ORIGINAL ARTICLE

IMPACT OF SOME HEMATOLOGICAL AND MICROBIOLOGICAL FACTORS ON TWO *NATRIX* SPECIES IN THE BIGA STREAM (ÇANAKKALE, TURKEY)

| Çiğdem Gül | Nurcihan Hacıoğlu Doğru * | and | Murat Tosunoğlu |

Çanakkale Onsekiz Mart University | Faculty of Arts and Sciences | Department of Biology | Canakkale | Turkey

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ABSTRACT

Background: The fresh water-dependent species like *Natrix* species are negative affected by fresh waters pollution. There are many investigations on microbial flora and hematological parameters of free-living reptiles, separately. But there is no information about the study on relation of hematological and microbiological parameters of snakes in Turkey. **Objectives**: In this study, some hematological and microbiological parameters of *Natrix natrix* and *Natrix tessellata* populations in the Biga stream (Canakkale, Turkey) were investigated and compared between two species. Antibiotic resistance and heavy metal susceptibility tests were also performed for the cloacal and oral samples bacterial flora from two species. **Methods:** Only healthy and mature animals were studied, and they were collected generally around the water resource with the method of hand capture. Their cloaca and oral samples were taken with sterile swabs. The blood samples into heparin containing vacutainer were places into Amies transport medium cooled with frozen gel packs and transported to laboratory for hematological analysis. Hematocrit was determined using the micro-hematocrit method **Results**: Twenty bacterial species representing three bacterial families were obtained from the oral and cloacal samples of *Natrix* sp. In the comparison between species *N. natrix* and *N. tessellata*, significant differences were detected in the WBC, MCV and MCH values. Significant differences were detected in the lymphocyte count and heterophil count. The glucose, cholesterol, uric acid, triglyceride, total protein, phosphorus, magnesium, potassium and chlorine values were higher in *N. tessellata* than in *N. natrix*. The urea, creatinine, albumin, calcium, HDL and sodium values were higher in *N. natrix* than in *N. tessellata*. **Conclusions**: It is thought that the bacteria likely to cause infection such as pathogens *Salmonella* sp. and *Aeromonas hydrophila*, which are detected more considerably in species *N. natrix*, might lead to an increase in the numbers of leukocytes and heterophils and a decrease in the number of lymphocytes. To the author's, this study is the first survey of relation between hematological and microbiological parameters of two *Natrix* species in Turkey. These findings are the basis for the knowledge of the biology of wildlife snakes and important future conservation efforts.

Keywords: *Biga stream, Hematology, Microbiology, Natrix natrix, Natrix tessellata, Turkey*

1. INTRODUCTION

Reptiles are often subjects of health assessment studies and investigations into naturally occurring diseases [30]. Checking blood parameters in reptiles may guide the evaluation of physiological and health conditions of populations and it may be used as an indicator in determining the environmental condition since these species are very sensitive to changes in habitat [11-19-32, 33-36]. Thus, it is important to examine blood parameters in reptiles and determine the changes in these species beforehand. Reptiles can harbor pathogenic microorganisms asymptomatically and serve as potential reservoirs of infection for humans, domestic animals, and other reptiles. Baseline information on the composition of microbial flora and the antimicrobial-heavy metal tolerance of these bacteria, which were isolated from the reptiles, is an extremely useful tool for the correct interpretation of bacteriological culture results, in order to better understand the role of bacteria as pathogenic agents in disease and the anthropogenic effects of domestic and industrial wastes on events among these animals [6-17,18-28]. Most studies have concentrated on a small subgroup of bacteria that are known to be zoonotic or on reptile species with commercial interest as pets [29]. Furthermore, it has been well established that many reptiles harbour Gram-negative bacteria as part of their normal flora and that these microbes are either commensal or opportunistic [28].

Hematological studies on different snake species are quite high in number [10-33,34,35-37,38]. Studies on plasma biochemistry in snake species have increased in the recent years [8-12-25-27]. The clinical hematology studies on species *N. natrix* and *N. tessellata* are rather few [10-32-37] and few studies on plasma biochemistry in these species has been encountered [13]. Reports on the occurrence of bacterial pathogens of snakes are quite few [6-16-14-28]. However, there is no data in which both the hematological and the microbiological data of snake species, particularly the *Natrix* species.

This study aims (1) to identify the Gram-negative bacterial flora of, and whether there is a difference in microbial flora, in two *Natrix* species, (2) to determine the antibiotic and heavy metal resistance of the isolated bacteria, (3) to give the hematological reference intervals in *N. natrix* and *N. tessellata* specimens, (4) to investigate whether there is a relationship between hematological and microbiological parameters of two *Natrix* species, and (5) to determine the baseline health data of free-ranging *N. natrix* and *N. tessellata* species to serve as a reference for conservation programs. To our knowledge, this is the first comprehensive and comparative report on the hematological values and baseline microbial flora of two *Natrix* species in the Biga Stream.

2. MATERIALS AND METHODS

2.1 Collecting Animal Samples:

The snake samples studied (eight *N. natrix* and eight *N. tessellata*) were captured around the Biga Stream (35T0520451; UTM4454127; sea level) at midday between April and June 2014. Only healthy and mature animals were studied, and they were collected generally around the water resource with the method of hand capture. All samples collected as a part of a Project (The Scientific and Technological Research Council of Turkey, with the guidelines of the local ethics committee; Canakkale Onsekiz Mart University, 2012/05–01) in Turkey.

Total body length (TBL) measurements of specimens were taken with a Mitutoyo digital caliper with 0.01 mm precision. Specimen's weights are determined by scale. And then, their cloacal and oral samples for microbiologic analyses were taken by means of sterile swabs. The blood required (1 mL) for hematological analyses were collected by means of a 5-mL syringe from the caudal vein. After all the processes, the samples were released back into their biotopes.

2.2 Microbiological Analyses:

Their cloacal and oral samples were taken with individually packed microbiological sterile swabs (Fıratmed BS13-0034) pharynx and from the cloaca by rotating the swab in the mouth or cloaca in order to obtain mucosal cells. The swab from the pharynx and one swab from the cloaca were places into Amies transport medium cooled with frozen gel packs and transported to laboratory for bacteriological analysis.

The cloacal and oral samples were placed into the buffered peptone water for enrichment at 35-37^{o}C for 24 hr. and then plated on MacConkey agar (MAC), Thiosulfate citrate bile salts sucrose agar (TCBS), Glutamate starch phenol red agar (GSP), Inositol brilliant green bile agar (IBG), and Chromogenic *E.coli* agar (CE) for the isolation of different Gram-negative bacterium species. The plates were incubated at 25-30°C for 24 to 48 hr. The isolated colonies were identified by Murray et al. (2011) [26]. For the isolates obtained, verification tests were performed according to Microgen ID-A panel-Gram negative (MID-64).

The antibiotic susceptibility test was performed using the method of standard disk diffusion [3]. The following antibiotics were used: tobramycin (TB10 µg/mL), kanamycin (K30 µg/mL), amoxicillin (AM10 µg/mL), oxytetracycline (O30 µg/mL), cefmetazole (CMZ30 µg/mL), gentamicin (G10 µg/mL), furazolidone (FR50 µg/mL), erythromycin (E15 µg/mL), cefoxitin (CN30 µg/mL), ampicillin (A10 µg/mL), cefotaxime (CE30 µg/mL), chloramphenicol (C30 µg/mL), trimethoprim (TR10 µg/mL), and cephalothin (CH µg/mL). The organisms were reported as resistant, intermediate or sensitive to each antimicrobial tested according to the Clinical and Laboratory Standard Institute [5].

The minimal inhibitory concentration (MIC) for each bacterial isolate for four heavy metals was determined using Mueller-Hinton agar (Merck) containing Pb^{+2}, Cu^{+2}, Cr^{+3}, and Mn^{+2} at concentrations ranging from 12.5 to 3.200 µg/mL. The metals added were $PbSO_4$, $CuSO_4.5H_2O$, $K_2Cr_2O_7$, and $MnCl_2.2H_2O$. The isolates were considered resistant if the MIC values exceeded that of the *Escherichia coli* K-12 strain, which was used as the control [23].

2.3. Hematological Analyses:

The blood samples into heparin containing vacutainer were places into Amies transport medium cooled with frozen gel packs and transported to laboratory for hematological analysis. The red blood cell counts (RBC) and white blood cell counts (WBC) were carried out using a Neubauer hemocytometer, where standard Hayem's solution for red blood cells and Turk's solution for white blood cells were used as a diluting solution. Hematocrit (HCT) was determined using the micro-hematocrit method [31]. The tubes were then spun in a micro-hematocrit centrifuge at 12.000 rpm for 5 min and the hematocrit (HCT) was calculated with a total blood level divided by the blood cell level. Hemoglobin concentration (Hb) was measured by the Sahli method with a Sahli's Hemoglobinometer [31]. The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated mathematically by taking the above-mentioned results into consideration [31]. Blood smears preparations stained with Wright's stain were used in determining the frequency and types of leucocytes.

For the analyses concerning plasma biochemistry, 0.3 mL of blood was centrifuged in a Cence L500 centrifuge at 4.000 rpm for 10 min and the plasma part was separated from the blood cells. From the plasma obtained, glucose, urea, cholesterol, creatinine, uric acid, albumin, calcium, triglyceride, total protein, phosphorus, magnesium and high-density lipoprotein (HDL) values were measured by means of an Elimat autoanalyzer, while potassium (K), sodium (Na) and chlorine (Cl) values were measured by means of a Cornley AFT-500 electrolyte apparatus.

The descriptive statistics were conducted using SPSS (v10.0). The Mann-Whitney U test is used to compare differences between hematological parameters of two different species.

3. RESULTS

Snake specimens that total body length from 75 to 85 cm and weighing 95-110 g were taken assessment for microbiological and hematological analyses.

Twenty bacterial species representing three bacterial families were obtained from the oral and cloacal samples of *Natrix* sp. Several members (total=70) of the Enterobacteriaceae, Vibrionaceae, and Pseudomonadaceae were isolated from the 16 animals studied (8 *N. tessellata* and 8 *N. natrix*) (Table 1). 31 bacteria were isolated from *N. natrix* (Oral:21 Cloacal:10) and 39 bacteria were isolated from *N.tesellata* (Oral:16 Cloacal:23). The most frequent isolates were *Aeromonas hydrophila* (20.51%), *Burkholderia pseudomallei* (10.25%), and *Citrobacter freundii* (10.25%) for *N. tessellata* (n=39) and *A. hydrophila* (25.80%), *Salmonella enterica* subsp. *arizonae* (IIIa) (22.58%), and *Enterobacter gergoviae* (12.90%) for *N. natrix* (n=31) (Table 1).

Table 1: Bacterial isolates of *N. tessellata* and *N. natrix.*

Bacteria		***N. tessellata* (n=39)**	***N. natrix* (n=31)**
Genus	**Species**	**Oral/Cloaca**	**Oral/Cloaca**
Burkholderia	*B. cepacia*	-	0/2(2)
	B. pseudomallei	1/3(4)	2/0(2)
Aeromonas	*A. hydrophila*	1/7(8)	5/3(8)
	A. caviae	1/2(3)	-
Plesiomonas	*P. shigelloides*	0/1(1)	-
Vibrio	*V. alginolyticus*	-	1/0(1)
	V. furnissi	0/1(1)	-
Pseudomonas	*Ps. flueroscens*	1/0(1)	-
	Ps. aeruginosa	1/1/(2)	-
Escherichia	*E. coli-inactive*	1/0(1)	-
Citrobacter	*C. diversus*	1/0(1)	0/1(1)
	C. freundii	2/2(4)	1/0(1)
Enterobacter	*E. gergoviae*	1/1(2)	3/1(4)
Cronobacter	*C. sakazaki*	0/1(1)	-
Klebsiella	*K. oxytoca*	1/2(3)	2/0(2)
	K. ozonae	1/0(1)	-
Proteus	*P. mirabilis*	1/0(1)	1/0(1)
Serratia	*S. marcescens*	0/1(1)	1/0(1)
Salmonella	*S.* subsp. *arizonae* (IIIa)	2/1(3)	5/2(7)
Yersinia	*Y. enterocolitica*	1/0(1)	0/1(1)

The results of the antibiotic susceptibility test revealed 10.52% (G120) - 92.10% (E15) and 19.35% (G120) - 100.00% (CN30) for the bacterial isolates of *N. tessellata* and *N. natrix*, respectively. Furthermore, it can be seen that the bacteria isolated from species *N. natrix* against all antibiotics had higher antibiotic resistance (Figure 1).

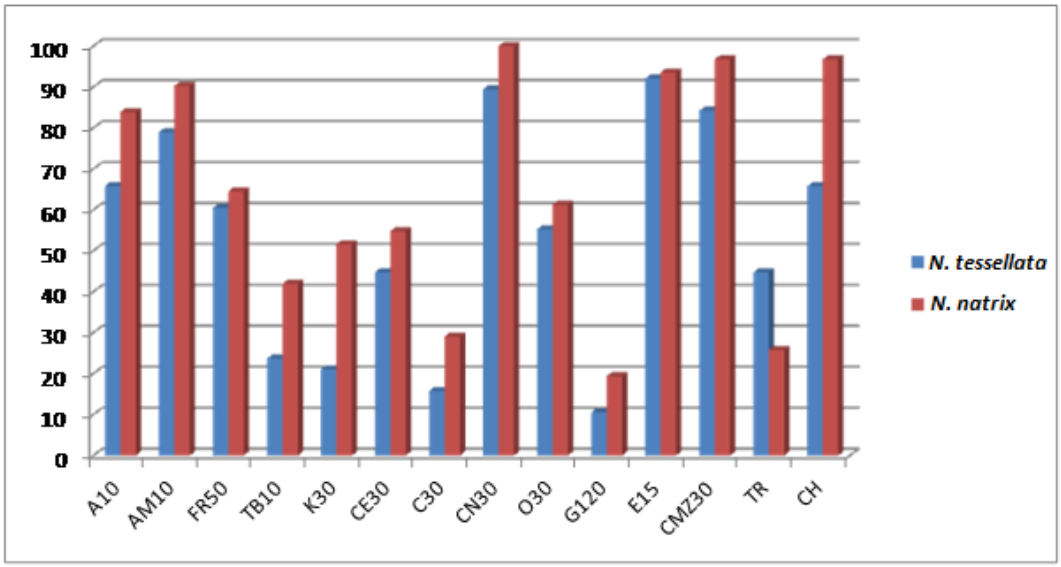

Figure 1: Antimicrobial resistant profile of isolated bacteria.

The trends in heavy metal resistance are shown in Table 2 from *N. tessellata* and *N. natrix*, respectively; Mn (48.71%) > Pb (43.58%) > Cu (35.89%) > Cr (17.94%); Mn (70.96%) > Pb (48.37%) > Cu (38.70%) > Cr (19.35%). Regarding heavy metal resistance profiles, it was determined that the bacteria isolated from species *N. natrix* also had higher heavy metal resistance in terms of the four metals (Table 2).

Table 2: Heavy metal tolerance in bacteria from cloacal and oral samples of *N. tessellata* and *N. natrix.*

Heavy metal	Total bacteria *N. tessellata/N. natrix*	Metal concentrations (µg/mL) with number of tolerant isolates							Resistant isolates	
	N= 39/31	100	200	400	800	1600	3200	>3200	n	%
Chromium		32/28*	4/2	3/1					7/3	17.94/19.35
Manganese						20/20*	15/9	4/2	19/11	48.71/70.96
Copper			25/19*	13/10	1/2				14/12	35.89/38.70
Lead		22/16*	15/11	1/2	1/2				17/15	43.58/48.37

The clinical and biochemical hematological values were given to Table 3.

Table 3: Clinical and biochemical hematological values in *N. natrix* and *N. tessellata.*

	Natrix natrix						*Natrix tessellata*					
Parameters	**N**	**Min**	**Max**	**Mean**	**SE**	**SD**	**N**	**Min**	**Max**	**Mean**	**SE**	**SD**
RBC (1mm^3)	8	600000	1120000	776625	69634.74	196956.8	8	435000	820000	589625	50610.18	143147.21
WBC (1mm^3)	10	6000	9000	7330	350.88	1109.6	8	4000	6000	4762.5	250.66	708.99
HB (g/dL)	8	4.40	9.80	6.76	0.72	2.05	8	5.80	8.20	6.86	0.27	0.77
HCT (%)	8	13.00	31.00	22.12	2.10	5.96	8	13.00	33.00	22.25	2.30	6.51
MCV (fL)	8	206.35	333.33	286.38	15.86	44.87	8	270.83	453.65	375.44	18.50	52.33
MCH (pg)	8	70.18	113.33	87.08	5.55	15.71	8	97.18	159.09	120.25	7.41	20.98
MCHC (%)	8	23.16	42.31	30.86	2.14	6.05	8	24.85	44.62	32.66	2.74	7.77
Lymphocytes (%)	8	33.00	43.00	38.25	1.19	3.37	8	38.00	51.00	44.75	1.76	4.97
Monocytes (%)	8	11.00	22.00	17.62	1.38	3.92	8	13.00	20.00	16.50	0.86	2.44
Heterophils (%)	8	19.00	26.00	22.50	0.86	2.44	8	16.00	22.00	19.37	0.65	1.84
Eosinophils (%)	8	6.00	10.00	8.12	0.44	1.24	8	4.00	11.00	8.50	0.77	2.20
Basophils (%)	8	11.00	17.00	13.50	0.80	2.26	8	6.00	15.00	10.87	1.00	2.85
Glucose (mg/dL)	10	23.10	200.60	70.59	18.27	57.78	15	32.88	265.80	81.34	16.34	63.31
Urea (mg/L)	11	12.60	63.70	38.96	4.47	14.84	9	4.70	23.70	14.26	2.26	6.78
Cholesterol (mg/dL)	11	141.00	639.20	341.14	42.38	140.58	13	150.50	982.20	438.20	68.07	245.43
Creatinine (mg/dL)	12	0.03	1.30	0.50	0.11	0.40	15	0.02	1.79	0.42	0.12	0.47
Uric acid (mg/L)	5	1.18	37.21	13.51	6.22	13.92	12	2.64	34.17	17.48	3.70	12.81
Albumin (g/dL)	10	0.17	3.32	1.84	0.28	0.90	14	0.16	3.06	1.73	0.22	0.84
Calcium (mg/dL)	10	5.15	13.95	11.73	1.10	3.51	12	5.15	13.95	7.15	1.05	3.64
Triglyceride (mg/dL)	9	63.62	335.70	205.95	35.14	105.44	12	52.75	896.10	485.86	109.21	378.32

Total protein (g/dL)	10	1.09	7.15	3.72	0.75	2.37	12	2.92	8.36	4.90	0.51	1.80
Phosphorus(mg/dL)	7	3.13	32.09	19.46	5.03	13.31	9	4.58	38.96	20.64	3.63	10.90
Magnesium(mg/dL)	5	0.46	3.35	2.11	0.50	1.13	4	0.51	6.82	2.53	1.44	2.88
HDL (mg/dL)	2	55.62	162.70	109.16	53.54	75.71	1	92.64	92.64	92.64	.	.
Potassium (mmol/L)	6	5.07	7.06	5.94	0.27	0.67	11	5.07	63.88	27.28	6.10	20.24
Sodium (mmol/L)	6	186.11	232.07	209.28	8.06	19.76	12	140.17	222.29	171.92	7.71	26.73
Chlorine (mmol/L)	6	148.02	181.60	161.55	5.36	13.14	12	108.67	251.27	167.03	10.61	36.77

The RBC values were found higher in *N. natrix*, whereas the HB and MCHC values were found higher in *N. tessellata* but these differences are not significant statistically ($p>0.05$). The hematocrit value was almost the same in both species. In the comparison between species *N. natrix* and *N. tessellata*, significant differences were detected in the WBC (z: -3.515, $p<0.05$), MCV (z: -2.731, $p<0.05$) and MCH (z: -2.626, $p<0.05$) values.

In the comparison between species *N. natrix* and *N. tessellata* according to leucocyte types counts, significant differences were detected in the lymphocyte count (z: -2.345; $p<0.05$) and heterophil count (z: -2,328; $p<0.05$).

The glucose, cholesterol, uric acid, triglyceride, total protein, phosphorus, magnesium, potassium and chlorine values were higher in *N. tessellata* than in *N. natrix*. The urea, creatinine, albumin, calcium, HDL and sodium values were higher in *N. natrix* than in *N. tessellata*. But these differences are not significant statistically ($p>0.05$). Significant differences in the urea (z: -3.077, $p<0.05$), calcium (z: -2.511, $p<0.05$), potassium (z: -2.162, $p<0.05$) and sodium (z: -2.435, $p<0.05$) values were detected between species *N. natrix* and *N. tessellata* ($p<0.05$).

4. DISCUSSION

As reptiles can harbour potential zoonotic bacterial pathogens, it was consequent to investigate these free-living reptiles, to get knowledge about the oral and cloacal bacterial flora [28]. Under normal conditions, the animals remain clinically healthy, but when stressed by crowding or unsanitary conditions, bacterial opportunists may overcome weakened immune barriers and cause disease. Infectious agents are a major cause of diseases and death in snakes, and bacterial infections are responsible for the majority of this mortality [14-16]. The most important pathogens involved are Gram-negative bacilli, particularly species of family Enterobacteriaceae and genera *Pseudomonas* and *Aeromonas* [7]. Stress, poor oral hygiene, malnutrition and the presence of oral bacterial flora, especially *A. hydrophila*, are thought to cause infectious stomatitis [16]. The detection of a higher rate of presence of *A. hydrophila* in both *Natrix* species than the other bacterium species indicates that both species may be confronted with infectious stomatitis depending on the change in ambient conditions.

Non-typhoidal *Salmonella* usually causes gastrointestinal disorders, although it may also cause severe clinical manifestations such as meningitis and septicemia, which can sometimes be fatal. They can also be caused by environmental or animal exposure. In the recent years, reports of salmonellosis from reptile reservoirs have gained more attention [4]. *S. enterica* has been previously isolated from free-living snakes [14,15-20-22]. Therefore, it is important that species *Salmonella enterica* subsp. *arizonae* (IIIa) was considerably isolated from species *N. natrix* in particular.

The bacterial flora is comprised of Gram-negative *Salmonella* sp., *Citrobacter* sp., *Escherichia* sp., *Klebsiella* sp., *Pseudomonas* sp., *Serratia* sp., and *Edwardsiella* sp., all of which were isolated in this study and are all considered opportunistic pathogens in reptiles, and their presence in association with overt clinical signs should be considered significant [2-14-21-28]. The isolation of various Gram (-) bacteria – an important cause of infection – from both snake species in our study reveals that they were intensively exposed to these microorganisms in their habitat. The values of bacterial density and diversity we obtained from *N. natrix* in our research were found higher than the values obtained in the previous study by Hacioglu & Tosunoglu (2014)[17]. It is thought that this might be an indication of the fact that the same species was exposed to a more intensive pollution stress in this region.

Bacterial resistance to antibiotics is an emerging public health concern because of the wide availability of antibiotics and their improper usage without proper prescription [9]. The introduction of heavy metals, in various forms in the environment, can cause considerable modifications in the structure and function of microbial communities. In the last decade, a number of studies reported that antibiotic resistant bacteria might arise in the environment through co- or cross-resistance to metals or co-regulation of resistance pathways [1-24]. The increase in heavy metal and antibiotic resistance for some pathogens is a significant global problem, complicating the battle against infectious diseases. Studies in which the antibiotic and heavy metal resistance of the bacterial species isolated from the *Natrix* species was determined could not be encountered. Nevertheless, Hacioglu and Tosunoglu (2014) obtained the highest antibacterial activity against antibiotics CN30 and E15 but the lowest antibacterial activity against antibiotic G120 in 11 different bacterial groups they had isolated from the *N. natrix* species in the Kavak Delta [17]. Regarding heavy metal resistance, Hacioglu and Tosunoglu (2014) obtained the rates of Cu > Cr > Mn, while the rates of Mn > Pb > Cu > Cr occurred in

our study. This might indicate that both regions were exposed to the intensive and wrong use of antibiotics CN30 and E15 [17].

According to these results, the significant occurrence of bacteria in the internal organs of snakes, with a high incidence of resistance against antibiotics and heavy metals, may risk aquatic animals and the public health. These data appoint the importance of epidemiological surveillance and microbiological monitoring.

Hematological results of *N. natrix* and *N. tessellata* specimens were compared with previously studies [13- 32-37] and it was determined that most of the parameters are different. In previous studies were examined effects on hematological parameters of factors such as seasons, hibernation, gender, age and captivity of factors [8-33-38]. Based on this information, variations in hematological parameters of the same species are normal and it is important to determine the specific reference intervals in this species.

Some hematological parameters of giant garter snakes (*Thamnophis gigas*) and valley garter snakes (*Thamnophis sirtalis fitchi*) specimens were investigated by Wack et al. (2012) [36]. Heterophil, lymphocyte and azurophil counts were found higher in the *T. gigas* and may be indication of chronic inflammation [36]. According to statistically results, leucocyte number and heterophil count of *N. natrix* were found higher than *N. tessellata*. These results may be show that there are an enfection in the *N. natrix* specimens.

5. CONCLUSION

It is thought that the bacteria likely to cause infection such as pathogens *Salmonella* sp. and *A. hydrophila*, which are detected more considerably in species *N. natrix*, might lead to an increase in the numbers of leukocytes and heterophils. The data obtained in this study appoint the importance of hematological and microbiological monitoring to freshwater snakes in the wildlife. These findings are the basis for the knowledge of the biology of wildlife animals and important future conservation efforts.

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