Age, sex and seasonal variation in the shape and size of erythrocytes of the alpine accentor, *Prunella collaris* (Passeriformes: Prunellidae)

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Abstract

The aim of our study was to analyse red blood cells from the high-altitude avian species alpine accentor (*Prunella collaris*). We caught 79 alpine accentors in the High Tatra Mountains (the West Carpathians, Slovakia) from 2001 to 2008. Blood samples were collected from the *vena ulnaris cutanea*. The cells and nuclei of juveniles tend to be wider and larger while adults, especially males, tend to have elongated (narrow) cells. The largest erythrocytes were found in winter and the smallest in summer, in June. Red blood cells with larger, elongated nuclei but overall smaller in size occurred at the beginning and the end of the breeding season, while larger, rounded cells with smaller nuclei were found in the middle of the breeding season. This kind of reshaping of erythrocytes of alpine accentors in summer is probably a response to hypoxia and elevated summer temperature in high mountains. As regards the length/width ratio, in August and September, erythrocytes were more elongated (both cell and nucleus) than in the other months. This study is probably the first assessment of seasonal effects on size and shape of red blood cells of bird species permanently living and breeding at high altitudes of the Palearctic mountains.

Keywords: Alpine accentor, blood, erythrocyte, size, shape

Introduction

The environmental stressors on endothermic vertebrates at high altitude are well understood: cold and hypoxic conditions interact to create a metabolically challenging environment within which constant energy production must be maintained in the face of reduced oxygen availability and increased thermal stress (Cheviron et al. 2008). In general, birds tolerate the conditions of high altitudes better than mammals do. Regarding flight demands, birds exhibit several adaptive features such as an efficient cross-current system in their airway, large tidal volume, short pulmonary circulatory time which maximizes the utilization of hematological factors in gas uptake, large respiratory surface areas and a thin air–blood barrier (Maina 2000a, 2000b; Figueroa et al. 2007).

The alpine accentor *Prunella collaris* (Scopoli, 1769) is a high-altitude bird species. It breeds in the alpine zone of mountains in the West Palearctic at an approximate elevation range of 1800 to 5000 m above sea level (asl), but is found in the West Himalayas and Pakistan at 3600–5000 m asl and is observed at almost 8000 m asl on Mount Everest (Hatchwell 2005). In the West Carpathians, it breeds from 1600 to 2600 m asl, often near the highest peaks (Dyrcz & Janiga 1997). Regardless of region, alpine accentors are closely associated with high mountains where habitats are not continuous, but rather have a patchy distribution. The size of the habitat patches available to accentors is determined by the physical and geographic characteristics of each individual mountain range. Because the alpine accentor is highly adapted to the high-mountain environment, hypoxia and low temperature might play an important role in the development and evolution of the respiratory and cardiovascular system of this bird species. Reconstruction of the biogeographic history of Prunellidae by Drovettskii et al. (2013) suggests that the origin of the species and its
initial diversification occurred within the Himalayan region. Then the genus dispersed out of the Himalaya and across the Palearctic from the mid to late Pliocene. Thus, *Prunella collaris* is today one of the oldest species of the Eurasian family Prunellidae.

The respiratory and cardiovascular modifications and the energetic demands in the high mountains may generate changes in hematological parameters (Carey & Morton 1976; Rosenmann & Ruiz 1993; Ruiz et al. 1995). Decrease in mean corpuscular volume (MCV) and increase in erythrocyte number are reported as the most common adaptations of blood to cold environment (Lechner 1976; Breuer et al. 1995; Ruiz et al. 1995). The variation in the number of erythrocytes usually generates changes in their size and shape. Measurement of erythrocyte size gives some idea of the surface area offered for exchange of gases with the plasma (Hartman & Lessler 1963). Small cell size and, as a consequence, a high surface area:volume ratio have been associated with a facilitated oxygen diffusion, an increase of the hemoglobin (Hb) concentration and an increase in erythrocyte count without increasing the blood viscosity (Promislow 1991; Rosenmann & Ruiz 1993). The Hb content of erythrocytes increases in direct proportion to their size (Hawkey et al. 1991). Larger erythrocytes contain more Hb than smaller ones. On the other hand, a strong negative association between the number and size of erythrocytes exists, and small erythrocytes require a greater circulating concentration of oxygen in blood in order to meet standard oxygen demand. The concentration of Hb differs across species (Kostelecka-Myrcha 1997), but depends on many other factors, e.g. age (Kostelecka-Myrcha et al. 1971), hormonal level (Puerta et al. 1995), season (Ronald & George 1988; Breuer et al. 1995), exposure to hypoxia (Maxwell et al. 1990b). Hb also participates in the innate immune response to pathogens (Bishlawy 1999; Liepke et al. 2003; Jiang et al. 2007).

Changes in the shape of erythrocytes can be deduced from nucleus:cell (N/C) and length:width (L/W) ratio. The red blood cells of birds are elliptical in shape with similarly shaped nuclei. Changes in shape of bird erythrocytes have been studied by many authors, focusing on species (Hartman & Lessler 1963; Ronald & George 1988; Palomeque et al. 1991; Bonatto et al. 2009; Gallo et al. 2015), age (Kostelecka-Myrcha et al. 1973, 1997), sex (Nowaczewski & Kontecka 2012) and body mass (Kostelecka-Myrcha et al. 1993; Kostelecka-Myrcha & Cholostiakow-Gromek 2001).

Red blood cells (RBC) also undergo a change in shape during development (Barrett & Scheinberg 1972). In immature forms the nucleus is rounder and paler (Claver & Quaglia 2009), and becomes more condensed with age. This study is probably the first in-depth assessment of seasonal effects on size and shape of RBCs of bird species permanently living and breeding at high altitudes. Moreover, we address the variation in cell morphology in relation to the sex and age of alpine accentors.

### Materials and methods

We caught 79 alpine accentors in the High Tatra Mountains (the West Carpathians, Slovakia) from 2001 to 2008 (number of birds caught: 2001 – 2, 2002 – 1, 2003 – 14, 2004 – 0, 2005 – 15, 2006 – 32, 2007 – 8, 2008 – 7). Birds were captured using mist nets, measured, weighed and colour-ringed. The juveniles (nestlings) were examined in the nest. Blood was collected by puncturing the *vena ulnaris cutanea*. A drop of blood was transferred onto a glass slide and thin blood smears were prepared in the field. Blood smears were air dried, fixed in methanol and stained according to Pappenheim (Doubek et al. 2003). The perimeter, length and width of 50 randomly chosen, but not deformed, erythrocytes and their nuclei, sampled from each individual, were measured using Micro Image software under 1000 x magnification. Statistical analysis was done using Statistica software v. 12. Erythrocyte perimeter, length and width, and nucleus perimeter, length and width were used in principal component analysis (PCA), which was calculated using a correlation matrix. PCA is widely used in morphological studies (Bartholomew 2007). It is a variable reduction technique that maximizes the amount of variance accounted for in the observed variables by a smaller group of variables called components. In morphological studies, PCA is a technique of static allometry which is purely morphological and concerns the measures of size and shape (Rohlf & Bookstein 1987). The first principal component (PC1) of a set of linear measurements provides an appropriate structural size measure. PC2 describes the largest shape variance. Other components follow similarly but in other directions of data variation than PC2. Closely related is the problem of how many PCs should be interpreted. In this type of morphometry, the procedure does not depend on a file of limiting assumptions (Yazdi 2014). We retained three explainable components. PC1 preserves the size relationships between the cells from different groups, while PC2 and PC3 mainly indicated the shape variability between the cells or changes in N/C and L/W ratios, respectively. Because the mean score showed a skewed distribution in some of the sample groups, a non-parametric approach to the analysis of
the data was necessary. Significant differences between groups of cells were verified using a Mann–Whitney rank sum test ($p < 0.05$).

### Results

A total of 3930 cells were measured (Table I) and subjected to PCA. Correlations with the original variables (component weights) and percentage of variance associated with the components are shown in Table II. Weights on PC1 represent general size. The most important information on shape is probably present in PC2, which shows a sharp antagonistic relationship between cell (cytoplasm) and nucleus variables. Additional information on shape, accompanied by 16% of variance, is present in PC3 and shows the antagonistic relationship between the length and width variables.

The erythrocytes of juveniles were significantly larger than those of adult birds (Figure 1) and they also differed in shape (Table III). The smaller, narrow cells of adults possess narrow but relatively large nuclei in comparison to the larger, round cells with smaller round nuclei of juveniles (PC2). Round cells of juveniles were relatively large compared to their relatively small, round nuclei.

Besides the effects of nucleus elongation during maturity (PC2), juveniles and adults significantly differed in nucleus and cell width variation (PC3). Juveniles had the widest erythrocytes and nuclei; in mature females they were slightly longer and adult males had the most elongated cells and nuclei (Table III, PC3).

Considering the general size in RBCs (PC1), the largest cells were found in adults in winter and the smallest in summer, in June (Table IV, PC1; Figure 2). Larger cells were more ellipsoid than smaller summer cells. This variation in shape was related to size variation.

Cells also varied in shape, independently of the general size. In adults, a bipolar variation between cell and nucleus characters was observed in seasons. Small elongated cells with relatively large elongated nuclei occurred in adults in winter, while large rounded cells with rounded but relatively small nuclei occurred in mid-summer (Table IV, PC2; Figure 3).

We observed a second change in cell shape, independent of that described above (PC2), which mainly involved a change in the width of the cell as well as the nucleus. PC3 (Tables II and IV; Figure 4) reflects another antagonistic variation – between widths and lengths of cells and nuclei. In August and September (the moulting period of adults in

| Variable          | EP ± SE | EL ± SE | EW ± SE | NP ± SE | NL ± SE | NW ± SE |
|-------------------|---------|---------|---------|---------|---------|---------|
| Juveniles n = 897 | 36.77 ± 1.37 | 14.38 ± 0.05 | 7.96 ± 0.05 | 16.22 ± 0.06 | 6.02 ± 0.02 | 3.43 ± 0.02 |
| Males n = 1883    | 34.35 ± 0.05  | 13.68 ± 0.02  | 6.80 ± 0.01  | 16.65 ± 0.03  | 6.50 ± 0.01  | 2.97 ± 0.01  |
| Females n = 1050  | 34.01 ± 0.07  | 13.43 ± 0.03  | 6.86 ± 0.02  | 16.52 ± 0.04  | 6.43 ± 0.02  | 2.96 ± 0.01  |

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Table I. Erythrocyte perimeter (EP), length (EL) and width (EW), and nucleus perimeter (NP), length (NL) and width (NW) of the alpine accentor (*Prunella collaris*) in µm. n = number of measured erythrocytes (two adult individuals of unrecognized sex are not included in the measurements); SE = standard error.

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Table II. Component weights (principal component scores – PC1, PC2, PC3) and percentage of variance associated with components indicating the size and shape of erythrocytes of alpine accentors (79 measured animals, total of 3930 measured cells).

| Variable          | PC1 (size) | PC2 (shape, Nucleus/Cell relationship) | PC3 (shape, Length/Width relationship) |
|-------------------|------------|----------------------------------------|----------------------------------------|
| Cell perimeter    | 0.86       | −0.36                                  | −0.301                                 |
| Cell length       | 0.76       | −0.23                                  | −0.555                                 |
| Cell width        | 0.69       | −0.50                                  | 0.369                                  |
| Nucleus perimeter | 0.64       | 0.72                                   | 0.081                                  |
| Nucleus length    | 0.43       | 0.84                                   | −0.14                                  |
| Nucleus width     | 0.72       | 0.004                                  | 0.607                                  |
| Variance (%)      | 48.6       | 28.1                                   | 15.5                                   |
Table III. Comparison of RBC size and shape between age–sex categories of alpine accentors. (77 birds; two adults of unrecognized sex were excluded from the matrix used in Table I). Size and shape are represented by means and standard deviation (SD) of principal component scores (PC1, PC2, PC3). Means with different letter indexes in a column are significantly different according to a Mann–Whitney rank sum test ($p < 0.05$).

| Variable (no. cells) | PC1 (SD) (size) | PC2 (SD) (Nucleus/Cell relationship) | PC3 (SD) (Length/Width relationship) |
|----------------------|----------------|--------------------------------------|--------------------------------------|
| Juveniles (897)      | 0.56 (1.41)$^a$ | −0.84 (1.14)$^a$                     | 0.31 (1.2)$^a$                       |
| Males (1883)         | −0.15 (0.72)$^b$ | 0.23 (0.58)$^b$                     | −0.22 (0.78)$^b$                     |
| Females (1050)       | −0.26 (0.69)$^c$ | 0.18 (0.62)$^b$                     | −0.05 (0.86)$^c$                     |

Table IV. Seasonal difference in the size and shape of erythrocytes of adult alpine accentors (61 animals, including two of unrecognized sex). Size and shape are represented by means (standard deviation, SD) of principal component scores (PC1, PC2, PC3). Means with different letter indexes in a column are significantly different according to a Mann–Whitney rank sum test ($p < 0.05$).

| Variable (no. cells) | PC1 (SD) (size) | PC2 (SD) (Nucleus/Cell relationship) | PC3 (SD) (Length/Width relationship) |
|----------------------|----------------|--------------------------------------|--------------------------------------|
| January (50)         | 0.26 (0.78)$^a$ | 0.24 (0.70)$^a$                     | 2.00 (1.10)$^a$                      |
| March (200)          | −0.05 (0.60)$^b$ | 0.34 (0.56)$^b$                     | 0.39 (0.65)$^b$                      |
| April (150)          | −0.05 (0.61)$^b$ | 0.11 (0.53)$^b$                     | −0.04 (0.62)$^b$                     |
| May (250)            | −0.05 (0.70)$^b$ | −0.05 (0.63)$^a$                   | 0.31 (0.67)$^b$                      |
| June (99)            | −0.56 (0.54)$^c$ | 0.11 (0.60)$^b$                     | −0.003 (0.84)$^c$                   |
| July (399)           | −0.23 (0.61)$^bc$ | 0.20 (0.58)$^a$                     | 0.10 (0.67)$^bc$                     |
| August (450)         | −0.04 (0.60)$^b$ | 0.10 (0.60)$^b$                     | −0.53 (0.81)$^b$                     |
| September (999)      | −0.29 (0.58)$^bc$ | 0.29 (0.58)$^a$                     | −0.38 (0.78)$^b$                     |
| October (436)        | −0.07 (1.03)$^b$ | 0.32 (0.56) $^a$                   | 0.003 (0.87)$^c$                     |

the Tatras), RBCs were more elongated (both cell length and nucleus length) than in the other months (PC3). In January, the cells and nuclei were round.

Discussion

Size and shape related to size

In altricial birds RBCs of juveniles are generally rounder, with less oval to round nuclei, and polychromatophilic cytoplasm. Immature erythrocytes of altricials change from a spherical to a flat form following the final mitotic division during development. Many studies provide evidence that initial changes in size and, mainly, in shape are influenced by conditions that affect the availability of oxygen. Flattening and deoxygenation of Hb are highly correlated (Barrett & Scheinberg 1963). In general, hematocrit (Hct), Hb, total surface area of erythrocytes (TSAE) and number of erythrocytes increase with increasing age, while size and MCV decrease (Kostelecka-Myrcha et al. 1971, 1973; Kostelecka-Myrcha & Myrcha 1989; Kostelecka-Myrcha & Jaroszewicz 1993, 1997; Potti 2007). The smallest erythrocytes were found in females of alpine accentors. Comparable differences between sexes of birds were noticed by Nowaczewski and Kontecka (2012). The hormone oestrogen inhibits erythropoiesis, reducing Hct, which means that the lower number of erythrocytes has to be compensated by changes in their size and shape. Erythrocytes are smallest in females; therefore, they should be able to exchange oxygen the most quickly and effectively.
Bird species of small body mass and relatively quicker and more intensive metabolic rate have small erythrocytes (Kostelecka-Myrcha et al. 1993). The erythrocyte difference between sexes depends, of course, on many other factors, such as metabolic demands during nesting or moulting, or hormonal levels. Probably this is the reason why some authors do not report differences in the size of RBCs between sexes of birds (Keçeci & Çöl 2011; Albokhadaim 2012; Dolka et al. 2014).

Besides sex and age, we found that season has an effect on the size and shape of RBCs. The largest erythrocytes were found in January, with a significant change in spring months. Recent theory predicts that the sizes of cells will evolve according to fluctuations in body temperature. Smaller cells speed metabolism during periods of warming but require more energy to maintain and repair. At a low temperature, when metabolism proceeds slowly, large cells should provide a sufficient surface to transport the metabolites required to sustain life (Adrian et al. 2016). Birds are in relative metabolic rest in winter. Demands on the supply of oxygen to the tissues are significantly lower than during the rest of the year. Erythrocyte size is at its upper limit, but food restriction may reduce size, MCV and mean corpuscular Hb (MCH) (Breuer et al. 1995; Kostelecka-Myrcha 1997; Kostelecka-Myrcha et al. 1997). This has been experimentally confirmed in chickens (Maxwell et al. 1990a). In alpine accentors, we found the largest erythrocytes in winter. There are experimental studies that demonstrate foraging preferences of *P. collaris*. In winter, it prefers food rich in lipids (Janiga & Novotná 2006). Lipids are a source of energy with a gradual release, so birds can survive longer without food. The results of our study thus confirm indirectly, via measurement of the size of RBCs, that there is not a major negative energy balance in *P. collaris* in winter.

In spring there is a significant increase in levels of gonadotropin hormone in the hypothalamus and stimulation of the gonads. The strong decrease in size of erythrocytes in March can be explained by the increased activity of birds in the early spring. This is also a time of photoperiod protraction; the level of sex hormones increases, males start singing (Janiga & Romanová 1997), metabolism accelerates and tissues need to be supplied with oxygen more effectively. A further decrease in size is recorded in June, just after the mating season and when days are longer. Hormonal changes in birds are most affected by photoperiod (lengthening or shortening of daylight).

In the breeding season (May–June), the size of RBCs is reduced even more. Egg-laying probably affects Hct. For a female, it is an energy-demanding period. It depends of course on the availability of food; when food is missing birds enter a negative energy balance and this condition may reduce Hct. Another theory attributes the decrease in Hct (before egg-laying) to vitellogenin, a yolk precursor in the plasma causing an increase in blood volume and a (relative) decrease in Hct (Morton 1994; Reynolds & Waldron 1999; Salvante & Williams 2002). The magnitude of this change in Hct is comparable to putative “adaptive” adjustments of Hct proposed to facilitate oxygen uptake and transfer during periods of intense metabolic activity (Saino et al. 1997a,1997b; Wagner et al. 2008).

It is not easy to explain why the erythrocytes of endothermic Alpine accentors are more spherical in summer than in winter. Shape change of nucleated erythrocytes from elliptical to circular form was investigated by Coiro et al. (1978) and Cohen et al. (1998). A 100% change of ellipsoid to spherical shape was attained with erythrocytes under high-temperature treatment. The authors report that temperature was responsible for depolymerization of microtubules in cells. In spherical erythrocytes the microtubules were not found, meaning that they are essential to maintain the ellipsoidal shape of cells (Coiro et al. 1978). Although extremely low environmental temperatures can reduce the internal temperature even of endothermic animals, the temperature drop is not so strong. Birds perform a physically demanding activity: flying. Cooling from the external environment is therefore compensated by the production of heat from working skeletal muscle. Reshaping of probably new erythrocytes in summer, as found in our study, should be affected by changing environmental temperature and consequently by change in the body temperature of birds.

**Nucleus:cytoplasm relationship and cell shape versus nucleus shape**

PC2 variation is not the size as in PC1; this type of shape change is independent of PC1 size variation. The shape of cells varies based on the change of the nucleus:cytoplasm ratio. One type of erythrocytes has smaller, round nuclei and more cytoplasm (lower N/C ratio) and cell shape is more spherical. We recorded these cells especially in juveniles. The second type of RBCs, an oval cell with oval and relatively large nuclei (higher N/C ratio), is present in adult birds.

The results of our study show that RBCs of juvenile birds adapt to their most important role in the body, the transport of oxygen through Hb, in bone marrow. Erythropoiesis has an important role in this
process. The erythroid cells can be found in the bone marrow of juveniles, whereas in adults they gradually decrease and the myeloid and thromboid cells increase (Taieb 2009). This is probably because in growing juveniles, it is important to increase the oxygenation of tissues, thus increasing the number of erythrocytes and changing their shape. It is possible that juvenile RBCs still contain remnants of ovarian Hb, which has a higher affinity for oxygen. This would explain a sufficient supply of oxygen to the tissues, although spherical erythrocytes do not appear to be very effective in the exchange of oxygen compared to oval and smaller RBCs of adults. As erythrocytes continue to mature, the cell and nucleus become more elongate (Campbell 1995).

A lower N/C ratio was mainly found for adults in May, and generally in summer. Three general factors determine cell shape: the state of the cytoskeleton, the amount of water that is pumped into a cell, and the state of the cell membrane. Nuclear shape changes can result from changes in: (i) cytoplasmic osmotic pressure, (ii) the activity of cytoskeletal motors (Kim et al. 2015), and (iii) the environmental conditions (Zhelev et al. 2006). Moreover, altered nuclear shape may be due to changes in the nuclear lamina (Webster et al. 2009).

RBCs of “summer” birds have more cytoplasm and a smaller nucleus compared to those of “winter” birds. It has been noted that a smaller and more condensed nucleus permits the existence of a proportionally larger cytosome and hence a greater content of Hb (Lucas & Jamroz 1961). The relatively large erythrocytes contain more Hb in summer than do the small erythrocytes in winter (Breuer et al. 1995); the content of Hb in bird cells also increases in the low-oxygen conditions (Maxwell et al. 1990b). We assume that this type of reshaping of erythrocytes of Alpine accentors in summer represents a response to hypoxia and elevated summer temperature in high mountains, since the Alpine accentor is an alpine species, which is well adapted to the high-altitude climate of continental Eurasian mountains. The species does not live in the arctic region. Some authors have shown Hb concentrations to be higher in native high-altitude birds than in birds adapted to low altitude (Carey & Morton 1976; Black & Tenney 1980).

Ellipsoid versus spherical cells – L/W relationship in RBCs (PC3)

The spherical small and spherical large cells with spherical nuclei were found mainly in juvenile birds. In adults, cells take on an ellipsoid shape that is most effective in the exchange of oxygen. Compared to that of males, female blood is probably less viscous because RBCs are more elongated in adult females than in males. This type of erythrocyte shape could distinguish individuals with an accelerated metabolism and an active way of life. Alpine accentors breed in polygynandrous groups; the role of females is crucial in the rearing of young birds. Birds nest and fly to high altitudes due to food availability; females moult 1 month later than males and they are very active metabolically.

In adults, the spherical cells were observed in winter. It has been proven that a prevalence of spherical erythrocytes is related to the presence of enough oxygen in the atmosphere and in the blood (Barrett & Scheinberg 1972). In January, due to food availability, P. collaris stay at lower altitudes. Here, partial pressure of oxygen in the atmosphere is higher compared to that at altitudes where they live in spring, summer and autumn. In winter, these individuals also slow down their metabolism (Janiga & Romanová 1997). In summer, and mainly in autumn, cells take on a shape that is most effective in exchange of oxygen. In this period, the blood is less viscous than in winter.

Collectively, this study on alpine accentors suggests that seasonal changes in RBC size and shape appear to be better correlated with the prevailing summer and winter conditions than with the particular altitudinal distribution of the examined populations. But synergic effects of temperature and low oxygen availability in the high mountains may be reflected in the N:C ratio of red blood cells.

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