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SHORT COMMUNICATION

Higher *in vitro* resistance to oxidative stress in extra-pair offspring

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Abstract

Oxidative stress is considered to act as a universal physiological constraint in life-history evolution of animals. This should be of interest for extra-pair paternity behaviour, and we tested here the prediction that offspring arising from extra-pair matings of female great tits show higher resistance to oxidative stress than within-pair offspring. Resistance to oxidative stress, measured as the whole blood resistance to a controlled free-radical attack, was significantly higher for extra-pair offspring as predicted although these were not heavier or in better body condition than within-pair offspring. Since resistance to oxidative stress has been suggested to enhance survival and reproductive rates, extra-pair offspring with superior resistance to oxidative stress, be it through maternal effects or paternal inheritance, may achieve higher fitness and thus provide significant indirect fitness benefits to their mothers. In addition, because oxidative stress affects colour signals and sperm traits, females may also gain fitness benefits by producing sons that are more attractive (sexy-sons hypothesis) and have sperm of superior quality (sexy-sperm hypothesis). Heritability of resistance to oxidative stress as well as maternal effects may both act as proximate mechanisms for the observed result. Disentangling these two mechanisms would require an experimental approach. Future long-term studies should also aim at experimentally testing whether higher resistance to oxidative stress of EP nestlings indeed translates into fitness benefits to females.

Introduction

Extra-pair paternity is a common phenomenon among socially monogamous species, but its adaptive significance remains a debated issue (Akcay & Roughgarden, 2007; Griffith, 2007). It is widely accepted to increase male fitness (Trivers, 1972; Birkhead & Møller, 1992) whereas the nature of the benefits to females remains elusive (Westneat & Stewart, 2003; Kempenaers, 2009). Since oxidative stress is suspected to affect various fitness-related traits and shape the evolution of life-history traits (Dowling & Simmons, 2009; Monaghan *et al.*, 2009; Costantini *et al.*, 2010), it may also influence extra-pair mating strategies.

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Oxidative stress is an imbalance between reactive species and antioxidants in favour of the former (Sies, 1991), leading to an excess of reactive species, which increases the rate at which oxidative damage to biological molecules (lipids, nucleic acids, proteins) is generated (Costantini & Verhulst, 2009). Oxidative stress is an important factor all along an animal's life because virtually all activities generate oxidative stress. Among them are reproductive activities (Salmon *et al.*, 2001; Alonso-Alvarez *et al.*, 2004) and immune activation (Sorci & Faivre, 2009). Oxidative stress is also hypothesised to be a major proximate cause of ageing and senescence (Finkel & Holbrook, 2000) and of reduced survival (Bize *et al.*, 2008) and is thus a universal threat against which all organisms have evolved several lines of defence (Halliwell & Gutteringe, 2007). However, within-species, individuals show significant variation in their ability to resist oxidative stress (Costantini & Verhulst, 2009; Monaghan *et al.*, 2009), which may result in substantial fitness variation. Finally, resistance to oxidative stress is suspected to have some

genetic basis (Vermeulen *et al.*, 2005; Khazaeli *et al.*, 2007), and substantial heritability of the antioxidant capacity has recently been reported in the yellow-legged gull (Kim *et al.*, 2010a).

Therefore, females may accrue indirect benefits by producing offspring with higher resistance to oxidative stress through extra-pair copulations with males having higher resistance to oxidative stress. Owing to the universality of oxidative stress as a pervasive threat in all living organisms, producing extra-pair offspring possessing greater resistance to oxidative stress could be a widespread benefit driving the evolution of female alternative reproductive strategies. An advantage for EP offspring in the ability to resist oxidative stress is to be predicted since many studies have identified various fitness-related traits for which EP offspring outperform their WP half-siblings (Kempenaers *et al.*, 1997; Garvin *et al.*, 2006; Dreiss *et al.*, 2008; Fossoy *et al.*, 2008; Magrath *et al.*, 2009). We studied in a natural population of great tits (*Parus major*) the hypothesis that extra-pair (EP) offspring have higher *in vitro* resistance to oxidative stress than within-pair (WP) offspring.

Materials and methods

Data on nestling resistance to oxidative stress and growth were collected during spring 2008 and 2009 in a natural population of great tits breeding in nest boxes in a forest near Bern, Switzerland (46°7′N, 7°8′E). Nest boxes were regularly visited from the beginning of the breeding season to finally determine the start of egg laying and hatching dates. All parents were captured on day 15 post-hatch and blood-sampled for paternity analyses. No adults, neither males nor females, were captured in both years, thus avoiding pseudo-replication.

Nestling morphological measurements

In 2008, we sampled all 287 nestlings from 50 nests on day 15 post-hatch. In 2009, we sampled all 677 nestlings from 79 nests on day 13 post-hatch. We measured their body mass (±0.1 g) and tarsus length (±0.05 mm), and collected a 20 μL blood sample from the brachial vein to assess their *in vitro* resistance to oxidative stress.

Paternity analyses

Blood samples of the nestling and the parents were transferred into 500 μL of absolute Ethanol. Fifty (2008) and 79 (2009) families were analyzed for paternity at 11 microsatellite loci (PmaC25, PmaCAn1, PmaD105, PmaD22, PmaGAn27, PmaGAn30, PmaTAGAn71, PmaTAGAn86, PmaTGAn33, PmaTGAn42 and PmaTGAn45; (Saladin *et al.*, 2003). We used CERVUS 3.0 software package (Kalinowski, 2007) to calculate allele frequencies, heterozygosity values, exclusion probabilities, and deviation from Hardy–Weinberg equilibrium based on the genetic data of 231 (2008) and 141 (2009) adult great tits of both sexes captured in the same study area. Our population did not deviate significantly from Hardy–Weinberg equilibrium at any of the 11 loci. Exclusion power of all loci was 0.99983 (2008) and 0.99980 (2009) for the first parent and 0.999998 (2008) and 0.999998 (2009) for the second parent. Nestlings were categorized as within-pair if all their loci matched those of their candidate social father, or if we found maximally one mismatch. They were considered extra-pair if their genotype mismatched their putative social father's genotype at two or more loci. Nestlings were sexed using the sexing primers 2917/3088 (Ellegren, 1996). We did not find any cases of intra-specific brood parasitism, and all nestlings within a brood shared the same mother.

Nestling resistance to oxidative stress

Nestling whole blood resistance to a controlled free-radical attack was assessed using the KRL test purchased from Brevet Spiral (Couternon, France; http://www.nutriteck.com/sunyatakrl.html) adapted to bird physiological parameters (Alonso-Alvarez *et al.*, 2004). This assay reflects the current availability of total antioxidant defences (enzymatic and non-enzymatic; Lesgards *et al.*, 2002), the past oxidative insults experienced by red blood cells (Esterbauer & Ramos, 1996; Brzezinska-Slebodzinska, 2001), and the degree of lipid peroxidation in the erythrocyte membrane (Zou *et al.*, 2001). This assay thus likely integrates both a measure of the oxidative damage undergone by blood cells in a recent past and a measure of antioxidant capacity. In other words it measures the current ability of red blood cells to resist oxidative stress owing to their current susceptibility to oxidative stress. Briefly, immediately after sampling, 7 μL of whole blood were diluted in 255.5 μL of KRL buffer (150 mM Na^+, 120 mM Cl^-, 6 mM K^+, 24 mM HCO_3^-, 2 mM Ca^{2+}, 340 mOsm, pH·7.4) and stored at 4°C before analysis that occurred 6.2 ± 4 h after blood collection. The time elapsed before performing the analyses did not influence the results ($F_{1,315} = 0.02$, $P = 0.90$). 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; Rojas Wahl *et al.*, 1998). 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 (Bertrand *et al.*, 2006). Readings were made every 3.5 min for 80 min and the microplate was shaken immediately before each reading to prevent cells from settling at the bottom of the wells. The initial amount of red blood cells, measured as the initial optical density, was not correlated with cell

half-life i.e. our measure of whole blood resistance to oxidative stress ($F_{1,315} = 0.001$, $P = 0.97$), and also did not differ in relation to paternity status ($F_{1,315} = 1.84$, $P = 0.18$).

Statistical analyses

We used restricted maximum-likelihood linear mixed-effects models to analyse nestling body mass, tarsus length and natural log-transformed *in vitro* resistance to oxidative stress. We only included broods that contained at least one EP nestling in order to compare EP to WP half-siblings. Models included year, paternity status, sex of the nestling, and both the interaction of paternity status × year and paternity status × sex as fixed factors, brood size and laying date as covariates, and nest identity as a random factor. Following a backward elimination procedure, the two-way interactions included in these models were subsequently removed if not significant (significance level set to 5%).

Results

Over the 2 years, 107 out of 964 nestling (11%) were sired by EP males in 44 of 129 broods (34%). In 2008, 13 of 50 broods (26%) contained at least one EP nestling, and in 2009 31 of 79 broods (39%). Nestling *in vitro* resistance to oxidative stress significantly depended on nestling paternity status ($F_{1,\ 274} = 6.75$, $P = 0.01$, see Table 1) with EP nestlings showing significantly higher resistance to a controlled free-radical attack than their half-siblings (see Fig. 1). Nestling body mass and tarsus length were not influenced by paternity status (see Table 1).

Discussion

Our results provide the first evidence that extra-pair offspring have better resistance to oxidative stress, measured as the whole blood resistance to a controlled free-radical attack, than their within-pair half-siblings. Since many fitness-related traits likely depend on individual ability to circumvent oxidative stress, a higher *in vitro* resistance to oxidative stress by EP offspring may translate into higher fitness of the offspring and their mothers.

Oxidative stress is known to affect various fitness-related traits (see 'Introduction') and individuals with higher resistance to oxidative stress should be selected (Benzie, 2000; Dowling & Simmons, 2009; Monaghan *et al.*, 2009). Considering that at least some components of adult resistance to oxidative stress are determined early in life (Blount *et al.*, 2003), higher resistance to oxidative stress of EP offspring may translate into higher fitness in both sexes, and further translate into direct and indirect fitness benefits to their mothers. In addition, because oxidative stress affects both sperm quality (Helfenstein *et al.*, 2010) and carotenoid-based traits (Mougeot *et al.*, 2010), females may also gain indirect fitness benefits through higher attractiveness (sexy-sons hypothesis; Weatherhead & Robertson, 1979) and higher sperm competitive ability (sexy-sperm hypothesis; Keller & Reeve, 1995) of extra-pair sons. Given that resistance to oxidative stress can show relatively high heritability (Kim *et al.*, 2010a, 2011), we can reasonably expect a genetic contribution to the difference we observed between EP and WP offspring. However, only few studies investigated the genetic heritability of resistance to oxidative stress and the genetic correlations between resistance to oxidative stress and life-history traits (Olsson *et al.*, 2008; Kim *et al.*, 2010b) and further investigations are thus needed to determine exactly whether and how resistance to oxidative stress is heritable.

Alternatively, maternal effects might also explain the observed result. First, differential allocation of antioxidants into the eggs by females (Sheldon, 2000) may lead

Table 1 Linear mixed models testing for an effect of nestling paternity status on whole blood resistance to oxidative stress, body mass and tarsus length in mixed broods including 349 nestlings. Terms retained in the final model are highlighted in bold. *F* and *P* values of the interactions are those immediately prior removal. Sample size varies between the analyses because not all individual could be measured for oxidative stress.

	Resistance to oxidative stress			Body mass			Tarsus length		
Effect	Estimate ± SE	F_{df}	*P*	Estimate ± SE	F_{df}	*P*	Estimate ± SE	F_{df}	*P*
(Intercept)	0.74 ± 0.88	–	–	16.55 ± 2.20	–	–	20.70 ± 1.13	–	–
Laying date	**0.02 ± 0.01**	**$2.40_{1,36}$**	**0.13**	**0.005 ± 0.03**	**$0.03_{1,39}$**	**0.86**	**−0.01 ± 0.015**	**$0.92_{1,39}$**	**0.34**
Brood size	**0.02 ± 0.04**	**$0.27_{1,36}$**	**0.61**	**−0.38 ± 0.11**	**$12.79_{1,39}$**	**<0.001**	**−0.06 ± 0.06**	**$1.13_{1,39}$**	**0.29**
Paternity*	**0.21 ± 0.08**	**$6.75_{1,274}$**	**0.01**	**0.11 ± 0.12**	**$0.78_{1,304}$**	**0.38**	**0.08 ± 0.08**	**$1.10_{1,304}$**	**0.29**
Sex†	**0.09 ± 0.07**	**$1.77_{1,274}$**	**0.18**	**0.70 ± 0.10**	**$47.32_{1,304}$**	**<0.001**	**0.55 ± 0.07**	**$69.29_{1,304}$**	**<0.001**
Year	**−0.36 ± 0.20**	**$3.11_{1,36}$**	**0.09**	**3.22 ± 0.48**	**$45.76_{1,39}$**	**<0.001**	**0.35 ± 0.24**	**$2.00_{1,39}$**	**0.17**
Paternity* × Year	−0.24 ± 0.19	$1.57_{1,273}$	0.21	−0.52 ± 0.30	$2.95_{1,303}$	0.09	−0.19 ± 0.20	$0.94_{1,303}$	0.33
Paternity* × Sex†	0.12 ± 0.15	$0.60_{1,272}$	0.44	−0.12 ± 0.23	$0.29_{1,302}$	0.59	−0.06 ± 0.15	$0.164_{1,302}$	0.69

*Relative to the within-pair siblings.
†Relative to the female nestling.

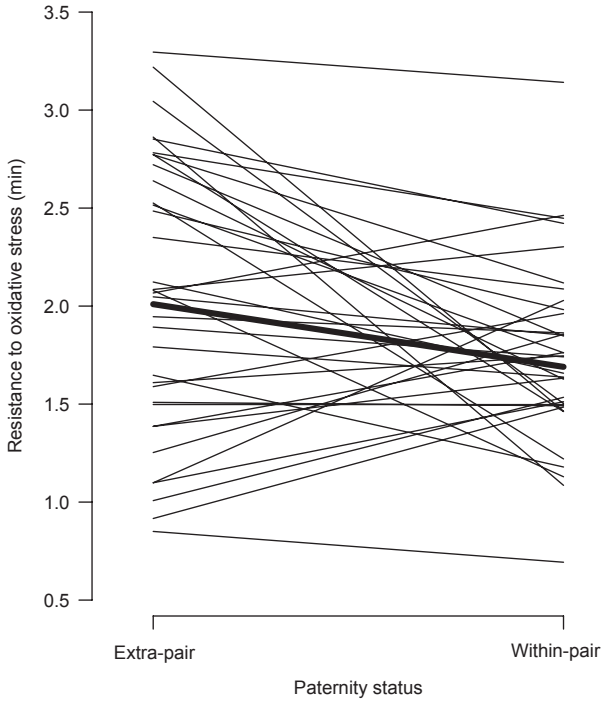

Fig. 1 Average within-nest nestling resistance to oxidative stress (natural log-transformed whole blood resistance to a controlled free-radical attack) in relation to their paternity status; extra- and within-pair. Each *line* represents one nest. The *line in bold* represents the average for both groups.

to higher levels of circulating antioxidants and thus higher resistance to oxidative stress (Surai, 2007) in EP nestlings. Second, EP offspring seem to be over-represented early in the laying sequence (Magrath *et al.*, 2009) and therefore to be developmentally more advanced than WP half-sibling of the same age. Hatching asynchrony may thus allow EP offspring to better resist oxidative stress since antioxidant systems develop gradually and are crucially influenced by early conditions (Surai, 2002; Blount *et al.*, 2003). Paternal inheritance of resistance to oxidative stress and maternal effects are two potential, non-mutually exclusive mechanisms for the observed result. Disentangling these two mechanisms would require an experimental approach.

If the higher resistance to oxidative stress of extra-pair offspring was at least partly inherited from their extra-pair father, this would select for female choice of extra-pair mates based on signals of male resistance to oxidative stress. Interestingly in great tits, males advertise their resistance to oxidative stress *via* the intensity of their carotenoid-based colouration (Losdat *et al.*, 2011). The benefits females could accrue by mating with more colourful males advertising their resistance to oxidative stress are of several types. First, females may gain direct benefits by having their eggs fertilized with sperm carrying less oxidative damages, therefore avoiding the risk of producing infertile eggs. Females may also produce more viable embryos since oxidative damages to sperm DNA can translate into deleterious mutations in the zygote (Tremellen, 2008; Velando *et al.*, 2008). Second, assuming heritability of the resistance to oxidative stress (Kim *et al.*, 2010a), females may enjoy indirect fitness benefits by producing offspring with superior resistance to oxidative stress that might translate into higher survival and reproduction (see above).

Here, we measured nestling resistance to oxidative stress as the erythrocyte resistance to a ROS-induced haemolysis. This measure was found to significantly correlate with reproductive effort and to predict survival in zebra finches (Alonso-Alvarez *et al.*, 2004, 2006), as well as survival of male and fecundity of female alpine swifts (Bize *et al.*, 2008), and thus appears to be ecologically and evolutionary relevant. However, it has to be kept in mind that additional markers of oxidative status are needed to draw a comprehensive picture of an individual's ability to face oxidative stress (Costantini & Verhulst, 2009; Hõrak & Cohen, 2010). Long-term experimental studies including both the oxidative damage to bio-molecules and the rate at which free-radicals are generated should allow to determine whether nestlings with higher resistance to oxidative stress indeed enjoy higher reproductive and survival prospects.

In conclusion, our results provide the first evidence that extra-pair offspring may have better resistance to oxidative stress than their within-pair half-siblings. Given that oxidative stress is a physiological constraint in most aerobic organisms, female extra-pair matings may be more widely explained by the benefits arising from extra-pair young with superior resistance to oxidative stress.

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