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Genetic factors implied in melanin-based coloration of the Italian wall lizard

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Abstract

As largely demonstrated for a wide range of vertebrates, the melanin-based coloration can be the effect of both mutations in the melanocortin-1 receptor (*MC1R*) gene and of differential expression of the same gene. However, in lizards, this mechanism is poorly known and some populations exhibit a high variation of melanism. Some populations of *Podarcis siculus* show a gradual melanization, suggesting a case of a quantitative phenomenon rather than a qualitative one. Here, we objectively quantify the continuous colour variation by spectrophotometric analysis demonstrating that the changes in the skin reflectance are not associated to mutations in the coding region of the *MC1R* gene but seem to be related to a concomitant variation of expression for this gene.

Introduction

Chromatic variation is one of the most perceptible phenotypic diversifications in animals. In general, it can be promoted by natural selection, sexual selection or genetic drift (Panhuis *et al*., 2001; Sobel *et al*., 2010; Butlin *et al*., 2012), while the habitat heterogeneity and the geographic isolation usually contribute at population level (Rundle & Nosil, 2005; Maan & Seehausen, 2011).

In natural populations, the colour patterns are involved in several biological processes, such as thermoregulation (De Jong, Gussekloo & Brakefield, 1996; Forsman, 1997; Vences *et al*., 2002), intraspecific communication (Majerus, 1998) and predation avoidance (Thayer, 1909; Cott, 1940; Fulgione *et al*., 2014). Moreover, some evidence show that the colour pattern represents a compromise between the sexual selection, for signalling functions (e.g. sexual/territorial signals and recognition of species), and the natural selection (Endler, 1978; Lythgoe, 1979; Andersson, 1994; Houde, 1997; Espmark, Amundsen & Rosenqvist, 2000; Dreher & Pröhl, 2014).

Melanin pigments provide the most widespread source of coloration in vertebrates. Most studies about pigmentation focus on the melanin system, primarily regulated by the melanocortin-1 receptor (*MC1R*) (Marklund *et al*., 1996*a*; Kijas *et al*., 1998, 2001; Rieder *et al*., 2001; Theron *et al*., 2001; Schmutz, Berryere & Goldfinch, 2002; Ducrest, Keller & Roulin, 2008; Fang *et al*., 2009; Skoglund & Höglund, 2010; Abitbol, Legrand & Tiret, 2014; Huang *et al*., 2014; Våge *et al*., 2014; Zhang, Xu & Luo, 2014). Although, there are few documented cases in which this gene is not implicated in the pigmentation process, such as some dog breeds (Kerns *et al*., 2004), mustelids (Hosoda *et al*., 2005) and some populations of pocket mice (Nachman, Hoekstra & D'Agostino, 2003).

Generally, the melanism can be the effect of mutations in the *MC1R* codogene region, alterations in the production of its ligands (*MSH*), or antagonists (*ASiP*), as well as the result of different gene expressions (Robbins *et al*., 1993; Klungland *et al*., 1995; Marklund *et al*., 1996*b*; Takeuchi *et al*., 1996; Våge *et al*., 1997; Rieder *et al*., 2001; Eizirik *et al*., 2003).

In mammals, a wide number of genes involved in the coat colour have been isolated and their functions have been well characterized (Bennett & Lamoreux, 2003). Coat colour shows many phenotypic variants regulated by a variety of genes often involved in genetic developmental defects, as observed in mouse, dog and human (Yen *et al*., 1994; Aberdam *et al*., 1998; Sturm *et al*., 2003; Anderson *et al*., 2009). Candille *et al*. (2007) reported that melanism in dogs is caused by a different component in the melanocortin pathway involving the *K* locus (*K* for blacK), in which a β-defensin protein encoded by *CBD103* gene acts as an alternative ligand for the *MC1R*. Overall, there are seven genes that influence the colour patterns in mammals and are involved in the complex pathways of the pigmentation process of the coat: *MC1R*, *AGEX*, *COMT*, *SILVER*, *TYRP*, *HTR* and *K* locus

(Abdel-Malek *et al*., 2001; Hoekstra *et al*., 2006; Candille *et al*., 2007). Nevertheless, all alleles have not yet been identified for each locus.

In birds, melanism is a ubiquitous component of plumage coloration implicated in a broad variety of functions (Barrowclough & Sibley, 1980; Bergman, 1982). The *MC1R* gene is a key regulator of melanin synthesis in feather melanocytes (Takeuchi *et al*., 1996; Theron *et al*., 2001; Mundy, 2005). In fact, this gene is associated with striking melanic plumage polymorphism in many avian taxa: bananaquit *Coereba flaveola* (Theron *et al*., 2001), snow geese *Anser caerulescens* (Mundy *et al*., 2004), arctic skua *Stercorarius parasiticus* (Mundy *et al*., 2004), white-winged fairywren *Malurus leucopterus* (Doucet *et al*., 2004), Eleonora's falcon *Falco eleonorae* (Gangoso *et al*., 2011) and domestic chicken *Gallus gallus* (Takeuchi *et al*., 1996; Andersson, 2003). In the tawny owl *Strix aluco*, colour variation is mainly due to the pheomelanin causing *MC1R* to account for 68% of the total variance in plumage coloration (Gasparini *et al*., 2009; Roulin & Ducrest, 2013; Scriba *et al*., 2013). Only two documented cases show the *MC1R* variation not associating with plumage polymorphism in blue-crowned manakin *Lepidothrix coronata* and leaf warblers *Phylloscopus trochiloides* (MacDougall-Shackleton, Blanchard & Gibbs, 2003; Cheviron, Hackett & Brumfield, 2006; Ducrest *et al*., 2008).

Teleosts have retained several colour genes as duplicates following the fish specific genome duplication (Braasch, Schartl & Volff, 2007). Several genetic pathways were well described in zebrafish *Danio rerio* and medaka *Oryzias latipes* (Parichy, 2003), for example, MSHs and ACTHs are implicated in physiological colour changes (Fujii, 2000; Richardson *et al*., 2008). The *MC1R* gene coding region has recently been characterized in this class and similarly to other vertebrates consisting of a single-copy, single-exon gene that encodes for a receptor with seven conserved transmembrane regions (Logan *et al*., 2003). A novel role for *MC1R* alterations was described for parallel evolution in cavefish *Astyanax mexicanus*, in which two distinct mutations cause reduced pigmentation associated with the brown mutant phenotype (Gross, Borowsky & Tabin, 2009).

Amphibians exhibit a wide colour variation within and between species due to the genetic basis of pigmentation (Hoffman & Blouin, 2000). The common frog *Rana temporaria* shows a sequence variation in the *MC1R* gene and does not explain the variation in the level of melanism (Herczeg, Matsuba & Merilä, 2010). Moreover, in some species, the melanization has been positively correlated to the heating rate (Vences *et al*., 2002).

In reptiles, the structural colours may be influenced at several levels, including testosterone-mediated melanin production, stress condition and spacing in iridophore platelets (Elkan & Cooper, 1980; Bagnara, Fernandez & Fujii, 2007). In some lizards, melanin-based coloration is due to *MC1R* (Rosenblum, Hoekstra & Nachman, 2004; Manceau *et al*., 2010; Kronforst *et al*., 2012), although the relationships between the genetic changes (mutation vs. regulation) and the colour pattern variability are largely unexplored. Sometimes pigmentations can result from convergent evolution. In fact, the effect of mutations in the *MC1R* gene shows that receptor functions, in two species of blanched forms (eastern fence lizard *Sceloporus undulatus* and little striped whiptail *Aspidoscelis inornata*), are altered in different ways (Rosenblum *et al*., 2010). Specifically, in *S. undulatus*, the mutation affects the receptor integration into the melanophore membrane, whereas in *A. inornata*, a different mutation affects the receptor signalling. All known mutations in the *MC1R* locus, many of which are adaptive, occur in the coding region either as amino acid changes or small deletions. However, it is important to note that the regulatory mechanisms that govern the expression of *MC1R* in lizards are poorly known.

We recently demonstrated that the *MC1R* gene expression and the MSH hormone plasmatic levels are higher in melanic lizards *Podarcis siculus* than its pale relative (Raia *et al*., 2010; Monti *et al*., 2013). Moreover, we did not find any association between the *MC1R* gene polymorphism and the skin coloration. Thus, the relationship between the melanic variants, both patterns of mutation, as well as the levels of gene expression was never tested. Here, we investigated the relationship between the colour (using skin reflectance) and the genetics (sequences vs. expression) in gradually differing melanic lizards.

Methods

Sampling

The Italian wall lizard is typically characterized by a white abdomen and a green back, whereas some melanic populations show a blue coloration on the abdomen. It is a species morphologically high variable so that a considerable number of subspecies has been described. The review of Henle & Klaver (1986) recognized a total number of 52 subspecies (Podnar, Mayer & Tvrtković, 2005).

We caught 30 individuals by nylon loop and analysed continuously melanic phenotypes, from three living populations, including the wild-type (WA-white abdomen, $n = 14$), bright blue (BB, $n = 10$) and the dark blue (DB, $n = 6$) lizards (Fig. 1).

Lizards were collected in three different sites of South Italy: WA from Campania mainland (40°50′N, 14°15′E), BB from the small islet of Licosa (40°15′N, 14°54′E) and DB from Faraglione of Capri (Scopolo – 40°32′N, 14°15′E). The latter two populations were already described as distinct subspecies: *Podarcis siculus klemmeri* Lanza & Capolongo, 1972 (BB) and *Podarcis siculus coeruleus* Eimer, 1872 (DB). All individuals were released at the point of capture after the measurements of reflectance and the sampling of tail by induced autotomy. Lizards were collected with the permissions of the county authorities. The experimental procedures were approved by the Ethical Committee for Animal Experiments, University of Naples Federico II (ID: 2013/0096988), and were according to Italian law (DL 26/2014).

Figure 1 Different levels of easily recognizable melanization in three individuals representing the range of a continuously varying colour trait in the Italian wall lizard *Podarcis siculus*: WA, wild type with a white abdomen and green back; BB, bright blue phenotype; DB, dark blue phenotype.

Skin reflectance

As demonstrated by previous studies (Shekar *et al.*, 2008), reflectance is one of the best indirect methods to objectively quantify the level of melanization on the skin. The animals' body coloration was determined by spectrophotometry (250–1000 nm, AvaSpec-2048-USB2-UA-50; Avantes, Apeldorn, Netherlands) for all samples. The measurement probe was held perpendicular to the body surface. The diameter of the spectrophotometer hole probe end covers a smaller surface area (0.2 mm) than of a single scale. A reflectance percentage tile (*R* %) was assayed among each individual. Lizard ventral skin coloration was measured by the reflectance at three positions on the throat. The average of reflectance was estimated for each phenotype. The spectral range was considered between 300 and 700 nm (according to Vroonen *et al.*, 2012). All measurements were expressed in relation to a white reference tile (WS2; Avantes).

The difference in the skin colour is outlined as a curve of reflectance in which the mode points out in the visible colour. Synthetic quantification of skin reflectance is the mathematical integral (*I*) under the spectrophotometric curve (Delegido *et al.*, 2010; Fulgione *et al.*, 2014). It was computed for the curve of each individual.

MC1R polymorphism

Total genomic DNA was extracted from 25 mg of tail tissue using QIAGEN DNeasy extraction kit (QIAGEN, Valencia, CA, USA), according to manufacturer's recommendations, for all samples. We amplified a region of 1500 base pair (bp), which includes the UTRs of the *MC1R* gene, using primer pair *p-MC1R-5′Pol-F* (5′-CTCACAGCTCTGTTTGAGAC ATC-3′) and *p-MC1R-3′Pol-R* (5′-CAGGTGCAGCAACAT TTTCC-3′) (Raia *et al.*, 2010). Polymerase chain reactions (PCR) were performed in a final volume of 20 μL, with 0.2 μL of *Pfu* DNA polymerase (Thermo Scientific, Waltham, MA, USA), 4 μL of 4X Tris buffer with $MgCl_2$, 1.6 μL of dNTPs (each dNTP 2.5 μM), 0.2 μL of 50 μM of each primer and 100 ng of DNA template under the following conditions: an initial denaturing step of 98°C for 3 min; 35 cycles of 10 s at 98°C, 30 s at 66°C and 1 min at 72°C; and a final extension step of 5 min at 72°C. PCR products were purified from unincorporated primers using Exonuclease I and Fast Alkaline Phosphatase (Thermo Scientific) or Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). The sequencing reaction was performed using the BigDye™ Terminator Cycle Sequencing chemistry (Applied Biosystems, Foster City, CA, USA). Sequences were purified using AutoSeq G-50 (Amersham, Uppsala, Sweden) spin columns and analysed by an ABI 3100 automated sequencing instrument (Perkin-Elmer, Genetic Analyzer, Foster City, CA, USA). Chromatograms were assembled and analysed using software Geneious version 5.4.3 (Biomatters, Auckland, New Zealand, available from http://www.geneious.com/). All sequence data generated in this study were deposited in GenBank (accession numbers KP119155–KP119160).

Real-Time PCR (RT-PCR) of *MC1R* gene

To determine the *MC1R* gene expression, total cellular RNA was isolated from tail tissue by using TRI Reagent (EuroClone, Milan, Italy) according to manufacturer's recommendations. Quantity and quality of the total RNA were determined by spectrometry and agarose gel electrophoresis, respectively. For cDNA synthesis with integrated removal of the genomic DNA contamination, QuantiTect® Reverse Transcription Kit (QIAGEN) was used as described by the manufacturer. We conducted a RT-PCR by using SYBR® Premix Ex Taq™ II (Tli RNaseH *Plus*) RR820A (TaKaRa, Dalian, China). PCR was performed in a final volume of 25 μL, with 2.5 μL of cDNA, 1 μL of each primer 10 μM, 8 μL of sterile distilled water and 12.5 μL of SYBR Premix Ex Taq II (2X) (TaKaRa). PCR cycling profile consisted of a cycle at 95°C for

30 s and 45 two-step cycles at 95°C for 15 s, at 60°C for 30 s and at 72°C for 20 s. Quantitative RT-PCR analysis was conducted by using the 2(−ΔΔC(T)) method (Livak & Schmittgen, 2001). RT-PCR was performed in a Rotor-Gene Q cycler (QIAGEN). For each RT-PCR experiment, data were normalized to the expression of the *β-actin* housekeeping gene. We analysed a fragment of 101 bp (from 275 to 375 nucleotide position) of the coding region of the *MC1R*. The primers used for each gene were as follows: *MC1R* forward 5′-TGGAGAC CCTCTTCATGCTTCT-3′, reverse 5′-GCTGCAGATCAG CATGTCCA-3′; *ACTB* forward 5′-GATCTGGCACCAC ACCTTCT-3′, reverse 5′-TCTTTTCTCTGTTGGCTTTGG-3′. Each sample was tested and run in duplicate. RT-PCR efficiency and dissociation curve are provided in the Supporting Information.

Results

The animals' body coloration, determined by spectrophotometry, showed three levels of the reflectance curve. White abdomen lizard exhibited the highest reflectance value, decreasing in blue bright and in dark blue gradually (Fig. 2).

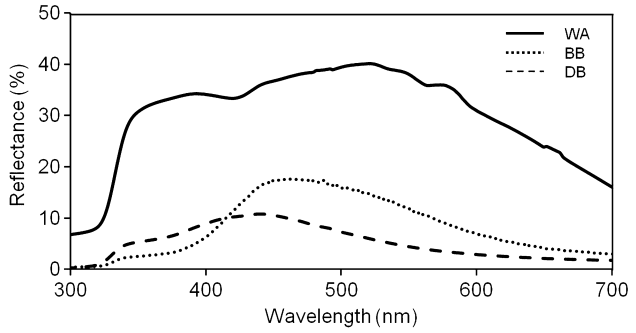

Figure 2 Pattern of skin reflectance (%) between 300 and 700 nm in the three phenotypes of lizards. Continuous line represents wild type with a white abdomen (WA); dotted line, bright blue phenotype (BB); dashed line, dark blue phenotype (DB). See Fig. 1 for direct comparison.

The average value of the integral, for each phenotype, shows significant differences [$I_{WA} = 12156.4 \pm 1215.6$; $I_{BB} = 3349.5 \pm 334.9$; $I_{DB} = 2099.7 \pm 209.9$; one-way analysis of variance (ANOVA) test $F = 428.166$, degrees of freedom (d.f.) = 2, P-values << 0.001].

The complete *MC1R* locus (945 bp) was analysed and sequenced in 30 individuals belonging to three phenotypes showing different colour patterns. A larger region of 1500 bp was amplified covering the whole coding region of the gene. Through the alignment of the complete coding region sequence (Accession No. GU225767.1), we identified the start and stop codons of the gene. Twelve polymorphic sites were detected, resulting in a total of six alleles (Table 1). All substitutions were synonymous and found in the third nucleotide position. There were no deletions/insertions in the *MC1R* coding region, and all of the mutations did not show any association to the different colour pattern groups (Fisher's exact test, $P > 0.05$). Wild-type populations (white abdomen) showed a larger number of alleles.

The expression of the *MC1R* gene was analysed by RT-PCR on RNAs from the skin of three phenotypes. The wild type was used to normalize the amount of mRNA in the two melanic populations. The expression pattern proved to be the inverse of the reflectance values. In the bright blue phenotype, the levels of expression were about 7-fold higher than the wild type, whereas in the dark blue, we found 20-fold amount of mRNA more than the wild type (Fig. 3).

Discussion

The Italian wall lizard populations showed several colour patterns due to the different degrees of melanin pigments in the skin (Fulgione *et al*., 2004; Bagnara *et al*., 2007; Corti *et al*., 2011). Our aim is to broaden the knowledge about the factors involved in the melanization in the Italian wall lizard. This process appears gradually quantitative and it can be objectively measured through the spectrophotometric technique. The gradual nature of the melanin-based coloration is an important peculiarity of lizard, which could be caused by gene regulation rather than single mutations in the *MC1R*

Table 1 Polymorphism in the *MC1R* coding region (945 bp)

	bp	42	54	108	123	150	180	270	351	390	594	732	930	#*N*
White abdomen		**A**	**A**	**G**	**C**	**G**	**C**	**C**	**C**	**A**	**C**	**G**	**G**	11
		G	C	A	–	–	G	–	–	G	T	–	C	2
		G	C	–	–	A	–	–	T	G	–	A	C	1
Bright blue		–	–	–	–	–	–	–	–	–	–	–	C	7
		–	–	–	–	–	–	T	–	–	–	–	C	3
Dark blue		–	–	–	T	–	–	–	–	–	–	–	C	6
Triplet codon		CC**A**	GC**A**	CA**G**	GG**C**	CT**G**	GC**C**	AA**C**	AA**C**	TC**A**	AT**C**	CT**G**	GT**G**	
Amino acid		Pro	Ala	Gln	Gly	Leu	Ala	Asn	Asn	Ser	Ile	Leu	Val	
Total														30

Sequences are grouped into three phenotypes: white abdomen, bright blue and dark blue. Allelic variants of the three populations are compared with the most frequent sequence (78% among the white abdomen population) selected as reference (highlighted in bold).

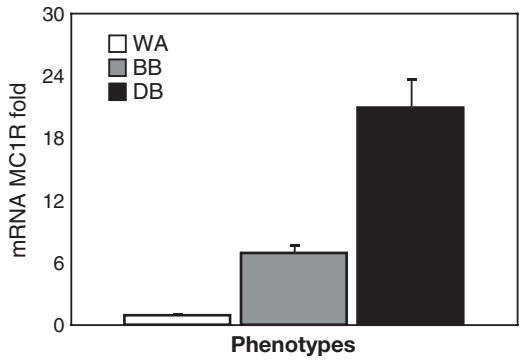

Figure 3 Expression levels (and 95% confidence intervals) for the *MC1R* gene determined by real-time polymerase chain reactions for the three phenotypes of lizard in skin tissues. WA, wild type; BB, bright blue; DB, dark blue. Values have been adjusted for the expression levels of WA phenotype.

codogene region. However, the function of the protein may be affected by mutations in different ways, causing phenotypes of different colour intensities (Moro, Ideta & Ifuku, 1999). To dispel this doubt, we analysed the sequence variations in *MC1R* gene, which did not show any associations between mutation patterns and level of melanin-based coloration. Therefore, we tested the alternative hypothesis. Our data from quantitative mRNA analysis show the differential expression of *MC1R* linked to the gradual variation in skin reflectance. The dark blue phenotypes possess just over 10-fold more mRNA than bright blue ones, and about 20-fold more than wild type. We suggest that either other genes or regulatory regions outside the coding sequence of *MC1R* might be responsible for the expression of melanism in *P. siculus* as well as in other vertebrates (Yen *et al*., 1994; Aberdam *et al*., 1998; Sturm *et al*., 2003; Candille *et al*., 2007; Anderson *et al*., 2009; Han *et al*., 2012).

Our study contributes to the understanding of genetic causal factors affecting pigmentation in vertebrates. In order to infer the variability among populations and its adaptive meaning, it is particularly important to improve case studies in which regulatory mechanisms drive colour changes.

According to our results, we hypothesize that new mutations allow populations to spread in environments in which different phenotypes results to be better adapted, with improved fitness, for example, mutations allowed the adaptation in rocket pocket mouse (Nachman *et al*., 2003) and white sands lizard (Rosenblum *et al*., 2010). Differently, when an evolutionary constraint acts in a way that the tempo and mode of the mutation are not advantageous, an alternative molecular mechanism could emerge (including the regulation of gene expression). Melanism in isolated blue lizard (Raia *et al*., 2010), sexual selection in blue-crowned manakin (Cheviron *et al*., 2006) and leaf warblers (MacDougall-Shackleton *et al*., 2003), and social interaction in tawny owl (Emaresi *et al*., 2013) could be focused on this scenario. In cases above, there is no evidence that sequence variation at *MC1R* is associated with colour variation.

Overall, our experimental approach can be applied to many colour phenotypes. In future studies exploring the regulatory functions and connecting the selection to colour variations among populations, it can complete the knowledge about the evolution of colour in vertebrates.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Dissociation curve for samples in real-time PCR for *MC1R* and *β-actin* showing a single peak, suggesting that only a specific PCR product was generated with this set of primers.
Figure S2. The efficiency value of real-time PCR is 0.94.