REPORT FROM A BOU-FUNDED PROJECT

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Linking variation in individual red blood cell size and mitochondria to variation in life history in the Collared Flycatcher *Ficedula albicollis*

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BACKGROUND

The rate at which animals oxidize substrates for energy production, the so-called metabolic rate, is expected to have strong cascading effects on the rate at which animals grow, reproduce and age. However, we still know very little about proximate mechanisms linking metabolism to life history (Flatt and Heyland, 2011).

A first well-known source of variation in animal metabolism and life history is body size, with larger species having a lower metabolism and slower pace of life (i.e. slower growth and reproduction). Interestingly, variation at the whole organism level may be partially explained by variation in size (and number) at the cellular level. Indeed, an increase in cell size leads to a decrease in cell surface/volume ratio, which in turn can lead to a decrease in energetic needs required for the maintenance of ionic gradient maintenance across cell membrane (Szarski, 1983). Accordingly, numerous studies have reported strong allometric scaling rules in vertebrates from cell size, usually using red blood cells (RBCs) as proxy, to developmental rate, body size and mass-corrected metabolic rate (Promislow, 1991; Gregory, 2002; Kozłowski et al., 2003). Those studies have been carried mostly across species, and thus it remains unclear whether similar relationships between RBC size and number and life history are found within-species (Maciak et al., 2014).

Secondly, because mitochondria are the cellular powerhouse of the organism, variation in mitochondria number and function is also expected to have strong cascading effects on metabolism from the cell to the whole organism level, and in turn on life history traits. Surprisingly, we know very little on the links between cell size and mitochondria number and function. Furthermore, because measures of mitochondria number and function is often very invasive (i.e. it usually requires the harvesting of tissues on culled individuals), the links between mitochondrial function and life history have rarely been investigated (Jimenez et al., 2014a,b).

This project builds on the recent finding that birds, in contrast to mammals, possess functional mitochondria in their RBCs (Stier et al., 2013), thus offering unique opportunities to examine mitochondrial attributes (i.e. number and function) using low invasive procedures (i.e. blood sampling instead of surgery or culling). Of course, blood sampling gives also prime access on RBC size and number. In the present study, we measured RBC size and mitochondria in a natural population of Collared Flycatchers (*Ficedula albicollis*) in order to (*i*) test for relationships between cell size and mitochondrial number and function, (*ii*) investigate the importance of genetics and environment in shaping variation in RBC size and mitochondria, and finally (*iii*) study links between variation in RBC size,mitochondria and life history.

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METHOD SUMMARY

Data were collected from May to July 2015 in a population of collared flycatchers breeding in the southern part of the Swedish island of Gotland (57°10'N, 18°20'E). This population is part of a long-term monitoring (> 35 years), and more information on the study site can be found in Doligez et al. (1999). Nests were monitored throughout the breeding season to collect information on reproduction.
On the second day after hatching, broods hatched on the same day were matched in pairs, and half of the hatchings were swapped within a pair of nests. This cross-fostering experiment allows comparing siblings raised in different nests and in so doing to separate genetic and early maternal effects occurring before hatching from environmental effects occurring after hatching (see also Voillemot et al., 2012).

Adult parents were caught and blood sampled during food provisioning of nestlings. Nestlings were measured and blood sampled at 12 day-of-age. Blood samples were collected from the brachial vein using a heparin-lithium coated Microvette® (CB300 LH, Sarstedt). Immediately after collection, 15µL of whole blood were transferred into a 500 µL of 1× Hunk's Buffered Salt Solution (HBSS) and gently homogenized before being stored at 5°C in the field until being processed at the end of the day in a field laboratory. RBCs in HBSS solution were washed at their arrival in the laboratory by centrifuging samples for 10 min to pellet cells, discarding the supernatant, and re-suspending cells in 1mL fresh 1× HBSS. RBC size and number were determined using a *Scepter™ 2.0 Cell Counter* (Merck Millipore, Germany; Martin-Ramirez *et al*., 2012). RBCs in HBSS solution were then stored at -80°C before being shipped on dry ice to Aberdeen, UK, for further laboratory analysis of mitochondrial attributes. We measured ATP levels within RBCs using the ATP Bioluminescence Assay HS II Kit (Roche Applied Science, Mannheim, Germany, cat# 699709001), following the manufacturer's protocol. We measured mitochondrial density using the specific fluorescent probe nonyl acridine orange (NAO, cat# A1372, Life Technologies). NAO is specifically binding to the polyunsaturated acidic phospholipid cardiolipin that is only found in the inner mitochondrial membrane. Additional measurements of mitochondrial attributes are currently under development.

In this report, we used measures of RBC size and number collected during fieldwork in adults and nestlings. We restricted our analyses of RBC mitochondrial attributes (ATP concentration and mitochondrial density) to samples on adults measured after fieldwork in the laboratory in Aberdeen. Blood samples of nestlings still need to be analysed in the laboratory.
The cross fostering experiment and blood sampling were conducted under a licence from the Swedish National Board for Laboratory Animals, and bird catching and manipulating under a ringing licence from the Bird Ringing Centre of the Swedish Museum of Natural History (Stockholm, Sweden).

MAIN FINDINGS

In nestling and adult collared flycatchers, the mean number of RBC cells per mL significantly declined with increasing RBC size (Figure 1). It indicates that individuals with larger RBC cells have fewer circulating cells, which is similar to what have been previously reported across species in various taxa (Stier al., 2015). Note that nestlings have fewer cells than adults.

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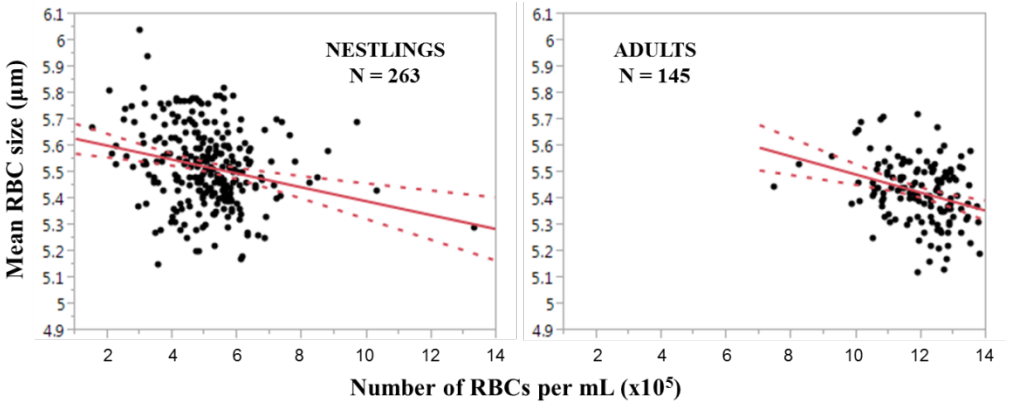

Figure 1. Relationship between RBC number and size in 12-day-old nestlings and in adult collared flycatchers. The best fitted regression lines (solid) and 95% confidence intervals (dashed) are presented in red. Nestlings: estimate ± s.e. = -2.64 e-7 ± 6.75 e-8, t = -3.91, p = 0.0001, r^2 = 0.06. Adults: estimate ± s.e. = -3.43 e-7 ± 8.53 e-8, t = -4.02, p < 0.0001, r^2 = 0.10.

Interestingly, in adult flycatchers (data on nestlings are not available yet) we found a significant negative relationship between ATP levels and mitochondrial density (Figure 2). It indicates that RBCs with greater amount of mitochondria were enclosing lower amount of ATP. This result is counterintuitive: ATP is produced by mitochondria, and thus a positive relationship was expected. Mitochondrial density was measured with the NAO fluorescent dye, and some studies pointed out that this dye can be influenced by the inner mitochondrial potential (Jacobson et al., 2002). Hence, an alternative explanation is that individuals with highly uncoupled mitochondria (high NAO signal) had lower amount of ATP (Brand, 2000). This scenario is however unlikely because cells and membranes were lysed before the measurements, thus cancelling out effects of mitochondrial potential. Additional markers of mitochondrial number and function are needed to get a better understanding of this relationship.

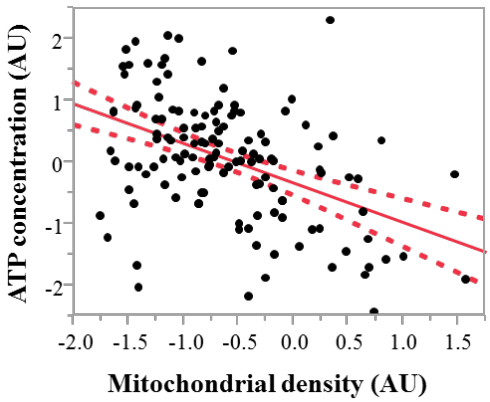

Figure 2. Relationship between ATP concentration and mitochondrial density. Data are from 133 adult flycatchers. The best fitted regression line (solid) and 95% confidence interval (dashed) are presented in red. Estimate ± s.e. = -0.64 ± 0.11, t = -5.79, p <0.0001, r^2 = 0.20. AU: arbitrary unit.

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The amount of mitochondria increased with RBC size, whereas the amount of ATP showed the opposite relationship (Figure 3). We found no evidence that the number of RBCs influenced mitochondrial density and ATP concentration (results not shown). Larger cells are expected to require less energy for ionic maintenance (Szarski, 1983), which is concordant with the finding that ATP levels are declining with cell size.

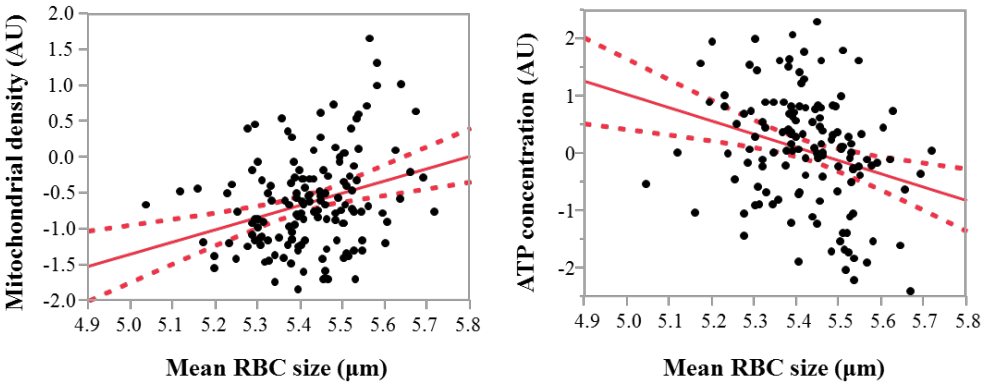

Figure 3. Influence of RBC size on mitochondrial density (left panel) and ATP concentration (right panel). The best fitted regression lines (solid) and 95% confidence interval (dashed) are presented in red. Mitochondrial density: n = 143, estimate ± s.e. = 1.72 ± 0.47, t = 3.69, p = 0.0003, r^2 = 0.10. ATP : n = 134, estimate ± s.e. = -2.32 ± 0.70, t = -3.29, p = 0.0013, r^2 = 0.08. AU: arbitrary unit.

Table 1. Results of random models where nestling tarsus length, weight, RBC diameter and number were entered as dependent variable, and where the nest of origin, the nest of rearing and the pair of nest were entered as random explanatory variables. Statistical analyses were performed on 222 nestlings distributed in 25 pairs of nests. Abbreviations: V_P: total phenotypic variance, V_{origin}: variance explained by the nest of origin, $V_{rearing}$: variance explained by the nest of rearing, h^2 = heritability estimate, c = variance explained by the rearing environment.

	V_P	V_{origin}	$V_{rearing}$	pair	error	h^2	c
Tarsus length	0.765	0.172	0.196	0.024	0.201	0.451	0.255
Body weight	3.497	0.289	1.753	0.000	1.166	0.166	0.501
RBC diameter	0.027	0.003	0.005	0.006	0.012	0.187	0.178
RBC count	2.08E+10	4.39E+08	5.33E+09	6.53E+08	1.4E+10	0.042	0.256

Blood sampling of nestlings in the cross-fostering experiment was restricted to pairs of nests where both nests had still nestlings alive 12 days after hatching (n = 222 nestlings in 25 pairs of nests). Preliminary results show low to moderate heritability level (h^2 = 19%) in RBC diameter; the heritability of RBC number was closed to 0 (h^2 = 4%). For comparison, heritability levels were 45% for tarsus length and 17% for body weight. Research in captive animals showed high and significant heritability of most RBC parameters, with h^2 comprised between 41 and 72% (Stier et al., 2015).The present study provides the first estimates from a wild population. The lower estimates reported in this study when compared

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to captive species are likely to come from important effects of the environment in shaping RBCs parameters. Accordingly, the nest of rearing explained 18% of the variance in RBC size and 26% in RBC count. The important amount of variance in RBC size, associated with significant amount of additive genetic variance, indicates that this trait can respond to selection.

Finally, we found that variation in nestling RBC size was significantly related to tarsus length but not body weight (Figure 4), whereas RBC number was related to nestling weight (and body condition) but not tarsus length (Figure 5). The positive association between body condition and RBC number indicates that the latter can be interpreted as measure of "condition". The negative relationship between RBC size and tarsus length indicated that smaller nestlings have larger RBCs. Because cellular metabolic rate is declining with cell size, and because RBC size may reflect cell size in other tissues (Kozłowski et al., 2010), this negative relationship may be explained by individuals with larger cells having the slowest metabolism and developmental rate.

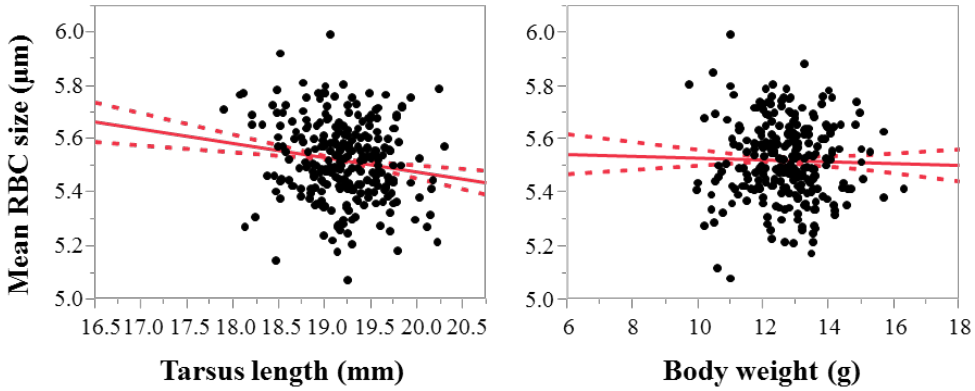

Figure 4. Relationship between RBC size and tarsus length (left panel) and body weight (right panel) in 12-day-old nestling collared flycatchers. The best fitted regression lines (solid) and 95% confidence interval (dashed) are presented in red. Tarsus length: estimate ± s.e. = -0.054 ± 0.014, t = -3.75, p = 0.0002. Body weight: t = -0.61, p = 0.54.

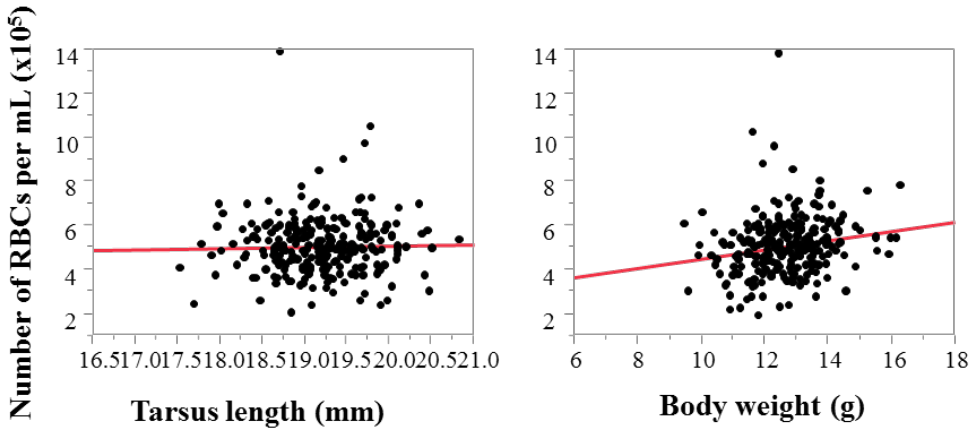

Figure 5 Relationship between RBC number and tarsus length (left panel) and body weight (right panel) in 12-day-old nestling collared flycatchers. The best fitted regression lines (solid) is presented in red. Tarsus length: t = -0.45, p = 0.65. Body weight: estimate ± s.e. = 21039 ± 5908, t = 3.56, p = 0.0004.

CONCLUSION

In the present study, we describe novel methods to measure cell size and number and mitochondrial attributes in RBCS. This approach allowed us to shed new lights on inter-dependence between cell size and mitochondrial function, and on the cascading effects of cell size and mitochondria on life history in birds.

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