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Seeing through the skin: dermal light sensitivity provides cryptism in moorish gecko

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Abstract

Concealment by means of colour change is a pre-eminent deceptive mechanism used by both predators and prey. The moorish gecko *Tarentola mauritanica* is able to blend into the background by either darkening or paling according to the substrate darkness. Here we examined the functioning of background perception in moorish gecko. We experimentally excluded the involvement of melanophore-stimulating hormone in camouflage. Blindfolded individuals change their colour consistently with the background. Surprisingly, individuals with covered flanks were not able to change colour, no matter whether they were allowed to see the substrate or not. Accordingly, we found high levels of opsin transcript and protein in the flank region of the gecko. These observations suggest that *T. mauritanica* skin melanophores are able to activate a process of colour change autonomously. This study yields the first evidence of crypsis mediated by dermal light sensitivity in amniotes.

Introduction

It has long been recognized that camouflage through background colour matching is an important adaptation that helps prey to evade predators and predators to ambush prey (Cott, 1940; Darwin, 1974; Wallace, 1985; Stevens & Merilaita, 2011). Although animals living in stable and uniform habitats can achieve concealment by fixed colours or colour patterns, a more flexible camouflage system is better suited when background colours exhibit spatial or temporal heterogeneity, or when concealment conflicts with other functions of integumentation, such as communication, or thermoregulation (Stuart-Fox & Moussalli, 2009). Many animals change their own body colour in response to ontogenetic or seasonal variation in the environment. Yet, the most spectacular examples of camouflage occur in species that modify their body colour within minutes or even seconds. Famous examples include octopuses, which alter their colour pattern within fractions of a second (Hanlon, Forsythe & Joneschild, 1999; Hanlon *et al*., 2009) as well as in fiddler crabs (Thurman, 1988; Llandres *et al*., 2013). Rapid colour change, serving social display, was reported in African dwarf chameleons (Stuart-Fox, Whiting & Moussalli, 2006; Stuart-Fox & Moussalli, 2008).

Although 'fixed' concealment colours could be explained by simple fixation of particular colour patterns by means of natural selection (Norris & Lowe, 1964), rapid camouflage requires that the animal somehow perceives its environment and adequately adjusts its colour. In previous experiments, we showed that the moorish gecko *Tarentola mauritanica* adjusts its skin darkness to match the substrate tone, and requires light to achieve this goal (Vroonen *et al*., 2012). Light triggering possibly acts directly on skin melanophores, or indirectly through autonomic or humoral responses (Cooper & Greenberg, 1992; Oshima, 2001). However, the scientific literature is quiet on which stimuli and perceptual systems are involved in the assessment of background colouration, particularly in lizards.

Darkening in moorish gecko might be a general response involving the activation of melanophores via alpha-melanophore-stimulating hormone (α-MSH), or under nervous control as in chameleons (Stuart-Fox, Moussalli & Whiting, 2008). Alternatively, darkening might be a local response exerted directly by melanophores (i.e. without any involvement of either the nervous or endocrine system). The perception of background shade change should have an almost instantaneous effect if the nervous system is involved, whereas colour change mediated by either the endocrine system or local cell response happens within minutes to hours (Fujii, 2000; Ban *et al*., 2005). Which of these systems, or combination of, affects melanophores functioning in the moorish gecko is unknown.

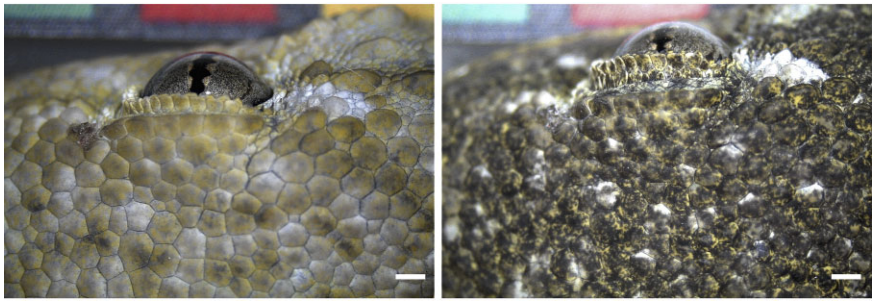

Figure 1 Change in colour of moorish gecko's skin surface. Images show the same spot of the gecko on light substrate (left) and on dark substrate (right). Scale bar = 1 mm.

Here, we address the question of how the moorish gecko obtains and processes information on background shading to modify its body colour. We specifically tested the contribution of the eyes, the central nervous system and the endocrine system in detection of light reflectance by the substrate.

Our results suggest that moorish gecko uses a previously unreported system based on dermal light sensitivity to perceive background coloration.

Material and methods

Sampling

We caught 40 *T. mauritanica* individuals by noose near Cilento, Italy (40°15′N, 14°54′E). During field work, we repeatedly observed the presence of barn owl *Tyto alba* as well as other predators that usually affect gecko population (Costantini *et al.*, 2005; Roulin & Dubey, 2012).

After experimentation, 30 individuals were released at the point of capture and 10 individuals were sacrificed for tissue analysis.

The animals were collected with the permissions from the county authorities (Cilento, Vallo di Diano and Alburni National Park prot. 0010678/ 2013). The animals were kept according to ministerial authorization (prot. 165/2006). The experiment was approved by the Ethical Committee for Animal Experiments University of Naples Federico II (ID: 2013/0096988) and according to Italian law (DL 116/92).

Skin reflectance

The animals' body colouration was determined by spectrophotometry on 15 individuals (Avantes, AvaSpec – 2048-USB2-UA-50, 250–1000 nm). The measurement probe was held perpendicular to the body surface. The diameter of the spectrophotometer hole probe end covers an area (0.2 mm) smaller than the surface of a single scale (see Fig. 1).

A reference percentage tile (R%) was assayed among each individual. Geckos' dorsal skin coloration was measured by the reflectance at six positions: (1) on the head; (2) between the forelimbs; (3) mediosagital; (4) mediolateral left and (5) right; and (6) between the hind limbs. Reflectance for wavelengths was considered between 300 and 700 nm (according to Vroonen *et al.*, 2012). The average of the integrals subtended the reflectance curve within the range of wavelength considered was assumed to be representative of the whole back.

Enzyme-linked immunosorbent assay (ELISA) detection of α-MSH

To test the idea that darkening in the moorish gecko involves the activation of melanine production in melanophores by α-MSH (i.e. via the endocrine system), we determined hormonal variation in individual geckos before and after the experimental induction of skin darkening. α-MSH levels in gecko sera were determined by ELISA assay, as described in Monti *et al.* (2013). For α-MSH assay, a blood sample of *c.* 50 μL was taken from the tail vein. Each sample was fractionated by centrifugation at 2300 g. for 15 min. The individual variation in α-MSH levels under the two light conditions was compared by means of paired *t*-test.

Testing light sensitivity of different body regions

We tested different body regions for light sensitivity. At the beginning of each trial, the gecko individual was placed in a small terrarium (15.5 × 25 × 18 cm). The bottom and adjacent sides of the terrarium were covered with either black or white paper (white or black box, substrate colour treatment). The top side was left transparent. The terrarium was then placed in a large incubator (Mir253; Sanyo, Bensenville, IL, USA) at 30°C. After 3 h, the gecko was removed from the incubator and its dorsal skin reflectance was measured immediately. Geckos were handled as little as possible to avoid colour changes due to stress: spectrophotometric measurements were collected in less than a minute.

To assess the contribution of the eyes to the perception of background colour, we performed experimental trials with blindfolded animals. In particular, for every gecko tested, measurements were performed in the absence of bandages, by bandaging the gecko's eyes or by covering the animal's thorax (body treatment). On each gecko the experiment was performed at least twice. Fifteen different *T. mauritanica* individuals were tested overall.

Gene expression of photosensitive protein SWS1 in gecko

We tested for gene expression of photosensitive protein SWS1 in gecko. In many vertebrates, the perception of substrate colour via the skin (rather than the eyes) was found to be local (i.e. based on direct action of the melanophores, Bagnara, 1957; Van Der Lek *et al.*, 1958; Oshima *et al.*, 1998). In the eye tissue of other gekkonids (i.e. tokay gecko, *Gekko gecko*), photosensitive pigments such as SWS1 (short wavelength-sensitive opsin) were observed (Kojima *et al.*, 1992; Loew, 1994). SWS1 opsin is sensitive to short wavelengths and is therefore expected to be present in *T. mauritanica* eyes. In order to measure the amount of SWS1 opsin present in each analysed tissue sample, we performed a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) on SWS1 mRNA. To obtain specific primers to be used in the RT-PCR, SWS1 gene, *exon* 4, was aligned among homologous sequences of *G. gecko* (accession number AY024356), *Anolis carolinensis* (exon 4 accession number ACSWSOPS3) and *Iguana iguana* (accession number AB626972). Primers were designed using the Geneious tools, Primer3 v.0.4.0 (Untergrasser *et al.*, 2012) and used to amplify and sequence the 177 base pairs (bp) of the codogenic SWS1 exon 4 in moorish gecko. The PCR reaction was performed in a DNA thermal cycler (Perkin Elmer Life Science, Waltham, MA, USA) as follows: 1 cycle at 45°C for 45 min for the reverse transcription followed by 2 min at 95°C; 40 cycles, each including 30 s at 94°C, 1 min at 60°C and 2 min at 68°C. After the last cycle, samples were kept for 7 min at 68°C and then stored at 4°C. Amplified PCR products were sequenced using Big Dye Terminator 1.1 (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using Geneious Basic program 5.5.3 (created by Biomatters; available from https://www.geneious.com). Homologous primers for the RT-PCR were then designed. Primers were forward, 5′-CGGGAG GTGTCCCGGATGGT-3′; reverse, 5′-GTAGATGATG GGGTTGTAGA-3′ (moorish gecko SWS1 mRNA, GenBank accession number KF803233). Endogenous actin was used as housekeeping gene for normalizing RNA expression; primer sequences were forward, 5′-ATCACT ATTGGCAACGAGC-3′; reverse, 5′-GGTCTTTACGG ATGTCAACG-3′.

Tissues from belly, flank, back, eyes and heart from 10 geckos were sampled and lysed with RNeasy mini kit (Qiagen Turnberry Lane, Valencia, CA) to isolate total RNA as described by the manufacturer. Total RNA was resuspended in 80 μL of RNase-free water, quantified using a NanoDrop 1000 (Thermo Scientific Inc., Waltham, MA USA) and 200 ng was reverse transcribed using Access RT-PCR System kit (Promega Fitchburg, WI, USA). Cycling parameters included a single-step cycle at 45°C for 45 min followed by 95°C for 2 min. Afterwards, we performed a semi-quantitative PCR using 30 cycles at 94°C 30 s, 60°C 1 min and 68°C 2 min. A final extension step was performed at 68°C for 7 min. PCR products were visualized by 1.5% agarose gel electrophoresis.

Western blot analyses

SWS1 opsin expression was analysed on 10 individuals by Western blotting of proteins obtained from tissue samples taken from the belly, flank, back, eyes and from the heart. The tissues were resuspended in radioimmunoprecipitation assay buffer containing protease inhibitors (Roche, Sandhofer Straße Mannheim, Germany) and lysed on ice by homogenization (200 strokes per sample) and sonication (2 min). Lysates were obtained by centrifugation at 14 000 *g* for 30 min at 4°C. Following the determination of protein content by the Bradford assay (Sigma Aldrich St. Louis, MO, USA), 100 μg of total proteins was analysed by 15% polyacrylamide sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by Western blotting with anti-opsin antibodies (1:1000 dilution) (Thermo Scientific). Only in the case of eye's sample, the total amount of protein analysed was 20 μg. For each sample, the protein intensity level was normalized to endogenous actin using anti-actin polyclonal antibodies (1:1000 dilution) (Sigma Aldrich).

Immunohistochemistry

Skin pigmentation in reptiles depends on melanophores, which synthesize and/or store pigments or light reflecting structures (Leclercq, Taylor & Migaud, 2009). We analysed the skin of geckos to detect the morphology of melanophores and their relationship with the opsin photosensitive protein. All skin samples (belly, flank, back) were fixed in Lillie's solution for 48 h, which acts also as decalcifier. Samples were embedded in paraffin and sliced into 5 μm thick sections. Some of the slides were stained with haematoxylin-eosin to look at their morphology while other serial slides, after deparaffinization, were subjected to decolourization of the melanin in 10% H_2O_2 for 48 h. The latter slides were processed in 10 mM sodium citrate (pH 6.0) for heat-induced epitope retrieval. Slides were then rinsed in 0.05% Tween 20 (Sigma Aldrich) in 0.1 M phosphate buffered saline (PBS) pH 7.4 (PBS-T20) for 30 min and incubated for 15 min in PBS with 3% H_2O_2 to block endogenous peroxidases. After blocking in 2% goat serum in PBS-T20 with 1% bovine serum albumin (BSA), sections were incubated with polyclonal anti-opsin antibody (1:800 dilution in PBS-T20 with 1% BSA; Thermo Scientific) at 4°C overnight. Sections were then washed twice in PBS-T20 and incubated with a peroxidase-conjugated secondary antibody (IgG anti-rabbit, 1:300 diluted in PBS-T20 with 1% BSA; Sigma Aldrich) for 1 h. Binding sites were revealed by 4-chloro-1-naphthol reaction according to the manufacturer instructions. Finally, slides were mounted with Aqua-Mount mounting medium (ThermoFisher Scientific, Hudson, NH, USA).

Results

α-MSH determination

According to the literature, skin darkening in lizard can be imputable to plasmatic α-MSH (Raia *et al.*, 2010; Monti *et al.*, 2013). As a consequence, we first tested the correlation

between the plasma level of this hormone and skin reflectance of either black or white geckos. Individuals treated in the laboratory to darken their skin colour show no significant increase in α-MSH concentration (paired sample *t*-test, $P = 0.332$).

Light sensitivity of different body regions

The gecko's ability to change colour in the presence of different substrates was observed (Fig. 1) and estimated by spectrophotometry (Figs 2 and 3). As shown in Fig. 2, geckos without bandages adjusted their body colour to the black substrate on which they had resided for 3 h (see also Vroonen *et al.*, 2012). When blindfolded, the geckos retained the ability to adjust their colour to the black box, although no appreciable change in skin reflectance was observed when the thorax was covered (Fig. 2).

Therefore, we measured the skin reflectance of geckos after 3 h in white or black boxes under three different experimental conditions: without any bandages, blindfolded and with the

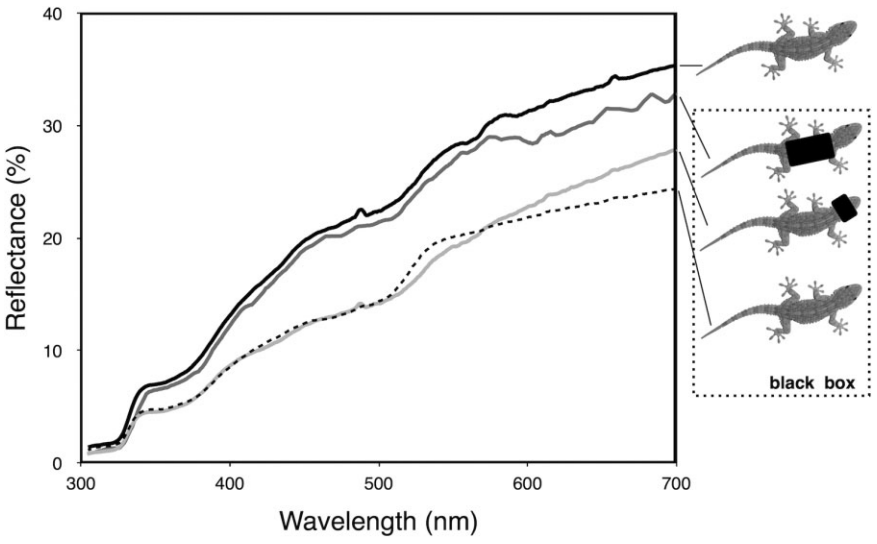

Figure 2 Mean reflectance spectra (%) of *Tarentola mauritanica* under different experimental conditions. From the top: pale gecko (just caught), covered thorax, bandaged eyes and without bandages (the last three measurements were performed after 3 h of immersion in a black box).

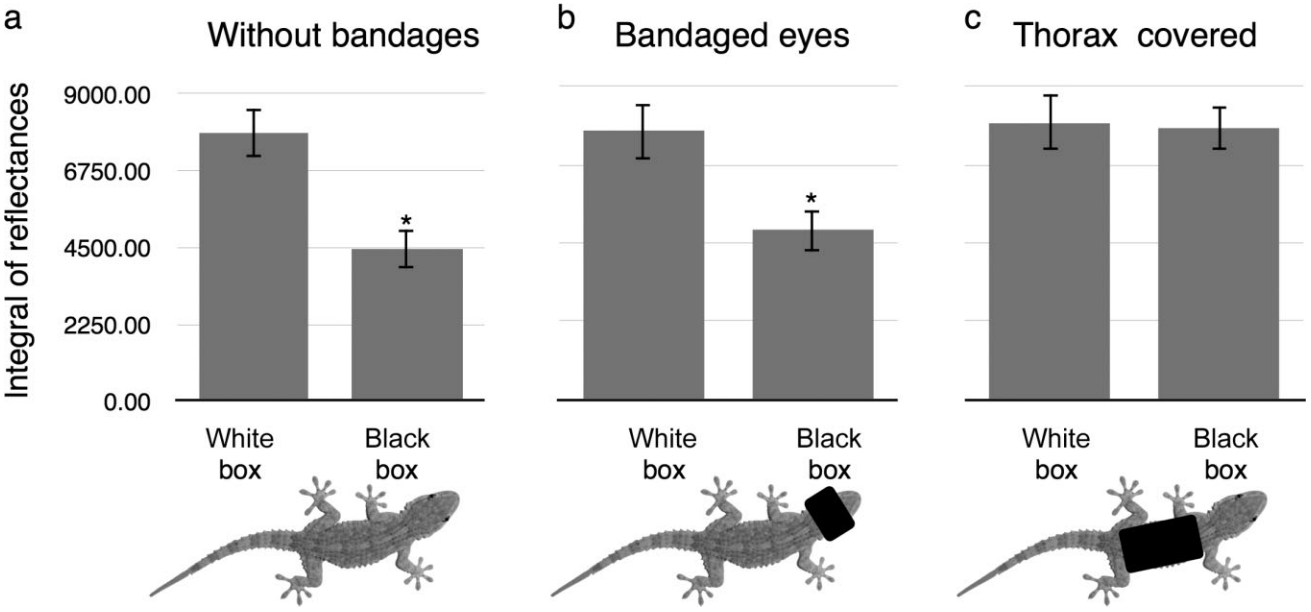

Figure 3 Spectrophotometric analysis of moorish gecko. Reflectance spectra were measured on geckos without bandages (a), with bandaged eyes (b) and with the thorax covered (both flanks and undersides) (c). Reflectance after 3 h of immersion in white and black boxes is reported. Histograms represent the mean values of the mathematical integrals under the spectrophotometric curves (significant differences were assessed using paired sample *t*-test; * indicates $P < 0.001$).

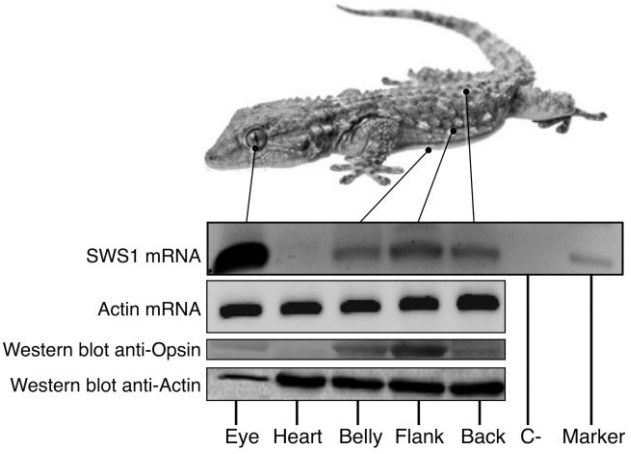

Figure 4 Opsin expression in moorish gecko. Reverse transcriptase polymerase chain reaction (RT-PCR; upper panels) and Western blot analysis (lower panels) of opsin SWS1 gene and protein expression, respectively. C-, negative control of the RT-PCR. For both RT-PCR and Western blot analyses, endogenous actin was used as an internal standard. Western blot was performed using anti-opsin and anti-actin antibodies. Total eye's proteins loaded in the Western blot were five times lower than in the other samples.

thorax covered. We observed that average skin reflectance of geckos without bandages was much higher after the animals had been exposed to white substrate than when kept on black one [Fig. 3a; paired sample *t*-test; $t = 15.6$, degrees of freedom (d.f.) = 14, $P < 0.001$]. A similar response was observed when the animals were blindfolded (Fig. 3b; paired sample *t*-test; $t = 8.07$, d.f. = 14, $P < 0.001$). Interestingly, no difference in reflectance between substrate treatments was observed when geckos had their thorax covered (Fig. 3c; paired sample *t*-test; $t = 0.47$, d.f. = 14, $P = 0.646$).

Expression of SWS1 opsin and immunohistochemistry of melanophores

As SWS1 opsin is sensitive to short wavelengths and is therefore expected to be present in *T. mauritanica* eyes, we performed a semi-quantitative RT-PCR analysis and observed a strong expression of the SWS1 opsin gene in geckos eyes (Fig. 4). Yet, SWS1 mRNA was also observed in skin tissues taken from belly, back and flanks. Heart tissue samples were used as negative controls for SWS1 RNA expression. In keeping with RT-PCR, Western blot analysis showed that opsin protein was present in eyes, as well as in skin cells of the trunk (belly, flank and back). In particular, we observed distinctively higher levels of opsin protein in the flanks than elsewhere (Fig. 4). As expected, no trace of either SWS1 mRNA or related protein was present in the heart. This important result was further borne out by immunohistochemistry analysis. Melanophores in the skin are rich in melanin (Fig. 5). Importantly, they are sensitive to opsin antibody. This suggests that the moorish gecko's melanophores are light sensitive and can therefore be held responsible for skin darkening.

Discussion

Non-fixed body colouration accrues to a number of species and body parts. Many of them serve the goal of concealment from predators or from vigilant prey, which is known as cryptism. Colour change in animals may involve either the nervous or endocrine system (a secondary response), or directly light-sensible cells in the outer layer of the organism, a primary response (Oshima *et al.*, 1998). The adaptive significance of such changes, whether it is cryptism or any feasible alternative, is little known in many cases. Cryptic colour change triggered by dermal light perception (i.e. based on direct action of the melanophores) has been noted in several non-amniotes, but seems to be quite rare in other vertebrates (Bagnara, 1957; Van Der Lek *et al.*, 1958; Lythgoe, Shand & Foster, 1984; Oshima *et al.*, 1998; Ban *et al.*, 2005; Kasai & Oshima, 2006). It has been demonstrated that in tilapia's (*Oreochromis niloticus*) skin multiple types of visual pigments are present, suggesting that dermal colour and pattern changes for camouflage and communication are regulated at the level of the integument (Ban *et al.*, 2005; Chen, Robertson & Hawryshyn, 2013). Recently, the expression of opsin in the skin and its putative role in 'distributed sensing' and camouflage has been reported in cuttlefish (Mäthger, Roberts & Hanlon, 2010). Tail darkening in hypophysectomized tadpoles held under dark conditions has an obvious cryptic function (Bagnara, 1957). Although few pioneering experiments (e.g. Parker, 1938; reviewed in Cooper & Greenberg, 1992) showed that this might be true for some lizards as well, little or no in-depth analysis of the mechanisms involved was surprisingly developed until now, despite the obvious importance of understanding cryptism as an anti-predatory strategy in amniotes (Meunier *et al.*, 2011). In a previous study, we demonstrated that the moorish gecko adjusts its skin darkness to match the substrate. This change in color only happens in daylight (Vroonen *et al.*, 2012). We started the present study thinking that the moorish gecko represents an ideal model to answer to some questions about colour perception and change. With this in mind, we investigated features involved in the sensorial dimension (visual) of this lizard, in order to clarify the mechanism and evolutionary constraints that induced its cryptism. The evidences we gathered in the present study (such as the coverage effects on substrate matching, the performance and the tissue-dependent opsin expression) indicate that background brightness is perceived by skin receptors, irrespective of circulating levels of α-MSH. In particular, we experimentally proved that the eye is not determinant in *T. mauritanica* skin colour change, as it is able to change colour even when blindfolded. Interestingly, we observed that the slow process of accommodation of the skin colour does not work when the thorax was completely obscured, suggesting that colour perception is mediated by this region of the body. Accordingly, we showed evidence for opsin expression in the skin, both at mRNA and

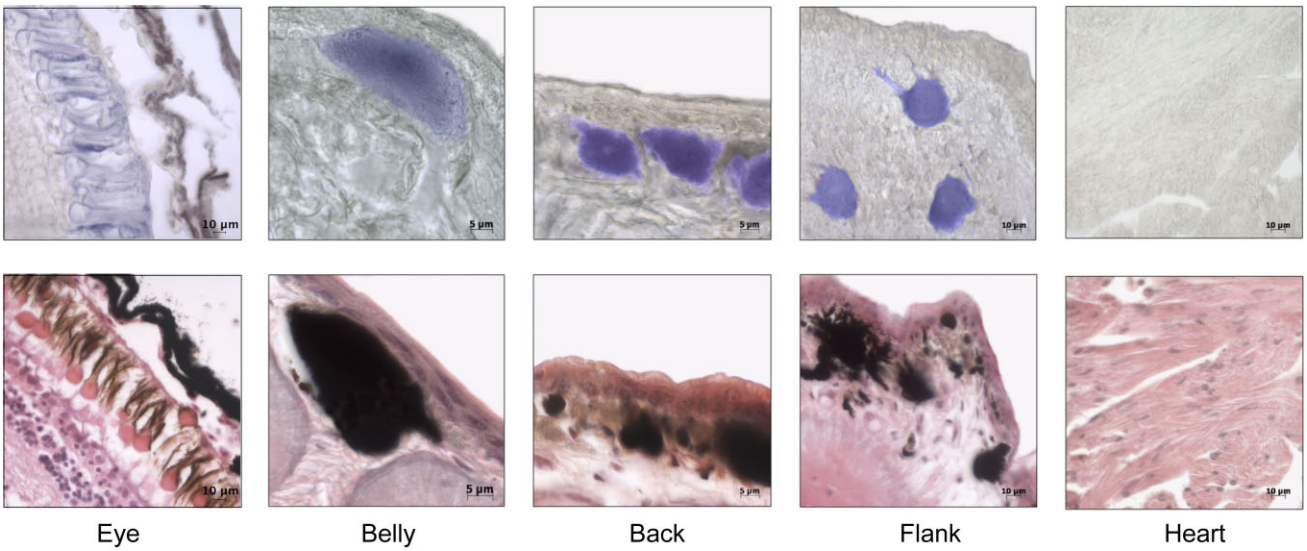

Figure 5 Sample sections in moorish gecko's eye, skin (belly, back and flank) and heart. Top: immunoreactivity with anti-opsin antibody shows the presence of opsin (blue) in cells of the eye and skin, but not in heart; bottom: haematoxylin–eosin analysis shows from left to right, retina with cone in eyes, skin (belly, back and flank) with brown melanophore and heart tissue.

protein levels. Histological analysis also showed the presence of opsin in the dermis, strongly linked to the melanophores. Our findings indicate that the gecko's skin acts as both a receptor and effector of skin darkening, independently from visual inputs. In other words, the gecko's skin is photosensitive, similar to that of other non-amniotes, and melanophores are able to activate skin darkening autonomously.

These results open room to further scientific questions, such as how the light brightness information, which is mainly processed at the level of the flanks, is transmitted to the back of the trunk, where colour change most prominently appears (Vroonen *et al*., 2012).

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