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BEHAVIORAL ECOLOGY - ORIGINAL RESEARCH

Morph-specific genetic and environmental variation in innate and acquired immune response in a color polymorphic raptor

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Abstract Genetic color polymorphism is widespread in nature. There is an increasing interest in understanding the adaptive value of heritable color variation and trade-off resolution by differently colored individuals. Melanin-based pigmentation is often associated with variation in many different life history traits. These associations have recently been suggested to be the outcome of pleiotropic effects of the melanocortin system. Although pharmacological research supports that *MC1R,* a gene with a major role in vertebrate pigmentation, has important immunomodulatory effects, evidence regarding pleiotropy at *MC1R* in natural populations is still under debate. We experimentally assessed whether *MC1R*-based pigmentation covaries with both inflammatory and humoral immune responses in the color polymorphic Eleonora's falcon. By means of a cross-fostering experiment, we disentangled potential genetic effects from environmental effects on the covariation between coloration and immunity. Variation in both immune responses was primarily due to genetic factors via the nestlings' *MC1R*-related color genotype/phenotype, although environmental effects via the color morph of the foster father also had an influence. Overall, dark nestlings had lower immune responses than pale ones. The effect of the color morph of the foster father was also high, but in the opposite direction, and nestlings raised by dark eumelanic foster fathers had higher immune responses than those raised by pale foster fathers. Although we cannot completely discard alternative explanations, our results suggest that *MC1R* might influence immunity in this species. Morph-specific variation in immunity as well as pathogen pressure may therefore contribute to the long-term maintenance of genetic color polymorphism in natural populations.

Keywords Genetic color polymorphism · Immune response · Melanocortin 1 receptor · Newcastle disease virus vaccine · Phytohemagglutinin skin-swelling test

Introduction

Although heritable melanin-based color polymorphisms are widespread in vertebrates, the processes governing the evolution, maintenance, and adaptive function of different morphs in the wild are rarely well understood. Pigmentation is often associated with variation in an extraordinarily diverse number of life history features (Roulin and Ducrest 2011). These widespread associations have been recently suggested to result from the pleiotropic effects of the main regulators of melanogenesis, at least in those vertebrate species for which inter-individual variation in melanin-based coloration is due to the melanocortin system (Ducrest et al. 2008). This system comprises the pro-opiomelanocortin gene (*POMC*), which encodes the melanocortins

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family: the peptides α-, β-, and γ-melanocyte-stimulating hormones (MSH) and the adrenocorticotrophic hormone (ACTH). All of these bind to five melanocortin receptors (MC1-5Rs) and their antagonists, agouti-signaling (ASIP) and agouti-related proteins (Brzoska et al. 2008; Wikberg and Mutulis 2008). The five MC-Rs are seven-transmembrane, G-protein-coupled receptors, which show tissue-specific expression levels and different affinities to the different melanocortin peptides and their antagonists. This system is particularly interesting because it is well conserved throughout vertebrates and so studies performed on any particular species will be of general interest.

Functional mutations at the *MC1R* gene often affect fur, feather, and skin color in many taxa (Theron et al. 2001; Eizirik et al. 2003; Mundy et al. 2004; Baião et al. 2007; Lin and Fisher 2007; Gross et al. 2009; Rosenblum et al. 2010; but see Roulin and Ducrest 2013) thereby providing support for the pivotal role of this locus in the evolution of plumage, pelage, and skin pigmentation. In homeothermic animals, the binding of α-MSH to its homonym receptor MC1R in melanocytes elicits the synthesis of eumelanin (producing brown-to-black pigmentation). In contrast, the binding of ASIP results in the synthesis of pheomelanin (producing yellow-to-reddish-brown pigmentation) (Lin and Fisher 2007). Unlike other genes such as *POMC*, *MC1R* is assumed to have no or few pleiotropic effects (Mundy 2005; Ducrest et al. 2008). However, there is now robust evidence to suggest that *MC1R* is constitutively expressed on non-melanocytic cells such as those of the immune system, thereby revealing a broad range of effects of melanocortin peptides on immunity (Becher et al. 1999; Neumann Andersen et al. 2001; Loser et al. 2010). The peptide α-MSH also interacts with immune cells and provokes antimicrobial, anti-inflammatory, and immunomodulatory activities (Catania 2007; Rennalls et al. 2010). The overall effect of the binding of α-MSH to MC1R or MC3R, or to MC5R appears to be anti-inflammatory. This effect may occur via the down-regulation of various pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α, IL-2, IFN-γ, IL-4, and IL-13, as well as the up-regulation of the production of immunosuppressive IL-10 in peripheral blood (Neumann Andersen et al. 2001; Maaser et al. 2006; Catania 2007; Ahmed et al. 2013). Moreover, the finding that *MC1R* is expressed not only on monocytes/macrophages, but also on dendritic cells and lymphocytes with antigen-presenting (B-lymphocytes) and cytotoxic (natural killer and a subset of T-cells) functions (Neumann Andersen et al. 2001; Rennalls et al. 2010) strongly suggests that the broad immunomodulatory effects of the melanocortin peptides are also mediated by lymphocyte-specific processes. Indeed, recent studies have shown that the binding of α-MSH to—probably—MC1R in dendritic cells impairs their functional activity to stimulate T-lymphocytes (Rennalls et al. 2010).

On the basis of the key regulatory role of the melanocortin system in many biological and physiological functions, numerous studies have reported covariations between melanin-based coloration and, for instance, reproductive strategies (Roulin et al. 2003), the ability to cope with variation in food supply and parasitism (Piault et al. 2009), resistance to oxidative stress (Galván et al. 2010; Roulin et al. 2011), and the regulation of stress responses (Almasi et al. 2010). Consequently, we can hypothesize that in species in which mutations at the *MC1R* are involved in the production of alternative melanin-based color morphs, different morphs will differ not only in coloration but also in the expression of some phenotypic traits such as those that are related to immunity. In this regard, Gangoso et al. (2011) reported correlative evidence of covariation between melanin-based coloration and the inflammatory cell-mediated response in wild Eleonora's falcon (*Falco eleonorae*) nestlings. This species exhibits two discrete melanin-based color morphs that are genetically determined by variation at the *MC1R* gene. Dark individuals are homozygous or heterozygous for a variant that leads to the deletion of four amino acids at position 114–117 in the extracellular domain of MC1R, which appears to be a constitutive form of the MC1R (Gangoso et al. 2011). To the best of our knowledge, this study suggested for the first time the possibility of pleiotropy at *MC1R* in a free-living animal and this hypothesis thus requires thorough testing. Our aim here was to disentangle potential genetic (nestling and/or biological parents genotype) from environmental (e.g., parental workload) effects on the covariation between coloration and immunity. We also assessed whether this covariation can be extended to the two main arms of the immune system: the non-specific innate (inflammatory) and acquired (humoral) immune responses (Roitt et al. 1998). We therefore conducted a cross-fostering experiment in which entire clutches were swapped between randomly chosen nests. There is an open debate about the existence of trade-offs between inflammatory and humoral immune responses (Lazzaro and Little 2009) that may be due to different reasons, such as energetic or nutritional factors (Hõrak et al. 2006; Hawley et al. 2007) or the parasite-antigen identity (Adamo 2004; Jacquin et al. 2012), Other authors, however, found weak evidence of these potential trade-offs between the two main immune branches (Ardia 2005; Matson et al. 2006). Nonetheless, to avoid any potential cross-regulation effect between these interacting branches of the immune system, we first challenged the inflammatory response via a phytohemagglutinin (PHA) skin-swelling test and then challenged the acquired humoral response to vaccination against Newcastle disease virus (NDV) in Eleonora's falcon nestlings. Given that there is a direct link between nestling color morph and *MC1R* mutations (Gangoso et al. 2011), coloration is a perfect surrogate of

MC1R. Based on the theoretical background, i.e., the role of *MC1R* in regulating both coloration and the inflammatory response (anti-inflammatory effect), we predicted that, compared to pale nestlings, dark eumelanic nestlings would have a reduced cell-mediated inflammatory response. The prediction regarding the humoral response is, however, not quite so straightforward. To date, no studies have ever reported an unequivocal relationship between melanocortin peptides/receptors and antibody production. Previous experimental evidence support an increase in lymphocyte proliferation in dark eumelanic individuals (Roulin et al. 2000). Nevertheless, this evidence refers to α-MSH bound to MC5R and not MC1R (Buggy 1998; Taylor and Namba 2001). Even so, recent studies on humans further suggest an immunosuppressive effect through the activation and binding of α-MSH to MC1R in dendritic cells (Rennalls et al. 2010), as well as α-MSH-dependent suppression of antigen-induced proliferation of T-lymphocytes via MC1R on monocytes/macrophages and B-lymphocytes (Cooper et al. 2005). Therefore, current knowledge does not allow us to make a direct prediction regarding the extent of the humoral response in differently colored Eleonora's falcon nestlings.

Materials and methods

Field procedure

Our study was carried out in 2010 on the islet of Alegranza (10.5 km^2, 289 m a.s.l.) in the Canary Islands (27º37′N, 13º20′W). The falcon colony consists of approximately 120 breeding pairs, 45 % of the total pairs breeding on these islands (Del Moral 2008); in all, 83 nests were intensively monitored. For each nest we recorded the morph of both parents using a spotting scope. Birds were sexed according to behavior and morphology: in adult males eye-rings, ceres and talons are bright golden, while in adult females these traits are dull grey or greenish-yellow (Walter 1979; Ristow et al. 1998). Color polymorphism is not a sexually dimorphic trait in this species with both morphs being as frequent in males as in females (Gangoso et al. 2011). Although there is a third color morph, the dark homozygous, its frequency is extremely low [0.7 % in nestlings (Gangoso et al. 2011) and less than 2 % in breeding adults], and thus, we grouped dark heterozygous and dark homozygous nestlings as "dark morph" in further analyses. Given the Mendelian inheritance of this trait (Wink et al. 1978; Gangoso et al. 2011), siblings may be either the same or different morphs, depending on the color morph of the parents. The incubation period lasts 30 ± 2 days and nestlings fledge at 35–40 days (Wink and Ristow 2000). Once the color morph of the individuals in the breeding pairs was confirmed, we carried out a cross-fostering experiment between randomly chosen nests. To avoid any potential adverse reaction from the birds, during the last week of incubation we exchanged eggs from closely located nests (mean distance = 200.38 m ± 225.16 SD) with the same number of eggs and similar laying date (±2 days). The resulting proportion of adult color morphs included in the experiment (26.56 % of dark adults vs. 73.43 % of pale adults) was representative of the frequencies found in our study population (see above). No dark homozygous adults could be included in the cross-fostering experiment. In all, we swapped 88 eggs from complete clutches from 32 nests (Online Resource Table S1). In this way, foster parents raised all nestlings and each nest contained nestlings of the same origin. Extra-pair paternity could have important implications for inferring the influence of parental genotype. However, although Eleonora's falcon breeds colonially and mates are often separated when away for hunting, extra-pair paternity was found to be almost inexistent in this species. By conducting multilocus DNA fingerprinting analyses in 60 fledglings from 17 families, Wink and Ristow (2000) found that social mothers and fathers were indeed the genetic parents in all cases. We can thus assume that all nestlings had the same genetic origin. Although there was no disruption to the female's incubation behavior, eggs failed to hatch in two nests (6.25 % of hatching failure). This failure rate was similar to that found in non-cross-fostered nests (7.84 %, $n = 51$).

After hatching, we recorded the number of nestlings (mean = 2.43 ± 0.61 SD). All birds were weighed using a Pesola scale (±5 g) and wing lengths were measured to the nearest millimeter. Wing length was used to estimate nestling ages (±1 day) as per Ristow and Wink (2004). A small blood sample from the brachial vein was collected and stored in absolute ethanol until molecular sexing (Py et al. 2006) and *MC1R* genotyping (see below). All birds were marked with a numbered aluminum ring and released after manipulation.

Ethics statement

Fieldwork was conducted in accordance with local legislation. Experimental research complied with institutional and national guidelines and has been approved by the institute ethics committee. Corresponding permission was granted by the Spanish Regional Administrations, the Dirección General del Medio Natural, Consejería de Medio Ambiente y Ordenación Territorial, Gobierno de Canarias (permit no. 316/2010, administrative file no. 2010/0680) and the Consejería de Medio Ambiente, Caza y Patrimonio, Cabildo de Lanzarote (permit no. ES-000694/2010).

Genetic control of color polymorphism

Genomic DNA was extracted from blood using the DNeasy Tissue Kit (Qiagen, Hombrechtikon, Switzerland) and the Biosprint robot 96 (Qiagen). We genotyped through fragment length analysis all individuals using the primers MC1R177fw (GCCATCCTGAAGAACAGGAA) and the hexachlorofluorescein 5′end-labeled MC1R1542rev (CGGTGCTGGCCAGCCAGA) (see details in Gangoso et al. 2011).

Immune challenges

Despite the controversy regarding the immune components involved in the PHA-induced swelling response (Martin et al. 2006; Vinkler et al. 2010), it seems clear that the PHA response involves the participation of both innate (granulocytes, inflammation) and adaptive (T-cells) components of the immune system (Palacios et al. 2009; Vinkler et al. 2010). Therefore, as a reliable surrogate of the general pro-inflammatory potential (Vinkler et al. 2010), we carried out the PHA-induced skin-swelling test (Smits et al. 1999) on 67 nestlings from 30 nests. When nestlings were 11–30 days old (mean = 23.74 days ± 3.81 SD), we injected subcutaneously 20 µL of the mitogen PHA-P (L8754 Sigma-Aldrich) dissolved in phosphate buffered saline, proportion 5 mg:1 mL, in the left patagium. Following the recommendation of Smits et al. (1999), we did not inject the right patagium with phosphate buffered saline solution as a negative control. The thickness of the patagium at the point of injection was measured successively three times with a pressure sensitive micrometer just prior to injection and then 24 h later (repeatability = 0.99, $F_{66,132} = 204.83$, $p < 0.001$). The PHA response was calculated as the difference between the mean of the measures taken 24 h after the PHA injection minus the mean of the measures taken just before the PHA injection.

To assess the humoral immune response, we vaccinated the same 67 nestlings against NDV and measured the NDV-specific antibody response to the vaccination. After measuring the skin-swelling response on the second day, we injected nestlings subcutaneously at the dorsal base of the neck with a solution that contained 0.1 mL of the commercial inactivated NDV vaccine (Hipraviar BPL2; HIPRA, Spain), plus 0.1 mL of the same oil adjuvant used in the commercial vaccine (total volume injected = 0.2 mL). Ten days after vaccination, nests were revisited to collect a second blood sample (1 mL) to quantify the specific antibody response to vaccination. We also weighed and measured the wing length of all nestlings again during this second visit. Blood samples were transferred into sterile tubes, kept cold for a few hours, and then centrifuged for 15 min at 3000 r.p.m. Serum was stored in liquid nitrogen until antibody titers were determined. We measured the anti-NDV antibody concentration in blood sera using the hemagglutination inhibition test (HI test), which is considered to be the reference test for the detection of NDV antibodies (OIE 2000). The humoral response to NDV can be measured from the first week after the challenge with an HI test (Broggi et al. 2013). Although antibody production increases in the fourth week, measuring individuals at this time is complicated by the fact that by then most can fly. The concentration of NDV antibodies (hereafter, "HI response") was determined by sequential dilution of the test sera from 1/2 to 1/640 and the addition of 4HA units of antigen HIPRAVIAR-CLON. E. Newcastle, clone CL/79, with VLDIA053 HAR-NDL, NDV strain La Sota and VLDIA030 SPF-CH-Chicken negative as controls.

Statistical procedure

We used structural equation models (SEM) to fully disentangle direct and indirect effects of genetic and environmental factors in explaining covariation between melanin-based coloration and the two interacting arms of the immune system. SEM encompass a wide range of multivariate statistical techniques, such as regression, factor analysis, and path analysis (Grace 2006), and allow for a statistical evaluation of multivariate hypotheses that contain multiple causal pathways including direct and indirect effects (mediated through correlations with other variables) and multiple dependent variables. These hypotheses can be concisely presented in a path diagram (Fig. 1), where arrows indicate the causal relationships between the different variables. We performed SEM in R software version 3.0.2 (R Core Team 2013) with the lavaan package (Rosseel 2012). The modeling process in SEM is guided by the researcher's a priori and theoretical knowledge and begins with a consideration of expected relationships based on mechanisms thought to operate in the system. To simplify the models and reduce the number of exogenous variables, we fitted generalized linear mixed models (GLMMs) as a preliminary step (see Online Resource Table S3 for details). In our case, the postulated causal relationships between variables in all competing models relied on information from previous studies (Gangoso et al. 2011), as well as the results obtained from GLMMs (Online Resource Table S3). Multi-collinearity was assessed by calculating generalized variance inflation factors (VIF) using the car package (Fox and Weisberg 2011); VIF values were ≤3 in all cases. We considered those explanatory variables with a $p \leq 0.05$ in the GLMMs and some non-significant ones that could underlie important relationships (such as the color morph of the biological parents) as exogenous variables in SEM. Both the PHA response and HI response were included as dependent variables in all competing models. Likewise, nestling body mass was included as a dependent

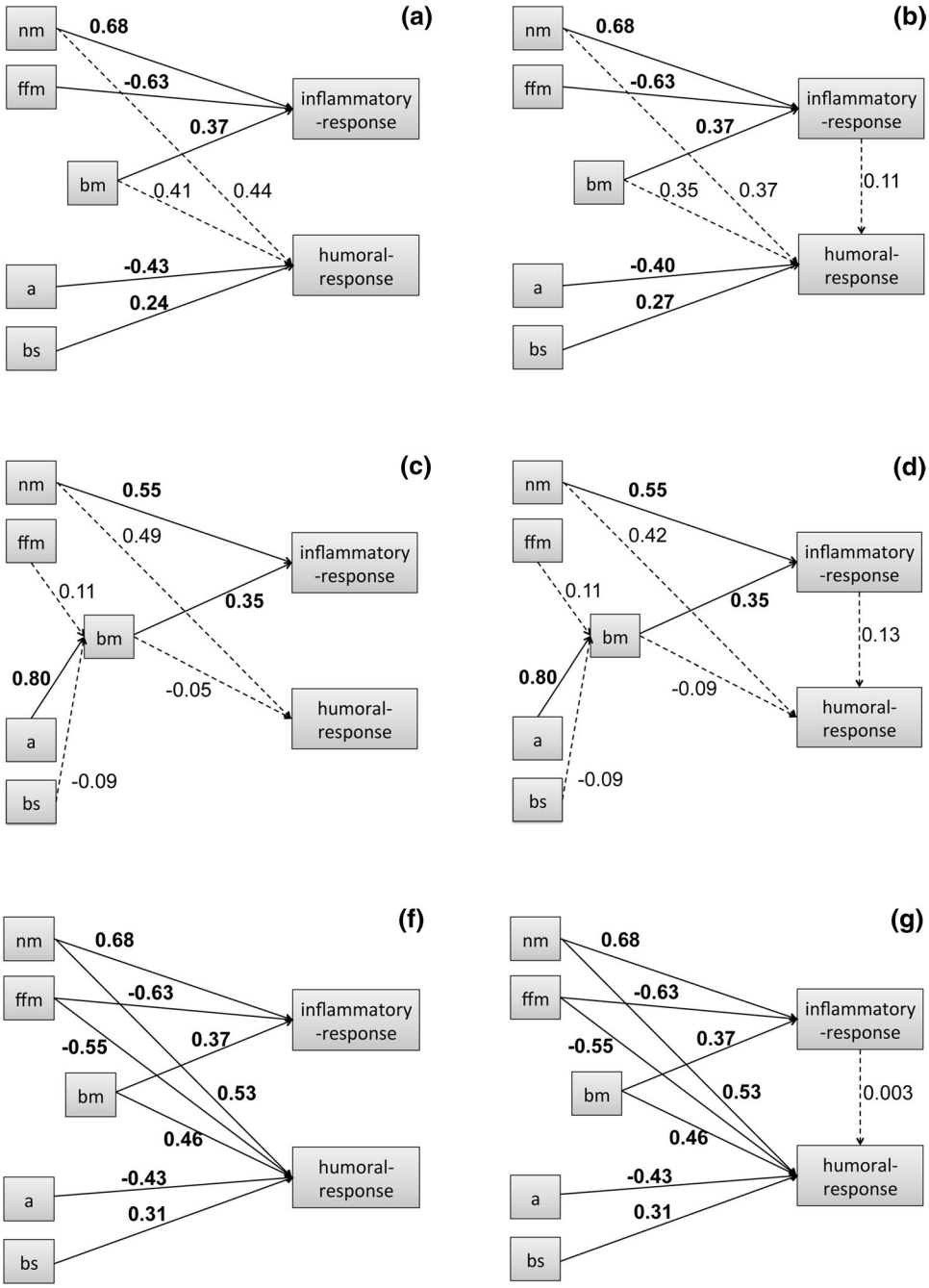

Fig. 1a–g Path diagrams showing all six competing models. *Numbers on arrows* represent regression coefficients. Significant relationships are highlighted in *bold* while *dashed arrows* represent non-significant relationships. *nm* Nestling color morph, *ffm* foster father color morph, *bm* nestling body mass, *a* nestling age, *bs* brood size

variable when analyzing indirect effects of body mass on nestling immune responses. Since one to three nestlings were clustered in different nests, we used the lavaan.survey package (Oberski 2014), which allows a clustering term to be included to control for pseudoreplication due to common nest origin. To perform the analysis, continuous variables were centered and scaled while factors were converted into dummy (0/1) variables. SEM is highly sensitive to incomplete data sets and so we removed from these analyses the three cases with missing data for some variables (thus the sample size was reduced to 64 individuals). The models were fitted using standard maximum likelihood and the parameters were estimated with robust SEs and a Satorra-Bentler scaled test statistic (Rosseel 2012).

We considered six competing models that included: direct genetic effects of nestling color morph and direct environmental effects on PHA and HI immune responses (models a, b, f, g); and direct genetic effects of nestling color morph but indirect environmental effects of the color morph of the foster father mediated by nestling body mass on PHA and HI immune responses (models c and d). Indirect effects (mediated through correlation with other

variables) are the product of two (or more) regression coefficients and so can be considered as interactions between these variables. In addition, all models were fitted assuming either no effect or a direct effect of the PHA response on the HI response (see Fig. 1). Finally, we fitted six new models in which the nestling color morph was replaced by the color morph of the biological parents to assess whether any other potential genetic relationship could be masked by the effect of nestling coloration (Fig. S1). Alternative models were compared using the Akaike's information criterion (AIC) and the Bayesian information criterion (BIC) (Burnham and Anderson 2002). Raw AIC and BIC values were further transformed to compare the relative performance of the models by means of Δ_i (AIC), Δ_i (BIC), as well as Akaike [w_i (AIC)] and Schwarz [w_i (BIC)] weights (Burnham and Anderson 2002) (see Online Resource Table S2).

Results

MC1R genotype-phenotype association and genotype frequencies

Forty-seven out of 67 genotyped nestlings (70.15 %; 27 males and 20 females) were homozygous for the wild-type (pale) *MC1R* allele (dd), 18 individuals (26.87 %; nine males and nine females) were dark heterozygous for the *MC1R* Δ12 variant (Dd), and two individuals (2.98 %; two males) were dark homozygous for the deletion (DD) (Online Resource Table S1).

Although the frequency of pale adult falcons is higher than the frequency of dark adults (80.52 vs. 19.48 % in 2010, $n = 154$, and 81.49 vs. 18.51 % in the period 2007–2012, $n = 1113$), pairing was random with regard to the color morph in 2010 ($\chi^2 = 0.44$, $df = 1$, $p = 0.501$, $n = 77$) and between years ($\chi^2 = 1.26$, $df = 1$, $p = 0.31$, $n = 552$). Genotype frequencies of nestlings (2010) are: DD = 1.14 %, Dd = 19.43, and dd = 79.43 ($n = 175$). Frequency of the dark allele (D) = 0.11 while the frequency of the pale allele (d) = 0.89. A χ^2 Hardy–Weinberg equilibrium test (Rodriguez et al. 2009) indicated that genotype frequencies are in equilibrium ($\chi^2 = 0$, $df = 1$).

Genetic and environmental effects on covariation between nestling color morph and immunity

Of the six competing models considered, the model including direct effects of genetic and environmental factors on both immune responses and without a direct effect of PHA response on HI response (model f) was selected based on its higher rounded w_i (AIC) and w_i (BIC) (Online Resource Table S2). Results for this model indicate that variation in both PHA and HI responses was primarily due to genetic factors via the nestlings' *MC1R*-related genotype (or color phenotype), although it was also influenced by environmental effects via the color morph of the foster father and, to a lesser extent, via the body mass and age of the nestling, and brood size (Fig. 1). The cell-mediated inflammatory response was strongly affected by the nestling color morph, with dark eumelanic nestlings developing lower responses than pale nestlings (see Fig. 2). The effect of the color morph of the foster father was also high, but in an opposite direction, since nestlings raised

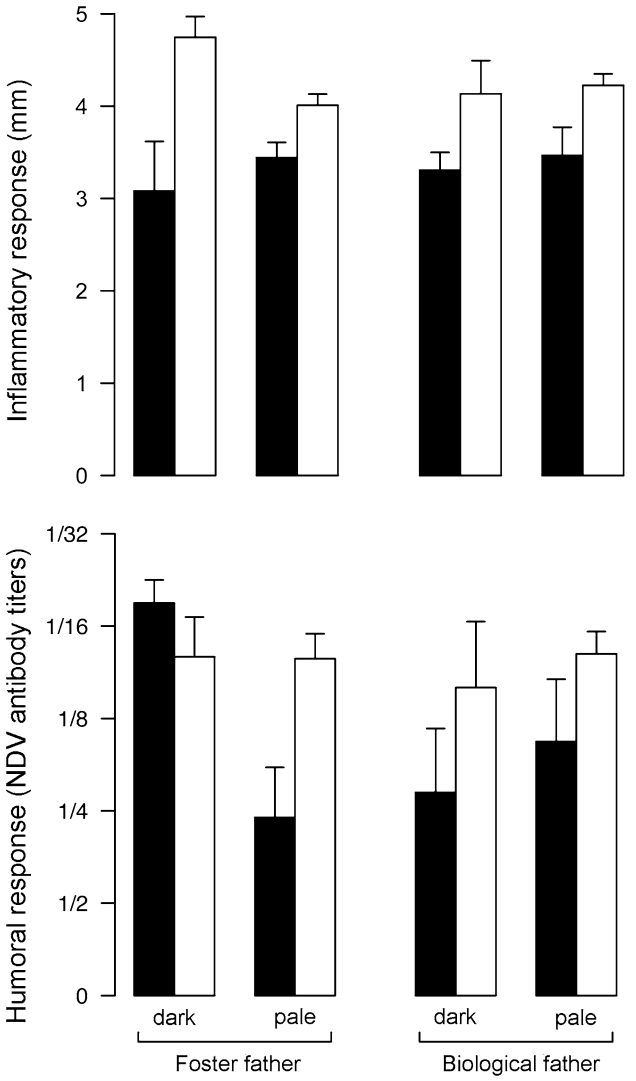

Fig. 2 *Upper panel* Inflammatory response (mm) to an injection of phytohemagglutinin of Eleonora's falcon nestlings. Results correspond to pale (*empty bars*, $n = 47$) and *dark* (*solid black bars*, $n = 20$) nestlings in relation to the color morph of their foster (*left*) and biological (*right*) father (*pale*, $n = 23$; *dark*, $n = 9$). *Lower panel* Humoral response measured as the concentration of antibodies against Newcastle disease virus (*ndv*) estimated from sequential dilutions that was positive in pale ($n = 46$) and dark ($n = 18$) Eleonora's falcon nestlings in relation to the color morph of their foster and biological father. Data are means from rough data ±SE

by dark eumelanic foster fathers had higher inflammatory responses than those raised by pale foster fathers (Fig. 2). The PHA response was also associated with nestling body mass, with heavier nestlings developing higher inflammatory responses. Nestling body mass was not associated with nestling or foster parents color morph (GLMM; sex, $F_{1,53.82} = 8.07$, $p = 0.006$; color morph, $F_{1,61.28} = 0.22$, $p = 0.64$; sex × color morph, $F_{1,56.39} = 0.89$, $p = 0.35$; age, $F_{1,62.52} = 82.60$, $p < 0.0001$; age^2, $F_{1,53.51} = 12.82$, $p = 0.0007$; foster father's morph, $F_{1,26.12} = 0.17$, $p = 0.68$; foster mother's morph, $F_{1,24.36} = 0.61$, $p = 0.44$). The humoral response was not affected by the inflammatory response elicited against the PHA challenge (the effect of the PHA response on the HI response was small and non-significant in models b, d, g; Fig. 1). As in the case of the PHA response, variation in the humoral response was associated with nestling color morph; dark eumelanic nestlings had lower humoral responses against the vaccine than pale nestlings (Fig. 2). In addition, environmental factors also influenced the humoral response. Nestlings raised by dark foster fathers had a higher humoral response than nestlings raised by pale eumelanic foster fathers (Fig. 2). Likewise, heavier nestlings and those raised in larger broods had higher humoral responses. On the contrary, older nestlings had lower humoral responses than younger ones (Fig. 1).

When the nestling color morph was replaced by the color morph of its biological parents, we found a significant effect of the morph of the biological father, but not of the biological mother (which was non-significant in all models). This effect was positive, but lower and in an opposite sense to that of the foster father (in models equivalent to models a, b), that is, nestlings born from dark eumelanic biological fathers showed lower inflammatory responses than those born from pale biological fathers. We found similar results in models equivalent to models f and g, where the effect of the color morph of the biological father also applied to the HI response. In this case, nestlings born from dark biological fathers had lower humoral responses than nestlings born from pale eumelanic biological fathers (Online Resource Fig. S1). All these six models returned higher AIC/BIC statistics than models that included the nestling color morph, thereby indicating that the nestlings' coloration is a better explanation of the genetic effects on the immune system than the biological parents' coloration.

Discussion

Strong evidence of pleiotropy between melanin-based pigmentation and innate immunity was reported in insects (see review in Wittkopp and Beldade 2009) and mammals, including humans (Teofoli et al. 1999; Getting et al. 2001; Reissmann and Ludwig 2013). In the present study, we experimentally confirmed the association between *MC1R*-dependent plumage coloration and direct genetic (via nestling color morph) and environmental effects (via foster father color morph and nestling body condition) on the immune system of Eleonora's falcon nestlings. Gangoso et al. (2011) have already reported correlative evidence (but with no cross-fostering experiment) of covariation between melanin-based coloration and the inflammatory cell-mediated immune response in nestlings of this species. Here, we expand that study by showing that such results remain significant when experimentally isolating genetic from environmental effects, and can also be extended to the humoral immune response. Since we cross-fostered entire clutches and so allocated genotypes randomly between environments (differential parental care), our results suggest that the magnitude of both immune responses is correlated to the nestling *MC1R* genotype. Nevertheless, we cannot dismiss other possible scenarios. For instance, other immune gene(s) may be physically linked to *MC1R* on the same chromosome. In addition, and despite the fact that the immune system is mostly affected by internal selection and hence, expected to be correlated to other phenotypic traits, the expression of immune phenotypes represents a complex summary of many organismal processes (Pigliucci and Preston 2004). Therefore, a trait covariation other than genetic is a true possibility. In this regard, Kim et al. (2013), by conducting pedigree-based quantitative genetic analyses in common kestrels (*Falco tinnunculus*), found a negative genetic covariance between melanin-based coloration and body mass in male adults as well as a positive genetic covariance between body mass and PHA response in fledglings. Despite the fact that all the three traits examined showed significant additive genetic effects on their expression, cell-mediated immunity of nestlings covaried only at the phenotypic (but not genetic) level with melanin-based coloration. In contrast to related kestrels (Fargallo et al. 2007), in our study species coloration is not condition dependent, but genetically determined by the same melanocortin system that is known to affect the development of an immune response. Therefore, we suggest that the relationship found between immunity and coloration is likely due to pleiotropy in the melanocortin system and so, a correlated response to selection on more than one trait (color) is expected to occur. Unfortunately, the limitations of our study do not allow us to unambiguously confirm this link.

As predicted, we found that dark eumelanic nestlings, regardless of their sex, mounted a lesser PHA response than pale nestlings. According to pharmacological evidence, this result could be mediated by higher activation and α-MSH binding to MC1R in dark eumelanic individuals bearing a gain of function mutation (Gangoso et al. 2011), since active MC1R has been shown to be necessary for inhibiting the liberation of pro-inflammatory cytokines, as well

as the over-production of immunosuppressive effectors in experimentally induced colitis in mice (Maaser et al. 2006). In addition, we found that environmental components during growth (nestling body mass and the color morph of the foster father) also played an important role. Environmental rearing conditions are complex and both adults are involved in parental care behavior. However, the role of males is mainly associated with food provisioning in this species. Therefore, it is not surprising that the coloration of the father rather than mother was positively associated with offspring immunity. The well-known positive effect of nestling body mass on PHA response (Alonso-Alvarez and Tella 2001) was, however, not mediated by parental workload, but instead exerted a direct effect on the PHA response. In this regard, Kim et al. (2013) showed a strong heritability of body mass that was also phenotypically and genetically correlated with the cell-mediated response of fledgling kestrels, which could be the case in our study species. However, these authors did not find a genetic or phenotypic covariation of body mass and melanin-based coloration. Even so, we found that nestlings raised by dark eumelanic foster fathers mounted higher PHA responses.

We found almost identical results in the humoral immune response and in the PHA response, that is, both genetic and environmental effects were involved in explaining the covariation between nestling melanin-based coloration and humoral response. Dark eumelanic nestlings had lower antibody titers towards a vaccine than pale eumelanic ones, irrespective of their sex and the color morph of their biological parents (Online Resource Table S1). As in the case of the PHA response, this could be the outcome of a similar mechanism, i.e., greater activity of MC1R in dark eumelanic fledglings. According to indirect evidence from recent pharmacological studies, this effect could be due to the immunosuppressive action of the activation and binding of α-MSH to MC1R in dendritic cells, i.e., antigen-presenting cells that play a crucial role in modulating responses by B and T lymphocytes, which results in a reduction in the ability of dendritic cells to induce allogenic T-lymphocyte proliferation. In addition to this, the peptide α-MSH suppresses antigen presentation/lymphocyte proliferation, which is of central importance for the differentiation of B-lymphocytes to antibody-secreting cells, and that this occurs via MC1R on monocytes/macrophages and/or B-lymphocytes and possibly also via MC3R on B-lymphocytes (Cooper et al. 2005), suggests that these processes might ultimately impair the acquired immune response to exogenous antigens. However, Cooper et al. (2005) found that the degree of variation in α-MSH-induced suppression of lymphocyte proliferation was similar in all the *MC1R* genotypes examined. Although this finding gives little support for our detection of *MC1R*-dependent morph-specific variation in the extent of the humoral immune response, it is worth noting that mutations at the same locus may drive different molecular mechanisms and give rise to different levels of trait expression (Rosenblum et al. 2010). Nonetheless, alternative explanations accounting for this pattern, such as the influence of other genes, physiological processes and/or environmental factors that differently affect alternative color morphs cannot be discarded.

In birds, it is widely assumed that the development of the immune system begins during the embryonic and post-hatching stages (Apanius 1998; Fellah et al. 2008). Therefore, the immune function of nestlings will be poorly developed in the first weeks after hatching (Apanius 1998) and maternal antibodies may well contribute to early humoral immunity (Hasselquist and Nilsson 2009; Arriero et al. 2013). Even if maternal effects did play an important role in the humoral response of Eleonora's falcon nestlings to NDV, this would not necessarily be mirrored by the effect of the color morph of the biological mother (which was non-significant in all models). However, recent evidence shows that in altricial birds, the intrinsic development of the immune function via the endogenous production of specific antibodies takes place within the first weeks after hatching (Palacios et al. 2009; King et al. 2010), which thus refutes the idea of limited endogenous humoral immunity during the first stages of life. Nevertheless, our approach does not allow us to distinguish between pre-hatching maternal and genetic effects. However, if this relationship was due to maternal effects, the magnitude of these effects is associated with a genetic marker (*MC1R*), which is in itself very interesting from an evolutionary point of view. Therefore, the probability that we are measuring antibodies against a vaccine produced by the nestlings themselves seems to be high, which gives credit to the hypothesis that the covariation between humoral response and nestling color morph may be the outcome of the pleiotropic effects of *MC1R*.

As in the case of the PHA response, environmental factors, in particular brood size, nestling age, and body mass, as well as the color morph of the foster father, also play an important role in the magnitude of the humoral immune response to vaccination. The development of the immune system is costly (Klasing and Leshchinsky 1999) and can be traded-off with the development of other biological functions such as somatic growth and rate of development (Brommer 2004; Pitala et al. 2010; Arriero et al. 2013), which is in agreement with our findings that show the positive effect of nestling body mass and the negative effect of nestling age on the HI response. Body mass may have a positive effect given the greater availability of resources than can be allocated to immunity. Yet the detection of younger nestlings with higher humoral responses seems counterintuitive, although this may indeed reflect the fact that somatic growth compromises immunity as both traits compete for the same resources (Norris and Evans

2000). Unfortunately, we were not able to detect whether the youngest nestlings still have some maternal antibodies that can be added to endogenously produced ones. The fact that nestlings from nests with larger broods had a higher HI response might merely reflect parental care capacity, i.e., better parents are able to raise larger broods. However, nestlings reared in larger broods had lower body mass (logistic regression, t-ratio $= -3.64$, $p = 0.0005$). In addition, and despite the effect of the color morph of the foster father on the HI response, dark foster fathers did not raise larger broods than pale foster fathers ($F_{1,29} = 0.64$, $p = 0.43$). Productivity and nestling body mass are strongly influenced by availability of food, which depends mainly on climatic conditions in our study system (Gangoso et al. 2013). It is possible that the superabundance of food experienced in 2010 may have obscured any potential asymmetry in hunting abilities and hence breeding output of differently colored males.

Morph-specific variation in immunity is important because it can influence differential vulnerability against pathogens. Selection imposed by pathogens may thus be involved in the evolution and long-term maintenance of genetic color polymorphism in natural populations. Assuming that an increased immune response is advantageous and that coloration and immunity are indeed correlated, the dark pale morph would attain higher fitness and could be selected for in those environments where pathogens are acting as powerful selective agents. Likewise, the dark eumelanic individuals could be outcompeted by pale ones through directional selection. However, maintenance and activation of the immune defense is energetically costly and traded-off with other biological functions and physiological demands (Schmid-Hempel 2003; Viney et al. 2005). Accordingly, recent evidence suggests that intermediate levels of immunity often result in higher lifetime fitness (Råberg and Stjernman 2003; Kim et al. 2013). This would lead to a different scenario, in which the pale morph is not necessarily favored over time and pathogen-driven balancing selection could facilitate the coexistence of alternative genotypes. It is important to note that different mutations at the same locus—even in cases of phenotypic (coloration) convergence—may lead to different levels of trait expression and, ultimately, to different evolutionary consequences (Rosenblum et al. 2010; Dessinioti et al. 2011). Therefore, the potential pleiotropic effects of *MC1R* mutations should not be generalized to other species in which the coding fragments affected by the mutations are different.

Conclusion

We showed that there is a genetic effect of *MC1R*-dependent coloration in the variation of the immune response, with lower innate and adaptive immune responses in those nestlings presenting the dark allele. In addition, immune response was also influenced in an opposite sense by environmental effects, with nestlings raised by dark fathers mounting higher immune responses. Morph-specific variation in immunity is important because it can influence differential vulnerability against pathogens and may therefore help understanding the processes governing the evolution, maintenance, and adaptive function of genetic polymorphism in the wild.

Author contribution statement L. G., A. R. and J. F. conceived and designed the experiments. L. G. and J. M. G. conducted fieldwork and performed the experiments. A. N. R. performed molecular analyses, L. G. analyzed the data and performed statistical analyses. L. G. wrote the first draft of the manuscript and all authors contributed to the final version.

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