Scimeca *et al. Biology Direct* (2025) 20:16
https://doi.org/10.1186/s13062-025-00602-5

Biology Direct

RESEARCH Open Access

Ultrastructural studies distinguish skin diversities among Galápagos iguanas

Manuel Scimeca[1*], Rita Bonfiglio[1], Giuliano Colosimo[2], Eleonora Candi[1,3], Glenn P. Gerber[4], Gregory A. Lewbart[5], Alessandro Mauriello[1], Gerry Melino[1], Christian Sevilla[6,7], Yufang Shi[8], Ying Wang[8] and Gabriele Gentile[2*]

Abstract

Iguanas exhibit diverse colors and behaviors reflecting evolutionarily adaptation to various habitats; in particular, the Galápagos iguanas represent unique color morphologies with distinct ecological niches. While external coloration in iguanas has ecological implications, comprehensive studies on the histological and ultrastructural aspects of their skin can provide insight into their adaptation to extreme environments, such as high UV exposure. Starting from these considerations the present study investigates the histological, ultrastructural and immunohistochemical features to comprehensively characterize the skin in adults of three species of Galápagos iguanas (*A. cristatus*, *C. subcristatus and C. marthae*). Morphological analysis revealed significant differences among the species, with the black-colored skin of *A. cristatus* showing a melanin-rich but vessel-poor dermis, while *C. subcristatus* and *C. marthae* displayed varying distributions of melanosomes and melanocytes. Notably, the absence of iridophores was consistent across all samples due to the absence of birefringent material under the optical microscope. Morphometric evaluations highlighted interspecific differences in the stratum corneum thickness, particularly between black- and non-black-colored (irrespectively if yellowish or pink) skin. The ultrastructural investigation confirmed the absence of iridophores in all analyzed samples. The cytokeratin expression assessed by immunohistochemistry showed stratified epithelium in the epidermis of *C. marthae* non-black-colored (pink) skin. The presence of a thickened stratum corneum and the stratification of the epidermis in non-pigmented skin could help the pink iguana to cope with the extreme conditions of the Wolf volcano, especially in relation to UV exposure. These skin characteristics may reduce the penetration power of UV rays into the superficial loose dermis, thereby attenuating potential UV-related damage such as DNA breaks and ROS generation. These findings offer insights into the adaptive strategies of these iguanas.

Keywords *Amblyrhynchus cristatus*, *Conolophus subcristatus*, *Conolophus marthae*, Skin color, Piebaldism, Reptiles, Iridophore, Chromatophore, Melanophore, Depigmentation

*Correspondence:
Manuel Scimeca
manuel.scimeca@uniroma2.it
Gabriele Gentile
gabriele.gentile@uniroma2.it
[1]Department of Experimental Medicine, University of Rome Tor Vergata, Rome 00133, Italy
[2]Department of Biology, University of Rome Tor Vergata, Rome 00133, Italy
[3]IDI-IRCCS, Rome 00166, Italy
[4]Beckman Center for Conservation Research, San Diego Zoo Wildlife Alliance, Escondido, CA, USA
[5]North Carolina State University College of Veterinary Medicine, Raleigh, NC, USA
[6]Galápagos Science Center GSC, Isla San Cristobal, Galápagos, Ecuador
[7]Galápagos National Park Directorate, Av. Charles Darwin, Puerto Ayora, Isla S. Cruz, Galápagos, Ecuador
[8]CAS Key Laboratory of Tissue Microenvironment and Tumor, Shanghai Institute of Nutrition and Health, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

Background

Iguanas are a diverse group of reptiles belonging to the family Iguanidae, exhibiting a complex pattern of colors and behaviors that reflect their adaptation to various habitats and different communication strategies. The different coloration patterns in the skin of iguanas and reptiles, in general, suggest that the integumentary system is particularly crucial in adapting to diverse habitats, acting as a dynamic organ that responds to environmental cues. The skin, an interface between an organism and its environment, plays a pivotal role in maintaining homeostasis, providing protection, and facilitating various physiological functions. In this scenario, the coloration of iguanas is often intricately linked to their habitat, representing evolutionary adaptations for thermoregulation, camouflage, and communication [1].

Iguanas are among the most spectacular representative species of the Galápagos Islands [2]. Four named species of Galápagos iguanas, all endemic, are currently recognized: *Amblyrhynchus cristatus* (marine iguanas), *Conolophus subcristatus* (common Galápagos land iguanas, hereafter yellow iguanas), *C. pallidus* (Barrington iguanas) and *C. marthae* (Galápagos pink land iguanas, or simply pink iguanas) [3, 4]. Whereas marine iguanas are widely distributed along coastlines throughout the archipelago [5], *C. subcristatus* occurs only on a few islands, and *C. pallidus* and *C. marthae* are limited to Santa Fe and Isabela islands, respectively [6]. Currently, many factors contribute to their threatened status at various levels of risk (IUCN Red List, https://www.iucnredlist.org/). Critically endangered pink iguanas [7] were described in 2009 [8]; their common name derives from their color, having become a symbol of the incredible biodiversity and evolutionary processes that have unfolded in the isolated ecosystems of the Galápagos archipelago [9].

Despite studies exploring external coloration and its ecological implications, the ultrastructural aspects of iguana skin remain less known, with not much information currently available for any of the Galápagos iguanas. In a recent paper [10], gross and microscopic analyses of skin structure provided histological details on the nature of coloration of three of the four named Galápagos iguana species, *A. cristatus*, *C. subcristatus* and *C. marthae*(Fig. 1A, B,C). Exclusively for *C. marthae*, the authors found that "pink" dermal areas are devoid of melanophores, and a rich network of confluent capillaries exists. A poor interaction between xanthophores and iridophores was also speculated to explain the peculiar pattern of coloration, as the study could not confirm the presence of iridophores. While this study provided solid evidence to explain the pink color of adult *C. marthae*, it also raised a question related to the recent discovery that *C. marthae* hatchlings are not pink upon emergence [11]. Like *C. subcristatus*, they are maculated; but contrary to *C. subcristatus*, *C. marthae* hatchlings show a green color pattern (Fig. 1D).

Generally, greenish and blueish colors in reptiles are primarily due to an interaction between different types of chromatophores in different skin layers. Chromatophores is a general term that includes melanophores, iridophores (granulophores or guanophores), xanthophores, and erythrophores. Iridophores are particularly important, as they contain guanine nanocrystals capable of reflecting and diffracting light. Iridophores may be in the superficial and deeper dermal layers. Whereas in the deeper layers, crystals protect against excessive UV radiation [1], crystals in the more superficial iridophores can diffract light, generating different colors, depending on the wavelength diffracted, which may depend on the density and organization of crystals. Green color emerges when the mostly diffracted blue wavelength interferes with carotenoids in xanthophores [1, 12]. Chromatophores are characterized in their ultrastructure; melanophores, iridophores, and xanthophores may be distinguished by Transmission Electron Microscopy (TEM) [13–15].

Another aspect of the iguana's skin, that can contribute to UV protection, is the thickness of the stratum corneum and the stratification of the epidermis. Indeed, the stratum corneum, generally composed of 6–7 layers in the skin of iguanas, as well as the epidermis's structure, represents the body's first line of defense against solar UV radiation [16]. Starting from these considerations, the present study investigates the histological, ultrastructural and immunohistochemical features to comprehensively characterize the skin in adults of three species of Galápagos iguanas (*A. cristatus*, *C. subcristatus* and *C. marthae*).

Methods

Sample collection

Skin samples were collected from individuals distinct from those studied by Lewbart and colleagues, although originating from the same populations [10]. Due to restrictions imposed by the Galápagos National Park Directorate (GNPD), the governmental authority overseeing Galápagos biodiversity, only two skin samples were obtained for *C. subcristatus* and *C. marthae*, both from a single individual iguana. For the marine iguana (*A. cristatus*), only one skin sample was available for collection. Skin samples were obtained from the left dorsolateral area of the body. Two regions (black- and non-black-colored areas) were sampled on *C. subcristatus* and *C. marthae*. The non-black-areas sampled in *C. subcristatus* were yellowish, while those from *C. marthae* were pink. The only skin sample available for *A. cristatus* was black. Iguanas were manually captured and restrained for the biopsy procedure according to a protocol approved by the GNPD. Sampling areas were disinfected using 96%

Fig. 1 Galápagos iguana species. (**A**) *C. marthae*, (**B**) C. *subcristatus*, (**C**) *A. cristatus*, (**D**) hatchling pink land iguana (*C. marthae*). Photos A, B, and C courtesy of Giuliano Colosimo; photo D courtesy of Johanes Ramirez-Kastdalen, published after Lewbart and collaborators [10]

ethanol-soaked gauze pads, followed by the application of lidocaine gel (Akorn Pharmaceuticals). After a few minutes, a 5 mm biopsy punch (Miltex®, Integra Life Sciences) was utilized to extract a full-thickness section of skin. The excised tissue sample was fixed in 4% neutral buffered formalin for 24 h and stored at room temperature. Hemostasis was achieved through direct pressure from a cotton-tipped applicator, followed by the application of lidocaine gel. Wounds were closed using a 4–0 polydioxanone suture (Ethicon Inc.) on a cutting needle employing a single horizontal mattress suture. This procedure was repeated for all three animals to obtain tissue samples from both visibly black-colored and non-black-colored areas, except for *A. cristatus*, for which a non-black-colored sample was not collected. Due to restrictions imposed by the GNPD, access to hatchlings of *C. marthae* was not possible. Thus, no hatchling skin samples were available for this investigation.

Histology

Iguana skin samples were incubated with 4% neutral buffered formalin for 24 h, dehydrated in ethanol (70°, 95° and 100°) and embedded in paraffin wax [17, 18]. Four-µm thick serial sections were stained with hematoxylin and eosin (H&E) [19], Movat pentachromic, Alcian blue or Periodic acid–Schiff (PAS) for morphological and morphometric analysis. For H&E, the slides were stained with Harris hematoxylin for 4 min, rinsed in running water for 8 min, and counterstained with alcoholic eosin for 1 min. Subsequently, the slides were dehydrated through ascending grades of ethanol, cleared in xylene, and coverslipped with synthetic resin. For Movat's Pentachrome staining, the slides were stained in 1% Alcian blue solution (in 3% acetic acid, pH 2.5) for 20 min, rinsed in running water for 10 min, incubated in 3% phosphotungstic acid for 10 min, rinsed briefly in distilled water, stained in Verhoeff's elastic stain for 10 min, rinsed in running water for 5 min, differentiated in 2% ferric chloride for

1 min, rinsed in running water for 5 min, stained in crocein scarlet-acid fuchsin solution for 2 min, rinsed briefly in distilled water, differentiated in 5% phosphotungstic acid for 5 min, rinsed in distilled water, and stained in alcoholic saffron for 5 min. Subsequently, the slides were dehydrated and mounted as described for H&E. For Alcian Blue staining, the slides were stained in 1% Alcian blue solution (3% acetic acid, pH 2.5) for 30 min, rinsed in running water for 2 min, counterstained with nuclear fast red for 5 min, and rinsed in running water for 2 min. Dehydration and mounting were performed as described for H&E. For PAS staining, the slides were treated with 0.5% periodic acid for 5 min, rinsed in distilled water, incubated in Schiff reagent for 15 min, and rinsed in running water for 5 min. Subsequently, the slides were stained with hematoxylin for 2 min, rinsed in running water for 5 min, and dehydrated and mounted as described for H&E.

The morphological analysis allowed us to evaluate differences in the number and localization of vessels and estimate the number of melanocytes and the dermis characteristics. The morphometric analysis, conducted on five serial sections stained with H&E, focused on evaluating the thickness of the stratum corneum. One measurement for each layer was taken on each slide, resulting in a total of five measurements per skin sample. Thickness was evaluated using digital images analyzed with Zen 3.9 software (Zeiss, Oberkochen, Germany). We then calculated ANOVA and post-hoc Tukey tests, under the reasonable assumption of normality being met. The software PAST 5.0.2 [20] was used for statistical analyses.

Transmission electron microscopy

Small fragments ($\sim 1 mm^3$) of each sample were fixed in 4% paraformaldehyde, post-fixed in osmium tetroxide and embedded in epoxy resins (EPON 812; Agar Scientific) as previously described [21–23]. Serial ultrafine Sect. (80 nm) have been cut with an ultramicrotome, collected on copper grids, stained with uranyl acetate and lead citrate and observed by TEM FEI 268D. The ultrastructural analysis (80Kv) mainly focused on identifying melanophores, iridophores, and xanthophores, including melanocyte granules (numerosity and differentiation), vessels, collagen fibers (presence) and stroma.

Immunohistochemistry

The immunohistochemical investigation focused on studying the expression of a marker of epithelial pan cytokeratin - CKPan) cells [24, 25]. Specifically, serial sections from paraffin blocks were used to study the expression of cytokeratin filaments. For antigen retrieval, sections were pretreated with EDTA citrate Ph 7.8 at 125 °C for 20 min by using low pressure cycle of a pressure cooker. Then, the sections were incubated for 30 min with the mouse monoclonal anti-Pan Keratin (clone: AE1/AE3/PCK26) primary Antibody (Ventana, Roche). Reactions were relieved by using the HRP-DAB detection system (UCS Diagnostics). Positive and negative controls were used for each reaction.

Results and discussion

Whereas the multilayered epidermis of Galápagos iguanas is in general composed by several different epidermal layers as well described by Alibardi and Toni [16], our results provide histological, immunohistochemical and ultrastructural compelling evidence of the peculiar differences among the skins of three species of Galápagos iguanas (*A. cristatus*, *C. subcristatus and C. marthae*). Consistently with previous work [10], morphological examination showed that the black-colored skin of *A. cristatus* was characterized by a superficial loose dermis rich in melanin but poor in large vessels (Fig. 2A), while black-colored skin samples from *C. subcristatus* displayed the presence of several melanosomes in both the epidermis and the superficial loose dermis (Fig. 2C). Large fibroblast-like cells arranged in a palisade under the epidermis were observed in both the black-colored (Fig. 2B) and non-black-colored (yellowish) (Fig. 2B) skin of *C. subcristatus*. Histological analysis of the skin of black-colored skin sample from *C. marthae* confirms the presence of few vessels and numerous melanocyte cells (Fig. 2E). In contrast, the non-black-colored (pink) skin samples were characterized by the absence of melanin and the presence of very large vessels in the superficial loose dermis (Fig. 2D).

The presence of a rich network of capillaries in the dermis of *C. marthae* could in principle play a relevant role in effective thermoregulation, especially in the absence of ventilation. This consideration would also be consistent with the fact that *C. marthae*, contrary to *C. subcristatus*, is typically found in the shade during the hottest hours of the day [2, 26, 27], with the network of capillaries likely helping to decrease and adjust body temperature.

Interestingly, no birefringent material was observed under light microscopy in either the H&E-stained sections or the sections stained with special histochemical stains such as Movat (Fig. 3) Alcian blue, and PAS (Fig. 4). This evidence suggests that no iridophores were present in the analyzed samples. In fact, under light microscopy iridophores generally appear as birefringent granules [28].

Morphometric evaluations uncovered significant differences in the thickness of the stratum corneum ($F = 103.018$; $p<<<0.001$). Specifically, while the black-colored areas of the skin in all three species exhibited similar thickness (mean ranging between 7.7 and 9.0 μm), larger values were observed in the non-black-colored skin of both *C. subcristatus* (14.8 μm) and *C. marthae*

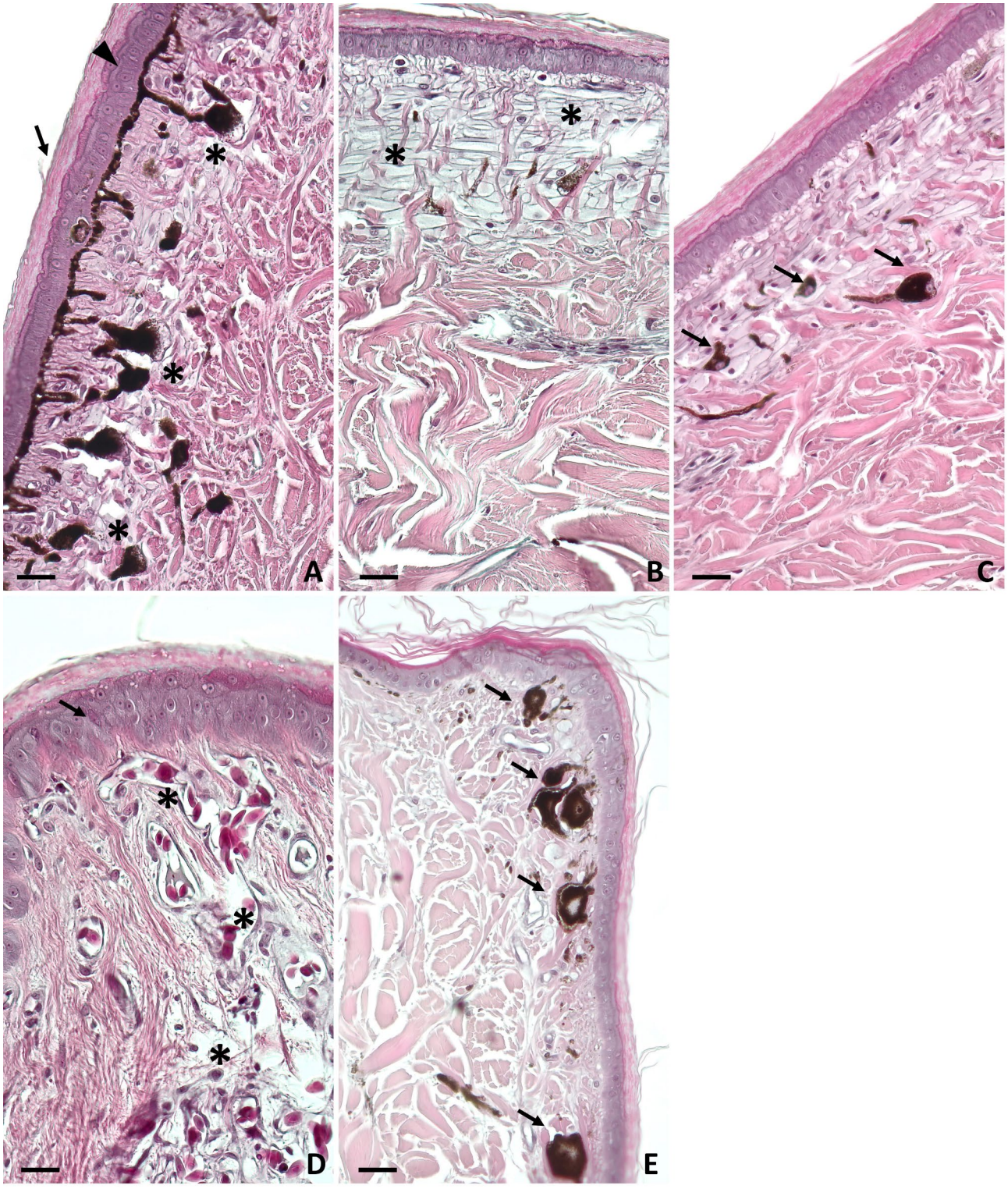

Fig. 2 Skin histological analysis of three Galápagos iguana species. (**A**) The haematoxylin and eosin-stained section of black-colored *A. cristatus* skin exhibits a thin stratum corneum (arrow), a single layer of columnar epithelium (arrowhead), and abundant melanin (asterisks). (**B**) The image displays numerous large fibroblast-like cells arranged in a palisade (asterisks) under the epidermis in the non-black-colored skin of *C. subcristatus*. (**C**) Black-colored skin of *C. subcristatus* shows numerous melanocyte cells (arrows). (**D**) Non-black-colored (pink) skin of *C. marthae* characterized by a thick superficial loose dermis (arrow) and massive blood vessels (asterisks). (**E**) Black-colored-skin of *C. marthae* displays numerous melanocyte cells (arrows). Scale bar = 100 µm

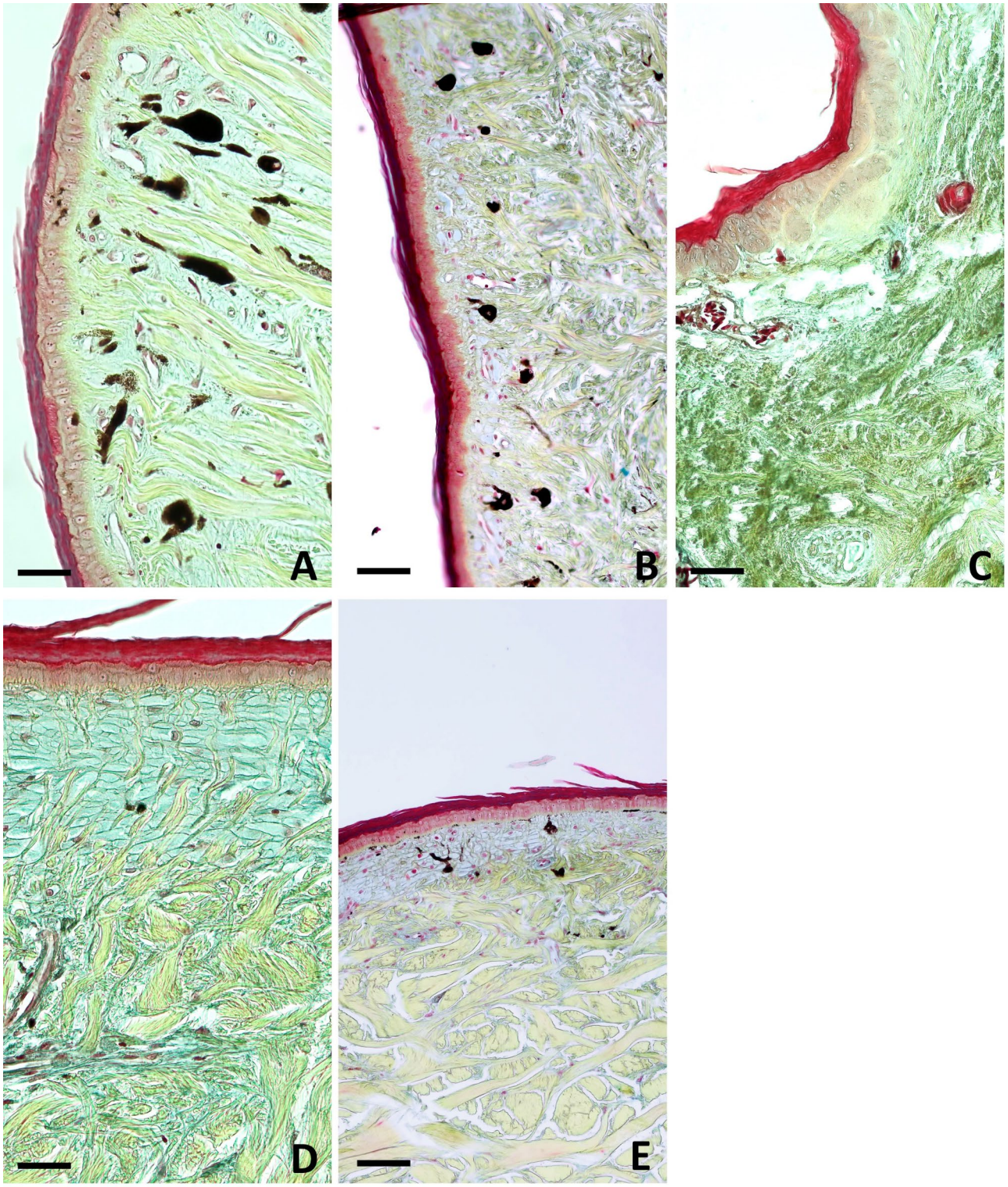

Fig. 3 Movat staining of skin samples from three Galápagos iguana species. (**A**) The image shows the presence of collagen fibers (yellow) in the superficial loose dermis of black-colored *A.cristatus* skin. (**B**) The image displays the presence of sparse collagen fibers (yellow) in the superficial loose dermis of black-colored *C. marthae* skin. (**C-E**) No/rare collagen fibers are present in the non-black-colored skin (pink) of *C. marthae* (**C**) and *C. subcristatus* (**D**) as well as in the black-colored skin of *C. subcristatus* (**E**). Scale bar = 100 µm

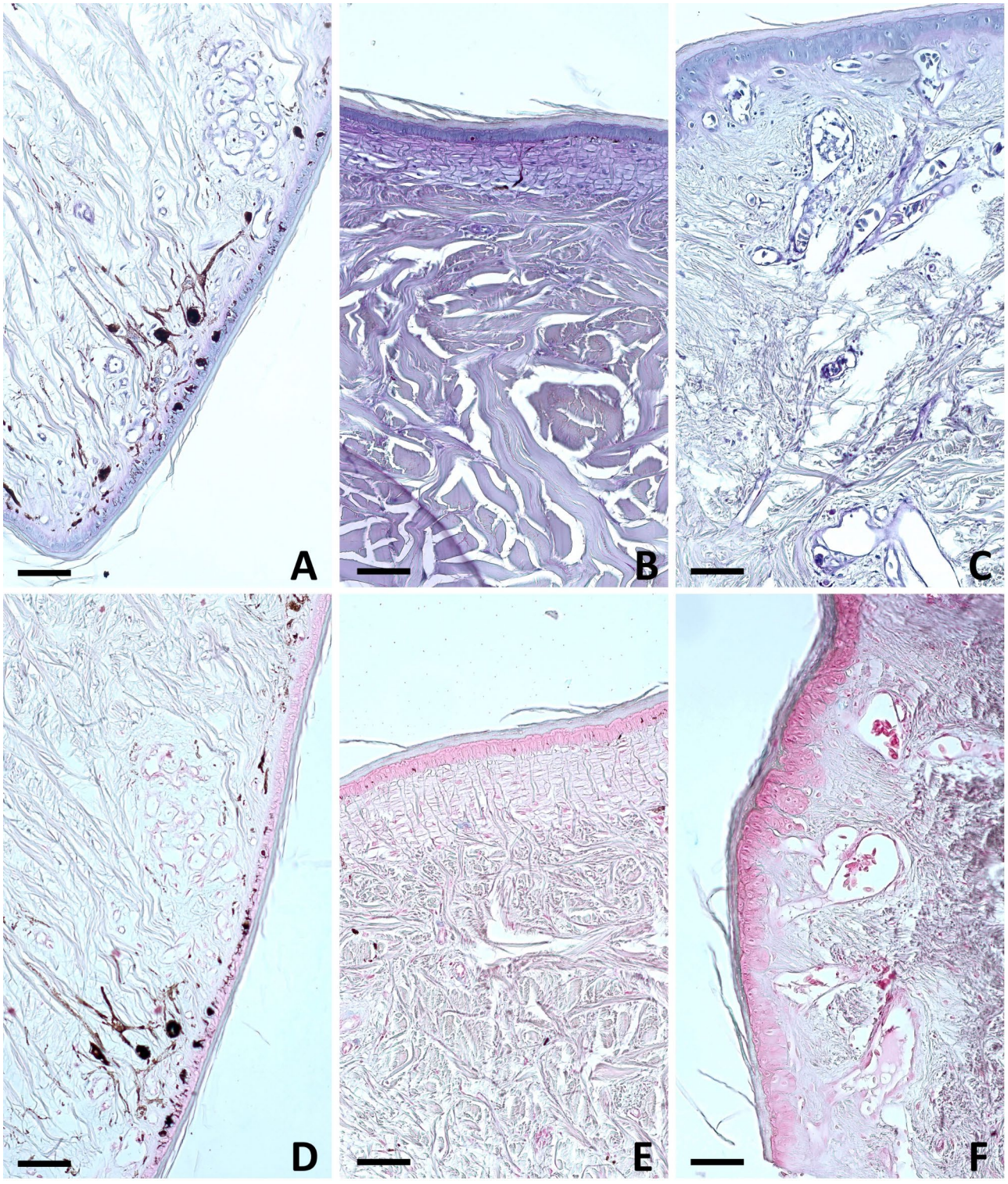

Fig. 4 Alcian blue and PAS staining of skin samples from three Galápagos iguana species. (**A-C**) Alcian Blue stains. Skin samples were negative for the presence of acid mucins. (**D-F**) PAS stains. Skin samples were negative for the presence of polysaccharides. Representative images from black-colored skin of *A.cristatus* (**A, D**), non-black-colored (yellowish) skin of *C. subcristatus* (**B, E**) and non-black-colored (pink) skin of *C. marthae* (**C, F**). Scale bar = 100 µm

(10.6 μm, see Table 1 for Tukey tests). Thus, the stratum corneum in black-colored skin was thinner compared to non-black-colored skin, regardless of the species. Additionally, the data revealed that the stratum corneum of dorsolateral skin, investigated in this work, is generally an order of magnitude thinner than that of the forelimb, studied by Lewbart and collaborators [10].

Further differences between species were also supplied by the immunohistochemical analysis, which recognized stratification of epithelial layers only in non-black-colored (pink) skin of *C. marthae*. Immunohistochemistry against the cytokeratin filaments (ckpan) allowed us to characterize the epidermis by marking the epithelial cells (Fig. 5). Ckpan-stained sections showed differences in the epidermis of *C. marthae* non-black-colored skin compared to all other iguana skin samples examined (black-colored and non-black-colored areas). Stratification of epithelial layers was therefore observed only in non-black-colored skin of *C. marthae* (Fig. 5A). Black-colored skin of *C. marthae* (Fig. 5B) and all the other analyzed skin samples (Fig. 5C-E) showed a single layer of epithelial cells.

The results of the ultrastructural analysis are shown in Fig. 6. In particular, the black-colored skin of *A. cristatus* was characterized by numerous melanocytes (Fig. 6A) with mature melanosomes, often arranged in clusters (Fig. 6B). Melanocytes were observed under the epidermis. Few vessels containing nucleated red blood cells were observed in the superficial loose dermis. The connective tissue layer consists of a sparse extracellular matrix of hyaluronan and proteoglycans supported by collagen fibrils and rare dermoepithelial junctions. Very few structures resembling xanthophores were observed in the superficial loose dermis of *C. subcristatus* (Fig. 6C).

Melanocyte cells rich in melanosomes were observed in the black-colored skin of both *C. subcristatus* (Fig. 6D) and *C. marthae* (Fig. 6E). The connective tissues of black-colored skin of *C. marthae* (Fig. 4F) displayed an extracellular matrix rich in collagen, as also evident from the Movat staining (Fig. 3B).

The non-black-colored skin of *C. subcristatus* showed a very dense extracellular matrix rich in collagen fibers and dermoepithelial junctions (Fig. 6G). Rare melanocytes were observed, and only a few melanosomes were present (Fig. 6H). Very large fibroblast-like cells with poorly electron-dense cytoplasm containing xanthophores were detected in the superficial loose dermis (Fig. 6I). The non-black-colored skin of *C. marthae* was devoid of both melanosomes and xanthophores (Fig. 6J, K). It was also characterized by large vessels containing nucleated red blood cells (Fig. 6L), indicating that these vessels form a network of vascular channels. Remarkably, iridophores were not detected in any of the analyzed skin samples. The thickening of the stratum corneum and the presence of a multi-stratified epidermis, and the absence of chromatophores in the non-black-colored skin of *C. marthae*, provide a basis for speculative considerations about the adaptation of Galápagos iguanas to the extreme conditions to which they are exposed, and the ontogenetic changes related to their peculiar skin color. Specifically, the characteristics of the skin of *C. marthae* may reflect the intricate interplay between morphology and the specific environmental challenges faced by this pink iguana in the Galápagos Islands. The examination of *C. marthae*, the iguana residing at high elevations, introduces intriguing possibilities, beyond the scope of the current investigation, for understanding the impact of UV radiation on skin health and, in general, on iguana's physiology. The substantial thickness of the stratum corneum in the skin of the pink iguana could be indicative of an adaptation to the challenges posed by the higher elevation environment characterized by strong ultraviolet radiation (UVI exceeding 16), although the absence of mature melanocytes appears paradoxical. Indeed, melanin is generally considered the perfect protection against ultraviolet-induced photodamage, and, therefore, it would be reasonable to expect the presence of abundant melanin pigments in iguanas found at high altitudes. In vitro studies focused on the possible toxic effect of photo-excited melanin could provide a rationale for a possible adaptive explanation of the *C. marthae* skin. In fact, melanin can also have toxic effects, especially after very high exposure to UV radiation [29]. UV-activated melanin seems to react with DNA and is known to act as a photosensitizer that produces oxygen radicals [30], which can lead to single-strand DNA breaks in skin cells in vitro [31, 32]. However, using the micronucleus test, Gustavino and coll [33]. showed that the rate of nuclear DNA damage in erythrocytes is much higher in pink iguanas than in other fully pigmented Galápagos species, including *A. cristatus*. This suggests that, even if the results of an adaptive response, in the pink iguana overall these traits are not as effective to limit DNA damage as they are in the other Galápagos iguana species. Clearly, this issue is worthy of further investigation.

Another interesting aspect of this study is the well-documented different distribution of melanophores

Table 1 Post-hoc Tukey tests. Cm, Cs, and Ac indicate *C. marthae*, *C. subcristatus*, and *A. cristatus*, respectively

	Cm black	Cs black	Ac black	Cm non black	Cs non black
Cm black	-	**0.04**	0.998	**<<<0.001**	**<<<0.001**
Cs black	**1.27**	-	**0.020**	**0.007**	**<<<0.001**
Ac black	0.13	**1.40**	-	**<<<0.001**	**<<<0.001**
Cm non black	**2.87**	**1.60**	**3.00**	-	**<<<0.001**
Cs non black	**7.01**	**2.74**	**7.14**	**4.14**	-

Probability is above the diagonal. The difference between means is under the diagonal. Statistically significant (α = 0.05) values are in bold

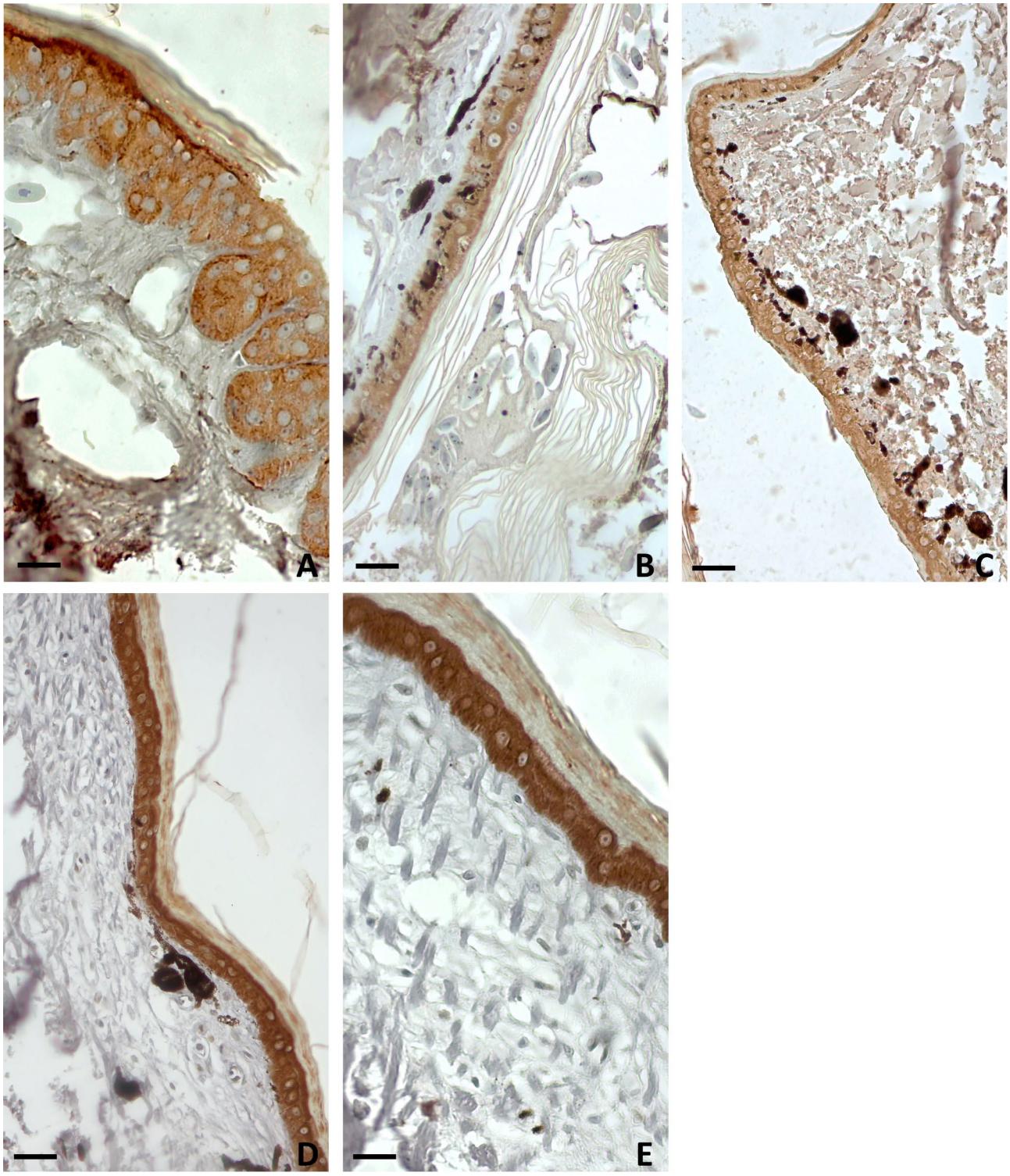

Fig. 5 Immunohistochemical analysis of cytokeratin filaments (ckpan) in skin samples from three Galápagos iguana species. (**A**) Immunohistochemical reaction highlights the presence of stratified epithelium in the non-black-colored (pink) skin of *C. marthae*. (**B**-**E**) No stratification of epithelial layers was observed in the black-colored skin of *C. marthae* (**B**) *A. cristatus* (**C**), and in both black-colored (**D**) and non-black-colored (**E**) skin of *C. subcristatus*. Scala bar = 100 µm

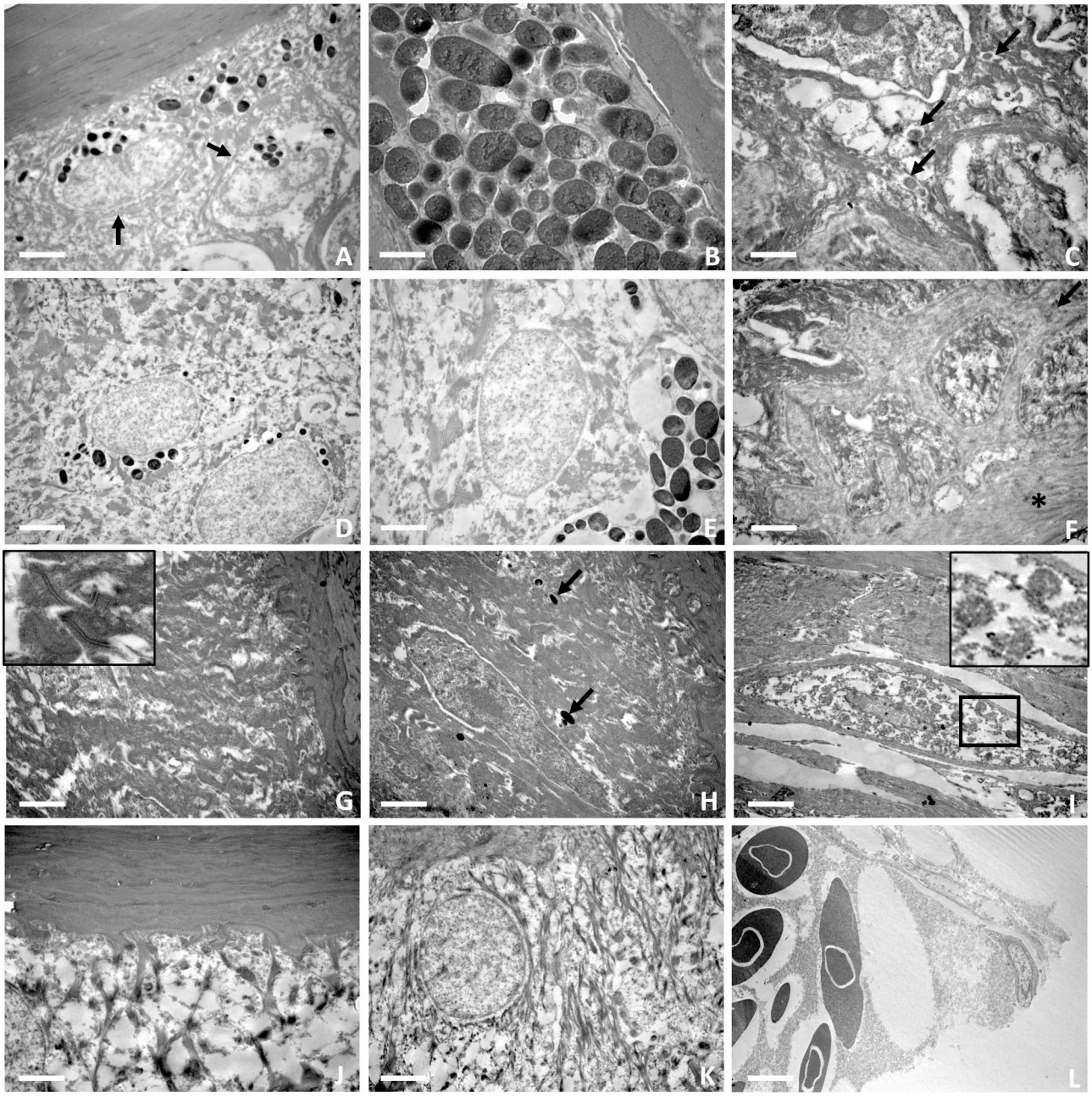

Fig. 6 Ultrastructural analysis of skin samples from three Galápagos iguana species. A-B) Black-colored skin of *A. cristatus*. (**A**) Electron micrograph of *A. cristatus* skin shows mature melanocytes (arrows). (**B**) Numerous melanosomes organized in clusters in the superficial loose dermis of black-colored skin of *A. cristatus*. Scale bar represents 1 µm. (**C**) The image displays structures resembling xanthophores (arrows) in cells present in the superficial loose dermis of the black-colored skin of the *C. subcristatus*. Scale bar represents 2,5 µm. (**D**) Melanosomes cells rich in melanosomes under the epidermis of the black-colored skin of the *C. subcristatus*. Scale bar represents 5 µm. (**E**) Melanosomes under the of the black-colored skin of *C. marthae*. Scale bar represents 2,5 µm. (**F**) The superficial loose dermis of black-colored skin of *C. marthae* characterized by numerous collagen fibers (asterisks). Scale bar represents 5 µm. (**G-I**) Non-black-colored (yellowish) skin of *C. subcristatus*. (**G**) Electron micrograph of *C. subcristatus* skin shows a very dense extracellular matrix rich in collagen fibers and dermoepithelial junctions (square). Scale bar represents 10 µm. (**H**) Rare melanosomes arrows. Scale bar represents 5 µm. (**I**) Very large fibroblast-like cells containing xanthophores-like structures (square) in the superficial loose dermis of a *C. subcristatus* skin. Scale bar represents 5 µm. (**J-L**) Non-black-colored (pink) skin of *C. marthae*. (**J**) Electron micrograph of *C. marthae* skin. Scale bar represents 5 µm. (**K**) Cell in the Epidermis without melanosomes. Scale bar represents 5 µm. (**L**) A vessel containing nucleated red blood cells. Scale bar represents 5 µm

in the black- and non-black-colored skin of all iguanas. This suggests that these types of chromatophores not only protect against high UV radiation in the Galápagos Islands but, in combination with xanthophores, may also substantially contribute to skin color variation. The homogeneous distribution of melanophores and xanthophores in the skin of any color would instead suggest that their contribution to color variation would be poor, if any. This explanation has been invoked in the case of *Phelsuma* geckos [12].

Notably, all analyses, including light and transmission electron microscopy investigation, did not provide evidence for the presence of iridophores. In fact, TEM sample preparation may cause artefacts in iridophores because staining may dissolve guanine crystals [13, 34]. However, even so, iridophores are still well detectable when present since they contain rows of membrane-bounded spaces, resulting in "white holes" where the reflecting crystals are placed. No such a pattern was identified in our samples. The general absence of a greenish/blueish coloration in adult Galápagos iguanas investigated here would be consistent with the absence of iridophores. The lack of iridophores would also be consistent with the fact that the color of Galápagos iguanas is relatively insensitive to temperature change, contrary to what was observed in the lizard *Urosaurus ornatus*, which has temperature-sensitive, physiologically active iridophores involved in a mechanism for rapid change of structurally derived color [35]. However, the lack of such features per se is not a good predictor of iridophore absence in reptiles. Comparative histological studies of reptile skin further underscore the peculiar characteristics of the Galápagos iguanas' skin. In the Fringe-toed lizard (*Acanthodactylus orientalis*), chromatophores are organized in a vertical hierarchy, with xanthophores on the surface, iridophores in the middle, and melanophores at the base. This arrangement contributes to the intricate pigmentation patterns responsible for the lizard's diverse skin colors [36]. Similarly, the Tokay gecko (*Gekko gecko*) exhibits a distinct regional variation in chromatophore distribution, with erythrophores predominating in orange/red areas and iridophores being more abundant in blue regions [15]. In this regard, Saenko and collaborators [12] showed that iridophores are present in the dermis of *Phelsuma* geckos, irrespective of whether the external coloration is light/dark brown or red. Additionally, Brejcha and collaborators [37] demonstrated that a combination of brown melanophores, yellow xanthophores, and white iridophores results in golden-brown colors in Deirochelyinae (Emydidae) turtles. While it is unclear if iridophores are present in *A. cristatus* and *C. subcristatus* hatchlings, *C. marthae* hatchlings should have iridophores, which conceivably disappear during subsequent developmental processes. Thus, an investigation that takes advantage of genome mapping and gene expression along a developmental gradient would be greatly beneficial for advancing the understanding of the genomic basis of coloration and adaptation in pink iguanas and reptiles in general. Accordingly, the mechanisms that may cause the lack of iridophores are still insufficiently known in squamates and are obscure in Galápagos iguanas. In squamates, only recently Garcia-Elfring and collaborators [38] demonstrated that reading frame mutations in the transcription factor EC (*tfec*) alter coloration and cause piebaldism in *Python regius* as well as the loss of iridophores in *Anolis lizards*, suggesting that *tfec* is necessary for the development of chromatophores. In an ultrastructure-imaging and genome-wide association study in the Asian vine snakes (*Ahaetulla prasina*), Tang and collaborators [39] documented a conserved amino acid substitution in the SMARCE1 gene associated with iridophores and levels of expression of more than 200 genes, including *tfec*. Although the evidence in these studies cannot be generalized to all reptiles and more work is necessary to address reptilian coloration diversities [40] fully, these investigations represent essential steps forward.

Conclusions

The findings presented in the current study provide valuable insights into the histological, ultrastructural, and immunohistochemical characteristics of the skin in three iguana species from the Galápagos Islands. This study notably confirmed the absence of iridophores in the skin of adult individuals across the three species examined, irrespective of their external coloration. Further research is needed to determine whether iridophores are present in pink iguana hatchlings and to explore potential ontogenetic changes, including genetic mechanisms, that might result in their absence in adults. Moreover, the factors underlying chromatic variation in the skin of *A. cristatus* across populations from different islands, not addressed in this study, remain unknown, warranting the expansion of this investigation to include those populations. The examination of *C. marthae* skin, the pink iguana residing at high elevations, introduces intriguing possibilities for understanding the impact of UV radiation on skin health. The absence of mature melanocytes, combined with a thickened stratum corneum, a multilayered dermis, and the presence of very large vessels, suggests an interplay of adaptive and non-adaptive traits, not fully understood, that could also be relevant to human skin research. Indeed, despite the very limited knowledge available, no skin cancers have been currently described in *C. marthae* despite exposure to extreme doses of UV radiation. Altogether, this evidence opens new avenues for exploring protective strategies against such environmental challenges in human skin.

Acknowledgements
We are indebted to the Galápagos National Park rangers for their invaluable support and friendship. We gratefully thank J Ramirez-Kastdalen for giving us permission to publish his photo of a *C. marthae* hatchling. This work is part of a long-term institutional agreement between the University Tor Vergata and the Galápagos National Park Directorate to conserve Galápagos iguanas.

Author contributions
GG and GM conceived the project; GG, CS LGA, GC, EC, GPG, AM carried out fieldwork; GG provided samples; MS and GG wrote the manuscript; MS, RB, YV performed the laboratory analyses; MS prepared figures. All the authors provided critical advice on an early version of the manuscript and approved this submitted version.

Funding
This work has been supported by National Geographic Grant NGS-68399 C-20 to GG; International Iguana Foundation 2017 Grant to GG; the European Union NextGenerationEU via MUR-PNRR M4C2-II.3 PE6 project PE00000019 Heal Italia (CUP: E83C22004670001) to GM, EC, AM, Associazione Italiana per la Ricerca contro il Cancro (AIRC) to GM (IG 2022 ID 27366; 2023–2027); Ministry of Health - HUB LIFE SCIENCE – Advanced Diagnostic- Italian network of excellence for advanced diagnosis (INNOVA) (PNC-E3-2022-23683266) to GM, AM, EC, MS; Fondazione Luigi Maria Monti IDI-IRCCS (RC to EC). This work has also been supported by Regione Lazio through LazioInnova Progetto Gruppo di Ricerca n 85-2017-14986; n 33 & 55-2021-T0002E0001.

Data availability
As per regulation enforced by the Ecuadorian Ministry of Environment, samples were entirely used for the present investigation.

Declarations

Ethics approval and consent to participate
Animal manipulation and tissue sampling were conducted following a protocol designed to minimize stress on the animals, adhering to established guidelines of all institutions involved and receiving approval from the Galápagos National Park Directorate, the governmental body responsible for biodiversity management in the Galápagos Islands of Ecuador. Samples were exported and imported under CITES export/import permits granted to GG.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 25 October 2024 / Accepted: 9 January 2025
Published online: 04 February 2025

References

1. Rutland CS, Cigler P, Kubale V. Reptilian skin and its special histological structures. In: Rutland CS, Kubale V, editors. Veterinary anatomy and physiology. IntechOpen. https://doi.org/10.5772/intechopen.84212
2. Di Giacomo C, Pucillo L, Sevilla C, Fucci G, Massoud R, Bernardini S, et al. 25-Hydroxyvitamin D plasma levels in natural populations of pigmented and partially pigmented Land Iguanas from Galápagos (*Conolophus* spp). Biomed Res Int. 2022;2022:7741397.
3. Lewbart GA, Grijalva CJ, Calle PP, Ingerman K, Muñoz-Pérez JP, Quezada G, et al. Health assessment of *Conolophus subcristatus*, *Conolophus pallidus*, and *C. subcristatus* X *Amblyrhynchus cristatus* hybrid (Galápagos land iguanas). PLoS ONE. 2019;14(10):e0222884.
4. Loreti P, Catini A, De Luca M, Bracciale L, Gentile G, Di Natale C. The design of an energy harvesting wireless sensor node for tracking pink iguanas. Sensors (Basel). 2019;19(5):985.
5. MacLeod A, Rodríguez A, Vences M, Orozco-terWengel P, García C, Trillmich F, et al. Hybridization masks speciation in the evolutionary history of the Galápagos marine iguana. Proc Biol Sci. 2015;282(1809):20150425.
6. Nieto-Claudín A, Sacristán C, Deem SL, Lewbart GA, Colosimo G, Esperón F, et al. Novel herpesvirus in the critically endangered Galapagos pink land iguana. Acta Trop. 2024;252:107127. https://doi.org/10.1016/j.actatropica.2024.107127
7. Gentile G. *Conolophus marthae*. The IUCN red list of threatened species. 2012. https://doi.org/10.2305/IUCN. UK. 2012- 1. RLTS. T1744 72A14 14375. En.
8. Gentile G, Snell H. *Conolophus marthae* sp.nov. (Squamata, Iguanidae), a new species of land iguana from the Galápagos archipelago. Zootaxa. 2009;2201:1–10.
9. Gentile G, Marquez C, Snell HL, Tapia W, Izurieta A. Conservation of a New Flagship species: the Galápagos Pink Land Iguana (*Conolophus marthae* Gentile and Snell, 2009). In: Angelici F, editor. Problematic Wildlife. Cham: Springer; 2016. https://doi.org/10.1007/978-3-319-22246-2_15
10. Lewbart GA, Colosimo G, Gaudette C, Negrão Watanabe TT, Parker J, Sevilla C, et al. 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*). Acta Zool. 2023;00:1–10. https://doi.org/10.1111/azo.12488
11. Ramirez-Kastdalen J, Cadena JP, Ortiz L. Habitat use, nesting biology, and demographic trends of the pink land iguana (*Conolophus marthae*) and Galápagos land iguana (*Conolophus subcristatus*) at Wolf volcano, Isabela island. IUCN SSC Iguana Specialist Group Annual Meeting. Soufriere, 2021.
12. Saenko SV, Teyssier J, van der Marel D, Milinkovitch MC. Precise colocalization of interacting structural and pigmentary elements generates extensive color pattern variation in *Phelsuma* lizards. BMC Biol. 2013;11:105.
13. Haisten DC, Paranjpe D, Loveridge S, Sinervo B. The Cellular basis of polymorphic coloration in common side-blotched lizards, *Uta stansburiana*. Herpetologica. 2005; 71(2). http://www.jstor.org/stable/24635515
14. Kuriyama T, Miyaji K, Sugimoto M, Hasegawa M. Ultrastructure of the dermal chromatophores in a lizard (Scincidae: *Plestiodon latiscutatus*) with conspicuous body and tail coloration. Zoolog Sci. 2006;23(9):793–9. https://doi.org/10.2108/zsj.23.793. PMID: 17043401.
15. Szydłowski P, Madej JP, Mazurkiewicz-Kania M. Histology and ultrastructure of the integumental chromatophores in tokay gecko (*Gekko gecko*) (Linnaeus, 1758) skin. Zoomorphology. 2017;136(2):233–40. https://doi.org/10.1007/s00435-017-0348-9. Epub 2017 Mar 17. PMID: 28553007; PMCID: PMC5423961.
16. Alibardi L, Toni M. Cytochemical, biochemical and molecular aspects of the process of keratinization in the epidermis of reptilian scales. Prog Histochem Cytochem. 2006;40(2):73–134. https://doi.org/10.1016/j.proghi.2006.01.001. Epub 2006 Mar 14. PMID: 16584938.
17. Tarantino U, Scimeca M, Piccirilli E, Tancredi V, Baldi J, Gasbarra E, et al. Sarcopenia: a histological and immunohistochemical study on age-related muscle impairment. Aging Clin Exp Res. 2015;27(Suppl 1):S51–60.
18. Bonfiglio R, Sisto R, Casciardi S, Palumbo V, Scioli MP, Giacobbi E, et al. Aluminium bioaccumulation in colon cancer, impinging on epithelial-mesenchymal-transition and cell death. Sci Total Environ. 2024;908:168335.
19. Scimeca M, Giocondo R, Montanaro M, Granaglia A, Bonfiglio R, Tancredi V et al. BMP-2 Variants in Breast Epithelial to Mesenchymal Transition and Microcalcifications Origin. Cells. 2020; 2;9(6):1381.
20. Hammer Ø, Harper DAT, Ryan PD. PAST: paleontological statistics software package for education and data analysis. Palaeontol Electron. 2001;4(1):9. http://palaeo-electronica.org/2001_1/past/issue1_01.htm
21. Scimeca M, Bonfiglio R, Varone F, Ciuffa S, Mauriello A, Bonanno E. Calcifications in prostate cancer: an active phenomenon mediated by epithelial cells with osteoblast-phenotype. Microsc Res Tech. 2018;81(7):745–8.
22. Mauriello A, Scimeca M, Amelio I, Massoud R, Novelli A, Di Lorenzo F et al. Thromboembolism after COVID-19 vaccine in patients with preexisting thrombocytopenia. Cell Death Dis. 2021; 3;12(8):762.
23. Bischetti S, Scimeca M, Bonanno E, Federici M, Anemona L, Menghini R, et al. Carotid plaque instability is not related to quantity but to elemental composition of calcification. Nutr Metab Cardiovasc Dis. 2017;27(9):768–74.
24. Bonfiglio R, Galli F, Varani M, Scimeca M, Borri F, Fazi S et al. Extensive Histopathological Characterization of Inflamed Bowel in the Dextran Sulfate Sodium Mouse Model with Emphasis on Clinically Relevant Biomarkers and Targets for Drug Development. Int J Mol Sci. 2021; 18;22(4):2028.
25. De Luca A, Rotili D, Carpanese D, Lenoci A, Calderan L, Scimeca M, et al. A novel orally active water-soluble inhibitor of human glutathione transferase exerts a potent and selective antitumor activity against human melanoma xenografts. Oncotarget. 2015;6(6):4126–43.
26. Porter WR, Witmer LM. Vascular Patterns in Iguanas and Other Squamates: Blood Vessels and Sites of Thermal Exchange. PLoS One. 2015; 14;10(10):e0139215.
27. Morgareidge KR, White FN. Cutaneous vascular changes during heating and cooling in the Galápagos Marine iguana. Nature. 1969;9(5206):587–91.

28. Monahan CF, Meyer A, Garner MM, Kiupel M. Gross, histologic, and immunohistochemical characteristics of cutaneous chromatophoromas in captive bearded dragons. J Vet Diagn Invest. 2021;33(5):932–8. https://doi.org/10.1177/10406387211025651
29. Kvam E, Tyrrell RM. The role of melanin in the induction of oxidative DNA base damage by ultraviolet A irradiation of DNA or melanoma cells. J Invest Dermatol. 1999;113(2):209 – 13. https://doi.org/10.1046/j.1523-1747.1999.00653.x. PMID: 10469305.
30. Korytowski W, Pilas B, Sarna T, Kalyanaraman B. Photoinduced generation of hydrogen peroxide and hydroxyl radicals in melanins. Photochem Photobiol. 1987;45(2):185 – 90. https://doi.org/10.1111/j.1751-1097.1987.tb05362.x. PMID: 3031709.
31. Marrot L, Belaidi JP, Meunier JR, Perez P, Agapakis-Causse C. The human melanocyte as a particular target for UVA radiation and an endpoint for photoprotection assessment. Photochem Photobiol. 1999;69(6):686–93. PMID: 10378007.
32. Sreedhar A, Aguilera-Aguirre L, Singh KK. Mitochondria in skin health, aging, and disease. Cell Death Dis. 2020;11(6):444. https://doi.org/10.1038/s41419-020-2649-z. PMID: 32518230; PMCID: PMC7283348.
33. Gustavino B, Terrinoni S, Paglierani C, Gentile G. (2014) *Conolophus marthae* vs. *Conolophus subcristatus*: does the skin pigmentation pattern exert a protective role against DNA damaging effect induced by UV light exposure? Analysis of blood smears through the micronucleus test. Paper presented at the Galápagos Land and Marine Iguanas Workshop, IUCN SSC Iguana Specialist Group Meeting, Puerto Ayora, 28–29 October 2014.
34. Rohrlich ST, Porter KR. Fine structural observations relating to the production of color by the iridophores of a lizard. *Anolis carolinensis*. J Cell Biol. 1972;53(1):38–52.
35. Morrison RL, Sherbrooke WC, Frost-Mason SK. Temperature-sensitive, physiologically active iridophores in the lizard *Urosaurus ornatus*: an ultrastructural analysis of color change. Copeia, 1996, 804.
36. Paray1 BA, Al-Sadoon MK. Ultrastructure of the dermal chromatophores in the Fringe-toed lizard, *Acanthodactylus orientalis*. ZOOLOGIA 34: e11923 | https://doi.org/10.3897/zoologia.34.e11923
37. Brejcha J, Bataller JV, Bosáková Z, Geryk J, Havlíková M, Kleisner K, et al. Body coloration and mechanisms of colour production in Archelosauria: the case of deirocheline turtles. R Soc Open Sci. 2019;6(7):190319. https://doi.org/10.1098/rsos.190319
38. Garcia-Elfring A, Sabin CE, Iouchmanov AL, Roffey HL, Samudra SP, Alcala AJ et al. Piebaldism and chromatophore development in reptiles are linked to the tfec gene. Curr Biol. 2023; 27;33(4):755–763.e3. https://doi.org/10.1016/j.cub.2023.01.004. Epub 2023 Jan 25.
39. Tang CY, Zhang X, Xu X, Sun S, Peng C, Song MH, et al. Genetic mapping and molecular mechanism behind color variation in the Asian vine snake. Genome Biol. 2023;9(1):46. https://doi.org/10.1186/s13059-023-02887-z
40. Tzika AC. On the role of TFEC in reptilian coloration. Front Cell Dev Biol. 2024;7:12:1358828. https://doi.org/10.3389/fcell.2024.1358828

Publisher's note