

## Basal frequency of micronuclei and hematological parameters in the Side-necked Turtle, *Phrynops hilarii* (Duméril & Bibron, 1835)

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**Abstract.** The present study aimed to evaluate basal frequency of micronuclei (MN) and hematological values in adult *Phrynops hilarii* in order to propose this aquatic turtle, broadly distributed in our region, as a biological monitor for future studies of environmental pollution assessment. Thirty-two adult turtles from a semi-natural environment located at the Zoological Experimental Station (Santa Fe, Argentina) were used. Blood samples were taken and the following parameters were determined: basal frequency of MN (BFMN), total red blood cells (RBC) count, hematocrit (Ht), hemoglobin (Hb), total and differential white blood cells (WBC). The BFMN determined for the species was  $3.56 \pm 1.39$ , while hematological parameters showed the following reference values:  $0.937 \pm 0.12 \times 10^6$  RBC/ $\mu$ l,  $27062.50 \pm 4565.43$  WBC/ $\text{mm}^3$ , hematocrit  $18 \pm 1.81\%$  and Hb concentration  $4.80 \pm 0.45$  g/dl. Differential WBC counts were:  $76 \pm 2.90\%$  for lymphocytes,  $20.12 \pm 2.56\%$  for heterophils,  $1.5 \pm 0.19\%$  for monocytes, and  $2.12 \pm 0.61\%$  for eosinophils, while no basophils were observed in any of the samples analyzed. No differences were observed between males and females in any of the variables analyzed. Data provided in this work could be useful as reference values for future studies of natural regions where *P. hilarii* occurs, employing this species as a sentinel organism for genotoxic and immunotoxic evaluation of environmental pollutants.

**Keywords.** Genotoxicity, immune system, hematological values, micronucleus test.

### INTRODUCTION

*Phrynops hilarii* (Duméril and Bibron, 1835), commonly called side-necked turtle, belongs to the Suborder Pleurodira, Family Chelidae. This species is distributed in the east of Uruguay, southern Brazil, almost all of Paraguay and Argentina (Cabrera, 1998; Van Dijk et al., 2012). In this country, it can be found in the provinces of Buenos Aires, Santa Fe, Entre Rios, Corrientes, Chaco,

Misiones, Formosa, Córdoba, Tucuman and Mendoza (Richard, 1999).

This species inhabits in streams and lagoons which in many cases constitute important areas of pollutants discharge, generally coming from agricultural and industrial activities. Evaluation of toxic effects produced by chemicals on hematological, immunological and genetic parameters in wild species is of fundamental importance for maintaining biodiversity, as they are

good indicators of the health of populations in potential exposure to different types of xenobiotics (Donald, 2004; Poletta et al., 2008).

The immune system (IS) is an excellent indicator of the health of an organism (Burns et al., 1996). Its integrity is essential for the defense against infectious organisms and their toxic products and, therefore, for the survival of all individuals. The IS is also very sensitive to chemicals exposure and considering the fast response of some immune parameters, many of them are used as markers of such exposure (Lafuente Giménez et al., 2001). In this sense, the exposure of wild or domestic animals to certain chemicals, whether acute or chronic, may affect the defense mechanisms. This information also allows the identification of associated diseases such as anemia, malnutrition, dehydration, inflammation, parasitemia, hematopoietic malignancies and disorders of hemostasis (Barboza et al., 2007).

White blood cells (WBC) are the cellular components of the IS and are involved in a significant number of processes. Certain situations may cause an increase or decrease of the values of selected blood components; in turn, these are used for the interpretation of physiological phenomena or accurate diagnosis of diseases and nutritional status (González Fernández, 2003). In addition, traditional hematological parameters may provide information about the general stress, especially in birds and reptiles (Gross and Siegel, 1983; Morici et al., 1997; Lance and Elsey, 1999). As it has been mentioned above, many individual factors (age, sex, stress, nutritional condition, hormones concentrations, body hydration levels, etc.) as well as environmental factors (temperature, pressure, oxygen concentrations, etc.) may affect hematological values and so they can be considered as biomarkers of exposure (Dessauer, 1970; Aguirre et al., 1995).

On the other hand, biomarkers of genotoxicity reveal alterations induced by various agents on genetic material of organisms. In particular, the MN test (Schmid, 1975) detects the effects of agents which modify the structure and/ or segregation of chromosomes by the identification of acentric fragments and/ or lagging chromosomes that remain separated from the main nucleus of the daughter cells during cell division. This technique allows the detection of early biological responses, before the damage is irreversible and causes imbalances in the health of an organism (Carballo and Mudry, 2006). Thus, the determination of the frequency of MN (FMN) is used as an indicator of the effects of various agents on the genetic material and the “machinery” associated with cell division (Schmid, 1975). The basal frequency of micronuclei (BFMN) is the result of “normal” errors in the processes of replication and/ or cell division, which are not caused

by the incidence of genotoxic agents. It is specific for each species and each cell population, and constitutes a reference value to determine the usefulness of a species as a sentinel or biological monitor. In reptiles, the MN test was applied to peripheral blood erythrocytes in different species (Zuñiga González et al., 2000; Poletta et al., 2008; Schaumburg et al., 2012), being a useful tool to determine the genotoxic potential of chemical (Poletta et al., 2009; Poletta et al., 2011; López González et al., 2013) and physical agents (Schaumburg et al., 2010).

Up to our knowledge, there is no report on the literature about the application of the MN test in this species, while the studies on hematologic values in *Phrynops hilarii* are extremely scarce (Pitol et al., 2007).

The aim of this study was to determine the BFMN and reference hematological values in adult *P. hilarii*, in order to propose this aquatic turtle of great importance in our region, as a biological monitor for future studies of environmental pollution assessment.

## MATERIALS AND METHODS

### *Animals*

All animals in this study were treated in accordance with the *Reference Ethical Framework for Biomedical Research: Ethical Principles for Research with Laboratory, Farm, and Wild Animals* (National Scientific and Technical Research Council, 2005), using non-invasive techniques of blood collection and minimizing stress and suffering by suitable management methods.

Thirty-two adults *Phrynops hilarii* (from 0.61 to 5.46 kg, Fig. 1) from a semi-natural environment at Zoological Experimental Station (Santa Fe, Argentina), were bled (1 ml) immediately from the external jugular vein (Rogers and Booth, 2004). All individuals were sexed, weighed and measured morphometrically.

### *Micronucleus test (MN)*

The MN test was conducted according to the technique described by Poletta et al. (2008) for its application in peripheral blood erythrocytes of another reptile species, with some modifications.

Two smears were performed per animal, fixed with ethanol and stained with 10% Giemsa solution previously centrifuged and filtered. The baseline frequency of MN (BFMN: number of cells with MN/1000 erythrocytes analyzed) was determined under an optical microscope at 1000x.

### *Hematological parameters*

For hematological study the following parameters were determined: RBC (red blood cells) and WBC (white blood cells)



**Fig 1.** Adult specimens of *Phrynops hilarii* (approximately 4-5 kg).

counts, differential leukocyte count (DLC), Ht and Hb.

The total leukocyte count was performed using a Neubauer chamber due to the presence of nucleated erythrocytes. In this species, like in all non-mammalian vertebrates, the use of automatic counter is impossible because the presence of nucleated erythrocytes gives wrong numbers. An aliquot of blood was diluted 1:200 with 0.6% NaCl (Lewis et al., 2008). The samples were observed under an optical microscope at 400x. For the DLC two smears were made per animal on clean slides, fixed with ethanol for 10 min and stained with May Grünwald (50%)-Giemsa (10%) solutions. The samples were observed under an optical microscope at 1000x. Hb concentration was determined using an autoanalyzer.

*Statistical Analysis*

The statistical analysis was conducted with the software SPSS 17.0 for Windows. Mean and Standard Error (SE) of each variable analyzed were calculated from data of all animals. All variables were analyzed in normality by Kolmogorov-Smirnov test and homogeneity of variances by Levene test. We used the

**Table 1.** Reference values for all variables studied in *P. hilarii*, including males and females particular values (BFMN, baseline frequency of micronuclei; RBC, red blood cells; SE, standard error; WBC, white blood cells).

Variables	(mean ± SE)	Males	Females
Total RBC (x10 <sup>6</sup> /μl)	0.937 ± 0.12	0.98 ± 0.23	0.91 ± 0.15
Total WBC (x10 <sup>3</sup> /μl)	27.06 ± 4.56	26 ± 2.75	27 ± 7.47
Hematocrit (%)	18 ± 1.81	20.17 ± 0.35	16.73 ± 2.84
Hemoglobin (g/dl)	4.80 ± 0.45	5.17 ± 0.16	4.58 ± 0.73
Lymphocytes (%)	76 ± 2.90	77.0 ± 7.02	75.40 ± 2.94
Heterophils (%)	20.12 ± 2.56	18.33 ± 5.84	21.20 ± 2.71
Monocytes (%)	1.5 ± 0.19	1.33 ± 0.33	1.60 ± 0.24
Eosinophils (%)	2.12 ± 0.61	1.67 ± 0.33	2.4 ± 0.98
BFMN	3.56 ± 1.39	3.08 ± 2.39	4.27 ± 1.92

Mann Whitney U-test to compare the BFMN and monocytes between males and females, while the rest of the variables were analyzed through the Student *t*-test. Linear regressions were carried out to analyze the relation between reference values and weight of the animals. A *P* value < 0.05 was considered statistically significant.

RESULTS

Reference values of all variables analyzed and the particular values for males and females are shown in Table 1 (Fig. 2). Concerning hematological parameters no basophils were observed in any of the DLC analyzed and no differences were observed between males and females in any of the variables (*P* > 0.05, Table 1).

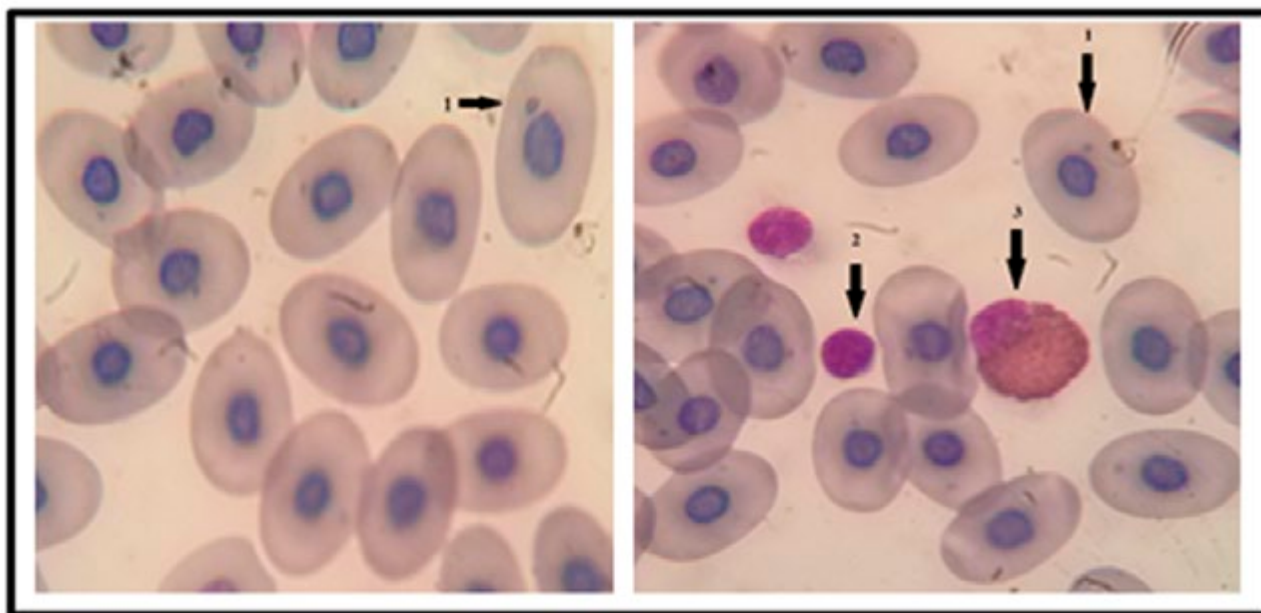
In the case of the BFMN, females showed higher values than males, but the difference was not statistically significant (*P* > 0.05, Table 1). No relationship was found between BFMN and weight of the animals (*P* > 0.05, *R*<sup>2</sup> = 0.001).

DISCUSSION

Currently, there is great interest in those studies that allow collecting information on the health status of wild populations. In the case of reptiles, hematological techniques have been evolving favorably (Wilmoth, 1994; Lowell, 1998), however, there are few hematology studies on the Testudines freshwater turtles (Metin et al., 2006; Pitol et al., 2007).

Reference hematological values found for different species of turtles (Troiano et al., 1998; Montilla Fuenmayor et al., 2006; Cabrera et al., 2011) differ from the values reported in our study, and this can be explained by the multiple factors that influence them, such as the species, the environment where they live and their geographic distribution. In the case of RBC and WBC counts, we found higher values than those reported by other authors (Troiano et al., 1998; Montilla Fuenmayor et al., 2006; Cabrera et al., 2011; Table 2). It has to be noted that the conditions and habits of the turtles were different in the mentioned works and this clearly exert a particular influence on certain hematological parameters.

Hematocrit of *Phrynops hilarii* was similar to those found in other turtle species (Troiano et al., 1998; Montilla Fuenmayor et al., 2006; Cabrera et al., 2011), while hemoglobin was lower than the values reported by Troiano et al. (1998) and Cabrera et al. (2011) (Table 2). Leukocytes populations showed different results. In the case of lymphocytes, our values were higher than those reported in similar species (Troiano et al., 1998, Montilla Fuen-



**Fig 2.** (1) Micronucleated erythrocyte, (2) lymphocyte and (3) eosinophil (arrows) of *Phrynops hilarii* stained with May Grunwald - Giemsa (1000x).

**Table 2.** Hematological parameters reported by different authors in turtles (Hb, hemoglobin; Ht, hematocrit; RBC, red blood cells; WBC, white blood cells).

	Hematological Parameters									Reference
	WBC (10 <sup>6</sup> /μl)	RBC (10 <sup>3</sup> / μl)	Ht (%)	Hb (g/dL)	Lymphocytes (%)	Heterophils (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)	
<i>Phrynops hilarii</i>	0.937	27.06	18	4.8	76	20.12	1.5	2.12	0	This study
<i>Chelonoidis chilensis chilensis</i>	0.70	9.2	25.2	11	26	28	5	32	0	Troiano et al. (1998)
<i>Chelonia mydas</i>	0.42	6.16	29.4	—	14.7	82.9	1.97	0.47	—	Montilla Fuenmayor et al., (2006)
<i>Geochelone denticulata</i>	0.44	7.82	20.3	7.0	25.5	55.6	0.4	15.8	1.5	Cabrera et al. (2011)

mayor et al., 2006; Cabrera et al., 2011) but the opposite was detected for heterophils. With respect to monocytes, our values were lower than those found in *Chelonia mydas* (Montilla Fuenmayor et al., 2006) and *Chelonoidis chilensis chilensis* (Troiano et al., 1998), but higher than those of *Geochelone denticulata* (Cabrera et al., 2011).

Unlike, eosinophils found in *Phrynops hilarii* were lower than those reported by Troiano et al. (1998) and Cabrera et al. (2011) in *Chelonoidis chilensis chilensis* and *Geochelone denticulata*, respectively, but higher than that of *Chelonia mydas* (Montilla Fuenmayor et al., 2006) (Table 2). Basophils were not observed in any of the ani-

mals studied; this could be due to the small number of this cell type present in the circulation of healthy animals (Work et al., 1998).

As we mentioned, the influence of factors such as age, sex, reproductive status, stress, temperature, time of year, capture techniques and methods of analysis could modify reference values substantially. Because of this, in order to be compared with those of other species, it is extremely important to describe in details most of the factors and conditions involved.

Similar to our study, some authors found no significant differences in hematological values between females

and males in *Caiman crocodilus* (Rossini, 2004), while other authors reported such differences in other reptiles species (Frair, 1977; Wood and Ebanks, 1984; Hart et al., 1991). We only found a moderate positive correlation between heterophils and weight.

Several studies in recent years have used the MN test as a biomarker of genotoxicity in various species of reptiles exposed to different chemicals (Poletta et al., 2009; 2011; Borratt et al., 2011; Capriglione et al., 2011; López González et al., 2013). Primarily, it is necessary to establish basal values of MN as it is species-specific and may vary with age, sex and health status of individuals. The results of our study have determined that BFMN of *P. hilarii* is  $3.56 \pm 1.39$ . This value is much higher than those found by our group in other endemic reptile species from Argentina. We found a BFMN of  $0.87 \pm 0.74$  in the broad-snouted caiman (*Caiman latirostris*; Poletta et al., 2008) and of  $0.95 \pm 0.27$  in the tegu lizard (*Tupinambis merianae*; Schaumburg et al., 2012). This finding could probably be attributed to factors intrinsic to the species, such as lifespan of circulating erythrocytes and removal time of senescent or damaged erythrocytes, or to phylogenetic distance (Caliani et al., 2014).

Moreover, Zúñiga-González et al. (2000; 2001) reported BFMN in species of lizards, snakes, and crocodiles between 0.10 and 0.30/1000, while in species of turtles (*Macrolemys temminckii*; n = 1 and *Kinosternon subrubrum*; n = 2) the value reported was 0/1000. The low values obtained in the cited study could be due to the small number of individuals used, as no more than one or two animals were used per species. However, similar data were obtained by Strunjak-Perovic et al. (2010) for the snake *Hierophis gemonensis* (BFMN = 0.30/1000; n = 10). Borratt et al. (2011) studied *Chelonia mydas* (marine green turtle) as a biological monitor to evaluate the level of pollution in a contaminated area (n = 60). In this study, animals used as controls were rehabilitated specimens with a FMN of 9/1000 erythrocytes, while animals coming from two contaminated sites showed a FMN of 42 and 627/1000 erythrocytes, respectively. Even when the control FMN was much higher than the one found in our study, it is important to highlight that animals used were rehabilitated, so comparison is not appropriate.

Finally, we found no relationship between BFMN and weight. These results agree with those reported in juvenile *Caiman latirostris* (Poletta et al., 2008) and adults *Tupinambis merianae* (Schaumburg et al., 2012); newborn tegu lizards showed instead a weak negative correlation between the BFMN and weight, possibly indicating a relation between poor nutritional state and deficiencies in the mechanisms of protection and/or repair of genetic damage (Schaumburg et al., 2014).

This study is the first report on the application of the MN test and determination of reference hematological values in *Phrynops hilarii*, considering individual variation due to factors such as sex, age and health conditions of individuals. This represents an important contribution to the knowledge of the biological characteristics of this species and is the initial step to propose it as a sentinel organism for future studies on the biomonitoring of pollutants present in its natural habitat.

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