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Haematologic reference values in free-living red-backed shrike (*Lanius collurio*) nestlings in an agricultural habitat

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Short communication

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Blood parameters are widely used to evaluate the condition of birds. Typically, only a few parameters are used, such as the haemoglobin concentration, haematocrit, or the erythrocyte and leukocyte counts. The aim of this study was to present reference values of the haematological parameters of a free-living population of red-backed shrike (*Lanius collurio*) nestlings in east-central Poland. We assessed various blood parameters (haemoglobin concentration, erythrocyte and leukocyte, on blood smears for the erythrogram, leukogram and thrombocyte count), and examined their relationships with the brood size, biometrics conditions and hatching date. To reduce dimensionality and address the potential collinearity among variables, we conducted a Principal Component Analysis (PCA) and used the resulting components in Generalised Linear Mixed Models. None of the models yielded statistically significant effects. These results suggest a relatively stable physiological status among these nestlings, likely supported by a food-rich agricultural landscape. The haematological profiles presented here offer valuable baseline data for assessing the health of red-backed shrike populations and may support future comparative studies in wild birds.

Key words: bird, blood cells, passerine, physiological condition, wild populations, avian health.

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Human activities pose an increasing threat to many wild animal species (Hoffmann *et al.* 2010). Various assessments can be used to evaluate the quality of a given population, the most common being tracking changes in the population size within a given area (Jellesmark *et al.* 2021; Willig *et al.* 2023), assessing individual survival rates (Canessa *et al.* 2016; Fernández-Chacón *et al.* 2021) and measuring breeding success (Hartway & Mills 2012; Rzępała *et al.* 2023). However, a body condition assessment provides an equally crucial perspective, as it reflects the energy reserves and overall health of individuals, which are directly linked to the survival and reproductive success (Labocha & Hayes 2012). There are numerous ways to assess the body condition of animals, applicable to the species, genera or broader taxonomic groups such as classes (Stevenson & Woods 2006). Some methods, such as the body size or Fulton's condition factor, are relatively straightforward (e.g. Bolger & Connolly 1989; Vanderkist *et al.* 2000), while others are more complex (e.g. Cattet *et al.* 2002; Marker & Dickman 2003).

Body condition, which is broadly defined as the physiological state of an individual in terms of its energy reserves and health status (Labocha & Hayes 2012), can be assessed using a variety of morphological and physiological indicators. Among the latter, blood parameters are commonly used as proxies of an avian body's condition (Masello & Quillfeldt 2004; Minias 2015). Typically, only a few parameters are measured, such as the haemoglobin, haematocrit, erythrocyte and leukocyte count (Dubiec & Cichoń 2001; Morrison *et al.* 2009; Golawski & Kondera 2023). The assumption is that birds which are in good condition exhibit higher values of the blood parameters (Ferrer 1990). However, determining these values is challenging due to the limited data available for many species. While poultry species (Khan & Zafar 2005; Etim *et al.* 2014) and captive birds kept as pets (Briscoe *et al.* 2010; Gaspar *et al.* 2021) are relatively well-studied, wild bird populations often lack such comprehensive data. The haematological data for birds of prey, often collected during the treatment of weakened individuals in rehabilitation centres (Dujowich *et al.* 2005; Black *et al.* 2011), may differ significantly from the data collected from wild populations, as shown in the short-toed snake eagle (*Circaetus gallicus*) (Baumbusch *et al.* 2021). Reference data is particularly scarce for wild species of limited economic importance, such as small passerines (e.g. Nava *et al.* 2001; Vinkler *et al.* 2010).

In this study, we present reference data for the blood parameters from a free-living population in a core range of red-backed shrikes (*Lanius collurio* L.) in east-central Poland. We investigated the blood parameters of nestlings in relation to the brood size, biometrics and hatching date, as these factors may influence the results (Dubiec & Cichoń 2001; Limiñana *et al.* 2009). Many shrike species are considered as endangered (Lefranc 2022), and for some species, special conservation actions have been carried out (Wiliams & Steiner 2008; Sachslehner *et al.* 2016). Some, including the red-backed shrike in Poland, have also been the focus of conservation actions (Tryjanowski 1999). Baseline blood parameter data is essential for monitoring a population's health and understanding future population trends, which we illustrate for this shrike species.

Methods

The study was carried out in compliance with the current Polish Law and was approved by the Ministry of the Environment (Permit No. 93/2021), the Regional Directorate for Environmental Protection in Warsaw (Permit No. WSTS.6401.92.2020.MO) and the Local Ethics Committee in Warsaw (No. 1164/2021).

The red-backed shrike is a small passerine widely distributed in Europe and western Asia, with a declining population trend (BirdLife International 2024). In eastern Poland, the majority of the population inhabits agricultural landscapes, breeding on the edges of woods, in clumps of trees, in orchards and near villages (Golawski & Meissner 2008). The nestlings remain in the nest for 14 days, with the fledglings staying nearby for an additional two weeks, and broods typically consist of 4-6 nestlings (Lefranc 2022). This shrike species is a mainly insectivorous species (Tryjanowski *et al.* 2003; Golawski *et al.* 2020). The study was conducted in 2021, in the agricultural area near the town of Siedlce in east-central Poland (52.14°N, 21.93°E), where red-backed shrikes occur at high densities (Golawski *et al.* 2020). A total of 30 nests were monitored approximately every five days during the laying, incubation and nestling periods until fledging (Golawski & Zduniak 2022). These inspections allowed for a determination of the hatching dates and facilitated an appropriate timing of the blood sampling.

Blood was sampled from 8-10-day-old nestlings (n = 40; once per nestling), by a brachial vein puncture with heparinised needles to heparinised Eppendorf tubes, then chilled in a fridge (+4°C) and delivered to the laboratory. The following analyses: measurement of the haemoglobin concentration (Hb), erythrocyte count (RBC) and leukocyte count (WBC) were determined using standard methods (Samour 2006; Lugowska *et al.* 2017; Kaminski *et al.* 2014). Blood smears (stained with May-Grünwald and Giemsa solutions) were prepared to evaluate the blood cell morphology. Smears were examined for the erythrogram (percentages of erythrocytes, erythroblasts and abnormal erythrocytes), leukogram (percentages of lymphocytes, heterophils, eosinophils, basophils and monocytes) and the thrombocyte count (thrombocytes per 100 leukocytes).

To reduce dimensionality and identify major axes of variation in the haematological dataset, we performed a Principal Component Analysis (PCA) on 20 blood parameters (see Table 1). The PCA was based on the correlation matrix, and the resulting component scores (PC1, PC2 and PC3) were retained for a further analysis. These three components collectively explained approximately 46% of the total variance in the blood parameters.

We then used Generalised Linear Mixed Models (GLMMs) with a normal distribution and identity link functions to test whether the variation in blood physiology (summarised by the PCA scores) was associated with nestling and brood-level traits. Three separate GLMMs were fitted, each using PC1, PC2 or PC3 as the dependent variable (Suplementary Material Table S1). Fixed effects included the brood size, hatching date (day of the season, where 1 = 1st May) and the Scaled Mass Index (SMI) of the nestlings.

Table 1

Hematologic parameters of red-backed shrike's nestlings, n = 40

Parameter	Mean ± SD	Median
Hb [g/l]	101.1 ± 24.4	100.0
RBC [10^6/μl]	2.2 ± 0. 6	2.2
WBC [10^3/μl]	17.3 ± 10.3	16.2
erythrocytes [%]	91.6 ± 15.0	94.5
erythroblasts [%]	3.9 ± 2.2	3.5
abnormal erythrocytes [%]	2.3 ± 2.3	2.0
lymphoid [%]	31.9 ± 10.6	31.2
large lymphocytes [%]	11.7 ± 5.7	11.0
small lymphocytes [%]	20.2 ± 10.6	18.4
heterophilic [%]	35.7 ± 13.5	38.2
heterophilic myelocytes [%]	13.7 ± 5.5	14.0
heterophilic metamyelocytes [%]	8.5 ± 3.9	8.3
segmented neutrophils [%]	6.3 ± 3.9	5.2
band neutrophils [%]	8.9 ± 5.1	8.3
basophilic [%]	19.3 ± 15.4	16.9
young basophils [%]	7.6 ± 4.0	6.4
mature basophils [%]	9.8 ± 6.5	8.2
eosinophilic [%]	9.2 ± 6.7	7.4
monocytoid [%]	7.0 ± 5.1	6.5
thrombocytoid [10^3/μl]	9.2 ± 5.8	9.2

The formula for SMI is: $SMI_i = M_i (L_0/L_i)^{bSMA}$, where M_i and L_i are the body mass and wing length of an individual, respectively. L_O is the arithmetic mean of wing length for all birds being considered in the scaling calculations, and bSMA is the slope derived from the standardized major-axis (SMA) regression between body mass and wing length, excluding outliers (Peig and Green 2009). Wing length was measured with a ruler (± 1 mm) and mass was recorded with a Pesola spring balance (± 0.5 g). 'Nest ID' was included as a random factor in all models. The model assumptions (normality of residuals and homogeneity of variance) were tested using the Shapiro-Wilk test and Levene's test, respectively. The analyses were conducted using SPSS v.21.0 (IBM Corp., 2012); $p \leq 0.05$ was considered statistically significant.

Results

Thirteen nests were analysed: twelve contained 4 to 6 nestlings, and one contained a single nestling (40 individuals in total). The nestlings hatched between 12 and 24 June, with a median hatch date of 19 June. All the broods included in the study represented the first breeding attempt of the season. At the time of the sampling, the mean SMI was 23.1 g (SD = 3.68, range = 15.0-29.2, n = 40). SMI values were not correlated with the hatching date (Spearman rank correlation, r = 0.02, p = 0.913) or brood size (r = 0.23, p = 0.152). The blood parameters (Hb, RBC, WBC, erythrogram, leukogram and thrombocyte count) are summarised in Table 1. Peripheral blood smears revealed a predominance of mature erythrocytes (93.9%) over young erythrocytes (3.9%), with 2.3% exhibiting abnormalities (e.g. elongated or irregular shapes, as well as displaced nuclei). Lymphocytes accounted for 31.9% of all leukocytes, predominantly small lymphocytes. Heterophils were the most abundant white blood cells (35.7%), with juvenile heterophils (myelocytes and metamyelocytes) outnumbering the mature forms (bands and segmented heterophils). Only mature eosinophils (9.2%) were observed, while both mature (9.8%) and young (7.6%) basophils were present. Monocytes and thrombocytes were rare (7.0% and 9.2 × 10^3/μl, respectively).

The GLMMs revealed no significant associations between the principal component scores and the explanatory variables. Specifically, PC1 was not significantly related to SMI, brood size or the hatching date (GLMM, $F_{3,36} = 0.99$, $p = 0.404$), nor was PC2 ($F_{3,36} = 1.29$, $p = 0.223$) or PC3 ($F_{3,36} = 2.21$, $p = 0.103$). Detailed model outputs are presented in Supplementary Material Table S1 and Table S2.

Discussion

The lack of significant associations between the physiological condition (summarised via the PCA) and brood characteristics (body condition, brood size and hatching date) suggests that early-life variation in these parameters does not strongly influence the blood physiology in red-backed shrike nestlings at the stage of development that we studied. This could indicate a degree of physiological homeostasis, whereby the nestlings maintain stable blood profiles despite moderate variations in their body mass or sibling competition.

However, the limited variation in brood size (in the vast majority comprising 4-6 nestlings), and the narrow hatching window – with the nestlings from all broods hatching within a 13-day period – may constrain the detection of subtle physiological patterns. It is also possible that small differences in the nestling age affected our results. All the individuals were studied during a specific stage of growth, while earlier or later developmental windows might reveal stronger physiological differences. Daily measurements of selected blood parameters in great tits (*Parus major*) and house martins (*Delichon urbicum*) have shown age-related changes (Kostelecka-Myrcha *et al.* 1973; Kostelecka-Myrcha & Jaroszewicz 1993). However, since the red-backed shrike nestlings included in our study were all between 8 and 10 days old, such changes may have been too subtle to detect. On the other hand, the habitats in this region of Poland appear to be highly suitable for shrikes, as was indicated by the low partial brood loss (0.5 nestling per brood; Golawski 2006). Additionally, although the clutch size is relatively consistent in this species, the breeding season extends from mid-May to mid-July (Lefranc 2022), potentially influencing blood parameters in ways more significantly than were observed here. For instance, seasonal effects were found in great tits (Dubiec & Cichoń 2001; Kaliński *et al.* 2019), likely due to the diminishing food availability as the season progressed. Previously-hatched nestlings show a higher immune response and have more leukocytes circulating in the blood than nestlings from late laid clutches (Sorci *et al.* 1997; Dubiec & Cichoń 2001). In chicks, the amount of leukocytes increases during the first weeks after hatching, and the immune system develops (Klasing & Leshchinsky 1999), so the quantity and quality of the food received at this time seems to be of particular importance for their normal development. Hoi-Leitner *et al.* (2001) noted that a high food abundance in the vicinity of the nest enhanced the cellular immune response in nestlings in serin (*Serinus serinus*); whereas food limitation suppressed the immune function and growth of lymphoid organs, e.g. in the bursa of Fabricius (Lochmiller *et al.* 1993; Birkhead *et al.* 1999). It may lead to a depressed immunity of the undernourished chicks (Klasing *et al.* 1987) and may also cause higher levels of infection.

An inadequate intake of essential nutrients may also reduce the synthesis of erythropoietin (a hormone responsible for red blood cell production) in nestlings. In addition, a diet poor in nutrients may suppress erythropoiesis in birds (Campbell 1994), which significantly results in decreased red blood parameters. Many authors have indicated that a reduction in haemoglobin levels in nestlings is associated with a limited access to food (e.g. Minias 2015; Lidman *et al.* 2020; Zaremba *et al.* 2021) or a poor food quality (e.g. Bańbura *et al.* 2007; Kaliński *et al.* 2019). The haemoglobin concentration was found to be positively affected by the quality of the diet in both nestlings (Pryke *et al.* 2011; Pryke & Rollins 2012) and adults (Pryke *et al.* 2012) on experimental studies on captive passerines. Furthermore, Merino & Potti (1998) and Potti *et al.* (1999) noted that high haematocrit values are correlated with an environmental abundance of food and the absence of infections and parasites.

Haematological data for free-living nestlings of the red-backed shrike is absent from the subject literature. Data on passerine nestlings is scarce overall (Puerta *et al.* 1995; Heatley *et al.* 2013), even for adults (e.g. Hauptmanova *et al.* 2002, 2004; Davis *et al.* 2004; Friedl & Edler 2005; Ricklefs & Sheldon 2007; Davis *et al.* 2008; Maney *et al.* 2008; Vinkler *et al.* 2010). However, the available data indicates that in wild passerine birds, Hb ranges from 11.0-25.1 g/dl, and the RBC is within the limits of 2.95-5.77 x 106/mm^3 (Fourie & Hattingh 1983; Puerta *et al.* 1995; Llacuna *et al.* 1996; Heatley *et al.* 2013; Millaku *et al.* 2015; Nimra *et al.* 2023), which is slightly higher than our findings for the red-backed shrike nestlings. Juvenile birds typically have lower Hb, Ht and RBC values than adults (Heatley *et al.* 2013; Kaminski *et al.* 2014), reflecting the age-related increases in oxygen transport efficiency (Montesinos *et al.* 1997; Gayathri *et al.* 2004).

The WBC counts fell within the broad range reported for passerines (4.5-57.9 × 10^3/mm^3; Puerta *et al.* 1995; Nimra *et al.* 2023). Young birds often exhibit higher leukocyte counts than adults, possibly due to their initial immune system activation (Puerta *et al.* 1995). This study's findings provide valuable reference data for monitoring the health and viability of red-backed shrike populations in their core

European range (Lefranc 2022). With a stable population in Poland (Wardecki *et al.* 2021) but a declining trend across Europe (PECBMS 2024), such data is crucial for conservation efforts.

Author Contributions

Research concept and design, Collection and/or assembly of data, Data analysis and interpretation, Writing the article, Critical revision of the article, Final approval of article: E.K., A.G.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

Supplementary Materials to this article can be found online at:
http://www.isez.pan.krakow.pl/en/folia-biologica.html
Supplementary files:

SM.01. Table S1. Results of Generalised Linear Mixed Models (GLMMs) testing the effects of the brood size, Scaled Mass Index (SMI) and hatching date on the first three principal components (PC1-PC3).

SM.02. Table S2. Factor loadings of individual blood parameters on the first three principal components (PC1-PC3).

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