did not differ significantly based on estimations from the element content of the following large bones: tibia-fibula, metatarsal

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bones. Thus, toe bones may represent the other parts of the skeletal system well in this respect. In the urbanization study the following elements were analyzed in the toe bones of *Bufo bufo*: Ca, P, Mg, Zn, B. The urban and two rural ponds separated on the basis of the elemental contents of toad toe bones. We found significant differences between the concentrations of elements at the urban and the rural sites. Our results showed that the elemental contents of bones can be estimated using toe bones and frogs and toads could be useful indicators in the assessment of environmental contamination. Our study demonstrated that the effects of urbanization could be studied by using the elemental contents of toe bones. The analytical method we developed added a further way to use toe bones and it is appropriate for the elemental analysis of small samples. As a consequence, the toe bone based monitoring procedure is especially useful because it does not require the killing of live specimens and still can be used to assess the environmental load and contamination.

INTRODUCTION

During the past decades the ecology and ecotoxicology of amphibians started to get attention [Sparling et al. 2000] because of global amphibian population declines [Houlahan et al. 2000]. Based on the lists of the International Union for the Conservation of Nature (IUCN) there are 787 rare or endangered amphibian species [Frost et al. 2006] and about 1,900 species known to be threatened [Stuart et al. 2008]. Frogs and toads are about 90% of all amphibians [McDiarmid and Micthell 2000] Therefore, they are an important link between human and ecosystem health [Hayes et al. 2002] and they are main components of aquatic and terrestrial ecosystems [Unrine et al. 2007]. Most adult frogs and toads feed on invertebrates, so they are important, energy-efficient trophic link between insects and other vertebrates [Sparling et al. 2000]. They are sensitive to environmental changes both in terrestrial and aquatic habitats because they have highly semi-permeable skins and different life cycle stages [Alford and Richards 1999]. Nevertheless, the information on the effects of environmental contamination on frogs and toads is little known [McDiarmid and Micthell 2000].

The declines of amphibian populations are caused by a number of factors, including habitat loss and fragmentation [Icochea et al. 2002, Beebee and Griffiths 2005], ultraviolet radiation and chemical pollution [Blaustein et al. 2003], climate change [Pounds 2001] and epidemic disease like chytric fungus [Pounds et al. 2006]. Some of these factors may also cause deformities and abnormalities in their development [Blaustein and Johnson 2003] lowering further the viability of populations. Effects of contamination may result in shorter body length, lower body mass, malformations of limbs or other organs [Sparling et al. 2000]. Thus, the risk of mortality and exposure to predation is increased by slowed down development, late metamorphosis, and small metamorph size [Rowe et al. 2001, Pahkala et al. 2002, 2003]. As a result, the use of anurans as bioindicators of accumulation of contaminants in pollution studies is increasing [Welsh and Ollivier 1998, Johansson et al. 2001, Loumbourdis et al. 2007].

The aim of this chapter is to review the element concentrations of frog and toad bones in different habitats and to demonstrate a new method for the assessment of environmental load using frogs and toads as bioindicators.

FROGS AND TOADS AS BIOINDICATOR ORGANISMS

Habitats of many frog and toad populations are small, temporary ponds and the surrounding forested area, which are usually suffered by many stressors such as UV-radiation [Cummins 2003, Hatch and Blaustein 2003], the use of pesticides [Gendron et al. 2006, Fellers et al. 2004] and industrial chemicals [Bishop and Gendron 1998, Sower et al. 2000], urbanization [Barrett et al. 2010], climate change [Corn 2005]. Since frogs and toads are sensitive to the alterations of their environment, they could be used as bioindicator organisms to follow changes in their habitats and in ecotoxicological studies [Henry 2000]. As their populations usually contains high numbers of individuals and they are good representatives of freshwater environments, they are good model organisms for pollution studies [Burger and Snodgrass 1998]. What is more, adult anurans play an important, usually intermediate role in food-webs because they are preys and predators as well but their position changes with their development, i.e. tadpoles also feed on algae [Murphy et al. 2000] making them even more sensitive to different stressors. Thus, frogs and toads may be used as biological indicators to assess the effects of environmental factors that may cause the declines of amphibian population.

In several earlier studies these animals were used to assess the effects of UV radiation [see e.g. Cummins 2003, Hatch and Blaustein 2003]. Its direct effects was demonstrated to cause embryonic mortality [Pahkala et al. 2002], abnormal larval development [Belden and Blaustein 2002], limb and muscular deformities [Weyrauch and Grubb 2006]. Similarly, different pesticides were also tested and deformities were detected [Pickrell 2002] but these studies were based on laboratory toxicity test [Cowman and Mazanti 2000]. Although most pesticides do not accumulate their toxicity is relative high [Kamrin 1997] which may cause paralysis [Fellers et al. 2004], decreased size of metamorphosis [Relyea and Diecks, 2008] and negative effects of liver and kidney [Khan et al. 2003].

The use of frogs and toads, as biological indicators of metal pollution is becoming more common [Burger and Snodgrass 1998]. The effects of metal accumulations were studied both under laboratory [Perez-Coll and Herkovits 1996, Herkovits and Helguero 1998, James and Little 2003] and field conditions [Puky and Oertel 1997, Demichellis et al. 2001, Flyaks and Borkin 2004, Fenoglio et al. 2006] but the number of field studies is low. In an earlier study the whole body of *Rana catesbeiana* tadpoles were analysed for different heavy metals (cadmium, chrome, manganese, arsenic, mercury) and the highest concentration of metals were found in the digestive tracts of tadpoles [Burger and Snodgrass 1998]. In another study, the elemental concentration of *Rana dalmatina, Bufo bufo* and *Rana ridibunda* tadpoles were compared and significant differences were found between the studied species. The heavy metal concentration of *R. dalmatina* in the whole body was significantly lower than in the other species which is caused by sediment contamination in the *R. dalmatina* habitat [Grillitsch and Chovanec 1995]. In the case of tadpoles Zhang et al [2007] reported that the ATPase activity increased with increasing of Cd and Pb concentrations in *Bufo raddei* tadpoles. This means that the ATPase activity may be a warning signal of pollutant-induced damages in the ionic and osmoregulatory system [Zhang et al. 2007]. In other studies differences were demonstrated between the different development stages [Baudo 1976, Puky and Oertel 1997]. Higher heavy metal concentration in tadpoles than adults may be caused by changes in feeding during development, tadpoles are detritivorus unlike adults, which are carnivorous and the detritivorous diet may be richer in metals [Baudo 1976]. Pavel and Kucera [1986] studied the accumulation of manganese, iron, copper and zinc in the whole body of *Rana esculenta* adults from three different localities. Their study demonstrated that in the case of manganese, iron and copper significantly different concentrations were found at the selected localities. However, studies including all development stages is needed because of the changing of susceptibility of frogs to heavy metals may depend on different stages of development [Perez-Coll and Herkovits 1996]. Puky and Oertel (1997) demonstrated e.g. that eggs contain a relatively low concentration of different heavy metals in comparison with a range of adult tissues such as muscle and parts of the body (e.g. kidney, liver).

ELEMENTAL COMPOSITION OF BONES

A lot of elements play an important role in the building of the skeleton. In general, 70% minerals, 20% collagen, 8% water and about 2% non-collagenous components are the composition of bones [Klepinger 1984]. Calcium and phosphorus are the most important elements in the bones, which were the main components in the formation of hydroxiapatite [Janus et al. 2008]. Oudadesse et al. [2004] reported that the main elements are calcium, phosphorus and magnesium in the bones. Smaller calcium and phosphorus contents cause smaller hydroxyapatite content in the bones which may cause more porous structure in the bones [Janus et al. 2008]. The Ca/P, Ca/Mg and the Zn/Ca rate are also important characteristics to be measured in bones (Busetto et al., 2008). Normally the Ca/P ratio is 2:1, but the optimal Ca/P ratio can not be reached with vitamin D deficiency, which leads to unhealthy bones [Nestler et al. 1948]. The optimal value of the Ca/Mg ratio is around 2:1,it is important for the calcification of the skeletal tissue [Nielsen 1973]. Zinc is necessary to produce the main organic components of bone matrix for normal calcification, the Zn/Ca ratio is higher than 0.5 in bones [Busetto et al. 2008],. However, if zinc is present in too high concentrations, it can interfere with hydroxyapatite crystal growth and lead to accelerated bone resorption [Kenney and McCoy 1997]. Bones are mineralized tissues so they may have high storage capacity of metals such as barium, lead, strontium and zinc [Linder and Grillitsch 2000]. Flyaks and Borkin [2004] studied the elemental concentration of femur of *Rana ridibunda*. Their result showed that iron (220 + 5 mg kg-1), zinc (88 ± 2 mg kg-1), manganese (6.8 ± 0.2 mg kg-1), copper (30 ± 1 mg kg-1), lead (150 ± 1), nickel (16 ± 1 mg kg-1) and cadmium (17 ± 2 mg kg-1) could accumulate in relative high concentrations in bones from the area where chemical and metallurgical factories released their run off waters [Flyaks and Borkin 2004]. In other studies strontium and barium concentrations were detected in bones [Klepinger 1984; Busetto et al. 2008]. These results show that bones may be applied as indicator organs to assess the environmental load.

NEW METHOD TO ASSESS ENVIRONMENTAL LOAD ON FROGS AND TOADS

Current analytical methods are relatively well developed to assess the effects of environmental load on amphibian populations. Nevertheless, in certain cases the available amount of sample is small. With the proposed micro analytical method different organs may be analysed without the need for killing live animals (Simon et al 2010). For our study toe bones of frogs and toads were chosen. The elemental analysis of toe clipped bone samples has several advantages: there is no need to kill the animals, the analysis has moderate chemical reagent demand and a high number of samples can be analysed by this method. Toe clipping is a commonly used standard method to identify individuals, particularly frogs and toads [McCarthy and Parris 2004]. This method is simple, safe and the toe clipped sample is applicable for histological examinations [Kriger et al. 2006, Hyatt et al. 2007], genetics [Noonan and Gaucher 2006, Amour and Lesbarréres 2007], or amphibian skeletochronological age determination [Bruce et al. 2002, Takashi and Masafumi 2009]. Hartel and Nemes [2006] reported that toe clipping is an acceptable method from a conservation viewpoint as it usually does not cause any serious effects.

In our earlier study [Simon et al. 2010] the trace element concentrations in *Rana esculenta* frog bones were investigated and a method was invented to estimate the elemental concentrations of frog bones based on phalanges. Before the elemental analysis of bones hydrogen peroxide may be used to clean the bones for two days. This period is enough to clean the bones from conjunctive tissue and it still does not cause elements to leach out from the bone tissue. There were good correlations between the elemental contents and dry weights of the bones: phosphorus (r^2 = 0.96), calcium (r^2 = 0.95), magnesium (r^2 = 0.97), sulfur (r^2 = 0.91), sodium (r^2 = 0.89), manganese (r^2 = 0.73), barium (r^2 = 0.63) and zinc (r^2 = 0.57). It means that the elemental contents of bones were commensurable to their weights. This correlation suggests that the concentration of trace elements was similar in the phalanges and in large bones. To test the usefulness of the method the measured elemental contents of phalanges were compared to the estimated elemental contents. Based on the tibiafibula and metatarsalis bones, front and hind limb digits, the measured and the estimated elemental contents of phalanges did not differ considerably. These results indicated that the elemental contents of phalanges reliably represented the elemental contents of tibiafibula, metatarsalis bones and digits. Thus, toe phalanges represent the other parts of the skeletal system and in spite of the small size of the phalanges, the elemental concentrations can be measured appropriately in them.

To test the developed new technique the effects of urbanization on the elemental concentration of the toad's skeletal system were investigated. The concentrations of trace elements in bones were higher in toads from the urban pond than from the rural ponds. Both calcium and phosphorus were significantly higher in samples collected at the rural ponds. Smaller amounts of boron and zinc were present in the toe bones from the rural ponds than from the urban one. These results demonstrated that different habitats may cause marked differences in the elemental contents of *B. bufo* toe bones. Although physiological differences and morphological deformities were not found in the toe bones, the concentrations of the main elements in the bone were markedly lower at the urban pond than at the rural ponds. In spite of the small size of toe bones, the elemental concentrations could be measured reliably. Thus, the developed technique based on toad toe bones may be useful for the assessment of contamination in pollution studies without killing frog and toad individuals.

CONCLUSION

Micro analytical methods give a new opportunity for using anurans as bioindicators. Small toe bones provide an appropriate amount of tissue sample for estimating the elemental composition of bones. Our results showed that the elemental composition of toe bones and large skeletal bones were in good correlation. Anthropogenic activity in urbanized areas has an effect on the elemental composition of toad toe bones. The concentration of major elements (Ca, P, Mg) was higher in the toe bones in rural sites, while Zn concentration was the highest at urban sites. The analysis of the anuran's toe bones is a new non-destructive method, which could be applied on live specimens without seriously harming their health. It may open further ways in environmental load assessments facilitating further monitoring to assess the effects of environmental pressure both on amphibians and their habitat.

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