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## Environmental and genetic components of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*)

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**Abstract** In this study, we estimated the environmental and genetic components of two variables related to avian oxidative stress using wild nestlings of the Eurasian kestrel (*Falco tinnunculus*). The study was carried out during two breeding seasons. In the first season, we assessed the between- and within-nest resemblance in serum reactive oxygen metabolites (ROMs) and total serum antioxidant barrier (OXY). In the second season, we carried out a cross-fostering experiment to determine the importance of environmental and genetic factors on ROMs and OXY. The 23.5% of ROMs variance was explained by the nest of origin, indicating a main genetic component. In contrast, the 52.8% of OXY variance was explained by the nest of rearing, indicating that this variable was more influenced by environmental components. These findings suggest that variations in ROMs and OXY could reflect, respectively, the expression of different genetic polymorphisms and differences in dietary uptake of antioxidants.

**Keywords** Antioxidants · Cross-fostering · Free radicals · Heritability · Oxidative damage

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### Introduction

The cumulative oxidative damage to macromolecules caused by pro-oxidant compounds is considered causally related to cellular senescence and death (Harman 1956, 1972; Beckman and Ames 1998). Organisms cope with pro-oxidant (or oxidant in chemical terms) production by means of antioxidant enzymes and molecules, many of which are derived from the diet (e.g. vitamins and carotenoids). The balance between pro-oxidants and antioxidant defences determines the degree of oxidative stress, which is closely related to ageing, life span and fitness (Finkel and Holbrook 2000). Metabolic activity continuously generates highly reactive pro-oxidant compounds (e.g. reactive oxygen species, ROS), which in turn generate reactive oxygen metabolites (ROMs), i.e. early peroxidation products of the exposure of biological macromolecules (lipids, proteins, nucleic acids) to pro-oxidants.

The levels of oxidative stress can be affected by environmental components. For example, surplus food intake has been shown to increase free radical production and promote peroxidation and degenerative processes, while dietary restriction has been shown to extend life span (Weindruch and Walford 1988; Simic and Bergtold 1991; Sohal and Weindruch 1996; Masoro 2002). In fact, dietary restriction, i.e. reduction of nutrient intake without malnutrition, is the only known manipulation able to lower the ageing rate, by decreasing mitochondrial ROS generation (Sohal et al. 1994; Gredilla et al. 2001) without substantially changing antioxidants (Sohal et al. 1994).

The genetic architecture (i.e. the number of loci influencing a trait and the nature of the interactions between alleles within and between loci; Merilä and Sheldon 1999) has also been shown to affect oxidative stress. For example, genetic polymorphisms in mammalian biotransformation enzymes can affect the individual’s ability to metabolise different compounds and are correlated with increased risk of toxicity owing to

their generation of free radicals (reviewed in Daly et al. 1993).

The above-mentioned studies have shown a variety of potential mechanisms underlying oxidative stress. However, the relative contribution of the environment and genetic architecture in birds are not known. Therefore, we carried out a study to estimate the effects of the environmental and genetic components on the variance of two variables related to avian oxidative stress (Costantini et al. 2006): the serum concentration of reactive oxygen metabolites (i.e. early oxidative damage; ROMs) and the total serum antioxidant barrier (OXY). We used wild nestlings of the Eurasian kestrel (*Falco tinnunculus*), a small monogamous raptor for which the relationship between these two variables and their correlates have been recently studied (Costantini et al. 2006). This study was carried out during two breeding seasons: in the first season, we evaluated the between- and within-nest resemblance in ROMs and OXY values and in the second breeding season, we performed a cross-fostering experiment to partition the variance of ROMs and OXY into environmental and genetic components.

## Materials and methods

### Field study

The study was carried out during the breeding seasons of 2003 and 2005 in a 1,200-km<sup>2</sup> area around Rome, where the population of kestrels (*Falco tinnunculus*) breeding, in nest boxes mounted on the utility lines of two local electric power companies, has been studied since 1998. Nests were distributed in different habitats, but mostly in cultivated (cereals fields) and uncultivated areas (fallow fields and pastures). In both the breeding seasons, the nests were visited regularly starting from the end of March in order to assess occupation and to record clutch size, hatching date and brood size. In 2003, we evaluated the natural variability and degree of resemblance (within-nest vs. among-nest variance) among siblings for ROMs and OXY by collecting data from 67 nests [the correlates of oxidative stress are presented in Costantini et al. (2006)]. In 2005, we performed a cross-fostering experiment to partition the variance of ROMs and OXY into environmental and genetic components. The nestlings were cross fostered when the oldest chick in the brood was 7 days old, which assured us all chicks had hatched and avoided disturbance during the hatching span which could provoke stress (Wiebe and Bortolotti 1994). The exchanged chicks were of similar size and the broods were matched by hatching date and habitat. The remaining nestlings were also manipulated to control for potential handling stress. The brood size was not altered, so as to maintain a constant breeding effort and intranest nestling competition (Potti et al. 1999). Fifteen days later, a sample of blood (400 µl) was

drawn from the brachial vein. The samples were kept cool (0–5°C) until centrifugation, which occurred within a few hours, and the serum was kept at -20°C until laboratory analyses. In total, we used 16 nests (76 nestlings), which provided 68 samples for ROMs (8 samples were discarded because the sera clotted) and 76 samples for OXY.

### Measurement of reactive oxygen metabolites

Reactive oxygen species (ROS) are extremely reactive with macromolecules such as lipids, proteins and nucleic acids. These form derivatives that can maintain oxidising properties such as reactive oxygen metabolites (e.g. hydroperoxides, ROOH), which are able to propagate the oxidation cascade. The increase of ROMs concentration in the circulating system is a consequence of the release of pro-oxidants from intracellular compartment or an inflammatory process via an immune response (e.g. Ames et al. 1993). The toxicity of ROMs is promoted by the presence of metals such as iron (Fe<sup>2+</sup> and Fe<sup>3+</sup>) and copper (Cu<sup>+</sup> and Cu<sup>2+</sup>). These metals catalyse the cleavage of ROOH, leading to the generation of two highly reactive and histolesive pro-oxidants, namely the alkoxyl (R-O•) and alkylperoxyl (R-OO•) radicals (e.g. Halliwell and Gutteridge 1989; Leffler 1993). The serum concentration of ROMs was measured by the d-ROMs test (Diacron, Grosseto, Italy). The serum (10 µl in 2003 and 20 µl in 2005) was first diluted with 200 µl of a solution containing 0.01 M acetic acid/sodium acetate buffer (pH 4.8) and *N,N*-diethyl-*p*-phenylenediamine as chromogen and then incubated for 75 min at 37°C. When the metabolites react with an alkyl-substituted aromatic amine solubilised in the chromogen, they produce a complex whose colour intensity is directly proportional to their concentration. After incubation, the absorbance was read with a spectrophotometer (Microplate Reader Model 550) at 490 nm and the concentration of ROMs was calculated by comparison with a standard curve. The results of the d-ROMs test are expressed in arbitrary units called “Carratelli units” (CARR U), where 1 CARR U corresponds to 0.08 mg of H<sub>2</sub>O<sub>2</sub>/100 ml. The standard solution (after adequate dilutions) was equivalent to a H<sub>2</sub>O<sub>2</sub> concentration ranging from 0.18 to 5.88 mM. The measurements were expressed as mM of H<sub>2</sub>O<sub>2</sub>. Assessment of measurement repeatability in the 2003 samples showed a high reliability of the methods used [pooled sample repeatability:  $r=0.96$ ,  $P<0.001$ ; intraplate repeatability ranged from 0.70 to 0.96, all  $P<0.001$ ; see Lessells and Boag (1987)].

### Measurement of serum antioxidant barrier

The serum antioxidant barrier includes both exogenous (e.g. ascorbate, tocopherols, carotenoids) and endogenous (e.g. uric acid) compounds. It was measured by the

OXY-Adsorbent test (Diacron, Grosseto, Italy). This dedicated kit uses a colorimetric determination to quantify the ability of the antioxidant barrier to cope with the oxidant action of hypochlorous acid (HOCl; pro-oxidant of pathologic relevance in biological systems). The serum (10 µl) was diluted 1:100 with distilled water. A 200 µl aliquot of a titred HOCl solution was incubated with 5 µl of the diluted serum for 10 min at 37°C. Then, 5 µl of the same chromogen solution used for the ROMs determination was added. An alkyl-substituted aromatic amine solubilised in the chromogen is oxidised by the residual HOCl and transformed into a pink derivative. The intensity of the coloured complex, which is inversely related to the antioxidant power, was measured with the same spectrophotometer at 490 nm. In 2003 and 2005, calibration was achieved by means of a reference serum able to neutralise, respectively, 680 µmol HOCl/ml (Standard curve: range 0.425–6.8 µmol/ml; all values are subsequently multiplied  $\times 100$  to correct for dilution) and 440 µmol HOCl/ml (Standard curve: range 0.275–8.8 µmol/ml; all values are subsequently multiplied  $\times 100$  to correct for dilution). Measurements were expressed as mM of HOCl neutralised. The pooled 2003 sample repeatability was  $r = 0.88$ ,  $P < 0.001$ ; intraplate repeatability ranged from 0.53 to 0.65, all  $P < 0.001$  (see Lessells and Boag 1987).

#### Statistical analyses

For the 2003 samples, we calculated the intraclass correlation coefficient (Lessells and Boag 1987) to estimate the fraction of total phenotypic variance of ROMs and OXY attributable to factors causing resemblance, i.e. between-sibling similarity in both variables (Falconer 1989). We calculated the variance components by one-way ANCOVA using nest as grouping factor and age as covariate because our sample encompassed a 22-day range (nestlings were 9–31 days old). The heritability was estimated as twice the intraclass correlation coefficient (Falconer 1989).

For the cross-fostering experiment in 2005, the environmental and genetic effects on ROMs and OXY were estimated by a widely used approach (e.g. Smith and Wettermark 1995; Merilä 1996). We performed a two-factor nested ANOVA with General Linear Models using a type III sum of squares due to unequal family size. The main effects were duplicate (a pair of nests matched by brood size and hatching date) and nest of origin (nested within duplicates). The term duplicate accounts for any differences between pairs of nests. Within duplicates, the variation due to the nest of origin estimates variation attributable to genetic transmission ( $\frac{1}{2}V_A$ ), but also includes a quarter of the dominance variance ( $\frac{1}{4}V_D$ ) and any pre-manipulation parental effects ( $V_P$ ). The term duplicate estimates the effects of the common environmental variance ( $V_{EC}$ ), and error variance equals random environmental variation plus  $\frac{1}{2}V_A + \frac{3}{4}V_D$ . Both duplicate and nest of origin were

included as random factors (for more details, see Merilä 1996). All analyses were carried out with STATISTICA 6.0 (StatSoft 2001, Tulsa, USA).

## Results

Table 1 reports the descriptive statistics regarding the natural variability of ROMs and OXY measured in the kestrel nestlings. For the data collected in 2003 (67 nests, 261 nestlings), the intra-class correlation coefficients for both variables indicated good resemblance between full siblings (ROMs:  $r = 0.300$ ,  $P < 0.001$ ,  $h^2 = 0.600$ , Confidence interval (CI) = 0.472–0.728; OXY:  $r = 0.248$ ,  $P < 0.001$ ,  $h^2 = 0.496$ , CI = 0.368–0.624).

The cross-fostering study carried out in 2005 showed that the variation in ROMs concentration was mostly explained by the nest of origin (23.5% of the variance;  $P = 0.027$ ) and secondarily by the nest of rearing (8.0% of the variance;  $P = 0.32$ ; Table 2). In contrast, the variation in OXY was explained by the nest of rearing (52.8% of the variance;  $P = 0.0005$ ), while no effect was found for the nest of origin (0.1% of the variance;  $P = 0.64$ ; Table 2). The whole model regression equation was significant for both variables (ROMs:  $R^2 = 0.46$ ,  $F_{15,52} = 2.93$ ,  $P = 0.002$ ; OXY:  $R^2 = 0.59$ ,  $F_{15,60} = 5.77$ ,  $P < 0.001$ ). There was no effect of the manipulation on both the variables, as they did not show any difference between non-fostered and fostered nestlings (ROMs:  $t = -1.42$ ,  $P = 0.16$ ; OXY:  $t = 0.30$ ,  $P = 0.77$ ).

## Discussion

There is much debate about whether the additive genetic variance of a trait is more likely to be expressed under stressful or benign conditions (Hoffman and Parsons 1991). In the season, in which, we performed the cross fostering, the fledging rate (95.8%) was slightly lower than that in the former years (e.g. 98.0% in 2003), although it was average for the clutch size (5.13; 7-year data set; Dell’Omo et al. 2006). This feature might strengthen our results because the heritabilities of a number of traits in wild bird populations have been found to be lower when conditions are poor (e.g. Gebhardt-Heinrich and van Noordwijk 1991; Merilä 1996;

**Table 1** Descriptive statistics regarding the natural variability of serum reactive oxygen metabolite concentration (ROMs; mM of  $H_2O_2$ ) and serum total antioxidant barrier (OXY; mM of HOCl neutralised) measured in kestrel nestlings of a Mediterranean population during the 2003 and 2005 breeding seasons

	<i>n</i>	Mean $\pm$ SE	–95%	+95%	Min	Max
2003						
ROMs	261	0.21 $\pm$ 0.01	0.19	0.23	0.05	0.92
OXY	261	286.79 $\pm$ 5.18	276.58	296.99	82.00	506.00
2005						
ROMs	68	0.31 $\pm$ 0.02	0.27	0.35	0.08	0.74
OXY	76	347.06 $\pm$ 8.14	330.83	363.28	145.00	440.25

**Table 2** Results of a two-factor mixed nested ANOVA performed to test the effects of duplicate (i.e. environmental component) and nest of origin (nested within duplicate) on serum reactive oxygen metabolites concentration (ROMs; mM of H<sub>2</sub>O<sub>2</sub>) and total serum antioxidant barrier (OXY; mM of HOCl neutralised) in kestrel nestlings

Variable	Source of variation	SS	df	MS	F	P
ROMs	Origin	0.35	8	0.04	2.14	0.027
	Duplicate	0.42	7	0.06	1.40	0.32
	Error	0.94	52	0.02		
OXY	Origin	15,751	8	1,969	0.76	0.64
	Duplicate	201,777	7	28,825	14.63	0.0005
	Error	154,784	60	2,580		

SS sum of squares, MS mean squares

but see Merilä et al. 1999); thus, the genetic contribution to a trait can be expected to be better expressed in good breeding seasons.

Low heritability of a trait is usually related to its positive contribution to fitness, since strong directional and constant selection is supposed to decrease the genetic variation, with all remaining variation being environmentally determined (Fisher 1930; Gustafsson 1986; Mousseau and Roff 1987; McCleery et al. 2004). This is because directional selection tends to decrease the additive genetic variance of a trait, since the alleles conferring the highest fitness are expected to be driven quickly to fixation by natural selection (e.g. Kimura 1958). However, this view seems to be in contrast with our results. Even if there is no evidence on the association between ROMs and fitness components in our study system, the production of oxygen metabolites is considered a fitness trait in light of its causal relationship with ageing and the onset of many pathologies in mammals (Beckman and Ames 1998). In blue tits (*Parus caeruleus*), nestling condition (fitness trait) was found to be under weak directional selection, allowing significant additive genetic variation to persist (Merilä et al. 1999). Our results, together with those of Merilä et al. (1999), agree with a relatively recent reversal of the current view that fitness traits (under strong directional selection) should have lower levels of genetic variability than non-fitness traits [under weak directional selection (Price and Schluter 1991; Merilä and Sheldon 1999)]. The seeming lack of genetic variation in fitness components compared to non-fitness traits is supposed to be the result of higher environmental variance, which is able to mask the genetic component (Price and Schluter 1991; Merilä and Sheldon 1999). As a possible explanation, it was suggested that because many life-history traits are complex and influenced by a number of other features, they capture more environmental variance than morphological traits (Price and Schluter 1991). Although current evidence suggests that environmental and non-additive genetic effects are the main determinants of fitness in nature, it has been hypothesised that fitness traits harbour high absolute levels of additive genetic variation, possibly because they can acquire genetic variation and

accumulate mutations from many loci (Merilä and Sheldon 1999).

Reactive oxygen metabolites are the expression of free radical generation and their subsequent interaction with antioxidants and bio-macromolecules. There are four main sources of pro-oxidant generation, namely mitochondrial electron transport, peroxisomal fatty acid metabolism, cytochrome *P*-450 and phagocytic cells (Ames et al. 1993; Beckman and Ames 1998). Therefore, the high contribution of the genetic component could result from the “per-locus” additive contribution of the many loci involved in determining pro-oxidant production in individual birds. The perspective that the variation of pro-oxidant production in birds could reflect the expression of different genetic polymorphisms occurring within the population is partly supported by studies on fish and mammals (Daly et al. 1993; Roy and Wirgin 1997). These studies have shown that allelic variants of some enzymes (e.g. cytochrome P450) can affect the individual’s ability to metabolise different compounds. Moreover, it is known that both prokaryotic and eukaryotic organisms show different genetic loci of potential relevance to the oxidative damage theory of ageing [e.g. structural and regulatory genes modulating production of free radicals; Martin et al. (1996)].

Unlike the pro-oxidants, serum antioxidants were affected by the nest of rearing. In the American kestrel (*Falco sparverius*), a cross-fostering experiment showed that the variance in blood carotenoids was largely explained by the nest of rearing via differences in food quality (Bortolotti et al. 2000), resulting in a low genetic component. According to studies showing that the enzymatic activity is primarily intracellular (Halliwell and Gutteridge 1990; Lin et al. 2004a, b), our results suggest that antioxidants from food intake (e.g. vitamins) and compounds such as uric acid (Lin et al. 2004a, b) could be the most important chemicals used to cope with pro-oxidants in the avian circulation.

This study presents novel results on the relative importance of the environmental and genetic background of variables related to oxidative stress. Establishing the relative contribution of the environmental and genetic components to the expression of a trait such as avian oxidative stress can help to better understand avian senescence, as well as the mechanisms underlying the pattern and tempo of senescence in vertebrates and the oxidative stress’ potential response to natural selection. Finally, we would like to stress that future studies should consider also the levels of pro-oxidants and antioxidants in different tissues to get a more comprehensive view of the individual oxidative stress.

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