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RESEARCH ARTICLE

Hematologic and Genotoxicological Research on *Pelophylax ridibundus* and *Bufotes variabilis* Living Around the Çan (Çanakkale, Turkey)

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Abstract

Hematologic parameters play an important role in the determination of the general health status as well as the physiology of amphibian species and the effects of various stress and poor conditions on some species. With this study, changes in the hematological parameters (red blood cell count, white blood cell count, hemoglobin concentration, hematocrit value, mean cell volume, mean cell hemoglobin and mean cell hemoglobin concentration) on an aquatic (*Pelophylax ridibundus*) and a terrestrial (*Bufotes variabilis*) amphibian living around the Çan (Çanakkale, Turkey) were presented. Also, to reveal the DNA damage on these two amphibian species, various nuclear abnormalities like micronucleus, binucleated nucleus, lobbed nucleus, blebbed, and notched nucleus were analyzed.

As a result of this study, the aquatic amphibian species when compared with the terrestrial amphibian species, significant differences in hemoglobin concentration (U= 22.5; W=88.5; Z= -3.446; p= 0.001), and heterophil count (U=34.00; W=205.00; Z= -3.79; p=0.00) were determined. The hemoglobin concentration of the terrestrial species was found higher while the heterophil count of the aquatic species was higher. Total nuclear abnormalities were found higher on the *B. variabilis* species but there was not a significant statistical difference. The micronucleus and other nuclear abnormalities of these two amphibian species when compared, the frequency of the notched nucleus was found statistically different. The frequency of the notched nucleus was higher in the *B. variabilis* species.

Keywords: Amphibia, Hematology, Micronucleus, *Pelophylax ridibundus, Bufotes variabilis*.

Introduction

In recent years, natural resources are in danger of contamination due to the increasing human population and development in technology and industry. Drying of the wetlands and pollution have had a negative effect on species dependent on fresh-water. Understanding poor environmental conditions is important for the sustainability of ecosystems and continuity of species and their quality of life (Çördük *et al.*, 2018). Amphibians and reptiles are adversely affected by pollution and habitat destruction. Amphibians are very sensitive to all types of changes in their aquatic habitats (Cunnigham & Saigo, 1999; Gül *et al.*, 2011).

The number of blood cells in amphibians varies between species (Hutchison & Szarski, 1965; Szarski & Czopek, 1966; Rouf, 1969; Sinha, 1983; Atatür *et al.*, 1999; Cabagna *et al.*, 2005). It has also been reported to be related to body weight, age, sex (Arvy, 1947; Schermer, 1958; Goniakowska, 1973; Sinha, 1983; Banerjee, 1988; Wojtaszek & Adamowicz, 2003), seasons (Zhukova & Kubantsev, 1979; Sinha, 1983; Wojtaszek *et al.*, 1997; Arserim & Mermer, 2008), and the distribution of species by altitude (Ruiz *et al.*, 1989; Gül *et al.*, 2011).

Pollution can also damage the genetic material of organisms. If the damage rate is high, the repair mechanism remains incapable and several mutations can occur in the cells (Dar *et al.*, 2016; Çördük *et al.*, 2018).

Assessment, monitoring, and investigation of the effects of pollutants on the genetic material of organisms has great importance. Genotoxicity can be defined as the ability of chemical, physical or biological agents to cause damage to genetic material. In vitro genotoxicity tests for various organisms have been developed, for monitoring contaminants that may cause mutation on genetic material. With the micronucleus (MN) test, the number of MN formed in cells due to the effects of environmental pollutants can be specified and in this way the genotoxical effects of pollutants can be determined and monitored. The MN test is one of the most widely used biological markers for on-site monitoring of genotoxic pollution (Al-Sabti & Metcalfe, 1995; Bolognesi *et al.*, 2006; Schaumburg *et al.*, 2012; Strunjak-Perovic *et al.*, 2010; Udroiu, 2006). In recent years, nuclear abnormalities (such as the kidney-shaped nucleus, lobbed nucleus, notched nucleus, and blebbed nucleus) and MN test was used for the determination of the effects of pollutants on some species (Ergene *et al.*, 2007; Guilherme *et al.*, 2008; Napierska, 2009; Strunjak-Perovic *et al.*, 2010; Çördük *et al.*, 2018). Also, various studies have identified the presence of MN and nuclear abnormalities in some amphibian species that were exposed to genotoxic pollutants (Marques *et al.*, 2009; Lajmanovich *et al.*, 2014; Josende *et al.*, 2015).

In this study, the red blood cell count (RBC), white blood cell count (WBC), hematocrit value (PCV), mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC), of the aquatic (*P. ridibundus*) and terrestrial (*B. variabilis*) amphibian that live around the Çan, were determined. These parameters were selected to assess the general health status of the two amphibia species and to determine whether or not they carry any infections.

Material and Methods

Study Site and on Site Sampling

Field studies were carried out in 2019 October and 2020 March and May; 16 *P. ridibundus* specimens and 18 *B. variabilis* specimens were collected from around the thermal power plant near Çan. All specimens were captured by hand and scoops. The localities where the specimens were caught is given in Fig. 1.

Morphological Analysis

Within the morphological parameters of collected specimens sex, head+body length (HBL) (with 0.01 mm precision Mitutoyo digital caliper) and body weight (with Sinbo digital scales, max. 3 kg 4570 mg) were measured. For the standardization of the hematological and genotoxicological analyses, similar sized specimens were used. For the hematologic and genotoxicological analysis,

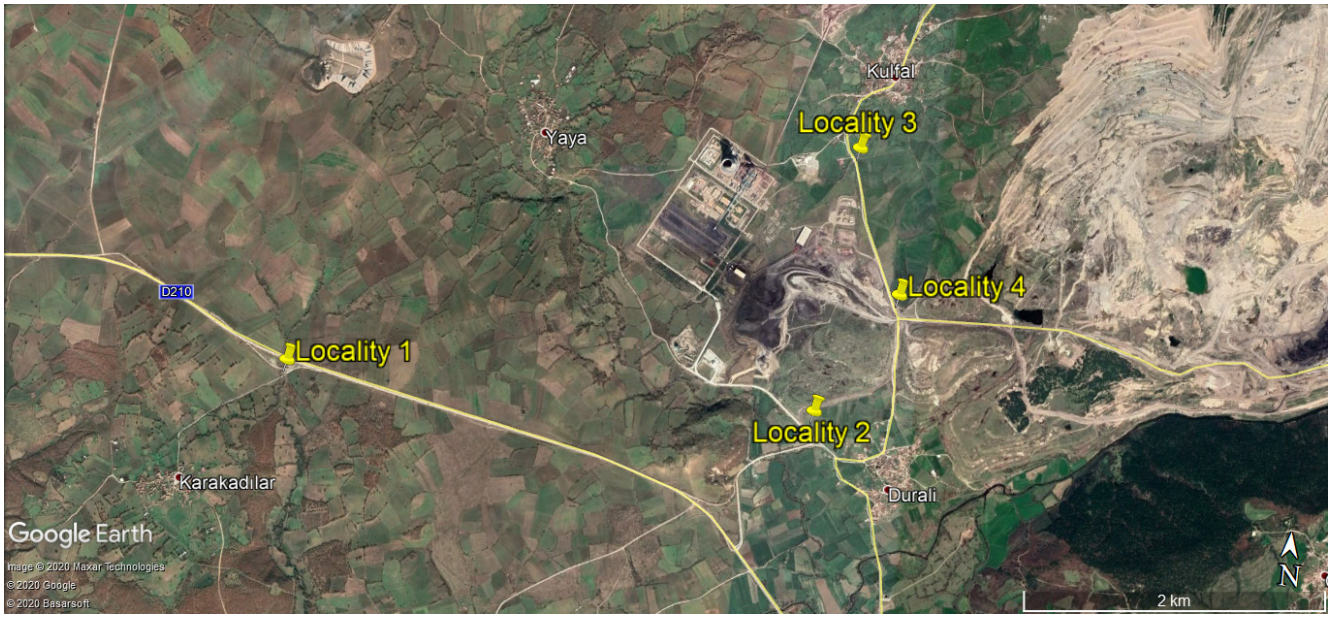

Figure 1. Localities for the specimens (Çan-Çanakkale).
Locality 1: 40° 00' 30.21" N, 26° 56' 43.34" E, 130 m above sea level (a.s.l.); Locality 2: 40° 00' 28.24" N, 26° 59' 04.16" E, 103 m a.s.l.; Locality 3: 40° 01' 28.89" N, 26° 59' 09.93" E, 175 m a.s.l.; Locality 4: 40° 00' 55.63" N, 26° 59' 27.39" E, 166 m a.s.l.

animals were dosed with ether, and blood samples were taken from the middle abdominal vein by heparinized microhematocrit tubes. For these procedures, the necessary permissions were obtained by the 2019/02-09 numbered decision of the local Ethics Committee of Animal Experiments of Çanakkale Onsekiz Mart University.

Hematological Analysis

As part of the hematologic studies, blood smears of these specimens were examined and the percentage of leukocytes in the blood was calculated. The clinical hematology, RBC count, WBC, PVC, MCV and MCHC were examined. In the erythrocyte and leukocyte count, Hayem's solution for erythrocytes and Turk's solution for leukocytes was used as a dilution solution. Blood cell count was measured by the Neubauer hemocytometer under the Olympus 1-15x micrometric oculars. Sahli method was used for determination of hemoglobin concentration (Tanyer, 1985). For the determination of hematocrit value, the blood sample was put into a capillary tube and centrifuged at 2000 rpm for 5 minutes in the microhematocrit centrifuge (Elektro-Mag). The MCV, MCH, MCHC were calculated mathematically by using the obtained results (Tanyer, 1985). Blood smears were prepared for the leukocyte types and these smears were stained with the Wright's stain (Başoğlu & Öktem 1984).

Genotoxicological Analysis and MN Test

For genotoxicological analysis, blood smears were prepared with peripheral blood from each species onto a coverslip cleaned with alcohol. These blood smears were dried at room temperature after fixated with ethanol for 20 minutes. After this procedure, smears were fixed with methanol for 15 minutes and stained with Giemsa stain (10% v/v) (Josende *et al.*, 2015; Çördük *et al.*, 2018). Each preparation of blood smear was examined by microscope (Olympus Cx21 Zeiss Primoster). The MN test was used to determine the number of MN and other nuclear abnormalities in the erythrocytes as a result of environmental pollutants and to monitor the genotoxic effects. The micronucleus detection was done by considering criteria such as; MN must be smaller than one-third of the main nucleus, MN must not be in contact with the main nucleus, MN must be the same color and density as the main nucleus and not be refractive (Heddle & Countryman, 1976; Fenech, 2000; Çördük *et al.*, 2018). Other nuclear abnormalities such as a kidney-shaped nucleus, lobbed nucleus, notched nucleus, blebbed nucleus, and binucleated nucleus were also identified and counted on the blood smears.

Statistical Analysis

In order to perform parametric tests, the data must show normal distribution and be homogeneous (Özdamar, 2004). The Mann-Whitney U test, which is one of the nonparametric tests, was used for data that did not show normal distribution in hematological analyzes. SPSS 20.0 program (IBM) was used for descriptive statistics of genotoxicological and hematological data. In all cases, $p \leq 0.05$ value was considered statistically significant.

Results

When the *P. ridibundus* and *B. variabilis* were measured, the average body weight was found 108 g in *P. ridibundus* and 72 g in the *B. variabilis*. The HBL was found 66.57 mm in *P. ridibundus* and 65.44 mm in *B. variabilis*. (Fig. 2). All data was evaluated together since there were no significant differences between males and females.

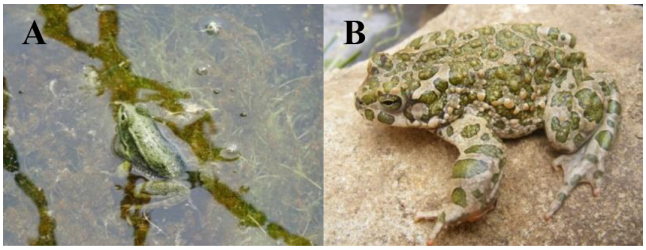

Figure 2. *Pelophylax ridibundus* (A) and *Bufotes variabilis* (B).

Hematological parameters of *P. ridibundus* and *B. variabilis* were given in Table 1. When the hematological parameters between these two amphibian species were compared, the only statistical difference was significant in hemoglobin value (U= 22.5; W=88.5; Z= -3.446; p= 0.001). It was determined that the hemoglobin value was lower in the *P. ridibundus*. When leukocyte percentages of these two specimens were compared, statistical differences were found in the monocyte (U=71.00; W=207.00; Z= -2.51; p=0.01) and heterophil (U=34.00; W=205.00; Z= -3.79; p=0.00) values. It was determined that the number of heterophil was higher in the *P. ridibundus* than the other species, while the number of monocyte and eosinophil was higher in the *B. variabilis*. Leukocyte types in the *P. ridibundus* and *B. variabilis* are given in Fig. 3.

Table 1. Statistics of some hematological parameters and percentage of leukocyte values of *Pelophylax ridibundus* and *Bufotes variabilis* (RBC: the red blood cell count, WBC: white blood cell count, PCV: hematocrit value, MCV: mean cell volume, MCH: mean cell hemoglobin, MCHC: mean cell hemoglobin concentration, Lymph: Lymphocyte, Mon: Monocyte, Het: Heterophil, Bas: Basophil, Eos: Eosinophil, N: number of specimens, SD: Standard Deviation).

	Bufotes variabilis					*Pelophylax ridibundus*				
	N	**Minimum**	**Maximum**	**Mean**	**SD**	**N**	**Minimum**	**Maximum**	**Mean**	**SD**
RBC (mm^3)	15	100000.00	920000.00	186133.33	204569.88	9	104000.00	140000.00	119555.55	11737.87
WBC (mm^3)	16	2600.00	4300.00	3209.37	534.85	8	2200.00	4200.00	3250.00	772.75
Hb (g/dL)	18	7.80	12.60	9.65	1.26	11	3.00	10.20	7.25	1.88
PCV (%)	17	16.00	56.00	38.47	11,05	13	11.00	59.00	36.07	13.08
MCV (μ^3)	14	456.52	4250.00	2609.74	986.34	8	2578.13	4214.29	3565.66	524.56
MCH (μμg)	15	91.30	4887.50	1008.44	1097.12	7	528.57	788.46	663.49	116.53
MCHC (%)	17	19.58	60.00	27.08	10.61	10	12.54	27.27	20.36	5.21
Lymph (%)	18	31.95	78.57	49.70	12.83	16	39.71	67.96	49.88	7.48
Mon (%)	18	3.19	18.12	11.03	4.20	16	2.03	17.09	7.18	4.57
Het (%)	18	1.12	12.28	5.94	4.25	16	6.41	25.82	14.02	5.26
Bas (%)	18	4.59	43.08	23.79	10.51	16	12.28	78.91	24.98	15.87
Eos (%)	18	2.44	25.56	9.44	5.77	16	1.56	9.82	5.56	2.54

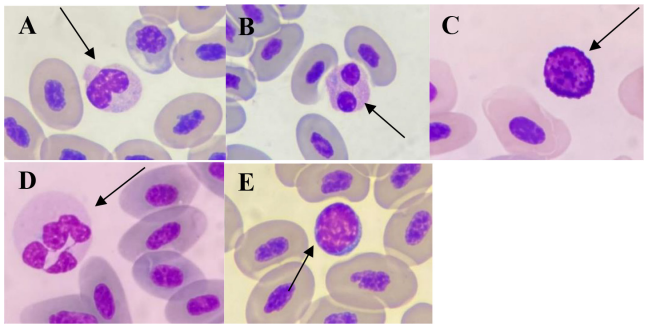

Figure 3. Leukocyte types in the *Pelophylax ridibundus* and *Bufotes variabilis*; Monocyte (A), Eosinophil (B), Basophil (C), Heterophil (D), Lymphocyte (E) (Leukocytes were examined at 1000X magnification).

Table 2. Percentages of the micronucleus and nuclear abnormalities in the *Pelophylax ridibundus* and *Bufotes variabilis* (Mean ± Standard Deviation).

	Bufotes variabilis	*Pelophylax ridibundus*
Micronucleus	0.21±0.16	0.27±0.21
Lobed Nucleus	0.35±0.24	0.32±0.27
Notched Nucleus	2.45±0.70	1.49±0.76
Kidney Shaped Nucleus	1.12±0.71	0.76±0.41
Blebbed Nucleus	1.94±1.20	2.03±0.86
Binucleate	0.05±0.07	0.21±0.60
Total nuclear abnormalities (%)	**6.12±0.99**	**5.08±0.75**

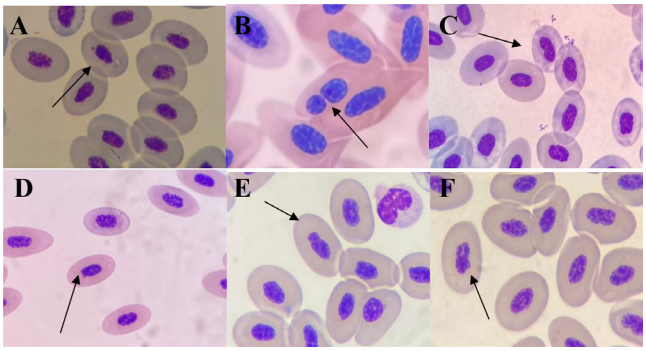

Figure 4. Micronucleus and nuclear abnormalities in *Pelophylax ridibundus* and *Bufotes variabilis*. Micronucleus (A), Binucleate (B), Kidney Shaped Nucleus (C), Blebbed Nucleus (D), Lobed Nucleus (E), Notched Nucleus (F) (Total of 1000 erythrocytes were counted at 1000X magnification.).

The frequency of the MN and nuclear abnormalities in the erythrocytes of *P. ridibundus* and *B. variabilis* were given in Table 2 and Fig. 4. When the MN and nuclear abnormalities of these two species were compared, it was determined that the notched nucleus (U=50.00; W=170.00; Z= -3.07; p=0.002) differ statistically. It was determined that the frequency of notched nucleus in the *B. variabilis* was higher than *P. ridibundus*.

Discussion

The data of the hematological parameters we obtained are following other studies with similar species. In previous

studies (Gül *et al.*, 2011), it has been reported that, among the five anuran species with different habitat choice, the number of erythrocytes is higher in terrestrial (*Bufotes variabilis*) and aquatic (*Pelophylax ridibundus*) species when compared to the semi-aquatic (*Rana dalmatina*) species. Also, in the previous studies, hemoglobin concentration, hematocrit value and some erythrocyte indices (MCV and MCHC) have found higher on the terrestrial species (*Bufotes variabilis*). Similar results were obtained in our study on the terrestrial specimens (*B. variabilis*).

Sils (2008) stated that in his study, *Pelophylax ridibundus* have high levels of heterophil that live in an urban and anthropogenically contaminated environment, when compared with the other representatives of the genus. He also stated, the increase observed in the heterophil and eosinophil ratios can be considered as activation of the protective functions of the blood. According to these results, the effect of the general heterophil increase on amphibians may be seen as an adaptive mechanism that enhances the defensive function of blood while living in a contaminated environment. Considering this information in the literature, the high number of heterophil in aquatic specimens was similar to the previous studies and it could be explained by the fact that the aquatic specimens were more affected by pollution.

Çördük *et al.* (2018) conducted a study for genotoxicological analyses due to pollution in *Pelophylax ridibundus* at different stations. Çördük *et al.* (2018) reported that the total nuclear abnormalities were high in the highly contaminated areas, where industrial, domestic and animal wastes were present, and pollutants in the water damages the organisms' genetic material. They identified the total nuclear abnormality as 8.64 in the highly contaminated area and 3.16 and 3.41 in the less contaminated areas. In our study, total nuclear abnormality of the same species was found as 5.08 so it is between the high and low contaminated areas.

In previous studies, a higher frequency of micronucleus in contaminated areas in *Pelophylax ridibundus* have been found. Gürkan *et al.* (2012) identified the micronucleus frequency as 0.3 in *P. ridibundus* in the area exposed to the genotoxic pesticides used in intensive agricultural activities. In our study, the percentage of MN value of the species collected from the Çan vicinity was found as 0.27. It has been reported that the micronucleus and other nuclear abnormality frequencies in the blood of *P. ridibundus* in contaminated areas were significantly higher than in the clean zone species, high heavy metal levels can lead to toxicity, and the detected genotoxicity may be related to the industrial, agricultural and domestic activities (Şişman *et al.*, 2015). The micronucleus frequency, which has been reported to increase due to pollution in previous literature (Gürkan *et al.*, 2012; Şisman *et al.*, 2015; Çördük *et al.*, 2018), has also been found high in our study region and is thought to be caused by the thermal power plant. There is no detailed study about the MN and other nuclear abnormalities found in the *B. variabilis*; therefore, no comparison has been made.

The results of this study, hematologic and genotoxicological parameters of the terrestrial and aquatic amphibian species showed changes when compared with the literature. Within the hematological parameters of the *P. ridibundus* and *B. variabilis*, the statistical difference was only in the hemoglobin value. When the percentage of leukocyte values were examined, it was determined that there was a significant increase in the number of heterophil in the aquatic *P. ridibundus* when compared to the terrestrial *B. variabilis*. This situation was determined as a cause of pollution. Total nuclear abnormality was observed more in the *B. variabilis* than in the *P. ridibundus*, but there was no statistically significant difference. When the micronucleus and nuclear abnormalities were examined between these two species, a statistical difference was found in the notched nucleus value. And the notched nucleus frequency was higher in the terrestrial species (*B. variabilis*) than in the aquatic species (*P. ridibundus*).

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