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Analysis of peripheral blood white blood cell parameters in European White Storks (*Ciconia ciconia* L.) chicks that varies by sex

Introduction

Biology and veterinary sciences have established health indicators for haematological parameters. The analysis of white blood cells provides some insight into the health status of the animal at the moment when it was sampled, and it can reflect habitat quality, nutrition, and other environmental stressors. Because of the ease in obtaining them, they are generally some of the most important data available for wildlife health monitoring (Bounous et al., 2000; Campbell, 2015). Avian leukocytes include granulocytes (heterophils, eosinophils and basophils) and agranulocyte cells (lymphocytes and monocytes) (Lashev et al., 2005; Szabó et al., 2010). The quantity and quality of leukocyte cells are generally used to determine immune reactions and diseases (Mitchell, Johns, 2008; Shen et al., 2009). Furthermore, changes in leucocytes also occur when birds are subjected to stress and when environmental quality is degraded (Fiorello et al., 2009; Kamiński et al., 2014). Some authors used varied haematological values as parameters to determine the effect of stress caused by soil pollution in white storks (Kamiński et al., 2015).

The relation between heterophils and lymphocytes (H/L ratio) has been used as a reliable stress indicator in birds. When a bird is stressed, an increase in the number of heterophils and a decrease in the number of lymphocytes is usually observed (Krams et al., 2012). Because of the increasing interest of zoos and public parks in wild species, some studies about wild and captive animals can be found in the literature (Aengwanich et al., 2002; Narkkong et al., 2011).

The white stork (*Ciconia ciconia* L.) is classified in the genus *Ciconia*, family *Ciconiidae,* which includes 19 species around the world (Norton, Whiteside, 2015). In

recent years, a noticeable change in the white stork population has been observed in Europe, together with a rapid decrease in reproductive success and an increase in mortality rates (Bocheński, Jerzak, 2006; Gilbert et al., 2016). Limited data exists regarding blood cells characteristics, blood cells size, and the haematological value of white storks (Jerzak et al., 2010; Kamiński et al., 2014).

The aim our study was the qualitative and quantitative analysis of white blood morphometric elements of peripheral blood (determining the quantity, blood cells dimension and several haematological values) in white stork chicks. One of the aims was to indicate whether the sex relevantly influences the variety of the examined white blood indicators.

Material and methods

Object

Samples were collected during the 2016 breeding season (June/July) from 53 juvenile white storks (25 males and 28 females) in Krapkowice (50°28′30″N, 17°57′55″E). The age of the nestlings varied from 18 to 53 days. Age (with accuracy of 1–2 days) was determined by measurement of the bill length following the Kania method (1988); therefore, the hatch date was found by back calculation.

Blood collection

Blood was collected from the *cutaneous ulnar* vein by 20G peripheral venous catheter and 10 ml syringe (Campbell, 2015). Material from each specimen was transferred to tubes with anticoagulant immediately after collection. Standard tubes containing K_2EDTA for 2 ml of blood (2 mg of anhydrous K_2EDTA per 1 ml of blood) (Medlab-Products) have been used.

Laboratory analysis

Haematological parameters were assessed using routine manual methods. Total WBC were counted using the Natt and Herrick method (Campbell, 2015). Blood smears were prepared immediately after collection, using the push slide technique. Smears of blood were air-dried, fixed in methanol and stained with the May–Grünwald stain method (Robertson, Maxwell, 1990). Slides were observed under a Nikon Eclipse Ni microscope with a Digital Camera (Nikon DS-Fi2). Cells were observed under a 1000× magnification and classified as heterophils, eosinophils, basophils, lymphocytes, and monocytes, according to criteria specified by others authors (Clark et al., 2009; Jamroz, Lucas, 1961).

The H/L ratio was then calculated by dividing the number of heterophils by the number of lymphocytes (Lentfer et al., 2015). The stress factor was eliminated during

the process of obtaining blood samples from the birds by covering the head of the chicks. Photos of the blood cells were taken using a Nikon Eclipse Ni microscope with Digital Camera (Nikon DS-Fi2) and processed using the image software NIS-Elements Basic Research.

To determine the genetic sex of young white storks, the multiplex PCR method was applied. Two sets of primers were used. First were the primers specific for W chromosome for amplification of the female-specific sequence on W chromosome. The second set of primers was used to replicate the 18S ribosome gene, which serves as a positive control of the PCR reaction (Kamiński et al., 2014; 2015).

Statistics analysis

Statistical analyses were performed using Statistica 13.1 software (*Dell Inc.*, 2016). The length and area of each type of leukocytes were measured on the blood smear. The mean, standard deviation, and maximum and minimum values for each variable were calculated. The results were given as mean ± SD. The shape ratio was marked, where 1/1 means round. Haematological values between males and females were compared by Kruskal-Wallis tests, and the level of significance set at $p < 0.05$.

Results

Heterophils (Fig. 1A) were single, round, or irregular cells (shape ratio 0.95/1 – Tab. 1) with spindle or oval shaped cytoplasmic granules, representing 41.2 ± 7.36% of leukocytes for males and 44.4 ± 7.16% for females. The nucleus had 2–3 lobules with contained dense and dark-staining chromatin for both sexes. They were round and measured 11.14 ± 0.65 μm in diameter for males and 11.01 ± 0.48 μm for females (Tab. 1).

Basophils were the smallest granulocytes, averaging 9.56 ± 0.78 μm in diameter for males and 9.13 ± 0.84 μm for females (Tab. 1). With May–Grünwald stain, their granules were vacuolated or clear, due to the bleaching effect of methanol fixation (Fig. 1B).

Eosinophils (Fig. 1C) had the diameter of 10.72 ± 0.49 μm for males and 10.97 ± 0.53 μm for females (Tab. 1). The granules were brighter in colour when compared to the heterophil granules. The nucleus was lobed and mostly stained clear blue and contained red-orange, round or rod shape granules. The shape ratio was 0.90/1, which indicates that the cells varied in shape.

Monocytes were not frequently observed, and their diameter was 8.8 ± 4.87% and 7.6 ± 3.93%, respectively, for males and females (Tab. 2). Monocytes were the largest type of leukocytes found in the blood film. They were round or irregular in shape with a violet, kidney shaped nucleus and a light, pale blue cytoplasm (Fig. 1D), measuring 13.40 ± 0.97 μm in diameter for males and 13.09 ± 1.05 μm for females (Tab. 1).

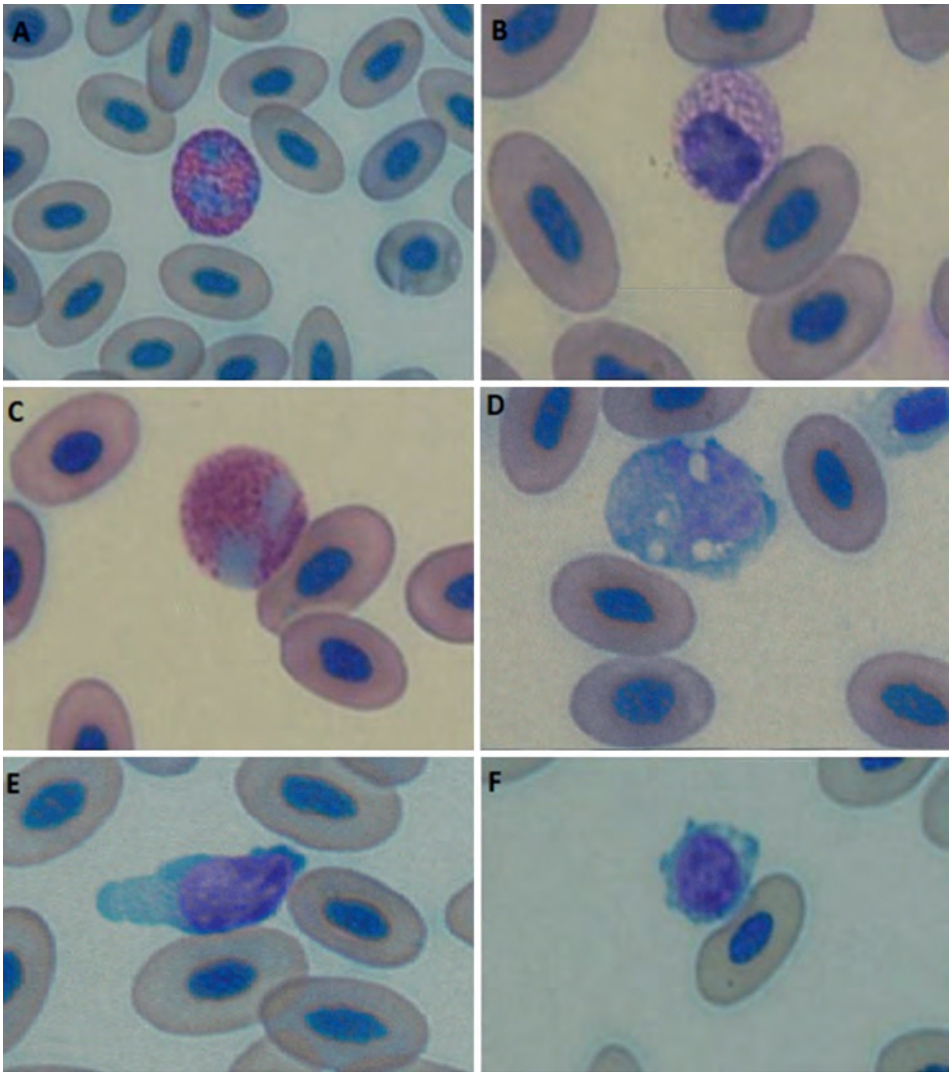

Fig. 1. May – Grünwald stained leukocytes in the blood film of a white stork (*Ciconia ciconia* L.); A – heterophil, B – basophil, C – eosinophil, D – monocyte, E – large lymphocyte, F – small lymphocyte

Lymphocytes are second only to the heterophils in frequency in most species (Tab. 2). Lymphocytes (Fig. 1E, F) had compact, dark nuclei and thin cytoplasm fringes of a blue colour. They were small and well differentiated with an average of 8.10 ± 0.66 μm and 8.28 ± 0.74 μm in diameter for large lymphocytes, respectively, for males and females, and 5.31 ± 0.65 μm and 5.57 ± 0.59 μm, respectively, for males and females for small lymphocytes (Tab. 1). Small lymphocytes had a shape ratio of 0.99/1, which indicated that the cells are round, while large lymphocytes had a shape ratio of 0.87/1, which proves that they are very diverse in shape. The H/L ratio was 4/4 for both sexes.

Tab. 1. Blood cell diameters in μm (mean ± SD) in white stork (*Ciconia ciconia* L.) chicks

Parameter	Male Mean ± SD	Female Mean ± SD	Shape ratio	Number of cells
Heterophils	11.14 ± 0.65	11.01± 0.48	0.95/1	100
Eosinophils	10.72 ± 0.49	10.97 ± 0.53	0.90/1	100
Basophils	9.56 ± 0.78	9.13 ± 0.84	0.98/1	75
Monocytes	13.40 ± 0.97	13.09 ± 1.05	0.93/1	100
Lymphocytes (small)	5.31 ± 0.65	5.57 ± 0.59	0.99/1	100
Lymphocytes (large)	8.10 ± 0.66	8.28 ± 0.74	0.87/1	100

There were no statistically significant differences in male and female white stork

Tab. 2. White blood cells (WBC) in male and female white stork (*Ciconia ciconia* L.) chicks

Parameter	Male n = 25	Female n = 28	Reference range from our study
	Mean ± SD	Mean ± SD	
WBC [$\times 10^9$/L]	18.44 ± 3.69	18.52 ± 3.33	13.56 – 30.22
Heterophils			
Number [$\times 10^3$/μL]	7.51± 1.53	8.05 ± 1.56	4.97 – 12.60
Relative [%]	41.20 ± 7.36	44.00 ± 7.16	26.00 – 57.00
Eosinophils			
Number [$\times 10^3$/μL]	1.73 ± 0.86	1.97 ± 0.83	0.45 – 3.47
Relative [%]	9.40 ± 4.41	10.50 ± 3.68	3.00 – 17.00
Basophils			
Number [$\times 10^3$/μL]	0.34 ± 0.30	0.38 ± 0.37	0.00 – 1.19
Relative [%]	1.80 ± 1.42	1.90 ± 1.86	0.00 – 6.00
Monocytes			
Number [$\times 10^3$/μL]	1.75 ± 1.38	1.43 ± 0.85	0.00 – 6.65
Relative [%]	8.80 ± 4.87	7.60 ± 3.93	0.00 – 22.00
Lymphocytes			
Number [$\times 10^3$/μL]	7.03 ± 1.89	6.70 ± 1.80	3.36 – 12.01
Relative [%]	37.20 ± 7.61	36.00 ± 6.48	22.00 – 52.00

There were no statistically significant differences in male and female white stork

Discussion

The WBC counts of white storks (Tab. 2) were similar to those living at large, as reported by others authors studying storks (Alonso et al., 1991; Kamiński et al., 2014; Kasprzak et al., 2006; Lashev et al., 2005; Montesinos et al., 1997; Puerta et al., 1989; Szabó et al., 2010). The white stork is a monomorphic species, and morphological differences between chicks of different sex do not exist. Female white storks had a higher estimated WBC number compared to males examined in this study, but the difference was not significant (Tab. 2). No significant differences were also observed between both of the sexes in white blood cell parameters (WBC frequencies). In our study, the heterophils were

the most frequent cellular type, representing 42.7 ± 7.32% of leukocytes for both sexes, as found by other authors (Tab. 3) in storks (Alonso et al., 1991; Montesinos et al., 1997; Santos, Serra, 2006; Szabó et al., 2010). Nevertheless, some authors marked lymphocytes as the predominant cells type in storks, as presented in table 3 (Aengwanich et al., 2002; Lanzarot et al., 2005; Puerta et al., 1989; Salakij et al., 2003). As a result, juvenile white storks are another exception to the rule that lymphocytes are the most abundant white cell type in birds, together with the Rheas *Rheidae* (Gallo et al., 2017), Cinereous vulture *Aegypius monachus* L. (Seok et al., 2017), White-naped cranes *Antigone vipio* Pallas (Rayhel et al., 2015), Red-tailed amazon parrot *Amazona brasiliensis* L. (Vaz et al., 2015), Barn owls *Tyto alba* Scopoli (Szabó et al., 2014), Cormorants *Phalacrocorax* (Gallo et al., 2013), Black cockatoos *Calyptorhynchus banksi* Latham (Le Souëf et al., 2013), Swans *Cygnus* (Milani et al., 2012), Hyacinth macaws *Anodorhynchus hyacinthinus* Latham (Kolesnikovas et al., 2012), Bearded vultures *Gypaetus barbatus* L. (Hernández, Margalida, 2010). Eosinophils, basophils and monocytes occurred in smaller proportions and were in similar numbers to the white storks reported by other authors (Alonso et al., 1991; Lashev et al., 2005; Puerta et al., 1989; Szabó et al., 2010).

Tab. 3. Comparative differential frequency of white blood cells in family *Ciconiidae* J.E. Gray (average ± SD); A – juvenile, B – adult; N – sample size; H – Heterophils, E – Eosinophils, B – Basophils, M – Monocytes, L – LymphocytesW

Authors of studies	Our study	Puerta et al. 1989	Montesinos et al. 1997*	Lashev et al. 2005	Alonso et al. 1991	Szabó et al. 2010	Santos and Serra 2006	Lanzarot et al. 2005	Aengwanich et al. 2002	Salakij et al. 2003
Species	White stork *Ciconia ciconia* L.						Black stork *Ciconia nigra* L.		Painted stork *Mycteria leucocephala* Pennant	
Age	A			B			A	B	A	B
N	53	24	23	6	7	80	48	36	10	12
H	42.70 ± 7.00	15.70 ± 2.10	67.90	41.80 ± 1.05	72.30 ± 5.20	61.00 ± 9.80	45.40 ± 15.90	41.60 ± 13.90	10.60 ± 9.49	40.30 ± 5.80
E	9.94 ± 4.03	23.70 ± 1.90	4.70	3.17 ± 0.48	4.80 ± 1.20	0.75 ± 0.91	5.60 ± 4.50	6.19 ± 3.91	12.00 ± 8.54	10.30 ± 1.10
B	1.84 ± 1.66	1.30 ± 0.20	0.30	0.67 ± 0.21	0.50 ± 4.60	0.38 ± 0.56	1.00 ± 2.50	–	1.00 ±1.33	–
M	8.17 ± 4.40	3.10 ± 0.40	1.30	0.50 ± 0.22	1.90 ± 0.40	3.44 ± 2.30	3.50 ± 3.30	1.15 ± 0.95	–	1.50 ± 0.50
L	36.60 ± 6.50	55.30 ± 2.40	23.00	53.80 ± 1.05	20.40 ± 4.60	34.30 ± 9.10	44.40 ± 20.20	50.30 ± 13.10	76.10 ± 10.28	48.40 ± 4.50
H/L	4/4	2/6	7/2	4/5	7/2	6/3	4/4	4/5	1/8	4/5

*Authors did not give ± SD

Heterophils to lymphocyte ratio (H/L) (Tab. 3) varies depending on the level of the stress reaction (Krams et al., 2012). In this research, the ratio was 4/4, and it was the same as reported by other authors in juvenile Black stork *Ciconia nigra* L. (Puerta et al., 1989; Santos, Serra, 2006) and adult Painted stork *Mycteria leucocephala* Pennant (Salakij et al., 2003). A similar ratio (4/5) was also noticed in an adult white stork (Lashev et al., 2005) and in a juvenile Black stork (Lanzarot et al., 2005). The high ratio indicated a stress reaction; whereas, the ratio reported by Alonso et al. (1991) was 7/2 for adult white storks. Such a large variation in the H/L ratio indicates that it should only be used in comparisons of similar bird life conditions.

Our results provide comparative morphological characteristics and a guide for the identification of blood cells in white stork chicks. These may be useful for health management in the white storks, which are endangered species and it is beneficial for further study and related research.

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Abstract

The aim our study was qualitative and quantitative analysis of white blood morphometric elements of peripheral blood (determining the quantity, blood cells dimension and several haematological values) in white stork chicks. One of the aims was to indicate whether the sex relevantly influences the variety of the examined white blood indicators. White blood cells parameters of 53 white stork chicks, with molecularly marked sex, were examined. Blood samples were collected in southern Poland (around Krapkowice town, near Opole city). Lymphocytes of white storks (mean 37% for females and males) were identified as round

cells with dark purple non-lobed, eccentrically positioned nucleus. Among the whole population we differentiated small lymphocytes with diameters of 5.31 ± 0.65 μm in males and 5.57 ± 0.59 μm in females, and large lymphocytes with a diameter of 8.10 ± 0.66 μm and 8.28 ± 0.74 μm, respectively in females and males. Monocytes (mean 8% for female and males) were the largest leukocytes found in the blood film of white storks, measuring 13.40 ± 0.97 μm for males and 13.09 ± 1.05 μm for females in diameter. The cytoplasm was abundant, and it stained blue-grey and very often contained vacuoles. Heterophils (mean 42.7%) were the largest in granular leukocytes group. They were round and 11.14 ± 0.65 μm for males and 11.01 ± 0.48 μm for females in diameter. The nucleus of heterophils was lobed, usually with two or three lobes. The cytoplasm contained brick-red, elongated granules. Eosinophils (mean 9.44%) were round cells, with a diameter of 10.72 ± 0.49 μm and 10.97 ± 0.53 μm, respectively, in males and females. The nucleus was lobed and mostly stained clear blue and contained red-orange, round or rod-shaped granules. Basophils (mean 1.84%) were round and contained dark blue granules, with an average of 9.56 ± 0.78 and 9.13 ± 0.84 μm in diameter, for males and females. The nucleus was usually non-lobed. The H/L ratio was 4/4 for both sexes. No significant differences in levels and types of leukocytes between male and female juvenile storks have been observed.

Key words: white blood cells size, white stork, hematology, morphometric

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Analiza parametrów białych krwinek krwi obwodowej piskląt bociana białego (*Ciconia ciconia* L.) w zależności od płci

Streszczenie

Celem naszego badania była analiza jakościowa i ilościowa morfometrycznych elementów krwi obwodowej (określenie ilości, wymiarów krwinek i kilku wartości hematologicznych) u piskląt bociana białego. Jednym z celów było wskazanie, czy płeć ma istotny wpływ na różnorodność badanych wskaźników krwi białej. Zbadano parametry białokrwinkowe 53 piskląt bociana białego, z molekularnie oznaczoną płcią. Próbki krwi pobierano w południowej Polsce (okolice miasta Krapkowice, w pobliżu miasta Opola. Limfocyty bocianów białych (średnio 37% dla samic i samców) zostały zidentyfikowane, jako okrągłe komórki z ciemnofioletowym, ekscentrycznie usytuowanym jądrem. Spośród całej populacji wyodrębniliśmy małe limfocyty: o średnicy 5,31 ± 0,65 μm u samców i 5,57± 0,59 μm u samic oraz limfocyty duże o średnicy 8,10 ± 0,66 μm i 8,28 ± 0,74 μm, odpowiednio u samic i samców. Monocyty (średnio 8% dla samic i samców) były największymi leukocytami znalezionymi we krwi obwodowej bociana białego, o średnicy 13,40 ± 0,97 μm u samców i 13,09 ± 1,05 μm u samic. Cytoplazma była obfita i zabarwiona na niebiesko-szaro, często zawierała wakuole. Heterofile (średnio 42,7%) były największymi krwinkami wśród granulocytów. Były okrągłe i miały średnicę 11,14 ± 0,65 μm u samców i 11,01 ± 0,48 μm u samic. Jądro heterofilów było płatowate, zwykle z dwoma lub trzema płatami. Cytoplazma zawierała ceglasto-czerwone, wydłużone ziarnistości. Eozynofile (średnio 9,44%) były okrągłymi komórkami o średnicy 10,72 ± 0,49 μm i 10,97 ± 0,53 μm, odpowiednio u samców i samic. Jądro było płatowate i przeważnie wybarwione na jasnoniebiesko, cytoplazma zawierała czerwono-pomarańczowe, okrągłe ziarnistości. Bazofile (średnio 1,84%) były okrągłe i zawierały ciemnoniebieskie ziarnistości o średniej średnicy 9,56 ± 0,78 μm i 9,13 ± 0,84 μm u samców i samic. Współczynnik H/L wynosił 4/4 dla obu płci. Nie zaobserwowano istotnych różnic w liczbie jak i frekwencji poszczególnych rodzajów krwinek białych między młodymi bocianami płci męskiej i żeńskiej.

Słowa kluczowe: krwinki białe, bocian biały, hematologia, morfometria

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