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Importance of melanin-based colouration and environment in shaping intracellular glutathione levels in nestling and adult tawny owls *Strix aluco*

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Resources allocated in reproduction are traded off against those invested in self-maintenance such as antioxidant response. Glutathione (GSH) is an intracellular antioxidant defence that scavenges reactive oxygen species, the deleterious byproducts of oxygen consumption. Given the role of intracellular GSH in pheomelanogenesis, a trade-off in GSH allocation between resistance in oxidative stress and melanin production may take place. To investigate how intracellular GSH is regulated in differently coloured individuals at the time of reproduction (in adults) and of intense melanogenesis (in nestlings), we measured the total pool of GSH produced ($_{t}$GSH), consumed ($_{ox}$GSH) and available ($_{red}$GSH) in adult tawny owls and their offspring which were cross-fostered between randomly chosen nests. Our goal was to describe potential correlations between resistance in oxidative stress and colour morphs in natural conditions. Nestling GSH levels were correlated with GSH levels in their genetic and foster parents suggesting that producing GSH is genetically and environmentally determined (although the effect of the foster nest seemed stronger). This species shows continuous variation in pheomelanin reddish colouration, which is associated with life-history strategies. Based on the hypothesis of GSH dependence of pheomelanism, we expected a greater amount of $_{ox}$GSH in dark compared to light pheomelanic nestlings, which was not the case. In contrast, light melanic breeding adults had higher levels of $_{t}$GSH and $_{red}$GSH than dark breeders probably because of a higher investment in antioxidant capacity. The link between pheomelanism and GSH may therefore be due to the fact that differently colored individuals have different life-history strategies rather than because the production of pheomelanin pigments requires GSH.

Keywords: colour polymorphism, glutathione, melanin, oxidative stress, pheomelanin

Introduction

Living organisms are constantly confronted by the by-product of metabolism, i.e. free radicals causing oxidative stress (Monaghan et al. 2009). It is therefore crucial for organisms to keep the homeostasis between the production of deleterious reactive oxygen species (ROS) and antioxidant defence systems. This ratio is critical as an excess

of ROS production over antioxidants exposes organisms to oxidative stress and important intracellular damages, such as DNA point mutations (Twigg et al. 1998), cell membrane rupture (Halliwell 1992) and oxidation of amino acids in proteins (Halliwell and Gutteridge 1989). As reproductive activities increase metabolism, resources invested in current reproduction are traded off against somatic self-maintenance and repair mechanism of oxidative balance (Cohen et al. 2010, Isaksson et al. 2011, Kim et al. 2011). In conditions where resources are limited (Williams 2018), individuals investing heavily in reproduction are likely to produce more ROS, constraining them to adopt antioxidant responses. The major regulator of redox state and antioxidant defences in animals cells, including red blood cells, is glutathione (i.e. $_{red}$GSH, Reddy et al. 1982, Halliwell and Gutteridge 1989, Stier et al. 2015).

Found at moderate to high cellular concentration in most organisms, GSH is a tripeptide (i.e. glutamic acid, cysteine, glycine) (Hopkins 1929) that scavenges ROS in a reaction catalysed by the glutathione peroxidase (GPx, Meister 1994). This redox process leads to the formation of glutathione disulfide (GSSG), derived from two $_{ox}$GSH molecules. Hence, the global pool of intracellular glutathione ($_{t}$GSH) is composed by reduced and oxidized GSH fractions (i.e. $_{t}GSH = _{red}GSH + _{ox}GSH$), both providing an accurate estimation of cellular redox state. $_{red}$GSH gives information on the amounts of available antioxidants, while $_{ox}$GSH indicates how much $_{t}$GSH was recently used either to scavenge ROS or in alternative metabolic or biochemical processes (e.g. protein turnover and pheomelanogenesis). The $_{t}$GSH pool is composed of more than 90% of $_{red}$GSH in healthy mammalian cells (Pastore et al. 2003), whereas the proportion of $_{red}$GSH is about 55–70% in bird red blood cells (Romero-Haro and Alonso-Alvarez 2014, Messina et al. 2020). Although GSH synthesis requires the action of two distinct enzymes, i.e. glutamate-cysteine ligase and GSH synthase (Meister and Anderson 1983, Anderson 1998, Lu 2000), its production can be physiologically limited by the conditionally essential amino acid cysteine (Meister and Anderson 1983). Accordingly, one can raise the hypothesis that the production of intracellular GSH is not only genetically controlled (Board et al. 1974, Rizzi et al. 1988, Krogmeier et al. 1993) but also condition-dependent due to the need of acquiring cysteine from the diet (Lu 2000). Surprisingly, very few studies investigated the genetic vs environmental determinism of intracellular GSH regulation. Specifically, our knowledge of how different genotypes or phenotypes adjust their overall GSH pool (i.e. $_{t}$GSH), especially in terms of recent (i.e. $_{ox}$GSH) and future (i.e. $_{red}$GSH) expenditures, remains surprisingly scarce in wild populations.

In this context, melanin-based colouration is a promising system to consider the relationship between genotype and phenotype for important ecological or physiological traits, such as variation in GSH antioxidant response. This widespread pigmentation system is composed of two pigments, namely eu- (grey to black) and pheomelanin (yellow to reddish-brown) and their synthesis can be under strong genetic control (Hubbard et al. 2010, Roulin and Ducrest 2013, San-Jose Garcia and Roulin 2017). Interestingly, recent studies pointed out the plausible role of intracellular GSH levels in the synthesis of melanin pigments (Benedetto et al. 1981, Ozeki et al. 1997). Within melanocytes, the concentration of sulfhydryl compounds, in particular GSH, can modulate tyrosinase activity (Benedetto et al. 1981, 1982, Land and Riley 2000), which is the key enzyme controlling the switch between eu- and pheomelanogenesis (Barsh 1996, Ito et al. 2000). This biochemical link led researchers to formulate predictions on the expression of GSH in relation to melanin-based colouration. For instance, they expect dark pheomelanic individuals to present higher GSH levels than lighter pheomelanic (but darker eumelanic) morphs due to the fact that GSH can be used as a substrate for pheomelanogenesis (Galvan and Solano 2009). In line with this hypothesis, melanocytes have lower intracellular levels of GSH when producing eumelanin, rather than pheomelanin pigments (Benathan et al. 1999). Moreover, experimental inhibitions of GSH levels in nestling great tits *Parus major*, red-legged partridges *Alectoris rufa* and greenfinches *Carduelis chloris* induced the production of smaller eumelanin-based plumage traits and conversely larger pheomelanic ones (Galvan and Alonso-Alvarez 2008, 2009, Horak et al. 2010). The observation that natural variations in the degree of eu- and pheomelanin-based colouration can be associated with resistance to oxidative stress (Roulin et al. 2011a) is suggestive that GSH may be implicated in both the oxidative balance and melanin synthesis. This stimulated researchers to propose that the use of GSH in the production of melanin pigments is traded off against resistance to oxidative stress (Galvan and Alonso-Alvarez 2008).

Even if GSH is implicated in melanogenesis, associations between GSH, melanin-based colouration and oxidative stress are not necessarily the outcome of trade-off resolution in GSH allocation. Indeed, differently coloured individuals may adopt alternative life-history strategies (Roulin 2004, Emaresi et al. 2014), each requiring specific levels of antioxidant defences (Roulin et al. 2011a). Under this scenario, one can predict that, if melanin-based colouration signals alternative life-history strategies, the covariation between GSH levels and melanin-based morphs could be the outcome of colour-specific behaviour, of a pleiotropic effect of genes involved in melanogenesis and oxidative stress (Ducrest et al. 2008) or of metabolites involved in melanogenesis and antioxidant defences such as GSH. Both scenarios illustrate the complexity of predicting whether the relationship between eu- and pheomelanin-based colouration and GSH levels should be positive or negative, particularly in adults at the time when they do not produce melanin pigments. Indeed, the sign of this relationship will depend on five factors, i.e. 1) the nature of the colour polymorphism (eu- vs pheomelanin-based colouration), 2) if there is a genetically based polymorphism in the production of GSH, some individuals being programmed to produce more GSH than others, 3) if the production of GSH is condition-dependent (i.e. more GSH produced when needed to resist oxidative stress), 4) if there is a trade-off in GSH allocation between melanin colour traits

and resistance to oxidative stress and finally 5) if differently coloured individuals adopt alternative GSH-independent life-history strategies that generate various levels of oxidative stress (in the present paper, we examined the four last possibilities, see below). These factors being non-mutually exclusive and their relative importance unknown, it is difficult to propose a priori predictions regarding how melanin-based colouration should covary with GSH. Descriptive studies where colouration and GSH are measured under natural conditions (i.e. without drastic manipulation of GSH levels) are therefore needed (see Galvan et al. 2010 for an example, although the colour polymorphism in this study remains unclear). Particular attention on the global pool of GSH produced ($_{t}$GSH), and specifically on the amount of GSH that has already been consumed ($_{ox}$GSH) vs the amount of GSH still available ($_{red}$GSH), should help improve our understanding of the role and the use of GSH in differently coloured individuals (with respect to their life histories). Figure 1 illustrates the main synthesis pathways and points out the main molecules.

Herein, we examined the covariation between resistance to oxidative stress and melanin-based colouration in the colour polymorphic tawny owl *Strix aluco*. This descriptive approach is an important first step to understand how feather colouration can signal the way animals deal with stressful factors such as oxidative stress. This owl is appropriate because it shows continuous variation in the degree of plumage reddishness (from light to dark), which is positively correlated to the amount of pheomelanin pigments and to a lesser extent to eumelanin pigments (Gasparini et al. 2009). This colour polymorphism is segregated in accordance with Mendelian's law of inheritance (Karell et al. 2011a) and is therefore highly heritable (Gasparini et al. 2009). There is accumulating evidence that, in the tawny owl, inter-individual variation in melanin-based colouration is associated with indices of individual performance, such as immunocompetence (Galeotti and Sacchi 2003, Gasparini et al. 2009) and offspring growth (Roulin et al. 2004, 2008, Piault et al. 2009), potentially signalling alternative physiological strategies or metabolic needs to cope with heterogeneous environments (Galeotti and Cesaris 1996, Brommer et al. 2005, Emaresi et al. 2011, Karell et al. 2011a). Of particular interest, recent findings indicated that variation in plumage colouration in breeding male tawny owls was associated with differences in ROS production and in red blood cell $_{t}$GSH levels (Emaresi et al. 2016). In the present study, we therefore specifically focused on GSH levels (i.e. $_{t}$GSH, $_{red}$GSH and $_{ox}$GSH) of non-moulting adults and their cross-fostered nestlings (rather than considering exclusively $_{t}$GSH in breeding adults), two life stages characterized by low and high melanogenic activities, respectively, in this species.

The main objectives of the present study were 1) to establish whether inter-individual intracellular GSH levels are genetically inherited or environmentally mediated and 2) to determine the sign of the potential relationship between GSH levels and colouration in both breeding adults and

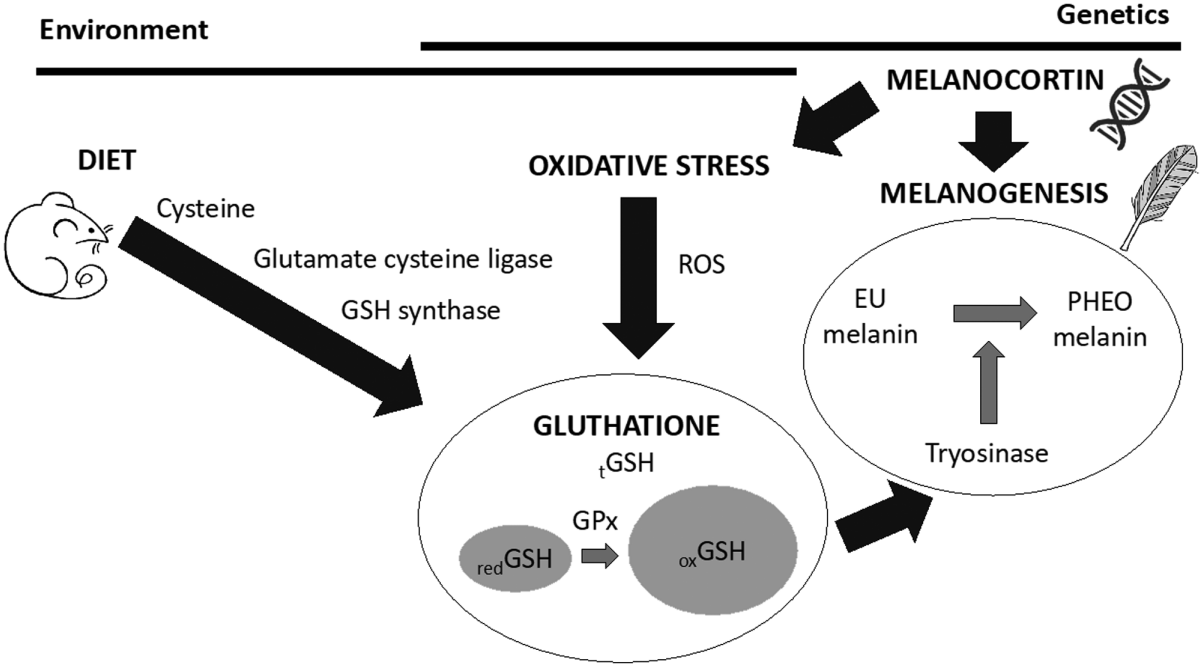

Figure 1. Schematic pathways showing how glutathione can account for covariations between diet, oxidative stress and melanogenesis. The essential amino-acid cysteine that is acquired through the diet is needed for glutathione synthesis. Glutathione is the major cellular antioxidant defence against oxidative stress. Glutathione can influence melanogenesis through its action on the tyrosinase enzyme controlling the production of eu- and pheo-melanin. Diet and oxidative stress can be influenced by environmental factors. The melanocortin genetic system can have pleiotropic effects on oxidative stress and melanogenesis. Alternative life-history strategies displayed by differently melanin-based coloured individuals can lead to gene-by-environment interaction. See the main text for details and abbreviations.

growing nestlings. To test for genetic GSH inheritance, we performed a cross-fostering experiment where eggs or hatchlings were swapped between randomly chosen nests in order to allocate genotypes randomly among different rearing environments. In addition, we manipulated brood size to induce various levels of stress, potentially oxidative stress (Wiersma et al. 2004, Christe et al. 2012), individuals raised (i.e. nestlings) or rearing (i.e. parents) an experimentally enlarged broods experiencing higher levels of stress than individuals allocated to experimentally reduced broods. Because environmentally induced covariation between colouration and GSH levels could be due to condition-dependent expression of colouration and/or GSH levels, we first tested whether the expression of melanin-based colouration is indeed not condition-dependent in our local population of tawny owls. To this end, we investigated whether colouration of breeding adults rearing an experimentally enlarged brood changes differently compared to adults rearing an experimentally reduced brood (Griffith 2000). This test was possible because we performed brood size manipulation experiments since 2005, providing therefore a large data set to investigate the change in colouration between two successive breeding seasons. Because nestlings but not parents were growing their feathers (i.e. showed strong melanogenesis) when blood was sampled, we also investigated whether the covariation between colour traits and GSH levels differed between nestlings and adults and was differentially affected by the brood size manipulation. We always tested plumage colouration of the father and mother, even though we did not have specific predictions regarding sex-specific effects.

Methods

The tawny owl is a medium-sized bird, commonly found in woodlands across Eurasia (Galeotti 2001). It is a monogamous and philopatric nocturnal species, living up to 20 years. Females are 16–20% larger than males (Baudvin and Dessolin 1992), both sexes showing a strong partition of the reproductive roles (Sunde et al. 2003). Females incubate their clutch for 28 days and remain inside the cavity with their owlets until they are thermo-independent at 15–20 days of age. Then, the mother stays around the nest, protecting the offspring against potential predators. Males are primarily engaged in supplying food to their brood. Nestling growth rate and survival strongly depend on prey availability, especially wood mice (*Apodemus* sp.; Roulin et al. 2009). In our 911 km^2 Swiss study area, 2011 was characterized by a small number of breeding pairs (n = 57) compared to 2007 (n = 125) or 2010 (n = 139). Clutches were composed of one to five eggs (2.7 eggs ± 0.77), deposited between 3 March and 22 April (mean ± SD = March 19 ± 12 days), while the mean number of nestlings that fledged per breeding pair was small (mean ± SD = 1.36 fledglings ± 1.34). Offspring leave the nest at 25–30 days of age but are fed by their parents until 90–120 days of age (Sunde 2008).

Experimental design

To test experimentally whether the expression of melanin-based colouration is sensitive to investment in reproductive activities (Griffith 2000) or to rearing conditions experienced at the nestling stage (see Piault et al. 2012 for example), we manipulated brood size between 2005 and 2010. As previously shown, this experimental approach has a significant effect on the adult (Roulin et al. 2011b) and nestling body condition (Roulin et al. 2008).

In 2011, we repeated the same brood size manipulation experiment to test whether the covariation between GSH and colouration differs between rearing environments. We matched 42 nests in pairs based on the criteria that clutches were initiated on a similar date (Pearson's correlation: $r = 0.92$, $p < 0.0001$). Among pairs of nests, brood sizes were randomly manipulated, leading to an exchange of 2.11 hatchlings or eggs on average (SD = 0.6) from nest E (experimentally enlarged) and placed in nest R (experimentally reduced), while 3.11 hatchlings or eggs on average (SD = 0.6) underwent the opposite exchange (i.e. from nest R to nest E). Out of the 42 manipulated broods, 8 broods were predated leading to a small imbalance between the two treatments (16 experimentally reduced broods vs 18 experimentally enlarged broods). As expected, this brood size manipulation (BSM) had the intended effect on brood size soon after hatching and thereby on rearing conditions; parents assigned to the enlarged brood treatment were rearing a larger number of nestlings than those assigned to the reduced brood treatment (mean ± SE number of nestlings per enlarged vs reduced brood: 3.17 ± 0.13 vs 2.37 ± 0.14; Student's t-test: $t_{32} = -4.07$, $p = 0.0003$). As previously described (Emaresi et al. 2014), adults and their offspring were captured post-manipulation, when nestlings were 10 days of age (mean ± SD = 9.73 ± 2.7). Females and their offspring were captured in the nest box during daylight (8 am–6 pm), while males were captured at night (when provisioning their brood; 10 pm–6 am). Individual body mass, tarsus length and wing length were measured to the nearest 0.1 g, 0.1 mm and 1 mm, respectively. Adult wing length and tarsus length were not significantly associated with the brood size manipulation experiment (Student's t-tests, $p > 0.3$). For each individual, we collected 60–200 µl of whole blood from the brachial vein, using heparin microtubes, which were immersed in dry ice in the field and transferred at −80°C within 8 hours until later analyses in the laboratory.

Assessment of plumage colouration

Although tawny owls vary continuously in the degree of reddishness, this species is usually considered as colour polymorphic in the literature (Glutz von Blotzheim and Bauer 1980, Galeotti 2001, Brommer et al. 2005) and hence we also employ this terminology. Adults were classified into one of five colour morphs (from light to dark reddish; Roulin et al. 2005), based on plumage colouration from different body areas (i.e. breast, flanks, back, head and wings). This visual

determination of adult plumage colouration is a highly reliable scoring method ($r = 0.89 \pm 0.02$, $F_{174,383} = 13.76$, $p < 0.0001$; Emaresi et al. 2014), providing a good estimation of overall colouration (Brommer et al. 2005). Moreover, colour scores assigned visually were found to be strongly correlated with brown chroma scores derived from spectrometric measurements (Pearson's correlation: $r = -0.84$, $n = 270$, $p < 0.0001$; Emaresi et al. 2014). At the nestling stage, plumage colouration is much less variable, which makes the classification of nestling plumage colouration into one of five colour morphs difficult. For this reason, nestling plumage colouration was assessed by spectrometric measurements. To this end, three feathers collected on the back of each nestling were overlaid on black paper to capture reflectance spectra at four distinct positions using the S2000 spectrophotometer (Ocean Optics, Dunedin, FL) and a dual deuterium and halogen 2000 light source (Mikropackan, Mikropack, Ostfildern, Germany). Based on these spectra, a mean brown chroma score was calculated for each nestling as described by Montgomerie (2006).

Adult plumage colouration was neither associated with clutch size, brood size before or after the manipulation (Student's t-tests, p-values > 0.39) nor with the experimental brood size treatment (Student's t-test by sex, $p > 0.59$). Moreover, adult wing and tarsus lengths were not associated with colouration in both sexes (Student's t-test, p-values > 0.3). Pairing with respect to plumage colouration was random, since there was no correlation between male and female colouration within breeding pairs ($r = 0.01$, $n = 28$, $p = 0.96$). Within pairs of experimental nests, foster and biological parents did not resemble each other with respect to plumage colour scores (females: $r = -0.02$, $n = 20$, $p = 0.94$; males: $r = -0.58$, $n = 9$, $p = 0.1$). Nestling body mass was unaffected by the experimental brood size treatment ($F_{1,8.7} = 0.56$, $p = 0.47$) and did not covary with plumage colouration ($F_{1,45.3} = 1.28$, $p = 0.26$; Supporting information). By cons, the brood size manipulation experiment influenced adult body mass, owls rearing a reduced brood being significantly heavier than those rearing an enlarged brood ($F_{1,31.03} = 9.01$, $p = 0.005$) but independently of plumage colouration ($F_{1,45.63} = 0.2$, $p = 0.66$; treatment by colouration interaction: $F_{1,39.77} = 1.29$, $p = 0.26$; Supporting information). It suggests that parents rearing enlarged broods were forced to work harder as previously reported in many different bird species (Santos and Nakagawa 2012).

GSH measurements

We measured intracellular total and oxidized intracellular glutathione levels ($_{t}$GSH and $_{ox}$GSH, respectively) in red blood cells using the Glutathione Colourimetric Detection Kit (Arbor Assays), following instructions provided by the kit manufacturer. For each blood sample, a minimum of 10 µl of blood collected in the heparin tube was diluted with an equal volume of 5-sulfosalicylic acid dihydrate (SSA) solution at 5% weight/volume (1 g of SSA per 20 ml of water) to remove proteins. After 15 min of incubation on ice, the diluted samples were centrifuged at 14 000 rpm and 4°C for 15 min to collect the supernatant. This latter solution was then diluted 1:2.5 with Assay Buffer and stored in two aliquots for final analyses. After a final dilution of 1:20 with Sample Diluent, 50 µl of extracted samples from the first aliquot were loaded in duplicates on a 96 wells microplate to assess total glutathione concentrations (i.e. $_{t}$GSH). The second aliquot of SSA-diluted samples was treated with 2-vinylpyridine to block any free GSH (5 µl of 2VP solution for every 250 µl of the sample) and incubated at room temperature for one hour. This solution was then diluted 1:20 with Sample Diluent and loaded in duplicates on 96 wells microplates to assess oxidized glutathione concentrations (i.e. $_{ox}$GSH). Based on the best standard curves, optical densities (OD) were measured at a wavelength of 405 nm after 10 min. $_{t}$GSH and $_{ox}$GSH concentrations were finally calculated from OD data using the standard dilution curve, while $_{red}$GSH levels were simply obtained by subtracting $_{t}$GSH and $_{ox}$GSH values. Inter-plate repeatability of $_{t}$GSH and $_{ox}$GSH scores demonstrated the reliability of this colourimetric assay ($_{t}$GSH: $r = 0.97 \pm 0.005$, $F_{12,29} = 96.95$, $p < 0.0001$; $_{ox}$GSH: $r = 0.99 \pm 0.0005$, $F_{12,29} = 955.6$, $p < 0.0001$; Lessells and Boag 1987).

Statistical analyses

In adults, based on data collected between 2005 and 2011, we tested whether plumage colour traits changed between consecutive breeding seasons, as a consequence of rearing a reduced or an enlarged brood. Using an ANCOVA model with visual colouration scores as response variable ('ColourMorph'), we entered adult sex, colouration scores determined the year before ('$ColourMorph_{X-1}$') and the brood size manipulation experienced the year before ('BSM_{X-1}') as explanatory variables, plus the interaction between both factors. In this model, we controlled for the year, individual identity and nest site by including them as random variables. In nestlings, we tested whether rearing conditions affected their plumage colour traits on the basis of data collected between 2008 and 2011. In this ANCOVA model, we entered nestling brown chroma ('Brownchroma') as a response variable, while brood size manipulation treatment (hereafter BSM), nestling sex, wing length (i.e. a reliable estimator of nestling age; $F_{1,73} = 470.65$; $p < 0.0001$) and residual body mass before fledging (i.e. corrected for wing length; $F_{1,73} = 261.8$; $p < 0.0001$) were added as explanatory variables. We controlled for an effect of the foster nest site and year by including both variables as random factors.

Preliminary analyses showed that melanin-based colouration of adults or nestlings were neither associated with date and time of the day when individuals were sampled (Pearson's correlations: $-0.14 < r < 0.08$, p-values > 0.29) nor with brood size before treatment, individual age (in years for adults and in days for nestlings) or wing length (Student's t-tests, p-values > 0.39). Hence, to avoid over-parametrizing our models when analysing GSH traits, we did not include capture date, time of the day, age and wing length as covariates. We computed body condition as residual body mass

corrected for sex in adults and wing length in nestlings. Note that including the covariates mentioned above in the models presented hereafter lead to similar results and interpretations. We had measured GSH traits for 26 male and 32 female adults (31 reds, 18 intermediates and 9 greys) and 75 cross-fostered nestlings distributed in 32 broods.

To test for genetic and environmental components of GSH traits, we compared GSH concentrations measured in cross-fostered nestlings with the GSH concentrations of either their biological parents (i.e. effect of origin probably explained by genetic inheritance) or their foster parents (i.e. environmental effect). We did not implement values of biological and foster parents in the same analyses because of the limited number of broods with complete data for both biological and foster parents. However, since $_{t}$GSH, $_{ox}$GSH or $_{red}$GSH levels of biological parents were not correlated with those of foster parents (Pearson's correlations: $-0.42 < r < 0.57$, p-values > 0.14), a relationship between GSH values found in the offspring and their biological parents is unlikely to be inflated by the values measured in their foster parents and vice versa. Therefore, we ran separate linear mixed models for each nestling GSH traits (i.e. $_{t}$GSH, $_{ox}$GSH or $_{red}$GSH levels as response variables), and in each model, we entered as explanatory variables BSM and GSH values of either biological (i.e. $_{t}$GSH, $_{ox}$GSH or $_{red}$GSH levels of Genetic Father and Genetic Mother) or foster parents (i.e. $_{t}$GSH, $_{ox}$GSH or $_{red}$GSH levels of Foster Father and Foster Mother), plus the two-way interactions between those variables. We included as a random effect the genetic brood identity when investigating effects of biological parents and the foster brood identity when investigating effects of foster parents on nestling GSH traits.

To investigate the link between melanin-based colouration and GSH traits (i.e. $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH), we first tested whether GSH levels measured in a given individual are correlated with its own colouration (within-individual comparisons). Then, we performed parent-offspring comparisons, investigating whether GSH levels measured in nestlings are associated with colouration scores of their biological (i.e. colour-genetic determinism) or foster parents (environmental determinism). For the within-individual comparisons, we performed separate ANCOVA models with either adult or cross-fostered nestling $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH levels as response variables. In each model, we entered as explanatory variables adult or nestling plumage colouration (i.e. 'ColourMorph' or 'Brownchroma', respectively), BSM and residual body mass (corrected for sex in adults and wing length in nestlings) as a covariate, plus the interaction between BSM and colouration. In these six mixed models, we controlled for the effect of the breeding site by including nest identity as a random factor. For the parent-offspring comparisons where nestling GSH traits were the response variables, we entered as explanatory variables the plumage colouration of either their genetic parents ('GFatherMorph' and 'GMotherMorph') or foster parents ('FFatherMorph' and 'FMotherMorph'), BSM and nestling residual body mass, plus the two-way interactions between BSM and parent colorations. We controlled for the effect of the nest of origin or of rearing by including genetic or foster brood identity as a random factor.

The final most parsimonious models were selected using a backward stepwise procedure where we sequentially removed non-significant terms ($p > 0.05$), starting with the least significant two-way interactions. For each model, we visually inspected that the distribution of errors was homogenous and normally distributed. Statistical analyses were performed using JMP IN 8.0.

Results

Expression of pheomelanin-based colouration is not condition-dependent

Based on data collected between 2004 and 2011, adult plumage colouration scores were closely associated with those determined the year before (i.e. ColourMorph$_{X-1}$; $n = 228$, $F_{1,226} = 825.27$, $p < 0.0001$), independently of sex ($F_{1,225} = 1.28$, $p = 0.26$) and the brood size manipulation treatment experienced the year before (BSM_{X-1}; $F_{1,199} = 0.4$, $p = 0.53$). The two-way interaction between ColourMorph$_{X-1}$ and BSM_{X-1} was not significant ($F_{1,198} = 2.2$, $p = 0.14$). In the same vein, nestling colouration scores were independent of sex ($n = 329$, $F_{1,306.9} = 0.94$, $p = 0.33$), wing length ($F_{1,320.4} = 0.36$, $p = 0.55$) and brood size manipulation treatment experienced during growth ($F_{1,190.1} = 1.12$, $p = 0.29$). There was a slight tendency for heavier nestlings to be more darkly melanic ($F_{1,325.5} = 3.0$, $p = 0.08$). Altogether, these results suggest that in the tawny owl the expression of melanin-based colouration is not or only weakly sensitive to environmental effects.

Glutathione levels in adult and nestling tawny owls

Adult tawny owls showed larger amounts of GSH than their offspring. Adults had on average 1578.5 µM of $_{t}$GSH (SD: 488.5 µM, range: [555.5–2702.0]), divided in 683.9 µM of $_{ox}$GSH (SD: 189.8 µM, range: [228.8–1120.1]) and 910.0 µM of $_{red}$GSH (SD: 366.9 µM, range: [326.7–2050.6]). Nestlings had on average 1290.6 µM of $_{t}$GSH (SD: 376.0 µM, range: [197.0–2127.4]), divided in 466.2 µM of $_{ox}$GSH (SD: 162.2 µM, range: [97.0–1051.7]) and 825.5 µM of $_{red}$GSH (SD: 251.9 µM, range: [100.1–1339.1]). The proportions of $_{t}$GSH levels in the reduced form (i.e. $_{red}$GSH/$_{t}$GSH) in red blood cells were similar to values reported in other bird species (i.e. 55–70%, Romero-Haro and Alonso-Alvarez 2014, Messina et al. 2020) and differed between adults and nestlings (Student's t-test, $t_{126} = -5.2$, $p < 0.0001$), with $_{red}$GSH constituting 56.7% of $_{t}$GSH levels in adults and 63.9% of $_{t}$GSH levels in nestlings.

Comparison between parental and offspring GSH levels

$_{t}$GSH levels measured in cross-fostered nestlings were significantly and positively related to $_{t}$GSH levels of their genetic

father (estimate ± SE = 0.29 ± 0.12, $F_{1,15.4} = 6.07$, p = 0.03; Table 1A, Fig. 2A) and both foster parents (father: estimate ± SE = 0.38 ± 0.11, $F_{1,20.7} = 6.83$, p = 0.02; mother: estimate ± SE = 0.16 ± 0.11, $F_{1,18.7} = 5.37$, p = 0.03; Table 1B, Fig. 2B). Note here that we found also a significant relationship between nestling $_{t}$GSH levels and the average $_{t}$GSH levels of foster parents (n = 54, $F_{1,19} = 13.64$, p = 0.002), but not with the average levels of genetic parents ($F_{1,8.4} = 0.76$, p = 0.41). Similarly, $_{ox}$GSH levels measured in these nestlings were significantly associated with $_{ox}$GSH levels only of their genetic father (estimate ± SE = 0.32 ± 0.13, $F_{1,13.5} = 5.92$, p = 0.03; Table 1C, Fig. 3A) and foster father (estimate ± SE = 0.35 ± 0.13, $F_{1,25.9} = 7.15$, p = 0.01; Table 1D, Fig. 3B). However, no significant relation was found when considering the average $_{ox}$GSH levels of genetic and foster parents (p-values > 0.28). Finally, $_{red}$GSH levels measured in these nestlings were significantly associated with $_{red}$GSH levels of their foster mother (estimate ± SE = 0.27 ± 0.08, $F_{1,22.5} = 11.41$, p = 0.003; Table 1F, Fig. 4B), but not with $_{red}$GSH levels of their foster father ($F_{1,19.2} = 0.94$, p = 0.34), nor those of their genetic parents (Table 1E, Fig. 4A). When considering the average $_{red}$GSH values of genetic and foster parents, our model revealed a significant relation between nestling $_{red}$GSH levels and the average $_{red}$GSH levels of foster parents (n = 52, $F_{1,18.2} = 10.59$, p = 0.004), but not with the average levels of genetic parents ($F_{1,10.23} = 0.16$, p = 0.70). Apart from one marginal relationship (nestlings showed higher levels of $_{ox}$GSH when experiencing enlarged rather than reduced broods, p = 0.06; Table 1D), we found no evidence that brood size manipulation experiment influenced nestling $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH levels (p-values > 0.26; Table 1).

Table 1. Results of linear mixed models testing the origin-related and environmental components of GSH expression in nestling tawny owls. In separate models, we investigated the relationship between $_{t}$GSH, $_{ox}$GSH or $_{red}$GSH levels of cross-fostered nestlings and the levels of their biological (i.e. $_{t}$GSH, $_{ox}$GSH or $_{red}$GSH levels of Genetic Father and Genetic Mother, models A, C, E) and of their foster parents (i.e. $_{t}$GSH, $_{ox}$GSH or $_{red}$GSH levels of Foster Father and Foster Mother, models B, D, F). In all models, we also tested for an effect of the brood size manipulation treatment (BSM) on nestling GSH concentrations, plus the two-way interactions between BSM and corresponding parental GSH levels. We controlled for an effect of the genetic (models with biological parents) or foster nest site (models with foster parents) by including either genetic or foster brood identity as random factor. Backward stepwise procedure was used to remove non-significant terms (p > 0.05), until obtaining best-fitted models (bold values).

	n	df	F	p
A. Nestling $_{t}$GSH levels				
$_{t}$GSH GeneticMale	**48**	**1,15.4**	**6.07**	**0.03**
BSM		1,16.2	0.84	0.37
$_{t}$GSH GeneticFemale		1,11.5	0.01	0.91
BSM × $_{t}$GSH GeneticMale		1,10.9	2.96	0.11
BSM × $_{t}$GSH GeneticFemale		1,11.1	0.07	0.8
B. Nestling $_{t}$GSH levels				
$_{t}$GSH FosterMale	**54**	**1,20.7**	**6.83**	**0.02**
$_{t}$GSH FosterFemale		**1,18.7**	**5.37**	**0.03**
BSM		1,19.7	0.79	0.38
BSM × $_{t}$GSH FosterMale		1,20.3	0.69	0.42
BSM × $_{t}$GSH FosterFemale		1,17.5	0.18	0.68
C. Nestling $_{ox}$GSH levels				
$_{ox}$GSH GeneticMale	**49**	**1,13.5**	**5.92**	**0.03**
BSM		1,14.0	1.38	0.26
$_{ox}$GSH GeneticFemale		1,10.7	1.23	0.29
BSM × $_{ox}$GSH GeneticMale		1,10.2	1.12	0.31
BSM × $_{ox}$GSH GeneticFemale		1,9.0	0.01	0.91
D. Nestling $_{ox}$GSH levels				
$_{ox}$GSH FosterMale	**61**	**1,25.9**	**7.15**	**0.01**
BSM		1,24.5	3.94	0.06
$_{ox}$GSH FosterFemale		1,18.7	0.45	0.51
BSM × $_{ox}$GSH FosterFemale		1,18.1	1.38	0.25
BSM × $_{ox}$GSH FosterMale		1,17.2	0.001	0.98
E. Nestling $_{red}$GSH levels				
$_{red}$GSHGeneticMale	48	1,19.2	1.34	0.26
BSM		1,17.3	0.16	0.69
$_{red}$GSH GeneticFemale		1,11.6	0.01	0.91
BSM × $_{red}$GSH GeneticFemale		1,9.5	1.73	0.22
BSM × $_{red}$GSH GeneticMale		1,14.2	0.48	0.50
F. Nestling $_{red}$GSH levels				
$_{red}$GSH FosterFemale	**62**	**1,22.5**	**11.41**	**0.003**
$_{red}$GSH FosterMale		1,19.2	0.94	0.34
BSM		1,17.4	0.01	0.92
BSM × $_{red}$GSH FosterFemale		1,15.5	2.00	0.18
BSM × $_{red}$GSH FosterMale		1,15.1	1.24	0.28

Covariation between pheomelanism and GSH levels: within-individual comparison

Nestling $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH levels were not significantly related to plumage colouration ($_{t}$GSH: $F_{1,44.0} = 1.93$, p = 0.17; $_{ox}$GSH: $F_{1,42.7} = 0.98$, p = 0.33; $_{red}$GSH: $F_{1,47.9} = 1.59$, p = 0.21; Table 2A–C, Fig. 5). There was however tight association between nestling GSH concentrations and body mass ($_{t}$GSH: $F_{1,56.9} = 9.84$, p = 0.003; $_{ox}$GSH: $F_{1,55.2} = 8.70$, p = 0.005; $_{red}$GSH: $F_{1,63.0} = 6.33$, p = 0.015), with heavier nestlings having higher levels of $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH (Fig. 6).

In adults, $_{t}$GSH levels were significantly explained by their plumage colouration, light melanic breeding adults showing higher $_{t}$GSH levels than darker melanic ones ($F_{1,52.7} = 4.35$, p = 0.04; Table 2D, Fig. 6). Interestingly, we found that adult melanin-based colouration was not significantly associated with $_{ox}$GSH levels ($F_{1,49.0} = 0.49$, p = 0.49; Table 2E, Fig. 6), but marginally with $_{red}$GSH levels ($F_{1,52.0} = 3.80$, p = 0.057; Table 2F, Fig. 6). Note also that we did not find any effect of the BSM experiment on GSH (alone or in interaction with colouration, p-values > 0.09; Table 2), even when removing residual body mass from these models.

Covariation between pheomelanism and GSH levels: parent-offspring comparison

Plumage colouration of the biological mother was not associated with $_{t}$GSH levels measured in their cross-fostered offspring (linear mixed-model, $F_{1,15.4} = 0.16$, p = 0.70), and

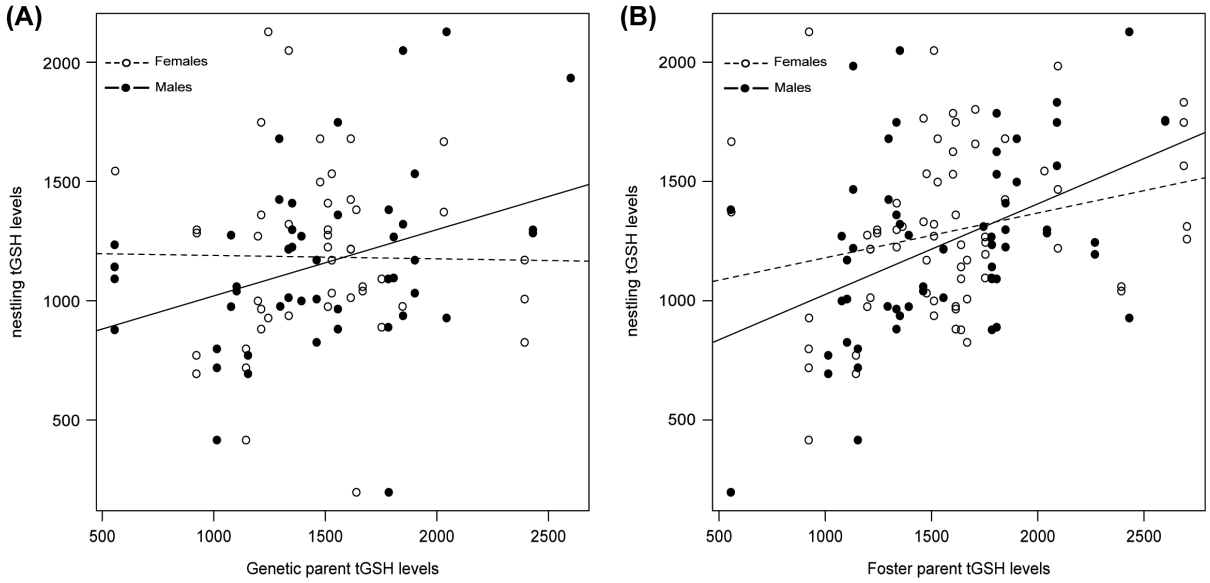

Figure 2. Relationship between nestling $_{t}$GSH levels and those of their (A) genetic father (closed circles and straight regression line; n = 48, estimate ± SE = 0.29 ± 0.12, $F_{1,15.4} = 6.07$, p = 0.03) and mother (open circles and dash regression line; n = 46, estimate ± SE = −0.02 ± 0.17, $F_{1,11.5} = 0.01$, p = 0.91) or (B) foster father (closed circles and straight regression line; n = 60, estimate ± SE = 0.38 ± 0.11, $F_{1,20.7} = 6.83$, p = 0.02) and mother (open circles and dash regression line; n = 66, estimate ± SE = 0.16 ± 0.11, $F_{1,18.7} = 5.37$, p = 0.03).

nestlings born from light melanic males showed non-significant higher $_{t}$GSH levels ($F_{1,18.3} = 3.10$, p = 0.10, Supporting information). However, we found no covariation between colouration of both biological parents and nestling $_{ox}$GSH (another linear mixed-model, father: $F_{1,18.6} = 1.99$, p = 0.17; mother: $F_{1,16.5} = 0.02$, p = 0.88) and $_{red}$GSH levels (another linear mixed-model, father: $F_{1,19.5} = 2.85$, p = 0.11; mother: $F_{1,15.9} = 0.31$, p = 0.59; Supporting information). Note also that no relationships were found when considering the mean colouration of both genetic parents in these models (p-values > 0.38). Similarly, plumage colouration of the foster parents was neither related to nestling $_{t}$GSH (linear mixed-model, father: $F_{1,22.9} = 0.005$, p = 0.95; mother: $F_{1,25.9} = 1.97$, p = 0.17), nor to $_{ox}$GSH (another linear mixed-model, father: $F_{1,22.72} = 0.08$, p = 0.78; mother: $F_{1,25.9} = 2.37$, p = 0.14) and $_{red}$GSH levels (another linear mixed-model, father: $F_{1,23.8} = 0.01$, p = 0.91; mother: $F_{1,28.2} = 1.29$, p = 0.26; Supporting information). Again, no relationships were found

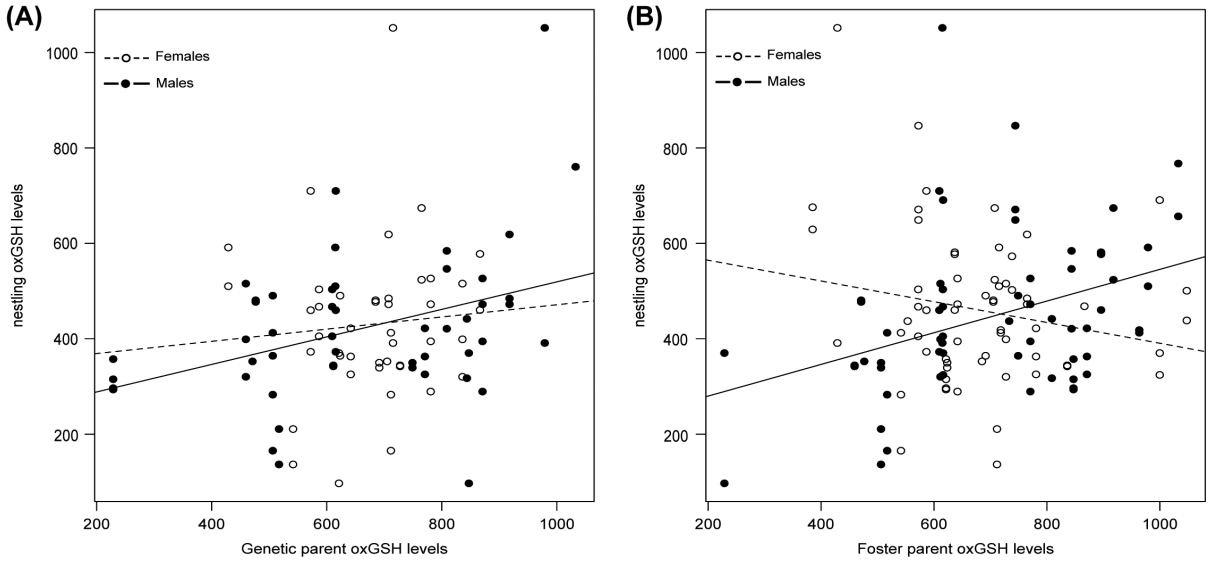

Figure 3. Relationship between nestling $_{ox}$GSH levels and those of their (A) genetic father (closed circles and straight regression line; n = 49, estimate ± SE = 0.32 ± 0.13, $F_{1,13.5} = 5.92$, p = 0.03) and mother (open circles and dash regression line; n = 43, estimate ± SE = 0.16 ± 0.3, $F_{1,10.7} = 1.23$, p = 0.29) or (B) foster father (closed circles and straight regression line; n = 61, estimate ± SE = 0.35 ± 0.13, $F_{1,25.9} = 7.15$, p = 0.01) and mother (open circles and dash regression line; n = 62, estimate ± SE = −0.24 ± 0.17, $F_{1,18.7} = 0.45$, p = 0.51).

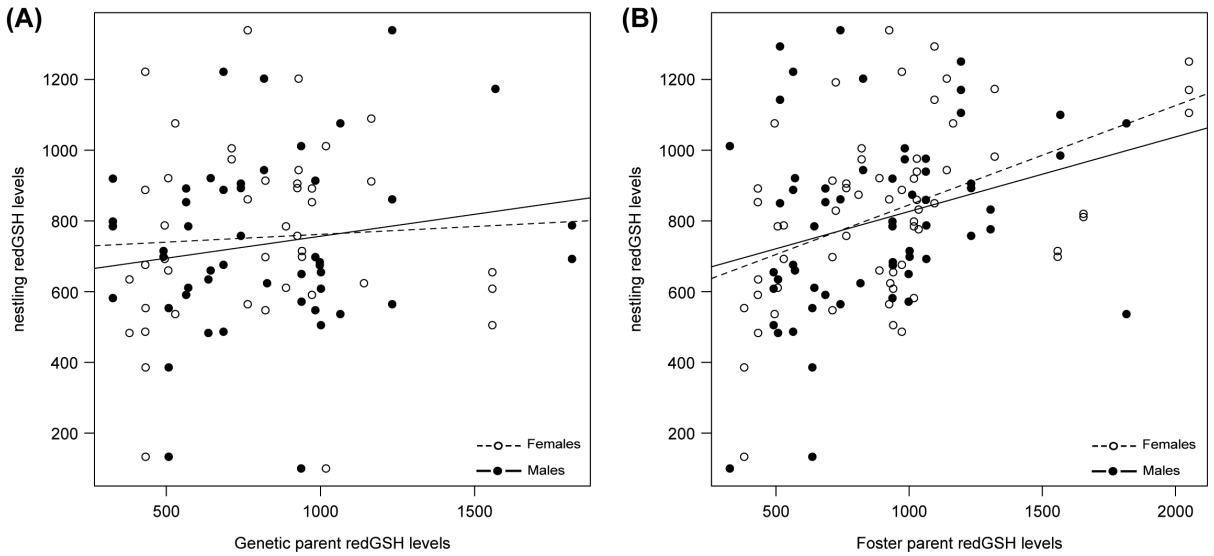

Figure 4. Relationship between nestling $_{red}$GSH levels and those of their (A) genetic father (closed circles and straight regression line; n = 48, estimate ± SE = 0.13 ± 0.11, $F_{1,19.2} = 1.34$, p = 0.26) and mother (open circles and dash regression line; n = 43, estimate ± SE = 0.05 ± 0.14, $F_{1,11.6} = 0.01$, p = 0.91) or (B) foster father (closed circles and straight regression line; n = 60, estimate ± SE = 0.21 ± 0.11, $F_{1,19.2} = 0.94$, p = 0.34) and mother (open circles and dash regression line; n = 62, estimate ± SE = 0.27 ± 0.08, $F_{1,22.5} = 11.41$, p = 0.003).

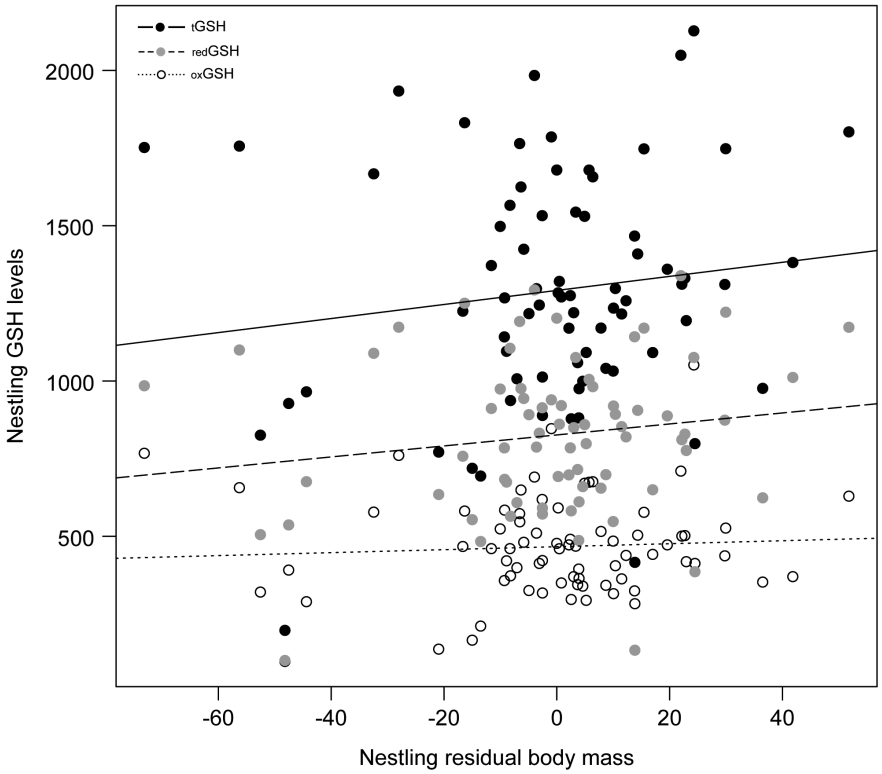

Figure 5. Nestling residual body mass (corrected for wing length) in relation to their $_{t}$GSH (closed circles and straight regression line; $r^2 = 0.02$, n = 73, $F_{1,56.9} = 9.84$, p = 0.003), $_{ox}$GSH (open circles and small dash regression line; $r^2 = 0.004$, n = 74, $F_{1,55.2} = 8.70$, p = 0.005) and $_{red}$GSH (gray circles and long dash regression line; $r^2 = 0.02$, n = 73, $F_{1,63.0} = 6.33$, p = 0.015).

Table 2. Within-individual linear mixed models investigating the link between melanin-based colouration and the levels of $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH in nestling (A–C) and adult (D–F) tawny owls. In addition of residual body mass (corrected for wing length in nestlings and for sex in adults) as covariate, we entered BSM and nestling or adult plumage colouration (i.e. 'Brownchroma' in models A–C and 'ColourMorph' in model D–F or, respectively) as explanatory variables, plus the two-way interaction between both variables. We controlled for the effect of the breeding site by including nest identity as random factor. Backward stepwise procedure was used to remove non-significant terms ($p > 0.05$), until obtaining best-fitted models (bold values).

	n	df	F	p
A. $_{t}$GSH levels in nestlings				
Residual Mass	**73**	**1,56.9**	**9.84**	**0.003**
Brownchroma		1,44.0	1.93	0.17
BSM		1,23.9	0.34	0.56
BSM × Brownchroma		1,52.4	0.02	0.89
B. $_{ox}$GSH levels in nestlings				
Residual Mass	**74**	**1,55.2**	**8.70**	**0.005**
Brownchroma		1,42.7	0.98	0.33
BSM		1,23.0	0.57	0.46
BSM × Brownchroma		1,51.7	0.31	0.58
C. $_{red}$GSH levels in nestlings				
Residual Mass	**73**	**1,63.0**	**6.33**	**0.015**
Brownchroma		1,47.9	1.59	0.21
BSM		1,24.8	0.13	0.72
BSM × Brownchroma		1,53.7	0.02	0.88
D. $_{t}$GSH levels in adults				
ColourMorph	**56**	**1,52.7**	**4.35**	**0.04**
BSM		1,23.8	3.15	0.09
Residual Mass		1,48.4	0.01	0.91
ColourMorph × BSMA		1,48.0	0.02	0.90
E. $_{ox}$GSH levels in adults				
ColourMorph	54	1,49.0	0.49	0.49
Residual Mass		1,40.4	0.13	0.72
BSM		1,21.5	0.02	0.90
ColourMorph × BSMA		1,44.4	0.34	0.56
F. $_{red}$GSH levels in adults				
ColourMorph	54	1,52.0	3.80	0.057
BSM		1,26.5	2.93	0.10
Residual Mass		1,47.0	0.13	0.72
ColourMorph × BSMA		1,45.7	0.03	0.85

between GSH concentrations and the mean colouration of both foster parents (p-values > 0.56). As mentioned above, nestling GSH levels were primarily dependent on their body mass, but were unaffected by the BSM (alone or in interaction with parental colour scores, p-values > 0.1; Supporting information).

Discussion

Although not questioning its genetic basis, our study demonstrated that the expression of GSH response was condition-dependent in our local population of tawny owls, conversely to melanin-based colouration. Indeed, we showed that nestling $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH levels were strongly related to body mass, while nestling $_{t}$GSH and $_{red}$GSH levels were mainly shaped by foster rearing conditions (positive relationship between GSH levels in foster parents and their foster offspring). Based on the idea of GSH dependence of pheomelanin-based colour traits, we expected a greater consumption of GSH (i.e. higher $_{ox}$GSH levels) in dark melanic nestlings, which were producing melanic feathers at the time when we blood-sampled them. This was however not the case. In contrast, we found that in adults, which were not moulting and hence not in a state of intense melanisation, melanin-based colouration was negatively correlated with $_{t}$GSH and $_{red}$GSH levels, dark melanic individuals showing lower $_{t}$GSH and $_{red}$GSH concentrations. In the following, we discuss potential mechanisms leading to covariations between adult $_{t}$GSH and $_{red}$GSH levels and their plumage colouration.

Genetic versus environmental determinism of GSH expression

Covariations between melanin-based colouration and GSH may arise because of condition-dependent expression of either colouration and/or GSH. Our results in adult and nestling tawny owls revealed that the expression of melanin-based colouration was not or only weakly sensitive to environmental effects.

To sustain adequate levels, GSH is synthesized by a two-step biosynthetic pathway (Meister and Anderson 1983, Anderson 1998, Lu 2000), raising the hypothesis of strong genetic inheritance of GSH expression (Board et al. 1974). In Holstein cows *Bos taurus* for instance, heritability of GSH concentration in erythrocytes was estimated at 0.61 ± 0.16 in red blood cell samples and at 0.67 ± 0.17 in whole blood samples (Krogmeier et al. 1993). But this antioxidant response is also physiologically dependent on cysteine availability, which is partly regulated by food intake (Lu 2000). Yet, there are (to our knowledge) few evidences of genetic or environmental determinism of GSH expression. The present study aimed to estimate the proportion of variation explained by these two types of determinism in the tawny owl. Our results revealed that nestling $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH levels were strongly associated with body condition, but not with brood size manipulation (after taking into account for body condition). Heavier nestlings were in better condition and probably more active, leading them to increase their metabolic rate and, in turn, their GSH concentrations. Alternatively, nestlings with higher GSH levels could afford a higher metabolic rate and costly behaviour. Despite the suggestive existence of a genetic basis (e.g. significant relation between nestling and genetic father $_{t}$GSH levels), average GSH concentrations measured in parents nevertheless emphasized that nestling $_{t}$GSH and $_{red}$GSH levels were primarily associated with those of their foster parents, highlighting the strong influence of foster rearing conditions on nestling GSH expression. In this context, access to the essential dietary amino acid cysteine though food supplied by the foster male is likely to play an essential role in the regulation of GSH expression, especially during poor breeding season like in 2011. First, because it provides resources (or energy)

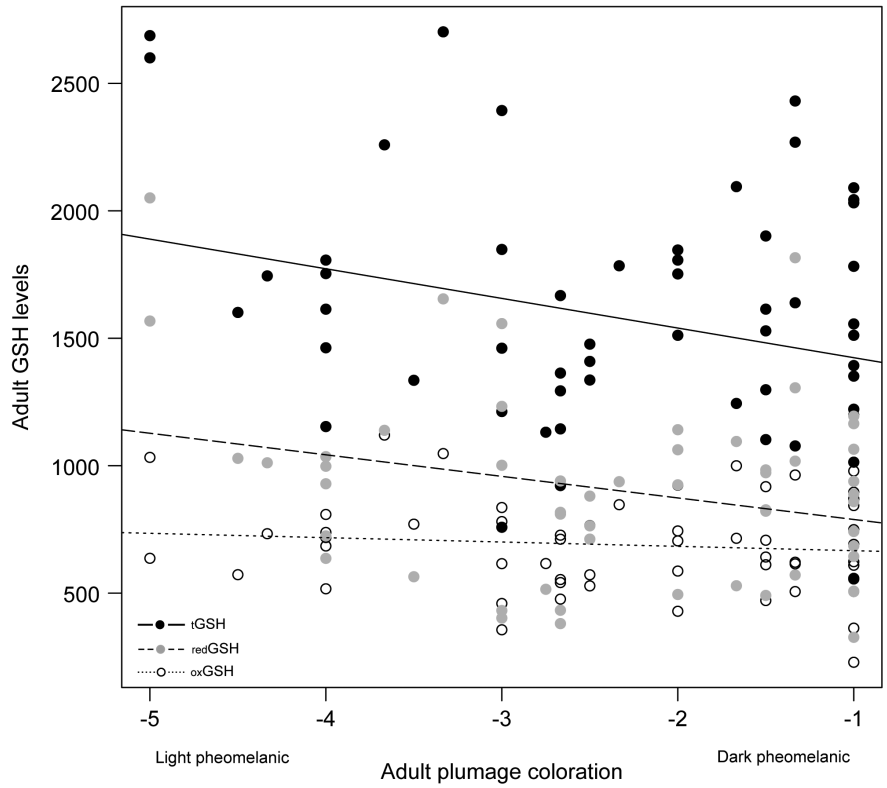

Figure 6. Plumage reddishness in relation to $_{t}$GSH (closed circles and straight regression line; $r^2 = 0.077$, $n = 56$, $F_{1,52.7} = 4.35$, $p = 0.04$), $_{ox}$GSH (open circles and small dash regression line; $r^2 = 0.01$, $n = 54$, $F_{1,49.0} = 0.49$, $p = 0.49$) and $_{red}$GSH levels (gray circles and long dash regression line; $r^2 = 0.075$, $n = 54$, $F_{1,52.0} = 3.80$, $p = 0.057$) in adult tawny owls.

that can be allocated to different maintenance traits, favouring thereby their overall condition. Second, because it enables the replenishment of nestling GSH pool by increasing cysteine availability, a limiting factor in the GSH biosynthesis. In line with this hypothesis, a laboratory study showed that rats supplied with fish oil complements (i.e. a source of cysteine) presented higher levels of GSH in their brain (Denny Joseph and Muralidhara 2012), attenuating thereby the oxidative stress and mitochondrial dysfunctions induced by a neurotoxicant. Moreover, in humans, a restricted dietary supply of methionine and cysteine mixture slowed the rate of whole blood GSH synthesis (Lyons et al. 2000). Nevertheless, the role and expenditure of dietary cysteine in GSH expression still need to be tackled further in natural systems, with experimental manipulations of either food supply or dietary cysteine levels (see for instance Badaloo et al. 2002).

Melanin-based colouration, glutathione and morph-specific life history strategies

The results of the present study suggest that differently colored tawny owls invest differentially in reproduction and in antioxidant defenses. Indeed, light pheomelanic fathers invest more in reproduction and in turn have higher ROS levels under stressful conditions (i.e. when raising an experimentally enlarged brood), whereas dark fathers are more consistent in their reproductive investment independently of environmental quality (Emaresi et al. 2014, 2016). Interestingly, in the present study larger amounts of antioxidants ($_{t}$GSH) were observed in light colored individuals only when rearing reduced broods, whereas no variation according to coloration was observed in enlarged broods (and thus in those broods with higher ROS levels). Therefore, the observed higher levels of $_{t}$GSH and $_{red}$GSH in light colored individuals could be the result of an overall higher investment in antioxidant defenses for oxidative shielding in light colored individuals regardless of reproductive investment. In other words, light colored individuals invest more effort in reproduction which triggers the production of high levels of free radicals but, independently of reproductive investment, they invest more resources into resistance to oxidative stress. Given the absence of effect of the brood size manipulation experiment on color-specific oxidative resistance, we suggest that the higher antioxidant defenses in light colored compared to dark individuals is not so linked to investment under stressful conditions but rather to morph-specific physiological profiles.

The tawny owl has become a study model in several research groups providing a wealth of data that we can discuss in light of the results presented in this paper. Previous results also suggested that more pheomelanic individuals invest overall more effort in breeding effort under prime environmental conditions and have a different physiological profile. For example, pheomelanic offspring increase more in mass

per food intake when fed ad libitum (Piault et al 2009) and are heavier (Morosinotto et al. 2020). Physiological measures also suggest that dark pheomelanic adults might pay high physiological costs in reproduction and body maintenance (e.g. telomere shortening, Karell et al. 2017) and have costly immune responses (Gasparini et al. 2009, Karell et al. 2011b) and more extensive moult (Karell et al. 2013). It thus appears that pheomelanic individuals may pay higher physiological costs and invest more in reproductions (except under very stressful conditions, where light colored individuals cope better and invest more; Emaresi et al. 2014). This would be in agreement with the lower $_{t}$GSH levels observed here in more pheomelanic individuals because, if they are unable to invest as much as light colored individuals in antioxidant defenses, we will observe indeed lower GSH levels, independently on brood manipulation.

Melanogenesis and glutathione

In agreement with previous study (Emaresi et al. 2016), we found that $_{t}$GSH levels and marginally $_{red}$GSH levels quantified in adult tawny owls were associated with melanin-based colouration, light melanic individuals showing higher levels than dark melanic ones. This indicated that light melanic breeding tawny owls generated greater GSH pool and had more GSH reserved for future use (i.e. $_{red}$GSH levels) compared to dark melanic ones. However, it is of particular interest to note that the different colour morphs were using similar amounts of GSH (i.e. $_{ox}$GSH levels) during this breeding season. Whereas we expected to find a greater GSH consumption (i.e. higher $_{ox}$GSH levels) in dark reddish nestlings because of the GSH dependence of pheomelanogenesis, we found no significant covariations between nestling $_{t}$GSH, $_{ox}$GSH and $_{red}$GSH levels and melanin-based colouration (with respect to their own plumage colouration or those of their genetic parents), contradicting the claim that such an association should be universal (Galvan and Alonso-Alvarez 2008, 2009). This latter outcome suggests that the expression of melanin-based colouration is not dependent on the pool of GSH available ($_{red}$GSH) and does not require specific amount of GSH ($_{ox}$GSH) in this species. Altogether, our results pointed out the GSH-independence of pheomelanogenesis in nestling tawny owls, but colour-specific differences in $_{t}$GSH and $_{red}$GSH concentrations in adults. Although we did not measure adult ROS production in 2011, greater $_{t}$GSH levels in light melanic adults are likely to be an adaptive response to confer oxidative shielding regardless of reproductive investment. Light colored individuals appear to invest more in antioxidant defenses (overall higher $_{t}$GSH and tendency to higher pool of $_{red}$GSH), whereas dark individuals appear to be less able to invest as much in maintenance. Another hypothesis is that differently coloured individuals own different physiological strategies or metabolic rates, which require specific protein turnover. GSH being involved in protein metabolism as a source of cysteine, colour-specific difference in $_{t}$GSH and/or $_{red}$GSH concentrations may thus arise because of different needs in cysteine availability rather than antioxidant activity. Finally, another interesting hypothesis is the role of GSH reductase (i.e. GR) in regenerating $_{red}$GSH from $_{ox}$GSH molecules. Under specific conditions, differently coloured tawny owls may have different patterns of GR expression, leading to colour-specific rates of GSH regeneration. If true, one can raise the idea that the colour morph regenerating faster its $_{red}$GSH stock may require consequently a smaller GSH pool ($_{t}$GSH levels). Additional studies are called to further investigate these alternatives, but non-mutually exclusive hypotheses on the proximate reasons leading to colour-specific variations in GSH expression.

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Author contributions

Guillaume Emaresi: Conceptualization (equal); Formal analysis (equal); Writing – original draft (equal); Writing – review and editing (equal). **Pierre Bize**: Conceptualization (equal); Writing – original draft (equal); Writing – review and editing (equal). **Alexandre Roulin**: Conceptualization (equal); Funding acquisition (lead); Writing – original draft (equal); Writing – review and editing (equal).

Transparent Peer Review

The peer review history for this article is available at <https://publons.com/publon/10.1111/jav.02908>.

Data availability statement

Data are available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.tb2rbp022> (Emaresi et al. 2021).

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