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Effect of the *MC1R* gene on sexual dimorphism in melanin-based colorations

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Abstract

Variants of the melanocortin-1 receptor (*MC1R*) gene result in abrupt, naturally selected colour morphs. These genetic variants may differentially affect sexual dimorphism if one morph is naturally selected in the two sexes but another morph is naturally or sexually selected only in one of the two sexes (e.g. to confer camouflage in reproductive females or confer mating advantage in males). Therefore, the balance between natural and sexual selections can differ between *MC1R* variants, as suggest studies showing interspecific correlations between sexual dimorphism and the rate of nonsynonymous *vs.* synonymous amino acid substitutions at the *MC1R*. Surprisingly, how *MC1R* is related to within-species sexual dimorphism, and thereby to sex-specific selection, has not yet been investigated. We tackled this issue in the barn owl (*Tyto alba*), a species showing pronounced variation in the degree of reddish pheomelanin-based coloration and in the number and size of black feather spots. We found that a valine (V)-to-isoleucine (I) substitution at position 126 explains up to 30% of the variation in the three melanin-based colour traits and in feather melanin content. Interestingly, *MC1R* genotypes also differed in the degree of sexual colour dimorphism, with individuals homozygous for the II *MC1R* variant being 2 times redder and 2.5 times less sexually dimorphic than homozygous individuals for the VV *MC1R* variant. These findings support that *MC1R* interacts with the expression of sexual dimorphism and suggest that a gene with major phenotypic effects and weakly influenced by variation in body condition can participate in sex-specific selection processes.

Keywords: adaptive coloration, barn owl, genetic basis of coloration, natural selection pigmentation, sexual selection

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Introduction

The melanocortin-1 receptor (MC1R) is a classical example of a close match between genotype and phenotype. This receptor is involved in the biochemical cascade leading to the production of melanin pigments, and it is frequently associated with intra- and interspecific variation of pigmentation in wild (Theron *et al.* 2001; Rosenblum *et al.* 2004; Baião & Parker 2012; reviewed in Roulin & Ducrest 2013) and domestic animals (reviewed in Linderholm & Larson 2013). In wild animals, missense mutations at different sites of the *MC1R* gene result in abrupt colour changes that lead to the occurrence of alternative colour morphs within or between populations (Mundy 2005; Uy *et al.* 2009; Dessinioti *et al.* 2011; Nowacka-Woszuk *et al.* 2013). New mutations can be naturally selected particularly in response to selection for colour background matching and, thereby, in response to predator–prey relationships (Kaufman 1974; Hoekstra *et al.* 2004, 2006). This process seems to occur in different taxa (mammals; Nachman *et al.* 2003; birds; Cibois *et al.* 2012; and reptiles; Rosenblum *et al.* 2004), supporting the hypothesis that alternative colour morphs might have evolved in a convergent

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manner through mutations at the *MC1R* gene and potentially as a result of strong variation in natural selective pressures (Manceau *et al.* 2010).

Comparisons between species indicate that the evolution of *MC1R* may not only depend on natural selection but also on sexual selection (Nadeau *et al.* 2007), which further supports that colour evolution through *MC1R* may finally depend on the balance between these two selective forces. Nadeau *et al.* (2007) showed that the rate of nonsynonymous *vs*. synonymous amino acid substitutions (d*N*/d*S*) at the *MC1R* positively correlates with the degree of sexual dimorphism in melanin-based colour traits of galliforms. However, the mechanism through which *MC1R* could affect sexual dimorphism remains unknown, particularly because the potential link between *MC1R* and sexual selection has been largely overlooked for several reasons. First, the major effects of *MC1R* on the expression of colour morphs are not or scarcely sensitive to environmental variation (i.e. *MC1R*-related variation in colour unlikely functions as a sexually selected condition-dependent signal; Cotton *et al.* 2004). Second, the occurrence of assortative mating with respect to colour morphs suggests that no *MC1R* variant is expected to have a higher reproductive advantage (Mundy *et al.* 2004). Third, a system of discrete colour morphs encoded by *MC1R* has often been shown to play a major role in camouflage, photoprotection (Jablonski & Chaplin 2010) and, probably, thermoregulation (Clusella Trullas *et al.* 2007) and therefore, natural selection may have a more important role than sexual selection in the evolution of variation at the *MC1R* gene. Finally, although variation at the *MC1R* has been observed to underlie colour polymorphism in sexually dimorphic species (Doucet *et al.* 2004), most of the species studied until now show no sex differences in coloration and relatively simple, discrete colour variation.

However, because of its fundamental role in melanin synthesis, we predict that certain mutations at the *MC1R* may entail correlated changes in the extent to which colour differs between males and females. From a proximate point of view, a mutation that, for instance, induces an increase in *MC1R* activity may produce dark coloured traits where melanin concentration is closer to saturation (as for instance in black morphs of artic skuas, *Stercorarius parasiticus*; Mundy *et al.* 2004). If sexual dimorphism is based on factors inducing a higher or a lower melanin synthesis only in one sex, these factors may have a less evident effect when jointly expressed with a more active *MC1R* (i.e. both sexes are already close to saturation in melanin content) than with a less active *MC1R* variant. From an ultimate point of view, if *MC1R* affects the degree of sexual dimorphism, *MC1R* variants allowing for larger sexual dimorphism could be selected because a dark or pale coloration is sexually selected in one sex and/or because natural selection is stronger in one sex (for instance, for cryptic coloration in females). In contrast, if natural selection to be cryptic is similar in both sexes, *MC1R* variants inducing similar adaptive coloration will be positively selected in both sexes. When natural and sexual selection forces are more or less balanced, intralocus sexual conflict at the *MC1R* may occur given that a given variant will be positively selected in one sex (e.g. a variant allowing for noncryptic colour in the sexually selected sex) and an alternative variant in the other sex (e.g. a variant allowing for cryptic colours in the sex that takes care of the offspring).

Understanding the role of *MC1R* in the expression of sexual dimorphism is key to understand potential conflicts arising between natural and sexual selections during the evolution of melanin-based colour traits. Here, we investigated whether *MC1R* is polymorphic in the barn owl (*Tyto alba*) and whether this polymorphism is associated with pheomelanin-based coloration (varying from white to dark reddish) and with the number and size of black eumelanic spots located on the tip of the ventral feathers (Fig. 1A). Although each sex can express any phenotype, females have on average a redder pheomelanic plumage with more and larger black spots than males (Roulin 2003; Dreiss & Roulin 2010). The reddish pheomelanic coloration seems to have evolved in response to local selective pressures (Antoniazza *et al.* 2010, 2014), maybe as an adaptation to different physical habitats and/or to prey on different rodent species (Roulin 2004; Charter *et al.* 2012; Dreiss *et al.* 2012). Eumelanic black spots are sexually antagonistically selected, with females and males being selected to display large and small spots, respectively (Roulin 1999; Roulin *et al.* 2010; Roulin & Ducrest 2011).

We first examined whether *MC1R* is associated with pheomelanin and eumelanin feather contents and with the three melanin-based colour traits. We measured each plumage colour trait on different body parts: the breast, belly, flank and underside of the wings, given that there exists substantial variation among these body parts (Table 1), and therefore, they could be differently associated with *MC1R* and sex. We then specifically tested whether alleles at the *MC1R* are differentially related to the degree of offspring sexual dimorphism measured as the difference in plumage coloration between male and female nestlings of the same genotype. In the barn owl, the degree of sexual dimorphism changes with age because males and females show different patterns of plumage maturation (Dreiss & Roulin 2010). In both sexes, reddish plumage coloration becomes lighter with age, but males lose spots and females exhibit larger spots with age. Thus, we also

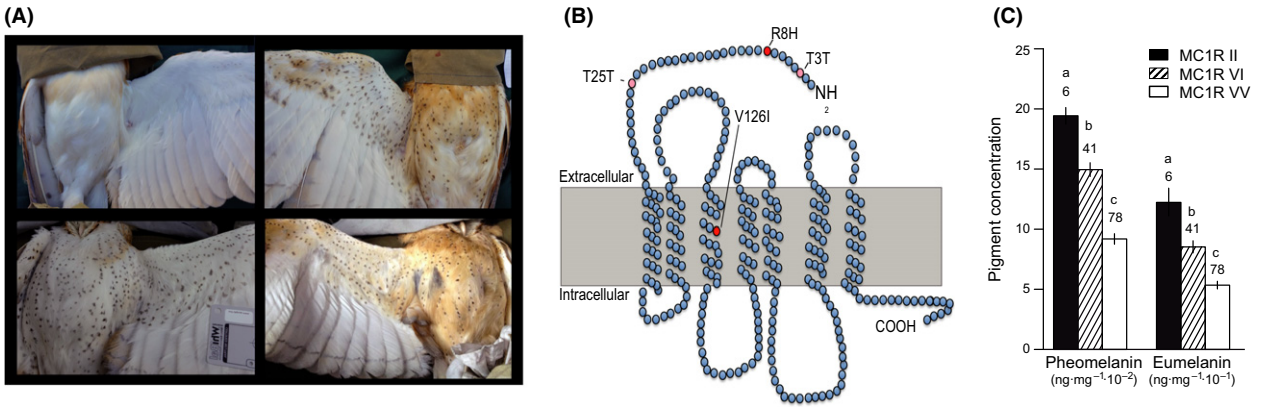

Fig. 1 Variation in melanin-based plumage traits, location of *MC1R* variants in the protein and effects of *MC1R* on melanin feather content in the barn owl. (A) Variation in the reddish pheomelanic coloration and in the number and size of eumelanic spots across and within nestling barn owls. (B) Two-dimensional model of the MC1R protein of the barn owl with polymorphic sites highlighted in red (nonsynonymous substitution) and light red (synonymous substitution). (C) Differences between *MC1R* genotypes for the mutation V126I in feather deposition of pheomelanin and eumelanin. For each pigment, mean (± SE) are reported, letters (a, b, c) indicate significant differences among *MC1R* genotypes, and numbers above bars indicate sample size.

analysed whether *MC1R* alters age-related changes in melanin-based traits and whether such changes induce variation in the degree of plumage sexual dimorphism.

Material and methods

Colour measurements and assessment of melanin pigments

The study was performed in western Switzerland in a population of wild barn owls breeding in nest boxes. Between 1996 and 2013, we collected blood and feather samples and measured melanin-based plumage traits on 2803 nestlings close to the fledging age (*c.a.* 50 days; for further details on sample size see Table S1 and S2, Supporting information). Nestlings were sired by 367 different males and 434 females (579 different pairs), and their sex was identified using molecular markers (Py *et al.* 2006). Because melanin-based traits are differentially expressed on the ventral body parts (Table 1), we measured plumage traits on the breast, belly and flank and on the underside of the wings. The pheomelanic reddish coloration, which is homogeneous on each body part, was scored using eight-colour chips ranging from -8 (white) to -1 (dark reddish), a method that highly correlates with objective spectrophotometric measurements ($r = -0.78$, $P < 0.0001$, $N = 1107$; Dreiss & Roulin 2010). The eumelanic black spots were counted within a 60 × 40 mm frame, and their diameter was measured to the nearest 0.1 mm. Measurement of all plumage traits are highly repeatable (for further details see Roulin 2004). A total of 783 adults (335 males and 448 females, Table S3, Supporting information) for which we have repeated measures over several breeding seasons were used to investigate the effect of *MC1R* on age-related changes in plumage traits. Some individuals ($n = 417$) were ringed as adults, and their age was estimated based on their moulting pattern (see Dreiss & Roulin 2010); however, statistical analyses (not shown) were qualitatively the same when only individuals of known exact age (i.e. ringed as nestlings) were used.

We analysed the amount of pheomelanin and eumelanin pigments in feathers in a subset of 125 nestlings (58 males and 73 females) from 43 nests using the same protocol as described in Roulin *et al.* (2013) for the barn owl (see also; Wakamatsu *et al.* 2002; Ito & Wakamatsu 2011).

MC1R sequencing

Genomic DNA was extracted from blood or dried feathers using DNeasy Blood Tissue or QiAmp DNA Micro kits (Qiagen, Hombrechtikon, Switzerland). Primers MC1R_44Fw and MC1R_944Rev designed based on *Gallus gallus* sequence (for sequences and protocols, see Table S4 and Appendix S1, Supporting information) amplified 900 bp of the *MC1R* coding sequence under the following conditions: 25 ng of genomic DNA, 250 nM of MC1R_43Fw and MC1R_944Rev, 200 μM dNTPs, 1× Qiagen buffer, 1× Q solution, 0.5 U of Taq polymerase (Qiagen, Hombrechtikon, Switzerland) at 95 °C for 5 min, followed by 34 cycles at 94 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min and final elongation at 72 °C for 10 min in 50 μL. The amplicons of 23 individuals of the extreme colour morphs (dark reddish and heavily spotted vs. white and immaculate) were purified with MinElute PCR purification kit (Qiagen, Hombrechtikon, Switzerland), TA-cloned in pGEMT

Table 1 Observed mean (± SE) plumage trait values in nestling barn owls of different *MC1R* genotypes. Values are given for each *MC1R* genotypes for the mutation V126I (II, VI and VV), each body part and each plumage trait

	MC1R genotype II				*MC1R* genotype VI				*MC1R* genotype VV			
	Breast	Belly	Flank	Wing	Breast	Belly	Flank	Wing	Breast	Belly	Flank	Wing
Reddish colour												
Male nestlings	2.64 ± 0.08	3.40 ± 0.09	2.74 ± 0.08	3.62 ± 0.11	3.12 ± 0.04	4.56 ± 0.07	3.51 ± 0.05	4.99 ± 0.07	5.15 ± 0.04	7.70 ± 0.02	5.90 ± 0.04	7.68 ± 0.02
Female nestlings	2.19 ± 0.08	2.76 ± 0.09	2.18 ± 0.08	2.88 ± 0.10	2.58 ± 0.03	3.43 ± 0.04	2.80 ± 0.04	3.53 ± 0.05	3.65 ± 0.02	6.18 ± 0.04	4.43 ± 0.03	5.98 ± 0.04
Number of black spots												
Male nestlings	60.96 ± 3.06	23.60 ± 2.53	51.04 ± 2.41	28.64 ± 2.27	61.33 ± 1.44	24.20 ± 1.04	53.54 ± 1.15	36.07 ± 0.92	50.28 ± 1.11	16.83 ± 0.63	52.31 ± 0.75	29.71 ± 0.53
Female nestlings	67.65 ± 3.22	25.48 ± 2.86	57.57 ± 2.92	35.40 ± 2.46	65.08 ± 1.16	29.09 ± 1.01	58.37 ± 1.05	41.34 ± 0.80	68.73 ± 0.90	36.16 ± 0.77	66.19 ± 0.64	51.25 ± 0.53
Spot diameter (mm)												
Male nestlings	1.20 ± 0.04	1.13 ± 0.07	1.44 ± 0.05	0.96 ± 0.04	1.10 ± 0.02	1.16 ± 0.03	1.39 ± 0.02	1.13 ± 0.02	0.94 ± 0.01	0.91 ± 0.02	1.40 ± 0.02	1.29 ± 0.02
Female nestlings	1.38 ± 0.04	1.25 ± 0.05	1.63 ± 0.05	1.12 ± 0.03	1.31 ± 0.02	1.31 ± 0.02	1.57 ± 0.02	1.24 ± 0.02	1.23 ± 0.01	1.44 ± 0.02	1.74 ± 0.01	1.59 ± 0.01

(Promega, Duebendorf, Switzerland) and plasmids sequenced in a 3130XL Genetic Analyzer (Life Technologies, Zug, Switzerland) with a special protocol that is in 10 μL with 2 μL of Big Dye V 3.1, 2 μL of 5× Q solution (Qiagen, Hombrechtikon, Switzerland), 1 μL of 10 μM of Primer T7 or SP6, 2 μL of plasmid diluted to 100 ng/ul and amplification at 98 °C for 2 min, 35 cycles at 96 °C for 15 s, 55 °C 15 s and 60 °C for 3 min. Sequences were aligned in CodonCode Aligner 3.7.1.2 (CodonCode Corporations, Dedham, MA, USA). To complete the coding sequence (CDS) and obtain the upstream and downstream UTR of *MC1R* sequences, we used RACE and genome walking assays using GeneRacer kit (Life Technologies, Zug, Switzerland) and GenomeWalker universal kit (Clontech, Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France), respectively (see Appendix S1, Supporting information). We then directly sequenced (without cloning) the whole CDS using MC1R-34Fw and MC1R_969Rev (located at the 5′ of the start codon and the 3′ of the stop codon, respectively). When DNA quality was not good enough to get the whole CDS, we separately amplified the first and second half of the gene with two distinct PCRs: one amplicon of 606 bp with the specific primers MC1R-34Fw and MC1R_568Rev and one of 565 bp with MC1R_404Fw and MC1R_969Rev (3 min at 95 °C; 35 cycles 30 s at 95 °C, 1 min at 62 °C, 1 min at 72 °C; 10 min at 72 °C). Sequencing was performed as described previously. The ancestral–derived status of *MC1R* alleles was defined by comparison with the *MC1R* sequence of the tawny owl, *Strix aluco* (Access number: KF201577.1), and chicken, *Gallus gallus* (NM_001031462).

Using allelic discrimination, we genotyped all the individuals for the mutation V126I, the most frequent nonsynonymous mutation found at the *MC1R* gene of the barn owl (see Results). Probably due to the high GC content of *MC1R*, a pre-amplification PCR was necessary before performing the allelic discrimination assay. Each individual was genotyped twice using two independent PCR products (for further details, see Appendix S1 and Table S4, Supporting information).

Statistical procedure

We first investigated whether *MC1R* genotypes for the nonsynonymous mutation V126I (i.e. homozygotes VV and II and heterozygotes VI) differ in the amount of pheomelanin and eumelanin pigments deposited in breast feathers collected in fledglings. We fitted separated linear mixed models for pheomelanin and eumelanin concentrations including nest of origin as random factor and *MC1R* genotype and sex as fixed factors. We then investigated the effect of *MC1R* on the expression

of the reddish coloration and the number and size of the black spots in fledglings. Each plumage trait was analysed as dependent variable in separate linear mixed models. We accounted for within-subject colour variation among body parts (breast, belly, flank and the underside of the wings) by fitting mixed models for longitudinal data with nestling identity as random effect (Pinheiro & Bates 2000). Models also included the random effect of year of birth and of maternal and paternal identities as well as *MC1R* genotype for the V126I mutation, sex, and body part (and all their interactions) as fixed factors.

To specifically investigate whether *MC1R* accounts for differences in the degree of offspring sexual dimorphism in different plumage traits, for each breeding pair we calculated mean plumage trait values of brothers and then of sisters who shared the same *MC1R* genotype. For each plumage trait and body part, genotype and family, sexual dimorphism was calculated as 'daughter value – son value' (i.e. positive values indicate female-biased melanization and negative values male-biased melanization). Values of sexual dimorphism were then standardized for the statistical analysis. Degree of sexual dimorphism was analysed using linear mixed models using *MC1R* genotype, body part, and their interaction (fixed factors) and maternal and paternal identities (random factors). Finally, we investigated whether colour plumage maturation (Dreiss & Roulin 2010) differs between *MC1R* genotypes. Using breeding individuals recaptured over consecutive years (Table S3, Supporting information), we fitted repeated-measures linear mixed models for each colour trait with individual identity and year as random variables and *MC1R* genotype, sex, body part, age (in years) and all their interactions as fixed variables. For this analysis, the sample size for II individuals was low (see Table S3, Supporting information) and only VI and VV individuals were considered. All the analyses were run in R v.3.0.2 (R Core Team, Vienna, Austria), all tests were two-tailed, and significance was set at $\alpha = 0.05$.

Results

Genetic variability at MC1R

We sequenced 1334 bp of the *MC1R*, which comprises 343 bp of the 5′ UTR, 945 bp of the exon that contains the whole coding sequence (CDS) and 46 bp of the 3′ UTR. The sequence is highly GC rich with a GC content of 69% (ENDMEMO, http://www.endmemo.com/bio/gc.php). We sequenced 1003 bp (MC1R-34Fw, MC1R_969Rev), 900 bp (43–944), 603 bp (-34–569) and 565 bp (404-969) of the CDS of 17, 23, 76 and 5 barn owls, respectively. We found two synonymous transitions c.9G>A (T3T) and c.75G>A (T25T), and two nonsynonymous transitions c.23G>A and c.376G>A with the following frequencies of the derived alleles 4.3%, 3.0%, 0.5% and 15.4%, respectively (Fig. 1B). The c.23G>A transition caused an arginine-to-histidine substitution at position 8 of *Gallus* sequence (NM_001031462) (R8H), and that would be located within the first outer loop of the MC1R protein. The most frequent nonsynonymous mutation (c.376G>A) corresponded to a valine-to-isoleucine substitution at position 126 (V126I) and would be located in the third transmembrane of the MC1R. Hereafter, the 'valine' allele is quoted V and the isoleucine allele, I.

MC1R genotypes and melanin feather concentration

Pheomelanin and eumelanin feather contents significantly differed between *MC1R* genotypes ($F_{2,79} = 105.91$, $P < 0.0001$ and $F_{2,79} = 43.06$, $P < 0.0001$, respectively), which explained 47.2 and 34.1% of the total variance in each pigment content, respectively (Table 2). VV nestlings deposited significantly less pheomelanin and eumelanin in their feathers than VI nestlings, and VI nestlings significantly less than II nestlings (Fig. 1C). Pheomelanin and eumelanin feather contents were lower in males (mean ± SE: 1796.38 ± 79.06 ng/mg and 74.37 ± 0.08 ng/mg, respectively) than in females (mean ± SE: 2089.79 ± 68.34 ng/mg and 95.63 ± 0.07; $F_{1,79} = 29.44$, $P < 0.0001$, $F_{1,79} = 27.06$, $P < 0.0001$, respectively). Nest of origin modelled as random effect accounted for 15.2% and 31.1% of the variance in pheomelanin and eumelanin feather contents, respectively.

Effect of MC1R-genotypes on melanin-based plumage traits

The impact of *MC1R* on all plumage traits was sex specific and differed between body parts (significant interactions between *MC1R*, sex and body parts in Table 3). As it can be seen in Fig. 2, the effect of *MC1R* was stronger on the pheomelanin-based reddish coloration than on the number and size of the black spots, which was further confirmed by statistical analysis comparing the relative impact of *MC1R* on the three plumage traits (see Appendix S2, Supporting information).

MC1R explained 33.7% of the total variance of the reddish coloration (Table 2). In the two sexes and for all body parts, II nestlings were significantly but slightly darker reddish than VI nestlings, whereas VV nestlings were clearly lighter coloured than the other two *MC1R* genotypes (see contrasts in Fig. 2A). This effect was stronger in males than in females (Fig. 2A). *Post hoc* contrasts showed that, for all body parts, differences in reddish coloration between II and VV nestlings

Table 2 Variation in melanin-based plumage traits in nestling barn owls explained by the *MC1R* gene. Shown is the percentage of variance explained by *MC1R* genotypes for the mutation V126I relative to the total variance of the trait (i.e. the four body parts of males and females combined in the same analysis) and relative to the variance within each sex and each body part. Nestling plumage dimorphism refers to the difference in melanin-based plumage traits between male and female siblings. Explained variance for adult coloration (estimated at mean adult age in our sample, i.e. 2 years old) was calculated from models accounting for age variation (see Methods)

		Breast		Belly		Flank		Wing	
Trait	% Of total variance	% Of male variance	% Of female variance	% Of male variance	% Of female variance	% Of male variance	% Of female variance	% Of male variance	% Of female variance
Melanin pigment feather content									
Pheomelanin	47.17	44.60	60.01	—	—	—	—	—	—
Eumelanin	34.11	35.27	49.79	—	—	—	—	—	—
Nestling plumage traits									
Reddish coloration	33.71	40.00	34.55	76.22	54.74	55.96	45.85	71.27	49.59
Number of spots	0.15	4.11	0.04	5.24	0.88	0.57	1.47	4.73	5.88
Spot diameter	0.05	5.72	2.22	5.78	0.15	0.54	0.76	2.25	13.35
Nestling plumage sexual dimorphism									
Reddish coloration	8.69	19.17	—	3.64	—	15.62	—	2.48	—
Number of spots	8.50	5.35	—	10.32	—	5.36	—	15.38	—
Spot diameter	2.10	1.61	—	1.24	—	4.24	—	2.09	—
Adult plumage traits									
Reddish coloration	22.72	53.34	10.32	77.75	30.49	61.22	16.00	79.08	27.88
Number of spots	0.54	1.35	1.39	0.50	0.06	2.45	0.42	0.33	0.92
Spot diameter	0.62	3.79	0.09	0.37	0.94	2.34	0.27	0.20	0.50

Table 3 Effect of *MC1R*-genotypes on reddish coloration, number and size of black spots in nestling barn owls. Linear mixed models to test whether *MC1R* has differential effect on males and females, and on the four different body parts (breast, belly, flank and underside of the wings)

	Reddish colour	Number of black spots	Spot diameter
Nestling identity	39.97%	29.30%	36.70%
Maternal identity	9.51%	13.49%	14.51%
Paternal identity	13.32%	18.54%	20.88%
Year	2.06%	5.04%	7.41%
MC1R	$F_{2,2676} = 1788.39$***	$F_{2,2622} = 4.96$**	$F_{2,2675} = 0.54$
Sex	$F_{1,2591} = 364.95$***	$F_{1,2386} = 60.96$***	$F_{1,2449} = 84.68$***
MC1R x Sex	$F_{2,2589} = 47.45$***	$F_{2,2399} = 47.12$***	$F_{2,2462} = 22.41$***
Body part	$F_{3,8328} = 1274.89$**	$F_{3,7162} = 1185.94$***	$F_{3,6894} = 445.64$***
MC1R x Body part	$F_{6,8331} = 333.20$***	$F_{6,7206} = 18.07$***	$F_{6,6938} = 168.09$***
Sex x Body part	$F_{3,8328} = 23.39$***	$F_{3,7225} = 1.15$	$F_{3,6963} = 4.31$**
MC1R x Sex x Body part	$F_{6,8331} = 19.82$***	$F_{6,7236} = 3.23$**	$F_{6,6971} = 19.68$***

We indicate the percentage of variance explained by the random variables (nestling, maternal and paternal identities as well as year). The symbols ** and ***P-values below 0.01 and 0.001, respectively.

and between VI and VV nestlings were significantly larger in males than in females (all $t_{2589} > 2.58$, all $P < 0.015$). Differences in reddish coloration between II and VI nestlings were also larger in males than in females but only on the underside of the wings and on the belly (all $t_{2589} > 2.54$, all $P < 0.015$) but not on the flank or on the breast (all $t_{2589} < 0.77$, all $P > 0.47$; Fig. 2A).

With respect to the number of black spots, *MC1R* explained 0.2% of the total variance. This small percentage is in part due to the fact that the effect of *MC1R* differed between sexes and body parts (Table 3, Fig. 2B). When taken this into account, *MC1R* explained between 0.04% to 5.9% of the variance that was specific to each sex and body part (Table 2). *MC1R* sometimes showed even opposite effects in males compared to females. For

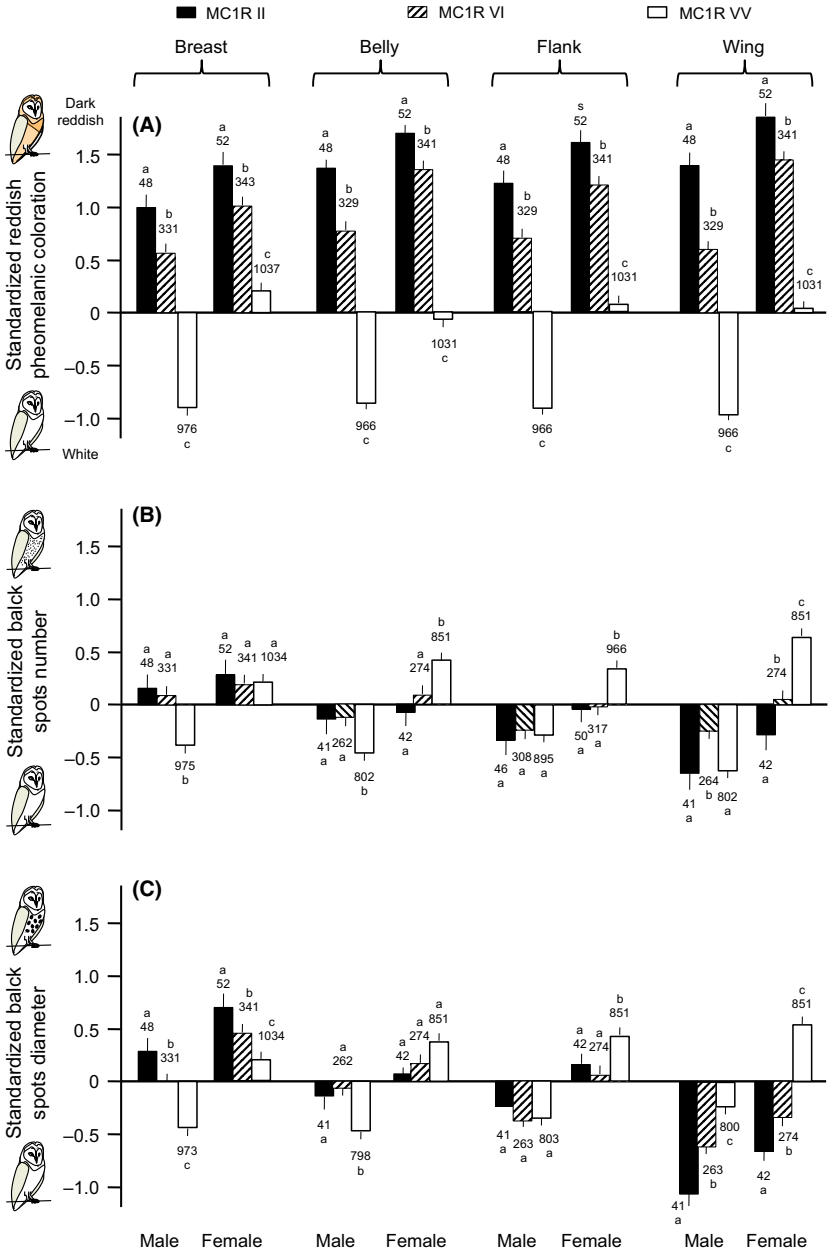

Fig. 2 Effect of *MC1R* on three melanin-based plumage traits in nestling barn owls. For each plumage trait (A. reddish pheomelanic coloration, B. number of black spots, C. diameter of black spots) and body part, we standardized values ([value - mean]/standard deviation) by pooling males and females. Reported are mean (± SE) predicted values obtained from linear mixed models including maternal and paternal identities as random variables and sex, *MC1R* and the interaction 'sex x *MC1R*' as dependent variables. Letters (a, b, c) indicate for each plumage trait and sex whether predicted means of the four body parts are different from each other in individuals sharing the same *MC1R* genotype; when two means have the same letter, it indicates that they are not significantly different from each other. Numbers above bars indicate sample size.

instance, on the belly, VV male nestlings displayed significantly fewer spots than II and VI males, whereas VV female nestlings showed significantly more spots than II and VI females (Fig. 2B). On the breast, significant differences among *MC1R* genotypes were found in males but not in females (VV males showed fewer spots than the other genotypes), whereas the opposite pattern was found on the flank (VV females showed more spots than the other genotypes and no differences existed in males; Fig. 2B). Furthermore, *MC1R* had a heterosis effect on the underside of the wings, because in males (but not in females), homozygous II and VV displayed fewer spots than heterozygous VI (Fig. 2B).

MC1R explained 0.05% of the total variance in spot diameter although *MC1R* explained between 0.2% and 13.4% of the variance that was specific to each sex and body part (Table 2). The effect of *MC1R* differed between body parts in interaction with sex (Table 3). On the breast, II nestlings displayed larger spots than VI nestlings that displayed larger spots than VV nestlings, an effect that was more pronounced in males than in females ($t_{2462} = 2.61$, $P = 0.009$; Fig. 2C). On the underside of the wings, the effects of *MC1R* reversed: VV nestlings displayed larger black spots than VI nestlings (particularly in females; $t_{2462} = 4.53$, $P < 0.001$), and II nestlings exhibited smaller black spots than VI

(Fig. 2C). On the belly, *MC1R* genotypes differed in the size of the black spots only in males (VV males showed smaller spots than the other genotypes), whereas on the flank, *MC1R* genotypes differed only in females (VV females showed larger spots than the other two genotypes; Fig. 2C).

Effect of MC1R genotypes on nestling sexual dimorphism

The degree of sexual dimorphism in nestlings differed significantly between *MC1R* genotypes and body parts (Table 4). For all body parts, sexual dimorphism was more pronounced in VV than in VI and II genotypes with respect to reddish coloration (all contrasts $t_{1400} > 2.17$, all $P < 0.031$), spot diameter (all contrasts $t_{1244} > 2.09$, all $P < 0.037$) and number of spots (all contrasts $t_{1194} > 2.84$, all $P < 0.005$; Fig. 3). Sexual dimorphism between II and VI nestlings was only significantly different for the reddish coloration of the underside parts of the wings ($t_{1400} > 2.45$, all $P = 0.014$) but not for the reddish coloration of the other body parts or for the number and size of the black spots (all contrasts $P > 0.068$).

Table 4 Effect of *MC1R* genotypes on sexual dimorphism in reddish coloration, number and size of black spots in nestling barn owls. Results from linear mixed models testing whether *MC1R* has differential effect between males and females, and between the four different body parts (breast, belly, flank and underside of the wings)

	Sexual dimorphism in nestlings		
	Reddish colour	Number of black spots	Spot diameter
Paternal identity	20.12%	15.91%	30.73%
Maternal identity	24.22%	40.09%	40.81%
MC1R	$F_{2,1664} = 60.24$***	$F_{2,1611} = 63.35$***	$F_{2,1601} = 42.68$***
Body part	$F_{3.1400} = 2.24$	$F_{3.1246} = 5.77$***	$F_{3.1194} = 1.63$
MC1R x Body part	$F_{6,1400} = 15.74$***	$F_{6,1244} = 4.11$***	$F_{6,1192} = 6.83$***

We report the percentage of variance explained by the random variables (paternal and maternal identities). ***P-values are smaller than 0.001.

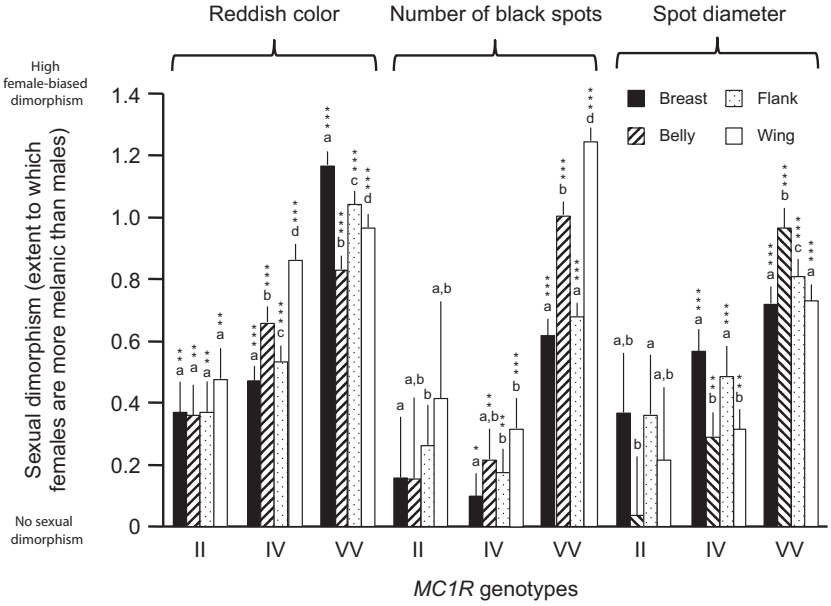

Fig. 3 Effect of *MC1R* genotypes on sexual dimorphism of three melanin-based plumage traits in nestling barn owls. For each colour trait and body part, we calculated sexual dimorphism as the difference between mean values of sons and daughters with the same *MC1R* genotype and use the standardized values for the statistical analyses. Means ± SE are reported. For each genotype and plumage trait, small letters indicate whether mean nestling sexual dimorphism is significantly different between body parts using paired *t*-test (two body parts with the same letter have similar means, whereas sexual dimorphism of two body parts having different letters have different means). Stars above letters indicate whether nestling sexual dimorphism is significantly different from zero using sign test (* for $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

In homozygous II nestlings, sexual dimorphism did not differ significantly between plumage traits (comparing dimorphism between reddish coloration and number of spots, between reddish coloration and spot diameter and between number of spots and spot diameter; paired *t*-tests: *P*-values > 0.30) although only the reddish coloration was significantly sexually dimorphic (contrasts in Fig. 3). In VI nestlings, sexual dimorphism in reddish coloration was significantly stronger than in the number of spots (t_{135} = 4.79, $P < 0.0001$) but of similar magnitude as sexual dimorphism in spot size (t_{135} = 1.31, $P = 0.19$) and sexual dimorphism was more marked in the size than in the number of black spots (t_{135} = 6.78, $P < 0.0001$). Finally, in VV nestlings, sexual dimorphism was stronger in reddish coloration than in the number and size of black spots (t_{325} = 10.33, $P < 0.0001$, t_{325} = 8.04, $P < 0.0001$, respectively) and sexual dimorphism was significantly more pronounced in spot size than in spot number (t_{325} = 2.24, $P = 0.026$).

Effect of MC1R genotypes on adult sexual dimorphism

Age-related changes in all plumage traits were significantly dependent on the *MC1R* genotype in interaction with sex, *MC1R* and body part (Table 5; Fig. 4). Reddish coloration became lighter with age in all genotypes, body parts and sexes (all t_{4850} > 3.70, all $P < 0.001$). Males and females differed in the rate at which reddish colour became lighter with age (i.e. the degree of sexual dimorphism changed with age), and such differences were largely dependent on genotype and body part. In VV breeding birds, colour changed more intensely in females than in males (i.e. steeper slopes for the relationship between age and reddish colour in Fig. 4A) for all body parts (all t_{4850} > 2.71, all $P < 0.007$) except for the breast, where males and females changed with the same rate (t_{4850} = 1.11, $P = 0.26$). In contrast, in VI adults, male reddish colour changed more intensely than female colour on the belly and flanks (all t_{4850} > 3.55, all $P < 0.001$) but not on the breast or the underside parts of the wings (all t_{4850} < 1.65, all $P > 0.09$).

The number of spots significantly decreased with age in all male body parts and for all genotypes (all t_{4621} > 3.58, all $P < 0.001$), except for the underside parts of the wing in VI males, where no significant change was detected (t_{4621} > 1.82, $P = 0.068$). In females, it significantly decreased in all body parts of VV adults (all t_{4621} > 3.70, all $P < 0.001$), whereas in VI females, it significantly increased with age on the wings and flanks (all t_{4621} > 2.28, all $P < 0.023$) and no significant change occurred on the breast and belly (all t_{4850} < 1.34, all $P > 0.18$). Further contrasts showed that, in VV adults, the degree of sexual dimorphism increased with age given that number of spots decreased more pronouncedly in males than in females for all body parts (all t_{4621} > 2.66, all $P < 0.008$) except on the underside parts of the wings (t_{4850} = 0.87, $P = 0.38$). In VI adults, sexual dimorphism is less pronounced (see also Fig. 3) and only on the underside part of the wings, it was

Table 5 Effect of *MC1R* on age-related changes in reddish coloration, number and size of black spots in adult barn owls. Results from linear mixed models testing the relationship between *MC1R*, sex and body part (breast, belly, flank and underside of the wings) on age-related changes in plumage traits.

	Reddish colour	Number of black spots	Spot diameter
Individual identity	17.20%	43.50%	45.71%
Year	1.61%	1.75%	8.04%
MC1R	$F_{1,776.4}$ = 1195.98***	$F_{1,769.2}$ = 6.71**	$F_{1,795.6}$ = 0.08
Sex	$F_{1,774.4}$ = 533.51***	$F_{1,767.4}$ = 128.44***	$F_{1,794.4}$ = 155.93***
MC1R x Sex	$F_{1,774.5}$ = 3.29	$F_{1,767.4}$ = 37.89***	$F_{1,794.5}$ = 10.23**
Body part	$F_{3,4850}$ = 2922.39***	$F_{3,4628}$ = 1331.28***	$F_{3,4194}$ = 1344.24***
MC1R × Body part	$F_{3,4850}$ = 43.04***	$F_{3,4628}$ = 41.17***	$F_{3,4200}$ = 101.87***
Sex x Body part	$F_{3,4850}$ = 53.83***	$F_{3,4628}$ = 57.23***	$F_{3,4200}$ = 5.43***
MC1R × Sex × Body part	$F_{3,4850}$ = 174.73***	$F_{3,4628}$ = 7.27***	$F_{3,4200}$ = 2.76*
Age	$F_{1,1560}$ = 1238.58***	$F_{1,5239}$ = 140.52***	$F_{1,1735}$ = 1.20
MC1R × Age	$F_{1,5591}$ = 0.55	$F_{1,5239}$ = 42.61***	$F_{1,4601}$ = 18.18***
Sex × Age	$F_{1,5582}$ = 39.04***	$F_{1,5232}$ = 26.50***	$F_{1,4588}$ = 45.29***
MC1R × Sex × Age	$F_{1,5586}$ = 78.65***	$F_{1,5232}$ = 4.16*	$F_{1,4594}$ = 7.71**
Body part × Age	$F_{3,4850}$ = 57.25***	$F_{3,4621}$ = 20.02***	$F_{3,4181}$ = 9.31***
MC1R × Body part × Age	$F_{3,4850}$ = 77.48***	$F_{3,4621}$ = 5.44***	$F_{3,4184}$ = 0.64
Sex × Body part × Age	$F_{3,4850}$ = 34.26***	$F_{3,4621}$ = 3.02*	$F_{3,4183}$ = 0.18
MC1R × Sex × Body part × Age	$F_{3,4850}$ = 22.54***	$F_{3,4621}$ = 1.58	$F_{3,4183}$ = 3.31*

We report the percentage of variance explained by the random variables (individual identity and year). *,** and ***P-values below 0.05, 0.01 and 0.001, respectively.

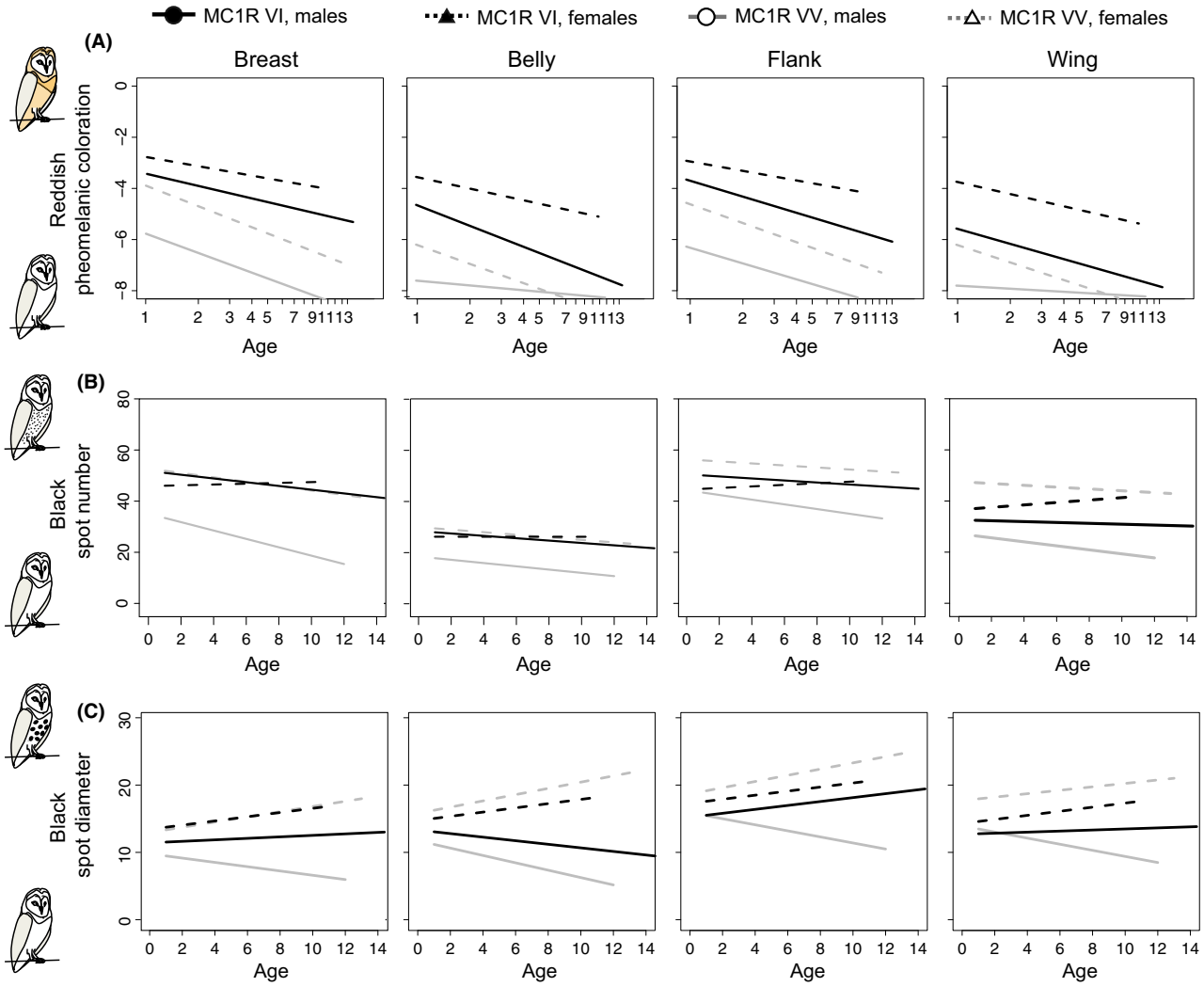

Fig. 4 Effect of *MC1R*-genotypes on age-related changes in three melanin-based plumage traits in adult barn owls. Shown are the predicted regression lines of age on reddish pheomelanin coloration (A), black spot number (B) and black spot diameter (C). For pheomelanic coloration (A), age is plotted with a logarithmic scale. Thick lines indicate slopes that were significantly different from zero.

observed a marked significant increase in sexual dimorphism with age ($t_{4850} = 4.23$, $P < 0.001$).

Spot diameter decreased with age in all body parts of VV males (all $t_{4183} > 3.18$, all $P < 0.002$). In VI males, spot diameter decreased with age on the belly and increased on the flanks (all $t_{4183} > 1.99$, all $P < 0.046$), whereas spot diameter remained unchanged on the wings and on the breast (all $t_{4183} < 0.81$, all $P > 0.42$). In VV females, spot diameter significantly increased in most body parts (all $t_{4183} > 2.20$, all $P < 0.027$) except on the belly ($t_{4183} = 0.29$, $P = 0.78$), whereas it remained unchanged in VI females (all $t_{4183} < 1.85$, all $P > 0.06$). Sexual dimorphism in spot diameter increased with age on all body parts of VV adults (all $t_{4183} > 4.34$, all $P < 0.001$), whereas in VI adults, sexual dimorphism increased on the belly ($t_{4183} = 2.82$, all $P = 0.005$) but remained constant on the other body parts (all $t_{4183} > 1.07$, all $P > 0.06$).

Discussion

Our study shows that polymorphism at the *MC1R* gene is associated with variation in pheomelanin- and eumelanin-based plumage traits as well as with feather pheomelanin and eumelanin contents in the barn owl. More importantly, our results indicate that *MC1R* genotypes differ in the degree of nestling sexual dimorphism and in age-related changes in the degree of adult sexual dimorphism. These findings are consistent with the hypothesis that even if natural selection is the major force promoting the evolution of MC1R-related variation in coloration (Kronforst *et al.* 2012), this gene may

also play a role when selection on coloration is sex specific by allowing for colour variation between sexes.

Polymorphism at the MC1R gene relates to colour variation in the barn owl

In Swiss barn owls, the *MC1R* sequence presents one relatively frequent nonsynonymous mutation at the position 126 (V126I). Recently, we have also confirmed the presence of this mutation (as well as its association with plumage coloration) in 21 other barn owl populations across Europe (R. Burri, S. Antoniazza, A. Gaigher, A. L. Ducrest, C. Simon, The European Barn owl Network, L. Fumagalli, J. Goudet, A. Roulin, unpublished data). The same mutation with similar effects on the phenotype has been reported in other bird species, which supports the existence of convergence at both genetic and phenotypic levels (Manceau *et al.* 2010). As observed here in the barn owl, the mutation V126I is present in the Gyrfalcon (*Falco rusticolus*) and in the domestic duck (*Anas platyrhynchos*), where this valine–isoleucine substitution is also associated with darker plumage colorations (Johnson *et al.* 2012; Zhan *et al.* 2012; Yu *et al.* 2013). The same mutation has been also observed in chickens (*Gallus gallus*), although no clear association with plumage coloration has been reported, probably because of the masking effect of closely linked mutations at the MC1R (Kerje *et al.* 2003; Dávila *et al.* 2014). The V126I mutation found here in the barn owl is located in the third transmembrane domain of the MC1R (Fig. 1B), which (together with the second domain) plays a key role in MC1R activation (García-Borrón *et al.* 2005). Actually, in humans, mutations at this location (e.g. M128T) induce a partial loss of function of the MC1R (MC1R exhibits a lower affinity to bind alpha-MSH and low coupling activity to cAMP; Pérez Oliva *et al.* 2009). These findings suggest that the V126I mutation found here could have a functional impact on the MC1R although, obviously, biochemical analyses are still needed, particularly because of the expected conservative changes (the two amino acids share physicochemical properties). We found a second nonsynonymous mutation at position 8 (R8H), which has been previously detected in the artic skua in association with plumage coloration (Janssen & Mundy 2013). The H-allele (associated with pale coloration in skuas) occurred at very low frequency (0.5%) in our studied population and in other European populations (R. Burri, S. Antoniazza, A. Gaigher, A. L. Ducrest, C. Simon, The European Barn owl Network, L. Fumagalli, J. Goudet, A. Roulin, unpublished data), although whether it could be at higher frequencies at other world populations deserves further attention (Roulin *et al.* 2009).

In the barn owl, the mutation V126I is strongly associated with plumage traits and, particularly, with the pheomelanin-based plumage. MC1R explained around the 33% of the variance in the reddish plumage coloration (~40% of the genetic variation; Roulin & Jensen 2015) and 47% of the variance in feather pheomelanin content (Table 2), which indicates that other genes involved in coloration are yet to be discovered in this species in contrast to other species where MC1R accounts for all variation in coloration (e.g. Gangoso *et al.* 2011). The *MC1R* gene accounts for a similar amount of variance in other species where adaptive melanin-based colour variation exists (e.g. in the beach mouse, *Peromyscus polionotus*; Hoekstra *et al.* 2006). Previous studies also support that variation in the pheomelanin-based coloration in the barn owl could have evolved as an adaptation to local selective pressures (Antoniazza *et al.* 2010, 2014), which is also in line with previous findings showing that alternative colour morphs exploit different physical habitats (red individuals tend to occupy less forested habitats and white individuals open landscapes) and prey on different rodent species (Roulin *et al.* 2004; Charter *et al.* 2012; Dreiss *et al.* 2012). The *MC1R* gene could be therefore an important part of the genetic underlying basis of such adaptive process, although the question that remains to be tackled is the implication that other loci may have in interaction with *MC1R* and whether variation at the *MC1R* gene drove local adaptation across Europe by merely altering the reddish coloration or also by pleiotropically affecting other traits (Mogil *et al.* 2003; Gangoso *et al.* 2011).

Variation at the *MC1R* gene was less markedly associated with eumelanic traits (Fig. 2), explaining between 0.04 and 5.9% of the variance in the number of spots (between <1% and 9.5% of the genetic variance) and 0.2 and 13.4% of the variance in spot size (between <1% and 5% of the genetic variance; Roulin & Jensen 2015). In the breast, *MC1R* affects the production of eumelanin pigments and spot number and size in the similar sense as for reddish plumage coloration (I-allele leads to a higher expression of eumelanin and pheomelanin; Figs 1C and 2), rather than to a higher expression of pheomelanin at the expense of eumelanin as observed in other species (Hubbard *et al.* 2010). In the other body parts, MC1R differentially affects the expression of eumelanic plumage traits, suggesting that other genes than *MC1R* may influence the overexpression of eumelanin at the specific time points when these spots are produced. The additive or epistatic action of other genes might be responsible for the large variation observed in the effect of *MC1R* on different body parts. While the effects of *MC1R* seem to be always incompletely dominant for the reddish plumage coloration, we observed in the number of spots the existence of dominance effects (heterozygous VI and homo-

zygous II were rather similarly coloured, whereas homozygous VV was clearly lighter coloured), opposite effects in males compared to females (on the belly, homozygous VV displays fewer spots in males but more in females compared to other genotypes) and effects only on heterozygous (heterosis) (on the wings, heterozygous males displayed more spots than homozygous II and VV males).

With respect to spot diameter, the impact of MC1R was exactly the opposite on different body parts, with the V-allele inducing larger black spots on the underside of the wings but smaller spots on the breast. Variation in plumage traits is pronounced not only between individuals but also within individuals. Thus, our results show that it is indeed the case with, for example for reddish coloration, the effect being less strong on the breast than on the belly, flank and wing, being stronger on the belly than on the flank and wing and being stronger on the wing than on the flank (Fig. 2). Similar variation in the strength of MC1R effects across body parts has been previously reported (e.g. Hoekstra *et al.* 2006), but, to our knowledge, variation in the direction of *MC1R* effects has never been reported in other species. This supports that *MC1R* can have an intricate effect on the expression of different plumage traits on different body parts, which suggests the existence of epistatic or additive effects between *MC1R* and other melanogenic genes.

Polymorphism at the MC1R gene and sexual dimorphism

We observed that the different genotypes at the *MC1R* gene differ in the degree of sexual dimorphism. In fledglings, we observed that homozygous VV individuals are more sexually dimorphic in all plumage traits than in the other genotypes (Fig. 3). Our results therefore suggest that the *MC1R* interacts in a nonadditive manner with the factors that determine colour variation between sexes in the barn owl. Otherwise, no significant effects of *MC1R* on the degree of sexual dimorphism would have been observed, which would have supported an additive effect (i.e. the *MC1R* gene affects coloration but with the same effect size on each sex). Nonadditive effects can result from epistatic effects (e.g. the phenotypic effects of the genes determining differences between sexes and age classes depend on the genotype at the *MC1R*) or from *MC1R* genotypes differing in their sensitivity to environmental conditions. The fact that colour traits are highly heritable in the barn owl and only very weakly sensitive to the environment (Roulin & Dijkstra 2003; Roulin *et al.* 2010) supports the existence of epistatic effects between the *MC1R* and genes inducing sexual dimorphism in coloration, although further studies are still needed to fully discard the existence of genotype-by-environment interactions.

By affecting sex-related colour variation, the way that the *MC1R* gene can drive the evolution of coloration grows in complexity. For instance, as observed here in the barn owl, *MC1R* affects the degree of sexual dimorphism of breast spots, a trait that has been shown to be under sexually antagonistic selection (large breast spots are favoured in yearling females but deselected in yearling males; Roulin *et al.* 2010). Homozygous females for the allele I exhibit larger spots and VV males exhibit smaller spots than other genotypes (Fig. 2C), suggesting that the I-allele and V-allele could be advantageous in females and males, respectively, and, moreover, that the *MC1R* could be responsible for the unsolved sexual conflict. However, we also observed that the V-allele allows for larger differences between sexes in breast spot size, supporting that this allele could still have a slightly higher advantage as it allows producing more sexually dimorphic offspring. Under this scenario, we would expect the V-allele to be more successful than the I-allele under sexual selection (or sex-specific natural selection). However, other factors should still be considered, particularly at the light of the multiple phenotypic effects of the alternative *MC1R* alleles shown by our study. Thus, as suggested above, the I-*MC1R* and V-*MC1R* variants may be subjected to local selection because of their effects on the reddish plumage coloration and, thus, the net selection on *MC1R* cannot be simply understood by its impact on spot size or in any single colour trait (the three plumage traits are genetically correlated; Roulin & Jensen 2015).

Moreover, we showed that *MC1R* genotypes also exhibit different patterns of colour maturation, affecting the degree of sexual dimorphism at different ages (Fig. 4). For some traits, for instance the diameter of breast black spots, sexual dimorphism increased with age in VV breeding birds but remained constant in VI individuals (Fig. 4C), reinforcing the pattern observed in nestlings (Fig. 3). Interestingly, *MC1R* age-related colour changes also led to opposite effects on sexual dimorphism in nestlings and in adults. For instance, differences between males and females in the reddish coloration of the belly (larger in VV than in II nestlings; Fig. 3) tend to disappear with age in VV adults but to increase in II adults (Fig. 4A). Therefore, net selection on *MC1R* has to be understood in a life history context, considering at what moment of the life, cycle selection is acting on coloration and the potential changes in the direction of selection that may occur across an individual lifetime. Although age-related changes in coloration are widespread, studies investigating selection in relation to coloration at different ages are generally lacking (although see Saino *et al.* 2013) and, to our knowledge,

no study investigated whether selection on *MC1R* varies across an individual's lifetime.

The *MC1R* gene is a remarkable example to understand the genetic basis of convergent evolution on melanin-based traits, particularly in response to strong natural selection, for instance, for background matching (Manceau *et al.* 2010). Here, we investigate the effects of *MC1R* on plumage colour traits of the barn owl but also its impact on sexual dimorphism, which is ubiquitous in animal populations. We showed that the *MC1R* gene explains a substantial part of variation in plumage traits in the barn owl but, moreover, that it has nonadditive effects on the degree of sexual dimorphism. These findings support that the evolution of colour variation through the *MC1R* gene is likely subjected to the interplay between multiple selective forces. Future studies are therefore needed to understand how often such forces conflict between each other and, for instance, whether pre-existing selection for sex-related colour variation hinders the evolution of adaptive colour variation through the *MC1R* gene. Such conflict is likely to occur given that often selection favours a concealed sex (usually females) and a more conspicuous sex (usually males).

Our study also provides answers to previous studies evidencing that the *MC1R* is somehow involved in the evolution of sexual dimorphism. Nadeau *et al.* (2007) showed that bird clades that evolved a more marked sexually dimorphic melanin-based coloration present a higher rate of amino acid changes (dN/dS) at the *MC1R* but not at other melanogenesis-related genes such as tyrosinase (*TYR*), tyrosinase-related protein-1 (*TYRP1*) and DOPA-chrome tautomerase (*DCT*). As observed here for the V-allele, some variants of *MC1R* allow for larger differences between sexes, suggesting that increased sexually dimorphism can evolve through the accumulation of *MC1R* mutations of similar effects. Our study offers a more complex picture of the potential effects of *MC1R* in coloration and highlights the need to approach the study of *MC1R* considering the action of the multiple selective forces acting on coloration.

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

Data accessibility

MC1R sequences: GenBank Accession nos.: KR018388, KR018389, KR018390, KR018391, KR018392. Phenotypic and genotypic data: Dryad repository: doi:10.5061/dryad.202f5.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Sample size in nestling barn owls of different *MC1R*-genotypes.

Table S2. Number of male and female nestling barn owls sampled between 1996 and 2013.

Table S3. Sample size of individual barn owls recaptured over several years.

Table S4. Sequences of the Primers used in this study.

Appendix S1. Supplementary Material and Methods

Appendix S2. Supplementary Results.