



# Exposure to sulfur in soil explains pigmentation by pheomelanin in birds inhabiting Iceland

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## Abstract

Melanins are the most common pigments in birds and mammals. The synthesis of the sulfurated form of melanin, termed pheomelanin, is promoted by high levels of thiols in melanocytes. Exposure to elemental sulfur in the environment can potentially influence intracellular thiol levels in terrestrial animals, but this has never been investigated in the wild. This is particularly relevant in areas with a high volcanic activity that facilitate sulfur exposure. We explored for an association between the pheomelanin content of feathers in 35 breeding birds from 13 phylogenetically diverse species and 29 locations in Iceland, and the sulfur content of nearby soil samples. After controlling for the effect of phylogenetic relatedness between species, we found that the pheomelanin content of feathers was significantly correlated with the sulfur content of soil, indicating that pheomelanin production increases with sulfur exposure. Our results suggest that environmental exposure to relatively high levels of elemental sulfur promotes the expression of pheomelanin-based pigmentation phenotypes in birds, likely by an influence on intracellular thiols during development. This is the first evidence that soil chemistry can affect the phenotype of animals. Highly volcanic areas such as Iceland are thus potential sources of global phenotypic diversity by promoting sulfur exposure in animals.

**Keywords** Bird coloration · Phenotypic plasticity · Soil chemistry · Sulfur exposure · Volcanic activity

## Zusammenfassung

### Der Schwefelgehalt des Bodens erklärt die Phäomelaninpigmentierung in Island lebender Vögel.

Melanine gehören zu den häufigsten Pigmenten bei Vögeln und Säugetieren. Die Synthese des als Phäomelanin bezeichneten schwefelhaltigen Form des Melanins wird durch einen hohen Thiolspiegel in den Melanozyten begünstigt. Kontakt mit elementarem Schwefel in der Umwelt kann potenziell den intrazellulären Thiolspiegel terrestrischer Tiere beeinflussen, allerdings wurde dies im Freiland bislang nie untersucht. In Regionen mit hoher vulkanischer Aktivität und der damit einhergehenden Schwefelexposition ist dies von besonderer Relevanz. Wir forschten nach einem Zusammenhang zwischen dem Phäomelanin Gehalt der Federn von 35 Brutvögeln aus 13 phylogenetisch verschiedenen Arten von 29 Orten in Island und dem Schwefelgehalt lokaler Bodenproben. Nach statistischer Berücksichtigung phylogenetischer Verwandtschaft zwischen den Arten stellte sich heraus, dass der Phäomelanin Gehalt der Federn signifikant mit dem Schwefelgehalt des

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Bodens korrelierte, was darauf hinweist, dass die Phäomelaninproduktion mit zunehmender Schwefelexposition ansteigt. Unsere Ergebnisse legen nahe, dass ein relativ hoher Gehalt an elementarem Schwefel in der Umwelt die Expression phäomelaninbasierter Pigmentierung bei Vögeln fördert, vermutlich aufgrund des Einflusses intrazellulärer Thiole während der Entwicklung. Dies ist der erste Beweis dafür, dass die Bodenchemie den Phänotyp von Tieren beeinflussen kann. Stark vulkanische Gebiete wie Island stellen daher potenzielle Quellen globaler phänotypischer Diversität dar, da sie Tiere verstärkt Schwefel aussetzen.

## Introduction

With few exceptions, the body surface of all organisms is coated with pigments that play a diversity of vital functions. Pigments determine the appearance of organisms to a large extent, and organic evolution is mediated in a significant part by this appearance. External coloration greatly determines the capacity to adapt to the environment and reinforces the differentiation of incipient species (Seehausen et al. 2008; Manceau et al. 2011). Highly diversified animal clades are indeed often associated with the evolution of conspicuous color traits, because sexual selection, which plays an important role in the generation of isolating mechanisms by adaptive radiation, usually favors conspicuous phenotypes (Maan and Seehausen 2011).

Melanins are the most common pigments in birds and mammals (Galván and Wakamatsu 2016). These pigments are synthesized within lysosome-like organelles called melanosomes in melanocytes. These cells extend dendrites to transfer the melanin-containing melanosomes to the target cells, mainly keratinocytes in the hypodermis and the pigmentary units of feather or hair follicles, via a shedding vesicle system (Ando et al. 2012). This confers pigmentation to the skin and associated structures such as feathers and hairs.

The synthesis of melanins in melanosomes consists in the oxidation of the amino acid tyrosine and the subsequent polymerization of the products that are formed. In the absence or under a certain threshold concentration of thiol compounds (i.e., compounds that can transfer sulfhydryl groups, -SH) in melanosomes, the resulting pigment is called eumelanin, a dark polymer of indole units. However, when thiol compounds, mainly the amino acid cysteine, are under a certain threshold concentration in melanosomes, sulfhydryl groups are incorporated into the structure of the polymer. The resulting pigment is then called pheomelanin, oligomers of sulfur-containing heterocycles (García-Borrón and Olivares Sánchez 2011). With the exception of psittaciform birds, the only organisms in which an evolutionary loss of mixed melanogenesis has been reported (Neves et al. 2020), eumelanin and pheomelanin are present at different proportions in skin, feathers and hairs, largely contributing to the diversity in pigmentation phenotypes observed in birds and mammals. While eumelanin is a dark pigment producing black, grey and dark brown colors, pheomelanin

produces reddish, orange and yellowish colors (Galván and Wakamatsu 2016).

As both tyrosine and cysteine are semi-essential amino acids, meaning that they are partly acquired in the diet, there is a potential environmental effect on the ability to synthesize melanin pigments through the availability of food resources. This potential effect, however, has been investigated only in a few studies. Dietary amino acids have been shown to affect hair eumelanin production in cats (Anderson et al. 2002) and the color (Poston et al. 2005) and size of the black bib of male House Sparrows *Passer domesticus*, a trait dependent on the levels of pheomelanin in feathers (Galván and Alonso-Alvarez 2017). Fargallo et al. (2007) showed that the proportion of male Eurasian Kestrel *Falco tinnunculus* nestlings that display the greyest plumage patches, which are produced by a low pheomelanin/eumelanin ratio in feathers, was higher in years of high prey abundance. In nestlings of another raptor species, the Northern Goshawk *Accipiter gentilis*, the abundance of available prey has been shown to predict plumage pheomelanin content (Galván et al. 2019).

These dietary effects are particularly relevant to pheomelanin, because the amino acid precursor that is limiting for pheomelanin synthesis (cysteine) plays essential biological roles but also causes oxidative stress if in excess (Janaky et al. 2000; Dilger and Baker 2008). Once incorporated into the process of pheomelanin synthesis, sulfhydryl groups from cysteine are not released back to melanocytes and keep being part of the pigment structure, meaning that pheomelanin production can contribute to cysteine homeostasis (Galván et al. 2012). This has experimentally been demonstrated in developing Eurasian Nuthatches *Sitta europaea*, in which dietary excess cysteine induces changes in DNA and RNA methylation in some genes that regulate cysteine metabolism in feather follicular melanocytes, favoring pheomelanin-based feather pigmentation and avoiding oxidative stress (Rodríguez-Martínez et al. 2019). The need for cysteine homeostasis, determined by the level of dietary intake of cysteine, may therefore constitute an evolutionary pressure affecting the expression of pheomelanin-based pigmentation in birds and mammals. Indeed, juvenile plumage expressing pheomelanin-based coloration has evolved more frequently in species of birds that have strict carnivorous diets, which have more abundant and physiologically available proteins, than in species with other diets (Rodríguez-Martínez and Galván 2020). Therefore, kinetic conditions

in melanocytes promote pheomelanin production under high thiol levels (Ito and Wakamatsu 2008), and this may then be favored by selection due to the benefits of cysteine homeostasis.

However, environmental effects on thiol levels in melanocytes that influence pheomelanin synthesis and pigmentation may not be exerted only through an effect of dietary cysteine. Exposure to elemental sulfur can also affect intracellular thiols. The biological effects of exposure to elemental sulfur are poorly studied, but it is known that terrestrial animals can be exposed to environmental sulfur via sulfur-containing ingested food, through inhalation of sulfur dust, by skin contact, and from exposure to sulfur in the surrounding environment (Kuklińska et al. 2013). Thus, the exposure to high levels of environmental sulfur can potentially exert physiological effects on birds and mammals. These effects influence intracellular thiol levels, which can produce toxicity as observed in rats inhaling sulfur particles and in guinea pigs ingesting sulfur-containing food (Kuklińska et al. 2013). These effects can also influence the development of integumentary structures that contain sulfur, as observed in sheep fed with sulfur-containing food and experiencing an increase in wool growth (Starks et al. 1954). A high increase in intracellular thiol levels can thus be toxic. From a kinetic perspective, this promotes pheomelanin synthesis in melanocytes (Ito and Wakamatsu 2008). Additionally, the use of thiols for pheomelanin synthesis can avoid the toxicity of high thiol levels (Rodríguez-Martínez et al. 2019). Therefore, exposure to high levels of environmental sulfur chemically promotes the production of large amounts of pheomelanin, and this may also be adaptive for birds and mammals. This would mean that exposure to environmental sulfur may contribute to the evolution of pheomelanin-based pigmentation, as pheomelanin-based color phenotypes should evolve more frequently in species inhabiting environments exposed to high sulfur levels. This possibility has never been contemplated.

Iceland is an ideal natural model to test this hypothesis. This 103,000 km<sup>2</sup> North Atlantic Island is one of the most volcanically active areas in the world, with about 30 active volcanic systems and volcanic eruptions occurring every 3–5 years on average (Schmidt et al. 2014). As sulfur dioxide (SO<sub>2</sub>) is typically the third most emitted volcanic gas after water and carbon dioxide, the daily emission of sulfur in the form of SO<sub>2</sub> into the atmosphere during volcanic eruptions in Iceland can exceed by several orders of magnitude daily SO<sub>2</sub> emissions from all anthropogenic sources in Europe (Schmidt et al. 2015). Volcanic SO<sub>2</sub> is then deposited on soils, which thus act as SO<sub>2</sub> sinks (Garland 1977). Additionally, there is an intense aeolian activity in Iceland due to the suspension of enormous amounts of volcanic dust particles in the atmosphere, which adsorb SO<sub>2</sub> on their surface and thus contribute, when depositing on soil, to spreading SO<sub>2</sub>

and transform it into sulfites and sulfates (Urupina et al. 2019). Although both volcanic SO<sub>2</sub> gas and dust particles can be transported over long distances before being settled on soil, most SO<sub>2</sub> deposition occurs around the volcanic cloud and causes extreme loading of the local ecosystems (Parnell and Burke 1990; Delmelle et al. 2001), mainly on waters, soils and vegetation (Gíslason et al. 2015). Also, SO<sub>2</sub> adsorption on the dust surface most likely occurs in the part of the cloud closest to the vent where ash concentration is the highest (Urupina et al. 2019). Lastly, natural hydrothermal waters, which contain high levels of sulfates, thiosulfates, polythionates and sulfide (Kaasalainen and Stefánsson 2011), represent an important input source of sulfur for hydrological systems in Iceland (Robinson et al. 2009). Animals inhabiting Iceland might consequently experience a high exposure to environmental sulfur.

However, sulfur distribution in the soil of Iceland is not homogeneous, hence sulfur exposure is expected to spatially vary along the island. This is reflected by the distribution of soil types. Icelandic soil types are categorized, on a decreasing level of carbon content, into Histosols and Andosols, in addition to Vitrisols and Leptosols (rock/scree) which have a near absence of organic matter and Cryosols (permafrost) (Arnalds 2015). Carbon content differences in Icelandic soils roughly provide information about sulfur distribution, because the availability of sulfur is generally related to the organic content of soils (e.g., Tabatabai 2005).

The aim of this study is to test for an association between the intensity of pheomelanin-based pigmentation in birds and the sulfur content of soil in Iceland. For this, we sampled for feathers 35 specimens from 13 species deposited in the bird collection of the Icelandic Institute of Natural History that had been collected during the breeding season in 29 locations in Iceland and obtained soil samples from nearby locations. We predicted that, if exposure to environmental sulfur affects the evolution of pheomelanin-based pigmentation, the relative content of pheomelanin in feathers should increase with the relative content of sulfur in the soil where birds developed the feathers, after controlling for phylogenetic effects. This is the first study investigating a possible effect of environmental sulfur exposure on the evolution of pigmentation.

## Materials and methods

### Species selection and sampling of feathers

Colors produced by pheomelanin can readily be distinguished from those produced by the other form of melanin (eumelanin), as the former produces dull yellow and orange colors while the latter produces black, grey and dark brown colors (Galván and Wakamatsu 2016). On this basis, we

assessed the plumage pigmentation phenotype of the species of birds that breed in Iceland regarding the presence of pheomelanin pigment in feathers (Hilmarsson 2011) and identified 17 species that show plumage patches expressing colors indicative of the highest pheomelanin concentrations in feathers (i.e., color categories 4–6 in Galván and Wakamatsu 2016). Any possible effect of soil sulfur content on pheomelanin-based plumage pigmentation may therefore most likely be detected in these species. In the bird collection of the Icelandic Institute of Natural History, we searched for specimens collected during the breeding season across Iceland for which we could also obtain soil samples near the bird sampling locations, and that covered the diversity of soil types in Iceland (Arnalds 2015).

We finally selected 13 species, and we could sample for feathers a total of 35 bird specimens. The selected species and numbers of sampled specimens were the Eurasian Wren *Troglodytes troglodytes* (1 specimen), the Redwing *Turdus iliacus* (1 specimen), the Red-throated Loon *Gavia stellata* (5 specimens), the Horned Grebe *Podiceps auritus* (2 specimens), the European Golden Plover *Pluvialis apricaria* (5 specimens), the Black-tailed Godwit *Limosa limosa* (4 specimens), the Dunlin *Calidris alpina* (1 specimen), the Red-necked Phalarope *Phalaropus lobatus* (3 specimens), the Common Snipe *Gallinago gallinago* (2 specimens), the Harlequin Duck *Histrionicus histrionicus* (2 specimens), the Eurasian Teal *Anas crecca* (3 specimens), the Gadwall *Anas strepera* (3 specimens) and the Eurasian Wigeon *Anas penelope* (3 specimens). The species thus belong to seven different families, covering a wide phylogenetic spectrum. All specimens corresponded to adult birds in breeding plumage, except two European Golden Plover specimens that were downy chicks. We included these chicks because their downy feathers contain the same yellow color produced by pheomelanin that is observed in adult feathers (Galván and Wakamatsu 2016; this study).

Although eight of these species are migratory and/or conduct the pre-breeding molt that leads to the breeding plumage on the wintering grounds or during migration (i.e., the Red-throated Loon, the Horned Grebe, the European Golden Plover, the Black-tailed Godwit, the Dunlin, the Red-necked Phalarope, the Common Snipe and the Harlequin Duck; Cramp et al. 1978, 1983), it is expected that chronic exposure to soil sulfur in the breeding grounds (approximately half a year) affects their physiology all year round. Although little is known about the duration of physiological effects of elemental sulfur exposure in animals (Kuklińska et al. 2013), studies on humans show long-term effects after exposure to toxic sulfurated agents (Jafari and Ghanei 2010) and after early diets with sulfur-containing amino acids (Rees 2002). Additionally, studies in Iceland and abroad show a high interyear fidelity to the breeding sites in several of these species such as the Horned Grebe

(Konter and Konter 2006), the Golden Plover and the Dunlin (Klima and Johnson 2005), the Black-tailed Godwit (Kruk et al. 1998), the Red-necked Phalarope (Schamel and Tracy 1991) and the Harlequin Duck (Bengtson 1972). Thus, differences are expected to arise when comparing the plumage pigmentation of birds sampled in sites with different sulfur contents in soil.

We collected body feathers from the 35 bird specimens. The target plumage patches were those displaying yellow and orange colors produced by the presence of pheomelanin in feathers (Fig. 1). These plumage patches were previously analyzed by Galván and Wakamatsu (2016) in three out of the 13 species included in this study, showing high pheomelanin concentrations as compared with other species. Considering the levels of thiazole-2,4,5-tricarboxylic acid (TTCA), a specific marker of the pheomelanin moiety (benzothiazole) whose levels are predicted by color intensity (Galván and Wakamatsu 2016), pheomelanin concentrations in these three species were as follows (in  $\mu\text{g}/\text{mg}$  feather): gadwall (55.05), red-throated loon (42.60) and black-tailed godwit (12.82). As we used samples from museum collection bird specimens, our study did not require an ethical approval.

### Measurement of pheomelanin content of feathers

The feathers collected from bird specimens were analyzed by micro-Raman spectroscopy to determine their relative content of pheomelanin, which exhibits a distinctive Raman signal that can be used for its non destructive identification and quantification (Galván et al. 2013a, 2018; Galván and Jorge 2015; Galván and Rodríguez-Martínez 2018). We used a Thermo Fisher DXR confocal dispersive Raman microscope (Thermo Fisher Scientific, Madison, WI, USA) with a point-and-shoot Raman capability of 1  $\mu\text{m}$  spatial resolution and using a near-infrared excitation laser of 780 nm. We analyzed two barbs and two barbules chosen at random for each feather. Barbs were analyzed using a 50 $\times$  confocal objective and setting laser power at 7 mW, while barbules were analyzed using a 100 $\times$  objective and setting laser power at 2.5 mW. Integration time was 3 s, and the number of accumulations was 12. The system was operated with Thermo Fisher OMNIC 8.1 software. Calibration and alignment of the spectrograph were checked using pure polystyrene.

We calculated the average Raman spectrum for each bird specimen and fitted it to an amplitude Gaussian deconvolution function using Origin v.9.1 (OriginLab Corporation, Northampton, MA, USA). We considered the three diagnostic Raman bands of pheomelanin, at about 500, 1500 and 2000  $\text{cm}^{-1}$  (Galván et al. 2013a,b). The mean ( $\pm$  SE) adjusted  $R^2$  of fitting these functions to the spectra was  $0.91 \pm 0.01$ . From the deconvolution functions, we calculated the position, area, intensity and width of the





**Fig. 1** Images of species of birds included in the study. **a** Eurasian Wren *Troglodytes troglodytes*; **b** Redwing *Turdus iliacus*; **c** Red-throated Loon *Gavia stellata*; **d** Horned Grebe *Podiceps auritus*; **e** European Golden Plover *Pluvialis apricaria*; **f** Black-tailed Godwit *Limosa limosa*; **g** Dunlin *Calidris alpina*; **h** Red-necked Phalarope *Phalaropus lobatus*; **i** Common Snipe *Gallinago gallinago*; **j** Harlequin Duck *Histrionicus histrionicus*; **k** Eurasian Teal *Anas crecca*; **l**

*Gadwall* *Anas strepera*; **m** Eurasian Wigeon *Anas penelope*. The reddish/chestnut plumage patch observed in the image of each species corresponds to the plumage patches pigmented by pheomelanin that were sampled from the bird specimens. All images were taken during the breeding season of birds. Photo credits and locations: **a**, **i**, **k** and **l** Rafael Palomo Santana (Spain); **b**, **d**, **e**, **f** and **m** Ólafur K. Nielsen (Iceland); **c** Sindri Skúlason (Iceland); **g**, **h** and **j** Greg Barsh (Alaska)

three Raman bands. As it was not possible to include the band at about  $2000\text{ cm}^{-1}$  in all deconvolution functions due to low intensity, we used the area of the band at about  $1500\text{ cm}^{-1}$  for the relative quantification of pheomelanin

in feathers, as this is the most important and repeatable predictor of pheomelanin concentration in both bird feathers and mammalian hairs (Galván et al. 2013a).

All samples were analyzed under the same conditions by a technician blind to the aims of the study and of the species identity of samples. There were no technical circumstances related to Raman spectroscopy that affected only some samples.

### Sampling of soil

We used the geographical coordinates associated with the bird specimens to obtain information on soil types for the bird sampling sites in Iceland. We obtained this information from the *Nytjaland* database, a GIS Icelandic Farmland Database created by the Agricultural University of Iceland. This database includes a good resolution supervised classification of soils and vegetation classes based on satellite images (Arnalds 2015) and allowed us to classify bird sampling sites into the following soil categories: Brown Andosols (differentiating those in grassland, richly vegetated heathland, poorly vegetated heathland, birch shrubland and forestry areas), Brown Andosols and Leptosols (in mossland), Gleyic and Histic Andosols (differentiating those in cultivated land and semi-wetland), Gleyic and Histic Andosols and Histosols (in wetland), Vitrisols (differentiating those in partially vegetated land and sparsely vegetated land), and absence of soil or poorly developed soil (lakes and rivers, glaciers and others) (Arnalds 2015).

We then searched for sites within a 10 km-radius circular area around bird sampling sites that had the same soil category and for which there were soil samples available at the Agricultural University of Iceland. We chose the soil sampling site that was closest to the bird sampling site, within the 10 km area, if more than one was available. The geographical correspondence between bird and soil sampling sites is shown in Fig. 2. Therefore, we maximized the

number of bird specimens available at the Icelandic Institute of Natural History for which there were soil samples available at the Agricultural University of Iceland that coincided with the collecting sites.

We used about 10 g of available samples of soil that had previously been collected from each soil collecting site. These samples had been dried in a stove and sieved to <2mm particle size.

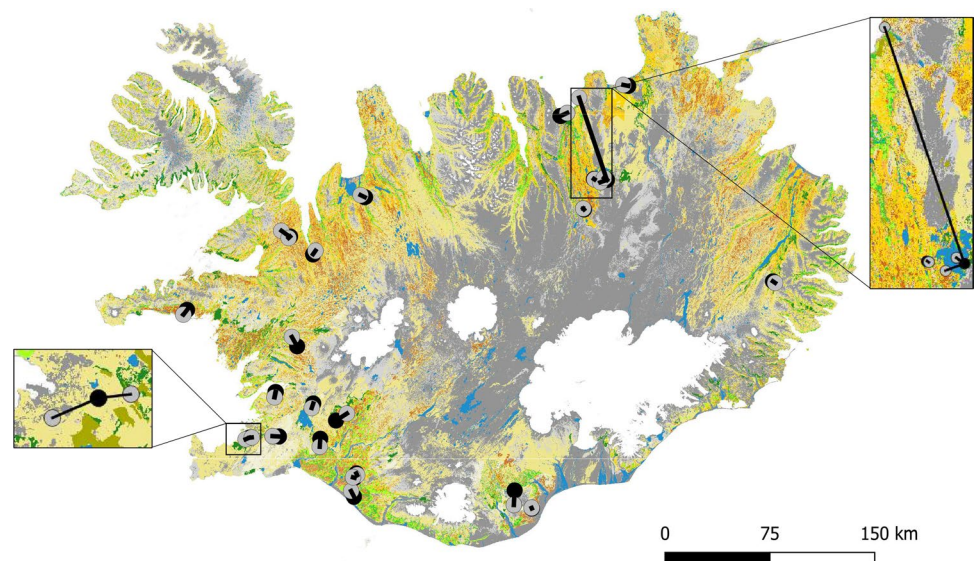
### Measurement of sulfur content of soil

A semi quantitative analysis of elemental sulfur in the soil samples was made by dispersive X-ray irradiation in an Inspect-S environmental scanning electron microscope (ESEM) (FEI Company, Hillsboro, OR, USA). The ESEM spectra of 10 g sub-samples of soil were obtained with an acceleration voltage, an acquisition time of 20 s, and under a pressure of 0.40 Torr in vacuum. For each 10 g sub-sample of soil, measurements were taken at four points chosen at random on the soil surface, and the average value was then calculated. The apparent concentration of sulfur provided by the equipment, calibrated with an iron disulfide (FeS<sub>2</sub>) standard, was used as a semi quantitative measurement of total sulfur in the soil samples. The repeatability of these analyses, considering the four measurements taken per sub-sample, was high ( $r = 0.86$ ,  $F_{34,105} = 25.94$ ,  $P < 0.001$ ).

### Phylogenetic analyses for testing feather pheomelanin-soil sulfur covariation

Species are evolutionarily related through phylogeny; thus they cannot be treated as independent sample units in statistical analyses (Felsenstein 1985). Therefore, to test whether the pheomelanin content of the feathers of birds breeding in

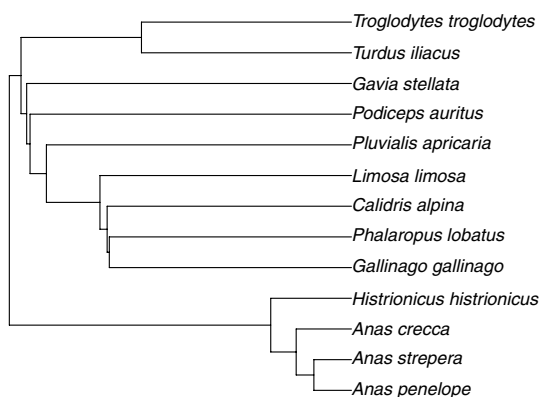
**Fig. 2** Distribution of bird sampling points (grey circles) and soil sampling points (black circles) across Iceland. Lines show the correspondence between both sampling points. Each color in the map represents a category of soil, excepting blue and light grey patches that represent lakes and rivers, and white patches that represent glaciers





Iceland depends on the sulfur content of soil, we conducted phylogenetic linear regression models pooling the data from all species. In the models, the pheomelanin content of feathers (in  $\log_{10}$ ) was the response variable and the sulfur soil content (in  $\log_{10}$ ) was a covariate. As we had multiple observations per species because we were interested in exploring intraspecific variation in pheomelanin-based pigmentation in different species of birds, we used the *Rphylopars* package in the R environment to estimate the phylogenetic covariance of the two traits across species while considering within-species covariance (Goolsby et al. 2017). We fitted the models to data considering different modes of evolution (Brownian motion (BM), Ornstein–Uhlenbeck (OU) and early burst (EB)), in addition to the model without phylogeny (i.e., considering independent sample units), and used the Akaike information criterion (AIC) to choose the best model.

To incorporate in the models information about the phylogenetic relationships between the 13 species of birds used in the study, we obtained 1000 probable phylogenies with the *Phylogeny subsets* tool of [www.birdtree.org](http://www.birdtree.org), and then constructed a consensus tree using the R package *phytools* (Revell 2012) (see Fig. 3). We ultrametrized the consensus tree by using a value of 0.1 for the smoothing parameter  $\delta$  in the R packages *ape* (Paradis et al. 2004) and *phangorn* (Schliep 2011). We measured the amount of phylogenetic signal in the response variable using the *fast.SSC* tool in *Rphylopars* (Goolsby et al. 2017). This tool calculates the scaled sum of squared changes (SSC) between ancestral and descendant nodes as described in Klingenberg and Gidaszewski (2010), using a fast ancestral state reconstruction algorithm. We also used this tool to calculate the corresponding  $P$  value for  $H_0$ :  $SSC = 1$ , using 1000 phylogenetic permutations.  $SSC = 1$  is indicative of a Brownian motion model of evolution in which changes in the trait along the phylogeny are proportional to the degree of relatedness among species, while  $SSC < 1$  and  $> 1$  are indicative of less or more phylogenetic signal,



**Fig. 3** Consensus phylogenetic tree for the 13 species of birds breeding in Iceland included in the study. Branch lengths are proportional to nucleotide substitutions

respectively, than expected under Brownian motion. SSC was calculated considering the average pheomelanin content of feathers per species.

## Results

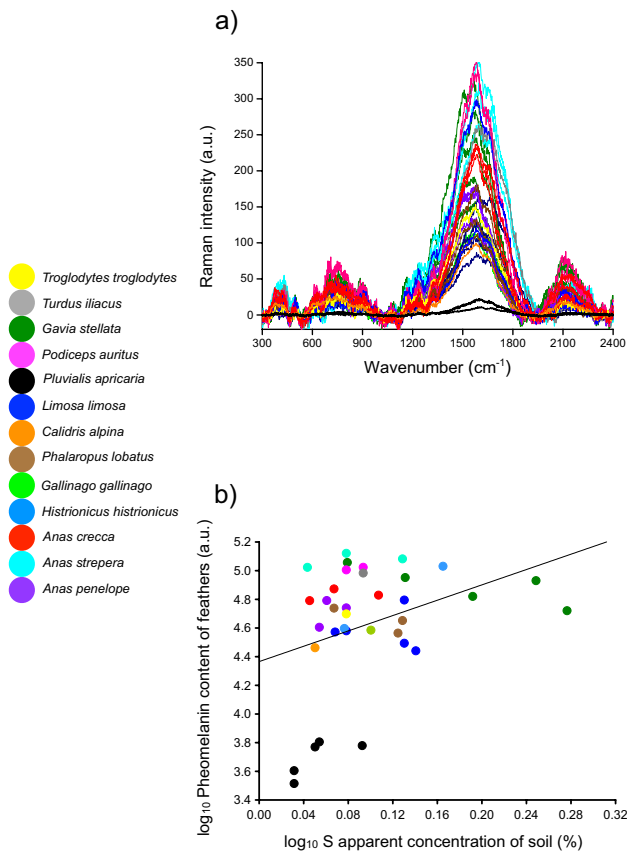
The model assuming a Brownian motion mode of evolution was the best model at explaining the covariation between the pheomelanin content of feathers and the sulfur content of soil, and it was significantly better ( $\Delta AIC = 7.50$ ) than the second model in importance, represented by that assuming an early burst mode of evolution (Table 1). The Brownian motion model revealed a significant and positive relationship between the pheomelanin content of feathers and the sulfur content of soil where the birds were collected ( $b = 6.21$ ,  $F_{1,11} = 5.18$ ,  $P = 0.044$ ). This indicates that the amount of pheomelanin pigment produced by birds in Iceland depends on the level of elemental sulfur in soil to which they were exposed during breeding (Fig. 4). As there were no technical circumstances related to Raman spectroscopy that affected only some samples (Fig. 4a), the pattern of correlation between pheomelanin feather content and sulfur soil content (Fig. 4b) can be considered to be the result of natural interspecific variability. It must be noted, however, that the pattern of covariation between pheomelanin feather and sulfur soil contents is dependent on the inclusion of the Golden Plover in the analysis (Fig. 4b).

The value of SSC for the pheomelanin content of feathers was  $> 1$  ( $SSC = 1.89$ ), but not statistically different from 1 ( $P = 0.395$ ). This is indicative of a strong phylogenetic signal, but still expected under Brownian motion, i.e., changes in the pheomelanin content of feathers along the phylogeny are proportional to the degree of relatedness among species. The expression of pheomelanin-based pigmentation

**Table 1** Results of phylogenetic linear regression models testing for covariation between the pheomelanin content of feathers in 35 bird specimens from 13 species, and the sulfur content of soil samples taken near the bird collection sites

Evolutionary model	AIC	$b$	$F$	df	$P$
Brownian motion	7.59	6.21	5.18	1,11	0.044
Early burst	15.09	6.50	5.68	1,11	0.036
No phylogenetic effects	41.12	2.68	4.46	1,33	0.042
Ornstein–Uhlenbeck	80.88	6.21	5.18	1,11	0.044

The models were conducted assuming three modes of evolution (Brownian motion, early burst and Ornstein–Uhlenbeck) and one model assumed that species were independent (no phylogenetic effects). The Akaike Information Criterion (AIC) for each model is given, together with the parameter estimate ( $b$ ), the  $F$ -statistic ( $df =$  degrees of freedom) and the  $P$  value for the sulfur content covariate effect



**Fig. 4** **a** Raw Raman spectra of pheomelanin obtained from feathers of 35 bird specimens from 13 species collected during breeding in Iceland. The feathers were taken from the reddish/chestnut plumage patches observed in Fig. 1. Each spectrum represents the average spectrum from two feathers (laser beam focused on four barbs and four barbules) of a single specimen. **b** Relationship between the pheomelanin content of feathers and the total content of sulfur in the soil of the sites where bird specimens were collected in Iceland. Pheomelanin content values were obtained by deconvoluting the Raman spectra in (a) and calculating the area of the main band at about  $1500\text{ cm}^{-1}$  from the deconvolution function. Sulfur content values were obtained by dispersive X-ray irradiation of soil samples in an environmental scanning electron microscope (ESEM) at vacuum. The line is the best fit line

phenotypes in birds that breed in Iceland is thus an evolutionarily conserved trait.

## Discussion

Environmental exposure to sulfur can affect intracellular thiol levels in organisms (Kuklińska et al. 2013). Pheomelanin synthesis in melanocytes is promoted by high thiol levels (Ito and Wakamatsu 2008). Accordingly, our results show that birds with phenotypes profusely pigmented by pheomelanin breeding in Iceland develop their plumage in sites with soils with higher sulfur contents

than sites of birds with phenotypes produced by lower amounts of pheomelanin. This is observed considering both intraspecific and interspecific covariation between pheomelanin content of feathers and soil sulfur content, at least across the 13 species of birds included in this study.

This covariation is dependent on the inclusion of samples from the European Golden Plover, but there are no reasons that may justify their exclusion. First, the physiological mechanism hypothesized here (i.e., the sulfur content of soil in a given area affects the pheomelanin content of feathers of birds inhabiting that area) is not species-specific, but bird-specific. This means that a given sulfur content of soil should be expected to lead to a given pheomelanin content of feathers in a bird exposed to the soil, regardless of the species this bird belongs to. Thus, although birds are necessarily related through a phylogeny and this requires controlling for phylogenetic effects in the analyses, the nature of the physiological effect of soil exposure on pigmentation should not be expected to differ between species. Furthermore, the species included in this study are those with the highest pheomelanin concentrations inhabiting Iceland (color categories 4–6 in Galván and Wakamatsu 2016), meaning that the exclusion of any of the species may lead to wrong subset of data for the tested hypothesis. Therefore, the association pattern between sulfur soil and feather pheomelanin contents should be considered by including data from the European Golden Plover.

This suggests that exposure to high sulfur soil levels in Iceland has favored the expression of pheomelanin-based plumage phenotypes, likely through an increase in thiol levels during development. Selection may have favored the maintenance of such phenotypes due to the benefits of pheomelanin production to avoid sulfur/thiol toxicity (Rodríguez-Martínez et al. 2019), thus explaining the spatial pattern presently observed in birds inhabiting Iceland. This is also supported by our results, as we found that the plumage pigmentation phenotype of Icelandic birds is a phylogenetically conserved trait, suggesting that it has strongly been favored by natural selection. It is then likely that the susceptibility of the pigmentary system of birds to be affected by sulfur exposure leads to phenotypic plasticity, producing intense pheomelanin-based pigmentation phenotypes when the degree of exposure to sulfur in soil is high, and natural selection benefits such phenotypes due to their adaptive benefits under high sulfur exposure.

None of the species studied here have a breeding distribution range restricted to Iceland. It is thus expected that the association between the expression of pheomelanin-based pigmentation and soil sulfur content has arisen as a consequence of chemical and selective pressures acting on the phenotype of birds along their whole breeding ranges, and can be observed outside Iceland. Future studies should test this hypothesis in other regions to determine its capacity to explain bird phenotypic diversity globally, but the



association may be particularly likely to arise in Iceland due to the high levels of environmental sulfur to which organisms might be exposed (see “Introduction” section). The effect of soil characteristics on the expression of pigmentation phenotypes in birds is thus likely to be specially strong in places with high volcanic activity such as Iceland. Indeed, 5 out of the 13 species studied here present subspecies whose breeding distribution is restricted to Iceland or nearby islands and Scandinavia (*Calidris alpina schinzii*, *Gallinago gallinago faeroeensis*, *Limosa limosa islandica*, *Turdus iliacus coburni* and *Troglodytes troglodytes islandicus*) and whose plumage pigmentation is more intensely reddish/chestnut, which is indicative of higher pheomelanin production (Galván and Wakamatsu 2016) than that of other subspecies (Cramp et al. 1983, 1988).

Although some studies have related bird species distribution with some characteristics of soils (e.g., Kålås et al. 1997; Gunnarsson et al. 2015; Wilson et al. 2005), the implications of developing in certain soil types on the external phenotype remained unexplored in birds and any other vertebrates. Our study therefore unveils an effect that soil sulfur content can exert on the pigmentation phenotype of animals, likely by affecting the amount of pheomelanin produced by melanocytes of feather follicles during development.

The activity of wild vertebrates has a known influence on soil formation and chemistry (Bancroft et al. 2005; Eldridge and James 2009; Platt et al. 2016), but this is the first study showing an opposite effect, i.e., an influence of soil chemistry on phenotypic characteristics of vertebrates. The expression of the pigmentation phenotype, in particular that generated by melanins which are the most common pigments in birds and mammals, can be influenced by environmental factors such as food availability (Galván et al. 2019), solar and terrestrial radiations (Galván et al. 2018), predation risk (Galván 2018), social interactions (McGraw et al. 2003), parasites (Jacquin et al. 2011) and several conditions during development that can potentially affect melanin synthesis (Hubbard et al. 2015). Our study shows that soil characteristics represent an additional environmental factor affecting the expression of melanin-based pigmentation in birds, likely by influencing exposure to sulfur and intracellular thiol levels, meaning that soil arises as a novel source of vertebrate phenotypic diversity. This is particularly relevant for regions with high volcanic activity such as Iceland, which may thus exert a significant contribution to animal pigmentation diversity by enhancing phenotypic plasticity through effects on sulfur exposure.

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**Data availability** The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Conflict of interest** The authors have no competing interests to declare that are relevant to the content of this article.

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