

RESEARCH ARTICLE

Contrasting response of haematological variables between long-term training and short exercise bouts in zebra finches (*Taeniopygia guttata*)

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ABSTRACT

Physical aerobic activity is oxygen demanding, but – particularly for birds – there is still little understanding of how blood contributes to oxygen supply under various activity levels. In a two-factorial experimental design, we investigated the long-term effect of daily flight training and the immediate effect of a short exercise bout on a set of haematological variables: haemoglobin (Hb) content, haematocrit (Hct), and red blood cell number (RBC_{count}) and size (RBC_{area}) in zebra finches (*Taeniopygia guttata*). For a period of 6 weeks, birds were either trained daily for 3 h in a flight arena or remained untrained. Subsequently, half of each group was blood sampled either in the resting condition or after a 5 min exercise bout in a flight-hover wheel. We found significantly lower Hb content, Hct and RBC_{count} compared with that in untrained controls in response to training, while RBC_{area} did not differ between treatments. Response to an exercise bout revealed the opposite pattern, with significantly higher Hb content and Hct compared with that in non-exercised birds. Additionally, RBC_{area} was significantly smaller immediately after exercise compared with that in non-exercised birds, and such short-term flexibility represents a novel finding for birds. This contrasting response in erythrocyte characteristics with respect to long-term training and short exercise bouts appears as a clear pattern, presumably underlain by changes in water balance. We infer alterations of blood flow to be involved in adequate oxygen supply. During an exercise bout, RBC_{area} flexibility may not only enhance oxygen delivery through improved erythrocyte surface area to volume ratio but also improve blood flow through a compensatory effect on blood viscosity.

KEY WORDS: Birds, Exercise training, Acute exercise, Aerobic flight activity, Red blood cell variables

INTRODUCTION

Aerobic metabolism is the main pathway by which vertebrates meet their energy requirements, highlighting the importance of adequate oxygen supply to the tissues by cardiovascular and respiratory systems. In particular, blood directly contributes to oxygen transport in the bloodstream and its release into the cells (Snyder and Sheafor, 1999). Several variables tightly connected to red blood cells (RBCs) give an indication of the oxygen-carrying capacity of the blood: haemoglobin (Hb) content, haematocrit (Hct, the fraction of erythrocytes in whole blood), and the number and size of

erythrocytes (RBC_{count} and RBC_{area}, respectively). An adequate oxygen supply depends heavily on Hb as it is the oxygen-binding molecule in erythrocytes (Hawkey et al., 1991). However, the volume and size of erythrocytes influences the amount of Hb either per volume of erythrocytes or per cell (Kostelecka-Myrcha, 2002). All these haematological variables represent phenotypically flexible traits and their modifications are considered to be associated with changing energy requirements and hence oxygen demands in support of overall oxygen requirements (Norte et al., 2009; Piersma et al., 1996; Swanson, 1990).

Physical aerobic activity is an obvious example that imposes substantial oxygen demands above resting conditions. Sports medicine provides vast knowledge on adjustments in the cardiovascular system, including haematological variables (Bassett and Howley, 2000; Hellsten and Nyberg, 2016), with two common but contrasting responses: firstly, effects in response to regular long-term training and, secondly, immediate effects in direct response to exercise bouts (El-Sayed et al., 2005; Mairbörl, 2013). Regular training of different modes and intensity frequently leads to decreases in haematological variables, particularly Hct and Hb content, compared with pre-training values or those in untrained individuals (Puhl and Runyan, 1980; Dressendorfer et al., 1981; Green et al., 1991). By contrast, elevation of Hct, Hb content and/or RBC_{count} occurs as an immediate response to a single and intensive exercise bout (Novosadová, 1977; Konstam et al., 1982; Ernst et al., 1991). This latter pattern is often found in other mammal groups, in particular in dogs and horses, which have been extensively studied in terms of cardiovascular adaptations to physical activity. However, more variable responses are reported for training (Arokoski et al., 1993; Flaminio and Rush, 1998; Rovira et al., 2007). In both humans and other mammals, variation in the size of erythrocytes, measured as mean corpuscular volume, was observed after a single exercise event (Costill et al., 1974; van Beaumont et al., 1981; Smith et al., 1989). The response of haematological variables to either a short exercise bout or regular training appears largely consistent among mammals, with higher values of Hb content and Hct, and a greater number of cells in direct response to short exercise and lower values of the same variables in response to long-term training.

Flight is an energetically efficient mode of locomotion as birds fly a given distance with lower costs compared with running (Butler, 2016). However, per unit time, flying is more costly than running or swimming, making birds an ideal taxon to study the response of haematological variables to aerobic physical performance (Butler and Bishop, 2000). Nevertheless, how blood variables respond to training or exercise conditions is far less clear in birds than in mammals because of the low number of controlled studies and also as a result of fundamental differences in erythrocytes. On the one hand, zebra finches (*Taeniopygia guttata*) and homing pigeons (*Columba livia*) subjected to long-term training reveal responses

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similar to those observed in mammals, i.e. lowered Hct and Hb content (Riera et al., 1983; Birkhead et al., 1998). Accordingly, low values of Hb content, Hct and RBC_{count} are found during the migration period, when birds are generally challenged with energetic demands during long-lasting flights (Piersma et al., 1996; Landys-Ciannelli et al., 2002). On the other hand, the immediate response to single exercise performance involves either a decrease in Hct or no change, which was observed either after flights up to 1 h or after prolonged flights reaching 22 h in pigeon (*Columba livia*) and red knot (*Calidris canutus*) (Butler et al., 1977; Bordel and Haase, 1993; Carmi et al., 1993; Peters et al., 2005; Jenni et al., 2006). Finally, a recent report considering the response of haematological variables to increased energy in birds stands in contrast to the known responses for mammals (Niedojadlo et al., 2018). The study, performed on the same model species as here, suggests different constraints on oxygen supply through blood and showed lower Hct, Hb content and RBC_{count} in birds kept at a cold versus thermoneutral temperature, the opposite response to that in mammals. This discrepancy between birds and mammals might be driven by the presence of a nucleus in avian erythrocytes in contrast to the anucleate mammalian red blood cells (Niedojadlo et al., 2018). The cold-induced changes in haematological variables thus resemble those reported by us for the long-term response to training rather than those regarding short-term exercise bouts and clearly call for more studies on birds under controlled conditions of different energy use or requirements.

Here, we performed a two-factorial design experiment on zebra finches, *Taeniopygia guttata* (Vieillot 1817), under controlled conditions aimed at testing for long-term effects of daily flight training on haematological variables (versus untrained controls) and for the effect of birds being sampled either under resting conditions or immediately following a short bout of strenuous exercise. Based on the available data, we hypothesize that birds change haematological variables in response to short exercise bouts and long-term training: birds lower haematological variables (Hb content, Hct and RBC_{count}) in response to long-term training but increase haematological variables (Hb content, Hct and RBC_{count}) as an immediate response to short exercise bouts. For RBC_{area} , we predict smaller erythrocytes in response to long-term training and short exercise bouts compared with those of untrained controls and birds at rest.

MATERIALS AND METHODS

Experimental animals

We maintained 98 adult female zebra finches in two outdoor cages (1 m×1 m×1 m) with free access to millet grain and water from June to mid-September. In a two-factorial design, birds were subjected to either a 6 week period of flight training ($N=56$) or remained untrained ($N=42$). At the end of the 6 weeks, half of each group was blood sampled under resting conditions, while the other half was sampled immediately after a short exercise bout. All the procedures were approved by the Local Ethical Committee on Animal Testing in Krakow, Poland (no. 69/2014).

Training and exercise bouts

Training consisted of repeated short take-off flights in a special flight arena with a perch at each end (6 m×2 m×2.5 m; Bauchinger et al., 2010) for 90 min each in the morning and the afternoon (at 09:00 h and 15:00 h). During these training sessions, a researcher walked in the flight arena in circles in a single direction and chased the birds to perform take-off flights from one perch to the other simply by approaching the perch the birds had just landed on. A

single training session resulted in ca. 450 rounds, equating to 900 take-off flights of 6 m in length. For each training session, only the trained group of birds was released into the flight arena. Release of the birds into the flight arena and their return to the housing cage did not require any handling as birds were either gently encouraged or learned to fly between their housing cage and flight arena when the passage between them was opened. Neither trained nor untrained controls had access to food and water during this time and untrained birds stayed in their cage during training sessions. After the 6 week training period, half of each experimental group, trained and untrained control, was subjected to a 5 min exercise bout in a flight-hover wheel (Chappell et al., 1999; Wiersma et al., 2007). In our birds, this short exercise type represents a peak physical activity of on average 7 times basal metabolic rate (BMR) (A.B., J.N., E.T.S., M.C. and U.B., unpublished data). In contrast, we expected the flight training activity to be in a lower BMR range, probably lower than 5–7 times BMR, as is the case for prolonged flights performed in a wind tunnel (Lindström et al., 1999; Klaassen et al., 2000; Schmidt-Wellenburg et al., 2007). On the one hand, our experimental birds experienced 1–2 s of rest between take-off flights; on the other hand, such a mode of flight is more costly than cruising flight. All birds were naive to the flight-hover wheel to avoid potential habituation and behaviours that might result in flight avoidance or performing voluntary flight rather than intense physical effort. Before the start of the exercise bout, each individual bird was placed inside the wheel and covered with a cloth for 2 min. Then, the cloth was removed and an experimenter started to manually rotate the wheel until it reached a speed at which the bird could just manage to maintain its position within the wheel through hop and hover activity. Five pingpong balls inside the wheel additionally prompted birds to use active wing flapping. After exactly 5 min, we stopped the exercise and immediately removed the bird from the chamber and collected a blood sample. Blood collection (see below) from birds sampled after the exercise bout and in the non-exercised (at rest) state was carried out in the morning hours, so that birds from the trained group skipped their morning training session. Each day, only seven birds were sampled, resulting in 14 consecutive sampling days with one or two birds of each of the four experimental groups.

Blood sampling and analysis

Blood samples of 100 μl were collected from the jugular vein using syringes (0.33 gauge) and transferred to Eppendorf tubes using heparinized capillaries. Haematological analysis was conducted within 20 min of blood sampling. Four haematological variables were assessed: Hb content, Hct, RBC_{count} and RBC_{area} . In addition, we calculated Hb content per cell (pg) based on two variables: Hb content and RBC_{count} . Hb content (g l^{-1}) was measured using a portable HemoCue Hb 201+ photometer (HemoCue AB, Angelholm, Sweden). The validity of this method was assessed by cross-calibration with reference to Drabkin's method ($r=0.82$, $P<0.001$, $n=26$). To obtain Hct, capillaries filled with $\sim 9 \mu\text{l}$ of blood were centrifuged for 6 min at 11,500 rpm (Eickemeyer, Tuttlingen, Germany) and measured using a calliper (to the nearest hundredth of a millimetre). Number of cells (per μl) was assessed by use of a Bürker's haemocytometer chamber. In order to assess RBC_{area} , we used blood smears fixed in methanol for 5 min. To visualize cells, we performed standard haematoxylin and eosin staining, using eosin Y (Analab, Warszawa, Poland) and Gill II hematoxylin (Sigma Aldrich, Taufkirchen, Germany). Erythrocytes were then photographed under 100-fold magnification of a light microscope (Nikon Eclipse 801, Japan) connected to a camera (Nikon

DS-Ri2, Tokyo, Japan) and NIS Elements software (Nikon). Finally, we used ImageJ software (v1.45, USA) to analyse the area of the RBCs. The software was set to automatically measure cell area after image thresholding. Cells with distorted shape or cell membrane and those touching other cells were excluded from the measurements. For each bird, we determined the area for 40 cells and averaged the results. If possible, two repeats were performed for Hb content, Hct and RBC_{count} measurement and the mean was used in further analysis.

Statistical analysis

A mixed model approach was employed to investigate changes in haematological variables in response to a short exercise bout and long-term training. Each haematological variable was treated as a dependent variable in a separate model. Two treatments were included as independent categorical factors, namely whether birds were trained or untrained and whether they went through a short exercise bout. Additionally, the date of blood sampling (there were 14 days of sampling) was added as a random effect to the model. In each analysis, we tested the interaction between main factors, which was removed from the model as it was insignificant in all models. Prior to the analyses, normality of residuals was assured. Statistical analyses were performed in SAS (v9.4, SAS institute Inc., Cary, NC, USA).

RESULTS

Hb content, Hct and RBC_{count} were significantly lower in the trained group than in the untrained controls [trained versus control means \pm s.d. for Hct: $51.9 \pm 3.8\%$ and $54.3 \pm 4.3\%$, 4.5% difference, $F_{1,81}=9.88$, $P=0.002$, Fig. 1A; for Hb content: 193.8 ± 13.5 and 202.1 ± 15.2 g l⁻¹, 4.1% difference, $F_{1,81}=9.68$, $P=0.002$, Fig. 1B; for RBC_{count}: 5.4 ± 0.6 and 5.7 ± 0.8 ($\times 10^6$) μ l⁻¹, 5% difference, $F_{1,80}=4.40$, $P=0.039$, Fig. 1C]. There was no difference in RBC_{area} between trained and untrained birds (58.6 ± 2.8 and 57.9 ± 3.6 μ m², respectively, $F_{1,82}=1.11$, $P=0.296$, Fig. 1D). Birds sampled immediately after a short exercise bout had significantly higher Hct and Hb content than non-exercised control birds (exercised versus control means \pm s.d. for Hct: $54.3 \pm 4.4\%$ and $51.5 \pm 3.6\%$, 5.3% difference, $F_{1,81}=11.94$, $P<0.001$, Fig. 1E; for Hb content: 202.7 ± 15.3 and 192.0 ± 12.2 g l⁻¹, 5.2% difference, $F_{1,81}=15.73$, $P<0.001$, Fig. 1F) and this finding was independent from long-term

training, as indicated by the non-significant interaction term (for Hct: $F_{1,80}=0.88$, $P=0.349$, for Hb content: $F_{1,80}=0.01$, $P=0.925$). RBC_{count} was not different between the groups, but tended to resemble the pattern of Hct and Hb content (5.6 ± 0.8 and 5.4 ± 0.6 ($\times 10^6$) μ l⁻¹, $F_{1,80}=2.57$, $P=0.112$, Fig. 1G). RBC_{area} was significantly smaller in birds sampled after the short exercise bout than in individuals sampled at rest (57.4 ± 2.9 and 59.1 ± 3.2 μ m², 2.8% difference, $F_{1,82}=4.51$, $P=0.036$, Fig. 1H); this was independent from long-term training as indicated by the non-significant interaction term ($F_{1,81}=0.03$, $P=0.856$). Lastly, there was no difference in Hb content per cell, either between training or among the short-term exercise treatment birds, after removal of the non-significant interaction term (for training: $F_{1,80}=0.02$, $P=0.88$; for exercise: $F_{1,80}=0.22$, $P=0.643$).

DISCUSSION

Haematological variables of zebra finches revealed a contrasting response to long-term training on the one hand and to a short exercise bout on the other hand. Daily training resulted in lower Hb content, Hct and RBC_{count} compared with that of untrained controls, whereas birds sampled immediately after a short exercise bout revealed higher values of Hct and Hb content in comparison to birds sampled at rest. The effects of training and exercise did not interact; both untrained and trained birds changed rather similarly, suggesting a uniform physiological response to short bouts of exercise. A novel finding was that erythrocytes were significantly smaller in birds subjected to a short and single exercise bout than in those that had not experienced short-term physical activity, presumably caused by changes in water balance. We interpret this change as a flexible response in RBC size following the definition of phenotypic flexibility by Piersma and Drent (2003). Erythrocyte size can be portrayed as a reversible trait just like other blood compounds and properties including blood viscosity (Ernst et al., 1991; El-Sayed et al., 2005).

Our results are in line with the limited data on Hct and/or Hb content in trained birds, performed either in a flight arena or after regular homing flights (Birkhead et al., 1998; Riera et al., 1983). Our findings also resemble reports for low Hct and Hb content for trained versus sedentary mammals (El-Sayed et al., 2005; Davis, 2009). The mammalian response in haematological variables to

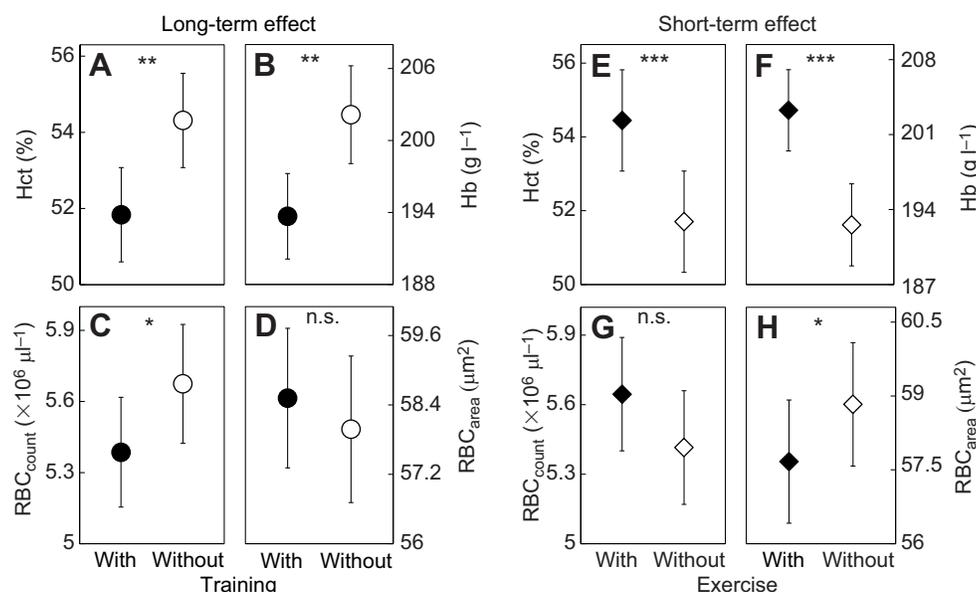


Fig. 1. Long- and short-term effects of training and exercise, respectively, on haematological variables in zebra finches. (A–D) Zebra finches were trained for 6 weeks in a flight arena or untrained. (E–H) Birds were subjected to a short exercise bout or allowed to rest. Hb, haemoglobin content; Hct, haematocrit; RBC_{count}, red blood cell count; and RBC_{area}, red blood cell area. Data are presented as least square means \pm 95% confidence interval. Asterisks indicate significant differences between groups: * $P<0.05$, ** $P<0.01$ and *** $P<0.001$; n.s., not significant. $N=56$ trained zebra finches, $N=42$ untrained controls, $N=49$ birds in the short exercise condition, $N=49$ birds in the resting condition.

training might be due to plasma volume expansion (Hellsten and Nyberg, 2016), a process that improves cardiovascular functioning especially through more efficient work of the heart (Convertino, 1991). Plasma volume expansion probably also underlies the observed decrease in avian haematological variables. Taken together, we infer a similar response of haematological variables to regular daily training in all endotherms studied in this respect.

The response to short-term exercise in zebra finches also resembles that reported for mammals (Cordova et al., 1993; Connes et al., 2006; Zobba et al., 2011), i.e. mammals likewise showed elevated Hct and Hb content immediately after exercise compared with that of individuals at rest. However, our study is in contrast to previously reported decreases in avian Hct in response to single flights (Butler et al., 1977; Peters et al., 2005), which might be due to the specific type and intensity of exercise (wind tunnel and homing flights versus our flight-hover wheel exercise). Energy requirements are dependent on flight mode (Butler, 2016) so RBC variables may be differently adjusted to flight-specific energy expenditures. Besides the response in Hb content and Hct, we also revealed a concomitant decrease in erythrocyte size in birds sampled immediately after a short bout of exercise. Such a decrease in cell size, measured as mean corpuscular volume, has previously only been shown for humans immediately after a short or even prolonged, but single exercise (Costill et al., 1974; van Beaumont et al., 1981; Stäubli and Roessler, 1986). Water homeostasis that involves fluid withdrawal from the blood vessels, and subsequently also from erythrocytes, is the most likely cause for our results on zebra finches, but we cannot fully exclude other factors such as psychological stress.

The haematological properties – described herein by four haematological variables – probably affect aerobic performance either directly through effects on the amount of transported oxygen per volume of blood or indirectly through effects on blood viscosity and ultimately blood flow properties. A direct positive association between RBC_{count} (also applies to Hct and Hb content) and oxygen transport per volume of blood through a higher binding capacity for oxygen can generally be assumed. In the case of RBC_{area}, however, a negative relationship to oxygen supply of the blood can be expected based on changes in surface area to volume ratio, which in turn is proposed to affect oxygen uptake and release (Kozłowski et al., 2003). Such a negative relationship between erythrocyte size and maintenance metabolism is indeed documented at the interspecific level for birds and geckos (Gregory, 2002; Starostová et al., 2009). Concerning the indirect effects, both erythrocyte number (also applies to Hct) and size would be positively related to blood viscosity. Higher numbers and larger particles increase the thickness and thus viscosity of a liquid (Nguyen et al., 2007). Such effects on blood viscosity may be viewed in the context of the known training and/or exercise effects on other cardiovascular traits such as heart beat frequency and stroke volume (Hellsten and Nyberg, 2016). Diluting the blood may be important for optimizing oxygen transport through the cardiovascular system. Training in this study translated into a 5% lower viscosity compared with that of the untrained birds (calculated based on the regression equation after Zhou et al., 1998). This effect is reversed during short-term exercise, which also involves the shrinkage of the RBCs (see Fig. 1E,H).

Our two-factorial study design allowed us to isolate clear and contrasting responses of blood variables to either long-term training or short exercise bouts. We put forward two different effects associated with the observed patterns. The first represents an additive effect of higher Hct, RBC_{count} and smaller erythrocytes in terms of enhanced oxygen supply at the moment of peak aerobic metabolism during

short intense exercise. The second effect is based on compensation through different haematological variables affecting blood viscosity and, in consequence, blood fluidity. Increased viscosity through higher RBC_{count} (or Hct) might be offset by decreased viscosity due to smaller erythrocytes. RBC variables probably respond differently to challenges in oxygen demands in order to adjust the oxygen carrying capacity of the cardiovascular system.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.B., J.N., E.T.S., U.B., M.C.; Methodology: A.B., J.N., E.T.S., U.B., M.C.; Validation: U.B.; Formal analysis: A.B., E.T.S., U.B., M.C.; Investigation: A.B., J.N., E.T.S.; Resources: E.T.S., U.B., M.C.; Writing - original draft: A.B.; Writing - review & editing: A.B., J.N., E.T.S., U.B., M.C.; Visualization: A.B., E.T.S.; Supervision: E.T.S., U.B., M.C.; Project administration: E.T.S., U.B.; Funding acquisition: U.B.

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Data availability

The raw data are available from figshare (Bury et al., 2019): <https://doi.org/10.6084/m9.figshare.c.4392689.v1>

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