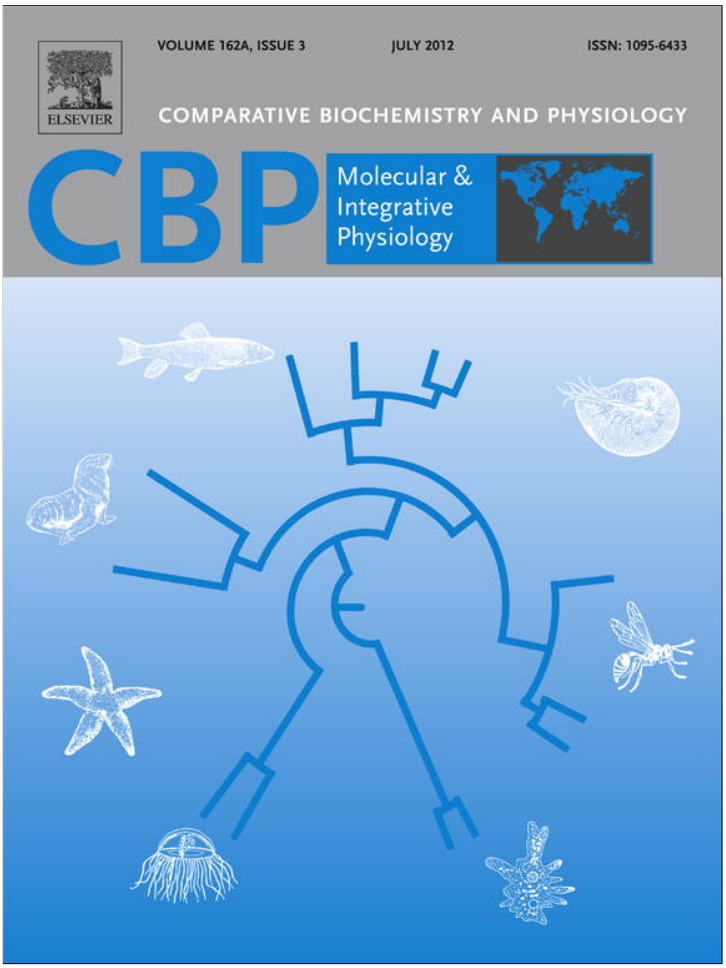

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High prevalence of cataracts in birds with pheomelanin-based colouration

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ABSTRACT

The crystalline lens of the eyes of vertebrates focuses light on the retina. Therefore, maintaining the lens clear is necessary for proper visual function. However, oxidative damage to proteins of the lens leads to opacification and lens dysfunction, termed cataract. Antioxidants thus have a role in avoiding the development of cataracts through their reduction of oxidative stress, and glutathione (GSH), a key intracellular antioxidant, belongs to the primary antioxidant defence mechanism of the lens. Other physiological mechanisms that require GSH may compete with the antioxidant mechanism of the eye. Pheomelanin is a main type of melanin, the most common pigment in vertebrates, and its synthesis consumes GSH. Here, we use data on 81 bird species to test the hypothesis that species producing large amounts of pheomelanin should have diminished capacity to use GSH to protect their eyes and, as a consequence, higher prevalence of cataracts. As predicted, the proportion of pheomelanic plumage was positively associated with the proportion of individuals with cataracts across species, suggesting that production of pheomelanin may have profound fitness consequences, as birds with cataracts have limited ability to perform vital activities. This constitutes the first comparative study of cataracts in wild animals.

1. Introduction

A cataract is an opacification in the crystalline lens of the eye in vertebrates, which obstructs the passage of light, leading to lens dysfunction. The lens collects and focuses light on the retina, from which the collected light is sent to the brain through the optic nerve. This makes maintenance of clarity of the crystalline lens of key importance for proper visual function. Indeed, cataracts are the main cause of human blindness and visual impairment (Congdon et al., 2003). The eye lens is almost entirely composed of proteins, whose oxidation and subsequent aggregation and precipitation generate the opacities characteristic of cataracts (Taylor et al., 1995). These proteins are subject to minimal turnover throughout life, making them especially susceptible to oxidative insults (Taylor et al., 1995). Thus, although some cases of cataracts are congenital (Asherie, 2011), the main causes of human cataracts are agents that produce oxidative stress (i.e., the imbalance between levels of reactive oxygen species and state of the antioxidant and repair machinery) such as ultraviolet (UV) radiation and smoking, as well as accumulation of effects of free radicals with ageing (Collman et al., 1988; Taylor et al., 1995). In humans, therefore, the prevalence of cataracts is higher in low latitude areas where sunlight exposure is high (Collman et al., 1988).

Given the importance of oxidative stress as a factor generating cataracts, different antioxidant mechanisms have evolved because of their capacity to counteract damaging effects of pro-oxidants. It is well known that depletion of antioxidants such as carotenoids and vitamin E by pro-oxidative agents is related to higher risk of cataracts, and that the risk is decreased with dietary supplementation of these antioxidants in humans (Jacques and Chylack, 1991; Christen et al., 2008). This has also been demonstrated in other mammals (Haque and Gilani, 2005), birds (Ferguson et al., 1956) and fish (Waagbø et al., 2003). However, the primary antioxidant defences of the crystalline lenses are not only composed of antioxidants that must be acquired in the diet, but also by antioxidants of endogenous origin such as glutathione (GSH) (Congdon et al., 2003).

GSH is a key intracellular antioxidant whose levels decrease with age in different animals (Bains and Shaw, 1997), thus representing a main cause of age-related cataracts (Nechita et al., 2006). In rats, age-related decreases in levels of protein sulfhydryl groups are associated with cataracts (Swamy and Abraham, 1987), and decreases in GSH levels and associated cataracts due to exposure to pro-oxidants can be avoided by a source of dietary antioxidants (Haque and Gilani, 2005). Indeed, GSH is considered of major importance to prevent cataract formation because it maintains protein thiol groups in the reduced state, thereby preventing the formation of high molecular weight protein aggregates (Reddy, 1990) that are the origin of the opacifications characteristic of cataracts (Taylor et al., 1995). Therefore, any physiological process that consumes GSH may compete for this antioxidant resource with the antioxidant machinery of the crystalline

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lenses. If such a GSH-consuming process has a partially genetic basis (i.e., is not entirely due to exogenous factors), interspecific differences in expression of the process may lead to interspecific variability in susceptibility to development of cataracts. In wild animals, this variability could be important from an evolutionary perspective because opacities in the crystalline lens can strongly impair visual function, with profound consequences for fitness. Birds represent a group in which cataracts may have dramatic effects on fitness, with strong mechanisms to avoid cataracts having evolved because of their advanced visual system and high dependence on visual activity, as well as their aerial and mostly diurnal habits that expose them to higher levels of ultraviolet radiation than any other class of animals. Indeed, although investigations of avian cataracts are almost anecdotal and limited to the field of veterinary science, variability has been observed in prevalence of cataracts among birds (Holmes and Austad, 1995; Brooks, 1997). However, the evolutionary basis for this variability has not been determined.

Here we hypothesise that interspecific variability in prevalence of cataracts should be due to physiological competition for the antioxidant machinery of the crystalline lens with other GSH-demanding processes. In particular, it may compete with the synthesis of pheomelanin, which is one of the two forms of melanin, the most common pigment in vertebrates. Its synthesis requires as a substrate the amino acid cysteine, of which GSH is the main physiological reservoir. This means that pheomelanogenesis consumes cysteine, in contrast to the synthesis of eumelanin, the other melanin form (Meyskens et al., 1999; Galván and Alonso-Alvarez, 2009). Indeed, pheomelanin may have evolved because of the adaptive benefits of the removal of cysteine, which can be toxic if in excess, during the synthesis of this pigment (Galván et al., in press). Therefore, species that have evolved the molecular basis to produce large amounts of pheomelanin may have a diminished capacity to protect their eyes from oxidative damage generated by exogenous factors such as ultraviolet radiation or through the effects of ageing. Thus, we predicted a positive correlation between the proportion of pheomelanic plumage and the proportion of individuals with cataracts across species of birds. Since latitude is related to degree of exposure to ultraviolet radiation and explains variability in cataract prevalence in humans (Collman et al., 1988), we tested our prediction controlling for the potentially confounding effect of latitude. With this aim, we used a dataset of 81 species of birds, representing the first comparative study of cataracts in wild animals.

2. Material and methods

2.1. Detection of cataracts in birds

By visually examining the crystalline lenses of freshly killed specimens delivered to a taxidermist (J.E.), we considered that they presented cataracts when they were opaque in their inner part and it was impossible to accurately perceive the design of millimetre paper through the lens (Fig. 1). Lenses with pale red colour were discarded because this is indicative of eye damage due to collision.

2.2. Quantification of melanin-based plumage colour expression

Eumelanic and pheomelanic traits are generally of distinctive colours, the former being responsible for black and grey colours and the latter for yellowish, reddish, chestnut and brown colours (Toral et al., 2008). Eumelanin and pheomelanin normally occur simultaneously in the tissues (Ozeki et al., 1997), but the darker colours conferred by eumelanin (Toral et al., 2008) make evident the lower content of this pigment in chestnut and brown colours as compared to black and grey colours (Galván and Alonso-Alvarez, 2009). Furthermore, many bird species have feather melanin contents of high purity (>90% of either eumelanin or pheomelanin; McGraw and Wakamatsu, 2004; J.J. Negro pers. com.). Therefore, we assumed that black and grey plumage colours were predominantly generated by eumelanin, while chestnut and brown colours were predominantly generated by pheomelanin. We did not consider conspicuous yellow or red colourations assumed to be generated by other pigments (i.e. carotenoids), unless chemically identified as melanin-based by Toral et al. (2008). Although a rough approximation to the real proportion of eumelanic and pheomelanic plumage, the assumption that black–grey colours are eumelanic and brown–chestnut colours are pheomelanic should be adequate for comparative purposes (Owens and Hartley, 1998). Indeed, this is the most appropriate method for

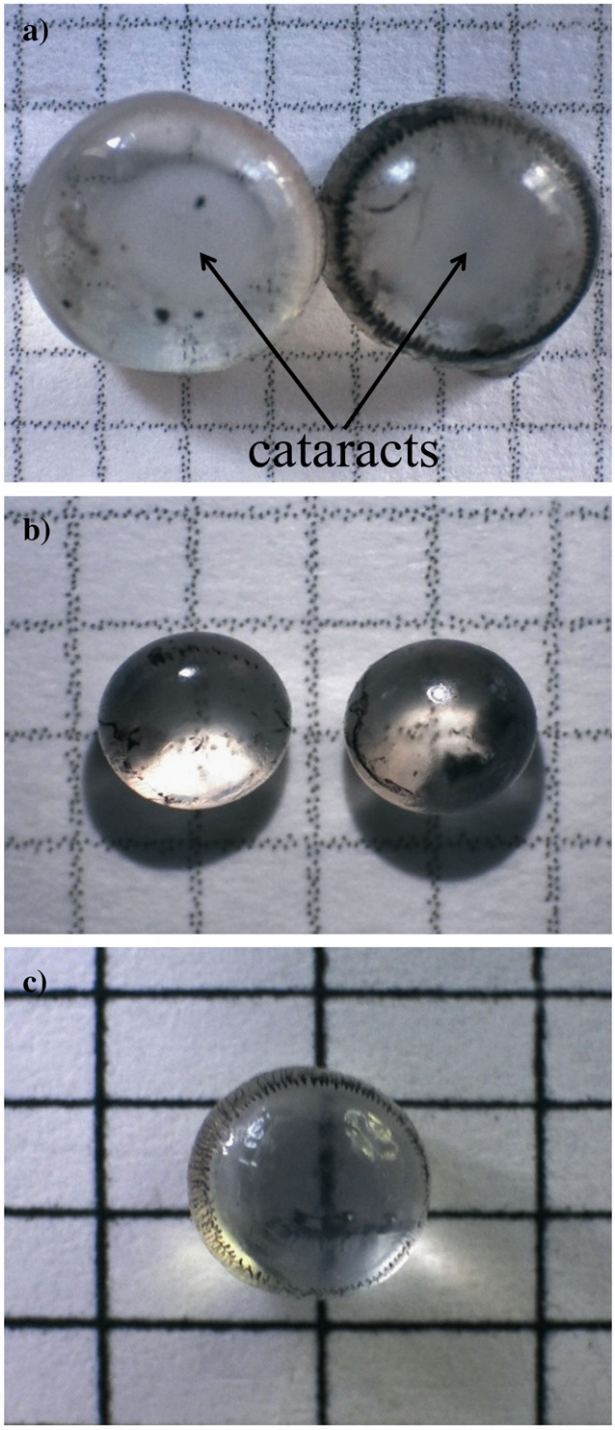

Fig. 1. Images showing crystalline lenses of birds with (a: Eurasian sparrowhawk *Accipiter nisus*) and without (b: house sparrow *Passer domesticus*; c: blackbird *Turdus merula*) cataract.

comparative analyses, quantifying eu- and pheomelanin contents so that they can be compared. This is because melanin content varies between plumage patches in any given species, so to obtain estimates of melanin content that are comparable among species, these measurements should be taken for every feather of a number of individuals of each species, a task that has never been undertaken.

Thus, we quantified the proportion of melanic plumage parts by examining illustrations in Cramp and Simmons (1977–1992) and Cramp and Perrins (1993–1994). Illustrations of both resting and flying adults in breeding plumage birds were examined. The method used by Beauchamp and Heeb (2001) and Galván (2008) was followed to obtain estimates of the proportion of eu- and pheomelanic colour present in the plumage of each species, assigning scores that ranged from 0 (total lack of melanic colour) to 5 (all melanic). When a species was sexually dichromatic regarding melanin-based coloration, eumelanic and pheomelanic scores were the average obtained for males and females. The feathers of owls (Order Strigiformes) present porphyrins, which generate similar colours to those of pheomelanin. However, recent findings show that the contribution of porphyrins to the colour of plumage is minimal as compared to the effect of pheomelanin, and that porphyrins photodegrade rapidly after being deposited in feathers (Negro et al., 2009). Therefore, owls were included in the analysis. Additionally, the barn owl *Tyto alba* shows extensive geographical variation in degree of pheomelanism, but since our specimens were all from Denmark, where the most intensively coloured individuals occur (Antoniazza et al., 2010), we scored the barn owl according to the most intense colour category observed in the species (Roulin, 2003). The tawny owl *Strix aluco* has both a eumelanic and a pheomelanic morph (Roulin et al., 2003), so we took the average score of both morphs. Similarly, we took the average score of the morphs in the ruff *Philomachus pugnax*, a highly polymorphic species. Fig. 2 shows examples of the different scores for pheomelanic coloration.

Chemical analyses confirmed that eu- and pheomelanic plumage colour scores were actually related to the content of eu- and pheomelanin, respectively, in feathers (see below). Thus, melanic

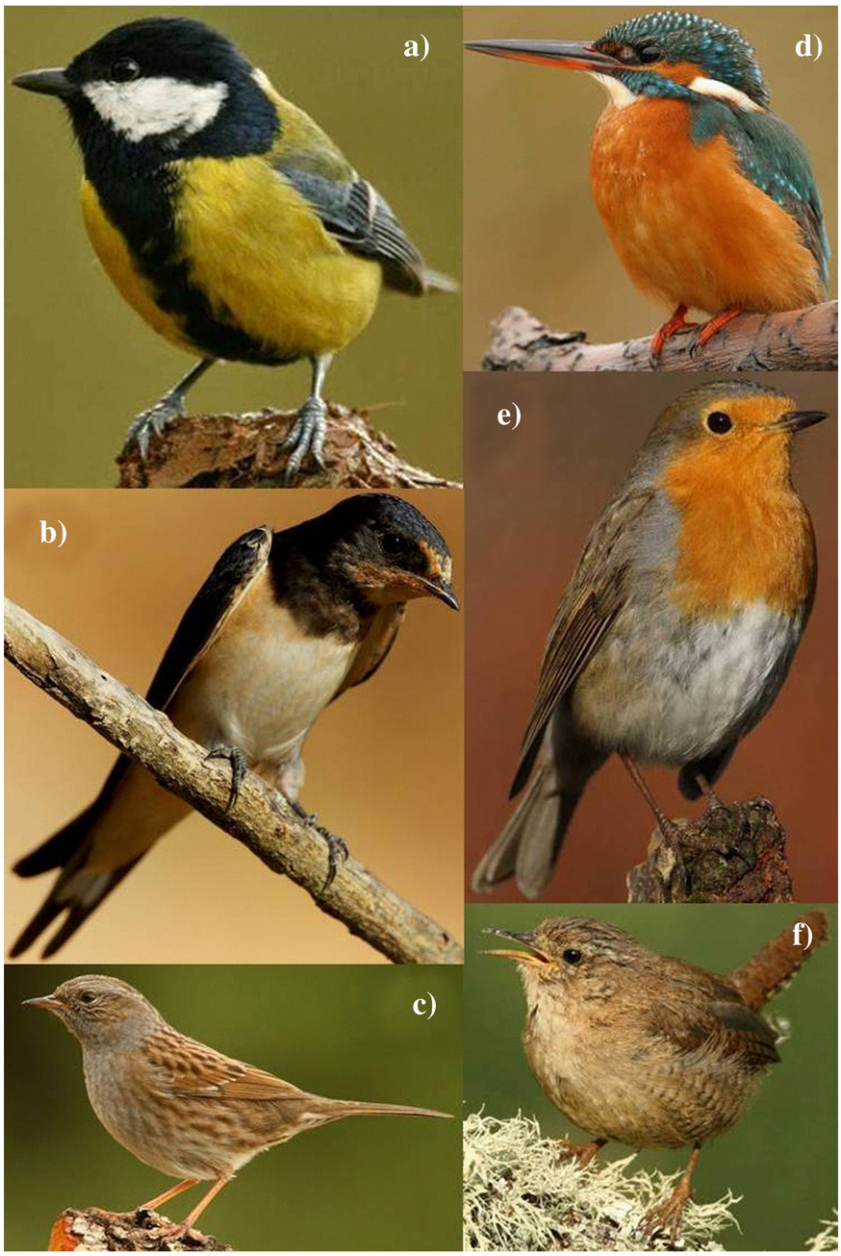

Fig. 2. Examples of species included in the study with different assigned scores for pheomelanic plumage colouration. a: great tit *Parus major*; b: barn swallow *Hirundo rustica*; c: dunnock *Prunella modularis*; d: common kingfisher *Alcedo atthis*; e: European robin *Erithacus rubecula*; f: Northern wren *Troglodytes troglodytes*. The scores for these species are shown in Supplementary Appendix 1. All photographs are courtesy of Rafael Palomo Santana.

colour scores were reliable proxies for the content of melanins in the plumage of birds.

2.3. Analyses of melanin content in feathers

We collected feathers from birds captured with mist nests during 25 May–5 June 2010 in woodland in six different sites around Chernobyl, Ukraine, as part of an ongoing study in the area (Møller et al., 2011). Feathers were collected from a total of 16 species, comprising 152 individual birds (Table 1). A total of 11 of the species were also included in the dataset on prevalence of cataracts, and the melanic plumage colour of the rest of the species was scored as described above (Table 1). A total of 10–20 feathers were plucked from the two most conspicuous colour patches of the plumage of birds, and stored in plastic bags in the dark until analyses were made. The analyses of melanin content in feathers were thus made on mixtures of feathers from different colour patches to get a general index of the melanin content in the plumage of the species.

The microanalytical methods to quantify the amounts of eumelanin and pheomelanin were based on the formation of specific degradation products, pyrrole-2,3,5-tricarboxylic acid (PTCA) by alkaline H_2O_2 oxidation of eumelanin and 4-amino-3-hydroxyphenylalanine (4-AHP) by reductive hydrolysis of pheomelanin with hydriodic acid (HI) (Wakamatsu et al., 2002; Ito et al., 2011). Eumelanin and pheomelanin were calculated by multiplying PTCA and 4-AHP values with 25 and 9, respectively.

2.3.1. Sample preparation

Feather samples were homogenised with Ten-Broeck glass homogenizer at a concentration of 10 mg/mL water.

2.3.2. Alkaline H_2O_2 oxidation to measure eumelanin (PTCA and PDCA) (Ito et al., 2011)

Sample homogenate (100 μL) was taken in a 10 ml screw-capped conical test tube, to which 375 μL 1 mol/L K_2CO_3 and 25 μL 30% H_2O_2 (final concentration: 1.5%) were added. The mixture was mixed vigorously at 25 °C ± 1 °C for 20 h. The residual H_2O_2 was decomposed by adding 50 μL 10% Na_2SO_3 and the mixture was then acidified with 140 μL 6 mol/L HCl (caution to CO_2 evolution). After vortex-mixing, the reaction mixture was centrifuged at 4000 *g* for 1 min, and an aliquot (80 μL) of the supernatant was directly injected into the HPLC system.

Table 1
Eu- and pheomelanic colour scores for the species used in the analyses of melanin content in feathers.

Species	Male eumelanic colour score	Male pheomelanic colour score	Female eumelanic colour score	Female pheomelanic colour score	N
Anthus trivialis	0	4	0	4	10
Coccothraustes coccothraustes	1	4	1	4	4
Erithacus rubecula	1	4	1	4	8
Fringilla coelebs	3	4	2	4	11
Hirundo rustica	2	1	2	1	59
Lanius collurio	2	1	1	3	9
Luscinia luscinia	0	5	0	5	2
Parus major	3	0	3	0	6
Phoenicurus ochruros	4	1	4	1	5
Phylloscopus sibilatrix	1	0	1	0	4
Sylvia atricapilla	5	0	4	1	2
Sylvia communis	1	2	1	2	4
Sylvia nisoria	3	0	3	0	5
Turdus merula	5	0	0	5	14
Turdus philomelos	0	4	0	4	5
Turdus viscivorus	1	3	1	3	4

2.3.3. HI reductive hydrolysis to measure pheomelanin (4-AHP) (Wakamatsu et al., 2002)

Sample homogenate (100 μL) was taken in a 10 mL screw-capped conical test tube, to which 20 μL 50% H_3PO_2 and 500 μL 57% HI were added. The tube was heated at 130 °C for 20 h, after which the mixture was cooled. An aliquot (100 μL) of each hydrolysate was transferred to a test tube and evaporated to dryness using a vacuum pump connected to a dry ice-cooled vacuum trap and two filter flasks containing NaOH pellets. The residue was dissolved in 200 μL 0.1 mol/L HCl. An aliquot (10–20 μL) of each solution was analysed on the HPLC system.

2.3.4. HPLC analyses

H_2O_2 oxidation products were analysed with the HPLC system consisting of a JASCO 880-PU liquid chromatograph (JASCO Co., Tokyo, Japan), a Shiseido C_{18} column (Shiseido Capcell Pak MG; 4.6 × 250 mm; 5 μm particle size) and a JASCO UV detector. The mobile phase was 0.1 mol/L potassium phosphate buffer (pH 2.1):methanol, 99:1 (v/v). Analyses were performed at 45 °C at a flow rate of 0.7 mL/min. Absorbance of the eluent was monitored at 269 nm. A standard solution (80 μL) containing 1 μg each of PTCA (pyrrole-2,3,5-tricarboxylic acid), PDCA (pyrrole-2,3-dicarboxylic acid), TTCA (thiazole-2,4,5-tricarboxylic acid) and TDCA (thiazole-2,3-dicarboxylic acid) in 1 mL water was injected every 10 samples.

HI reductive hydrolysis products were analysed with an HPLC system consisting of a JASCO 880-PU liquid chromatograph, a JASCO C_{18} column (JASCO Catecholpak; 4.6 × 150 mm; 7 μm particle size) and an EICOM ECD-300 electrochemical detector. The mobile phase used for analysis of 4-AHP was 0.1 mol/L sodium citrate buffer, pH 3.0, containing 1 mmol/L sodium octanesulfonate and 0.1 mmol/L Na_2EDTA: methanol, 98:2 (v/v). Analyses were performed at 35 °C at a flow rate of 0.7 mL/min. The electrochemical detector was set at + 500 mV versus an Ag/AgCl reference electrode. A standard solution (10–20 μL) containing 500 ng each of 4-AHP (4-amino-3-hydroxyphenylalanine) and 3-AHP (3-amino-4-hydroxy- phenylalanine; 3-aminotyrosine from Sigma) in 1 mL 0.1 M HCl was injected every 10 samples.

2.3.5. Relationship between melanic colour scores and melanin content in feathers

We used Pearson *r*-values as estimates of the effect size of the association between melanic plumage colour scores and the content of pheomelanin (4-AHP) and eumelanin (PTCA) in the feathers of the 16 species of birds. The largest effect size corresponded to the relationship between pheomelanic colour scores and pheomelanin content in feathers of males ($r = 0.55$), followed by the same relationship in females ($r = 0.46$) and males and females pooled ($r = 0.43$; Fig. 3). The relationship between eumelanic colour scores and eumelanin content in feathers yielded lower effect sizes (males: $r = 0.33$, females: $r = 0.41$, males and females pooled: $r = 0.34$; Fig. 3).

It must be considered that the chemical analyses of melanin content quantify the concentration of eu- and pheomelanin in feathers, while the melanic plumage colour scores quantify the extent of plumage covered by colour supposedly conferred by eu- and pheomelanin. These two variables thus quantify different characteristics of birds. Therefore, our analyses of the relationship between melanic colour scores and melanin content in feathers are conservative. Even so, we found relatively large effect sizes for the association between colour scores and measurements of melanin content, with effect sizes especially large in the case of pheomelanic colour scores and pheomelanin content measurements. This indicates that our scoring method of plumage colour provides a reliable index of the degree of melanization of the plumage of birds.

2.4. Breeding latitude

We calculated the breeding latitude as the mean of the northernmost and the southernmost latitudes during the breeding season (Cramp and Simmons, 1977–1992; Cramp and Perrins, 1993–1994).

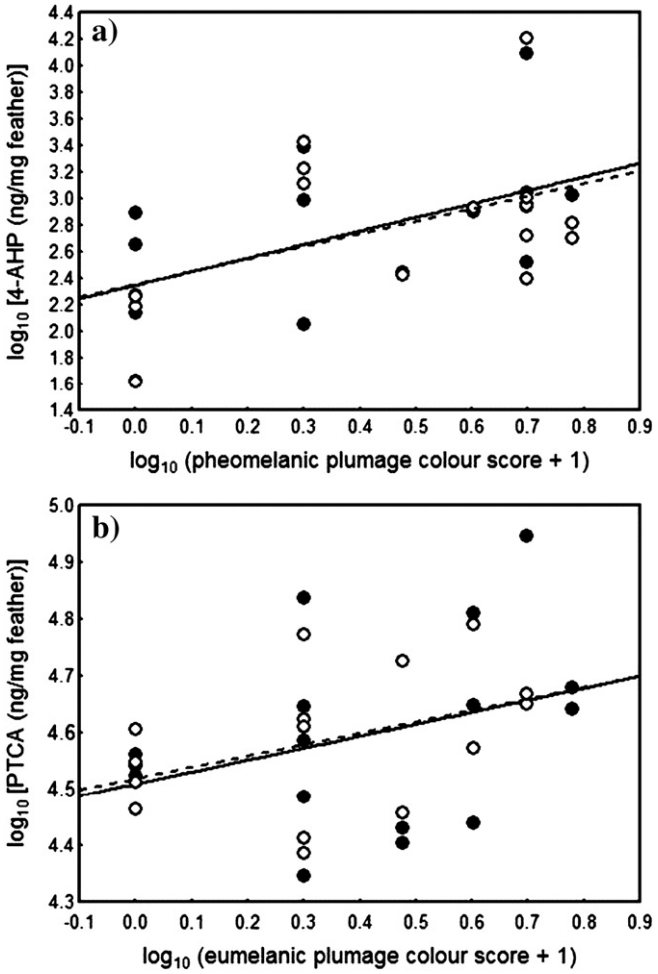

Fig. 3. Relationship between the content of pheomelanin (4-AHP) in feathers and pheomelanic plumage colour scores (a), and between the content of eumelanin (PTCA) in feathers and eumelanic plumage colour scores (b) in 16 species of birds. The lines are the regression lines. Males: solid symbols, continuous line. Females: hollow symbols, dashed line.

Information on the proportion of individuals with cataracts, eu- and pheomelanic plumage colour scores and breeding latitude for all species is given in Supplementary Appendix 1.

2.5. Data analyses

We regressed the proportion of individuals with cataracts per species (arcsin-transformed) against melanic plumage colour score and breeding latitude (both $\log_{10}$-transformed). We corroborated the results by also considering a binomial response variable (i.e., presence vs. absence of cataract) instead of the proportion of individuals with cataracts per species. Since species are not independent sample units, we used phylogenetic generalised least squares (PGLS) models with an unpublished function by R. Freckleton (pglm3.4.r) in R statistical environment. First we estimated the phylogenetic signal with the parameter λ, which ranges from 0 (phylogenetic independence) to 1 (species' traits covary in proportion to their shared evolutionary history; Freckleton et al., 2002). Then we calculated the maximum-likelihood value of λ, with which we made the phylogenetically corrected regression of cataract prevalence against melanic colour scores and latitude. Since there was large variation in number of specimens examined per species, we weighted the analyses by sample size following the recommendation of Garamszegi and Møller (2007). See Supplementary Appendix 2 for further details on phylogenetic analyses. The phylogenetic hypothesis used (Supplementary Appendix 3) was obtained from the phylogeny compiled by Galván and Møller (2011), assuming all branch lengths to equal unity.

3. Results

We found instances of cataract in 13 different species (Supplementary Appendix 1). The maximum-likelihood of λ was 0.872. At this value, the PGLS model was significant ($F_{1,78}=5.51$, $P=0.006$) and explained 12.5% of the variance in cataract prevalence among species. The effect of pheomelanic plumage colour score was significant and positive ($b=0.25$, $t=2.85$, $P=0.005$; Fig. 4), while the effect of breeding latitude was not significant ($b=-0.20$, $t=-1.56$, $P=0.123$). The effect of pheomelanic plumage colour score was also significant without weighting the model by sample size ($b=0.23$, $t=2.67$, $P=0.009$), or after considering a binomial response variable (i.e., presence vs. absence of cataract) instead of the proportion of individuals with cataracts per species ($\lambda=6.61\times10^{-5}$, $b=0.51$, $t=3.75$, $P<0.001$). By contrast, eumelanic colour score had no significant effect ($b=-0.07$, $t=-0.70$, $P=0.487$).

4. Discussion

Pheomelanogenesis consumes cysteine, of which GSH is the main reservoir (Benedetto et al., 1981; Meyskens et al., 1999). Accordingly, negative associations between degree of pheomelanization and other processes that require GSH protection have recently been found. For example, bird species with large proportions of pheomelanic colour have smaller brains, as brain development requires large amounts of energy and antioxidants (Galván and Møller, 2011), and suffer more strongly from the effects of ionising radiation, to which GSH is particularly susceptible (Galván et al., 2011). It has also been reported that more pheomelanic individual barn owls *T. alba* are more sensitive to physiological stress caused by corticosterone than less pheomelanic birds (Almasi et al., 2008), and that tawny owls *S. aluco* belonging to the pheomelanic morph have lower viability during adverse environmental conditions than conspecifics belonging to the eumelanic morph (Karell et al., 2011).

Here, the prevalence of cataracts was higher among species with a large proportion of plumage coloured by pheomelanin, as predicted, suggesting that species that produce large amounts of pheomelanin may have diminished capacity to protect their crystalline lenses from oxidative stress. GSH is an antioxidant of major importance for the prevention of cataract formation because it maintains protein thiols in the reduced state and thus prevents the formation of high molecular

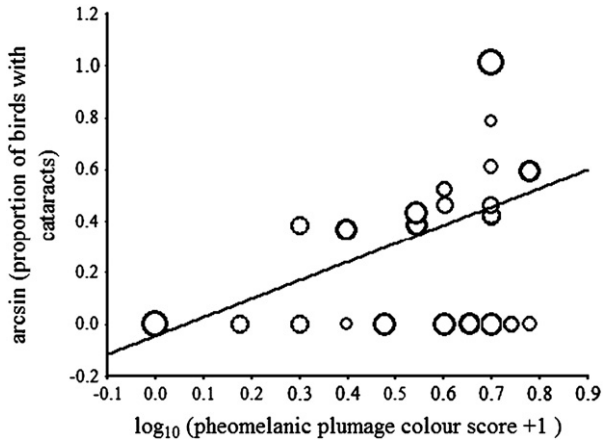

Fig. 4. Relationship between proportion of individuals with cataracts and pheomelanic colour score in 81 species of birds. The size of the symbols increases logarithmically with sample size for illustrative purposes only.

weight protein aggregates, which are the basis for lens opacification (Reddy, 1990). Therefore, competition for GSH resources may exist between pheomelanin synthesis and antioxidant protection of the eye. These results confirm from an evolutionary perspective the antioxidant cost of producing pheomelanin, which in humans translates into higher risk of skin cancer (Meyskens et al., 1999). These results should be taken with caution because we could not control for the age of birds in our analyses, and ageing is an important factor causing cataracts (Taylor et al., 1995). However, we probably collected a high variation of ages in the bird specimens that we examined, which makes unlikely that our results are biassed toward certain age classes.

This is the first report of cataracts in an extensive set of species. Given the importance of the visual system for birds, the fitness consequences of developing cataracts are likely to be profound. Wild birds are under permanent exposure to high levels of UV radiation, the main factor causing cataracts through the oxidative damage it produces (Collman et al., 1988; Taylor et al., 1995), and birds with cataracts are likely to have limited ability to hunt, forage or perceive visual signals and cues. Therefore, wild birds with cataracts might be limited in vital abilities and hence suffer elevated risk of mortality. Likewise, species with high prevalence of cataracts may have evolved antioxidant mechanisms to counteract their damaging effects (Holmes and Austad, 1995), which in turn may lead to higher costs of self-maintenance and, as a consequence, stronger constraints on life-histories. Future studies should investigate the fitness consequences of cataracts for individual birds, as well as possible antioxidant mechanisms that may have evolved to avoid them. This knowledge from wild birds, which are under permanent exposure to high levels of ultraviolet radiation and are able to develop antioxidant mechanisms to counteract the oxidative damage of their high metabolic rates (Holmes and Austad, 1995), may have implications for understanding patterns of variation in cataract prevalence in humans and other species.

Supplementary data to this article can be found online at doi:10.1016/j.cbpa.2012.03.012.

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