Hematological parameters of health status in the common toad *Bufo arenarum* in agroecosystems of Santa Fe Province, Argentina

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Abstract. We compared some hematological parameters and values of plasma cholinesterase activity in adult *Bufo arenarum* from a control site and agricultural sites. The mean values of the plasma cholinesterase activity did not vary among toads collected in pristine forest and agricultural sites. The blood parameters (hematocrit, hemoglobin concentration, white blood cells, and heterophils) from agricultural sites differed from the control site. The results suggest a high pesticide impact from intensive cropping in mid-eastern Santa Fe Province of Argentina.

Key words: Agroecosystem; Argentina; *Bufo arenarum*; hematological parameters; pesticide; plasma cholinesterase.

Introduction

Amphibians are often identified as a group of organisms that are particularly sensitive to environmental pollutants because their double life-cycles and relatively permeable skins provide more opportunities for exposure to pesticides than those of other vertebrates (e.g. U.S.EPA, 2002; Mann et al., 2003). Moreover, amphibian populations are declining worldwide and one of the many contributory factors proposed for this phenomenon is agricultural intensification (Kiesecker et al., 2001; Alford et al., 2001; Christin et al., 2003).

In Argentina a wide range of pesticides are commonly used in agricultural practices. Bullacio and Panello (1999) listed a broad group of agrochemicals such as pyrethroids (e.g. deltamethrin, cypermethrin, alfamethrin), chlorinated (e.g. endosulfan, acetochlor), organophosphorus (e.g. monocrotophos, fenitrothion) or carbamate (e.g. carbofuran, carbendazim) pesticides. Among these agrochemicals, organophosphorus, carbamate and pyrethroid compounds constitute an important group of pesticides with an intensive use in Argentina (CASAFE, 1999). There is not a reliable database on the consumption and application rate of pesticides in Argentina. However, studies conducted in the provinces of Entre Ríos and Santa Fe (Argentina) during 2000-2004 revealed residues of organochlorine pesticides in amphibian tissues (Lajmanovich et al., 2002) and high sensitivity to pyrethroids (Izaguirre et al., 2000). In addition, malformations (Peltzer et al., 2001) and inhibition of plasma cholinesterase (Lajmanovich et al., 2004) were reported in anurans collected in the same region. These studies indicate that investigations into the toxic effects of pesticides in amphibian populations from the areas surrounding crop fields in Argentina are necessary.

Biomarkers are essential to assess the environmental and health risks of exposure to potentially toxic chemicals (NRC, 1987). In particular, changes in blood parameters can be useful in some species as biochemical markers of toxicity for pesticide exposure (Haratym-Maj, 2002). As part of a continuing study to assess the adverse effect from pesticides on a population of anurans, the aim of this work was to establish some hematological parameters of adult *Bufo arenarum* from a control site and from agricultural sites. Plasma cholinesterase (ChE) activity was also assayed. *B. arenarum* was selected because it has an extensive distribution in Neotropical regions (Cei, 1980), and it is frequently found in agricultural lands and urban territories.

Materials and Methods

The study area was situated in the mid-eastern area of Santa Fe Province (Argentina) (fig. 1). Because intensive agriculture was introduced to this area in the 20^{th} century, the remaining forest is restricted primarily to riparian woodlands surrounded by croplands and pastures.

24 adult male *B. arenarum* were collected by hand from four sampling sites ($n = 6$ for each site); one control site, located in a pristine forest (PRB: Paraná River Boundary, 31°42′S 60°30′W), and three agricultural sites: Monte Vera (site A), Monte Vera (site B) and Angel Gallardo, situated in suburban woodlands (fig. 1). These three areas differed in the degree of human disturbance, successional stages and crop. Monte Vera (site A) (MVA, 31°32′S 60°41′W), Monte Vera (site B) (MVB, 31°30′S 60°40′W) and Angel Gallardo (AG, 31°33′S 60°40′W) were often used for intensive agricultural production with transgenic soybean. Vegetables such as lettuce, tomato, and spinach were also cultivated.

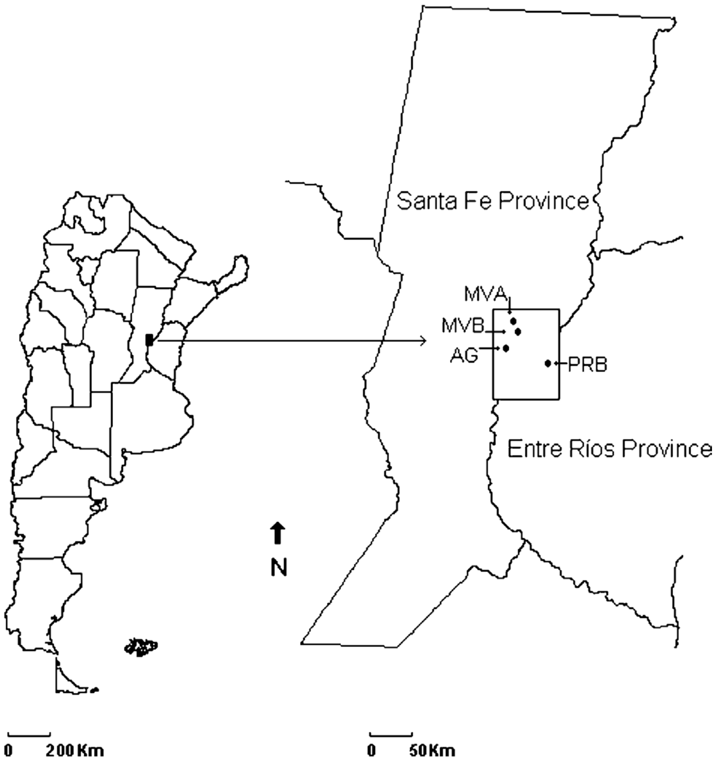

Figure 1. Locations of the sampling sites. PRB = Paraná River Boundary; MVA = Monte Vera (site A); MVB = Monte Vera (site B); AG = Angel Gallardo.

The toads were captured in January 2004, with a mean air temperature of 25°C and rainfall of 1000 mm. Animals were transported to the laboratory within 1 h, in a covered bucket with water, to avoid stress. Head-cloaca length and body weight were recorded. Duplicate samples of blood (ca. 500 μL) were taken from animals anaesthetized with 30% ethyl alcohol by cardiac puncture using a heparinized small needle (20 mm length) following location of the heart via palpation. The toads were released after external examination of general body condition, at the same sites where they had been captured. Plasma ChE activity was determined colorimetrically by the method of Ellman et al. (1961) with modifications for optimal assay conditions (Lajmanovich et al., 2004). The plasma was separated by centrifugation at 4500 r.p.m. for 10 min. Variations in optical density were measured at 405 nm for 2 min at 25°C. Slides were prepared and stained with the May Grunwald-Giemsa method (Dacie and Lewis, 1991). It is important to note that amphibian blood contains nucleated erythrocytes, leucocytes and thrombocytes. The red blood cells, erythrocytes, typically are nucleated and elliptical. The white cells are made up of agranular leucocytes (lymphocytes and monocytes) and granular leucocytes (basophiles, heterophils, and eosinophils) (Duellman and Trueb, 1986).

Hematocrit (Hct) was determined by centrifugation of whole blood at 12000 $\times$ g for 5 min. in heparinized capillary tubes. Hemoglobin (Hb) concentration was measured by the cyanmethemoglobin method (reading at 540 nm); the red blood cell (RBC) and white blood cell (WBC) counts were determined in a Neubauer chamber after diluting the samples in Natt and Herrick solution (Fudge, 2000). Mean cell hemoglobin (MCH), mean cell volume (MCV) and mean cell hemoglobin concentrations (MCHC) were calculated using standard methods for amphibian blood (Peri et al., 1998). The data were analyzed statistically using the non-parametric Kruskal-Wallis ANOVA; pairwise comparisons between samples from the four sampling sites were tested by the Dunn test for post-hoc multiple comparisons. A level of probability below 0.05 was considered to be significant.

Results and Discussion

Mean (± SD) length and body mass of male toads were 96 ± 13 mm and 90.3 ± 3.5 g, respectively. The mean values of the plasma ChE activity varied from 139.2 μmol $seg^{-1} L^{-1}$ for the toads collected from the pristine forest (PRB) to 166.4 μmol $seg^{-1} L^{-1}$ for individuals captured from an agriculture site (MVB) (table 1).

In amphibian and reptile populations exposure to anti-cholinesterase pesticides generally is evaluated by comparison of enzyme activity from animals collected in non-polluted areas with those from agricultural areas (Sparling et al., 2001; Sánchez-Hernández, 2003). In our study the means of ChE activity did not show significant differences ($P > 0.05$). Several authors have documented differences in the sensitivity to the anti-pesticides among enzymes of the same tissue from different species (Johnson and Wallace, 1987; Monserrat and Bianchini, 1998; Pan and Dutta, 1998) and suggested that species-related differences in enzyme susceptibility can primarily be due to dissimilar enzyme amounts and inhibitor affinity degree to cholinesterase receptors.

The blood parameters of toads from agricultural sites did not differ significantly from those of the control (table 1), with the exceptions of Hct, Hb, WBC and

Table 1. Mean values (± SD, $n = 6$) of some hematological parameters and plasma cholinesterase activity in specimens of *Bufo arenarum* sampled in control site (PRB) and agricultural sites (MVA, MVB and AG) in Argentina (fig. 1).

Parameter	Sampling site			
	PRB	MVA	MVB	AG
RBC ($\times 10^{12}$/L)	0.57 ± 0.11	0.46 ± 0.13	0.55 ± 0.06	0.55 ± 0.15
MCHC	19.95 ± 0.63	19.35 ± 1.71	22.40 ± 3.90	21.39 ± 2.47
MCH (pg)	143 ± 15.0	125 ± 15.6	181 ± 23.1	160 ± 46.0
MCV (fl)	718 ± 77	655 ± 125	650 ± 82	636 ± 101
Eosinophils (%)	17 ± 8.2	16 ± 9.8	12 ± 4.3	10 ± 10.0
Lymphocytes (%)	62 ± 7.4	71 ± 7.1	59 ± 11.9	64 ± 27.7
Monocytes (%)	1.0 ± 0.51	1.0 ± 0.89	1.0 ± 0.07	2.0 ± 0.5
ChE activity (μmol $seg^{-1} L^{-1}$)	139 ± 52	146 ± 72	166 ± 47	141 ± 39

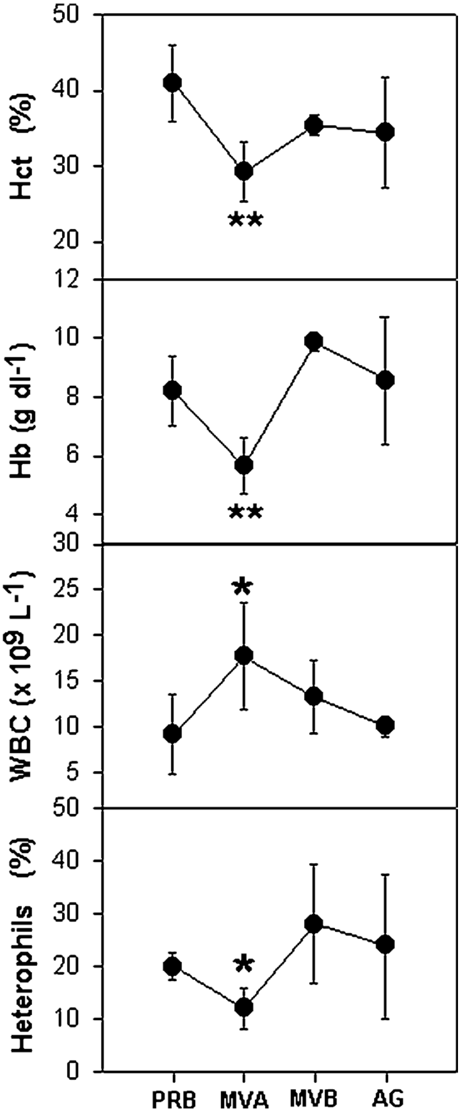

Figure 2. Mean (± SD) values of hematocrit (Hct), hemoglobin (Hb), white blood cells counts (WBC), and heterophils in common toad (*Bufo arenarum*) collected from control (PRB) and agricultural (MVA, MVB and AG) sites in Argentina. * $P < 0.05$, ** $P < 0.01$.

heterophils in MVA site (fig. 2). Blood parameters of control toads were similar to those from another study of *B. arenarum* (Peri et al., 1998). Lymphocytes were the dominant cell among the various types of leucocytes. We did not identify basophils. Our investigation showed in MVA samples a normochromic/normocytic anemia (Hct 29.16%, control 41; Hb 5.65 $g\,dL^{-1}$, control 8.19). Low values of Hct (anemia) may indicate bacterial infections and gastrointestinal disorders, including parasitism and hemorrhages (Dein, 1986). Elevated leucocyte number (leucocytosis) can be symptomatic of stress syndrome and inflammatory/infective and neoplastic processes (Ots et al., 1998). In this context, U.S. EPA (1982) and WHO (1996) suggested that pesticide exposure can cause leucocytosis. Finally, no abnormalities were observed in the erythrocytes and leucocytes.

Notably, the MVA site heterophils (%) were lower than in the other sites ($P < 0.05$). Recent studies have shown that pesticide exposure causes measurable alterations in the immune function of amphibians (e.g. Carey et al., 1999; Gilbertson et al., 2003). Other studies on humans working in the production of liquid pesticides, reported a significant decrease in the number of neutrophils (Klucinski et al., 1996). Neutropenia/Heteropenia is a serious disorder because it makes the body vulnerable to bacterial and fungal infections and is often the result of exposure to pesticides (Berkow, 1992).

In nature, wildlife is often exposed to mixtures of chemicals and their degradation products (Ensenbach and Nagel, 1995). The interactions between various combinations of pesticides can affect hematological parameters (Dolara et al., 1994). Indeed, another possible explanation for variation in the blood parameters of toads at the sites studied is contamination as a consequence of prolonged use of pyrethroids (Desi et al., 1985; Garg et al., 1997; Haratym-Maj, 2002). Although these results are preliminary, the differences in hemopoietic and leucocyte levels in the toads from sampling site MVA may possibly be due to a high pesticide impact from an intensive cropping in mid-eastern Santa Fe Province of Argentina. It is important to note that toads collected from MVA had been in contact with clothes and tools that farmers utilize to fumigate. Clearly, the interaction of human-induced habitat modifications and disease dynamics in agroecosystems deserves further study.

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