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Chronic exposure to low-dose radiation at Chernobyl favours adaptation to oxidative stress in birds

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Summary

1. Ionizing radiation produces oxidative stress, but organisms can adapt to their exposure with physiological adaptive responses. However, the role of radioadaptive responses in wild populations remains poorly known.

2. At Chernobyl, studies of birds and other taxa including humans show that chronic exposure to radiation depletes antioxidants and increases oxidative damage. Here, we present analyses of levels of the most important intracellular antioxidant (i.e. glutathione, GSH), its redox status, DNA damage and body condition in 16 species of birds exposed to radiation at Chernobyl. We use an approach that allows considering the individual bird as the sampling unit while controlling for phylogenetic effects, thus increasing the statistical power by avoiding the use of species means as done for most previous comparative studies.

3. As a consequence, we found a pattern radically different from previous studies in wild populations, showing that GSH levels and body condition increased, and oxidative stress and DNA damage decreased, with increasing background radiation. Thus, when several species are considered, the overall pattern indicates that birds are not negatively affected by chronic exposure to radiation and may even obtain beneficial hormetic effects following an adaptive response. Analysis of the phylogenetic signal supports the existence of adaptation in the studied traits, particularly in GSH levels and DNA damage.

4. We also show that, under equal levels of radiation, the birds that produce larger amounts of the pigment pheomelanin and lower amounts of eumelanin pay a cost in terms of decreased GSH levels, increased oxidative stress and DNA damage, and poorer body condition. Radiation, however, diminished another potential cost of pheomelanin, namely its tendency to produce free radicals when exposed to radiation, because it induced a change towards the production of less pro-oxidant forms of pheomelanin with higher benzothiazole-to-benzothiazine ratios, which may have facilitated the acclimation of birds to radiation exposure.

5. Our findings represent the first evidence of adaptation to ionizing radiation in wild animals, and confirm that pheomelanin synthesis represents an evolutionary constraint under stressful environmental conditions because it requires GSH consumption.

Key-words: adaptation, Chernobyl, ionizing radiation, oxidative stress, pheomelanin

Introduction

Ionizing radiation is composed of particles able to liberate electrons from atoms or molecules and thus creates partially reduced chemical species, the most common being reactive oxygen species (ROS), which are involved in chain reactions that are potentially damaging to cells (Riley 1994). Living organisms have evolved a diversity of antioxidant compounds that can eliminate these damaging effects by combating ROS, which are constantly produced in the body by cellular metabolism. ROS activate cell signalling

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pathways which may trigger adaptive responses (Viña *et al.* 2006), but when antioxidant levels are below the thresholds required to limit ROS production, it leads to states of oxidative stress (Finkel & Holbrook 2000). Ionizing radiation is therefore an important source of oxidative stress in cells (e.g. Simone *et al.* 2009). This means that ionizing radiation can have profound effects on the evolutionary ecology of organisms, as oxidative stress is the ultimate cause of the deterioration of phenotypes (i.e. senescence) and the death of organisms, and it is thus considered a major determinant of the evolution of life-history strategies (Dowling & Simmons 2009; Metcalfe & Alonso-Alvarez 2010; Galván *et al.* 2012a).

However, most research on the biological effects of ionizing radiation have been conducted with cells or with organisms under laboratory conditions, which limits the capacity to obtain information on consequences for the ecology and evolution of organisms. Studies on wild populations are necessary to obtain a comprehensive view of the evolutionary consequences of ionizing radiation because free-living populations may be limited or constrained in their ability to cope with effects of ionizing radiation. Natural background radioactivity levels show extreme variation of several hundred-fold and have recently been found to affect mutational input and the expression of certain phenotypic traits, but studies on natural radioactivity are still few and scattered (Galván & Alonso-Alvarez 2011; Møller & Mousseau 2013). Natural radiation and radiation accidents like those produced at the nuclear power plants of Chernobyl in 1986 and Fukushima in 2011 have had catastrophic environmental consequences, and the large levels of radioactivity released to the environment represent involuntary experiments and good opportunities for investigating the effects of ionizing radiation on wild populations of organisms. In Chernobyl, several studies have reported significant effects of radiation on the abundance, distribution, life history and mutation rates of plants and animals (Møller & Mousseau 2006), and effects on the abundance of animals have already been detected in Fukushima (Møller *et al.* 2012, 2013). In particular, radioactivity from Chernobyl has been found to produce oxidative stress by depleting antioxidants in humans (e.g. Ivaniota, Dubchak & Tyshchenko 1998; Neyfakh, Alimbekova & Ivanenko 1998; Romanenko *et al.* 2000; Vartanian *et al.* 2004) and other animals (Møller, Surai & Mousseau 2005; Møller, Karadaş & Mousseau 2008). Radiation levels in Chernobyl have also been found to covary with levels of cellular damage or dysfunction that may be mediated by oxidative damage (Sugg *et al.* 1996; Fenech, Perepetskaya & Mikhalevich 1997; Marozik *et al.* 2007; Bonisoli-Alquati *et al.* 2010, 2011), and with other physiological consequences of oxidative stress such as reductions in brain size (Møller *et al.* 2011) and the expression of eye cataracts (Mousseau & Møller 2013).

There seems to be some consistency in reporting reductions in antioxidant levels and increases in oxidative damage in animals exposed to radioactive contamination (see studies mentioned above). Some authors, however, have found in humans that the levels of some antioxidants can even increase at low doses of radiation, although high levels of radiation may deplete antioxidants (Ivanenko & Burlakova 2013), and a recovery of oxidative status can be produced over time (Skesters *et al.* 2010). Indeed, the high degree of radioactive contamination found in the region of Chernobyl and the relative long time (28 years) elapsed since the accident make this an excellent scenario for investigating possible mechanisms of adaptation to ionizing radiation in natural populations.

Radiation-induced adaptive responses have been well documented for decades in a diversity of species including humans through experiments in which cells or organisms are exposed to low doses of radiation (priming or conditioning dose) before receiving a higher, challenging dose (Olivieri, Bodycote & Wolff 1984; Iyer & Lehnert 2002). These studies have shown that chronic exposure to low, 'adapting' doses of different types of radiation increases the resistance of cells against subsequent acute exposure to challenging doses (Tapio & Jacob 2007). Correlative studies also report some evidence of radio-adaption in cells from humans chronically exposed to low levels of radioactivity in Chernobyl (Tedeschi *et al.* 1995). The ultimate mechanisms of the radio-adaptive response include complex patterns of cellular signalling and epigenetic changes that would favour the transmission of the response to the offspring (Kovalchuk *et al.* 2004). It seems that the starting point of the mechanism is not the direct effect of ionizing radiation on cellular structures, but an induction by ROS generated by radiation, which causes DNA damage (Tapio & Jacob 2007). As mentioned above, ROS generated by radiation is probably the cause of the observed decreases in antioxidant levels and increases in oxidative damage in humans and other animals from Chernobyl. Thus, there is evidence of physiological costs of radiation exposure in natural populations at Chernobyl, but not of adaptation to it. Alternatively, however, organisms may show adaptive responses to radiation at Chernobyl. This is potentially plausible given the observed positive effect of low-dose radiation on some antioxidants (Ivanenko & Burlakova 2013), the recovery in the antioxidant status a long time after exposure (Skesters *et al.* 2010), and the fact that many organisms in the Chernobyl region have been chronically exposed to low doses of radiation, conditions that may favour radio-adaption (Tapio & Jacob 2007). Searching for adaptive responses to radiation in natural populations is of key importance as it can potentially determine the capacity of species to evolve physiological adaptations and thus differential susceptibilities to overcome environmental challenges such as those that occurred in Chernobyl and Fukushima (Somero 2010).

Adaptive responses to radiation in natural populations at Chernobyl may not have been detected for several reasons. The large variability in radiation levels found in the entire Chernobyl zone represents a continuous environmental gradient, although there is a high temporal

consistency in the background radiation levels to which individual organisms are exposed within their home ranges at Chernobyl (see Materials and Methods), which may limit the capacity to detect adaptive responses in natural populations. In addition, it has been reported that the range of acute lethal doses of artificial ionizing radiation varies greatly among taxa, which is for example considerably greater in plants than in higher vertebrates, and lower in birds than in mammals (Newman & Unger 2003). This among-taxa difference in susceptibility to radiation has already been reported in animals from Fukushima and Chernobyl regarding effects on population abundance (Møller *et al*. 2013), and susceptibility to natural radioactivity also varies among taxa (Møller & Mousseau 2013). Studies on the biological consequences of radioactivity at Chernobyl have concentrated on a few taxa (Møller & Mousseau 2006), and in the particular case of studies that report antioxidant and oxidative damage levels, they are all intraspecific and limited to humans, two species of birds and one species of fish (see references cited above). Therefore, the among-taxa variability in susceptibility to radiation may represent an additional limitation in the capacity to detect radio-adaptive responses. In fact, although the effects of radioactivity on bird populations at Chernobyl are negative overall, some species' populations grow with increasing radiation (Galván, Mousseau & Møller 2011), lending support to the potential role of an adaptive response to chronic radiation exposure. Comparative studies may represent a solution for the two limitations mentioned above. Comparing several species that show different susceptibilities to radiation and that are subjected to a range of radiation levels enhances the capacity to detect effects. Comparative studies in which several phylogenetically distant species are investigated for antioxidant status have to our knowledge never been conducted in natural populations at Chernobyl, but are clearly necessary for developing insight into the potential role of radio-adaption for the evolution of organisms.

The aim of this study was to investigate covariation between levels of glutathione (GSH) and DNA damage with levels of background radiation in wild populations of several phylogenetically distant species of birds in the Chernobyl region. We focus on GSH because it is one of the antioxidants most susceptible to radiation (Riley 1994; Ivaniota, Dubchak & Tyshchenko 1998; Neyfakh, Alimbekova & Ivanenko 1998; Ivanenko & Burlakova 2013), the most important intracellular antioxidant, and its redox status (GSH/GSSG) represents a relevant index of cellular oxidative stress (Wu *et al*. 2004). We consider DNA damage as measured by the comet assay, which quantifies strand breaks, as this is the most common damage to DNA caused by ionizing radiation (e.g. Kovalchuk *et al*. 2004). In two species of birds from Chernobyl (the barn swallow *Hirundo rustica* and the great tit *Parus major*), circulating antioxidant levels have decreased and oxidative damage has increased with radiation levels (Møller, Surai & Mousseau 2005; Møller, Karadaş & Mousseau 2008; Bonisoli-Alquati *et al*. 2010, 2011). Thus, we predict that the same patterns should be found at the interspecific level regarding GSH and GSH/GSSG if birds exposed to radioactive contamination show a general and consistent physiological cost mediated by radiation. Alternatively, if there has been an adaptive response by birds to the chronic exposure of background radiation at Chernobyl, radiation should improve, at least up to certain level, the antioxidant response of birds. This should in turn prevent finding a decrease in levels of GSH and GSH/GSSG and an increase in DNA damage (which is probably caused by radiation-induced oxidative stress; Bonisoli-Alquati *et al*. 2010) and body condition (which predicts the survival of birds; Møller & Szep 2001) with increasing radiation. Such an adaptive response may however be costly to maintain, and such costs may be reflected in the population trends of birds. Therefore, we also analysed associations between the intensity of the physiological response and population trends of the species of birds at Chernobyl (Galván, Mousseau & Møller 2011).

When searching for possible differential capacities of species to adapt to chronic exposure to radiation, it is necessary to consider the production of melanins, the most common animal pigments. We have previously found that populations of species of birds expressing plumage colours typically provided by pheomelanin, a polymer of benzothiazine and benzothiazole units that constitutes one of the two main types of melanin, are more susceptible to the negative effects of radiation at Chernobyl (Galván, Mousseau & Møller 2011). The hypothesized mechanism behind this observation is that the sulphhydryl groups of cysteine and GSH are incorporated into the pheomelanin structure during its synthesis in melanocytes (García-Borrón & Olivares Sánchez 2011; Ito *et al*. 2011a). Therefore, pheomelanin synthesis represents a consumption of an antioxidant resource because GSH (which is also the main physiological reservoir of cysteine) can no longer exert its antioxidant role once incorporated into the structure of the pigment, which is then deposited in inert tegumentary structures such as feathers and hair (Pavel, Smit & Pizinger 2011). Thus, pheomelanin synthesis represents a physiological cost under exposure to environmental factors that produce high levels of oxidative stress, as these conditions lead to greater demands of GSH for antioxidant protection (Galván, Ghanem & Møller 2012). However, it has never been directly tested if pheomelanin production entails GSH depletion and oxidative stress in organisms exposed to ionizing radiation. This test is necessary to determine why species producing large amounts of pheomelanin are more susceptible to the effects of radiation (Galván, Mousseau & Møller 2011). Therefore, we predict that under equal levels of background radiation birds with higher levels of pheomelanin in feathers should have lower levels of GSH and higher oxidative stress and DNA damage than birds producing lower amounts of pheomelanin. In contrast, eumelanin, a polymer of 5,6-dihydroxyindole-2-carboxilic acid (DHICA) and 5,6-dihydroxyindole (DHI) units

that constitutes the other main type of melanin, is produced in the absence of cysteine and GSH (García-Borrón & Olivares Sánchez 2011; Ito *et al*. 2011a) and protects cell survival and decreases DNA damage under exposure to ionizing radiation (Kinnaert *et al*. 2004). We thus predict that the content of eumelanin in feathers should enhance oxidative status and reduce DNA damage in birds exposed to equal levels of background radiation.

Lastly, given that the two units of pheomelanin have different oxidation potentials, benzothiazine having a greater reducing ability than benzothiazole, which is rather stable towards oxidation (Wakamatsu, Ohtara & Ito 2009; Wakamatsu *et al*. 2012), we tested the possibility of a radiation-mediated conversion of benzothiazine into benzothiazole. Benzothiazole has a higher oxidation potential than benzothiazine, which makes the former produce more ROS when exposed to energetic radiation (Takeuchi *et al*. 2004; Ye *et al*. 2006). Thus, a conversion of benzothiazine into benzothiazole may protect birds at sites with radioactive contamination. We tested this by analysing the effect of background radiation on the ratio of thiazole-2,4,5-tricarboxylic acid (TTCA, a degradation product specific to the benzothiazole moiety; see Methods) to 4-amino-3-hydroxyphenylalanine (4-AHP, a degradation product specific to the benzothiazine moiety). We tested all predictions in wild populations of birds that were sampled in several sites around Chernobyl with a large range of background radiation levels.

Materials and methods

FIELD METHODS

We captured birds in mist nets at eight sites within and close to the Chernobyl Exclusion Zone on May 25 through June 5, 2010 from four pairs of relatively uncontaminated and contaminated sites (see Table S1 in Supporting Information). We used 35–50 mist nets 12 m long each for two consecutive days at each of the study site (i.e. one evening and one morning capture session at each site). In addition, for capturing barn swallows *Hirundo rustica* we used mist nets deployed across the doors and windows of barns in farms, both within and just outside the Chernobyl Exclusion Zone. All birds were banded with a unique aluminium band for individual identification and then sexed and aged according to standard criteria, sampled for feathers, blood and sperm, and released. Blood samples were obtained by venipuncture at the wing artery with a sterilized needle and collected using heparinized capillary tubes, preserved in RNALater (only those for DNA damage measurement; Ambion Life Technologies, Grand Island, NY, USA) and kept on ice in the field and stored at 4 °C upon arrival in the laboratory. The birds belonged to 16 different species (Fig. 1), although information on some variables was not available for some species (see below and Table S1).

MEASUREMENT OF BACKGROUND RADIATION LEVELS

We measured background radiation levels at the exact capture spot of each bird using a hand-held dosimeter (Model: Inspector, SE International, Inc., Summertown, TN, USA). We have previously measured the level of background radiation in the field in connection with bird census studies and cross-validated these measurements with those reported by the Ukrainian Ministry of Emergencies. Once having finished a 5-min point count we measured radiation levels 2–3 times at ground level directly in the field at each point where we censused birds, using a hand-held dosimeter. We cross-validated our measurements against measurements published by Shestopalov (1996), estimated at the midpoints of the ranges published in the Chernobyl atlas. This analysis revealed a very strong positive relationship [linear regression on log-log transformed data: $F_{1,252} = 1546{\cdot}49$, $R^2 = 0{\cdot}86$, $P < 0{\cdot}0001$, slope (SE) = 1·28 (0·10)], suggesting that our field estimates of radiation provided reliable measurements of levels of radiation among sites. These measures of residential background radiation levels also represent actual doses received by individual birds because background radiation levels and external and internal doses are strongly positively correlated (T.A. Mousseau, A.P. Møller, D. Tedeschi & A. Bonisoli-Alquati unpublished manuscript).

Repeatabilities in background radiation levels for the same individuals were estimated at three different intervals: among captures within the same day, among captures at the start and later on in the season, and between years. All three repeatability estimates were large and highly significant (during the same day: $r = 0{\cdot}997$, $F_{26,45} = 1041{\cdot}98$, $P < 0{\cdot}0001$; within season: $r = 0{\cdot}985$, $F_{26,33} = 129{\cdot}40$, $P < 0{\cdot}0001$; among years: $r = 0{\cdot}892$, $F_{60,61} = 17{\cdot}52$, $P < 0{\cdot}0001$). Repeatability estimates decreased with increasing intervals between the captures. Still the repeatability of background radiation was as high as 0·89 when based on estimates obtained in subsequent years. Repeatabilities of this magnitude are considered high by any yardstick (Becker 1984; Falconer & Mackay 1996). Individuals were almost always recaptured at the same site as where they were first captured. This is not surprising given the high degree of site philopatry among birds during the breeding season.

MEASUREMENT OF GLUTATHIONE LEVELS IN ERYTHROCYTES

GSH was measured by HPLC following a modified procedure of the technique developed by Araki & Sako (1987). Briefly, 20 μL of red blood cell concentrate was lysed with 50 μL of a 100 mL buffer solution containing 8·29 mg NH_4Cl, 37 mg Na_2EDTA/1 g $KHCO_3$. 50 μL of 10% TBP-tri-n-butylphosphine in dimethylformamide (DMF; reduction step = total glutathione) or 50 μL of DMF (reduced GSH) was then added following incubation for 30 min at 4 °C. Then, proteins were precipitated by 10% chilled trichloroacetic acid containing 1 mM EDTA, vortexed vigorously and centrifuged at 1000 g for 5 min at 4 °C. To 50–100 μL of the supernatant, 100–200 μL borate buffer (pH 9·5; 0·2 M) containing 4 mM Na_2EDTA and 100 μL of SBD-F (ammonium 7-fluorobenzo2-oxa-1, 3-diazole-4 sulphonate) 1 mg mL^{-1} in borate buffer were added. The mixture was incubated for 60 min at 60 °C under constant agitation. The tubes were then cooled on ice, passed through a 0·45 μm filter and 20 μL were separated on HPLC.

A Waters HPLC system (Waters Corporation, Milford, MA, USA) was used with separation on a RP C18-ODS column (Chrompack Intersil, 15 cm × 4·6 mm) using a phosphate buffer (pH: 6·0; 1/15 M) and methanol/H_2O (50/50) as the eluent under a gradient of 0–98% over 15 min with a flow rate of 0·5 mL min^{-1}. Fluorescence was measured using an excitation wavelength of 385 nm and an emission of 515 nm. HPLC data were analysed with the software MILLENNIUM 4.0 (Waters Corporation). We obtained information on GSH and GSSG for 13 species of birds.

MEASUREMENT OF DNA DAMAGE LEVELS

We used red blood cells, which are nucleated in birds, for analysis of genetic damage. We performed the comet assay using the

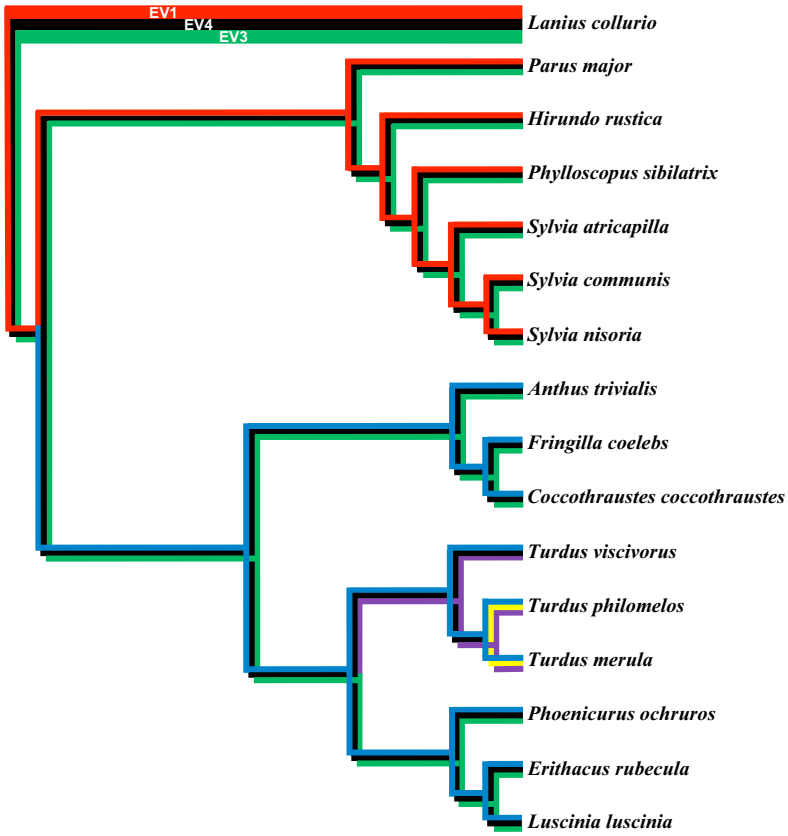

Fig. 1. Phylogenetic hypothesis for the species of birds used in the study. The sign of the scores of the species in the eigenvectors obtained from phylogenetic vector regressions (PVR) made on the response variables is depicted by contrasting pairs of different colours. The first phylogenetic eigenvector (EV1) was selected for the analysis of GSH levels and GSH:GSSG ratio, the fourth eigenvector (EV4) was selected for the analysis of DNA damage scores, and the third eigenvector (EV3) was selected for the analysis of body mass. Negative vs. positive eigenvector scores are represented by contrasting, respectively, red and blue branches (for EV1), yellow and black branches (for EV4) and purple and green branches (for EV3).

protocol described by Singh *et al.* (1988) with modifications. Avian red blood cells are highly susceptible to damage at alkali labile sites, so performing electrophoresis at the recommended pH of >13·1 inflates damage estimates considerably. Because of the increased sensitivity to alkaline conditions, we performed a modified comet assay with an alkaline unwinding step, but neutral electrophoresis conditions.

All steps were performed under incandescent light to prevent additional DNA damage. Single-frosted slides (VWR, Radnor, PA, USA) were prepared in advance by dipping the slides in 1·5% normal melting-point agarose (BioRad, Hercules, CA, USA) twice; the backs of the slides were then wiped clean and the slides were allowed to dry for at least 24 h prior to use for the comet assay. Approximately, 5 μL of hemolymph in 50 μL of 1× PBS was added to 450 μL of 1% low melting-point agarose (Amresco, Solon, OH, USA) and 100 μL of the agarose mixture was immediately layered onto the prepared slides and covered with a glass coverslip, then allowed to solidify for 5 min at 4 °C. The coverslip was then removed and a second layer of 100 μL of low melting-point agarose was layered on top of the first and covered again with a coverslip, which was removed after 5 min. Two samples were placed on each slide, with a total of four replicates for each individual. The slides were allowed to incubate for 1 h at 4 °C to allow the gel to fully solidify. The slides were then immersed in cold lysis buffer (1% sodium sarcosinate, 2·5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% Triton X-100, pH 10 with the Triton X-100 added immediately prior to use) and kept for 1 h at 4 °C. The slides were rinsed with cold ddH_2O, and then immersed in alkali buffer (300 mM NaOH, 1 mM Na_2EDTA, pH = 12·1) to allow the DNA to unwind for 30 min at 4 °C. Electrophoresis was conducted using neutral buffer (300 mM sodium acetate, 100 mM Tris, pH 10) for 30 min at 0·7 V cm^{-1} and 100 mA at 4 °C. We rinsed the slides three times for 5 min each in a neutralization buffer (0·4M Tris, pH 7·4) followed by 15 min in 70% ethanol for drying. The slides were then placed in a darkened cupboard and allowed to dry overnight before storage in a dark slide box.

Slides were stained using a 1 : 10,000 dilution of SYBR® Gold (Trevigen Inc., Gaithersburg, MD, USA) and images were captured using METASYSTEMS METAFER 4 software (Metasystems Group, INC, Newton, MA, USA), using a Zeiss Axioskop (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) fitted with an automated slide stage. We captured 100–300 cells for each slide analysed and used the CometScan module for automated analysis at 20× magnification.

Standard comet parameters were automatically captured for each cell, and percent DNA in tail was selected as the best parameter for analysis, which is one of the most widely used parameters for analysis (Kumaravel *et al.* 2009). We calculated our estimate of DNA damage based on the average percent of DNA in the tail of each comet from all cells measured on all side replicates for each individual. Distributions of tail damage within each individual are often not normally distributed, so we also considered the median as a measure of central tendency, as well as the 75th percentile because it is less sensitive to extreme values (Duez *et al.* 2003). We obtained information on these variables for 14 species of birds.

MEASUREMENT OF MELANIN LEVELS IN FEATHERS

Feathers were collected from a total of 16 species, comprising 152 individual birds. 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 and detection by HPLC of specific degradation products, 4-AHP by reductive hydrolysis of pheomelanin with hydriodic acid (HI) (Wakamatsu, Ito & Rees 2002) and pyrrole-2,3,5-tricarboxylic acid (PTCA) and TTCA by alkaline H_2O_2 oxidation of eumelanin and pheomelanin respectively (Ito *et al.* 2011b). Thus, 4-AHP and TTCA are specific to pheomelanin and PTCA is specific to eumelanin. Feather samples were first homogenized with Ten-Broeck glass homogenizer at a concentration of 10 mg mL^{-1} water.

For 4-AHP analyses, 100 μL of sample homogenate 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 M HCl. An aliquot (10–20 μL) of each solution was analysed on the HPLC system.

For PTCA and TTCA analyses, 100 μL of sample homogenate was taken in a 10 mL screw-capped conical test tube, to which 375 μL 1 M K_2CO_3 and 25 μL 30% H_2O_2 (final concentration: 1·5%) were added. The mixture was mixed vigorously at 25 ± 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 M HCl. 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.

HI reductive hydrolysis products were analysed with an HPLC system consisting of a JASCO 880-PU liquid chromatography, a JASCO C18 column (JASCO Catecholpak; 4·6 × 150 mm; 7 μm particle size) and an EICOM ECD-300 electrochemical detector (Eicom, Kyoto, Japan). The mobile phase used for analysis of 4-AHP was 0·1 M sodium citrate buffer, pH 3·0, containing 1 mM sodium octanesulphonate and 0·1 mM Na_2EDTA: methanol, 98:2 (v/v). Analyses were performed at 35 °C at a flow rate of 0·7 mL min^{-1}. The electrochemical detector was set at +500 mV vs. an Ag/AgCl reference electrode. A standard solution (10–20 μL) containing 500 ng each of 4-AHP and 3-AHP (3-amino-4-hydroxyphenylalanine; 3-aminotyrosine from Sigma) in 1 mL 0·1 M HCl was injected every 10 samples.

H_2O_2 oxidation products were analysed with the HPLC system consisting of a JASCO 880-PU liquid chromatography, a Shiseido C18 column (Shiseido Capcell Pak MG; 4·6 × 250 mm; 5 μm particle size, Shiseido Co., Ltd., Tokyo, Japan) and a JASCO UV detector. The mobile phase was 0·1 M 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^{-1}. Absorbance of the eluent was monitored at 269 nm. A standard solution (80 μL) containing 1 μg each of PTCA, PDCA (pyrrole-2,3-dicarboxylic acid), TTCA and TDCA (thiazole-2,3-dicarboxylic acid) in 1 mL water was injected every 10 samples. We obtained information on these variables for 16 species of birds.

DATA ANALYSES

We analysed the relationships between the response variables (GSH level, GSH:GSSG ratio, DNA damage level and body mass) and background radiation and melanin levels (predictor variables, which also included wing chord in the case of the model for body mass to account for variation in body condition independent of body size) by means of partial least squares regressions (hereafter PLSR; Carrascal, Galván & Gordo 2009). This statistical tool is an extension of multiple regression analysis where associations are established with components extracted from predictor variables that maximize the explained variance in the response variable. These components are defined as a linear combination of predictor variables, so the original multidimensionality is reduced to a lower number of orthogonal components to detect structure in the relationships between predictor variables and between these factors and the response variable. The extracted components account for successively lower proportions of original variance. When multiple response variables are used, PLSR creates a synthetic response variable from the linear combination of the original response variables. Results obtained with PLSR are similar to those from conventional multiple regression techniques. However, this method is extremely resilient to the effects of sample size and degree of correlation between predictor variables, which makes PLSR especially useful when the sample size is small and in cases of severe multicollinearity (Carrascal, Galván & Gordo 2009). There was a high degree of correlation among our predictor variables (Pearson's correlation test: TTCA-4-AHP: $r = 0{\cdot}72$, $N = 152$, $P < 0{\cdot}0001$; TTCA-PTCA: $r = -0{\cdot}18$, $N = 152$, $P = 0{\cdot}023$; PTCA-4-AHP: $r = -0{\cdot}19$, $N = 151$, $P = 0{\cdot}022$; TTCA-radiation: $r = -0{\cdot}46$, $N = 152$, $P < 0{\cdot}0001$; 4-AHP-radiation: $r = -0{\cdot}33$, $N = 151$, $P < 0{\cdot}0001$; PTCA-radiation: $r = 0{\cdot}35$, $N = 152$, $P < 0{\cdot}0001$), which makes PLSR the most appropriate analytical tool for our data. We also used PLSR to test the effect of radiation on TTCA:4-AHP ratio, and to test for the effects of the physiological responses found here on the population trends of the species. To estimate the intensity of the physiological responses, we obtained the studentized residuals of the regressions between the response variables (i.e. GSH levels, GSH:GSSG ratio, DNA damage score and body mass) and the scores of the PLSR components described above, and population trends were calculated as the slope estimates of the relationship between abundance and radiation levels (taken from Appendix 1 in Galván, Mousseau & Møller 2011). The latter test was made using the mean residuals of the regressions between the response variables and the scores of the PLSR components per species, which were predictor variables in PLSR models while slope estimates were the response variable.

The significance of the extracted PLSR components was determined with two criteria. First, a cross-validation test of the parameter Q^2 was carried out to determine if a component was significant. Then, we tested the significance of the correlation coefficient of the relationship between PLSR scores for the response variable and PLSR component scores, thus determining if the amount of variance explained in the response variable was significant. We also determined the contribution of predictors to the PLSR model with two complementary criteria. First, we calculated the relative contribution of each variable to the derived components by means of the square of the predictor weight, considering that a predictor was important when it accounted for more than 5% of the total variance in the response variable explained by the PLSR component (i.e. square weight >0·05). The second criterion consisted of testing the statistical significance of the regression coefficients of the predictors, thus determining the degree of correlation between the response variable and these predictors. The latter test was made by bootstrapping using 1000 replications. PLSR analyses were made with STATISTICA 8.0. (StatSoft, Inc., Tulsa, OK, USA) and TANAGRA 1.4 (Rakotomalala 2005).

Bird species are evolutionarily related through their phylogenetic history, which can lead to an overestimation of degrees of freedom if phylogenetic relationships are not taken into account (Felsenstein 1985). We used phylogenetic eigenvector regression (PVR) to correct for the effect of common ancestry in the analysis of the relationship between the response variables and background radiation and melanin levels (Diniz-Filho, De Sant'ana & Bini 1998). Diniz-Filho & Torres (2002) and Martins, Diniz-Filho & Housworth (2002) tested several comparative methods (Felsenstein's independent contrasts, autoregressive method, PVR and phylogenetic generalized least squares) and 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) type I and II errors. Moreover, PVR does not assume any evolutionary model *a priori* (an advantage if the true evolutionary model is unknown or if it is complex), and it gives similar statistical performance even for evolutionary processes that are distinct from Brownian motion (i.e. evolutionary changes are added to values present at the previous node on a phylogenetic tree, thus creating similarity between recently diverged lineages; e.g. Blomberg, Garland & Ives 2003). PVR is based on the eigenfunction decomposition of phylogenetic distance matrices, so that the phylogenetic relationships between species can be translated into predictor variables (phylogenetic eigenvectors) that capture phylogenetic effects (Diniz-Filho, De Sant'ana & Bini 1998).

To obtain the phylogenetic eigenvectors for the species of birds included in the study, we used the PVR approach in the software SAM 4.0 (Rangel, Diniz-Filho & Bini 2010) considering the species' mean values of our response variables and of melanin levels in feathers, thus considering correlations between variables while obtaining the eigenvectors. The phylogenetic hypothesis used (Fig. 1) was taken from the species-level supertree constructed by Davis (2008), assuming all branch lengths being equal to unity. SAM makes an approach to PVR that represents the only comparative method that can deal with non-normal variable distributions, thus being the most robust method to deviations from normality in the response variables (Dormann *et al.* 2007). However, PVR can ignore important phylogenetic information if traits evolve under Brownian motion (Rohlf 2001), so we first tested the evolutionary model of our response variables by using phylogenetic signal-representation (PSR) curves (Diniz-Filho *et al.* 2011). PSR curves represent the amount of divergence in traits (measured by PVR's R^2) along the eigenvectors against the cumulative eigenvalues of the eigenvectors. A linear relationship between these parameters is indicative of Brownian motion, and negative or positive deviations indicate that species resemble each other less or more, respectively, than expected under Brownian motion in an analogous way to Blomberg, Garland & Ives's (2003) *K*-statistic (Diniz-Filho *et al.* 2011). Mean deviations from the PSR curve also represent a measure of phylogenetic signal (Diniz-Filho *et al.* 2011). We constructed PSR curves for our response variables using the software PAM 0.9 (*Phylogenetic Analysis in Macroecology*; T.F. Rangel & J.A.F. Diniz-Filho, unpublished) considering the first eight eigenvectors. Mean deviations from the PSR curve were negative in the four response variables, indicating that closely related species are less similar regarding these traits than expected under Brownian motion evolution (Fig. 2). When traits follow this kind of nonlinear model of evolution, only part of the eigenvectors can be used to describe the phylogeny because not all eigenvectors are equally useful for this aim (Diniz-Filho *et al.* 2011). Thus, from the phylogenetic eigenvectors generated with SAM, we selected those that reduced the largest amount of autocorrelation in the residuals below an arbitrarily defined threshold for Moran's I or its statistical significance, which is the most appropriate selection method (Diniz-Filho *et al.* 2012).

Only one phylogenetic eigenvector was selected for the analysis of each response variable. The first phylogenetic eigenvector (EV1) was selected for the analysis of GSH levels and GSH:GSSG ratio, and discriminated birds from the families Turdidae, Muscicapidae, Motacillidae and Fringilidae (positive scores) from the rest of the phylogeny (negative scores) (Fig. 1). EV4 and EV3 were selected for the analyses of DNA damage and body mass, respectively, and discriminated the genus *Turdus* (negative scores) from the rest of the phylogeny (positive scores) (Fig. 1).

PVR has the additional advantage that the extracted phylogenetic eigenvectors can be used as predictor variables in any other statistical linear model to correct for phylogenetic effects on response variables (Diniz-Filho, De Sant'ana & Bini 1998; Dormann *et al.* 2007; Diniz-Filho *et al.* 2011, 2012). In our case, this feature allowed us to use the individual bird as the sampling unit in the PLSR models instead of the species, while controlling for the effect of common ancestry among groups of individual birds that belong to the same species (Martins & Hansen 1996). This permits the analyses to include the entire variability in background radiation levels, which would be highly reduced if the mean values of species were considered instead, which in turn would represent a limitation to finding possible relationships between the response variables and radiation levels as mentioned before (see Introduction). Thus, after obtaining the phylogenetic eigenvectors using the species mean values of the variables as described above, we assigned the same eigenvector score of each species to all individuals belonging to that species, hence constituting predictor variables that were added to the PLSR models. Therefore, we conducted the analyses considering that all individuals of the same species constitute a hard polytomy in the phylogeny (Purvis & Garland 1993), as they are all equally related to each other (in phylogenetic

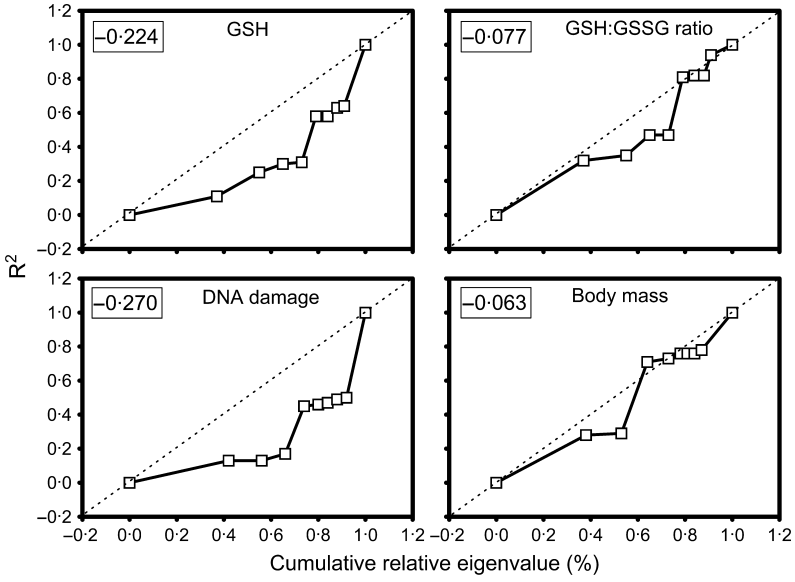

Fig. 2. Phylogenetic signal-representation (PSR) curves for the response variables analysed in the study constructed with the results of eight phylogenetic vector regressions (PVR) sequentially increasing the number of eigenvectors. R^2 indicates the amount of variance in the response variables that is explained by the phylogenetic eigenvectors, and is represented against the eigenvalues of the eigenvectors used in the PVR models expressed as proportion of the trace. The 45° dashed line represents the expected pattern under Brownian motion. Inserts are the mean values of the difference between R^2 and eigenvalue, which is indicative of the phylogenetic signal in the traits. DNA damage refers to the mean percentage DNA in the comet tail as measured by means of the comet assay.

terms) and are separated by the same phylogenetic distance from the other groups of conspecific individuals.

Although we have found GSH:GSSG ratios <1 (which indicate more oxidized than reduced glutathione and thus an impaired oxidative status) in other studies with birds (own observations), in this study we found GSH:GSSG ratios below 1 in several of our bird samples (see Results), so to determine if these cases were not normal values potentially affecting results, we repeated the analyses excluding samples with the lowest ratios (lower than or equal to 0·5). Furthermore, the sex of birds was added as a predictor variable to all PLSR models, but it never contributed importantly to them (i.e. accounting for more than 5% of the total variance in the response variable explained by the model), so it was removed from the analyses. We also included radiation level squared as a predictor to account for nonlinear relationships between the response variables and radiation, but it also was not an important predictor in any model. Lastly, to determine if results considering several species of birds differ from previous intraspecific studies on effects of radiation on antioxidant and oxidative damage levels at Chernobyl (Møller, Surai & Mousseau 2005; Møller, Karadaş & Mousseau 2008; Bonisoli-Alquati *et al.* 2010, 2011), we also show the results of analyses considering data from the barn swallow only, as only this species had sample sizes sufficient for robust statistical power in intraspecific tests, and it was one of the two species considered in the previous studies.

The data used for this study are archived in the Dryad Digital Repository (Galván *et al.* 2014).

Results

EFFECT OF RADIATION ON GLUTATHIONE LEVELS

The mean (±SE) GSH level in birds was 621·76 ± 44·38 ng mg^{-1} pellet, and ranged from 23·89 to 2598·20 ng mg^{-1}. The mean redox status of GSH, represented by the GSH:GSSG ratio, was 1·80 ± 0·14, and ranged from 0·03 to 8·47.

The PLSR model for GSH levels resulted in a significant component that explained 19·2% ($P < 0·0001$) of the variance in this variable. The effect of background radiation level was positive, indicating that GSH levels increase with radiation. Other important predictors were the markers for pheomelanin content of feathers (TTCA and 4-AHP), which had a negative effect on GSH levels (Table 1). This indicates that under equal levels of background radiation the birds that produce more pheomelanin have lower levels of GSH. The phylogenetic eigenvector (EV1) was also an important predictor, but the eumelanin content of feathers was not (Table 1). All important predictors except TTCA were also significant (Table 1). GSH levels were significantly positively correlated with the PLSR component ($r = 0·44$, $N = 120$, $P < 0·0001$; Fig. 3a). Results did not change when samples with GSH:GSSG ratios lower than 0·5 ($N = 20$) were excluded, as one significant PLSR component was obtained explaining 17·5% ($P < 0·0001$) of variance in GSH levels with the same important predictors as the model with all data (predictor weights: radiation: 0·53, TTCA = −0·28, 4-AHP = −0·55, EV1 = 0·56) and showing the absence of contribution of the eumelanin content of feathers (PTCA's predictor weight = 0·12). When only data for the barn swallow ($N = 56$) were considered, no significant PLSR component was obtained.

The PLSR model for the redox status of GSH also resulted in a significant component that explained 13·1% ($P < 0·0001$) of variance in the GSH:GSSG ratio. The effect of background radiation level was positive (Table 1), which means that oxidative stress in the cells of birds decreased as radiation increased. As in the model for GSH levels, the effect of pheomelanin content in feathers was negative, and the eumelanin content of feathers was also an important predictor with a positive effect on redox status (Table 1). Thus, under equal levels of radiation, the birds that produce more pheomelanin and less eumelanin have higher levels of oxidative stress. EV1 was the most important predictor, accounting for 41·4% of the total variance in the GSH:GSSG ratio of birds explained by the model. Radiation level and EV1 were also significant predictors (Table 1). The GSH:GSSG ratio was significantly positively correlated with the PLSR component ($r = 0·36$, $N = 118$, $P < 0·0001$; Fig. 3b). Again, results did not change when samples with GSH:GSSG ratios lower than

Table 1. Results of partial least squares regression (PLSR) models explaining the relationship between four response variables (GSH levels, GSH:GSSG ratio, DNA damage score and body mass) and pheomelanin (TTCA and 4-AHP) and eumelanin (PTCA) content of feathers, radiation levels at the capture sites and a phylogenetic eigenvector in birds from Chernobyl. Phylogenetic eigenvectors were selected from phylogenetic vector regressions (PVR) made with the response variables and pheomelanin and eumelanin levels considering the phylogeny of the species of birds. The DNA damage score is a synthetic response variable built in the PLSR, composed by the mean, median and 75th percentile percentage DNA in the comet tail as measured by means of the comet assay. The model for body mass includes wing chord as a predictor, thus actually analysing variation in the body condition of birds. Predictor weights (i.e. the contribution of each predictor variable to the PLSR component) and percentage of variance in the response variables explained by the PLSR models are shown

	$\log_{10}$ GSH (ng mg^{-1} blood)	$\log_{10}$ GSH:GSSG ratio	DNA damage score	$\log_{10}$ Body mass (g)
$\log_{10}$ TTCA (ng mg^{-1} feather)	**−0·27**	**−0·32**	**0·45*****	**−0·27*****
$\log_{10}$ 4-AHP (ng mg^{-1} feather)	**−0·43*****	**−0·25**	**0·26***	**−0·24*****
$\log_{10}$ PTCA (ng mg^{-1} feather)	0·18	**0·28**	**−0·40***	−0·07
$\log_{10}$ Radiation (μSv h^{-1})	**0·59*****	**0·58****	**−0·65*****	**0·30*****
Phylogenetic eigenvector score	**0·60*****	**0·64*****	**−0·38**	**−0·77*****
$\log_{10}$ Wing chord (cm)	–	–	–	**0·42**
% variance explained	19·2	13·1	12·0	63·0

Predictor weights explaining more than 5% of the total variance are marked in bold. Asterisks indicate predictors whose regression coefficients are significant: $^{*}P < 0·05$; $^{**}P < 0·01$; $^{***}P < 0·001$.

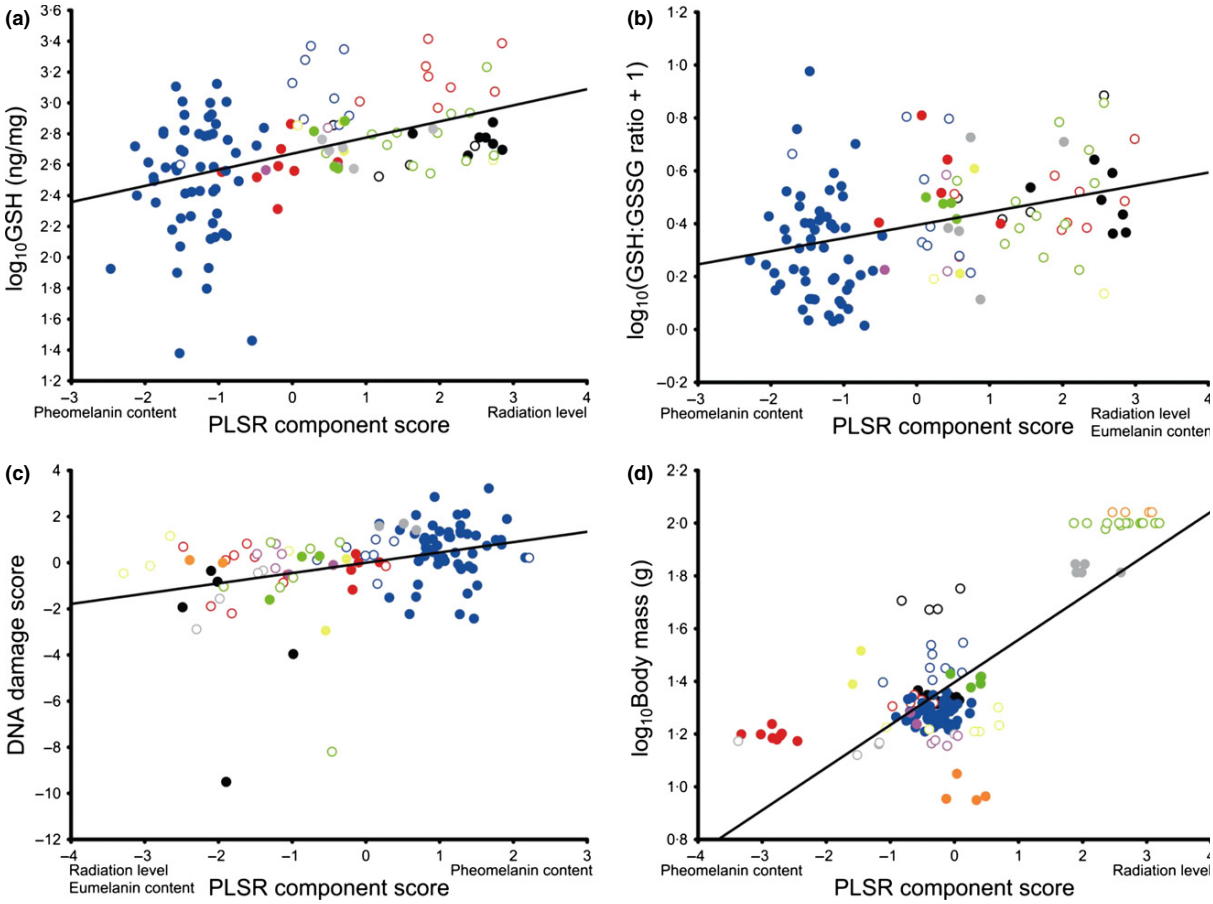

Fig. 3. Relationship between the scores of partial least-squares regression (PLSR) components and (a) GSH levels, (b) GSH:GSSG ratios, (c) DNA damage scores and (d) body mass in birds from Chernobyl. PLSR component scores represent the position of sampling units (i.e. individual birds) along an axis composed of the predictor variables pheomelanin content of feathers (measured as TTCA and 4-AHP levels), eumelanin content of feathers (measured as PTCA levels), and radiation levels at capture site and phylogenetic effects (accounted for as the scores of a selected eigenvector from phylogenetic vector regression (PVR) models). The names of predictors (excluding the phylogenetic eigenvector and also wing chord in (d) for the sake of simplicity) below the PLSR components indicate which side of the axes increased with increasing values. In (c), DNA damage score is a synthetic response variable built in the PLSR, composed of the mean, median and 75th percentile percentage DNA in the comet tail as measured by means of the comet assay. Results did not change when the two outlying points at the bottom of the graph were removed from the analysis (see text). In (d), wing chord is included as a predictor so that variation in body mass actually reflects variation in body condition. The lines are the regression lines. Colour codes: solid black: *Anthus trivialis*; hollow black: *Coccothraustes coccothraustes*; solid red: *Erithacus rubecula*; hollow red: *Fringilla coelebs*; solid blue: *Hirundo rustica*; hollow blue: *Lanius collurio*; solid light green: *Luscinia luscinia*; hollow light green: *Parus major*; solid pink: *Sylvia atricapilla*; hollow pink: *Sylvia communis*; solid dark green: *Sylvia nisoria*; hollow dark green: *Turdus merula*; solid grey: *Turdus philomelos*; hollow grey: *Phoenicurus ochruros*; solid orange: *Phylloscopus sibilatrix*; hollow orange: *Turdus viscivorus*.

0·5 were excluded, as one significant PLSR component was obtained explaining 7·6% ($P = 0·005$) of variance in the GSH:GSSG ratio in which only the eumelanin content of feathers was no longer an important predictor (predictor weights: radiation: 0·58, TTCA = −0·38, 4-AHP = −0·29, PTCA = 0·19, EV1 = 0·62), and no significant PLSR component was obtained when only data for the barn swallow ($N = 56$) were considered.

EFFECT OF RADIATION ON DNA DAMAGE

The mean, median and 75th percentile of percent DNA in tail were response variables in a PLSR model, which created a synthetic response variable from the linear combination of the three measures. These measures were all positively related to the synthetic response (response loadings for mean = 0·59, median = 0·55, 75th percentile = 0·59), which thus represent a general index of DNA damage. A significant PLSR component explaining 12·0% ($P < 0·001$) of the variance in DNA damage was obtained. The effect of radiation was negative (Table 1), indicating that DNA damage decreased with increasing background radiation. DNA damage increased with increasing pheomelanin content of feathers and decreased with increasing eumelanin content (Table 1). EV4 was an additional important predictor (Table 1). All predictors accounted for more than 5% of the total variance explained by this component, and all were significant except EV4 (Table 1).

The synthetic index of DNA damage was significantly positively correlated with the PLSR component ($r = 0{\cdot}35$, $N = 112$, $P < 0{\cdot}001$; Fig. 3c). When two outlying points (see Fig. 3c) were removed from the analysis, a significant PLSR component explaining 14·3% of the variance in DNA damage was obtained that included the same predictors than the model including those points. When GSH level was added to the model as a predictor variable, it constituted an important predictor accounting for 16·8% of the total variance in DNA damage explained by the model (12·2%, $P < 0{\cdot}001$), with a negative effect (predictor weight = −0·41) on this trait. Thus, DNA damage decreased with increasing GSH levels in cells.

When using data on barn swallows only ($N = 53$), a significant PLSR component was obtained that explained 6·4% ($P = 0{\cdot}067$) of the variance in DNA damage. The effect of radiation, as well as that of pheomelanin content in feathers, was positive (predictor weights: radiation = 0·47, TTCA = 0·71, 4-AHP = 0·49), while the eumelanin content was not an important predictor (PTCA's predictor weight = 0·19). This indicates that, when only barn swallows were considered and contrary to the pattern found for all species, background radiation levels and pheomelanin production increased DNA damage. It must be emphasized, however, that the variance in background radiation levels to which barn swallows were exposed (0·84) was significantly (506-fold) lower as compared to the variance considering all species (425·12; Levene's test: $F_{1,163} = 44{\cdot}78$, $P < 0{\cdot}0001$). Additionally, the maximum level of radiation to which barn swallows were exposed (2·90 μSv h^{-1}) was 32-fold reduced as compared to the maximum level in the entire data set of species (92·90 μSv h^{-1}).

EFFECT OF RADIATION ON BODY CONDITION

The PLSR model for body mass resulted in three significant components regarding Q^2, but only the first two components explained significant amounts of variance in that variable (component 1: 63·0%, $P < 0{\cdot}0001$; component 2: 10·2%, $P < 0{\cdot}0001$; component 3: 1·3%, $P = 0{\cdot}162$). We only selected the first component because the information generated by the second component was redundant with the first component. The effect of radiation was positive, indicating that the body condition of birds increased with increasing background radiation, but eumelanin content was not an important predictor (Table 1). The effect of pheomelanin content in feathers was negative (Table 1), indicating that under equal levels of radiation birds producing more pheomelanin were in poorer condition. EV3 and wing chord were additional important predictors. All important predictors except wing chord were also significant (Table 1). Body mass was significantly positively correlated with the PLSR component ($r = 0{\cdot}79$, $N = 152$, $P < 0{\cdot}0001$; Fig. 3d). When GSH level was added to the model as a predictor variable, it tended to covary positively with body mass, but it accounted for less than 5% of the total variance in body mass explained by the model (63·5%; predictor weight = 0·18). No significant PLSR components were obtained when only data for the barn swallow ($N = 59$) were considered.

EFFECT OF RADIATION ON PHEOMELANINS

When the effect of radiation on the TTCA:4-AHP ratio was analysed, a significant PLSR component that explained 6·5% of variance ($P = 0{\cdot}002$) was obtained. The model showed that, as predicted, the effect of radiation was significant ($P < 0{\cdot}001$) and positive and accounted for >5% of the variance explained by the component (weight = 0·81). The phylogenetic eigenvector also accounted for >5% of the variance (weight = −0·58), but it was not significantly related to the TTCA:4-AHP ratio ($P = 0{\cdot}061$).

PHYLOGENETIC SIGNAL IN ANTIOXIDANT STATUS, OXIDATIVE DAMAGE AND BODY CONDITION

Mean deviations from the 45° line in the PSR curves were negative for all response variables (Fig. 2), indicating that the species considered differed more than expected under Brownian motion regarding these traits. However, the magnitude of deviations differed between traits, being relatively large for GSH and DNA damage (−0·224 and −0·270 respectively) and small for GSH:GSSG ratio and body mass (−0·077 and −0·063 respectively) (Fig. 2). Thus, there was considerably more phylogenetic signal in the levels of reduced GSH than in oxidative stress levels represented by the redox status of GSH.

EFFECT OF PHYSIOLOGICAL RESPONSES ON THE POPULATION TRENDS OF SPECIES

The PLSR model with the mean residuals per species for GSH levels, GSH:GSSG ratio, DNA damage score and body mass as predictors of the population trends of the species resulted in a component that explained a marginally significant proportion of the variance in the slope estimates (27%, $P = 0{\cdot}047$), although the component was not significant ($Q^2 = -0{\cdot}19$). Thus, the physiological responses against radiation did not have negative consequences for the population trends of the species.

Discussion

EFFECTS OF IONIZING RADIATION ON OXIDATIVE STATUS, DNA DAMAGE AND BODY CONDITION

Birds improve their antioxidant levels and body condition and decrease their oxidative stress levels and DNA damage with increasing background radiation to which they are exposed at Chernobyl. Ionizing radiation creates ROS, depletes antioxidant levels and thus induces oxidative stress in cells, but as in any toxic compound, the magnitude of

these effects is largely dependent on the magnitude of the doses (Riley 1994). This also means that the dose of radiation determines the capacity of organisms to adapt to their exposure (Tapio & Jacob 2007). In our study area around Chernobyl, birds are exposed to background radiation levels ranging from 0·02 to 92·90 μSv h^{-1}, the mean value being 16·24 μSv h^{-1}. Thus, radiation levels are remarkably high in some sites, but most sites have low radioactivity, albeit significant as compared to non-contaminated control sites in the neighbourhood of Chernobyl. Furthermore, the accident at the nuclear power plant at Chernobyl took place 28 years ago, which has caused chronic exposure to low-dose radiation across many generations. These conditions should favour individual responses of physiological plasticity to achieve adaptation or 'acclimation' to these new environmental conditions, and variation in these responses may be affected by evolution (Woods & Harrison 2002). These conditions are also known to particularly favour physiological adaptation of organisms to ionizing radiation (Tapio & Jacob 2007). Our study provides evidence that birds have physiologically adapted to chronic exposure to radiation at Chernobyl, as radiation levels did not negatively affect their oxidative status, DNA integrity or physical condition.

Our analyses are for obvious reasons entirely correlational, implying that we cannot make strong inferences about causation. Likewise, we cannot assume that unknown variables may have not affected our analyses and conclusions. We find the latter assumption unlikely to apply because we included a range of variables that were known to correlate with our response variables. Our study sites were generally unaffected by human disturbance, which we can dismiss as a potentially confounding variable. We also consider food abundance or quality to be an unlikely confounding variable since animals generally are distributed across resource gradients in an ideal free fashion. The distance among the study sites is short and all sites can be reached by flying birds in less than an hour. Hence, resource abundance per capita should be similar across environments differing in level of background radiation. This is also supported by little or no effect of background radiation on success or condition of nestling birds in sites differing in background radiation level at Chernobyl (Møller *et al*. 2005; Møller, Karadaş & Mousseau 2008). Hence, it is likely that the mechanisms that we have hypothesized according to our review of the literature are a reliable cause of the findings reported here.

The analysis of phylogenetic signals in the studied traits supports the existence of physiological adaptation in birds. In fact, deviations from the expected Brownian motion model of evolution were negative for all the response variables (except body mass, which had a deviation close to zero as expected for interspecific variation in body size), and as these deviation values can be interpreted in an analogous way to Blomberg, Garland & Ives (2003) *K*-statistic (Diniz-Filho *et al*. 2011), with negative values indicative of adaptation in at least some of the species considered (Blomberg, Garland & Ives 2003). Interestingly, the deviation value was large for GSH levels, but low for the redox status of GSH, which suggests adaptation in the levels of the most important intracellular antioxidant (i.e. GSH), but not in oxidative stress levels. Thus, GSH levels seem to be more labile than its redox status, and the physiologically plastic response of birds to radiation would be mediated by reduced GSH and not by its oxidation rate. This makes sense, as birds may be able to mount adaptive responses by varying GSH synthesis, but not its susceptibility to oxidation. This is congruent with the view of antioxidants having the capacity to influence the evolution of life-history strategies in birds (Galván *et al*. 2012a). Similarly, the large deviation value found for DNA damage suggests that birds develop physiological adaptations to reduce this physiological cost. To our knowledge, this represents the first evidence of adaptation to ionizing radiation in wild populations of animals.

These results contrast with previous intraspecific studies on two species of birds (Møller, Surai & Mousseau 2005; Møller, Karadaş & Mousseau 2008; Bonisoli-Alquati *et al*. 2010, 2011) and also in humans and one species of fish (Sugg *et al*. 1996; Fenech, Perepetskaya & Mikhalevich 1997; Ivaniota, Dubchak & Tyshchenko 1998; Neyfakh, Alimbekova & Ivanenko 1998; Romanenko *et al*. 2000; Vartanian *et al*. 2004; Marozik *et al*. 2007), showing that antioxidant levels decrease and oxidative damage increases with radiation at Chernobyl. However, this apparent contradiction may just be the consequence of different taxonomic scales in the analyses. There is large variation among taxa in susceptibility to the effects of ionizing radiation (Newman & Unger 2003; Møller & Mousseau 2013; Møller *et al*. 2013), and in our study area there is high temporal consistency in background radiation levels to which individual birds are exposed (see Materials and Methods). Thus, studies that focus on single species may have limited capacity to detect adaptive responses to radiation. Indeed, when we restricted our analyses to the species with the largest sample size (i.e. the barn swallow), we found that, as previously reported (Møller, Surai & Mousseau 2005; Bonisoli-Alquati *et al*. 2010, 2011), DNA damage increased with radiation levels. But both the range and maximum level of background radiation for barn swallows was considerably reduced as compared to the values observed considering all species (a 506-fold increase in variance and a 32-fold increase in maximum level). It is actually expected that species differ in their capacity to adapt to changing environmental conditions (Somero 2010), which may explain why, although the effect of radiation on the population trends of birds in our study area at Chernobyl is overall negative, the populations of several species appear to be positively affected by radiation (Galván, Mousseau & Møller 2011). We have previously reported that background radiation negatively affects the survival of several species of birds in our study area (Møller *et al*. 2012), which also contradicts the results shown here. This is probably also explained by the

differential adaptive capacities mentioned above, as here we sampled surviving individuals and therefore only those that actually achieved adaptation to radiation. Therefore, our study stresses the importance of comparative studies to increase the amplitude of environmental conditions and potential responses to them, which thus increases the capacity to detect physiological adaptations.

Our results do not only show that GSH levels and body condition of birds were not negatively affected by background radiation, but that these traits even increased with radiation levels. One explanation may be that birds were responding to an oxidative challenge by transiently increasing the levels of antioxidants. However, this may be valid for acute exposure to ionizing radiation (Kovalchuk *et al*. 2007; Dauer *et al*. 2010), but not for chronic exposure as experienced by birds at Chernobyl. Furthermore, this could not explain the positive effect on body condition of birds, which actually suggests that exposure to radiation may increase survival of birds (Møller & Szep 2001). Additionally, and more importantly, background radiation levels covaried negatively with oxidative stress (GSH:GSSG ratio) and DNA damage levels, which can neither be explained by the effect of a transient exposure to radiation as shown in mice in which the GSH:GSSG ratio decreases after an acute exposure to X-ray radiation (Navarro *et al*. 1997). The positive effect of radiation on oxidative stress and DNA damage levels further supports the view that birds can benefit from chronic exposure to radiation, and the fact that these two different measures of physiological damage show the same pattern of covariation with radiation levels demonstrates congruence in this interpretation. We did not find an effect of the intensity of this physiological response on the population trends of the species, which suggests that birds do not pay a cost of maintaining such a response in the long term. The explanation for the overall beneficial effects of radiation found here may be that birds mount an adaptive physiological response (Dimova, Bryant & Chankova 2008) that results in individuals overcoming the initial challenge of ionizing radiation and achieving an improved antioxidant status, DNA integrity and body condition, which may be related to radiation hormesis (Luckey & Lawrence 2006). Albeit surprising, these results agree with recent findings in *Drosophila* that had been exposed to X rays as instar larvae, in which irradiation reduced the frequency of somatic mutations that may result from DNA damage but increased the frequency in mutants deficient in DNA repair (Koana, Takahashi & Tsujimura 2012). This suggests that low-dose radiation can activate DNA repair genes (Koana, Takahashi & Tsujimura 2012). Indeed, this may also explain a similar effect found in developing red-legged partidges *Alectoris rufa* that reduced their levels of oxidative damage after being chronically exposed to a pro-oxidant compound (Galván & Alonso-Alvarez 2009). Similarly, GSH levels in plasma of humans chronically exposed to radiation at Chernobyl increases only under low doses of radiation (Ivanenko & Burlakova 2013). In accordance with the adaptive nature of these plastic responses, it has been shown that a chronic exposure to low-dose γ radiation can lead to a prolongation of life span (Ina & Sakai 2004).

The plastic responses that can lead to adaptation to radiation exposure may be found in a broad diversity of organisms, as exemplified by studies carried out in several taxa (Dauer *et al*. 2010). For example, grasshoppers chronically exposed to low levels of radiation at Chernobyl have been reported to have lower DNA damage levels (measured as levels of 8-hydroxydeoxyguanosine) after an acute challenging irradiation than grasshoppers that had not previously been exposed to radiation (Mortensen 2013). In mice, the damaging effects (prevalence of thymic lymphomas) of a challenging X-ray irradiation were considerably reduced by previous low-dose irradiation, and this reduction was even greater when the mice had been continuously irradiated with γ-rays in the long term (more than 1 year) (Ina *et al*. 2005). Chronically irradiated mice also showed greater body mass (as found here in birds) and immune activity than controls (Ina *et al*. 2005). Protective effects of low 'adapting' doses of radiation before a challenging dose have also been reported for natural radioactivity levels in three species of ungulates (Ulsh *et al*. 2004). Radio-adaptive responses are also observed in humans. Lymphocytes from inhabitants of Ramsar, Iran, one of the world's places with the highest natural radioactivity levels, exposed to background radiation throughout life show lower frequency of chromosome aberrations than persons exposed to negligible radiation levels (Ghiassi-Nejad *et al*. 2002), although DNA damage measured by the comet assay has been reported to be considerably greater in lymphocytes of Ramsar inhabitants than in persons exposed to normal background radiation, the repair rate is higher in the former only if exposure to radiation was relatively low (Masoomi *et al*. 2006). In Chernobyl, lymphocytes of people chronically exposed to low doses from fallout did not show evidence of radio-adaptation regarding frequency of chromosome and chromatid aberrations after a challenging γ-ray irradiation (Padovani *et al*. 1995), but adaptation was shown to occur after a challenge with a glycopeptide that causes double strand DNA breaks (Tedeschi *et al*. 1995). There are several other studies reporting evidence of adaptation in humans occupationally exposed to X- and γ-rays (Tapio & Jacob 2007).

The hypothesized physiological adaptive responses that may explain our results could be transferred from adult birds to their offspring, thus being transmitted across generations producing the patterns that we observed (i.e. an adaptive maternal effect; Mousseau & Fox 1998). This is likely for radio-adaptive responses, as illustrated by Kovalchuk *et al*.'s (2004) report of adaptation of plants to radiation around Chernobyl. These authors demonstrated that the progeny of plants that had been chronically exposed to radiation (although they basically only compared one irradiated site with one non-irradiated site, so results should be taken with caution as unknown factors different from radiation may also account for these effects) was more

resistant to mutagens, showing a higher expression of genes that control the main enzymatic antioxidants and DNA-repair for several generations. They also determined that genome stabilization, measured as homologous recombination levels, was higher in plants collected from contaminated sites at Chernobyl than those from control sites. The global genome DNA of two generation of plants grown at laboratory conditions from seeds collected in contaminated sites was also considerably hypermethylated in comparison to control plants (Kovalchuk *et al*. 2004). As genome stabilization prevents reshuffling of the hereditary material and methylation is one of the main epigenetic mechanisms, their results represent important cues about the mechanisms that permit the inheritance of radio-adaptive responses. Studies on human cells also show similar mechanisms. Thus, lymphoblastoid cells exhibiting adaptive response after receiving a low dose of radiation before a challenging dose show an up-regulation of protein synthesis genes and down-regulation of metabolic and signal transduction genes (Coleman *et al*. 2005), and ROS production in fibroblasts increases with increasing radiation dose and this leads to changes in miRNA expression (Simone *et al*. 2009). Therefore, epigenetic mechanisms such as DNA methylation and miRNA expression could be key in the inheritance of the adaptive response to ionizing radiation, and may explain why we can observe physiological adaptation in some birds 28 years after the nuclear power plant accident at Chernobyl.

PHYLOGENETIC INERTIA IN OXIDATIVE STATUS, DNA DAMAGE AND BODY CONDITION

Physiological adaptation to low doses of ionizing radiation is thus possible, and our study suggests that it may have important evolutionary implications because physiological plasticity that allows variation in GSH and DNA damage levels seem to differ across species of birds as indicated by negative phylogenetic signals (Blomberg, Garland & Ives 2003; Diniz-Filho *et al*. 2011). This variability could favour the role of natural selection (Woods & Harrison 2002). Furthermore, phylogenetic eigenvectors were important predictors of variation in GSH levels, GSH redox status, DNA damage and body condition, supporting the interpretation of interspecific variation in capacity to mount radio-adaptive responses. In EV1, the phylogenetic eigenvector selected to account for phylogenetic effects in the analyses of GSH levels and redox status showed that birds from the families Turdidae, Muscicapidae, Motacillidae and Fringilidae were positioned at the positive part of the axis while the rest of the phylogeny (i.e. families Lanidae, Paridae, Sylviidae and Hirundinidae) were positioned at the negative part. The effect of EV1 on GSH levels and GSH:GSSG ratio was positive, meaning that species that belong to the families Lanidae, Paridae, Sylviidae and Hirundinidae are phylogenetically constrained to increase their GSH levels and decrease oxidative stress, thus being particularly limited to express plastic adaptive responses to ROS exposure. In fact, the two species of birds in which radiation at Chernobyl has been reported to deplete antioxidant levels and increase oxidative damage (the barn swallow and the great tit) belong to these families (Møller, Surai & Mousseau 2005; Møller, Karadaş & Mousseau 2008; Bonisoli-Alquati *et al*. 2010, 2011). EV4 and EV3, the eigenvectors selected for the analyses of DNA damage and body condition, were negatively related to these variables, and *Turdus* species were positioned at the negative part of these axes. As *Turdus* species were included in the positive part of EV1, this suggests that phylogenetic inertia causes the birds that belong to this genus to obtain a large benefit from radiation exposure in terms of increased GSH levels and body condition and decreased oxidative stress but also pay a cost in terms of increased DNA damage. This may have important conservation implications that should be considered in bird populations exposed to radioactive contamination or other pro-oxidant agents.

INFLUENCE OF MELANIN PRODUCTION ON IONIZING RADIATION EFFECTS

Production of pheomelanin, one of the two main types of the most abundant pigments in animals, represents a physiological cost under stressful environmental conditions. We found that under equal levels of background radiation birds that produce larger amounts of pheomelanin have lower levels of GSH, higher oxidative stress and higher levels of DNA damage, and they are in poorer condition than birds that produce lower levels of this pigment. We have previously found that the population trends of species of birds that show a higher expression of plumage colours typically conferred by pheomelanin are more negatively affected by radiation exposure at Chernobyl than populations of species with lower expression of these colours (Galván, Mousseau & Møller 2011), the hypothesized mechanism behind that (i.e. consumption of GSH during pheomelanogenesis) thus being consistent with the results of this study. Other studies of wild populations of animals also show that the expression of pheomelanin-based traits may limit the development of costly physiological processes or viability under adverse, stressful environmental conditions. Across species of birds, the expression of pheomelanin-based colour is negatively associated with brain size, whose production requires high GSH levels (Galván & Møller 2011), and positively related to the prevalence of cataract, which GSH critically contributes to prevent (Galván *et al*. 2012b). Western bluebirds *Sialia mexicana* with a greater expression of pheomelanic breast plumage coloration have been reported to be more likely to die of an epidemic (Keyser & Siefferman 2005). Tawny owls *Strix aluco* belonging to the pheomelanic morph have lower viability during adverse environmental conditions than conspecifics belonging to the eumelanic morph (Karell *et al*. 2011). Similar effects can also be found in mammals, as shown by a positive association between the degree of pelage pheomelanization and lipid oxidative damage in wild boars (Galván, Alonso-Alvarez & Negro 2012).

Our study now suggests that the mechanism behind the observed patterns between the production of pheomelanin and costly physiological processes or stressful environmental conditions is as previously hypothesized, that is, the incorporation of sulphhydryl groups from cysteine or GSH into the pheomelanogenesis pathway (García-Borrón & Olivares Sánchez 2011; Ito *et al*. 2011a), which represents a consumption of cysteine/GSH and thus a decrease in antioxidant capacity (Galván, Ghanem & Møller 2011; Pavel, Smit & Pizinger 2011; Galván, Ghanem & Møller 2012). Accordingly, Simone *et al*. (2009) showed that human fibroblasts exposed to ionizing radiation suffered from less radiation-mediated ROS production if they received a previous treatment with cysteine (which is depleted during pheomelanin production in melanocytes). Recently, it has been reported that pheomelanin production per se is a physiological cost that enhances melanoma development in mice (Mitra *et al*. 2012), and the consumption of cysteine during pheomelanogenesis has been attributed to such an effect (Morgan, Lo & Fisher 2013). Our results indicate that the consumption of GSH by pheomelanin production is accompanied by increased oxidative stress, DNA damage and decreased body condition in birds. This suggests that pheomelanin synthesis has profound implications for the physiological plasticity of organisms. Therefore, pheomelanogenesis represents an important physiological cost and thus a constraint to adaptation to stressful environmental conditions.

Eumelanogenesis occurs in the absence of or below a threshold level of sulphhydryl groups in melanocytes (García-Borrón & Olivares Sánchez 2011; Ito *et al*. 2011a). Accordingly, we found that, in contrast to pheomelanin, eumelanin levels in feathers did not affect GSH levels but were positively related to the GSH:GSSG ratio and negatively related to DNA damage levels. This means that, under equal levels of background radiation, the birds that produced more eumelanin suffered less oxidative stress and DNA damage than birds producing less eumelanin. This is consistent with the previously observed protective effect of eumelanin against DNA damage in human melanoma cells, which also show increased survival by producing this pigment (Kinnaert *et al*. 2004). The black pigments of fungi are also protective and enhance growth under exposure to ionizing radiation (Dadachova *et al*. 2007).

Interestingly, we also found that radiation had an effect on the structure of pheomelanin produced by birds. We predicted that ionizing radiation may degrade the benzothiazine moiety of pheomelanin (here represented by 4-AHP) to a benzothiazole moiety (represented by TTCA) because of the higher oxidation potential of the former (Wakamatsu, Ohtara & Ito 2009). It is known that UVA radiation produces pheomelanin radicals and solvated electrons, which induces a reduction of molecular oxygen to superoxide anions, thus making pheomelanin phototoxic (Takeuchi *et al*. 2004; Ye *et al*. 2006): another physiological cost of this pigment. However, this ROS production might be reduced in the benzothiazole moiety of pheomelanin as compared to the benzothiazine moiety due to the rather stable nature of the former, so pheomelanins with higher relative contents of benzothiazoles are less prooxidant under radiation exposure (Wakamatsu, Ohtara & Ito 2009). Photodamage of pheomelanin, which actually occurs in natural hair (Wakamatsu *et al*. 2012), may therefore have beneficial biological effects as suggested by increases in the production of melanin free radicals under low UVA doses but large decreases at high doses (Fernández *et al*. 2012). The conversion of benzothiazine to benzothiazole had never been reported for an effect other than UV radiation, but our results indicate that the TTCA:4-AHP ratio in the feathers of birds increases with background radiation, suggesting that ionizing radiation also induces a change in the structure of pheomelanin. The change towards the production of forms of pheomelanin more stable to oxidation may actually have facilitated the acclimation of birds to ionizing radiation despite the GSH consumption during pheomelanin production (see above). Although feathers are inert structures when mature, melanins are synthesized in melanocytes located in the feather follicles before being transferred to feathers (Yoshihara *et al*. 2011), and these melanocytes can be affected by ionizing radiation.

Conclusions

The inclusion of several species of birds in the analysis of effects of ionizing radiation on oxidative status, DNA damage and body condition allowed us to detect a pattern of covariation that differs from studies that focus on single species, probably because this permits inclusion of a greater range of variation in radiation levels to which birds are exposed and a greater variability in susceptibility to radiation. We considered the individual bird as the sampling unit instead of mean values per species for the studied variables, thus controlling for the effect of common ancestry among individuals that belong to the same species. This analytical approach, which was made by considering individuals as polytomies at the tips of the phylogeny, probably also increased our capacity to discern a general pattern. We thus found that GSH levels and body condition increased, and oxidative stress and DNA damage decreased, with increasing background radiation levels to which birds were exposed at Chernobyl. This suggests that birds have the capacity to adapt to chronic exposure to ionizing radiation, which may have resulted in a hormetic response. Our analysis of phylogenetic signal in the studied traits supports the existence of adaptation. Epigenetic mechanisms, as shown in other organisms, may favour such an inherited radio-adaptive response, which may lead to the pattern observed 24 years after the nuclear power plant accident at Chernobyl. Under equal levels of radiation, the birds that produce more pheomelanin have lower GSH levels, higher oxidative stress, more DNA damage and poorer

condition, while those that produce more eumelanin are better protected against oxidative stress and DNA damage. A radiation-induced change towards the production of more stable forms of pheomelanin may have facilitated the acclimation of birds to radiation exposure despite the cost of pheomelanin production.

Therefore, birds have the capacity to adapt to chronic exposure to low-dose ionizing radiation, although this capacity varies across species and is particularly reduced in those producing larger amounts of pheomelanin and those that are phylogenetically constrained to mount plastic responses for GSH levels. Our study thus stresses the importance of incorporating a multi-species approach to investigate the biological effects of ionizing radiation as conclusions derived from single-species studies may not represent general trends in taxonomic terms. However, it is necessary to be cautious and understand that the positive effects of radiation exposure that we are reporting here represent an overall pattern, which is very useful for inferring evolutionary implications that should be viewed at the fine scale for biodiversity conservation purposes because the capacity to develop adaptive responses depends on the species, and intraspecific variation is also possible (Luckey & Lawrence 2006). Indeed, the effects of radiation at Chernobyl on the populations of organisms, and for birds in particular, have been negative overall (Møller & Mousseau 2006; Møller *et al.* 2012), which does not preclude positive effects for populations of some species (Galván, Mousseau & Møller 2011) or physiological adaptations in the surviving individuals (this study). Thus, any conclusion about biological effects of ionizing radiation is likely to benefit from an integrated approach that considers intra- and interspecific studies as well as physiological and population studies.

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Data accessibility

Data deposited in the Dryad repository http://dx.doi.org/10.5061/dryad.rb5hr.

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Supporting Information

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

Table S1. Sample sizes for different species and sites.