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RESEARCH ARTICLE

# Sex- and melanism-specific variations in the oxidative status of adult tawny owls in response to manipulated reproductive effort

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## ABSTRACT

Oxidative stress, determined by the balance between the production of damaging reactive oxygen species (ROS) and antioxidant defences, is hypothesized to play an important role in shaping the cost of reproduction and life history trade-offs. To test this hypothesis, we manipulated reproductive effort in 94 breeding pairs of tawny owls (*Strix aluco*) to investigate the sex- and melanism-specific effects on markers of oxidative stress in red blood cells (RBCs). This colour polymorphic bird species shows sex-specific division of labour and melanism-specific history strategies. Brood sizes at hatching were experimentally enlarged or reduced to increase or decrease reproductive effort, respectively. We obtained an integrative measure of the oxidative balance by measuring ROS production by RBCs, intracellular antioxidant glutathione levels and membrane resistance to ROS. We found that light melanic males (the sex undertaking offspring food provisioning) produced more ROS than darker conspecifics, but only when rearing an enlarged brood. In both sexes, light melanic individuals had also a larger pool of intracellular antioxidant glutathione than darker owls under relaxed reproductive conditions (i.e. reduced brood), but not when investing substantial effort in current reproduction (enlarged brood). Finally, resistance to oxidative stress was differently affected by the brood size manipulation experiment in males and females independently of their plumage coloration. Altogether, our results support the hypothesis that reproductive effort can alter the oxidative balance in a sex- and colour-specific way. This further emphasizes the close link between melanin-based coloration and life history strategies.

**KEY WORDS: Brood size manipulation, Colour polymorphism, Glutathione, Life history traits, ROS production, Oxidative stress**

## INTRODUCTION

Life history traits can differ between species, populations or individuals because of subtle differences in how they trade-off central life history components such as growth, reproduction or maintenance. Although the existence of life history trade-offs is well documented, the underlying mechanisms remain elusive (Flatt and Heyland, 2011). This information is, however, of central importance for gaining insight into the constraints moulding organismal diversity and the evolution of alternative life history strategies.

In natural populations, energy and nutrients are often limited, implying that their allocation to one trait can occur at the expense of other traits relying on the same resources. Accordingly, there are numerous examples showing that experimental restriction or supplementation of organisms with energy or particular nutrients induces a change in their life histories (e.g. Catoni et al., 2008; Prevedello et al., 2013; Ruffino et al., 2014). The conversion of nutrients into energy usable by the organisms for trait allocation is also known to come at a cost. Indeed, in aerobic organisms, respiration has double-edged effects. It allows the production of energy as adenosine triphosphate (ATP) while simultaneously generating damaging reactive oxygen species (ROS) (Balaban et al., 2005). Although organisms have evolved diverse arrays of mechanisms to minimize the production of ROS or neutralize them, they are nonetheless exposed to oxidative stress (and in turn oxidative damage) when their production of ROS exceeds the level of antioxidant defences (Balaban et al., 2005). For instance, parent zebra finches (*Taeniopygia guttata*) tending an experimentally enlarged brood were more exposed to oxidative stress (Alonso-Alvarez et al., 2004; Wiersma et al., 2004) and oxidative damage (Reichert et al., 2014), which can in turn jeopardize future reproduction or survival (Bize et al., 2008; Stier et al., 2012; Blount et al., 2015). Although variation in the balance between ROS production and antioxidant defences is thought to be central in shaping the diversity of life histories (Costantini, 2008, 2014; Dowling and Simmons, 2009; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al. 2009), we still know little about the covariation between oxidative stress and life history traits.

The colour polymorphic tawny owl (*Strix aluco*) is an excellent model system to test whether life history strategies are associated with oxidative stress for two main reasons. Firstly, this monogamous raptor species shows pronounced sexual size dimorphism (females are 20% bigger than males) that is associated with a strong partition of the reproductive roles between sexes. The female parent incubates the clutch for 28 days, and remains close to the nest to brood the hatchlings, distribute food among the progeny and protect the nest against predators (Da Silva et al., 2013), whereas the male parent hunts most prey items delivered to the nest. Offspring leave the nest at 25–30 days of age but are fed and protected by their parents until 90–120 days of age (Sunde, 2008). Hence, males and females are likely to differ in their reproductive constraints and, in turn, in the regulation of their oxidative balance in relation to brood food demand. In particular, because females rely on their partner for food during most of the reproductive period, females might be more exposed than males to oxidative stress when environmental conditions deteriorate.

Secondly, tawny owls show continuous variation in melanin-based plumage coloration (from light to dark reddish; for pictures, see Da Silva et al., 2013), the expression of which is strongly genetically controlled and not sensitive, or only weakly so, to environmental conditions (Brommer et al., 2005; Gasparini et al., 2009; Emaresi et al., 2013). Interestingly, differently coloured adult males display alternative trade-off resolution and life histories

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(Emaresi et al., 2011, 2014). Light reddish males (hereafter referred to as 'light melanic' males) have a low annual survival and finely adjust reproductive effort in response to variation in offspring food demand, whereas dark melanic males have a high annual survival and allocate a fixed effort to raise their brood independently of offspring need (Emaresi et al., 2014). Hence, if oxidative stress is implicated in life history trade-offs, we predict light and dark melanic males to exhibit different regulation of their oxidative balance according to variation in offspring food demand.

To test the hypotheses of sex-specific and melanism-specific changes in the oxidative balance in breeding adult tawny owls, we experimentally enlarged or reduced brood sizes at hatching to increase or decrease reproductive effort, respectively (Alonso-Alvarez et al., 2004; Wiersma et al., 2004; Metcalfe and Monaghan, 2013; Reichert et al., 2014). We measured the effects of our experiment on three markers of the oxidative balance to account for the fact that oxidative status is a multifactorial trait that is best characterized by multiple markers of ROS production and defences (Hõrak and Cohen, 2010; Selman et al., 2012). Therefore, using red blood cells (RBCs) (Stier et al., 2015) we measured mitochondrial ROS production (Stier et al., 2013), total intracellular glutathione levels (tGSH, a major intracellular antioxidant; Halliwell and Gutteridge, 2007) and cell resistance to free radicals (Bize et al., 2008).

## MATERIALS AND METHODS

### Brood size manipulation experiment

The present study was conducted in 2010 on a population of tawny owls located in western Switzerland. In 2010, females produced between two and seven eggs (mean±s.d.: 5.13±0.94 eggs), which hatched between 21 February and 31 May (mean date 31 March, s.d.=13.3 days). On the basis of similar hatching dates, 94 nests were pair matched to decrease (reduced broods) or increase (enlarged broods) parental investment as described in two previous studies (Roulin et al., 2011; Emaresi et al., 2014). Among pairs of nests, brood size at hatching was randomly manipulated, leading to an exchange in which 1.74 hatchlings on average (s.d.=0.6) from experimentally enlarged nests ($N$=47) were placed in experimentally reduced nests ($N$=47), while 2.74 hatchlings on average were moved from the reduced to the enlarged nests. Each family was thus composed of hatchlings from two origins, disrupting the potential covariation between brood size and parental genotype and phenotype. This brood size manipulation at hatching had the intended effect on reproductive effort and subsequently rearing conditions; parents assigned to the enlarged brood treatment reared a larger number of 10 day old owlets than those assigned to the reduced brood treatment (mean±s.e. number of owlets per enlarged versus reduced brood: 4.92±1.27 versus 3.47±1.11; ANCOVA accounting for initial brood size: $F_{1,141}$=141.9, $P$<0.0001).

When nestlings were 10 days old, we captured both parents to investigate the consequences of the brood size manipulation experiment on their oxidative status. Adults can easily being sexed based on their body size (females are 20% bigger than males) and the presence of a brood incubating patch in females only. Females were captured in the nest box during daylight (08:00 h to 18:00 h, $N$=94), while males were captured at night when provisioning their brood (22:00 h to 06:00 h, $N$=88). Males captured before or after experimental manipulation did not differ with respect to melanin-based coloration (Student's $t$-test: $t_{83}$=0.83, $P$>0.40). Upon capture, each adult was weighed to the nearest gram, and their left wing length was measured to the nearest 1 mm and left tarsus to the nearest 0.1 mm. We found no differences between the two brood size treatments in adult wing length and tarsus length (Student's $t$-tests, $P$>0.42).

### Assessment of plumage coloration

Although tawny owls vary continuously in their degree of reddishness, this species is usually considered to be colour polymorphic in the literature (e.g. Glutz von Blotzheim and Bauer, 1980; Galeotti and Cesaris, 1996; Brommer et al., 2005; Karell et al., 2011) and hence we also employed this terminology. The rationale for this procedure is that melanin-based coloration is strongly heritable, implying that colour morphs are associated with different genes (Roulin, 2004). Scores of adult plumage coloration were determined either visually in the field or through spectrophotometric analysis in the laboratory. The visual scoring method, based on five distinct colour morphs (Roulin et al., 2004) is a reliable approach, evidenced by high inter-annual repeatability of colour scores visually assigned to the same individuals between 2005 and 2010 ($r$=0.89±0.02, $F_{174,383}$=13.76, $P$<0.0001; Lessells and Boag, 1987). In parallel, three feathers collected from the back of adults were stuck together on black paper to capture reflectance spectra at four distinct positions using an S2000 spectrophotometer (Ocean Optics, Dunedin, FL, USA) and a dual deuterium and halogen 2000 light source (Mikropackan, Mikropack, Ostfildern, Germany). From these spectra, we calculated a mean brown chroma score for each individual as described by Montgomerie (2006). In the collected data, the two scoring methods were tightly correlated (Pearson's correlation: $r$=0.84, $N$=270 individuals, $P$<0.0001), reassuring us that we could consider only visual coloration scores in the present study. This choice is based on the fact that visual colour scores provide a better overall estimation of plumage coloration (as explained in Brommer et al., 2005) than brown chroma assessed with three back feathers.

Adult plumage coloration was associated with neither hatching date of the first egg nor brood size before and after the manipulation ($P$>0.38). Although randomly assigned with respect to female coloration (78 out of 80 females being already captured once before their brood size was manipulated, Student's $t$-test, $t_{78}$=−0.66, $P$=0.51), it appeared that males rearing a reduced brood tended to be slightly darker than those rearing an enlarged brood (brood size was manipulated without prior knowledge of male plumage coloration, $t_{58}$=2.14, $P$=0.04). This is, however, not a major problem for the present study, as our aim was to investigate changes in oxidative status with coloration within the two brood size treatments. Within pairs of experimental nests, foster and biological parents did not resemble each other with respect to plumage colour scores (Pearson's correlations: −0.16<$r$<−0.03, $P$>0.49). Pairing with respect to male and female coloration was not assortative in either treatment (enlarged nests: $r$=0.025, $N$=32, $P$=0.89; reduced nests: $r$=−0.18, $N$=28, $P$=0.34). Adult wing and tarsus lengths were not associated with coloration in either sex ($P$>0.12).

### Measures of oxidative status

To get an adequate estimate of the oxidative status of breeding owls (Hõrak and Cohen, 2010; Selman et al., 2012), we measured three different markers in RBCs (Stier et al., 2015). To this end, we collected 60–200 µl of blood from the wing vein into EDTA tubes for measurement of ROS production and membrane resistance to free radicals and into heparin tubes for measurement of total intracellular glutathione levels (tGSH). Each 16 µl sample of whole blood collected in EDTA tubes was immediately diluted in 584 µl of isotonic buffer (KRL buffer, Brevet Spiral V02023, Courernon, France) and stored at 4°C before further analyses, which occurred within 24 h of blood collection.

RBCs possess functional mitochondria (Stier et al., 2013), and thus we measured the endogenous production of ROS by RBC mitochondria by flow cytometry in combination with the MitoSOX™ Red mitochondrial superoxide indicator (Invitrogen) (Mukhopadhyay et al., 2007). For each individual (55 males and 69 females), 420 µl of KRL-diluted whole blood was centrifuged at 300 rpm and 4°C for 4 min. After removing the supernatant, we added 400 µl of KRL buffer and 1 µl of probe MitoSOX Red. Samples were incubated at 37°C for 30 min and finally analysed by flow cytometry using a BD FACS Calibur, with excitation at 582 nm (FL2). Data were acquired and analysed with the software FACSDiva and CellQuest Pro. For each sample, the mean fluorescence of 50,000 cells was determined.

Glutathione is the most abundant intracellular antioxidant, and probably also the most important (Halliwell and Gutteridge, 2007). We measured the tGSH of RBCs using the DetectX® Glutathione Colorimetric Detection Kit (Arbor Assays). For each sample (60 males and 80 females), 25 µl of blood collected in heparin tubes was diluted with 100 µl of 5% metaphosphoric

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acid, stored for 15 min on ice and then centrifuged at 14,000 rpm and 4°C for 15 min to collect the supernatant. From this extraction, 6 µl of the sample was diluted with 114 µl of sample diluent and loaded in duplicate on a 96-well microplate, to which 25 µl of colorimetric detection reagent and 25 µl of reaction mix were added per well. Optical densities (OD) were measured at a wavelength of 405 nm after 20 min of incubation. Observed OD were Box–Cox transformed before being used in models with a Gaussian-distributed error.

RBC resistance to a free-radical attack provides a general assessment of oxidative status at the whole-cell level. Although this measure is non-specific, because it can be affected by numerous pathways, including changes in ROS production and intracellular antioxidant levels, it is interesting because it gives an integrative measure at the whole-cell level of the different mechanisms involved in oxidative stress (Bize et al., 2014). We measured RBC resistance to free radicals by assessing the time required to haemolyse 50% of RBCs exposed to a controlled free-radical attack using the KRL bioassay (Brevet Spiral V02023) (Alonso-Alvarez et al., 2006; Bize et al., 2008). KRL-diluted whole blood (90 µl; 58 males and 74 females), loaded in duplicate on a 96-well microplate (intra-plate repeatability: $r$=0.99, $P$<0.0001), was subjected to a free radical attack at 40°C by adding a solution of 150 mmol $l^{-1}$ of 2,2′-azobis-(aminodinopropane) hydrochloride diluted in 153 µl of KRL buffer (Alonso-Alvarez et al., 2006; Bize et al., 2008). The lysis of RBCs was monitored with a microplate reader device as the decrease of OD at 540 nm wavelength. The time between blood collection and measurement of ROS production (mean±s.d. 10:37±06:20 h:min, range 02:00 to 22:15 h:min) or RBC resistance to free radicals (mean±s.d. 10:04±06:20 h:min, range 01:30 to 22:00 h:min) was not associated with brood size manipulation ($P$>0.78) or with male and female plumage coloration ($P$>0.20).

### Statistical analyses

We investigated the effects of our brood size manipulation experiment (BSM) on post-manipulation mitochondrial ROS production, intracellular tGSH levels, RBC resistance to free radicals and body mass in four separate statistical models. For each model, BSM, sex of the corresponding parent and its plumage coloration were entered as explanatory variables, plus all possible interactions between these three variables. Time of blood sampling (in hours), body mass (corrected for wing length) and age or age$^2$ of each individual (in years) did not significantly covary with our oxidative measurements (all $P$>0.10), and thus these covariates were not included in our final models. In each model, we controlled for the effect of the nesting site on the response variable by including rearing nest identity as a random factor. For each statistical model, we first ran a full factorial model and then dropped non-significant terms (starting with non-significant interactions) in a stepwise manner in order to produce the minimal adequate final model. For each model, we verified that the distribution of errors was homogeneous and normal. Statistical analyses were performed using the statistical software JMP IN 8.0.

## RESULTS

ROS production measured in RBCs of breeding owls rearing 10 day old chicks was significantly explained by the interaction between BSM, sex and colour ($F_{1,115.7}$=8.03, $P$=0.005; Table 1). Analyses performed separately for each sex and each brood size treatment showed that this interaction resulted mainly from differences in males rather than females. Light melanic males produced significantly more ROS than dark melanic ones when rearing an enlarged brood (colour effect in enlarged broods: estimate±s.e.=−0.38±0.11, $F_{1,21}$=11.80, $P$=0.003), but not when rearing a reduced brood (colour effect in reduced broods: estimate±s.e.=0.03±0.10, $F_{1,30}$=0.07, $P$=0.80; Fig. 1). There was no relationship between female coloration and ROS production either in enlarged broods (slope±s.e.=0.07±0.09, $F_{1,31}$=0.57, $P$=0.46) or in reduced broods (slope±s.e.=−0.09±0.10, $F_{1,35}$=0.44, $P$=0.33; Fig. 1).

RBC tGSH levels were significantly explained by the two-way interaction between BSM and colour ($F_{1,133.8}$=5.13, $P$=0.025; Table 1). The factor ‘sex’ had no significant effect, either alone or in interaction with BSM (all $P$>0.24). Models performed separately for each treatment group showed that light melanic adults had higher tGSH levels than dark melanic ones when rearing an experimentally reduced brood (estimate±s.e.=−49.5±23.7, $F_{1,66}$=4.40, $P$=0.04), whereas no association between tGSH and coloration was apparent when adults were rearing an experimentally enlarged brood (estimate±s.e.=41.6±29.3, $F_{1,64}$=2.00, $P$=0.16; Fig. 2).

RBC resistance to free radicals was significantly explained by the two-way interaction between BSM and sex ($F_{1,75.6}$=8.85, $P$=0.004; Table 1). The factor ‘colour’ had no significant effect, either alone or in interaction with BSM (all $P$>0.19). Models performed separately for each sex showed that females had less resistant RBCs when rearing an enlarged rather than a reduced brood ($F_{1,72}$=11.76, $P$=0.001), whereas no significant difference was

**Table 1. Results of linear mixed models investigating the effects of brood size manipulation, sex, plumage coloration and their interactions on ROS production by RBC mitochondria, Box–Cox-transformed tGSH levels, RBC resistance to free radicals and body mass in adult tawny owls**

| Source of variation | $N$ | Estimate | s.e. | d.f. | $F$ | $P$ |
|---|---|---|---|---|---|---|
| ROS production | | | | | | |
| BSM (E) | 124 | 0.02 | 0.06 | 1,56.6 | 0.10 | 0.75 |
| Sex (F) | | −0.15 | 0.06 | 1,58.1 | 5.74 | 0.02 |
| BSM (E) × sex (F) | | −0.01 | 0.06 | 1,58.1 | 0.01 | 0.93 |
| Colour | | −0.09 | 0.06 | 1,115.8 | 2.78 | 0.10 |
| BSM (E) × colour | | −0.05 | 0.06 | 1,115.8 | 0.67 | 0.42 |
| Sex (F) × colour | | 0.09 | 0.06 | 1,115.7 | 2.64 | 0.11 |
| BSM (E) × sex (F) × colour | | 0.16 | 0.06 | 1,115.7 | 8.03 | 0.005 |
| Box–Cox-transformed tGSH | | | | | | |
| BSM (E) | 140 | −16.63 | 20.41 | 1,69.7 | 0.66 | 0.42 |
| Colour | | −3.60 | 18.71 | 1,133.8 | 0.04 | 0.85 |
| BSM (E) × colour | | 42.36 | 18.71 | 1,133.8 | 5.13 | 0.025 |
| RBC resistance to free radicals | | | | | | |
| BSM (E) | 132 | −0.51 | 0.53 | 1,75.6 | 0.93 | 0.34 |
| Sex (F) | | −1.50 | 0.53 | 1,75.6 | 8.04 | 0.006 |
| BSM (E) × sex (F) | | −1.57 | 0.53 | 1,75.6 | 8.85 | 0.004 |
| Body mass | | | | | | |
| Sex (F) | 143 | 97.97 | 3.78 | 1,85.2 | 678.84 | <0.001 |

We controlled for the effect of the nesting site on each response variable by including the rearing nest identity as a random factor in each model. Model selection followed a stepwise procedure.

ROS, reactive oxygen species; RBC, red blood cell; tGSH, total intracellular glutathione; BSM, brood size manipulation; E, enlarged; F, female.

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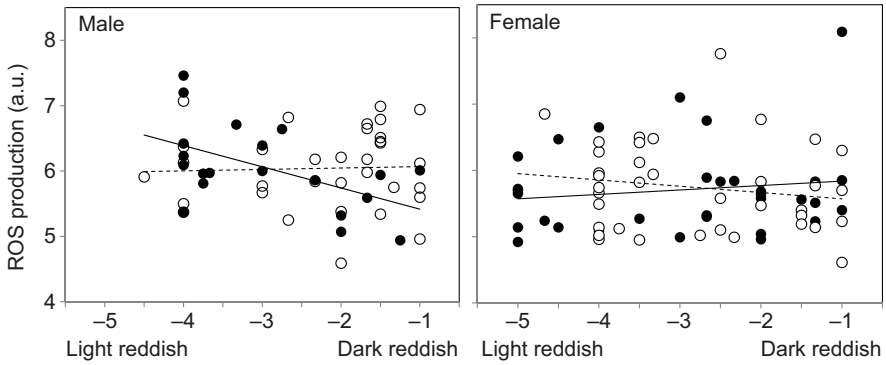


**Fig. 1. Endogenous production of reactive oxygen species (ROS) by red blood cell (RBC) mitochondria, in relation to melanin-based coloration in male and female tawny owls following brood size manipulation.** Reduced brood, open circles and dashed regression line; enlarged brood, filled circles and solid regression line. There is a significant relationship between ROS production and coloration in males rearing an enlarged brood (linear regression; slope±s.e.=–0.38±0.11, $F_{1,21}$=11.80, $P$=0.003) but not in males rearing a reduced brood (slope±s.e.=0.03±0.10, $F_{1,30}$=0.07, $P$=0.80) or in females rearing an enlarged (slope±s.e.=0.07±0.09, $F_{1,31}$=0.57, $P$=0.46) or a reduced brood (slope±s.e.=–0.09±0.10, $F_{1,35}$=0.44, $P$=0.33).

found between males rearing an enlarged or a reduced brood ($F_{1,56}$=1.36, $P$=0.25; Fig. 3).

Adult body mass was significantly explained by the effect of sex (linear mixed model: $F_{1,85.2}$=678.84, $P$<0.001; Table 1), with female owls being ca. 20% heavier than males. The factors 'colour' and 'BSM' had no significant effect, either alone or in interaction, and their interactions with sex were also not significant (all $P$>0.14).

## DISCUSSION

### Sex-specific links between oxidative status and life history strategies

Tawny owl parents display a clear division of duties during reproduction. Males deliver food to their offspring and partner (Sasvári et al., 2009), while females guard the brood and distribute the prey items among the progeny. Our brood size manipulation experiment is primarily expected to increase male parental effort and reduce female food resources. Thus, this treatment is likely to differentially affect male and female oxidative status in this species. An important consequence of an increase in parental investment in males (i.e. increased food supply) is an increase in metabolic rate (Nilsson, 2002) and potentially in ROS production (Balaban et al., 2005; but see Stier et al., 2014a,b). In the present study, we partly verified this scenario, because our brood size manipulation affected ROS in males rearing enlarged broods but in interaction with plumage coloration. However, we did not measure daily variation in metabolic rate of males rearing reduced and enlarged broods, and thus studies are still needed to demonstrate a clear association between increased metabolic rate in response to work load and ROS production.

Concordant with experimental studies on adult zebra finches in captivity (Alonso-Alvarez et al., 2004) and adult great tits (*Parus major*) in the wild (Losdat et al., 2011), our brood size manipulation experiment led to a significant decrease in RBC resistance in females (but not in males) rearing an enlarged compared with a reduced brood. This effect was independent of coloration. Because of their brooding behaviour, females rearing an enlarged brood could suffer from reduced food intake until owlets are thermo-independent at 15–20 days of age. Therefore, they could have experienced a shift in their diet that lowered their acquisition of antioxidants (i.e. carotenoids, vitamin E; Catoni et al., 2008; Cohen et al., 2009). Females rearing an enlarged brood could also have had to trade off their own maintenance at the expense of heat production to keep warm their numerous growing chicks. All those processes are likely to induce higher oxidative stress in females rearing

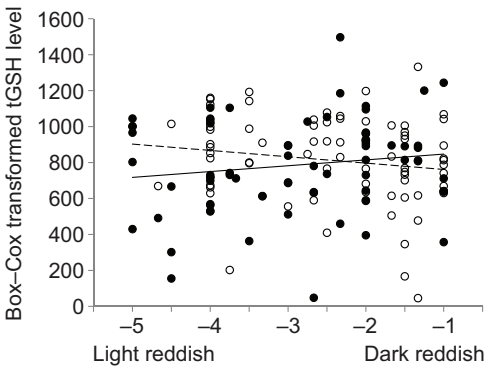


**Fig. 2. Total intracellular glutathione (tGSH) levels in relation to melanin-based coloration in breeding tawny owls following brood size manipulation.** Data for tGSH (μmol $l^{-1}$) were Box–Cox transformed. There was a significant relationship between tGSH and coloration for males rearing an experimentally reduced brood (linear mixed model; open circles and dashed regression line, slope±s.e.=–49.5±23.7, $F_{1,66}$=4.4, $P$=0.04) but not the enlarged brood (filled circles and solid regression line; slope±s.e.=41.6±29.3, $F_{1,64}$=2.0, $P$=0.16).

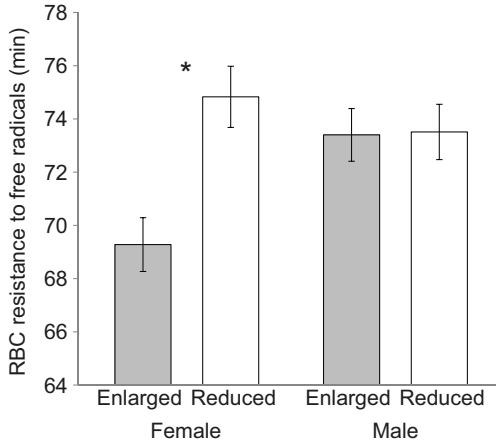


**Fig. 3. RBC resistance to a free radical attack in relation to brood size manipulation in adult tawny owls.** RBC resistance to free radicals was significantly lower in females rearing an enlarged than a reduced brood (ANOVA; $N$=74, *$P$=0.001), whereas there was no difference between males rearing an enlarged versus a reduced brood ($N$=58, $P$=0.25).

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enlarged broods, as suggested in this study by the observed decline in RBC resistance to free radicals after their brood had been enlarged. The exact proximate mechanisms driving the evolution of these sex-specific differences remain to be explored. One mechanism could be the opposite action of sex steroids on oxidative balance, namely the positive and negative effect of testosterone and oestrogen, respectively (Viña et al., 2005; Tobler and Sandell, 2009; Casagrande et al., 2012). Another hypothesis is the effect of body mass on oxidative status, with individuals generating more free radicals as a consequence of higher basal metabolic rate imposed by larger body mass. This hypothesis is, however, not supported by our data, because we failed to detect a relationship between body mass (corrected for wing length) and ROS production or RBC resistance to free radicals in adult tawny owls (G.E., unpublished results; but see Markó et al., 2011 for similar results in the collared flycatcher *Ficedula albicollis*).

### Colour-specific oxidative status

Melanin-based coloration displays tight and complex links with oxidative balance: antioxidant properties of melanin pigments, pleiotropic effects of the melanocortin system (Ducrest et al., 2008; Roulin and Ducrest, 2011) or the biochemical role of tGSH in melanogenesis (Galván and Alonso-Alvarez, 2008; Galván and Solano, 2009). These effects raised the hypothesis of colour-specific strategy in the regulation of tawny owl oxidative balance according to environmental conditions.

To our knowledge, the present study is the first to reveal covariation between ROS production and adult melanin-based coloration. Light melanic males produced more ROS than dark melanic ones when rearing enlarged broods, while no significant difference was found between differently coloured males rearing a reduced brood. Previous works on this species provided empirical evidence that light melanic individuals show stronger responses to stressful environmental conditions caused either by an experimental brood enlargement or by food deprivation (Roulin et al., 2008, 2011; Piault et al., 2009; Emaresi et al., 2014). As a consequence of this adaptive strategy, light melanic individuals are likely to suffer from physiological costs. In adults, for instance, food provisioning to larger broods can increase male metabolic rate (Nilsson, 2002), and such constraint can lead to an increase of ROS production (Balaban et al., 2005). Although we did not specifically measure paternal investment in the two brood size treatments, 7 years of population monitoring revealed that light melanic males invested more effort than darker conspecifics when their brood was experimentally enlarged (Emaresi et al., 2014). Higher ROS production observed in light melanic males rearing enlarged broods may therefore be a by-product of their specific life history strategy. Note that there was a light bias in our dataset, with males rearing a reduced brood showing significantly ($P$=0.04) darker melanism than those rearing an enlarged brood (see Materials and methods). Because the aim of this study was to investigate changes in the oxidative status with coloration within the two brood size treatments, this involuntary bias (the coloration of male owls is determined after we manipulated their brood) between the treatments should have little effects of the results. Additional research is nonetheless welcome to confirm the robustness of the links between melanin-based coloration and brood size manipulation reported in this study.

Intracellular antioxidants such as GSH have a key role in scavenging ROS, but can also interact biochemically within important molecular pathways such as pheomelanogenesis (Galván and Solano, 2009). This dual role has led to the hypothesis that melanocytes, in which eumelanogenesis prevails at the expense of pheomelanogenesis, suffer higher oxidative stress because they require low thiol group conditions, and thereby low tGSH concentrations (Ito, 2003; Galván and Solano, 2009). Observations from experimental studies on great tits and red-legged partridges (*Alectoris rufa*) during plumage development were consistent with this hypothesis (Galván and Alonso-Alvarez, 2008, 2009); nestlings with experimentally reduced tGSH levels expressed eumelanic traits to a larger degree, sometimes at the expense of pheomelanic traits (Galván and Alonso-Alvarez, 2009). These outcomes suggested a close association between the production of melanin pigments and tGSH during plumage development (but see Galván et al., 2010 for contrasting correlative results between tGSH and melanic colorations). However, whether an association between plumage coloration and tGSH is found outside the period of plumage development, and thus melanin pigment production, remains unclear. The present study focused on a life stage (i.e. adult reproduction) when moult is not occurring, and hence when pigment synthesis is probably not taking place. Interestingly, our results show that light (pheo)melanic owls exhibited higher levels of tGSH than dark (pheo)melanic conspecifics when rearing an experimentally reduced brood, whereas such covariation was not significant for tawny owls rearing an experimentally enlarged brood. Hence, although it supports the hypothesis of a close link between tGSH and melanin-based coloration, the negative relationship between tGSH and pheomelanin reported outside the period of plumage development in adult owls is opposite to the positive relationship between tGSH and pheomelanin during plumage development in growing partridges (Galván and Alonso-Alvarez, 2009). Our finding emphasizes that formulating general predictions on the links between melanin-based coloration and tGSH can be complex and may change during the life stage of an organism, in particular whether measurements are performed during or outside the period of melanin pigment synthesis. Indeed, outside the period of pigment synthesis, the link between coloration and oxidative status can be mediated by colour-specific life history strategies (Emaresi et al., 2014), with for instance light melanic adult owls investing more in reproduction but also in antioxidant defences for oxidative shielding (Stier et al., 2012; Blount et al., 2015). Given the global pool of GSH (tGSH) is composed of two forms, namely reduced GSH (amount of available antioxidants) and oxidized GSH (amount of tGSH recently used), futures studies are needed to address how each form covaries with melanin-based coloration to obtain insight into the importance of oxidative shielding and damage.

### Conclusions

Oxidative stress has been proposed to play an important role in shaping the cost of reproduction and life history trade-offs (Costantini, 2008, 2014; Monaghan et al. 2009; Metcalfe and Alonso-Alvarez, 2010; Stier et al., 2012; Metcalfe and Monaghan, 2013; Speakman and Garratt, 2014). By manipulating reproductive effort in the tawny owl, a melanin-based polymorphic bird species with a strong division of labour and melanism-specific life history strategies (Emaresi et al., 2014), we found results supporting the hypothesis that increased reproductive effort (in relation to sex or coloration) is associated with increased ROS production and decreased levels of antioxidant defences.

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The authors declare no competing or financial interests.

### Author contributions
G.E., A.R. and P.B. designed the study, analysed the data and wrote the paper. G.E., I.H., E.G., A.R. and P.B. collected the data.

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