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MC1R-dependent, melanin-based colour polymorphism is associated with cell-mediated response in the Eleonora's falcon

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Abstract

Colour polymorphism in vertebrates is usually under genetic control and may be associated with variation in physiological traits. The melanocortin 1 receptor (*Mc1r*) has been involved repeatedly in melanin-based pigmentation but it was thought to have few other physiological effects. However, recent pharmacological studies suggest that MC1R could regulate the aspects of immunity. We investigated whether variation at *Mc1r* underpins plumage colouration in the Eleonora's falcon. We also examined whether nestlings of the different morphs differed in their inflammatory response induced by phytohemagglutinin (PHA). Variation in colouration was due to a deletion of four amino acids at the *Mc1r* gene. Cellular immune response was morph specific. In males, but not in females, dark nestling mounted a lower PHA response than pale ones. Although correlative, our results raise the neglected possibility that MC1R has pleiotropic effects, suggesting a potential role of immune capacity and pathogen pressure on the maintenance of colour polymorphism in this species.

Introduction

In vertebrates, inter- and intraspecific variation in colouration often results from variation in the production of melanin pigments (Hill & McGraw, 2006). Although an environmental and genetic component can be found in the expression of melanin-based colouration in species where this trait varies continuously (Fitze & Richner, 2002; Horth, 2006; Jensen *et al.*, 2006; Fargallo *et al.*, 2007; but see Roulin *et al.*, 2010), the expression of discrete melanic morphs is usually genetically controlled with little effect of the environment (Roulin, 2004; but see Lepetz *et al.*, 2009). Thus, the widespread covariation between the degree of melanism and morphology, physiology, behaviour and reproductive parameters (Roulin, 2004) may be the outcome of pleiotropic effects of the key regulators of melanogenesis. For instance, the proopiomelanocortin gene (*POMC*) encodes for melanocortin peptide hormones (melanin-stimulating hormones α-, β-, γ-MSHs and the adrenocorticotropic hormone ACTH) that trigger the production of melanin pigments when binding to the melanocortin 1-receptor (MC1R) and other physiological and behavioural functions when binding to four other melanocortin receptors (MC2-5Rs) (Ducrest *et al.*, 2008).

In a number of vertebrates, the expression of alternative pale and melanic phenotypes is associated with mutations at the *Mc1r* or *extension* locus. Gain-of-function mutations responsible for constitutive active MC1Rs result in a higher production of eumelanin pigments and thus darker colouration in wild-type animals. On the contrary, loss-of-function mutations produce lighter melanin colouration (see reviews in Hoekstra, 2006 and Ducrest *et al.*, 2008). Apart from producing melanin pigments, *Mc1r* is commonly considered to induce few other physiological effects. As a consequence, the evolution and maintenance of MC1R-dependent colour morphs may often be the direct effect of natural selection exerted on colouration itself rather than being an indirect

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effect of selection acting on genetically correlated traits (Mundy, 2005). However, MC1R is not only expressed in the melanocytes of developing feather buds and hair follicles (Robbins *et al.*, 1993; Schiöth, 2001), but also in various cells of the immune system such as monocytes, macrophages (Taherzadeh *et al.*, 1999), neutrophils (Catania *et al.*, 1996), B lymphocytes, natural killer cells and a subset of cytotoxic and $CD8^+$ T cells (Neumann Andersen *et al.*, 2001; Loser *et al.*, 2010). This suggests that MC1R could have immunological consequences. Accordingly, the melanocortin α-MSH has potent and broad effects on inflammation, which are in part exerted when bound to MC1R (Luger *et al.*, 2003; Loser *et al.*, 2010). Activation of MC1R causes a collective reduction in the pro-inflammatory molecules (IL1, -2, -4, -6, -12, -13, TNF, NF-κB and prostaglandins) and an increase in the anti-inflammatory mediators involved in the inflammatory process (Catania *et al.*, 2004) and is critical for the induction of cytotoxicity in human and murine $CD8^+$ T cells (Loser *et al.*, 2010). Based on the hypothesis that MC1R is not only involved in the expression of melanin-based colouration but also in immunological processes, we derive the prediction that alternative colour morphs may differ in their cell-mediated response, i.e. pale morphs tending towards inflammatory types of immunity and dark morphs tending towards anti-inflammatory, in species in which variation in melanin-based colouration is due to mutations at *Mc1r*.

We evaluated this prediction in the Eleonora's falcon (*Falco eleonorae*) (Fig. 1), a species that exhibits a striking melanin-based colour polymorphism with individuals displaying a pale or dark morph with little variation within these two morphs. We first tested whether mutations at *Mc1r* are associated with the expression of a pale or dark morph and whether there is temporal variation in morph frequencies. In addition, we examined whether nestlings of the two morphs differed in cell-mediated response measured by the phytohemagglutinin (PHA)-induced skin-swelling test.

Fig. 1 A differently coloured Eleonora's Falcon (*Falco eleonorae*) breeding pair; the pale male (right) transferring a prey to the dark female (left). As can be seen, the difference in colouration between the two morphs may be mainly due to differential deposition in feathers of eumelanin pigments and to a lower extent of pheomelanin pigments.

Materials and methods

The study species

The Eleonora's falcon is a medium-sized migratory raptor that breeds colonially over the entire Mediterranean basin and winters in East Africa, Madagascar and other islands in the Indian Ocean (Ristow & Wink, 1995; Gschweng *et al.*, 2008). The bulk of the breeding population is concentrated in the Mediterranean Sea, and only a few colonies are found in the Atlantic Ocean. One to three (rarely four) eggs are laid on the ground from the beginning of July to mid-August (Walter, 1979; Cramp & Simmons, 1980). After 28–30 days of incubation, the first two chicks hatch within 36 h, whereas the third one hatches usually 2–3 days later (Wink & Ristow, 2000). Siblings may be either the same as different morphs, depending on the colour morph of the parents and the Mendelian inheritance of this trait (Wink *et al.*, 1978; Ristow *et al.*, 1998). Difference in colouration between morphs is apparently mainly due to eumelanin and, to a lower extent, to pheomelanin (Galván *et al.*, 2010).

Field procedure

Our study was carried out in the Alegranza Islet (10.5 km^2, 289 m a.s.l.), Canary Islands (27°37′N, 13°20′W), in 2007, 2008 and 2009. This colony consists of ca. 120 breeding pairs, which represent 45% of the pairs breeding in the Archipelago (Del Moral, 2008). For each nest, we observed the adults using a telescope to identify their morph and sex by behaviour and morphology: adult males have bright-golden eye-rings, cere and talons, whereas these traits are dull grey or greenish-yellow in adult females (Walter, 1979; Ristow *et al.*, 1998; authors' personal observation, see Fig. 1). L. G. and J.M G. independently reliably assigned 384 adults to the dark and pale colour morphs (see pictures in Ristow *et al.*, 1998). All 36 individuals captured in two different years were assigned to the same morph each time. In addition, colour morph assigned at the nestling stage in eight individuals was the same when recaptured at adulthood 1 or 2 years later. In nestlings, the colour morph was determined from the colour pattern of their undertail coverts, although this can only be achieved after these feathers are long enough around 25 days after hatching (see Ristow *et al.*, 2004). In those nests easily accessible by foot (63% of the population), we recorded clutch size (mean = 2.7, SD = 0.50), the number of hatchlings (mean = 2.26, SD = 0.69) and hatching date (mean 20.91 August, SD = 7.48) after measuring nestling wing length to estimate their age using the formula provided by Ristow & Wink (2004). All birds were weighed using a Pesola scale (± 5 g), and wing length was measured using

a 30-cm rule to the nearest mm. We collected 20 μL of blood from the brachial vein using 0.5-mL syringes from 72 adults and 394 nestlings (152 from 74 nests in 2007, 75 from 49 nests in 2008 and 167 from 77 nests in 2009, all of them between 15 and 29 days old). Blood samples were preserved in absolute ethanol until molecular sexing (Py *et al.*, 2006) and *Mc1r* sequencing. All birds were marked with a numbered aluminium ring and released after manipulation.

Genetic control of colour polymorphism

Genomic DNA was extracted from blood using the DNeasy Tissue kit (Qiagen, Hombrechtikon, Switzerland) and the Biosprint robot 96 (Qiagen). A 820-bp fragment of the MC1R gene was amplified using the following primers MSHR72 (5′-ATGCCAGTGAGGGCAACCA-3′) and MSHR9 (5′-CTGGCTCCGGAAGGCATAGAT-3′) (Mundy *et al.*, 2004). PCRs were performed in 50 μL contained 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 50 mM KCl, 20 mM Tris (pH 8.4), 250 nM of each primer, 0.2 U μL^{-1} of *Taq* DNA polymerase (Qiagen) and 12.5 ng genomic DNA with the following cycles: 95 °C for 5 min followed by 35 cycles at 94 °C for 45 s, 60 °C for 45 s and 72 °C for 45 s and then final extension at 72 °C for 5 min. The amplicons of 11 individuals of the two different morphs of *Falco eleonorae* were TA-cloned in pGEMT (Promega, Dübendorf, Switzerland) and plasmids sequenced in both directions in a 3130XL Genetic Analizer (Applied Biosystems, Zug, Switzerland) using Big Dye V 3.1 terminator chemistry. Sequences were aligned in CodonCode Aligner (CodonCode Corporations, Dedham, MA, USA). We then genotyped, through fragment length analysis, 554 individuals for the deletion using the primer MC1R177fw (GCCATCCTGAAGAACAGGAA) and the hexachlorofluorescein 5′end-labelled MC1R1542rev (CGGTGCTGGCCAGCCAGA) (94 °C for 5 min followed by 27 cycles at 94 °C for 30 s, 59 °C for 30 s, 72 °C for 30 s and then 72 °C for 5 min) on the genetic analyzer ABI3100 (Applied Biosystems).

Cell-mediated response

As a reliable surrogate of the individual general pro-inflammatory potential (Vinkler *et al.*, 2010), we carried out the phytohemagglutinin (PHA)-induced skin-swelling test (Smits *et al.*, 1999) in 88 nestlings from 41 nests in 2007 and in 42 nestlings from 28 nests in 2008. Ninety-six of them belonged to the pale morph (49 males, 44 females and 3 undetermined) and 34 to the dark morph (14 males and 20 females). When nestlings were 15–29 days old (median = 21.5 days), we injected subcutaneously in the left patagium 20 μL of the mitogen PHA-P (L8754; Sigma-Aldrich, Química SA, Madrid, Spain) dissolved in phosphate-buffered saline proportion 5 : 1 (Tella *et al.*, 2008). The thickness of the point of injection was reliably measured three times with a pressure-sensitive micrometre just prior injection and 24 h later (repeatability = 0.99, $F_{99,200} = 976.82$, $P < 0.001$). PHA response was calculated as the difference between the mean of the measures taken 24 h after PHA injection minus the mean of the measures taken just before PHA injection.

Statistical procedure

We tested whether there was temporal variation in morph frequencies using contingency tables. We investigated whether variation in PHA response was associated with plumage colouration using generalized linear mixed models (GLMMs) with normal error distribution and identity link function. PHA response was included as a dependent variable, and we added year and nest identity nested within year as random factors; their contribution to explain variation in PHA response was estimated by means of a restricted maximum-likelihood (REML) procedure (JMP 8 software; SAS Institute Inc., Cary, NC). Nestling sex and morph as well as the morph of the father and mother and rank in the within-brood hatching order were included in the model as fixed terms. Nestling body mass, hatching date and brood size were included as covariates. The interaction between nestling sex and morph was also included. We also tested for interactions between random and fixed terms, but they were not significant (results not shown). In addition, these interactions could not be included at the same time in the models because it is not possible to have combinations of all sexes and morphs in 2–3 nestling broods, so we decided to perform a simplified model. We followed a stepwise removal procedure, which resulted in a final model where only significant effects (< 0.05) were retained.

To assess the relationship between nestling body mass and colour morph, we used a similar GLMM model with body mass as the dependent variable and the same factors and interactions. In addition, we included age in days and age^2 as covariates, as well as the interactions age*sex and age*morph. All tests were two tailed, and *P*-values smaller than 0.05 were considered significant.

Results

Mc1r genotype–phenotype association

A 12 base pair in-frame deletion (*Mc1r Δ12*) corresponding to the following amino acids, methionine, aspartic acid, asparagine and valine at positions 114–117, were found in dark individuals, both in heterozygosis (dark heterozygous individuals) and in homozygosis (dark homozygous), whereas pale individuals were homozygous wild type (Fig. 2). First, we confirmed that all 72 adult birds assigned visually to dark or pale morph were, indeed, correctly assigned on the basis of *Mc1r* allelic variants. We genotyped 554 individuals (adults and nestlings), of which 427 (77.1%) were homozygous for the wild-type (pale) *Mc1r* allele, 123 individuals (22.2%) were dark heterozygous for the *Mc1r Δ12* variant and four individuals (0.7%) were

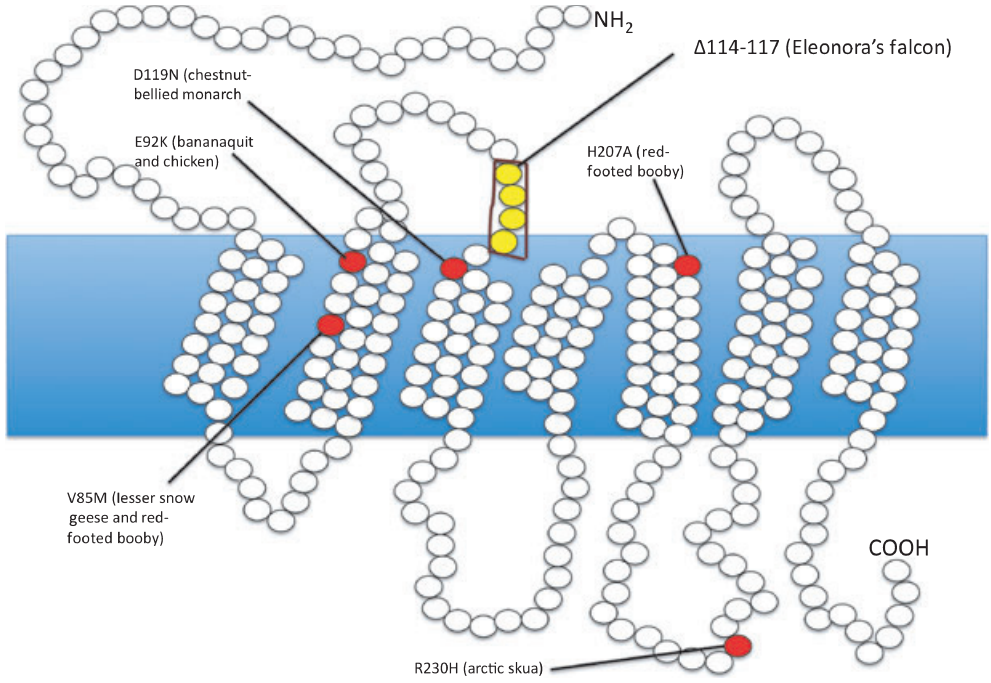

Fig. 2 Predicted secondary structure of *Mc1r* showing several mutations associated with melanism in birds. Each circle represents an amino acid residue. Point mutations are identified by their position in the molecule and the amino acid substitution related with the phenotype. Data from Theron *et al.*, 2001; Mundy *et al.*, 2004; Baião *et al.*, 2007; Uy *et al.*, 2009. Modified after Mundy, 2005.

dark homozygous for the deletion. Because dark and pale morphs segregate as a Mendelian trait (Wink *et al.*, 1978), the dark allele 'D' dominating over the pale one 'd', and the morph of the two parents was known in most cases, we can thus determine whether a particular allele of the nestling comes from the father or mother. Therefore, nestling genotypes were ascertained in 94.2% of cases, except for those dark heterozygous nestlings whose parents were dark (2%). We categorized genotypes as follows: (d^fd^m pale homozygous, pale alleles inherited from both parents), (d^fD^m dark heterozygous, pale allele from the father and dark allele from the mother), (D^fd^m dark heterozygous, dark allele from the father and pale allele from the mother) and (D^fD^m dark homozygous, dark alleles from both parents).

Morph frequency

During the study period, the frequency of dark falcons was lower than the frequency of pale birds for both adults and nestlings (Table 1). Colour polymorphism was not sexually dimorphic with the pale morph being as frequent in males as in females in adults (chi-square test: $\chi^2 = 0.01$, d.f. = 1, $P = 0.92$) and in nestlings ($\chi^2 = 1.20$, d.f. = 1, $P = 0.30$; Table 1). There was also no significant variation in morph frequencies among years in both adults ($\chi^2 = 0.32$, d.f. = 2, $P = 0.85$) and nestlings ($\chi^2 = 0.82$, d.f. = 2, $P = 0.66$). However, in nestlings, the pale morph was more frequent in males than in females in 2007 (87.2% vs. 65.8%; $\chi^2 = 9.72$, d.f. = 1, $P = 0.002$) but not in 2008 ($\chi^2 = 0.84$, d.f. = 1, $P = 0.42$) and 2009 ($\chi^2 = 0.19$, d.f. = 1, $P = 0.71$).

Table 1 Morph frequencies of breeding adult (a) and nestling (b) Eleonora's falcons from the Canary Islands between 2007 and 2009. Morph frequencies in nestlings were based on the analysis of MC1R

	Males		Females		Total			%	
	Pale	Dark	Pale	Dark	Pale	Dark	Sum	Pale	Dark
(a) Adults									
2007	89	27	100	16	189	43	232	81.46	18.53
2008	80	17	74	23	154	40	194	79.38	20.62
2009	92	19	88	23	180	42	222	81.08	18.92
Total	261	63	262	62	523	125	648		
(b) Nestlings									
2007	68	10	48	25	116	35	151	76.8	23.2
2008	31	10	19	10	50	20	70	71.4	28.6
2009	59	20	66	19	125	39	164	76.2	23.8
Total	158	40	133	54	291	94	385		

Nestling morph, cell-mediated response and body mass

Pale nestlings mounted a stronger PHA response than dark ones (Table 2). In addition, females mounted a stronger PHA response than males (Table 2). The

Table 2 Results of the mixed models explaining variability in cell-mediated response against PHA and body mass in relation to colour morph in nestling Eleonora's falcons. Significant results ($P < 0.05$) are shown in bold.

	Nestling PHA response			Nestling body mass		
	F	d.f.	*P*	*F*	d.f.	*P*
Fixed effects						
Nestling sex	6.72	1, 115.7	**0.01**	54.37	1, 221.5	**< 0.0001**
Nestling morph	4.11	1, 97.32	**0.04**	0.18	1, 284	0.67
Father's morph	6.14	1, 65.18	**0.02**	0.60	1, 178.6	0.44
Mother's morph	0.10	1, 63.09	0.75	0.19	1, 178.6	0.66
Nestling sex*morph	4.25	1, 118.9	**0.04**	0.03	1, 213.9	0.87
Nestling body mass	6.19	1, 101.7	**0.01**			
Rank within-brood	0.59	2, 81.25	0.55	3.89	2, 232.3	**0.02**
Hatching date	1.18	1, 63.71	0.28			
Brood size	2.25	1, 20.59	0.15	0.74	1, 218.9	0.39
Age				181.90	1, 345.8	**< 0.0001**
Age^2				24.21	1, 341.6	**< 0.0001**

interaction between nestling sex and morph was significant (Table 2), because in males pale nestlings mounted a stronger PHA response than dark individuals (test slices $F_{1,109.21} = 7.63$, $P = 0.007$), whereas in females pale and dark nestlings did not differ in PHA response (test slices $F_{1,112.25} = 0.08$, $P = 0.78$; Fig. 3). In the same model, the colour morph of the father was also significant: nestlings sired by dark fathers mounted a stronger PHA response than when sired by pale fathers (Table 2). Nestling body mass was positively related to the ability to mount a PHA response (Table 2).

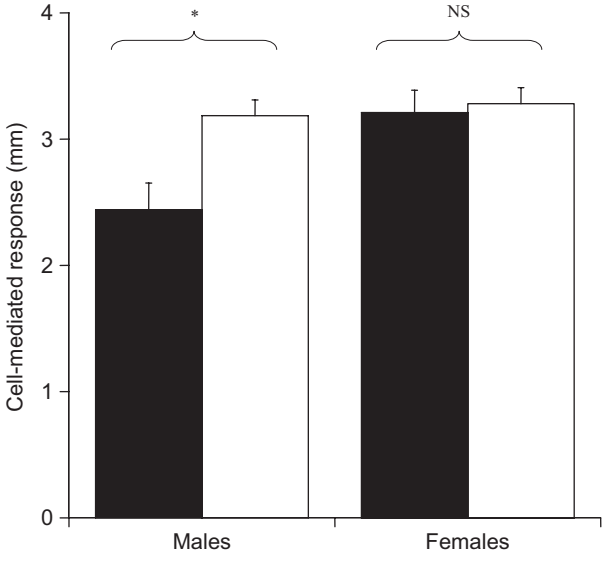

Fig. 3 Cell-mediated response against an injection of phytohemagglutinin (PHA) in pale (empty bars) and dark (filled bars) male and female Eleonora's falcon nestlings. Data are least square means from the model ± SE that indicate the extent to which the skin swelled (in mm) 24 h after a subcutaneous injection of PHA. *The PHA response was stronger in pale than dark males and NS that the difference was not significant in females.

Because both the morph of nestlings and that of their fathers, but not of their mothers, were related to nestling PHA response, but in opposite ways, we attempted to examine whether the effect of paternal morph was potentially genetic. If this is the case, we predict that *Mc1r* alleles passed on by the father have a different effect on PHA response than *Mc1r* alleles passed on by the mother. This was, however, not the case because nestling genotype (which indicates from which parent each *Mc1r* allele is inherited) was neither associated with PHA response alone nor in interaction with nestling sex (mixed model ANOVA; sex: $F_{1,106.7} = 5.83$, $P = 0.02$; genotype: $F_{2,91.11} = 0.67$, $P = 0.52$; interaction: $F_{2,113.1} = 1.07$, $P = 0.35$). In *a posteriori* planed comparison, we found that PHA response did not differ between dark heterozygous d^fD^m and dark heterozygous D^fd^m nestlings ($F_{1,108.70} = 0.88$, $P = 0.35$). This result also demonstrated that the effects of father morph and nestling morph were independent. Nestling body mass depended only on nestling sex and age and was not associated with nestling and parents colour morph (Table 2).

Discussion

Although at least 127 loci are known to affect pigmentation in vertebrates (Bennett & Lamoreux, 2003), the *Mc1r* gene has been found involved repeatedly to melanin-based pigmentation diversity in natural populations of several fish, birds, reptiles and mammals (Hoekstra, 2006; Ducrest *et al.*, 2008). There are some examples where intraspecific variation in the degree of melanism affecting the entire plumage or pelage shows a clear association with *Mc1r* allelic variants: jaguar *Panthera onca* and jaguarundi *Herpailurus yaguarondi* (Eizirik *et al.*, 2003), lesser snow goose *Anser c. caerulescens* and Artic skua *Stercorarius parasiticus* (Mundy *et al.*, 2004), banana-quit *Coereba flaveola* (Theron *et al.*, 2001) and red-footed booby *Sula sula* (Baião *et al.*, 2007). However, in a similar number of cases of partial melanism, *Mc1r* does not account for colour variation (e.g. blue-crowned manakin *Lepidothrix coronata* (Cheviron *et al.*, 2006), grey wolves *Canis lupus* (Anderson *et al.*, 2009), common frog *Rana temporaria* (Herczeg *et al.*, 2010), red-tailed hawk *Buteo jamaicensis* (Hull *et al.*, 2010) or, only partially, e.g. beach mouse *Peromyscus polionotus* (Hoekstra *et al.*, 2006)).

The discrete variation in melanin-based plumage colouration in Eleonora's falcons is perfectly explained by an in-frame deletion of four amino acids at the *Mc1r* gene. This simple phenotype–genotype association may have important implications because the *Mc1r*, unlike other genes involved in the production of melanin pigments, such as the *POMC* gene, is thought to have

few pleiotropic effects (Mundy, 2005). Indeed, the apparent lack of negative pleiotropic effects has been invoked to explain why this gene is so often associated with colour polymorphism in different taxa with colouration evolving as a direct response of selection being exerted on colouration itself (Mundy, 2005). However, there is a growing understanding of MC1R function beyond colouration, which has arisen from pharmacological studies on constitutive knock-out mice and *in vitro* studies of the biochemical function of MC1R. For instance, it has been shown that MC1R has a negative effect on different forms of inflammation and a positive effect on resistance to oxidative stress induced by UV radiation and apoptosis as well as nociception (reviewed in Luger *et al.*, 2003; Ducrest *et al.*, 2008; Loser *et al.*, 2010).

As in the Eleonora's falcon variation in colouration is due to MC1R, any phenotypic correlation between colouration and other phenotypic traits such as cell-mediated response may be also due to the variation at the *Mc1r*. The immune system is influenced by conditions experienced during development, which result from the combination of origin-related (genetic and/or maternal) and environmental/parental influences (Falconer, 1989). To fully disentangle these factors associated with plumage traits on immune response, cross-fostering experiments would be helpful. Our correlative approach is, nevertheless, valuable because it is the first study that has attempted to test whether there is a link between phenotypic traits and MC1R-based colouration in a natural population.

In the Eleonora's falcon, the ability to mount an inflammatory response was morph and sex specific. Although there is a large debate on the immune components involved in the PHA response and how the results may be interpreted (Martin *et al.*, 2006; Tella *et al.*, 2008; Vinkler *et al.*, 2010), it seems clear that PHA-induced swelling implies the participation of both innate (granulocytes, inflammation) and adaptive (T cells) components of the immune system (Palacios *et al.*, 2009; Vinkler *et al.*, 2010). We thus propose here that the differences in cell-mediated response between dark and pale nestlings may be due to pleiotropic effects of Mc1r. A number of pharmacological studies have established that MC1R and the agonist α-MSH have broad effects on the immune system (Catania & Lipton, 1993; Catania *et al.*, 1996; Lipton & Catania, 1997; Loser *et al.*, 2010), suggesting that cell-mediated immunity may differ between different *Mc1r* genotypes. As would be predicted by gain-of-function mutations, the dark morph is exhibiting a reduced inflammatory type of response. Alternatively, melanin-based colouration may be associated with a resource allocation strategy in key fitness components, such as immunity (Roulin *et al.*, 2000; Galeotti & Sacchi, 2003; Gasparini *et al.*, 2009a; Piault *et al.*, 2009). Therefore, dark nestlings would allocate fewer resources towards the cell-mediated response than pale individuals. In line with this, antioxidant (e.g. carotenoids Blount *et al.*, 2003; McGraw & Ardia, 2007; but see Costantini & Møller, 2008) allocation to redox homoeostasis may render immune system temporally deficient. Dark nestlings faced with devoting carotenoids to cope with oxidative stress may be consequently limited in their ability to use carotenoids as immunomodulators, especially during feathers growth, when the levels of intracellular antioxidants (glutathione) should be low enough to synthesize eumelanin (Galván *et al.*, 2010). To date, there are only two studies that have specifically tested for the effects of colour morph on PHA response and both studying continuously varying melanin-based colour traits. Our findings contrast markedly with those of Jacquin *et al.* (2011), which found that darker melanic Feral Pigeons (*Columba livia*) had a greater PHA response than paler ones and suggest that this difference may be explained by pleiotropic effects of genes coding for melanin pigmentation on the immune system. These contrasting results may be due, at least in part, to genes other than Mc1r are responsible for the continuous variation in melanin-based colouration in this species. In addition, Gasparini *et al.* (2009a) found that PHA response of dark reddish Tawny Owls (*Strix aluco*) was enhanced after a humoral immune challenge, whereas it was reduced in pale reddish owls. This pattern, however, was not found among control-unvaccinated individuals. But in the Tawny Owl, variation in colouration is mainly due to pheomelanin, accounting for 68% of the total variance in plumage colouration (Gasparini *et al.*, 2009b), and thus, one would expect exactly the opposite pattern than for eumelanin: if pale eumelanic individuals mount stronger PHA response than dark eumelanic individuals, dark pheomelanic should also mount stronger PHA response than pale pheomelanic individuals. As in the case of Feral Pigeons, the gene responsible for colour polymorphism in Tawny Owls has not been identified yet. Moreover, it is possible that different mutations at Mc1r have differential effects on immunity.

An outstanding question is why the relationship between melanin-based colouration and inflammatory response was found only in male nestlings. Weaker cell-mediated response in male than in female nestlings has been observed in other species (e.g. Fargallo *et al.*, 2002; Tschirren *et al.*, 2003). The mechanism behind this sex-specific variation has not been identified, although it has been associated with differential exposure to pathogens, competitive abilities, or with intrinsic morphological or physiological differences already present at early stage of life, such as body size (Fargallo *et al.*, 2002), sex hormones (Duffy *et al.*, 2000; Mougeot *et al.*, 2004) or other biochemical compounds that drive the expression of sexual traits (von Schantz *et al.*, 1999; Tschirren *et al.*, 2003). In addition, male and female nestlings may show different priorities for developing potentially competing vital functions; if food is limited, male nestlings may prioritize growth over the development of the immune

function (Fargallo *et al.*, 2002; Dubiec *et al.*, 2006). Interestingly, the only deviation found in the frequencies of morphs was a reduced occurrence of dark morph among males in one of the 3 years of the study. Unfortunately, no data are available to test the causes of these differences but we speculate that differences in immunity or individual quality may drive morph-specific sex differences in nestling survival.

The colour morph of the father, but not of the mother, was associated with offspring PHA response; nestlings sired by dark fathers mounted a stronger PHA response than when sired by pale fathers. However, the relationship with the paternal colour is not genetic, as the magnitude of nestling PHA response was similar when the dark allele was inherited from the father and mother. Therefore, the relationship between father genotype and offspring PHA response may be environmentally mediated through rearing conditions. Environmental components during growth, such as diet quality, brood size or parental effort, may decisively influence cell-mediated response (Tella *et al.*, 2000; Pitala *et al.*, 2007), because it is known to be correlated with body mass and dietary protein intake (Alonso-Álvarez & Tella, 2001). As male Eleonora's falcons provide most of the prey to the nest (Walter, 1979; L. Gangoso and J.M. Grande, personal observation), the effect of the colour of the father could be explained if, for instance, dark adults delivered more prey than pale adults or different prey species. In our study population, the productivity of nests sired by dark males was significantly higher than that of nests sired by pale males in 2009 (2.33 ± 0.22 vs. 1.75 ± 0.11, $F_{1,92}$ = 5.54, P = 0.02), but not in 2008 (0.79 ± 0.20 vs. 0.71 ± 0.10, $F_{1,93}$ = 0.12, P = 0.73) and 2007 (1.68 ± 0.21 vs. 1.59 ± 0.12, $F_{1,99}$ = 0.14, P = 0.71).

The long-term maintenance of polymorphism implies the absence of directional selection favouring one morph over the other or a selective balance between the alternative genotypes (Fisher, 1930) with colour variants representing equally fit strategies to cope with fluctuating environmental factors (Galeotti & Sacchi, 2003; Roulin *et al.*, 2008). This suggests that different phenotypes may be adapted to specific environmental conditions and thus occupy different habitats where they find the optimal environment (Ravigné *et al.*, 2004) or, alternatively, may occupy the same habitat but perform differently over time if the specific conditions vary temporarily (Kassen, 2002). Consequently, our results suggest that temporal shifts in resources to devote to the immune system and changes in pathogen pressure may affect the relative equilibrium between both colour morphs. Whatever the scenario, displaying different plumage colouration may advertise morph-specific phenotypic attributes. The potential selective value of dark vs. pale plumages may include concealment during hunting, resistance to plumage abrasion and/or indirect effects due to pleiotropy, such as a differential cell-mediated response. Clearly, more data are needed to examine whether selection may be acting on colouration itself or on genetically correlated traits such as immunity. To understand the maintenance of colour polymorphism, we need to focus not only on the direct consequences of colour polymorphism for individual fitness but also on the pleiotropic effects associated with the expression of genes involved in melanogenesis.

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