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When pink is a question: Comparative gross and microscopic skin structure analyses reveal the histological basis of skin colour in Galápagos pink land iguanas (*Conolophus marthae*)

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Abstract

One of the rarest and most unusual iguanas on the planet is the Galápagos pink land iguana (*Conolophus marthae*). There have been a number of hypotheses on the source of their pink coloration, including that the colour is from blood and a relative lack of dermal pigmentation. We obtained full thickness skin biopsies of three species and compared tissue from darkly pigmented areas and lightly pigmented surfaces. "Pink" areas of pink iguanas are devoid of pigment cells (e.g. melanophores) and the dermal tissue is rich with aggregates of confluent capillaries. This was in sharp contrast to the minimally vascular (only capillaries were observed) dermal areas of the marine and yellow iguanas. The dermal stratum laxum of every biopsy site contained melanophores except for the pink skin of pink iguanas. Interestingly, marine iguanas have a much thicker epidermal stratum germinativum/granulosum, between 2 and 10 cells thick depending on location, compared to the thinner epidermal stratum germinativum/granulosum of land iguanas (one to three cells thick with most areas possessing just one or two cell layers). These microscopic differences might reflect differences in habitat and ecology of the three species.

KEYWORDS

chromatophores, epidermis, melanophores, skin pigmentation, squamates

1 | INTRODUCTION

When Steven Tyler made his assertive statement "Pink is not even a question" (Aerosmith, 1997), he did not refer to the strange pink colour exhibited by one of the most enigmatic species of iguana on this planet: The Galápagos pink land iguana. Indeed, the Galápagos archipelago is home to four species of iguanas: marine iguanas (*Amblyrhynchus cristatus*), common Galápagos land iguanas (*Conolophus subcristatus*), Barrington land iguanas (*C. pallidus*) and Galápagos pink land iguanas (hereafter simply pink iguanas, *C. marthae*). This last species is restricted to Wolf Volcano on Isabela Island (Galápagos) and is listed as critically endangered in the IUCN Red List of Threatened Species (Gentile, 2012). The strange coloration of the species represents one of the most intriguing evolutionary open questions about this reptile since its discovery and description (Gentile et al., 2009; Gentile & Snell, 2009).

In general, coloration in animals may result from pigments that selectively absorb specific wavelengths of light and reflect light of other wavelengths. However, coloration may also be structural, being determined by the interaction between incident light and ordered tissues, leading to light interferences (Andrade & Carneiro, 2021; Olsson et al., 2013). In some cases, coloration may arise from an interaction between pigmentary and structural mechanisms (Shawkey & D'Alba, 2017). In the original description of pink iguanas, Gentile and Snell (2009) noted that when one pink scale was surgically removed, "blood flowed out of the tissue of the removed scale, which immediately lost its pink color" and became white. For this reason, it could be reasonably theorized that what appears as pink colour in the skin of *C. marthae* is blood visible through the skin, in the absence of melanin. Melanin, present in melanophores, is not the only molecule regulating skin pigmentation in reptiles. Several other kinds of substances exist in squamates, located in different chromatophores that lie in different skin layers. For example, transparent nanocrystals of guanine are in iridophores and contribute structural pigmentation, whereas pterines and carotenoids (in xanthophores and erythrophores) contribute pigmentary coloration (Andrade & Carneiro, 2021; Monahan et al., 2022; Nordlund et al., 2008; Rutland et al., 2019; Saenko et al., 2013). Previous studies confirmed the presence of carotenoids in the blood of *Conolophus*, with lutein and zeaxanthin found as the prevalent molecules (Costantini et al., 2005). Costantini et al. (2005) did not extend the investigation to skin, but lutein and zeaxanthin were the most abundant carotenoids in several tissue samples, including blood and skin of the squamate *Lacerta vivipara* (San-Jose et al., 2012). In humans, supplementation with lutein and zeaxanthin may significantly increase skin-lightening and reduce sallowness. These carotenoids filter blue light and protect skin from environmental factors including high-energy sources. However, they are also able to block the formation of melanin pathways (Juturu et al., 2022).

Pigmentation is certainly a character under selection in animals and the emergence of some phenotypic traits may also be favoured by selection acting on a small proportion of the variants associated with the trait (Ju & Mathieson, 2021). In reptiles, besides having several genetic and histological bases, skin pigmentation may have several functions (Kuriyama et al., 2019). Skin pigments may be involved in intraspecific and interspecific communication in different contexts such as reproduction, camouflage, aposematism and mimicry. It can also serve as health indicators (immunocompetence, parasite load, oxidative stress and ageing) and assist in thermal regulation. Last, skin pigments may provide protection from high solar radiation (Bagnara & Matsumoto, 2002). The lack of melanin or other pigments in large areas of the body of the pink iguana and the direct exposure of blood and tissues to the high UVB radiation of the area where the species lives (up to 600 $\mu W/cm^2$; Di Giacomo et al., 2022) could have severe consequences. Preliminary analyses indicated that UVB radiation at Wolf Volcano that may penetrate deep in the dermis of pink iguanas could be associated with a rate of DNA damage higher than in other, fully pigmented, Galápagos iguana species (Gustavino et al., 2014). It is then conceivable that, in pink iguanas, the detrimental effect of excessive UVB exposure may drive the regulation of basking behaviour and even habitat preferences of pink iguanas (Di Giacomo et al., 2022).

While awaiting genetic and ecological studies that fully address the selective advantage (if any) of being pink, it is important to clarify the histological basis of this peculiar pigmentation pattern of pink iguanas. For this reason, we conducted a complete and comparative microscopic examination of differently pigmented skin samples from three of the four Galápagos iguana species. *Amblyrhynchus cristatus*, *C. subcristatus* and *C. marthae*, are iguana species all endemic to the Galápagos islands. Whereas *A. cristatus* and *C. subcristatus* have a wide distribution throughout the archipelago, *C. marthae* is restricted to Wolf Volcano on Isabela Island. As part of a population health assessment authorized by the Galápagos National Park (GNP), wild iguanas from Isabela were captured in September 2019 (Colosimo et al., 2022). Veterinary health examinations that included sampling blood, ectoparasites and faeces were performed on each animal. During this sampling, skin biopsies were obtained from individuals of *A. cristatus*, *C. subcristatus*, and *C. marthae* to determine the microscopic skin anatomy. With consideration for animal welfare, and to make sure we were in compliance with the rules of the Galápagos National Park and our scientific

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permits, we only obtained skin biopsies from a very small number of iguanas.

2 | MATERIALS AND METHODS

2.1 | Sampling procedure

Three iguanas, one from each species, were hand captured and manually restrained for the biopsy procedure. Two areas on the left dorsal forelimb of each iguana were cleaned with 70% ethanol-soaked gauze pads followed by the application of topical lidocaine gel (Akorn Pharmaceuticals). After several minutes a 5mm Miltex® biopsy punch (Integra Life Sciences) was used to remove a full thickness section of skin. This tissue sample was immediately placed in 10% neutral buffered formalin and stored at ambient temperature. Haemostasis was achieved with direct pressure from a cotton-tipped applicator, a drop of lidocaine gel was applied, and the wound was closed with a 4–0 polydioxanone (Ethicon Inc.) on a cutting needle employing a single horizontal mattress suture. For all three animals this process was repeated in order to obtain tissue samples from visibly black-pigmented and non-black-pigmented areas.

2.2 | Sample preparation

Tissue samples were fixed in 10% neutral buffered formalin for 18 months and bisected perpendicularly to the point of the scale. Both bisected sections of all tissues were then processed routinely, embedded in paraffin, sectioned at 5 microns, stained with haematoxylin and eosin (HE), and three serial sections of each were examined by light and virtual microscopy up to a magnification of 600×.

3 | RESULTS

3.1 | Sample interpretation

Histology images and description of the sections from each animal are provided in Figures 1–3 and Table 1, respectively. The "pink" area of pink iguanas is devoid of pigmented cells (e.g. melanocytes) and the stratum laxum is rich with aggregates of confluent capillaries lined by endothelial cells. This was in sharp contrast to the low numbers, and much smaller diameter capillaries of the marine and yellow iguanas. The dermal stratum laxum of every biopsy site contained melanophores except for the pink skin sample of the pink iguana. The thickness and cell

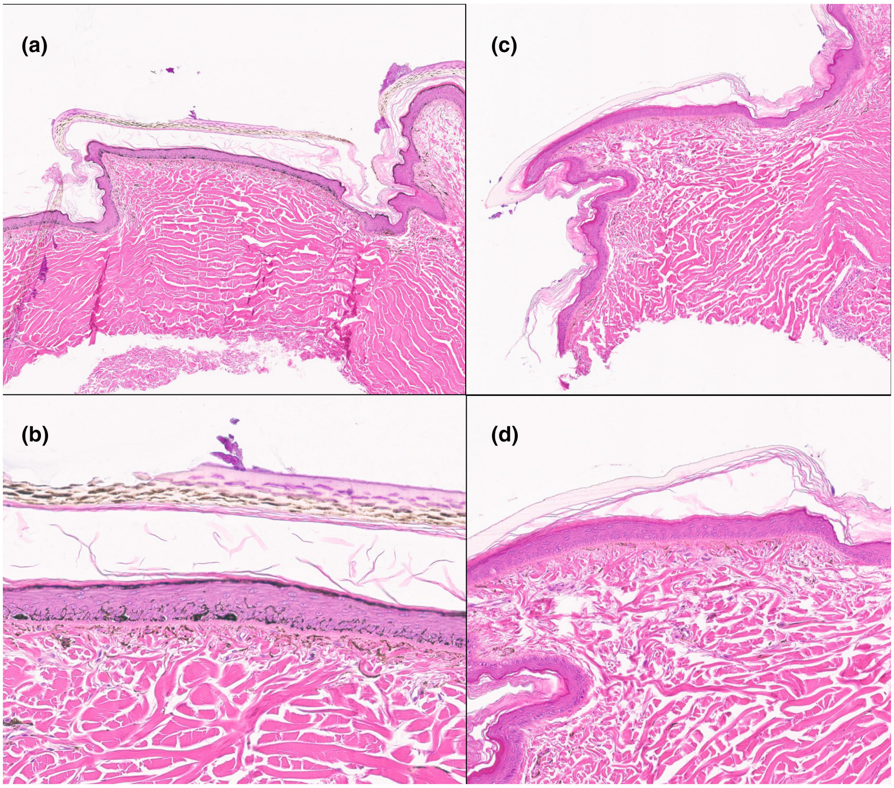

FIGURE 1 Histomorphology of the left forelimb skin sample of a marine iguana (*Amblyrhynchus cristatus*). (a, b) Black-pigmented region (c, d). White region (note the minimal number of melanocytes). Virtual sections of haematoxylin and eosin.

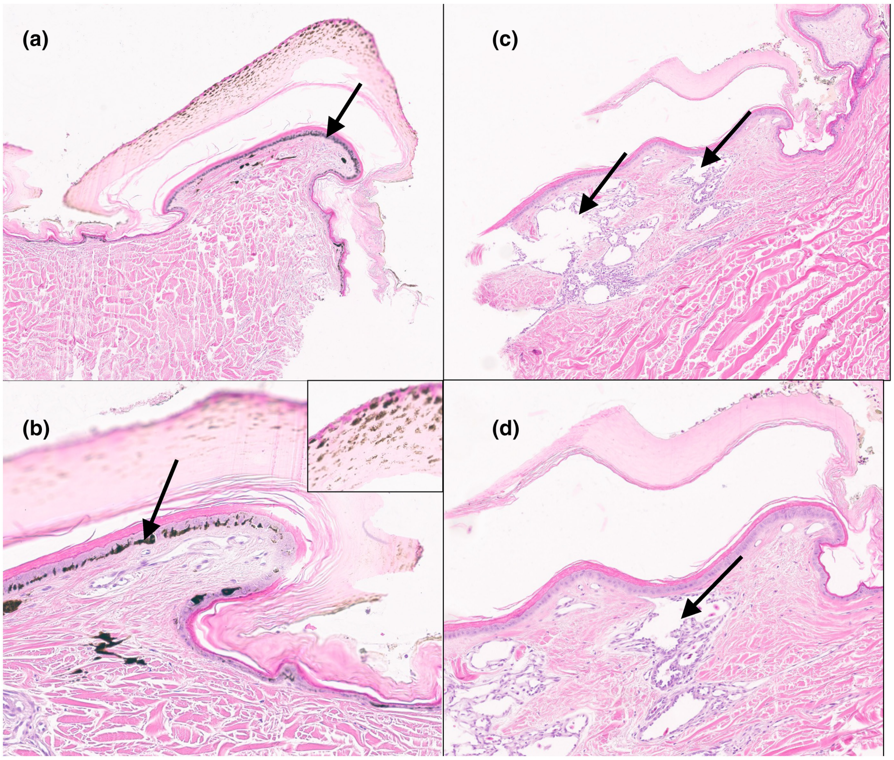

FIGURE 2 Histomorphology of the left forelimb skin sample from a pink land iguana (*Conolophus marthae*). (a, b, b "inset") Black-pigmented region with inset of stratum corneum with better resolution (black arrows indicate melanophores). (c, d) Pink region (black arrows indicate enlarged capillaries or vascular channels). Virtual sections of haematoxylin and eosin.

layers were measured at the narrowest and thickest sections observed, and the results were recorded in Table 1 as a range.

4 | DISCUSSION

In this work, we identified substantial differences in the histological dermal arrangements of Galápagos iguana species, with some peculiarities found in pink iguanas. Saurian and more specifically iguanid dermal gross and microscopic anatomy have been described elsewhere (Alibardi, 2003; Lillywhite & Maderson, 1982; Rutland et al., 2019). In general, (when resting) the epidermis is composed of three cellular levels (from outside to inside): (i) the stratum corneum; (ii) the stratum granulosum; (iii) the stratum germinativum (Rutland et al., 2019). Keratinocytes originate in the stratum germinativum, and the stratum granulosum possesses lipids that reduce water permeability (Rutland et al., 2019). The underlying dermis is made up of the stratum laxum and stratum compactum. Iguanid lizards all possess scales that originate from the outer keratinized epidermal layer. In iguanas, these scales form tight junctions that are important in preventing desiccation. In general, the heavily keratinized epidermis aids reptiles in preventing desiccation (Rutland et al., 2019). Pigmentation of reptilian skin is influenced by four dermal chromatophores (pigment cells): melanophores (blacks, browns), iridophores (iridescence), erythrophores (oranges, reds) and xanthophores (yellows, oranges). Chromatophores typically reside in the stratum laxum and occasionally in the stratum compactum; however, melanophores may also reside in the epidermis, as in mammalian species, but they are usually less abundant than in the dermis. Galápagos iguana species exhibit this pattern, with all three epidermis layers showing intracellular fine granular pigments. Such pigments are abundant only in the black regions of the skin in *A. cristatus* and *C. marthae*, whereas they seem less abundant in *C. subcristatus*. Occasional pigmentary cells are also found in the yellowish epidermis in *C. subcristatus*. Interestingly, the complete lack of pigmentary cells and large aggregates of capillaries within the stratum laxum in the "pink" sample from the pink iguana appear to be the cause for the species' unique colour, as suspected by some. The large capillaries of the pink iguanas share features with mammalian hemangiomas and angiomatosis, although no mitotic figures were observed (Meuten, 2020). It is also possible, yet would require more work, that these capillaries are analogous to vascular plexi and rete mirabile

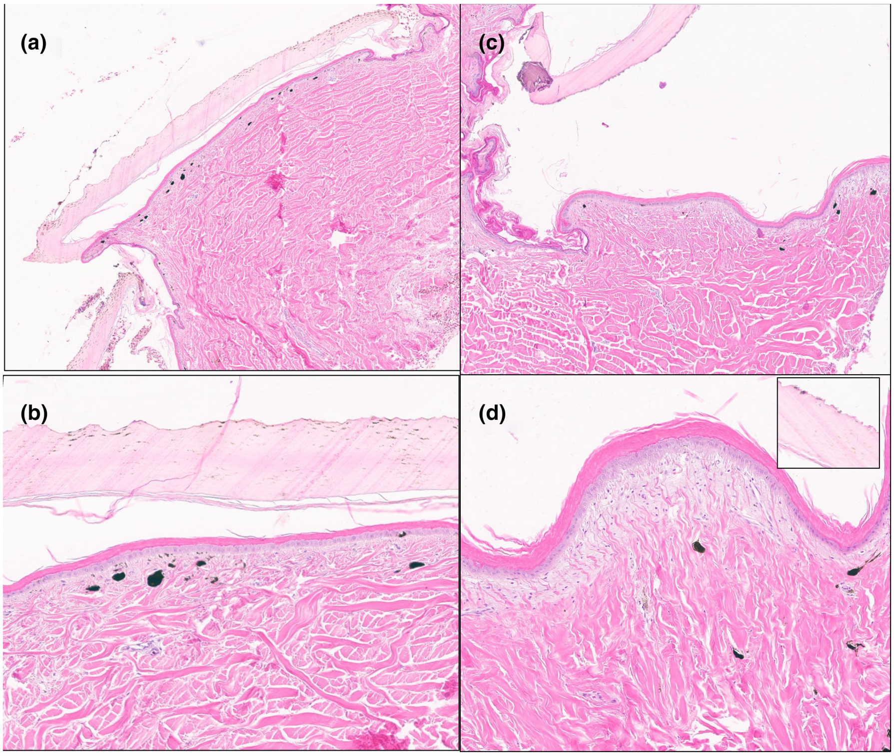

FIGURE 3 Histomorphology of the left forelimb skin sample from a yellow land iguana (*Conolophus subcristatus*). (a, b) Black-pigmented region. (c, d, d "inset") Yellow-pigmented region with inset of artefactually lost stratum corneum. Virtual sections of haematoxylin and eosin.

found in species of birds (Harms et al., 2016) and fishes (Witternberg & Wittenberg, 1974) respectively.

Our data indicates that the pink coloration (Figure 4) is not due to pigments but rather a lack thereof. This raises the question: is it evolutionary advantageous to be pink at 1700 m on the equator? Solar radiation is extremely high at the top of Wolf Volcano in Galápagos. Whereas potential detrimental effects caused by excessive exposure to solar radiation have already been suggested (Di Giacomo et al., 2022), it is not yet clear whether an evolutionary trade-off may exist, allowing the pink iguana to sustain the potential disadvantage associated to the lack of pigmentation in a wide area of the body. This issue is fascinating in light of the recent discovery of *C. marthae* hatchlings (Figure 5). *Conolophus marthae* and *C. subcristatus* hatchlings appear maculated. However, only in *C. marthae* hatchlings exhibit dark areas interspersed in a greenish background, replaced by a brownish background in *C. subcristatus*. Iridiophores can refract blue wavelengths of light, which may interact with yellow carotenoids to produce a green colour (Rutland et al., 2019; Sherbrooke & Frost, 1989). This adaptive trait, which allows camouflage and reduces hatchlings' predation, disappears in both species' adults. Admittedly, this study lacks an ontogenetic approach, which is currently impossible for the restrictions imposed by the Galápagos National Park Directorate. We can then only hypothesize that, for pink iguanas, the large confluent vascular channels within the stratum laxum found in the pink areas of the skin form during individual growth well after hatching. The lack of melanophores and the poor interaction between the rare xanthophores and iridophores prevent the formation of a darker coloration in this area. This would cause the skin to appear pink in the adults. We hope we can further clarify this issue in future studies.

The marine iguana has a much thicker epidermis, between two and 10 cells thick, depending on location, compared to the thinner epidermis of the land iguanas (one to three cells thick with most areas possessing just one or two cell layers). These microscopic differences might reflect differences in habitat and ecology of the three species. For marine iguanas, having a thick epidermis is probably beneficial to decrease water loss and assist with thermoregulation, given they spend so much time in the cool, hyperosmotic marine environment (Dawson et al., 1977; Hobson, 1965, 1969; Morgareidge & White, 1969). The two terrestrial species in this study have a similar number of cell layers in the epidermis.

In general, all Galápagos iguana species are counter shaded, meaning their dorsal surface is darker than their ventral surface (Burtt, 1981). This can obviously be associated with the fact that the ventral surface is not typically

TABLE 1 Details of the epidermal and dermal microscopic anatomy of the marine iguana (*Amblyrhynchus cristatus*), yellow land iguana (*Conolophus subcristatus*) and the pink land iguana (*Conolophus marthae*).

Iguana species	Skin colour sampled	Epidermis: Stratum corneum	Epidermis: Stratum Germinativum/ granulosum	Dermis: Stratum Laxum	Dermis: Stratum compactum
Amblyrhynchus cristatus	Black	40–90 microns thick Contains many to numerous keratinocytes with black to brown, finely granular pigment (heavily pigmented)	7–10 cell layers thick The stratum germinativum contains many to numerous pigment cells with associated dendritic processes and intracytoplasmic, black to brown, finely granular pigment (heavily pigmented) The superficial stratum granulosum contains numerous intracytoplasmic, black to brown, finely granular pigment forming a thin near-solid sheet (moderately pigmented)	25–50 microns thick Often small pigment cells with associated dendritic processes containing intracytoplasmic, black to brown, finely granular pigment (moderately pigmented) Composed of disorganized fine connective tissue Occasional small capillaries with intraluminal erythrocytes Endothelial cells have scant cytoplasm and a closed chromatin pattern with a greater than 2:1 nuclear to cytoplasmic ratio. Often nuclei bulge into the vascular lumen	600–700 microns thick Occasional, small, superficial pigment cells with intracytoplasmic, black to brown, finely granular pigment (mildly pigmented) Composed of dense connective tissue fibres that are oriented parallel to the epidermis Rare small capillaries with intraluminal erythrocytes Endothelial cells have scant cytoplasm and a closed chromatin pattern with a greater than 2:1 nuclear to cytoplasmic ratio. Often nuclei bulge into the vascular lumen
	White	30–40 microns thick Not pigmented	2–6 cell layers thick Not pigmented	50–100 microns thick Occasional small pigment cells with rare dendritic processes containing intracytoplasmic, golden brown to black, finely granular pigment (mildly pigmented) Composed of disorganized fine connective tissue Occasional small capillaries with intraluminal erythrocytes Endothelial cells have scant cytoplasm and a closed chromatin pattern that is occasionally more open with a greater than 2:1 nuclear to cytoplasmic ratio. Often nuclei bulge into the vascular lumen	300–400 microns thick Occasional, small, superficial pigment cells with intracytoplasmic, golden brown to black, finely granular pigment (mildly pigmented) Composed of dense connective tissue fibres that are oriented parallel to the epidermis Rare small capillaries with intraluminal erythrocytes Endothelial cells have scant cytoplasm and a closed chromatin pattern with a greater than 2:1 nuclear to cytoplasmic ratio. Often nuclei bulge into the vascular lumen.

TABLE 1 (Continued)

Iguana species	Skin colour sampled	Epidermis: Stratum corneum	Epidermis: Stratum Germinativum/granulosum	Dermis: Stratum Laxum	Dermis: Stratum compactum
Conolophus marthae	Black	100–150 microns thick Contains many to numerous keratinocytes with black to brown, finely granular pigment (heavily pigmented)	1–2 cell layers thick The stratum germinativum contains many to numerous pigment cells with intracytoplasmic, black to brown, finely granular pigment (heavily pigmented)	100–230 microns thick Occasional large pigment cells with intracytoplasmic, black to brown, finely granular pigment (mildly pigmented) Composed of disorganized fine connective tissue Often contains small capillaries with intraluminal erythrocytes. Capillaries very rarely form small clusters that occasionally interconnect Endothelial cells predominantly have scant cytoplasm and a closed chromatin pattern that is rarely more open with a greater than 2:1 nuclear to cytoplasmic ratio. Often nuclei bulge into the vascular lumen. Capillary clusters in this region have predominantly endothelial cells with an open chromatin pattern, occasional nucleoli, a 1:1 to 2:1 nuclear to cytoplasmic ratio, and moderate nuclei extension into the vascular lumen	700–800 microns thick Occasional, large, superficial pigment cells with intracytoplasmic, golden brown to black, finely granular pigment (mildly pigmented) Composed of dense connective tissue fibres that are oriented parallel to the epidermis and alternate in layers 90 degrees to each other Rare small capillaries with intraluminal erythrocytes Endothelial cells have scant cytoplasm and a closed chromatin pattern with a greater than 2:1 nuclear to cytoplasmic ratio. Often nuclei bulge into the vascular lumen
	Pink	100 to 150 microns thick Not pigmented	1–2 cell layers thick Not pigmented	100–460 microns thick Not pigmented Composed of disorganized fine connective tissue Many small to moderately sized capillaries with intraluminal erythrocytes that often form large aggregates. Capillaries within the aggregates often interconnect Endothelial cells within individual capillaries and within the aggregates predominantly have scant cytoplasm and a closed chromatin pattern that is occasionally more open with a greater than 2:1 nuclear to cytoplasmic ratio. Often nuclei markedly bulge into the vascular lumen. Occasionally endothelial cells within the capillary clusters of this region have a more open chromatin pattern, occasional nucleoli, and a 1:1 to 2:1 nuclear to cytoplasmic ratio	900–1200 microns thick Not pigmented Composed of dense connective tissue fibres that are oriented parallel to the epidermis and alternate in layers 90 degrees to each other Rare small capillaries with intraluminal erythrocytes Endothelial cells have scant cytoplasm and a closed chromatin pattern with a greater than 2:1 nuclear to cytoplasmic ratio. Often nuclei bulge into the vascular lumen

Continues)

TABLE 1 (Continued)

Iguana species	Skin colour sampled	Epidermis: Stratum corneum	Epidermis: Stratum Germinativum/ granulosum	Dermis: Stratum Laxum	Dermis: Stratum compactum
Conolophus subcristatus	Black	100–150 microns thick Often contains keratinocytes with black to brown, finely granular pigment (moderately pigmented)	1–2 cell layers thick The stratum germinativum contains occasional pigment cells with intracytoplasmic, black to brown, finely granular pigment (mildly pigmented)	25–50 microns thick Occasional large and small pigment cells with intracytoplasmic, black to brown, finely granular pigment (moderately pigmented) Composed of disorganized fine connective tissue. Rare regions composed of disorganized, very fine, wispy connective tissue and round to polygonal cells with moderate amounts of intracytoplasmic, amphophilic to clear, very finely granular material Rare small capillaries with intraluminal erythrocytes Endothelial cells have scant cytoplasm and a closed chromatin pattern that is occasionally more open with a greater than 2:1 nuclear to cytoplasmic ratio. Often nuclei bulge into the vascular lumen. Within the lumen and forming up to a single cell layer thick ring around the capillaries are often low numbers of round to polygonal cells with moderate amounts of intracytoplasmic, amphophilic to basophilic, finely granular material	650–850 microns thick Rare, small, superficial pigment cells with intracytoplasmic, black to brown, finely granular pigment (mildly pigmented) Composed of dense connective tissue fibres that are oriented parallel to the epidermis and alternate in layers 90 degrees to each other Rare small capillaries with intraluminal erythrocytes Endothelial cells have scant cytoplasm and a closed chromatin pattern that is occasionally more open with a greater than 2:1 nuclear to cytoplasmic ratio. Often nuclei bulge into the vascular lumen. Within the lumen and forming up to a single cell layer thick ring around the capillaries are often low numbers of round to polygonal cells with moderate amounts of intracytoplasmic, amphophilic to basophilic, finely granular material
	Yellow	100–150 microns thick Rarely contains keratinocytes with golden brown to brown, finely granular pigment (mildly pigmented).	1–3 cell layers thick The stratum germinativum contains occasional pigment cells with small amounts of golden brown to black, finely granular, intracytoplasmic pigment (mildly pigmented)	30–150 microns thick Occasional to rare large pigment cells with intracytoplasmic, golden brown to black, finely granular pigment (mildly pigmented) Composed of disorganized, very fine, wispy connective tissue and round to polygonal cells with moderate amounts of intracytoplasmic, amphophilic to clear, very finely granular material. Fine, widely spaced collagen bundles extend up from the stratum compactum to the epidermis Rare small capillaries with intraluminal erythrocytes Endothelial cells have scant cytoplasm and a closed chromatin pattern that is occasionally more open with a greater than 2:1 nuclear to cytoplasmic ratio. Often nuclei bulge into the vascular lumen. Within the lumen and forming up to a single cell layer thick ring around the capillaries are often low numbers of round to polygonal cells with moderate amounts of intracytoplasmic, amphophilic to basophilic, finely granular material	1000–2500 microns thick Occasional, small and large, superficial pigment cells with intracytoplasmic, black to brown, finely granular pigment (mildly pigmented) Composed of dense connective tissue fibres that are oriented parallel to the epidermis and alternate in layers 90 degrees to each other Rare small capillaries with intraluminal erythrocytes Endothelial cells have scant cytoplasm and a closed chromatin pattern that is occasionally more open with a greater than 2:1 nuclear to cytoplasmic ratio. Often nuclei bulge into the vascular lumen. Within the lumen and forming up to a single cell layer thick ring around the capillaries are often low numbers of round to polygonal cells with moderate amounts of intracytoplasmic, amphophilic to basophilic, finely granular material

FIGURE 4 Photograph of a mature male pink land iguana (*Conolophus marthae*) near the rim of the Wolf Volcano caldera. (Photo courtesy of Giuliano Colosimo).

FIGURE 5 Photograph of a hatchling pink land iguana (*Conolophus marthae*) taken in the Wolf Volcano caldera. (Photo courtesy of Johanes Kastdalen).

visible to other iguanas or terrestrial predators, or exposed directly to solar radiation. However, the milky colour of the ventral body surface of marine iguanas can also be interpreted as an adaptation to reduce visibility to aquatic predators, while swimming in open water. Predators from above may have trouble distinguishing dark-coloured prey from the dark ocean below, and predators from below may lose sight of light-coloured prey that blend in with the sky above (Burtt, 1981).

In conclusion, we elucidated, for the first time, the histological skin structure of three of the four described Galápagos iguana species. The comparative approach we used allowed us to identify similarities with other reptilian skin models and document the peculiarity of the skin structure of *C. marthae*. In this regard, we now agree with Steven Tyler that, at least from an anatomical and histological point of view for *C. marthae*, "pink" is not a question and that "pink it's like red but not quite." In fact, we provide solid evidence demonstrating that the pink colour of pink iguanas is actually due to blood flowing in the abundant confluent vascular channels in the stratum laxum, not protected by melanophores.

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