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Expression of glucocorticoid and mineralocorticoid receptor genes co-varies with a stress-related colour signal in barn owls

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ABSTRACT

Glucocorticoid hormones are important intermediates between an organism and its environment. They enable an organism to adjust its behavioural and physiological processes in response to environmental changes by binding to mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) expressed in many tissues, including the integument. The regulation of glucocorticoids co-varies with melanin-based colouration in numerous species, an association that might result from pleiotropic effects of genes in the melanocortin system and evolve within a signalling context. Most studies have focused on the circulating levels of glucocorticoids disregarding the receptors that mediate their action, and that might partly account for the covariation between the regulation of stress and melanin-based colouration. We investigated the association of the expression levels of *GR* and *MR* genes with melanin-based colouration in the growing feathers of nestling barn owls (*Tyto alba*). We also explored the association between *GR* and *MR* expression levels and the expression of genes related to the melanocortin system and melanogenesis to better understand the origin of the link between the expression of receptors to which corticosterone binds and melanin-based colouration. Nestling barn owls displaying larger eumelanic black feather spots expressed *GR* and *MR* at lower levels than smaller-spotted individuals. However, we found that the expression of the *GR* and *MR* genes was positively rather than negatively correlated with the expression of genes involved in the deposition of melanin pigments at the time we sampled the nestlings. This provides mixed evidence of the association between melanin-based traits and *MR* and *GR* gene expression. The finding that the expression of *GR* and *MR* was positively associated with the expression of the *PCSK2* gene (encoding one of the protein convertase responsible for the production of hormone peptide ACTH and α-MSH) suggests that the melanocortin system may be implicated in the establishment of the covariation between melanin-based colour and the expression of receptors to which glucocorticoids bind. However, further studies investigating the expression of melanin-based traits with stress-related endpoints at different time points of feather development will be necessary to understand better the proximate mechanism linking melanin-based traits with stress.

1. Introduction

Hormones play an essential role in translating external stimuli into physiological changes to enhance the survival prospects of an organism in the face of continuous changes in its environment (Ketterson and Nolan, 1992). The glucocorticoid hormones: corticosterone in amphibians, reptiles, birds, and rodents and cortisol in non-rodent mammals and fishes, are under the control of the hypothalamic-pituitaryadrenal (HPA) axis and affect most cells in the body (Ballard et al., 1974; Lattin et al., 2012). Thus, variation in the levels of glucocorticoid hormones (hereafter referred to as "corticosterone") plays a major role in many physiological and behavioural functions (reviewed in Sapolsky et al., 2000; Wingfield et al., 1998), and have important consequences on the development (Cottrell and Seckl, 2009; Henriksen et al., 2011), health (Costantini et al., 2011; Harris and Seckl, 2011), reproductive success (Bonier et al., 2009; Breuner et al., 2008; Vitousek et al., 2014) and survival of an individual (Goutte et al., 2010; Rivers et al., 2012; Romero and Wikelski, 2001). Such multiple links to aspects of an individual's fitness have been proposed to favour the evolution of animal ornaments that signal corticosterone levels or the capacity of an individual to withstand stressful conditions (Buchanan, 2000; Husak and Moore, 2008). Several empirical cases support the expected association between corticosterone levels and different types of ornaments (coloration and other visual displays, vocalisations, among others; Buchanan et al., 2016; Leary et al., 2006; Moore et al., 2016; Roulin et al., 2008; San-Jose and Fitze, 2013; Weiss et al., 2013). However, the proximate factors driving this association remain obscure for most types of ornaments, particularly for those whose expression shows little

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environmental plasticity, such as colourations based on the deposition of melanin pigments (Roulin, 2016).

In many vertebrate species, glucocorticoids are associated with melanin-based colouration (San-Jose and Roulin, 2018). Most studies that have investigated the covariation between melanin-based colour traits and the HPA axis have focused on glucocorticoid levels in plasma (*e.g.* Corbel et al., 2016; Kittilsen et al., 2009; Saino et al., 2013) or in the integument, such as feathers and hairs (Bennett and Hayssen, 2010; Ghassemi Nejad et al., 2017; Kennedy et al., 2013). Although the HPA axis is a complex system regulated at multiple levels and by multiple factors (Breuner and Orchinik, 2002; Funder, 2005; Holmes and Seckl, 2006; Romero, 2004), it is clear that the action of corticosterone is mainly mediated in a dose-dependent manner by the amounts of glucocorticoid (GR) and mineralocorticoid (MR) receptors that bind to corticosterone in different target tissues (Iida et al., 1985; Vanderbilt et al., 1987; Yang et al., 1989). MR and GR have different properties which are thought to independently mediate the action of baseline (MR) and stress-induced glucocorticoid levels (GR) (De Kloet et al., 1990; Funder, 1997; Conway-Campbell et al., 2007).

GR are well distributed throughout the body with GR being more ubiquitous than MR (Ballard et al., 1974; Funder, 2005; Lattin et al., 2012; Lattin et al., 2013). Their distribution can show seasonal variations across tissues (Breuner and Orchinik, 2001; Lattin and Romero, 2015; Lattin et al., 2013), and be influenced or not by circulating hormone levels or stress (Breuner et al., 2003; Dickens et al., 2009; Lattin and Romero, 2015). More importantly, GR are expressed in different skin cells, including melanocytes, where melanin pigments are produced (Serres et al., 1996). Measuring variation in the expression of glucocorticoid receptors (GR, MR) is thus key to not only understand how the activity and action of the HPA axis is regulated but also how corticosterone levels ultimately influence the development of melanin-based colouration and why corticosterone levels co-vary with melanin-based colouration. Despite their importance in the regulation of the HPA axis and that they have been shown to relate to fitness (*e.g.* receptors can vary in certain tissues between individuals adopting different reproductive tactics: Arterbery et al., 2010), only one study has investigated the link between melanin-based traits and the amount of GR and MR (Lattin and Romero, 2013). In this study, conducted in house sparrows (*Passer domesticus*), males with large black bibs showed a smaller amount of GR and MR in skin tissues than males with small bibs, but the generality of this finding remains untested.

Additionally, it has never been investigated by what means GR and MR and, thereby, corticosterone could co-vary with the synthesis of melanin in the integument. The association between corticosterone and melanin-based colouration has been proposed to originate from the pleiotropic effect of the melanocortin system (Ducrest et al., 2008; Roulin and Ducrest, 2011). Although other alternative pathways are possible (*e.g.* Wilkins et al., 2014), the melanocortin system is a good candidate governing the regulation of both colouration and the HPA axis. The genes of the melanocortin system lead to the production of hormonal peptides (α-melanocyte-stimulating hormone, α-MSH, and adrenocorticotropic hormone, ACTH) and membrane receptors (melanocortin receptors 1 and 2, MC1R, MC2R) that have major regulatory effects on melanin synthesis and corticosterone release (reviewed in Ducrest et al., 2008).

In the present study, we examined the relation between melanin-based traits and the expression levels of *GR* and *MR* genes in growing feathers of nestling barn owls. The barn owl (*Tyto alba*) displays melanin-based traits that are associated with various life-history, morphological, behavioural and physiological traits (Roulin and Ducrest, 2011). Barn owls displaying larger eumelanic spots on the feather's tip of their ventral body side are less sensitive to stress than smaller-spotted individuals because: i. they have a lower increase in blood corticosterone levels after a stressful event (Almasi et al., 2010), ii. are less affected by an artificial increase in blood corticosterone levels during growth and breeding (Almasi et al., 2008; Almasi et al., 2012), and iii. lose less weight under food depletion (Dreiss et al., 2010). Thus, given that large spotted individuals are less stress-sensitive than small spotted individuals, we predict that large spotted individuals should express less *GR* and *MR* in their feathers than small spotted individuals. In parallel, we measured the expression levels of different melanogenic and melanocortin-related genes in order to identify the potential proximate mechanism or mechanisms linking melanin-based colour traits and the HPA axis activity. If genes involved in melanogenesis and/or those of the melanocortin system contribute to the expected association between spot size and the expression levels of *GR* and *MR*, we expect to find a negative relationship between the expression of melanogenic and melanocortin-related genes with receptors involved in the binding of glucocorticoids (GR, MR). Thus, a negative association in line with the expected negative relationship between corticosterone and spot size and the expected positive association between the expression of melanogenic and melanocortin-related genes with spot size.

2. Methods

2.1. Study species and site

The barn owl is a medium-sized raptor that lives in open landscapes and preys on small mammals. Females lay two to eleven eggs at a rate of one egg every two to three days between February and the beginning of August. After an incubation of ca. 32 days, nestlings hatch asynchronously every two to three days, which leads to a within-brood age hierarchy (Roulin, 2004a). Females stay in the nest box until the elder nestlings are able to thermoregulate by themselves and to feed without maternal help. Pennaceous feathers start growing around 10–15 days of age and plumage is completed at approximately 55 days when nestlings are ready to fledge.

The study was carried out in 2014 in an open rural landscape in western Switzerland and based on a sampling of 39 male and 42 female nestlings from 25 breeding pairs. Nestlings were visited for the first time at an early stage of feather development, when they were between 15 and 27 days old (mean ± SD: 20.79 ± 3.4 days). During this visit, we ringed the nestlings, plucked three to five feathers from their breast and belly, and took a blood sample for molecular sex determination following the protocol described by Py et al. (2006). We plucked feathers at an early stage of development to pinpoint the time when nestlings produce the black eumelanic spots on the upper part of the feathers. In this study, feathers were plucked at an earlier age compared to a previous study where spots were already formed when feathers were sampled (San-Jose et al., 2017). Thus, in our study, spots were not visible at the time we sampled the nestlings' feathers. We re-visited the nest-boxes when nestling feathers were fully developed (at ca. 55 days of age) in order to assess plumage colouration (see below for the procedure) and used mean spot size from each body part as a proxy of the spot size for plucked feathers. At each visit, we also weighed the nestlings and measured the length of their left wing in order to determine their age precisely.

2.2. Assessment of plumage traits

In the Swiss population of barn owls, the overall colour of the ventral body side varies from white to rufous due to differential deposition of pheomelanin. The tips of the ventral feathers are covered with a varying number of black spots, whose diameter and number also vary between individuals. On average, females are redder and display larger and more eumelanic spots than males (Dreiss and Roulin, 2010). The expression of these plumage traits is strongly heritable and weakly sensitive to the environment (Roulin and Jensen, 2015). In the present study, we focused only on the size of the black spots given that in previous studies no associations have been found between spot number or the pheomelanin-based colouration with stress-related parameters (Almasi et al., 2010; Almasi et al., 2008). Within a 60 × 40 mm^2

frame placed on a standard position of the breast and the belly of all nestlings and adults, we measured the diameter of 10 to 15 black spots (and all spots if less than 10; minimum of spots: two [one individual]) to the nearest 0.1 mm using a slide calliper. We used the mean diameter of spots per body part for our statistical analyses. These methods have already been shown to be reliable (Roulin, 2004b).

2.3. RNA extraction

We extracted total RNA from the base of the developing feathers plucked from the breast and belly of the nestlings. We used breast and belly feathers because they can present very different colour patterns in terms of spots size. Additionally, by using feathers from two different body parts, we can assess whether an association between GR, MR and melanin-based traits is related to melanogenic genes (*MLANA*, *MITF-M* a melanocyte-specific isoform of *MITF, OCA2, PMEL, TYR*) and genes of the melanocortin system (*PCSK2*, *MC1R*) in a consistent manner between body parts. The feathers were collected within the hour following the capture of the nestlings and directly stored in liquid nitrogen before being stored at −80 °C until gene expression analyses.

For each nestling and body part, two to five feather bases were grounded with a pestle in liquid nitrogen, resuspended in 600 µL of lysis buffer containing 10 mM DTT and passed through a spin filter column (InviTrap Spin Universal RNA Mini kit, Stratec, Berlin, Germany) to remove genomic DNA, barbs and barbules. Following the manufacturer protocol (InviTrap Spin Universal RNA Mini kit), total RNAs were eluted in 50 µL of water. To further remove all possible genomic DNA contaminants, a second DNAse I treatment was applied by incubating the 50 µL of eluted RNA with 40 U of DNAse I (Roche Diagnostics Ltd, Basel, Switzerland), 50 U of RNase Inhibitor (Promega AG, Dübendorf, Switzerland) and 1× Roche buffer for 30 minutes at 37 °C, before rapidly freezing the solution at −80 °C. The RNA was then purified and concentrated to 20 µL with the GeneJet RNA purification Kit (Thermo Fisher Scientific, Ecublens, Switzerland). For each series of RNA extraction (12 samples), one random sample was analysed with the Fragment analyser of Advanced Analytical (Labgene, Châtel-St-Denis, Switzerland) to assess RNA quality. All RQN (RNA quality number) values were above 8.0 (scale from 1 to 10), which indicates that the RNA samples were of high quality (Müller et al., 2016; Zhang et al., 2015). Finally, 1 µg of DNase I-treated total RNA was reverse transcribed in a final volume of 20 µL, using 4 µL of 5× VILO reaction buffer and 2 µL of 10× SuperScript Enzyme Mix reverse transcriptase (Thermo Fisher Scientific) at 25 °C for 10 min, at 42 °C for 60 minutes, followed by reverse transcriptase inactivation at 85 °C for 5 minutes. The cDNA was diluted ten times with TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) to achieve a final quantity of 100 ng of RNA, which was finally stored at −20 °C until quantification.

2.4. Gene sequencing

To explore the potential proximate mechanism linking melanin-based traits and the HPA axis activity, we chose to determine the relationship between genes involved in the HPA axis (*GR*, *MR*) with genes involved in the melanocortin system (*PCSK2*, *MC1R*) and the synthesis and development of melanin pigments (*MLANA*, *MITF-M* a melanocyte-specific isoform of *MITF, OCA2, PMEL, TYR*). We did not measure the expression of the *POMC* gene, a main actor of the melanocortin system, because in barn owl feathers we only detect its truncated form, which may not be functional (San-Jose et al., 2017). The convertase PC2 encoded by the gene *PCSK2* cleaves the POMC prohormone into different melanocortin peptides (*e.g.* ACTH, α-MSH) that bind to and activates different melanocortin receptors, including MC1R and MC2R (Chretien and Mbikay, 2016; Eipper and Richard, 1980; Gallo-Payet, 2016; Pritchard and White, 2007). Binding of the ligand α-MSH to the MC1R receptor triggers melanogenesis by activating the expression of the microphthalmia transcription factor (*MITF*) gene. MITF is a transcription factor that upregulates the expression of different genes involved in the synthesis of melanins (*e.g.* tyrosinase, *TYR*) (Du et al., 2003; Vachtenheim and Borovansky, 2010) and in the development and maturation of the melanosomes (*e.g.* protein melan-A, *MLANA*, Pre-MELanosome protein, *PMEL*, melanocyte-specific transporter protein, *OCA2*). MLANA is a transmembrane protein expressed on the surface of melanosomes and plays an essential role in melanosome biogenesis, vesicular trafficking and melanosome maturation and autophagy (Aydin et al., 2012; Giordano et al., 2009; Hoashi et al., 2005). The *PMEL* gene codes for a transmembrane glycoprotein that is regulated by multiple proteolytic processes resulting in an amyloid fibrillar matrix, which enables the deposition of melanin pigments and potentially prevents the toxic effect of melanin derivatives in the melanosome (Bissig et al., 2016). The *OCA2* gene, also known as the pink-eyed dilution protein or p-gene in human, plays an important role in the biogenesis of melanosomes and regulation of eumelanin content in melanocytes through the processing and trafficking of the tyrosinase enzyme (Hirobe et al., 2002; Orlow and Brilliant, 1999; Ozeki et al., 1995; Tamate et al., 1989). We did not include the expression levels of *MC2R* gene in our analyses because the expression of this gene was at the limit of detection, even after preamplification.

The genes *GR* (*NR3C1*, accession numbers KX108748 – KX108749), *MR* (*NR3C2*, KX108754 – KX108757), *MLANA* (KY433283 – KY433284), *PMEL* (KY433285), *GAPDH,* (KU712279) and *TBP* (KY433286) were sequenced from polyA cDNAs following the protocols described in San-Jose et al. (2017). We sequenced the eight exons of the *GR* and *MR*. The 2380 bp of *GR* included the start codon (position 1) and the stop codon (2322 bp). In eight individual owls of the extreme reddish and whitish morphs that were sequenced, we found two synonymous mutations c.582C > A (I194, six individuals of genotype *CC*, 1 *AA* and 1 *CA*) and c.1557G > A (A519, 6 *GG*, 1 *AA* and 1 *GA*). The 3108 bp sequence of the *MR* gene contained the start and stop codons located at 112 bp and 3055 bp, downstream the beginning of the sequence, respectively. Out of eight to ten individuals sequenced we found four mutations, three of them were synonymous: c.261C > T (C87, three individuals of the genotype *CC*, 2 *T* and 3 *CT*), c.1353 T > C (S451, 8 *TT*, 0 *CC* and 2 *TC*) and c.1707G > T (T569, 7 *GG*, 0 *TT*, 3 *TG*), and one non-synonymous: c.292C > G (Q98E, 3 *CC*, 1 *GG* and 4*CG*). We sequenced 590 bp corresponding to the five exons of *MLANA* (*MART-1*) with a start and stop codon at 69 and 410 bp downstream of the beginning of the sequence, respectively. One non-synonymous mutation at position c.237C > T resulted in an arginine to threonine substitution were found in 47 barn owl individuals (R56T, with 43 homozygotes *CC,* one homozygous *TT* and three heterozygous *CT* individuals). Besides, we sequenced for five individuals ten exons corresponding to 1977 bp of the Pre-MELanosome protein gene (*PMEL*), also called *gp100* or *SILV*. We also sequenced 1483 bp of the TATA-binding protein (*TBP*) and 1054 bp of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) which were used with the ribosome protein L13 (*RPL13*) sequenced in a previous study (San-Jose et al., 2017), as reference genes for qPCR. The sequence of TBP contained the start and the stop codons at position 135 and 1043 bp and included seven exons and the GAPDH sequence contains at least ten exons but was missing the start codon. None of the mutations was related to barn owl plumage traits or gene expression (results not shown). Sequences of all the other genes (*MC1R, OCA2*, *PCSK2*, *MITF-M*, *RPL13*, *TYR*) are described in a previous study (for details see San-Jose et al., 2017).

2.5. Quantitative PCR

The specific qPCR primers and dual labelled fluorescent probes were designed with Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) and DNASTAR PrimerSelect software or directly by Microsynth (Balgach, Switzerland) (Table S1). Each primer and probe were blasted to verify its specificity before purchasing them from Microsynth (Balgach, Switzerland) and Eurogentec (Liège, Belgium). We then assessed

the concentrations for each gene primer pairs and probes in order to reach a qPCR amplification efficiency of 95 to 105 % (Table S1). Due to the low levels of gene expression of some genes (*MC1R, PCSK2*), we preamplified the cDNA with the TaqMan preamplification Master Mix kit (Thermo Fisher Scientific) for 14 cycles. We verified that the pre-amplification was uniform by calculating the delta–delta Cq values, which are ΔCq of the preamplified gene minus ΔCq of the cDNA (Cq values of the target genes minus Cq values of the reference gene *GAPDH*). The qPCR assays were run on a QuantStudio 6 real-time PCR system (Thermo Fisher Scientific) and each sample was run in triplicate with 2 µL of the diluted cDNA and 8 µL of master mix containing 1× qPCR MasterMix Plus Low ROX (Eurogentec, Liège, Belgium) or 1× TaqMan gene expression MasterMix (Thermo Fisher Scientific) and primers and probes. To correct for interplate variation, each pre-amplified cDNA sample was arbitrarily assigned to a plate, and three pools of different preamplified cDNAs were added to each plate and used to set up the threshold values between plates. To correct for any variation in cDNA content, Cq scores of the candidate genes were normalized using three reference genes: the ribosome protein L13 (*RPL13*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and TATA-binding protein (*TBP*). Cq scores were imported into qBasePLUS software 1.3 (Biogazelle, Zwijnaarde, Belgium) and reference genes quality analysed with geNorm (Vandesompele et al., 2002). We calculated the mean relative quantities (RQs) per sample and used these values for our statistical analyses. All the genes were transformed to fulfil the normality assumptions of parametric statistical tests.

2.6. Statistical analyses

Within each body part, we used Pearson's correlation coefficients to estimate all the pairwise correlations among the expression levels of *GR*, *MR*, melanocortin, and melanogenic genes (Table 1). We investigated to what extent the pattern of gene co-expression (*i.e.* the pairwise correlations among the glucocorticoid, melanocortin and melanogenetic genes) was similar between body parts by running a Mantel's test with 9,999 permutations using the *mantel.rtest* function from the *ade4* package (Dray and Dufour, 2007). The Mantel's test confirmed that gene co-expression patterns of melanogenic and melanocortin genes within each body part were strongly associated (0.62, *P*-value < 0.002). Therefore, to obtain a reduced set of genes and avoid collinearity, we performed a principal components (PC) analysis to summarize variation in the expression of melanogenic and melanocortin-related genes into a few orthogonal PCs with all body parts. We limited the number of components to the number that accounted for 80 % of the total variance (Kassambara, 2017; Table 2).

Finally, we used linear mixed effect models to test if the expression of *MR* and *GR* genes (*MR* and *GR* were considered as dependent variables in separate models) is related to the size of eumelanic spots displayed on the tip of ventral feathers and/or the expression of genes associated to the melanocortin system (*MC1R*, *PCSK2*) and melanogenesis (*MLANA*, *MITF-M*, *OCA2*, *PMEL*, *TYR*). In the models, we considered as covariates the mean spot diameter of the nestlings measured on the body part where the feathers were plucked from, *i.e.*, the breast and belly, and the three PCs of gene expression (PC 1, PC 2, PC 3, Table 2). As independent variables, we added in the models the date and time of the day (hour) when the feathers were sampled, as sampling latency (*i.e.* the time between a nestling was captured and its feathers were plucked), sex, body mass and age of the nestlings. The analyses showed no significant interactions between body parts and any of the three PCs and were removed from the final models (for each of the interactions the 95 % credible interval (CrI) overlapped with zero, see Table S2). Spot diameter was not significantly correlated to PC 1 ($r = 0.08$, 95 % CrI [−0.09; 0.24]) and PC 2 ($r = 0.05$, 95 % CrI [−0.12; 0.21]) but correlated to a low degree with PC 3 ($r = 0.23$, 95 % CrI [0.07; 0.38]). For this reason, the spot diameter was considered in the same model as the three PCs. The random effect of brood identity was considered in the models as well as the random effect of nestling identity in order to consider the repeated measurements taken on the nestlings (*i.e.* the observed values at the two different body parts), with the random effect "nestling identity" nested in the brood of origin.

Table 1
Pairwise correlation matrix between genes associated to the melanocortin system (*PCSK2*, *MC1R*), genes involved in melanogenesis synthesis (*MITF-M, MLANA*, *PMEL*, *OCA2*, *TYR*), eumelanic spot size and receptor of glucocorticoids (*GR*, *MR*), among breast and belly feather. Correlation analyses were based on 156 feather samples taken on breast and belly of 81 nestling barn owls from 25 broods. The reported values correspond to Pearson's correlations coefficients between genes within (A) breast and (B) belly.

A.

	PCSK2	*MC1R*	*MITF-M*	*MLANA*	*OCA2*	*PMEL*	*TYR*	*GR*	*MR*
PCSK2									
MC1R	-0.25								
MITF-M	0.17	0.04							
MLANA	-0.25	0.60	0.26						
OCA2	-0.34	0.31	0.37	0.49					
PMEL	-0.35	0.74	0.09	0.79	0.56				
TYR	-0.20	0.36	0.34	0.53	0.67	0.54			
GR	0.28	0.13	0.36	0.33	-0.06	0.20	-0.09		
MR	0.19	0.10	0.61	0.42	0.21	0.28	0.21	0.85	
Spot size	-0.02	0.05	0.02	0.05	0.29	0.15	0.28	-0.21	-0.15

B.

	PCSK2	*MC1R*	*MITF-M*	*MLANA*	*OCA2*	*PMEL*	*TYR*	*GR*	*MR*
PCSK2									
MC1R	-0.40								
MITF-M	0.26	-0.19							
MLANA	-0.25	0.60	-0.22						
OCA2	-0.10	0.01	0.29	0.17					
PMEL	-0.30	0.73	-0.13	0.79	0.10				
TYR	-0.12	0.38	0.05	0.57	0.42	0.54			
GR	0.28	-0.11	0.25	0.22	-0.05	0.15	-0.07		
MR	0.19	-0.07	0.40	0.28	0.16	0.26	0.23	0.83	
Spot size	-0.21	-0.07	-0.11	-0.10	0.11	-0.09	-0.03	-0.27	-0.25

Table 2
Principal components analysis of seven genes associated with the melanocortin system (*MC1R*, *PCSK2*) and melanogenesis (*MLANA*, *MITF-M*, *OCA2*, *PMEL*, *TYR*). The table represents the correlation between the original variables with their standard errors and the three first components (PC 1, PC 2, PC 3), eigenvalues and cumulative variance in percentage, the proportion of variance of each gene explained by each PC (square values of Pearson correlations, R^2) and the proportion of variance PCs explained by each gene. The coordinates of individuals of the first three components were used in the mixed effect models. Values in bold and *italic* represent parameters with, respectively, a high and moderate correlation (0.4 to 0.6) with components.

	Loadings ± SEM	Proportion of variance of each gene explained by each PC	Proportion of PC variance explain by each variable
PC1			
PCSK2	0.25 ± 0.05	0.21	0.06
MC1R	*−0.43 ± 0.13*	0.63	0.19
PMEL	*−0.49 ± 0.13*	0.82	0.24
MITF–M	−0.04 ± 0.07	0.00	0.00
MLANA	*−0.47 ± 0.5*	0.75	0.22
OCA2	−0.33 ± 0.72	0.33	0.10
TYR	*−0.42 ± 0.09*	0.00	0.00
Eigen value	3.40		
Cumulative variance	0.49		
PC2			
PCSK2	0.37 ± 0.03	0.19	0.14
MC1R	−0.24 ± 0.07	0.08	0.06
PMEL	−0.12 ± 0.09	0.02	0.02
MITF–M	**0.73 ± 0.05**	0.74	0.55
MLANA	−0.04 ± 0.68	0.00	0.00
OCA2	*0.44 ± 0.11*	0.24	0.18
TYR	0.25 ± 0.06	0.09	0.06
Eigen value	1.39		
Cumulative variance	0.68		
PC3			
PCSK2	**−0.77 ± 0.02**	0.50	0.60
MC1R	−0.27 ± 0.07	0.06	0.07
PMEL	−0.22 ± 0.1	0.04	0.05
MITF–M	−0.04 ± 0.06	0.00	0.00
MLANA	−0.26 ± 0.71	0.06	0.07
OCA2	*0.47 ± 0.4*	0.17	0.21
TYR	0.01 ± 0.09	0.00	0.00
Eigen value	0.84		
Cumulative variance	0.80		

All statistical analyses were done with R 3.5.1 (R Core Team, 2018). Linear mixed effect models were run with the *lme4* package (Bates et al., 2015) and principal components with the *prcomp* function from the *stats* package. The significance of predictors was assessed by simulating 50,000 random values from the joint posterior distribution of the model parameters using the *sim* function from the *arm* package (Gelman and Hill, 2007). We used the 2.5 % and 97.5 % quantiles of the simulated data as the lower and upper bands of our 95 % credible interval (CrI). Predictors with a credible interval that did not include zero were considered to be significantly associated with the expression levels of *GR* and *MR* receptor genes. To compare the effect sizes of individual model terms, all numerical variables were standardized to mean of zero and SD of one. For each of the models, we visually inspected the residuals to verify the assumptions of normality and homoscedasticity for mixed effect models.

For the figures, we simulated 5,000 random samples from the joint posterior distribution of the model parameters within the range of the predictor variables (or each factor level). From these simulations, we used the mean predicted values and the 2.5 % and 97.5 % quantiles as lower and upper limits of the 95 % CrI.

3. Results

The expression of the gene *GR* was strongly correlated with the expression of *MR* gene in the breast ($r = 0.85$, 95 % CrI [0.78; 0.91]) and belly feathers ($r = 0.82$, 95 % CrI [0.74; 0.91]; Fig. S1) The expression of the glucocorticoid gene *GR* was positively correlated to a low to moderate extent with the expression of the melanocortin gene *PCSK2* ($r = 0.28$ in both body parts), and the melanogenic genes *MITF-M*, *MLANA*, and *PMEL* ($0.20 \geq r \leq 0.36$; Table 1). The expression of the *MR* was positively and lowly correlated with the expression of the melanocortin gene *PCSK2* ($r = 0.19$ in both body parts) and positively and low-to-strongly correlated with the melanogenic genes *MITF-M*, *MLANA*, *OCA2*, *PMEL* and *TYR* ($0.16 \geq r \leq 0.61$; Table 1). The patterns of co-expression of the glucocorticoid genes with the melanocortin and melanogenic genes were significantly correlated between body parts (Mantel test: $r = 0.61$, with a randomization *P*-value of 0.003). The size of eumelanic black spots measured on the breast was positively and low-to-moderately associated with the melanogenic genes *OCA2*, *PMEL* and *TYR* in breast feathers ($0.15 \geq r \leq 0.29$; Table 1). In belly feathers, the size of eumelanic black spots measured on this body part was poorly associated with the expression of melanogenic genes (Table 1). In contrast, spot size was weakly and negatively correlated with the expression of the melanocortin gene *PCSK2* in belly feathers ($r = -0.21$) and not in breast feathers ($r = -0.02$).

The PCA yielded three components that explained 80 % of the variance in the expression of melanogenic and melanocortin genes (Table 2). The first PC was mainly represented by the melanogenic genes *PMEL, MLANA, TYR*, and *MC1R*. PC1 explained a large fraction of the variation observed in the expression of these genes (≥ 61 %) and, reciprocally, *PMEL*, *MLANA*, *TYR* and *MC1R* together, explained most of the variation (84 %) in PC 1 (Table 2). Note that the standard error for *MLANA* exceeded the coefficient value, and thus, the contribution of this gene to PC 1 has to be interpreted with care. The results were similar whether we removed *MLANA* or not from the analyses. The PC 2 was mainly represented by *MITF-M*, which explained up to 74 % of the variation in the expression in *MITF-M*. A large fraction of the variation in the PC 2 (55 %) was explained by *MITF-M*, with smaller contributions of other genes (Table 2). PC 3 was mostly represented by *PCSK2*, explaining 50 % of the variation observed in the expression of this gene. The 60 % of the variation in PC 3 was explained by *PCSK2* (Table 2).

The linear mixed effects models showed that the expression of *GR* was positively associated with PC 2 and negatively to PC 3, which was the variable with the largest effect size estimate (Table 3, Fig. 1). Therefore, individuals expressing *GR* at high levels highly express the gene *MITF-M* (PC 2) and *PCSK2* (PC 3), and moderately the melanogenic gene *OCA2* (PC 2). The relationship between PC 3 and the expression of *MR* suggests also a moderate negative relation between *OCA2* and *MR*. However, the standard error of *OCA2* loading on PC 3 was nearly as large as the coefficient value and the relationship between PC 3 and *MR* changed (0.4, 95 % CrI [−0.03; 0.10]) when removing *OCA2* from the analyses (the relationship between *GR* and PC 1 and PC 3 were similar whether we removed *OCA2* or not from the analyses). In consequence, the interpretation of the relationship between PC 3 and *MR* must be considered with caution. Individuals with small spots expressed *GR* at higher levels than conspecifics with larger eumelanic spots (Table 3, Fig. 2). Expression levels of *GR* were not differentially expressed among body parts (Table 1) and not associated with PC 1.

The expression of the *MR* gene was negatively associated with PC 1 and PC 3 and positively associated with PC 2 (Table 3, Fig. 3). Therefore, individuals expressing *MR* at high levels highly express the gene *PCSK2* (PC 3) and *MITF-M* (PC 2) and moderately the gene MC1R (PC 1), and melanogenic genes, *MLANA*, *OCA2*, *PMEL,* and TYR (PC 1). Individuals with small spots expressed the *MR* receptor gene at higher levels than conspecifics with larger eumelanic spots (Fig. 2). Expression of the *MR* receptor gene was not differentially expressed among body

Table 3
Relationship between the gene expression level of *GR* and *MR* receptors and genes associated with the melanocortin system and the melanogenesis pathway. Linear mixed effect model with brood identity and nestling identity nested in brood identity were introduced as random factors. Analyses were based on 156 feather samples taken on breast and belly of 81 nestling barn owls from 25 broods. Values in bold represent significant parameters.

	GR model	MR model
Fixed factors	estimates† (95 % CrI)	estimates† (95 % CrI)
Intercept	0.27 (0.18; 0.37)	0.18 (0.09; 0.27)
Date of sampling	0.02 (−0.05; 0.09)	0.03 (−0.04; 0.09)
Hour of sampling	−0.02 (−0.09; 0.05)	−0.03 (−0.1; 0.03)
Sampling latency	−0.02 (−0.08; 0.03)	−0.02 (−0.07; 0.04)
Nestling body mass	−0.02 (−0.15; 0.12)	−0.03 (−0.16; 0.1)
Nestling age	−0.03 (−0.16; 0.11)	−0.03 (−0.16; 0.1)
Nestling sex*	−0.05 (−0.17; 0.08)	0.01 (−0.11; 0.13)
Size of eumelanic spots	**−0.06 (−0.13; −0.001)**	**−0.08 (−0.13; −0.02)**
Body part**	−0.07 (−0.2; 0.06)	−0.07 (−0.19; 0.05)
PC 1	−0.03 (−0.07; 0.01)	**−0.08 (−0.11; −0.04)**
PC 2	**0.08 (0.03; 0.12)**	**0.16 (0.11; 0.2)**
PC 3	**−0.14 (−0.21; −0.08)**	**−0.09 (−0.15; −0.03)**
Random factors	Variance ± sd	Variance ± sd
Brood ID (*intercept*)	0.0 ± 0.0	0.0 ± 0.0
Nestling ID in Brood ID (*intercept*)	0.0 ± 0.0	0.0 ± 0.0
Residual variance	0.11 ± 0.33	0.10 ± 0.31

We indicate the variance explained by the random variables (individual and brood identities) and residual variance (± sd).
† Estimates are based on standardized predictors.
* Gene expression difference from female to male.
** Gene expression difference from breast to belly feathers.

parts (Table 1). Note that the sex, age and body mass of nestlings as well as sampling latency (*i.e.* time in between first disturbance and feather sampling), date and hour of sampling did not explain any part of the variation in the levels of expression of *GR* and *MR*.

4. Discussion

Nestling barn owls displaying large eumelanic spots showed lower expression of *GR* and *MR* genes in feathers compared to small-spotted conspecifics. Our results are consistent with a previous study in house sparrows showing that males displaying large melanic bibs show less GR proteins in the skin of their bib compared to males with small bibs (Lattin and Romero, 2013). These associations are also in line with previous findings showing that the regulation of corticosterone, the hormone that binds to GR and MR is negatively related with the size of feathers' black spots in the barn owl (Almasi et al., 2010; Almasi et al., 2008) and with melanin-based traits in other species (reviewed in San-Jose and Roulin, 2018).

The relationship between melanin-based traits and the expression of glucocorticoid receptors (*MR, GR*) could arise for different reasons (Fig. 4). One possibility is that the link between melanin-based traits and the expression of *MR* and *GR* arise because the genes regulating the synthesis and deposition of melanin pigments are co-expressed with *MR* and *GR* receptors. For instance, the expression levels of melanogenic genes and the receptors binding glucocorticoids (GR, MR) may be regulated by a common factor. Under this hypothesis, we would have expected to find an association between the expression levels of melanogenic genes and *MR* and *GR* genes of negative sign, given that the expression levels of the *MR* and *GR* genes in this study (Fig. 2) and the abundance of MR and GR in a previous study in house sparrows were lower in more melanised individuals (Lattin et al., 2013). However, we found a significant but positive association between glucocorticoids receptors (*MR, GR*) and genes involved in the production of melanin

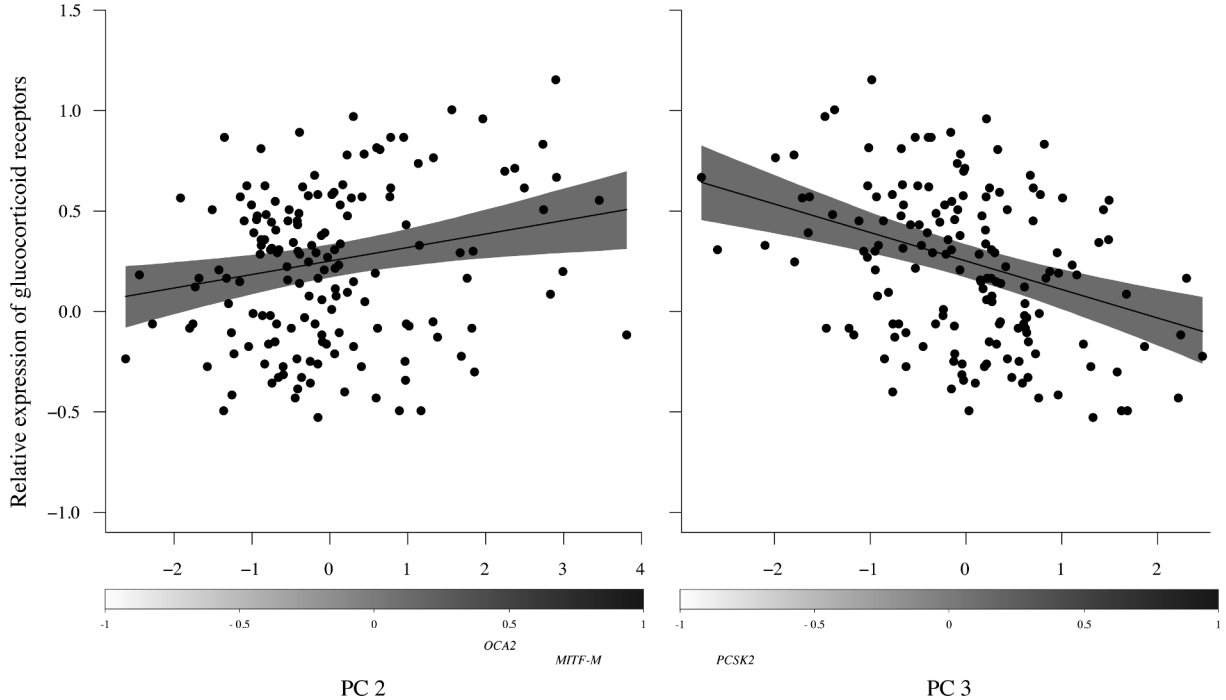

Fig 1. Relationship between principal components (PC 2, PC 3) and the log-transformed expression of *GR* in feathers of nestling barn owls. The dots represent the raw data, and the black lines represent the fitted values estimated from the GR model (Table 3). The grey shadings represent the 95 % CrI of the regression lines. The gradient bars below the X-axis represent the loadings (correlations) of each variable on each principal component. We only represent parameters with, respectively, a high and moderate correlation (0.4–0.6) with components (see Table 2).

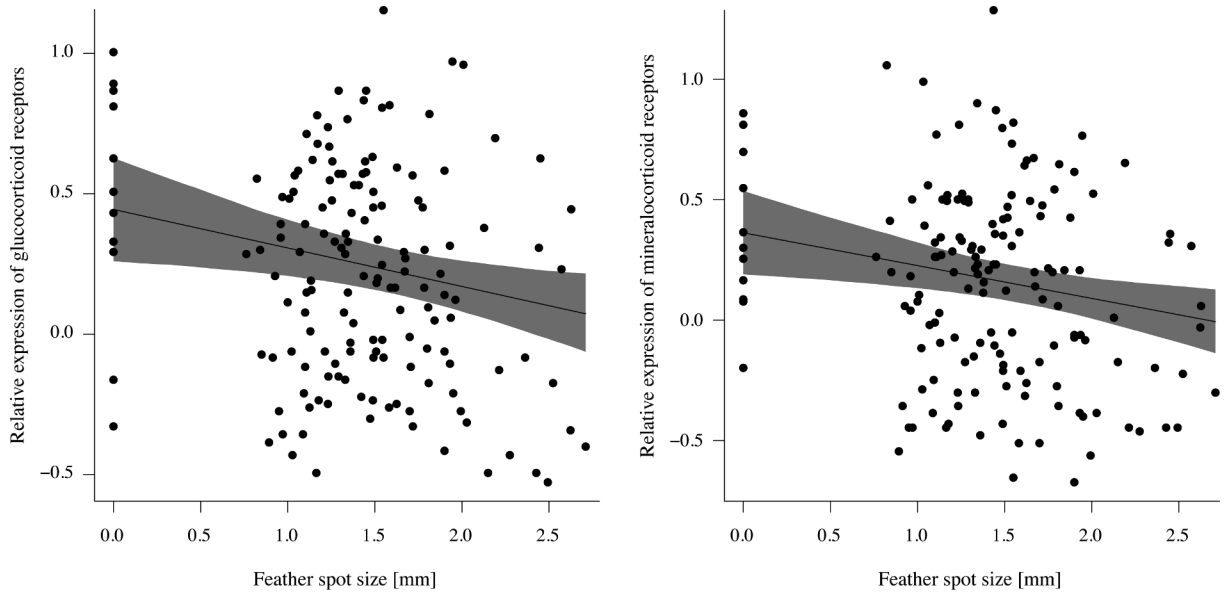

Fig 2. Relationship between the size of black feather spots and the log-transformed expression of *GR* and *MR* in feathers of nestling barn owls. The dots represent the raw data, and the black lines represent the fitted values estimated from the MR and GR model (see Table 3). The grey shadings represent the 95 % CrI of the regression lines.

pigments (*TYR* [PC 1], *MITF-M, OCA2* [PC 2], Figs. 1 & 2) and in the biogenesis and development of melanosomes (*MLANA, PMEL* [PC 1], Figs. 1 & 2). These positive rather than negative associations are in contradiction with the hypothesis that the link between melanin-based traits and the expression of *MR* and *GR* results from the co-expression of *MR* and *GR* receptors with the genes regulating the synthesis and deposition of melanin pigments.

The absence of evidence for a negative relationship between *MR* and *GR* with the expression of melanogenic-related genes might be due to the timing of feather sampling. Although we tried to pinpoint the time when individuals produce their black spots, we do not have the certitude that all individuals (and/or all feathers) were producing the spots at the time we plucked the feathers. This is supported by the fact that we found no evidence for the expected positive relationship between spot size and PC 1 ($r = 0.08$ 95 % CrI [−0.09; 0.24]) and PC 2 ($r = 0.05$ 95 % CrI [−0.12; 0.21]), which is the most representative PC of the expression of melanogenic genes (see Table 2). The lack of a relationship between black spots and the expression of melanogenic-related genes suggests that the expression levels of melanogenic genes at the time we collected the feathers are not entirely representative of the final plumage colouration. We expect that the expression of colour genes will at one point of the development correlate to the final spot size (unpublished data). However, it is difficult to foresee whether the degree of co-expression of *GR* and *MR* and melanogenic genes will remain positive (as reported here), become negative (as expected by the above-stated hypothesis) or even disappear throughout the plumage development. It is possible that the expression of *GR* and *MR* negatively correlate to eumelanic spot size but positively to the expression of melanogenic genes later during development. Confirming such scenario would suggest that the association between *GR* and *MR* and colouration

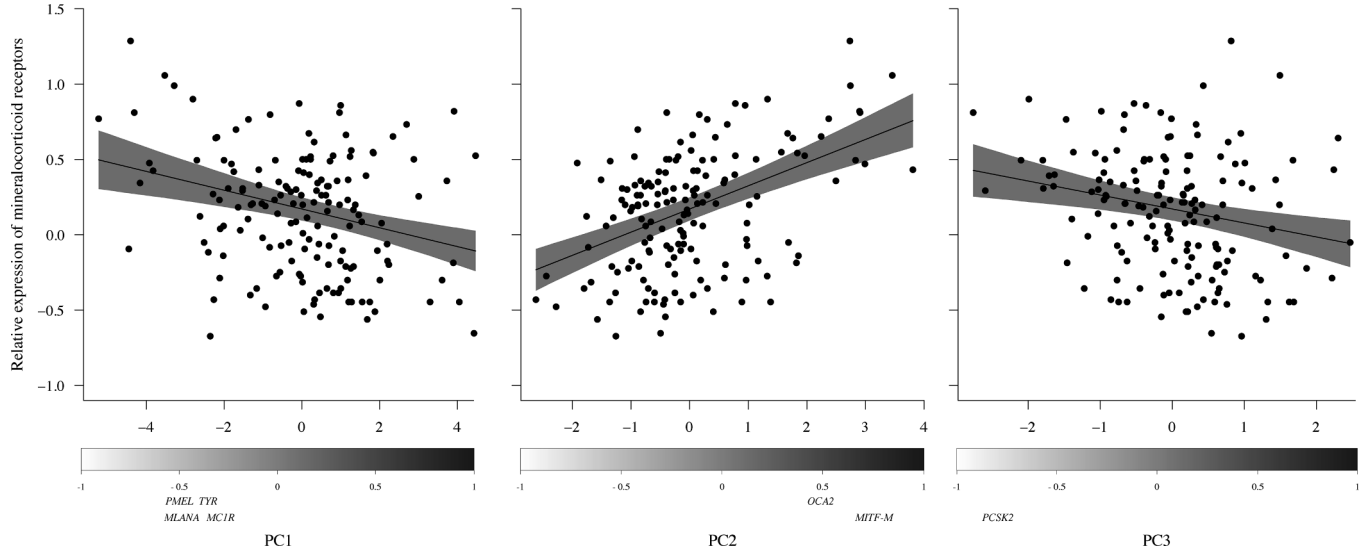

Fig 3. Relationship between principal components (PC 1, PC 2, PC 3) and the log-transformed expression of *MR* in feathers of nestling barn owls. The dots represent the raw data, and the black lines represent the fitted values estimated from the MR model (Table 3). The grey shadings represent the 95 % CrI of the regression lines. The gradient bars below the X-axis represent the loadings (correlations) of each variable on each principal component. We only represent parameters with, respectively, a high and moderate correlation (0.4–0.6) with components (see Table 2).

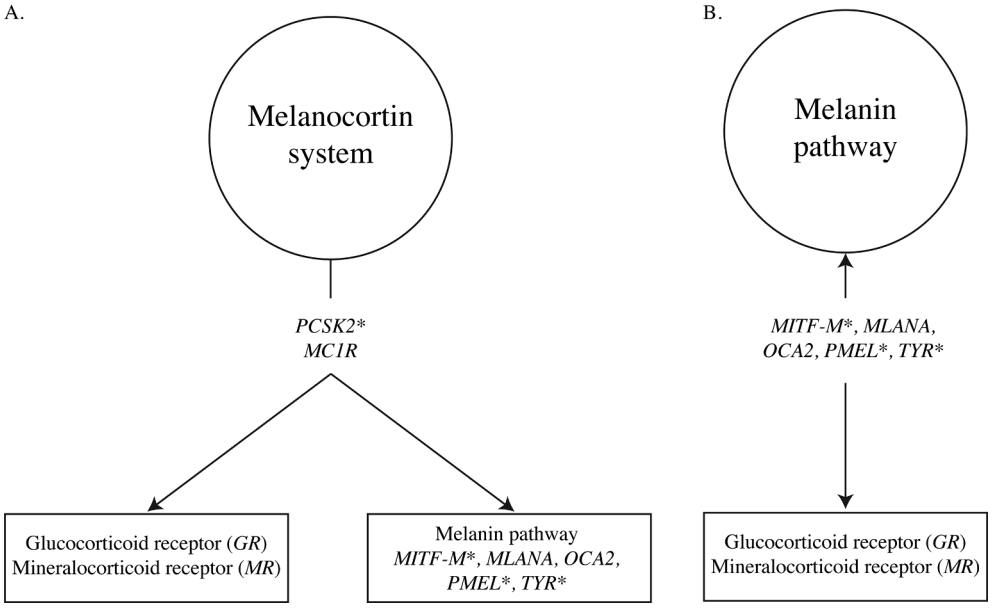

Fig 4. Hypothetical links between melanin-based traits and stress-related endpoints (*i.e.* in this case the expression of glucocorticoids receptors [GR, MR]). The link between melanin-based traits and the expression of glucocorticoid receptors (*MR, GR*) could arise because (A) of the multiped effects of the melanocortin system on melanic genes and stress-related endpoints (Ducrest et al., 2008; Roulin and Ducrest, 2011) or because of (B) the co-expression of genes involved in the production of melanin pigments with glucocorticoid receptors (*MR, GR*) genes. The asterisk (*) refers to genes that were found to be associated, via principal components analyses (Table 2) with the expression of either *GR* or *MR* or both (Table 3).

is not shaped by the effect of *GR* and *MR* genes on the expression of melanogenic genes, or *vice versa*. Future studies accounting for the temporal evolution of gene expression in feathers may help to understand better why we observed an association between the expression of *GR* and *MR* measured at an early age and melanin-based traits but not a similar association with the genes associated to the production of melanin pigments.

Alternatively, the link between melanin-based traits and the expression of *MR* and *GR* might arise through the melanocortin system and its multiple effects on the expression of melanogenic genes and the HPA axis. For instance, through the expression of the POMC prohormone or protein convertases (PCSK 1/3, PCSK2) involved in the cleavage of the POMC prohormone into different active peptides (Pritchard et al., 2002). The relationship might arise because the proteins involved in the processing of POMC, or the downstream effector products regulated by the melanocortin system, affect the expression of *GR* and *MR* and the expression of melanogenic genes simultaneously. The cleavage of the POMC prohormone results in ACTH that induces the production of corticosterone in adrenal glands and the skin (Fig. 5) (Eipper and Richard, 1980; Slominski et al., 2005; Smith and Funder, 1988). Corticosterone is known to affect positively or negatively the expression of glucocorticoid receptors depending on the tissue (Karandrea et al., 2002; Nishimura et al., 2004; Paskitti et al., 2000), which makes possible that ACTH indirectly influences the abundance of MR and GR in skin tissues. ACTH can be further cleaved by the convertase PC2 (encoded by the gene *PCSK2*) into corticotropin-like intermediate peptide (CLIP) and α-MSH, the latter being involved in the synthesis of melanin pigments once bound to MC1R. The relationships between PC 3 with *GR* and *MR* suggest that the melanocortin system and the upstream regulators, the convertase PC2, is somehow implicated in the link between *GR* and *MR* and melanin colouration. This link might be the result of a trade-off mediated by the convertase PC2 between the production of ACTH and α-MSH (Fig. 5), which consequently influence the production of corticosterone and the expression of glucocorticoid receptors (*GR*, *MR*), and the expression of melanogenic genes. Even though we did not measure the corticosterone levels of nestlings in this study, this hypothesis is supported by previous findings in barn owls showing that melanin-based traits are associated with circulating corticosterone levels (Almasi et al., 2010) and to the

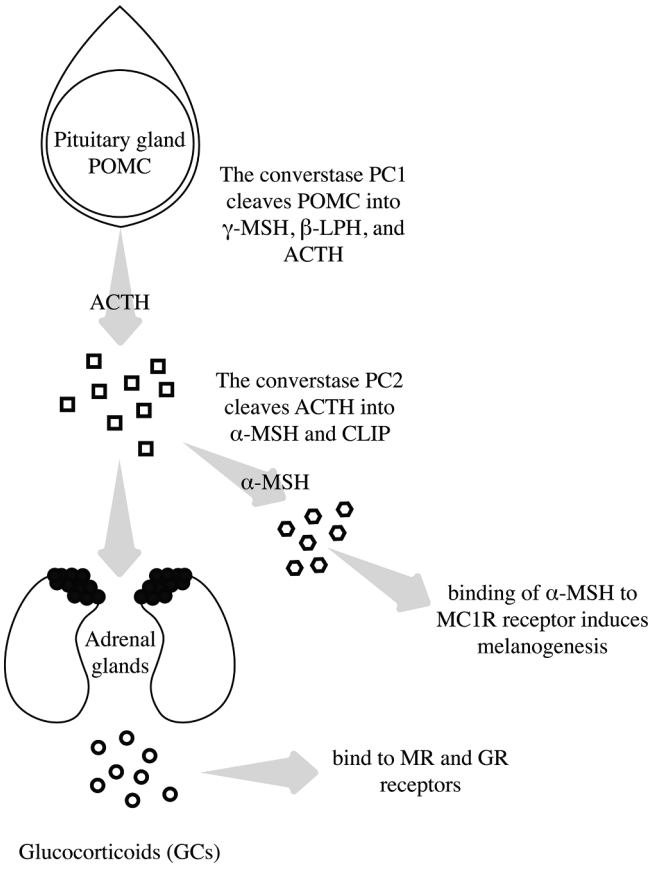

Fig 5. The link between melanin-based traits and the HPA axis. Both melanocortin hormones, α-MSH and ACTH, are derived from the cleavage of the POMC prohormone by the protein convertases PC 1 and PC 2 (respectively encoded by the gene *PCSK1/3* and *PCSK2*). In hair and feather follicles, binding of the melanocortin α-MSH to MC1R induces the synthesis of melanin pigments. Whereas, binding of the melanocortin ACTH to the MC2R expressed in the adrenal glands and the skin activates the downstream signalling for the production of corticosterone.

expression of *PSCK2* in our study (Table 1) and in older barn owl nestlings (San-Jose et al., 2017; Scriba et al., 2013). Finally, we cannot exclude that the expression of *GR* and *MR* are independent of the expression of *PCSK2* and that the relationship between those genes may occur through a pathway that is independent of the melanocortin system.

Overall, our study provides evidence for a negative association between the expression of receptors involved in the regulation of stress and melanin-based traits. We provide mixed evidence regarding the proximate causes driving such a link between melanin-based traits and stress-related endpoints. Moreover, although our results are in line with previous studies showing a link between melanin based-traits and parameters involved in stress regulation (*i.e.* corticosterone levels), we do not know how well the expression of *MR* and *GR* in feathers is representative of the expression of *MR* and *GR* in tissues that are more relevant to the regulation of stress (*e.g.* hippocampus, hypothalamus and amygdala). A previous study has shown that the abundance of GR and MR across tissues are correlated to some extent (Lattin et al., 2015) and although the correlations of MR and GR in the tissues tested were mostly weak (range of correlations between different tissues of house sparrows for GR [$r = 0.01–0.30$] and MR concentration [$r = 0.01–0.54$]), these results support the idea that GR and MR are similarly expressed throughout the body. Additional studies investigating across-tissues correlation in *GR* and *MR* expression in tissues involved in the regulation of the HPA axis, including metabolic (*i.e.* liver, kidney, subcutaneous fat and muscles) and brain tissues, will be necessary to help us draw a firm conclusion on the significance of negative associations between melanin-based traits and the expression of *GR* and *MR* in feathers.

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Author contributions

A.R. obtained funding. A.R.; P.B. and A.-L.D. conceived and designed the study; P.B. conducted fieldwork; P.B., A.-L.D. and C.S. conducted all the genetic analysis; P.B. conducted the statistical analyses; P.B and A.R. wrote the manuscript with important contributions of L.M.S.-J. and A.-L.D. All authors read and provided input on the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.113224.

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