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Evolutionary biology

Nestling erythrocyte resistance to oxidative stress predicts fledging success but not local recruitment in a wild bird

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Stressful conditions experienced by individuals during their early development have long-term consequences on various life-history traits such as survival until first reproduction. Oxidative stress has been shown to affect various fitness-related traits and to influence key evolutionary trade-offs but whether an individual's ability to resist oxidative stress in early life affects its survival has rarely been tested. In the present study, we used four years of data obtained from a free-living great tit population (*Parus major*; $n = 1658$ offspring) to test whether pre-fledging resistance to oxidative stress, measured as erythrocyte resistance to oxidative stress and oxidative damage to lipids, predicted fledging success and local recruitment. Fledging success and local recruitment, both major correlates of survival, were primarily influenced by offspring body mass prior to fledging. We found that pre-fledging erythrocyte resistance to oxidative stress predicted fledging success, suggesting that individual resistance to oxidative stress is related to short-term survival. However, local recruitment was not influenced by pre-fledging erythrocyte resistance to oxidative stress or oxidative damage. Our results suggest that an individual ability to resist oxidative stress at the offspring stage predicts short-term survival but does not influence survival later in life.

1. Introduction

Survival until reproduction, as a determinant of individual fitness, strongly depends on the conditions experienced by individuals in their early life [1]. In birds, post-fledging survival is well known to mostly depend on fledging date and body mass at fledging [2,3] but has also recently been shown to depend on cell-mediated immunity [4] or glucocorticoid-mediated stress response [5], thereby suggesting a strong influence of physiological processes at early age on future survival.

Oxidative stress, defined as an imbalance between the formation of reactive oxygen species and the antioxidant response in favour of the former [6], has been identified as a physiological constraint affecting fitness-related traits [7]. The ability of an individual to cope with oxidative stress during early development might have long-lasting consequences for survival, but this has rarely been tested.

At early stages of growth, individuals are hypothesized to be exposed to oxidative stress owing to their fast development and their exposure to intense sibling competition [8,9]. This was empirically demonstrated by manipulations of sibling competition or growth rate, which translated into increased oxidative damage [10], reduced antioxidant levels [11] or reduced erythrocyte resistance

Table 1. Summary of the GLMMs testing for an effect of (*a*) erythrocyte resistance to oxidative stress (253 nests, 1658 nestlings), and (*b*) oxidative damage (141 nests, 790 nestlings, year 2010 only), on fledging success and recruitment probability. Fledging success was not recorded in 2008. Models were reduced using a backward selection procedure. Terms retained in the final model are highlighted in italic. Values of terms not retained in the final model are those immediately prior removal.

	fledging success			recruitment probability		
	estimate ± s.e.	$\chi^2_{\text{d.f.}}$	*p*-value	estimate ± s.e.	$\chi^2_{\text{d.f.}}$	*p*-value
(a) erythrocyte resistance to oxidative stress	*2.64 ± 1.12*	*5.56_1*	*0.018*	*0.27 ± 0.33*	*0.66_1*	*0.42*
body mass	*0.55 ± 0.15*	*12.59_1*	*<0.001*	*0.22 ± 0.07*	*9.54_1*	*0.002*
brood size	0.34 ± 0.95	0.13_1	0.72	0.03 ± 0.08	0.14_1	0.71
laying date	0.05 ± 0.25	0.04_1	0.85	*−0.04 ± 0.02*	*3.08_1*	*0.08*
sex[a]	*−1.09 ± 0.64*	*2.93_1*	*0.09*	0.22 ± 0.23	0.93_1	0.33
year[b]	0.49 ± 3.76	0.02_1	0.90	*2.48 ± 1.07*	*9.36_2*	*0.01*
				1.93 ± 1.09		
year[b] × erythrocyte resistance to ox. stress	3.86 ± 7.11	0.30_1	0.59	−0.50 ± 4.18	1.32_2	0.52
				−1.28 ± 4.19		
(b) oxidative damage	*0.52 ± 2.03*	*0.07_1*	*0.80*	*1.37 ± 0.86*	*2.52_1*	*0.11*
body mass	*0.53 ± 0.15*	*11.88_1*	*<0.001*	*0.38 ± 0.12*	*9.71_1*	*0.002*
brood size	0.24 ± 0.66	0.14_1	0.71	0.13 ± 0.19	0.47_1	0.49
laying date	0.08 ± 0.16	0.23_1	0.63	−0.05 ± 0.05	0.85_1	0.36
sex[a]	*−0.99 ± 0.62*	*2.56_1*	*0.10*	0.30 ± 0.43	0.50_1	0.48

[a]Relative to female siblings.
[b]Estimates given for 2010 relative to 2009 (fledging success), and for 2009 and 2010 relative to 2008 (recruitment probability).

to oxidative stress [12], although some studies failed to detect such effects [13,14]. For these reasons, and because oxidative stress has been reported to predict life expectancy in some studies of birds [15,16], one might expect individual resistance to oxidative stress at an early age to explain variance in offspring fledging success and recruitment probability, both major correlates of offspring survival [17]. A link between pre-fledging oxidative damage and recruitment probability was reported in the long-lived European shag *Phalacrocorax aristotelis* and provides the first piece of evidence of a long-lasting effect of oxidative processes [18]. However, additional tests of this hypothesis on different biological systems are mandatory to draw general conclusions about long-lasting effects of oxidative stress on survival.

In the present study, we used data collected in a free-living great tit population (*Parus major*; $n = 1658$ nestlings) over a four-year period to test whether pre-fledging resistance to oxidative stress (measured as erythrocyte resistance to oxidative stress and oxidative damage to lipids) predicts fledging success and/or recruitment probability.

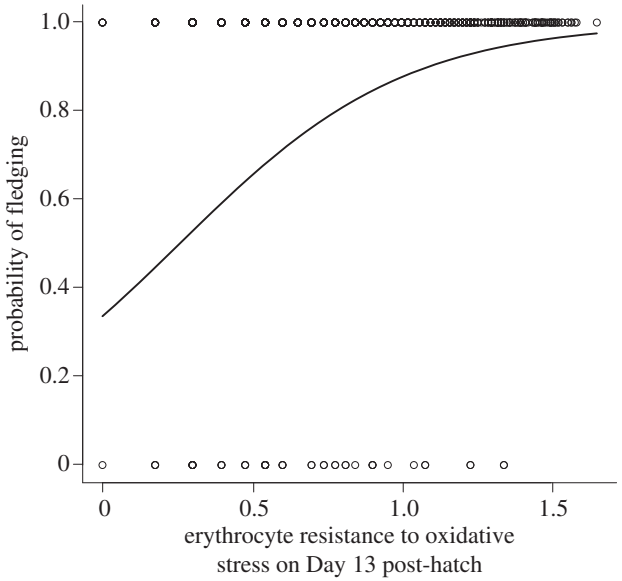

Figure 1. Probability of fledging in relation to offspring erythrocyte resistance to oxidative stress ($\log_{10}$-transformed minutes) on day 13 post-hatch. The fitted line shows the fledging probability estimated from GLMM.

2. Material and methods

Data on offspring resistance to oxidative stress and morphology were collected during spring 2008, 2009 and 2010 in natural populations of great tits breeding in nest-boxes in a forest near Bern, Switzerland (46°7′ N, 7°8′ E). On day 13 post-hatch, we measured offspring body mass (±0.1 g) and took a 30 µl blood sample from the brachial vein to assess their resistance to oxidative stress measured as erythrocyte resistance to oxidative stress (all years, $n = 1658$) and oxidative damage to lipids (2010 only, $n = 790$). Nestlings were also sexed using primers 2917/3088 [19]. Fledging occurs on day 17–20 post-hatch, and post-fledging care lasts for about 15–20 days [20].

Each subsequent breeding season (springs 2009, 2010 and 2011), we captured breeding adults while they were feeding their nestlings using clap-traps in the same forest to assess recruitment probability. Overall, we sampled 1658 nestlings, of which 99 (6.4%) recruited the following year into the same study populations. For data, see the electronic supplementary material.

(a) Erythrocyte resistance to oxidative stress

For all individuals, we assessed erythrocyte resistance to a free-radical attack using the KRL (Kit Radicaux Libres) test (see the electronic supplementary material for details).

(b) Oxidative damage to lipids

For the 790 individuals born in 2010, we also estimated the plasma levels of malondialdehyde (see the electronic supplementary material for details).

(c) Statistical analyses

We tested whether oxidative stress predicted (i) fledging success (whether a nestling fledged or died before fledging) and (ii) recruitment probability (whether a nestling recruited or not the subsequent year) using generalized linear mixed models with a binomial error distribution and a logit link function. Sex of the nestlings, erythrocyte resistance to oxidative stress, oxidative damage, body mass, brood size, laying date, breeding year and the interaction between year and erythrocyte resistance to oxidative stress were included as fixed factors. Identity of the nest was fitted as a random factor and was year-specific to avoid pseudo-replication. For each dependent variable, we ran one model including oxidative damage ($\log_{10}$ transformed), considering year 2010 only, and one model excluding oxidative damage, considering all years.

3. Results

Fledging success and recruitment probability were strongly positively related to nestling body mass prior to fledging (table 1). Erythrocyte resistance to oxidative stress significantly predicted fledging success with birds with superior pre-fledging erythrocyte resistance to oxidative stress being more likely to fledge (figure 1), but did not predict recruitment probability (table 1). Oxidative damage to lipids did not predict fledging or recruitment probabilities (table 1). Erythrocyte resistance to oxidative stress and oxidative damage to lipids were not significantly correlated ($F_{1,778} = 2.39$, $p = 0.12$), and nor was nestling body mass with any of the measures of oxidative stress ($F_{1,788-1640} < 1.08$, $p > 0.29$).

4. Discussion

In a four year study on free-living great tits, we showed that resistance to oxidative stress, measured in terms of erythrocyte resistance to free radical attack, significantly predicted individual fledging success, providing evidence that individual ability to resist oxidative stress predicts short-term survival. However, we did not detect a link between pre-fledging resistance to oxidative stress and local recruitment.

Offspring body mass was a strong predictor of fledging success and recruitment rate in our study, as shown before [2,3], which potentially reflects a competitive advantage before [21] and/or after [22] fledging, a higher capacity to resist food shortage [2] or social dominance [23].

We found a positive link between pre-fledging erythrocyte resistance to oxidative stress and fledging success, corroborating the few studies reporting a link between resistance to oxidative stress and survival [15,16]. This result has important evolutionary implications because fledging success is a main component of offspring (and parent) fitness [24], and because it reveals substantial selection pressure on juvenile resistance to oxidative stress. Furthermore, given that most nestling mortality occurs before day 13 post-hatch, a stronger relationship may be expected between fledging success and resistance to oxidative stress measured shortly after hatching. Nestling resistance to oxidative stress likely depends on their ability to monopolize antioxidant resources provided by the parents [13], but also on individual growth rate [12], or on the presence of parasites [25], or environmental pollution [26].

Pre-fledging resistance to oxidative stress did not predict recruitment probability, suggesting that greater nestling resistance to oxidative stress does not provide post-fledging selective advantage in our study system. This result contrasts with a recent study on the long-lived shag [18]. The discrepancy might reflect the strong post-fledging selection on body condition in our model species and/or the extremely high predation risk occurring shortly after fledging in passerines [27], which may mask any advantage of greater nestling resistance to oxidative stress. Alternatively, our results could reflect an absence of correlation between pre- and post-fledging resistance to oxidative stress, which deserves further investigation.

Our study cannot discriminate between dispersal and post-fledging survival. However, given the absence of evidence for dispersal beyond our studied local population despite intensive sampling in several surrounding populations over 20 years (H. Richner 1992–2012, unpublished data), the well-known preference of great tits for nest-boxes over natural holes [28], and the fact that some of the nest-boxes remain empty every year, it is unlikely that we missed many recruits. Therefore, local recruitment may be taken as a reasonable proxy for survival to breeding age.

In summary, our study provides evidence in a wild bird for selection acting before but not after fledging on the basis of nestling ability to resist oxidative stress and suggests that some components of resistance to oxidative stress are related to short-term survival during offspring growth.

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Electronic Supplementary Material: details of the assays used to assess resistance to oxidative stress

Erythrocyte resistance to oxidative stress

Individual ability to resist oxidative stress was assessed using the KRL test purchased from Brevet Spiral (Couternon, France; http://www.nutriteck.com/sunyatakrl.html) adapted to bird physiological parameters (1). This assay provides a quantitative measurement of the whole blood resistance to oxidative stress as it assesses the time required to haemolyse 50% of red blood cells of the sample when exposed to a controlled free radical attack. It reflects the current availability of total antioxidant defences (enzymatic and non-enzymatic) as well as the past oxidative insults experienced by red blood cells (2, 3), and also indicates the rates of lipid peroxidation in the erythrocyte membrane (4). Briefly, 7µl of whole blood were immediately after sampling diluted in 255.5 µl of KRL buffer (150 mM Na^+, 120 mM $Cl^–$, 6 mM K^+, 24 mM $HCO3^-$, 2 mM Ca^{2+}, 340 mOsM, pH 7.4) and stored at 4°C before analysis 6.2 ± 4 hours after blood collection. We loaded 80 µl of KRL-diluted whole blood into wells of a 96-well microplate. We subsequently added to each well 136 µl of a 150 mM solution of 2,2-azobis- (amidinopropane) hydrochloride (AAPH; a free radical generator; 646 mg of [2,2′-azobis-(amidinopropane) hydrochloride] diluted in 20 ml of KRL buffer (5)). The microplate was subsequently read with a microplate reader spectrophotometer (PowerWave XS reader, Witec Ag, Switzerland) at 40°C. The rate of haemolyse was determined by the change in optical density measured at 540 nm (6). Readings were made every 3.5 minutes for 80 minutes and the microplate was shaken immediately before each reading to prevent cells from settling at the bottom of

the wells. The repeatability of the method, assessed using samples from individual great tits that were not included in the present study, was high ($r = 0.78$, $p < 0.001$, $n = 80$).

Oxidative damage to lipids

Plasmatic concentrations of malondialdehyde (MDA), formed by the β-scission of peroxidized fatty acids, were assessed using HPLC with fluorescence detection, as described previously (7) with some modifications. All chemicals were HPLC grade, and chemical solutions were prepared using ultra pure water (Milli-Q Synthesis; Millipore, Watford, UK). Sample derivitization was done in 2 ml capacity screw-top microcentrifuge tubes. To a 5µl aliquot of sample or standard (1,1,3,3-tetraethoxypropane, TEP; see below) 5 µl butylated hydroxytoluene solution (0.05% w/v in 95 % ethanol), 40 µl phosphoric acid solution (0.44 M), and 10 µl thiobarbituric acid (TBA) solution (42 mM) were added. Samples were capped, vortex mixed for 5 seconds, then heated at 100°C for exactly 1 hour in a dry bath incubator to allow formation of MDA-TBA adducts. Samples were then cooled on ice for 5 minutes, before 80 µl n-butanol was added and tubes were vortex mixed for 10 seconds. Tubes were then centrifuged at 13,000 rpm and 4 °C for 4 minutes, before a 55 µl aliquot of the epiphase was collected and transferred to an HPLC vial for analysis. Samples (40 µl) were injected into a Dionex HPLC system (Dionex Corporation, California, USA) fitted with 260 a 2 µm pre-column filter and a Hewlett-Packard Hypersil 5µ ODS 100 x 4.6 mm column maintained at 37°C. The mobile phase was methanol-buffer (40:60, v/v), the buffer being a 50mM anhydrous solution of potassium monobasic phosphate at pH 6.8 (adjusted using 5M potassium hydroxide

solution), running isocratically over 3.5 min at a flow rate of 1 $ml.min^{-1}$. Data were collected using a fluorescence detector (RF2000; Dionex) set at 515 nm (excitation) and 553 nm (emission). For calibration, a standard curve was prepared using a TEP stock solution (5 µM in 40% ethanol) serially diluted using 40% ethanol. TEP standards were assayed in triplicate and showed high repeatability ($r = 0.99$, $P < 0.0001$, $n = 12$). We also observed high repeatability of the method on a subsample of individuals, including individuals that are not part of this study ($r = 0.98$, $p < 0.001$, $n = 32$). SL did all analyses blindly with respect to treatments.

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