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Development of red blood cells parameters in the White Stork *Ciconia ciconia* chicks, growing in different environments

Abstract

Blood samples were obtained from 111 White Stork *Ciconia ciconia* chicks (age 10 to 51 days), reared in 36 nests in south-western Poland in 2010. Nests were located at four different types of environment: a) breeding colony in the middle Odra River valley (Kłopot), b) solitary nests in the middle Odra River valley, c) solitary nests around the city of Zielona Góra, d) solitary nests near the Głogów copper smelter. Standard methods in hematology were performed for establishing the level of RBC, Hct, Hgb, MCV, MCH MCHC and WBC. The lowest RBC number was in the storks from solitary nests around the city, while the highest number was in the storks from the middle Odra River valley, (both solitary and colonial). Conversely, the level of Hct was higher in storks from around the city of Zielona Góra and the Głogów copper smelter than in storks from the middle Odra River valley. This could be an effect of dehydration in chicks growing near the cities. The highest Hgb concentration was found in storks from solitary nests in the middle Odra River valley and near the city of Zielona Góra, while the lowest Hgb was registered in storks growing in colonies and near the copper smelter. Our results indicate that there are physiological mechanisms which enable chicks' bodies to attain the proper state during their development in different environmental conditions, eg. a low RBC was compensated by a high Hgb concentration in the chicks' blood.

Keywords: White Stork chicks, blood, hematological parameters, environments.

Introduction

Hematology parameters are one of the valuable tools for analysing wild animals, whether evaluating health and condition, diagnosing disease or carrying out clinical monitoring (Lanzarot et al. 2005; Kamiński et al. 2014). Moreover, these animals are used as bioindicators to provide an early warning of pollution effects on organisms (Fox 2001, Kamiński et al. 2006, 2007). Bird hematology has revealed that interpretation of blood parameters is quite difficult, since variations in the blood are caused by diverse internal and external factors, including age, sex, geographical elevation, energy expenditure,

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parasitism, nutrition, pollutants and genetics (Fair et al. 2007; Llacuna S. et al. 1996). Normal ranges for various blood parameters in the White Stork have been established by different investigators in bird physiology and pathology (Puerta et al. 1989; Alonso et al. 1991; Howlett et al. 2002; Lashev et al. 2005; Szabó et al. 2010).

White Storks (*Ciconia ciconia*) can act as a biomonitoring indicator of pollution in the environment (Fox 2001; Kaługa 2006; Kamiński et al. 2006, 2007). Avian chicks can infer conditions in a particular habitat, since they do not move from the nest and they are fed based on surrounding aquatic prey, mainly fish and amphibians, exclusively from the nesting territory of the adults (Elliott 1992). Despite the potential to use hematology parameters to assess pollution levels few studies of birds have confirmed the existence of an association between hematology and condition. In this article we describe how hematology values differ among White Stork chicks, especially under exposure to various environment and pollutants.

According to the Inspectorate of Environmental Protection report assessing soil contamination by benzo[a]pyrene (B(a)P), copper (Cu), lead (Pb) and chrome (Cr), these indicators exceeded the concentration threshold in the soil around the Głogów smelter (Meinhardt et al. 2009). The action of lowering dust-gaseous emissions from the most crucial sources in the Głogów Copper Mining Region significantly improved air quality in this region. However, there are still air protection problems waiting to be solved, which are connected with the emission of gaseous-dust pollutants from the domestic-municipal sector i. e. so-called low emissions and road transport pollutant emissions (Strzelecka & Niedźwiecka 2012). The water of the River Odra in Kłopot village contained high levels of organic phosphorus and nitrites, in Połęcko (a village near Kłopot) these levels exceeded standards of BOD5 (biochemical oxygen demand) and non-ionic ammonia were reported (Lewicki 2011). Excessive concentrations of heavy metals are detrimental. They destabilize ecosystems because of their bioaccumulation effect in organisms, and may cause toxic effects on biota and even death in most of living beings (Govind & Madhuri 2014). However, the copper factory was not found to have any direct negative impact on the population dynamics of storks breeding in the Głogów area (Kujawa 1994).

Today, only a small part of Poland's White Stork population is living in breeding colonies (Tryjanowski et al. 2006). Solitary nesting is particularly frequent in Eastern population (Czajkowski et al. 2012). Traditionally, solitary nests were thought to be less successful, but their long-term success rate is remarkably constant (Thomas 1987). Moreover, bird colonies are not peaceful places (Stokes & Boersma 2000). Nest damage, material-stealing episodes or injuries caused unintentionally or through fights is the result of normal behavior in the colony (Wittenberger & Hunt 1985; Thomas 1986; Stokes & Boersma 2000). It is possible that for first-time breeders arriving later than experienced breeders, the colonies may not have been attracted because the nest-building and copulation stage had passed (Thomas 1986). Vergara et al. (2007)

showed that age correlated strongly with breeding success in that adults were more successful breeders than young birds. However, White Stork coloniality is an active area of research. Many advantages explain the maintenance of colonies in birds: an increase in mating opportunities, decreased predation, the possibility of extra-pair copulations by females and help in communicating information about the location of food sources (Vergara & Aguirre 2006). The potential benefits in breeding colonies include increased food-finding (Brown 1988; Wilkinson 1992) and reduced predation (Birkhead 1977; Hoogland & Sherman 1976).

The aim of this study was to determine the impact of the environment on hematological parameters in free-living White Stork chicks. We investigated RBC, Hct, Hgb, MCV, MCH, MCHC and WBC in wild animals living in colonies, solitary nests, and also in polluted and unpolluted environments. In our study, we also considered other factors affecting hematology status, such as the age of nestlings, the changes particularly associated with the quality of the environment and the type of nest location (in colonies and solitary nests).

Materials and methods

Samples were collected between June and July 2010. The study included 111 free-living White Stork nestlings. Fieldwork was conducted in four areas of south-western Poland:

- a) 13 nests in a colony in the middle Odra River Valley in Kłopot village (52°06'N, 14°42'E), hereafter called the Colony;
- b) 8 solitary White Stork nests in the middle Odra River Valley (52°02'N, 14°57'E), hereafter called Villages; both were located away from the factory, surrounded by natural areas of forests and arable fields;
- c) 8 solitary nests around the city of Zielona Góra (51°57'N, 15°30'E) – about 120,000 inhabitants and with no heavy industry; hereafter called the City;
- d) 7 solitary nests near the Głogów copper smelter (51°40'N, 16°05'E) – an area within several km of a visible source of pollution; hereafter called the Smelter.

All birds were examined and sampled between 10:00 AM and 1:00 PM. They were retrieved from the nest and placed in individual cotton sacks. Stork chicks were presented for physical examination, ringed and a blood sample was obtained. The nestlings' age was established (10-51 days) based on bill measurements (Kania 1988).

Blood sampling and hematology analysis

Blood samples (1.1 ml) were collected via venipuncture of the brachial vein using a 25-gauge cannula. After collection, the blood was mixed immediately in tubes with the anticoagulant dipotassium ethylenediaminetetra-acetic acid (EDTA 1.6 mg/ml of

blood). The samples were stored in darkness at 4-6°C. Blood samples were processed in the laboratory within 12 hours of sampling. Whole blood was used for the complete blood count. Red blood cell (RBC) and white blood cell counts (WBC) were performed with the Natt-Herrick solution (1 : 200 dilution) (Natt & Herrick 1952) and were determined using the improved Bürker hemocytometer. Hematocrit (Hct) was determined by centrifugation at 12 000xg for 3 minutes (Owen 2011). Hemoglobin (Hgb) concentration was estimated by the cyanomethemoglobin method (Drabkin 1945). Blood mixed with Drabkin's fluid was centrifuged at 3 500xg for 10 minutes and the Hgb was measured in the supernatant in a spectrophotometer at a wavelength of 540 nm. The mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated using the following equations: $MCV = Hct / RBC \times 10$; $MCH = Hgb / RBC \times 10$; $MCHC = Hgb / Hct \times 100$ (Samour 2006).

Ethics Statement

The experiments complied with the Guidelines of the European Union Council and current Polish laws, according to the Ethical Commission (permission number: DKFOP-ogiz 4200/III-109/2920/08/aj Warszawa 2008.04.24).

Statistical analysis

Statistical analyses of data were performed using Statistica 9.1 software (StatSoft Inc. 2010). Data are given as arithmetic means \pm standard deviation (SD) in tables. A significance level of $\alpha = 0,05$ was chosen for all statistical tests. Normality was assessed by the Shapiro-Wilks test. To determine environment-related changes, chicks were divided into four subgroups depending on their nest. Differences in the hematological values of White Stork chicks from different environments were examined by two-way ANOVA with Tukey's HSD post-test.

Results

All birds appeared to be in good condition and no abnormalities were noted during physical examination nor in hematologic analyses. The age difference of the chicks in each environment was not statistically significant. RBC, Hgb, Hct and WBC were positively correlated with the chicks' age as opposed to MCV, MCH and MCHC. There were no significant differences between age and nest location.

The RBC number had a normal distribution. RBC measured in samples collected from nestlings in the villages (Colony and Villages) was significantly higher than that measured in chicks near the city (City) ($p < 0.001$) (Fig. 1).

On the other hand, Hct collected from nestlings near the City and Smelter was significantly higher than from storks in the Colony and Villages (Fig. 2).

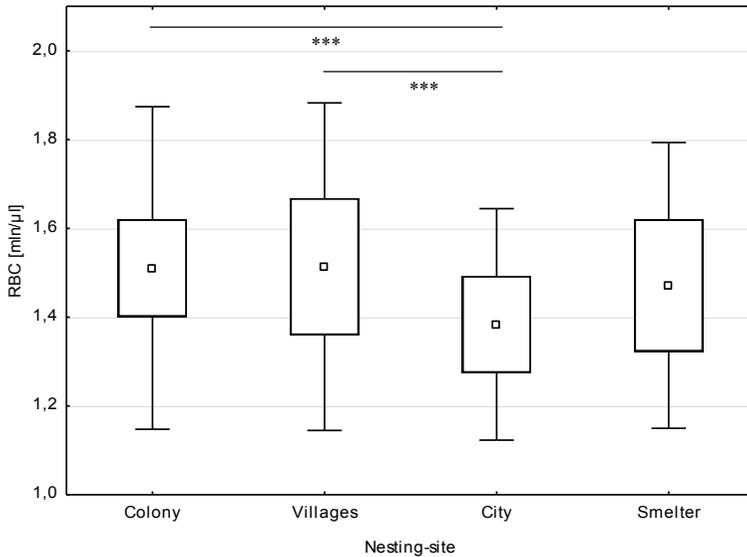


Figure 1. Red blood cell count of the White Stork *Ciconia ciconia* chicks from four different environments (Colony, Villages, City and Smelter). Values are means \pm SD. Lines between environments are statistically significant *** $p < 0.001$ (post-hoc HSD Tukey's test; Error: Between MS = 0.01232, $df = 106$, $P < 0.001$).

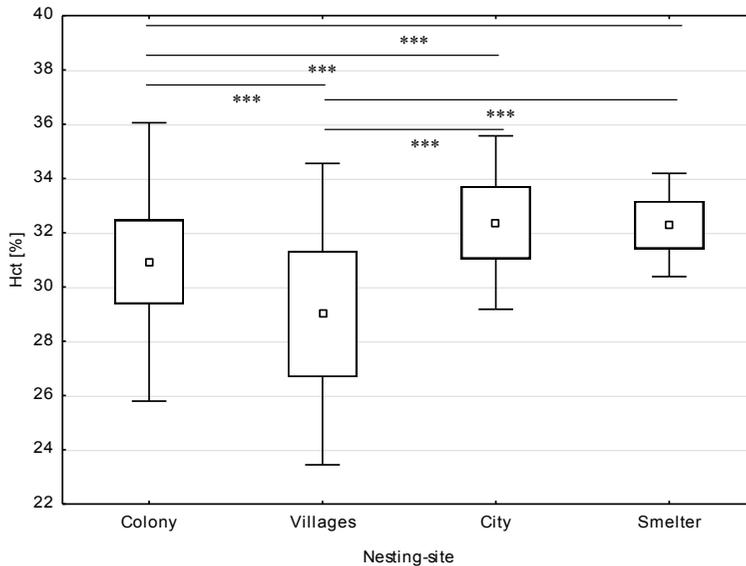


Figure 2. Hematocrit of White Stork *Ciconia ciconia* from different environments (Colony, Villages, City and Smelter). Values are means \pm SD. Lines between environments are statistically significant *** $p < 0.001$ (post-hoc HSD Tukey's test; Error: Between MS = 1.5705, $df = 106$, $P < 0.001$).

In both groups City and Villages, the Hgb concentration was significantly higher than that measured in storks growing in the Colony and near the Smelter (Fig. 3).

The MCV in all environments was significantly different. Like MCH, the highest value occurred in storks from solitary nests near the City and the lowest in the Colony. The MCH was significantly similar in storks from the Colony and near the Smelter in Głogów (Fig. 4, 5).

MCHC was significantly similar in storks from the Colony and near the Smelter, like solitary nests in the Villages and near the City (Fig. 6). WBC was significantly lower in storks from the Colony than in storks from the Smelter and Villages (Fig. 7).

Most statistically significant differences were found between chicks in the Colony and storks from solitary nests in the Villages and near the City. Moreover, the nestlings from the polluted area near the Smelter differed more than storks from the Villages. Blood levels of Hct, Hgb and WBC were higher between City locations and the Smelter, and generally a higher RBC count was reported in bird populations from other unpolluted areas (Colony, Villages). Contrary to our expectations, nestlings from the referenced Colony had lower hemoglobin concentrations than those sampled in other locations.

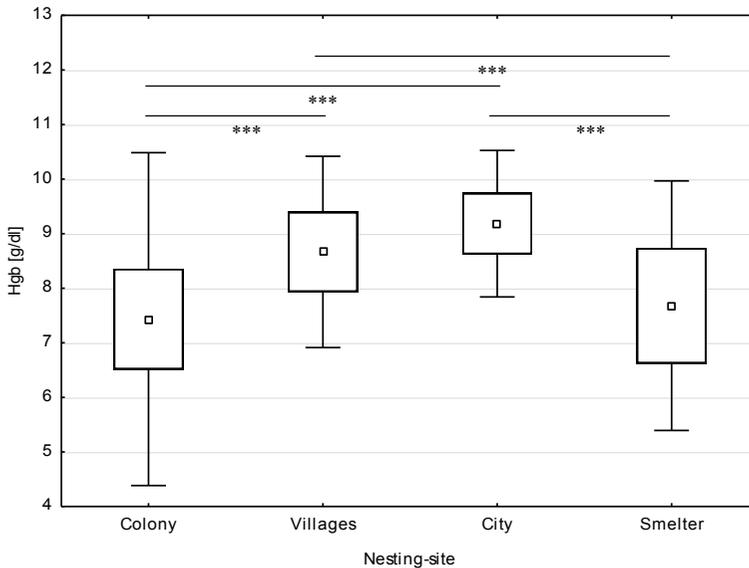


Figure 3. Hemoglobin concentration of White Stork *Ciconia ciconia* from different environments (Colony, Villages, City and Smelter). Values are means \pm SD. Lines between environments are statistically significant *** $p < 0.001$ (post-hoc HSD Tukey's test; Error: Between MS = 0.73431, $df = 106$, $P \leq 0.001$).

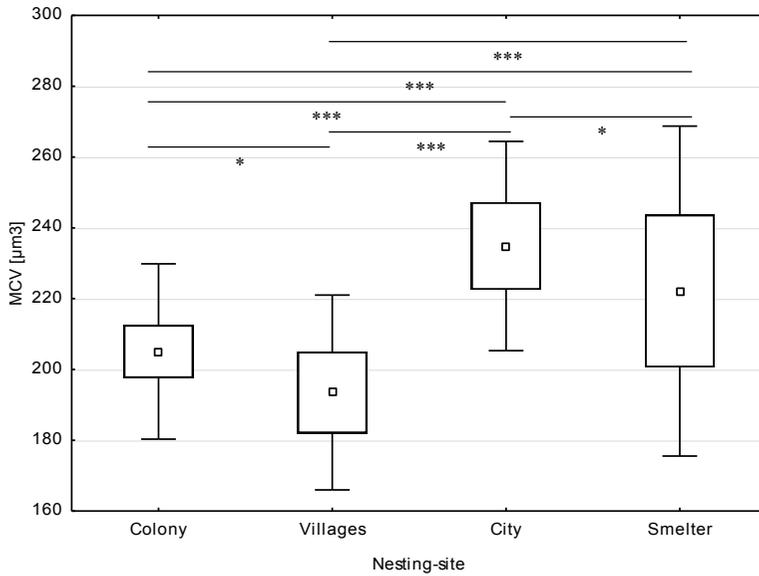


Figure 4. Mean cell volume of White Stork *Ciconia ciconia* from different environments (Colony, Villages, City and Smelter). Values are means \pm SD. Lines between environments are statistically significant *** $p < 0.001$, * $p < 0.05$; (post-hoc HSD Tukey's test; Error: Between MS = 203.57, df = 106, $P < 0.05$).

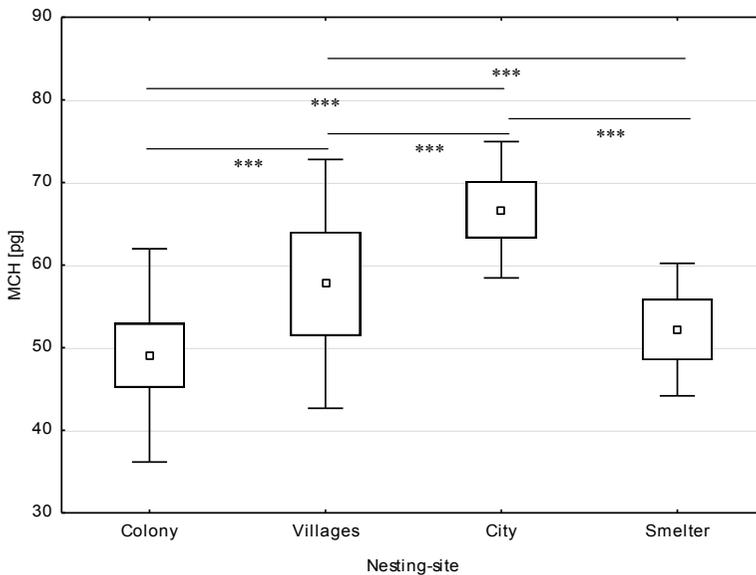


Figure 5. Mean corpuscular hemoglobin values of White Stork *Ciconia ciconia* from different environments (Colony, Villages, City and Smelter). Values are means \pm SD. Lines between environments are statistically significant *** $p < 0.001$ (post-hoc HSD Tukey's test; Error: Between MS = 30.469 and MS = 6.692, df = 106, $P < 0.01$).

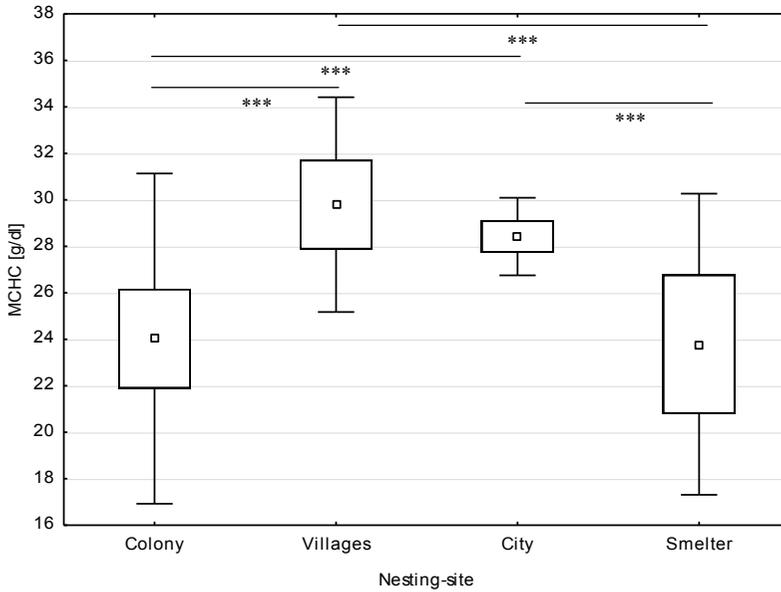


Figure 6. Mean corpuscular hemoglobin concentration of White Stork *Ciconia ciconia* from different environments (Colony, Villages, City and Smelter). Values are means \pm SD. Lines between environments are statistically significant *** $p < 0.001$ (post-hoc HSD Tukey's test; Error: Between MS = 6.692, df = 106, $P < 0.05$).

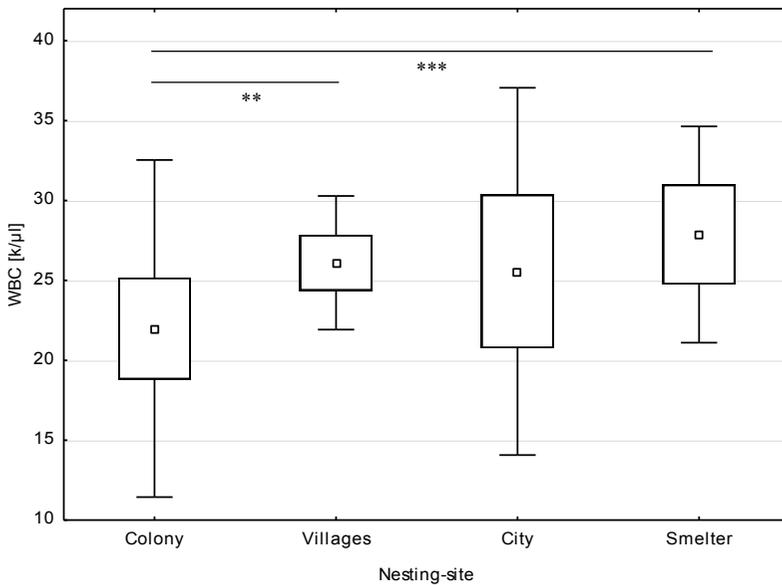


Figure 7. White blood cells of White Stork *Ciconia ciconia* from different environments (Colony, Villages, City and Smelter). Values are means \pm SD. Lines between environments are statistically significant *** $p < 0.001$; ** $p < 0.01$; (post-hoc HSD Tukey's test; Error: Between MS = 16.287, df = 106, $P < 0.01$).

Discussion

Research determined the hematologic values from a large population (N = 106) of free-living White Storks nestlings, providing reference intervals that can be used as baseline information for further studies. It is difficult to determine the health of individuals when studying field-caught wild species (Weber et al. 2002). However, the reference values for chicks of free-living storks are within the range of variation found in other publications (Puerta et al. 1989; Alonso et al. 1991; Aengwanich et al. 2002; Lashev et al. 2005; Szabó et al. 2010) (Table 1). The dissimilarity of our results with those found by other authors (Alonso et al. 1991; Lashev et al. 2005; Szabó et al. 2010) supported the theory that age, nesting locality and environment have a considerable influence on hematological parameters (Puerta et al. 1990; Kamiński et al. 2014). We assume that in diagnostics based on blood indices interpretation, healthy animals living under similar environmental conditions should always be compared.

Table 1. Mean of hematological parameters between storks according to different scientists (mean \pm SD); RBC - red blood cells number; Hct - hematocrit value; Hgb - hemoglobin concentration; MCV - mean cell volume; MCH - mean corpuscular hemoglobin; MCHC - mean corpuscular hemoglobin concentration; WBC - white blood cells number, n.i. = not investigated

Parameter	Our studies/ 10-51-day-old nestlings <i>Ciconia</i> <i>ciconia</i> /Poland N = 106	Puerta M.L./ 15-68-day-old chicks <i>Ciconia</i> <i>ciconia</i> /Spain N = 23	Alonso J.C./ adult <i>Ciconia</i> <i>ciconia</i> /Spain N = 7	Szabó Z./adult <i>Ciconia ciconia</i> Hungary N = 80	Salakij Ch./adult <i>Mycteria leucoce-</i> <i>phala</i> /Thailand N = 80
RBC [mln/ μ l]	1.47 \pm 0.17	1.87 \pm 0.17	2.59 \pm 0.12	2.28 \pm 0.35	2.44 \pm 0.11
Hct [%]	32 \pm 2	36.2 \pm 1.5	44.1 \pm 1.3	46.3 \pm 5.3	47.4 \pm 1.5
Hgb [g/dl]	7.7 \pm 1	11.1 \pm 0.6	13.9 \pm 0.8	12.8 \pm 2	17.8 \pm 0.4
MCV [fl]	222 \pm 22	226 \pm 21	172 \pm 8	203 \pm n.i.	213 \pm 13
MCH [pg]	52 \pm 9	70 \pm 6	54 \pm 4	56 \pm n.i.	75.4 \pm 3.8
MCHC [g/dl]	24 \pm 4	31.9 \pm 1	31 \pm 2	28 \pm n.i.	36.8 \pm 1
WBC [k/ μ l]	28 \pm 4	62.1 \pm 4.7	23.8 \pm 2.4	21.6 \pm 4.2	21 \pm 1.2

Age-related increases in hematologic parameters in White Storks (Puerta et al. 1989; Alonso et al. 1990; Montesinos et al. 1997) were similar to those from studies of domestic fowl, geese, quails, flamingos (Hawkey et al. 1984), psittacines (Clubb et al. 1991), ostriches (Palomeque et al. 1991; Fudge 1996), bustards (Alonso et al. 1990; Howlett et al. 2002), birds of prey (Lanzarot et al. 2001; Dujowich et al. 2005), and pigeons (Gayathri et al. 2004). These results may be partly explained by adaptation to flight, as the need for oxygen is greatly increased to prevent age-related tissue damage caused by changes in their blood (Kasprzak et al. 2006). We found that the hematological parameters of the storks significantly increase in accordance with their age and that many of these variations are similar to those reported by Montesinos et al. (1997). RBC counts, Hct and Hgb values were lower than those described by Puerta et al. (1989) for older white

or black stork chicks (15-68 days old). Increased hemoglobin content in adults may also be a consequence of decreased blood volume per unit of erythrocyte surface with age (Palomeque & Planas 1977; Celdran et al. 1994; Eklom & Lill 2006). The WBC counts were similar to those in adult and captured storks (Alonso et al. 1991; Salakij et al. 2003; Szabó et al. 2010). The mean WBC number for our free-living nestling storks is lower than the values reported in slightly older chicks (Puerta et al. 1989), possibly because of the different capture, restraint and analytical methods employed (Padilla et al. 2003).

However, age effects were small compared to nesting locality. This study provides information on the hematological response in stork nestlings in relation to different environments, namely, the location of fieldwork near a heavy metals source (copper smelter), near a city and in small villages situated far from industry. The results of Kamiński et al. (2006) studies show that concentrations of toxic heavy metals (Mg, Zn, and Cd) gradually increased in the blood of the storks in polluted areas near Głogów, and were significantly higher near Zielona Góra than in Kłopot village (Colony). Kamiński et al. (2008) observed an increase in antioxidant enzyme activity with an increase in heavy metals such as Cd and Pb in the blood of young storks from polluted environments (Smelter) and suburbs (City). Those investigations provide evidence that White Storks from the Colony and Villages have better conditions for growth and development than in polluted areas near Głogów and around the City of Zielona Góra.

Several studies concentrating on hematological parameters were useful in the detection of early or low-level responses to pollutants (Baos et al. 2006). The effects of toxic load are better manifested in nestlings than in adult birds (Belskii et al. 2005). The impact of pollution on animals was shown by other authors. A significant decrease in the erythrocyte count, an increase in MCV and MCH and a decrease in weight in *Turdus merula* from the polluted zone was observed by Llacuna et al. (1996). Our results indicated the following: the higher RBC count reported in bird populations from unpolluted areas (Colony, Villages), MCV and MCH significantly increased in the population of storks in the City and in the Smelter, but the mean weight of nestlings in polluted and other locations did not differ significantly. Most pollutants had a significant negative relationship with hemoglobin and RBC count, and a positive significant relationship with Hct, WBC in cockerels and in human blood (Dayal & Dubey 2012; Poursafa et al. 2011). In our study, Hct was higher in nestlings from City locations and the Smelter than in the Colony and Villages. A high Hct value could be an effect of dehydration in chicks growing near the City and Smelter. According to our expectations, storks near the Smelter had a lower hemoglobin concentration than chicks in solitary nests in the villages. The results of this study confirm that White Storks from cleaner areas have better conditions for growth and development than from polluted areas.

Interestingly, the location of the nest affects hematological parameters. Lack (1968) suggested that solitary and colonial nesting in the same species were an adaptation to different food densities. This is not the case with White Storks from our research and

maguari storks (Thomas 1986). Solitary (Villages) and colonial nesting had a similarly high level of RBC count and lower Hct and MCV in nestlings from the City and Smelter. More exposure to stressors in chick storks in single nests may be the cause of higher Hgb, MCH, MCHC and WBC levels in chicks from solitary nests in Villages. The increase in hemoglobin concentrations gives greater possibilities for energy generation and potentially better physiological conditions as it was shown in barn swallows (Kasprzak et al. 2011). The total white blood cell counts indicate the immune function and are routinely used as an index of illness in birds (Campbell 1995). The higher WBC counts obtained in chicks in solitary rather than in colony nests could provide information more about a bird under stress than the presence of parasites or infection.

Normal values for blood constituents vary widely for different species (Van Wyk et al. 1998). Thus to assess the physiological and pathological condition of wild birds it is of paramount importance to know the normal blood values for individual species. Acquaintance with the reference ranges of the White Stork's blood parameters will be useful in ensuring their general health and also establishing the cause of death in birds found dead in the wild. It is important to take into account the location of fieldwork in the vicinity of a point source of heavy metals, or the location near a city or villages situated far from factories.

This study also supports the view that we should pay closer attention to the problems occurring from interactions between different types of stressors. This can be noticed when working in a wild environment where the animals are not just exposed to one factor but are influenced by a whole range of stress factors. Since wild animals do not live in isolated conditions, the blood parameters are conditioned by different environmental factors (living in a colony, solitary nests and polluted areas).

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