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Testicular melanization has evolved in birds with high mtDNA mutation rates

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Abstract

Melanin is mainly found in the integument of animals, but it also appears in several extracutaneous tissues. The presence of melanin in testes has been anecdotally reported in all vertebrate groups, but the causes and functions of this melanin remain unknown. Similar to other extracutaneous melanins, testicular melanin may protect male germ cells from oxidative stress. Given the high respiratory activity of spermatozoa, oxidative stress generated by mitochondrial dysfunction as a consequence of mtDNA mutations directly affects sperm viability. Thus, natural selection may favour testicular melanization in males of species with high historical mutation rates in the mitochondrial genome. Here, we tested this hypothesis using information on occurrence of testicular melanization and mutation accumulation as reflected by cytochrome *b* mtDNA base pair substitution rates in a large set of 134 species of birds, controlling for the confounding effects of body mass, reproductive activity and phylogeny. We found that testicular melanization has evolved in species with high rates of accumulated mitochondrial mutations and propose that this is an adaptive response related to the protective capacity of melanin against oxidative stress. In support of this hypothesis, testicular melanization was more frequently observed during the breeding season of birds (i.e. when spermatogenesis is likely to occur) than during reproductive inactivity. In contrast to other extracutaneous melanins whose abundance seems to reflect skin and coat colour, we did not find a correlation between the proportion of plumage coloured by melanins and occurrence of testicular melanization. Whereas future experimental studies should test these hypotheses, our study highlights for the first time that melanization patterns in animals may evolve as a response to historical mutation rates.

Introduction

Melanins are the most common pigments in animals. In vertebrates, melanins are synthesized by specialized cells called melanocytes, from which they are transferred to the target tissues that are usually the skin and associated integumentary structures such as feathers and hairs (van den Bossche *et al.*, 2006). These melanocytes that are found in and confer colour to the integument are categorized as classical melanocytes, and most research on melanin synthesis and function has focused on them (Lin & Fisher, 2007). However, other melanocytes are found in the eye, inner ear, brain, bones, heart, skeletal muscle and visceral adipose tissue (Steel & Barkway, 1989; Hirano, 1990; Zucca *et al.*, 2004; Zadlo *et al.*, 2006; Yajima & Larue, 2008; Randhawa *et al.*, 2009; Tu *et al.*, 2009). These nonclassical melanocytes are not derived from melanoblasts that take the dorso-lateral pathway during development like classical melanocytes, but from

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melanoblasts that probably take other routes (Brito & Kos, 2008). Furthermore, the function of melanins produced by nonclassical melanocytes, except that of ocular and ear melanins (e.g. Steel & Barkway, 1989; Zadlo *et al.*, 2006), remains unclear (Ito, 2008).

Extracutaneous melanin can also be found in the testes. In vertebrates, the presence of testis melanin has been reported in fish (Louiz *et al.*, 2009), amphibians (de Oliveira & Zieri, 2005; Zieri *et al.*, 2007), reptiles (Guillette *et al.*, 1983), mammals (Scott & Fritz, 1979; Poole & Lawton, 2009) and birds (Serventy & Marshall, 1956). However, the frequency and causes of melanin appearance in the testes, as well as its possible function, have never been investigated in any of these groups, making testicular melanin one of the poorest known types of melanins.

Although the function of testicular melanin is unknown like most extracutanous melanins, the so far known functions of some melanins produced by nonclassical melanocytes are all related to protection against factors that generate physiological stress. First, melanin of the retinal pigment epithelium, iris and choroid protects the eye from oxidative damage caused by solar radiation (Liu *et al.*, 2005; Zadlo *et al.*, 2006; Zareba *et al.*, 2006). Second, melanin of the stria vascularis, in addition to playing an important role in normal cochlea development (Steel & Barkway, 1989), protects the inner ear from toxic compounds and noise-induced stress (Laurell *et al.*, 2007; Murillo-Cuesta *et al.*, 2010). Third, although the function of melanin associated with dopaminergic neurons (i.e. neuromelanin) is still not fully understood, this internal pigment seems to be important for the survival of neurons by protecting them from reactive quinones and toxic metals (Solano *et al.*, 2000; Hearing, 2009). Fourth, heart melanocytes may contribute to maintain the balance of oxidative stress in the myocardium, thus contributing to avoid atrial arrythmias (Levin *et al.*, 2009). Lastly, it has been suggested that the melanin of the visceral adipose tissue may protect adipocytes from oxidative damage (Randhawa *et al.*, 2009) and that melanin of the muscle may have an antioxidant role (Tu *et al.*, 2009). Given the importance that different types of extracutaneous melanin seem to have in protecting tissues from oxidative damage and toxic agents, it is therefore likely that testicular melanin fulfils a similar function.

Sperm is particularly sensitive to DNA damage caused by oxidative stress, i.e. the imbalance between the production of reactive oxygen species and antioxidant molecules tipped towards the former. This is because the male germ line suffers from large numbers of cell divisions, long cell production periods and a lack of repair mechanisms in the late stages of cell formation, and the spermatozoa demand a high respiratory activity and present large amounts of polyunsaturated fatty acids prone to oxidation in their plasma membranes (reviewed in Velando *et al.*, 2008). Oxidative stress can modify the structure of DNA and cause mutations in the genome of mitochondria and membranes of male germ cells (Velando *et al.*, 2008). Therefore, testicular melanin may protect male germ cells from oxidative stress and decrease the impact of mutations. Thus, testicular melanization would be open for natural selection. If this phenomenon protects individual males from the occurrence of mutations, selection would favour the evolution of melanization in species that experience high mutation rates. This is likely, as species differ in susceptibility to mutagens or ability to repair DNA (reviews in Hoffmann & Parsons, 1991; Friedberg *et al.*, 2006; Halligan & Keightley, 2009).

In birds, testicular melanin has been found in the interstitium, wall of seminiferous tubules, tunica albuginea and epididymis (Anthony & Buss, 1974; Aire, 1979; López & Paramio, 1980; Agrawal & Bansal, 1983). The occurrence of bird testicular melanin has been suggested to be associated with the gonadal cycle, melanogenesis being absent during the period of spermatogenic activity and appearing during the regression period (Anthony & Buss, 1974; Lake, 1981; Agrawal & Bansal, 1983; Hore *et al.*, 1994). However, the so far published reports of testicular melanization in birds can be considered almost anecdotal and limited to a few species as in the other classes of vertebrates, and our own observational experience with several birds indicates that there are species in which testicular melanization can be observed both during breeding and nonbreeding, others in which it is only observed in either of these periods, and others in which it has never been observed. Therefore, the suggested seasonal variation in testicular melanization may not be a general pattern in birds, and there is high variability in the occurrence of testicular melanization among species, which is what would be expected from natural selection acting with variable intensity among individuals of different species (Darwin, 1859).

The aim of this study was to investigate a possible relationship between mutation rates, measured as DNA base pair substitution rates in the mitochondrial cytochrome *b* gene (Nabholz *et al.*, 2008, 2009), and occurrence of testicular melanization in a large set of species of birds during different parts of their annual cycle. Although mitochondrial DNA (mtDNA) is strictly maternally transmitted, mutations in mtDNA accumulate in tissues and cause dysfunction of the mitochondria (e.g. Kujoth *et al.*, 2005), which in turn generates oxidative stress (Esposito *et al.*, 1999) and causes sperm dysfunction (Spiropoulos *et al.*, 2002). High mutation loads may also result from the accumulation of mutations during the lifespan of an individual, but a previous study found no effects of longevity on the mutation rate of mtDNA in birds (Møller *et al.*, 2010). Thus, males that have inherited through the female germline a mitochondrial genome that has suffered a high mutation rate will benefit from protecting their spermatozoa from oxidative stress and dysfunction that would induce the inherited

mutated genome on the numerous mitochondria carried by them. If testicular melanins can protect male germ cells from oxidative stress as other extracutaneous melanins do in different tissues, selection would favour testicular melanization in males of species with high historical mtDNA mutation rates. We thus predicted that substitution rate should explain the observed variability in testicular melanization across species and should be higher in species with testicular melanization.

As body size is an important predictor of substitution rate (reviews in Nabholz *et al.*, 2008, 2009), we tested for a relationship between testicular melanization and substitution rates while controlling for the potentially confounding effect of body size. In addition, we searched for a possible relationship between testicular melanization and melanin-based plumage colouration exhibited by the species, because associations between coat and skin melanin-based colour and heart and cochlear melanocyte abundance have been reported in mice and humans (Schrott & Spoendlin, 1987; Tachibana *et al.*, 1992; Motohashi *et al.*, 1994; Yajima & Larue, 2008). Therefore, a similar pattern may exist in other types of extracutaneous melanin in birds. Furthermore, plumage colour generated by other pigments (i.e. carotenoids) can signal the capacity to protect sperm against oxidative stress (Helfenstein *et al.*, 2010). We predicted that species with testicular melanization would also have more intense melanin-based plumage colouration than species that do not melanize their testes.

Materials and methods

Testicular melanization

By visually examining the testes of freshly killed specimens delivered to a taxidermist (J.E.), we considered that they contained melanin when their colour was black or grey (see Appendix S1). Previous authors have determined that the presence of these colours in the testes of birds is because of melanin granules (Anthony & Buss, 1974; Aire, 1979; López & Paramio, 1980; Lake, 1981; Agrawal & Bansal, 1983; Hore *et al.*, 1994). Otherwise, the testes were white or pale yellow, and such testes were considered to not contain melanin (Appendix S1). We only used information on adult males to avoid potential confounding effects of age. We asked an independent observer to score the colour of testes in photographs of 25 birds from different species, and there was a 100% of correct hits, which indicates that the repeatability of colour scores is very high.

We collected information on the breeding season of temperate birds from Cramp & Simmons (1977–1992), Cramp & Perrins (1993–1994), Ridgely & Tudor (1989), Howell & Webb (1995), del Hoyo *et al.* (1992–2002, 2003–2007) and Brazil (2009), and information on testicular melanization was thus divided as derived from testes during the breeding season, during the nonbreeding season, collected on unknown dates (i.e. date of collection was unavailable), and collected during any period (i.e. presence or absence of testicular melanization independent of collection date). Although the reproductive activity of some species of tropical birds presents certain levels of seasonality (Wikelski *et al.*, 2000), these species do not breed during particular annual season, but their reproductive activity depends strongly on local environmental conditions (Wyndham, 1986). Thus, we did not differentiate information collected during breeding and nonbreeding seasons for tropical birds, and to be conservative we considered that all information on testicular melanization for these species was collected on unknown dates.

Information on testicular melanization for the species considered in this study is provided in Appendix S2. We obtained information on both testicular melanization and substitution rate of adult males from a total of 134 species of birds. There was information from 49 species for the breeding season (testicular melanization was present in 12 species; 24.5%), 55 species for the nonbreeding season (testicular melanization was present in 15 species; 27.2%) and 62 species with collection dates that could not be assigned to a specific phase of the reproductive cycle (testicular melanization was present in 24 species; 38.7%).

Mitochondrial DNA substitution rates

Species-specific mtDNA base pair substitution rates were used as reported by Nabholz *et al.* (2008, 2009). Briefly, complete cytochrome *b* sequences for 1571 avian species were aligned, and changes in the third base pair of amino acid encoding codons were used as a measure of genetic divergence (Nabholz *et al.*, 2009). Such changes in the third base pair of amino acid encoding codons are most often synonymous with respect to amino acid changes and are thus not usually under selection. The third codon position substitution rate provides a good approximation of synonymous substitution rate for two different reasons. First, the vast majority of the cytochrome *b* divergence is synonymous (the ratio nonsynonymous/synonymous divergence is close to 2% (Stanley & Harrison, 1999; Nabholz *et al.*, 2008). Second, all the transition substitutions (A↔G and T↔C) of the cytochrome *b* third codon position are synonymous, and most of the substitution are transitions (the ratio transition/transversion being typical >15 in the dataset). Substitution rates for the species considered in the study are provided in Appendix S2.

Melanin-based plumage colouration

Using colour plates, we obtained information on melanin-based plumage colouration of the 134 bird species. Several authors have used this method previously (see, e.g. John, 1995; Yezerinac & Weatherhead, 1995;

Badyaev, 1997; Badyaev & Hill, 2000; Badyaev *et al.*, 2002; Galván, 2008; Biard *et al.*, 2009; Caro, 2009; Stang & McRae, 2009), and it has been shown to be a reliable method of quantifying different components of plumage colour that is even correlated with the avian perception of colour (del Val *et al.*, 2009; Seddon *et al.*, 2010).

In the integument of vertebrates, melanin is mainly produced in two forms, eumelanin and pheomelanin (Ozeki *et al.*, 1997). 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 when compared with black and grey colours (Galván & Alonso-Alvarez, 2009). Furthermore, many bird species present feather melanin contents of high purity (>90% of either eumelanin or pheomelanin; McGraw & Wakamatsu, 2004; J.J. Negro, pers. comm.). Therefore, we considered that black and grey plumage colours were predominantly generated by eumelanin, whereas chestnut and brown colours were predominantly generated by pheomelanin. We did not consider blue, green, yellow or red colourations assumed to be generated by other pigments (i.e. carotenoids, porphyrins or psittacofulvins) or by feather structures, unless chemically identified as being melanin-based by Toral *et al.* (2008). We neither considered the brownish crest patch of the violet turaco *Musophaga violacea* because it contains turacin (Moreau, 1958). Owls (Order Strigiformes) were not scored because their feathers contain both melanins and porphyrins and, although porphyrins photodegrade rapidly (Negro *et al.*, 2009), the colour effect of these pigments may remain for longer periods in owls than in diurnal birds whose feathers also contain both melanins and porphyrins such as bustards (Order Gruiformes) (With, 1978). Although the score developed here only provides 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 & Hartley, 1998).

Thus, we quantified the proportion of melanic plumage parts by examining illustrations in Cramp & Simmons (1977–1992), Cramp & Perrins (1993–1994), Ridgely & Tudor (1989), Howell & Webb (1995), del Hoyo *et al.* (1992–2002, 2003–2007) and Brazil (2009). Illustrations of both resting and flying adult males in breeding plumage birds were examined. The method used by Beauchamp & 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 had different subspecies or colour morphs differing in extent or type of melanin-based colouration, we used the nominate subspecies or the most common morph, respectively (e.g. the Gouldian finch *Chloebia gouldiae*; Franklin & Dostine, 2000). It must be noted that eu- and pheomelanic colour patches can coexist in the same feathers, and thus the sum of both colour scores, in a species that presents both colour types, is not always necessarily five, but higher values are also possible. Information on eu- and pheomelanic plumage colour scores for the species used in the study is provided in Appendix S2.

Body mass

J.E. weighed most of the specimens that were examined for testicular melanization. When body mass was unavailable for a species, we obtained it from Lislevand *et al.* (2007) and Dunning (2008). Body mass values for the species used in the study are provided in Appendix S2.

Data analyses

Bird species are evolutionarily related as reflected by phylogeny, and therefore, they should not be treated as independent sample units (Felsenstein, 1985; Harvey & Purvis, 1991). Thus, the effect of common ancestry among taxa can lead to an overestimation of degrees of freedom if phylogenetic relationships are not taken into account. We used phylogenetic eigenvector regression (PVR) to quantify the amount of phylogenetic signal and to correct for it in the analysis of the relationship between the occurrence of testicular melanization and substitution rates (Diniz-Filho *et al.*, 1998). Diniz-Filho & Torres (2002) and Martins *et al.* (2002) have tested several comparative methods [Felsenstein's independent contrasts, autoregressive method, PVR and phylogenetic generalized least squares (PGLS)] and have found that PVR yields good statistical performance regardless of the details of the evolutionary mode used to generate the data and provides similar results to other methods, with very good (i.e. low) error types I and II. Moreover, PVR does not assume any *a priori* evolutionary model (an advantage if the true evolutionary model is unknown or if it is too complex) and has similar statistical performance even under evolutionary processes distinct from Brownian motion. For these reasons, PVR is a comparative method that is widely used with a diversity of taxa and ecological questions (e.g. Giannini, 2003; Kriloff *et al.*, 2008; Montoya *et al.*, 2008; Bisson *et al.*, 2009).

We first performed a principal coordinates analysis (PCORD) on the matrix of pairwise phylogenetic distances between the 134 bird species (after a double-centred transformation). In a second step, we selected the first nine eigenvectors obtained by the broken-stick rule to account parsimoniously for the phylogenetic signal.

Eigenvectors extracted from double-centred phylogenetic distance matrices are able to detect the main topological features of the cladogram under different sample sizes or number of taxa used in the analyses (Diniz-Filho *et al.*, 1998). We found that the original matrix of phylogenetic distances between the 134 bird species and the reproduced matrix of distances estimated based on the first nine eigenvectors were very similar (Mantel test with 999 randomized matrices to estimate significance: $r = 0.828$, $P < 0.0001$; test carried out using PopTools 3.2.3; Hood, 2010). These eigenvectors were used as additional predictor variables in the generalized linear models (see below) to control for phylogeny.

The phylogenetic hypothesis (see Appendix S3) was constructed from the species-level supertree made by Davis (2008), with additional information from other sources for some species not covered by Davis (2008): Drovetski *et al.* (2004), Outlaw & Voelker (2006) and Alström *et al.* (2008). Although the vinous-throated parrotbill *Paradoxornis webbianus* was not covered by Davis (2008), we considered this species as the sister group of *Sylvia* warblers as reported for the other *Paradoxornis* species (*Paradoxornis gularis*, *Paradoxornis guttaticollis*, *Paradoxornis davidianus* and *Paradoxornis nipalensis*) in this phylogeny (Davis, 2008). Because we used different phylogenies that employed different methods, we set all branch lengths equal to unity in our compiled phylogeny, thus assuming a speciational model of evolution.

We regressed the occurrence of testicular melanization on substitution rate, body mass ($\log_{10}$-transformed), eu- and pheomelanic plumage colour scores ($\log_{10}$-transformed) and the first nine phylogenetic eigenvectors (EV1–EV9 hereafter), using a generalized linear model with a binomial response variable (for the response codes 0, absence of testicular melanization, and 1, presence of testicular melanization) and a logit link function. The phylogenetic signal (i.e. amount of variance or deviance exclusively explained by phylogeny) in testicular melanization and substitution rate was calculated by regressing these variables on EV1–EV9, using the above-mentioned generalized linear model for testicular melanization and a general linear model for substitution rate. Because there was a high variability among species in the number of individual birds that were examined for testicular melanization (see Appendix S2), and this could lead to violations of the assumptions of phylogenetic comparative analyses (Freckleton, 2009; Garamszegi & Møller, 2010), we weighted the models by number of individuals examined per species, thus given more importance to determinations of presence or absence of testicular melanization in species for which we had information for many individuals.

As some authors have suggested that testicular melanization is associated with the gonadal cycle of birds (Anthony & Buss, 1974; Lake, 1981; Agrawal & Bansal, 1983; Hore *et al.*, 1994; see however references in Anthony & Buss, 1974 for exceptions), we determined whether prevalence of testicular melanization was correlated between breeding and nonbreeding seasons in 20 species for which two or more adult males (range: 2–38 specimens) were examined in both periods. In these species, prevalence of testicular melanization in both periods was strongly positively correlated (Kendall $tau = 0.68$, $P < 0.0001$). Therefore, we combined information on all periods in subsequent analyses. However, we controlled for possible seasonal effects by assigning each species a score that resulted from giving to each specimen a score of 0 if collected during the breeding season, 1 if collected during the nonbreeding season, and 0.5 when the season of collection was unknown. This seasonality score ($\log_{10}$-transformed) was added as a covariate to the generalized linear model described earlier.

In our case, neither eumelanin- nor pheomelanin-based plumage colour score was significantly related to substitution rate (generalized linear regression with an ordinal multinomial response variable and $\log_{10}$ body mass as covariate: eumelanin-based colour score: $b = -5.34$, $\chi^2_1 = 2.25$, $P = 0.133$; pheomelanin-based colour score: $b = 5.47$, $\chi^2_1 = 2.88$, $P = 0.090$), thus avoiding problems of collinearity between these variables. Generalized models were checked for deviations from canonical assumptions using the over-dispersion coefficients. An over-dispersion value different from unity appears when the deviance of an observed response variable exceeds the nominal deviance, given the respective assumed distribution of errors and the proper link function. Therefore, parameter estimates and related statistics must be computed taking into account the over-dispersion. Over-dispersion coefficients were relatively high in some of our analyses (ranging from 3.5 to 3.8). This condition frequently occurs when fitting generalized linear models to categorical response variables, and the assumed distribution is binomial or ordinal multinomial. To solve this problem, we allowed the variance function of the binomial distributions to have a multiplicative over-dispersion factor computed as deviance χ^2/d.f. (Long, 1997). On the other hand, the assumption of normality in the general linear model was checked by exploring the distribution of residuals.

We corroborated the results of the PVR analyses explained above with generalized estimating equation (GEE) models corrected for phylogeny (Paradis & Claude, 2002). The GEE approach is an extension of PGLS (Martins & Hansen, 1997), but the dependence among observations is specified with a correlation matrix instead of a variance–covariance matrix as in PGLS, and non-normal distributions can be modelled. GEE models were performed in R statistical environment with the library 'geepack' and the function 'geeglm', specifying a binomial response distribution with a logit link function, and a correlation structure determined by the matrix of phylogenetic distances.

Results

From the total of 134 species, we found instance of testicular melanization in 42 different species (31.3%). The phylogenetic signal in prevalence of testicular melanization was relatively high (20.5%) and significant ($\chi^2_9 = 131.84$, $P < 0.0001$). The phylogenetic signal in substitution rate was also significant ($F_{9,124} = 3.82$, $P < 0.001$) and of similar magnitude (21.7%) to that for testicular melanization. Thus, both the prevalence of testicular melanization and substitution rate significantly depended on the effect of phylogenetic inertia.

The full model for prevalence of testicular melanization (Table 1) was significant ($\chi^2_{14} = 170.56$, $P < 0.0001$) and explained 28.8% of deviance. The effects of substitution rate and body mass were positive, i.e. species with testicular melanization had higher substitution rates and were larger than species without testicular melanization, whereas the seasonal effect was negative, i.e. testicular melanization tended to be more common during the period of reproductive activity (Table 1). The phylogenetic effect was significant, especially because of the influence of EV9 (Table 1). By contrast, neither eumelanin-based nor pheomelanin-based plumage colour scores significantly contributed to explain variation in testicular melanization across species (Table 1).

Table 1 Results of the generalized linear models explaining interspecific variation in prevalence of testicular melanization of adult male birds in relation to mtDNA substitution rate, body mass, eu- and pheomelanin-based plumage colour scores, seasonal effects and phylogeny. For each variable, the fitted regression coefficient (*b*) and its corresponding significance (*P*-values for partial effects) are shown. Phylogenetic effects are computed from the first nine eigenvectors (EV1–EV9) obtained from a principal coordinates analysis applied to the matrix of pairwise phylogenetic distances between bird species. The results of the full and reduced (i.e. without melanin-based plumage colour scores) models are shown. Significant effects are in bold.

	Full model		Reduced model	
Effect	*b*	*P*	*b*	*P*
Substitution rate	**10.21**	**0.034**	**10.44**	**0.023**
Body mass	**1.89**	**0.022**	**1.79**	**0.019**
Eumelanin-based plumage colour score	−1.91	0.155	–	–
Pheomelanin-based plumage colour score	−0.37	0.741	–	–
Seasonality score	**−9.93**	**0.003**	**−8.52**	**0.008**
Phylogeny		**<0.0001**		**<0.0001**
EV1	−0.13	0.345	−0.13	0.292
EV2	0.09	0.289	0.07	0.378
EV3	0.16	0.069	**0.17**	**0.045**
EV4	−0.05	0.691	−0.03	0.821
EV5	0.02	0.872	0.01	0.914
EV6	0.15	0.288	0.15	0.305
EV7	−0.12	0.507	−0.19	0.171
EV8	−0.09	0.654	−0.08	0.668
EV9	**−0.74**	**<0.0001**	**−0.78**	**<0.0001**

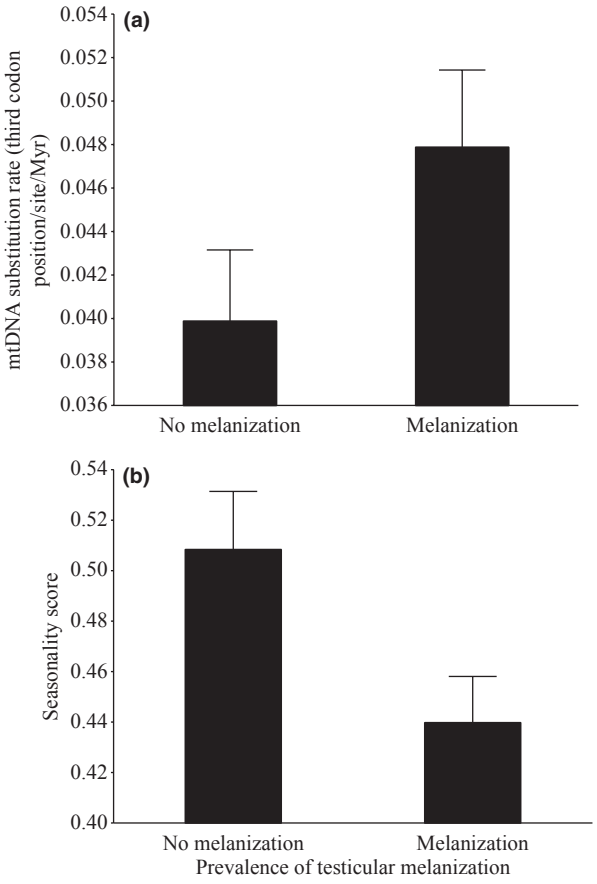

Fig. 1 mtDNA substitution rate (a) and seasonality score (b) in relation to occurrence of testicular melanization in adult males of 134 species of birds. The seasonality score decreases as the proportion of specimens examined during the breeding season increases. Means ± SE are shown. Data are weighted by sample size.

As nonsignificant terms may obscure the effects of other variables, we removed the melanin-based scores from the model. This reduced model was also significant ($\chi^2_{12} = 202.8$, $P < 0.0001$) and explained 31.5% of deviance in prevalence of testicular melanization. The effect of substitution rate was positive and significant with an increase in substitution rate by 20% when comparing species with and without melanization of the testes (Table 1; Fig. 1a), as well as the effect of body mass, whereas the seasonal effect was negative and significant (Table 1, Fig. 1b). EV9 was the main phylogenetic vector contributing to the significant effect of phylogeny (Table 1). The results of this model were corroborated by the GEE model, as mutation rate, body mass and seasonality score all contributed significantly (all $P < 0.001$) to explain variability in prevalence of testicular melanization. Furthermore, a model without

EV1–EV9 included significant effects of substitution rate ($b = 10.06$, $\chi^2_1 = 4.14$, $P = 0.041$) and body mass ($b = 0.87$, $\chi^2_1 = 8.64$, $P = 0.003$), implying that the positive effect of substitution rate on the occurrence of testicular melanization remained significant when the effects of phylogeny were not considered.

The most negative scores of EV9 corresponded to species of the families Strigidae (*Asio flammeus*, *Asio otus*, *Bubo bubo*, *Strix aluco*), Accipitridae (*Buteo buteo*, *Milvus milvus*), Muscicapidae (*Cyanoptila cyanomelana*, *Ficedula albicollis*, *Ficedula hypoleuca*, *Ficedula zanthopygia*, *Muscicapa striata*) and Turdidae (*Cossypha niveicapilla*, *Monticola solitarius*, *Stiphrornis erythrothorax*), and the most positive scores to species of the families Apodidae (*Apus apus*), Psittacidae (*Amazona finschi*, *Poicephalus gulielmi*), Coraciidae (*Coracias caudatus*), Laniidae (*Lanius excubitor*, *Lanius cristatus*), Corvidae (*Garrulus glandarius*, *Pica pica*, *Corvus frugilegus*), Paridae (*Lophophanes cristatus*, *Parus major*) and Estrildidae (*Chloebia gouldiae*, *Euplectes afer*, *Mandingoa nitidula*, *Pyrenestes sanguineus*, *Uraeginthus bengalus*, *Vidua chalybeata*). Thus, males of species of the families Strigidae, Accipitridae, Muscicapidae and Turdidae have a higher phylogenetic inertia to present testicular melanization, and this phylogenetic inertia was independent of their mitochondrial substitution rates.

Discussion

Testicular melanization is observed in bird species with high mtDNA substitution rates. This finding was obtained after controlling for the confounding effects of body mass (Nabholz *et al.*, 2008, 2009) and phylogeny (Felsenstein, 1985; Harvey & Purvis, 1991), and for seasonal effects in testicular melanization. This was the prediction on the basis that natural selection should favour testicular melanization in males of species with high historical rates of mtDNA mutations, as males that receive highly mutated mitochondrial genomes through the female germ line should have a higher probability of sperm dysfunction because of oxidative stress (Esposito *et al.*, 1999; Spiropoulos *et al.*, 2002), and testicular melanin may protect sperm from oxidative stress like other extracutaneous melanins do in other tissues (Solano *et al.*, 2000; Liu *et al.*, 2005; Zadlo *et al.*, 2006; Zareba *et al.*, 2006; Laurell *et al.*, 2007; Hearing, 2009; Levin *et al.*, 2009; Randhawa *et al.*, 2009; Tu *et al.*, 2009; Murillo-Cuesta *et al.*, 2010).

Although a certain level of oxidative stress is necessary for the maturation of male germ cells (Maiorino & Ursini, 2002), the presence of melanin may help control this mechanism by limiting oxidative stress experienced by the spermatozoa. The high variability in susceptibility to mutagens or ability to repair DNA that exists among species of animals (reviews in Hoffmann & Parsons, 1991; Friedberg *et al.*, 2006; Halligan & Keightley, 2009) should allow natural selection to act on individual birds and hence produce differences among bird species. The fact that the amount of phylogenetic signal was similar in testicular melanization and substitution rate indicates that the intensity of phylogenetic constraint is similar in both traits.

An alternative, nonexclusive explanation is that higher substitution rates in species with testicular melanization may not (only) constitute an adaptive strategy, but is a constraint imposed by oxidative stress. A close relationship between mtDNA mutation and oxidative stress is widely recognized (e.g. Esposito *et al.*, 1999; Barja, 2002; Wei & Lee, 2002), so it is likely that bird species with high mtDNA mutation rates experience high levels of endogenous oxidative stress. It is also known that oxidative stress depletes levels of an important intracellular antioxidant (i.e. glutathione, GSH), whose oxidation is indeed a requisite for the maturation of sperm cells (e.g. Maiorino & Ursini, 2002). GSH inhibits the synthesis of eumelanin (i.e. the darkest form of melanin) and enhances the synthesis of pheomelanin (i.e. the lightest form of melanin) (Ozeki *et al.*, 1997; Galván & Alonso-Alvarez, 2008, 2009; Galván & Solano, 2009). Although the chemical composition of testicular melanin has not been determined, its dark colour suggests that its pheomelanic content may not be relatively high (Toral *et al.*, 2008). Therefore, testicular melanization in species with high substitution rates may be a physiological consequence caused by high levels of endogenous oxidative stress in these species. This alone, however, cannot explain the evolution of testicular melanocytes, which requires a functional explanation like that provided above.

Some authors have suggested that testicular melanogenesis in birds does not take place during the period of spermatogenesis (Anthony & Buss, 1974; Lake, 1981; Agrawal & Bansal, 1983; Hore *et al.*, 1994; and see references in Anthony & Buss, 1974 for exceptions). Our more extensive study cannot confirm that pattern, as we did not only observe testicular melanization during both breeding and nonbreeding periods, but even detected a higher probability of occurrence during the period of reproductive activity (i.e. when spermatogenesis is likely to occur). Indeed, the presence of melanin in testes has been associated with reproductive activity in lizards (Guillette *et al.*, 1983) and mammals (Poole & Lawton, 2009). This finding is consistent with our hypothesized role of testicular melanin in protecting sperm cells from oxidative damage. Thus, the hypothesized adaptive pattern may be a general trend that can be detected beyond possible seasonal variation in testicular melanogenesis. It is worthwhile mentioning that variation in the abundance of extracutaneous melanin has been associated with stressful responses in other vertebrates. In fish, melano-macrophages (i.e. cells of the unspecific immune system that contain melanin) appear in testes when exposed to toxic compounds (Louiz *et al.*, 2009). In humans, melanin seems to appear in the prostate only when lesions are present (Farid & Gahukamble, 1995;

Di Nuovo *et al.*, 2002). Importantly, Parkinson disease is associated with a depletion of neuromelanin (Zucca *et al.*, 2004) and with high levels of neuronal mtDNA mutations (Bender *et al.*, 2006) in the human substantia nigra, so that neuromelanin may protect neurons from the effects of these mutations. These findings are consistent with our adaptive explanation for the observed relationship between testicular melanization and high mtDNA substitution rates in birds.

If testicular melanin protects male germ cells from oxidative stress, the question then arises why testicular melanization has not evolved in all species. The answer may be related to the fact that melanization also generates physiological costs, whose magnitude depends on levels of oxidative stress. Under conditions of low oxidative stress, eumelanogenesis can be costly because it would require the absence of GSH synthesis that therefore cannot be used for antioxidant activity (Galván & Alonso-Alvarez, 2009; Galván & Solano, 2009). Under conditions of high oxidative stress, pheomelanogenesis can be costly because it demands GSH resources that cannot be used in antioxidant activity by other processes (Galván & Alonso-Alvarez, 2009; Galván & Solano, 2009). Extracutaneous melanins can also sequester and accumulate toxic metals and this, while protecting cells from these compounds, can represent a toxicological risk because of later releases of the metals (Lindquist *et al.*, 1988; Mårs & Larsson, 1999). These factors may constitute selective pressures acting against testicular melanization that would only be avoided in cases of strong needs, for example as a consequence of high mitochondrial mutation rates.

We found that some species belonging to certain bird families are more prone to have evolved testicular melanization only through phylogenetic inertia. Because this effect was independent of mitochondrial substitution rates, phylogenetic inertia of genomic origin different from the mitochondria, or at least different from the mitochondrial cytochrome *b* gene, might be constraining the evolution of testicular melanization in birds. The origin of this inertia may be in the genes that control melanin synthesis. For example, nucleotide diversity in the melanocortin-1 receptor gene (MC1R), a nuclear gene that plays a key role in the regulation of melanogenesis, is independent from mtDNA diversity in birds (Bensch *et al.*, 2006), so that MC1R may be a candidate gene at the origin of the phylogenetic inertia detected in testicular melanization. This possibility should be explored in the future.

On the other hand, the proportion of plumage coloured by melanins did not predict variation in occurrence of testicular melanization across species. In mice and humans, heart and cochlear melanocyte abundance has been associated with coat and skin colour (Schrott & Spoendlin, 1987; Tachibana *et al.*, 1992; Motohashi *et al.*, 1994; Yajima & Larue, 2008). Thus, our results suggest that a correspondence between the activity of cutaneous and extracutaneous melanocytes may only occur in certain extracutaneous melanin types, and variation may exist in the degree of similarity between these melanin types regarding the molecular bases that control their functioning. Therefore, melanin-based plumage colour differs from colour generated by other pigments (i.e. carotenoids) that can signal the capacity to protect sperm from oxidative stress (Helfenstein *et al.*, 2010). This difference may be because of the fact that carotenoids, in contrast to melanins, are not synthesized by organisms, so there are no different pathways of carotenoid production in different tissues (Parker, 1996).

In conclusion, testicular melanization has evolved in relation to mtDNA substitution rates in birds. We realize that our study raises more questions than answers, but we emphasize this is the first time that the occurrence of testicular melanization in any animal group is investigated in an extensive comparative study. Our findings open new avenues for research, suggesting that the evolution of melanogenesis may be linked to historical mutation rates in the maternally inherited genome.

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References

Agrawal, S.C. & Bansal, G. 1983. Instance of melanosis in the gonads of male Indian Robin, *Saxicoloides fulicata* (Lin). *Poult. Sci.* **62**: 385–388.

Aire, T.A. 1979. The epididimal region of the japanese quail (*Coturnix coturnix japonica*). *Acta Anat.* **103**: 305–312.

Alström, P., Olsson, U., Lei, F., Wang, H.-T., Gao, W. & Sundberg, P. 2008. Phylogeny and classification of the Old World Emberizini (Aves, Passeriformes). *Mol. Phylogenet. Evol.* **47**: 960–973.

Anthony, R.G. & Buss, I.O. 1974. Relationship of gonadal recrudescence and testicular melanogenesis in California Quail. *Condor* **76**: 452–457.

Badyaev, A.V. 1997. Altitudinal variation in sexual dimorphism: a new pattern and alternative hypotheses. *Behav. Ecol.* **8**: 675–690.

Badyaev, A.V. & Hill, G.E. 2000. Evolution of sexual dichromatism: contribution of carotenoid- versus melanin-based coloration. *Biol. J. Linn. Soc.* **69**: 153–172.

Badyaev, A.V., Hill, G.E. & Weckworth, B.V. 2002. Species divergence in sexually selected traits: increase in song elaboration is related to decrease in plumage ornamentation in finches. *Evolution* **56**: 412–419.

Barja, G. 2002. Endogenous oxidative stress: relationship to aging, longevity and caloric restriction. *Ageing Res. Rev.* **1**: 397–411.

Beauchamp, G. & Heeb, P. 2001. Social foraging and the evolution of white plumage. *Evol. Ecol. Res.* **3**: 703–720.

Bender, A., Krishnan, K.J., Morris, C.M., Taylor, C.A., Reeve, A.K., Perry, R.H. *et al.* 2006. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. *Nat. Genet.* **38**: 515–517.

Bensch, S., Irwin, D.E., Irwin, J.H., Kvist, L. & Åkesson, S. 2006. Conflicting patterns of mitochondrial and nuclear DNA diversity in *Phylloscopus* warblers. *Mol. Ecol.* **15**: 161–171.

Biard, C., Gil, D., Karadaş, F., Saino, N., Spottiswoode, C.N., Surai, P.F. *et al.* 2009. Maternal effects mediated by antioxidants and the evolution of carotenoid-based signals in birds. *Am. Nat.* **174**: 696–708.

Bisson, I.-A., Safi, K. & Holland, R.A. 2009. Evidence for repeated independent evolution of migration in the largest family of bats. *PLoS ONE* **4**: e7504.

van den Bossche, K., Naeyaert, J.-M. & Lambert, J. 2006. The quest for the mechanism of melanin transfer. *Traffic* **7**: 769–778.

Brazil, M. 2009. *Birds of East Asia*. Christopher Helm, London.

Brito, F.C. & Kos, L. 2008. Timeline and distribution of melanocyte precursors in the mouse heart. *Pigment Cell Melanoma Res.* **21**: 464–470.

Caro, T. 2009. Contrasting coloration in terrestrial mammals. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **364**: 537–548.

Cramp, S. & Perrins, C.M. 1993–1994. *The Birds of the Western Palearctic*, 3 vols. Oxford University Press, Oxford.

Cramp, S. & Simmons, K.E.L. 1977–1992. *The Birds of the Western Palearctic*, 6 vols. Oxford University Press, Oxford.

Darwin, C. 1859. *The Origin of Species by Means of Natural Selection or the Preservation of Favored Races in the Struggle for Life*. Murray, London.

Davis, K.E. 2008. *Reweaving the Tapestry: A Supertree of Birds*. PhD Thesis, University of Glasgow, Glasgow.

Di Nuovo, F., Sironi, M.G. & Spinelli, M. 2002. True prostatic blue nevus associated with melanosis: case report, histogenesis and review of the literature. *Adv. Clin. Path.* **6**: 135–139.

Diniz-Filho, J.A.F. & Torres, N.M. 2002. Phylogenetic comparative methods and the geographic range size – body size relationship in new world terrestrial carnivora. *Evol. Ecol.* **16**: 351–367.

Diniz-Filho, J.A.F., De Sant'ana, C.E.R. & Bini, L.M. 1998. An eigenvector method for estimating phylogenetic inertia. *Evolution* **52**: 1247–1262.

Drovetski, S.V., Zink, R.M., Fadeev, I.V., Nesterov, E.V., Koblik, E.A., Red'Kin, Y.A. *et al.* 2004. Mitochondrial phylogeny of *Locustella* and related genera. *J. Avian Biol.* **35**: 105–110.

Dunning, J.B. Jr 2008. *CRC Handbook of Avian Body Masses*, 2nd edn. Taylor & Francis Group, Boca Raton, FL.

Esposito, L.A., Melov, S., Panov, A., Cottrell, B.A. & Wallace, D.C. 1999. Mitochondrial disease in mouse results in increased oxidative stress. *Proc. Natl Acad. Sci. USA* **96**: 4820–4825.

Farid, M.K. & Gahukamble, L.D. 1995. Melanosis of the prostate in an elderly patient—a case report. *Cent. Afr. J. Med.* **41**: 101–102.

Felsenstein, J. 1985. Phylogenies and the comparative method. *Am. Nat.* **125**: 1–15.

Franklin, D.C. & Dostine, P.L. 2000. A note on the frequency and genetics of head colour morphs in the Gouldian finch. *Emu* **200**: 236–239.

Freckleton, R.P. 2009. The seven deadly sins of comparative analysis. *J. Evol. Biol.* **22**: 1367–1375.

Friedberg, E.C., Walker, G.C., Siede, W. & Wood, R.D. 2006. *DNA Repair and Mutagenesis*. ASM Press, Washington, DC.

Galván, I. 2008. The importance of white on black: unmelanized plumage proportion predicts display complexity in birds. *Behav. Ecol. Sociobiol.* **63**: 303–311.

Galván, I. & Alonso-Alvarez, C. 2008. An intracellular antioxidant determines the expression of a melanin-based signal in a bird. *PLoS ONE* **3**: e3335.

Galván, I. & Alonso-Alvarez, C. 2009. The expression of melanin-based plumage is separately modulated by exogenous oxidative stress and a melanocortin. *Proc. Biol. Sci.* **276**: 3089–3097.

Galván, I. & Solano, F. 2009. The evolution of eu- and pheomelanic traits may respond to an economy of pigments related to environmental oxidative stress. *Pigment Cell Melanoma Res.* **22**: 339–342.

Garamszegi, L.Z. & Møller, A.P. 2010. Effects of sample size and intraspecific variation in phylogenetic comparative studies: a meta-analytic review. *Biol. Rev.* **85**: 797–805.

Giannini, N.P. 2003. Canonical phylogenetic ordination. *Syst. Biol.* **52**: 684–695.

Guillette, L.J. Jr, Weigel, J. & Flater, G. 1983. Unilateral testicular pigmentation in the Mexican lizard *Sceloporus variabilis*. *Copeia* **1983**: 155–161.

Halligan, D.L. & Keightley, P.D. 2009. Spontaneous mutation accumulation studies in evolutionary genetics. *Annu. Rev. Ecol. Evol. S.* **40**: 151–172.

Harvey, P.H. & Purvis, A. 1991. Comparative methods for explaining adaptations. *Nature* **351**: 619–624.

Hearing, V.J. 2009. The expanding role and presence of neuromelanins in the human brain – why gray matter is gray. *Pigment Cell Melanoma Res.* **22**: 10–11.

Helfenstein, F., Losdat, S., Møller, A.P., Blount, J.D. & Richner, H. 2010. Sperm of colourful males are better protected against oxidative stress. *Ecol. Lett.* **13**: 213–222.

Hirano, S. 1990. Observations of pigment granules in the bones of silky fowls. *Arch. Histol. Cytol.* **53**: 89–93.

Hoffmann, A.A. & Parsons, P.A. 1991. *Evolutionary Genetics and Environmental Stress*. Oxford University Press, Oxford.

Hood, G.M. 2010. PopTools version 3.2.3. URL http://www.poptools.org.

Hore, B.B., Mehrotra, P.N. & Hore, A. 1994. Testicular melanin pigments and the gonadal cycle in a tropical wild passerine bird, *Dicrurus adsimillis* (Passeriformes, Dicruridae). *Funct. Dev. Morphol.* **4**: 21–24.

Howell, S.N.G. & Webb, S. 1995. *A Guide to the Birds of Mexico and Northern Central America*. Oxford University Press, Oxford.

del Hoyo, J., Elliot, A. & Sargatal, J. (eds). 1992-2002. *Handbook of the Birds of the World*, Vols 1–7. Lynx Edicions, Barcelona.

del Hoyo, J., Elliot, A. & Christie, D.A. (eds). 2003-2007. *Handbook of the Birds of the World*, Vols 8–12. Lynx Edicions, Barcelona.

Ito, S. 2008. Melanins seem to be everywhere in the body, but for what? *Pigment Cell Melanoma Res.* **22**: 12–13.

John, J.L. 1995. Haematozoan parasites, mating systems and colourful plumages in songbirds. *Oikos* **72**: 395–401.

Kriloff, A., Germain, D., Canoville, A., Vincent, P., Sache, M. & Laurin, M. 2008. Evolution of bone microanatomy of the tetrapod tibia and its use in palaeobiological inference. *J. Evol. Biol.* **21**: 807–826.

Kujoth, G.C., Hiona, A., Pugh, T.D., Someya, S., Panzer, K., Wohlgemuth, S.E. *et al.* 2005. Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. *Science* **309**: 481–484.

Lake, P.E. 1981. Male genital organs. In: *Form and Function in Birds*, Vol. 2 (A.S. King & J. McLelland, eds), pp. 1–61. Academic Press, London.

Laurell, G., Ekborn, A., Viberg, A. & Canlon, B. 2007. Effects of a single high dose of cisplatin on the melanocytes of the stria vascularis in the guinea pig. *Audiol. Neurootol.* **12**: 170–178.

Levin, M.D., Lu, M.M., Petrenko, N.B., Hawkins, B.J., Gupta, T.H., Lang, D. *et al.* 2009. Melanocyte-like cells in the heart and pulmonary veins contribute to atrial arrhythmia triggers. *J. Clin. Invest.* **119**: 3420–3436.

Lin, J.Y. & Fisher, D.E. 2007. Melanocyte biology and skin pigmentation. *Nature* **445**: 843–850.

Lindquist, N.G., Larsson, B.S. & Lydén-Sokolowski, A. 1988. Autoradiography of [^{14}C]paraquat or [^{14}C]diquat in frogs and mice: accumulation in neuromelanin. *Neurosci. Lett.* **93**: 1–6.

Lislevand, T., Figuerola, J. & Székely, T. 2007. Avian body sizes in relation to fecundity, mating system, display behavior, and resource sharing. *Ecology* **88**: 1605.

Liu, Y., Hong, L., Wakamatsu, K., Ito, S., Adhyaru, B.B., Cheng, C.-Y. *et al.* 2005. Comparison of the structural and chemical properties of melanosomes isolated from retinal pigment epithelium, iris and choroid of newborn and mature bovine eyes. *Photochem. Photobiol.* **81**: 510–516.

Long, J.S. 1997. *Regression Models for Categorical and Limited Dependent Variables*. Sage, Thousand Oaks, CA.

López, R. & Paramio, A. 1980. Consideraciones sobre la presencia de melanina en testículos de aves de la raza White Plymouth Rock. *Rev. Cubana de Ciencia Avícola* **7**: 141–148.

Louiz, I., Ben-Attia, M. & Ben-Hassine, O.K. 2009. Gonadosomatic index and gonad histopathology of *Gobius niger* (Gobiidea, Teleost) from Bizerta lagoon (Tunisia): evidence of reproduction disturbance. *Fish. Res.* **100**: 266–273.

Maiorino, M. & Ursini, F. 2002. Oxidative stress, spermatogenesis and fertility. *Biol. Chem.* **383**: 591–597.

Mårs, U. & Larsson, B.S. 1999. Pheomelanin as a binding site for drugs and chemicals. *Pigment Cell Res.* **12**: 266–274.

Martins, E.P. & Hansen, T.F. 1997. Phylogenies and the comparative method: a general approach to incorporating phylogenetic information into the analysis of interspecific data. *Am. Nat.* **149**: 646–667.

Martins, E.P., Diniz-Filho, J.A.F. & Housworth, E.A. 2002. Adaptive constraints and the phylogenetic comparative method: a computer simulation test. *Evolution* **56**: 1–13.

McGraw, K.J. & Wakamatsu, K. 2004. Melanin basis of ornamental feather colors in male Zebra Finches. *Condor* **106**: 686–690.

Møller, A.P., Erritzøe, J., Karadas, F. & Mousseau, T.A. 2010. Historical mutation rates predict susceptibility to radiation in Chernobyl birds. *J. Evol. Biol.* **23**: 2132–2142.

Montoya, D., Zavala, M.A., Rodríguez, M.A. & Purves, D.W. 2008. Animal versus wind dispersal and the robustness of tree species to deforestation. *Science* **320**: 1502–1504.

Moreau, R.E. 1958. Some aspects of the Musophagidae. Part 3. *Ibis* **100**: 238–270.

Motohashi, H., Hozawa, K., Oshima, T., Takeuchi, T. & Takasaka, T. 1994. Dysgenesis of melanocytes and cochlear dysfunction in mutant microphthalmia (mi) mice. *Hear. Res.* **80**: 10–20.

Murillo-Cuesta, S., Contreras, J., Zurita, E., Cediel, R., Cantero, M., Varela-Nieto, I. *et al.* 2010. Melanin precursors prevent premature age-related and noise-induced hearing loss in albino mice. *Pigment Cell Melanoma Res.* **23**: 72–83.

Nabholz, B., Glemin, S. & Galtier, N. 2008. Strong variations of mitochrondrial mutation rate across mammals: the longevity hypothesis. *Mol. Biol. Evol.* **25**: 120–130.

Nabholz, B., Glemin, S. & Galtier, N. 2009. The erratic mitochondrial clock: variations of mutation rate, not population size, affect mtDNA diversity across birds and mammals. *BMC Evol. Biol.* **9**: 54.

Negro, J.J., Bortolotti, G.R., Mateo, R. & García, I.M. 2009. Porphyrins and pheomelanins contribute to the reddish juvenal plumage of black-shouldered kites. *Comp. Biochem. Physiol. B* **153**: 296–299.

de Oliveira, C. & Zieri, R. 2005. Testicular pigmentation in *Physalaemus nattereri* (Steindachner) (Amphibia, Anura) with anatomical observations on the extracutaneous pigmentary system. *Rev. Bras. Zool.* **22**: 454–460.

Outlaw, D.C. & Voelker, G. 2006. Systematics of *Ficedula* flycatchers (Muscicapidae): a molecular reassessment of a taxonomic enigma. *Mol. Phylogenet. Evol.* **41**: 118–126.

Owens, I.P.F. & Hartley, I.R. 1998. Sexual dimorphism in birds: why are there so many different forms of dimorphism? *Proc. Biol. Sci.* **265**: 397–407.

Ozeki, H., Ito, S., Wakamatsu, K. & Ishiguro, I. 1997. Chemical characterization of pheomelanogenesis starting from dihydroxyphenylalanine or tyrosine and cysteine. Effects of tyrosinase and cysteine concentrations and reaction time. *Biochim. Biophys. Acta* **1336**: 539–548.

Paradis, E. & Claude, J. 2002. Analysis of comparative data using generalized estimating equations. *J. Theor. Biol.* **218**: 175–185.

Parker, R.S. 1996. Absorption, metabolism, and transport of carotenoids. *FASEB J.* **10**: 542–551.

Poole, A. & Lawton, C. 2009. The translocation and post release settlement of red squirrels *Sciurus vulgaris* to a previously uninhabited woodland. *Biodivers. Conserv.* **18**: 3205–3218.

Randhawa, M., Huff, T., Valencia, J.C., Younossi, Z., Chandhoke, V., Hearing, V.J. *et al.* 2009. Evidence for the ectopic synthesis of melanin in human adipose tissue. *FASEB J.* **23**: 835–843.

Ridgely, R.S. & Tudor, G. 1989. *The Birds of South America*, Vol. 1. Texas University Press, Texas, TX.

Schrott, A. & Spoendlin, H. 1987. Pigment anomaly-associated inner ear deafness. *Acta Otolaryngol.* **103**: 451–457.

Scott, J.N. & Fritz, H.I. 1979. Nonpigmented tunica vaginalis testis in the opposum. *Ohio J. Sci.* **79**: 79–80.

Seddon, N., Tobias, J., Eaton, M. & Odeen, A. 2010. Human vision can provide a valid proxy for avian perception of sexual dichromatism. *Auk* **127**: 283–292.

Serventy, D.L. & Marshall, A.J. 1956. Factors influencing testis coloration in birds. *Emu* **56**: 219–222.

Solano, F., Hearing, V.J. & García-Borrón, J.C. 2000. Neurotoxicity due to o-quinones: neuromelanin formation and possible mechanisms for o-quinone detoxification. *Neurotox. Res.* **1**: 153–169.

Spiropoulos, J., Turnbull, D.M. & Chinnery, P.F. 2002. Can mitochondrial DNA mutations cause sperm dysfunction? *Mol. Hum. Reprod.* **8**: 719–721.

Stang, A.T. & McRae, S.B. 2009. Why some rails have white tails: the evolution of white undertail plumage and antipredator signaling. *Evol. Ecol.* **23**: 943–961.

Stanley, S.E. & Harrison, R.G. 1999. Cytochrome b evolution in birds and mammals: an evaluation of the avian constraint hypothesis. *Mol. Biol. Evol.* **16**: 1575–1585.

Steel, K.P. & Barkway, C. 1989. Another role for melanocytes: their importance for normal stria vascularis development in the mammalian inner ear. *Development* **107**: 453–463.

Tachibana, M., Hara, Y., Vyas, D., Hodgkinson, C., Fex, J., Grundfast, K. *et al.* 1992. Cochlear disorder associated with melanocyte anomaly in mice with a transgenic insertional mutation. *Mol. Cell. Neurosci.* **3**: 433–445.

Toral, G.M., Figuerola, J. & Negro, J.J. 2008. Multiple ways to become red: pigment identification in red feathers using spectrometry. *Comp. Biochem. Physiol. B* **150**: 147–152.

Tu, Y.-G., Sun, Y.-Z., Tian, Y.-G., Xie, M.-Y. & Chen, J. 2009. Physicochemical characterisation and antioxidant activity of melanin from the muscles of Taihe Black-bone silky fowl (*Gallus gallus domesticus* Brisson). *Food Chem.* **114**: 1345–1350.

del Val, E., Borràs, A., Cabrera, J. & Senar, J.C. 2009. Plumage colour of male Common Crossbills *Loxia curvirostra*: visual assessment validated by colorimetry. *Rev. Cat. Ornitol.* **25**: 19–25. URL http://www.ornitologia.org/publicacions/25_19_25.pdf.

Velando, A., Torres, R. & Alonso-Alvarez, C. 2008. Avoiding bad genes: oxidatively damaged DNA in germ line and mate choice. *Bioessays* **30**: 1212–1219.

Wei, Y.-H. & Lee, H.-C. 2002. Oxidative stress, mitochondrial DNA mutation, and impairment of antioxidant enzymes in aging. *Exp. Biol. Med.* **227**: 671–682.

Wikelski, M., Hau, M. & Wingfield, J.C. 2000. Seasonality of reproduction in a tropical rain forest bird. *Ecology* **81**: 2458–2472.

With, T.K. 1978. On porphyrins in feathers of owls and bustards. *Int. J. Biochem.* **9**: 893–895.

Wyndham, E. 1986. Length of birds' breeding seasons. *Am. Nat.* **128**: 155–164.

Yajima, I. & Larue, L. 2008. The location of heart melanocytes is specified and the level of pigmentation in the heart may correlate with coat color. *Pigment Cell Melanoma Res.* **21**: 471–476.

Yezerinac, S.M. & Weatherhead, P.J. 1995. Plumage coloration, differential attraction of vectors and haematozoa infections in birds. *J. Anim. Ecol.* **64**: 528–537.

Zadlo, A., Rozanowska, M.B., Burke, J.M. & Sarna, T.J. 2006. Photobleaching of retinal pigment epithelium melanosomes reduces their ability to inhibit iron-induced peroxidation of lipids. *Pigment Cell Res.* **20**: 52–60.

Zareba, M., Szewczyk, G., Sarna, T., Hong, L., Simon, J.D., Henry, M.M. *et al.* 2006. Effects of photodegradation on the physical and antioxidant properties of melanosomes isolated from retinal pigment epithelium. *Photochem. Photobiol.* **82**: 1024–1029.

Zieri, R., Taboga, S.R. & de Oliveira, C. 2007. Melanocytes in the testes of *Eupemphix nattereri* (Anura, Leiuperidae): histological, stereological, and ultrastructural aspects. *Anat. Rec.* **290**: 795–800.

Zucca, F.A., Giaveri, G., Gallorini, M., Albertini, A., Toscani, M., Pezzoli, G. *et al.* 2004. The neuromelanin of human substantia nigra: physiological and pathogenic aspects. *Pigment Cell Res.* **17**: 610–617.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Examples of bird testes with (a, from an eagle owl *Bubo bubo*) and without (b, from an icterine warbler *Hippolais icterina*) melanin.

Appendix S2 Information on testicular melanization, body mass (g), mitochondrial DNA substitution rate and eu- and pheomelanin-based plumage coloration in 134 species of birds.

Appendix S3 Phylogenetic hypothesis used in the study.

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