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RESEARCH ARTICLE

Unique evolution of vitamin A as an external pigment in tropical starlings

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ABSTRACT

Pigments are largely responsible for the appearance of organisms. Most biological pigments derive from the metabolism of shikimic acid (melanins), mevalonic acid (carotenoids) or levulinic acid (porphyrins), which thus generate the observed diversity of external phenotypes. Starlings are generally dark birds despite iridescence in feathers, but 10% of species have evolved plumage pigmentation comprising bright colors that are known to be produced only by carotenoids. However, using micro-Raman spectroscopy, we have discovered that the bright yellow plumage coloration of one of these species, the Afrotropical golden-breasted starling *Cosmopsarus regius*, is not produced by carotenoids, but by vitamin A (all-*trans*-retinol). This is the first organism reported to deposit significant amounts of vitamin A in its integument and use it as a body pigment. Phylogenetic reconstructions reveal that the retinol-based pigmentation of the golden-breasted starling has independently appeared in the starling family from dark ancestors. Our study thus unveils a unique evolution of a new class of external pigments consisting of retinoids.

KEY WORDS: Biological pigments, Birds, Carotenoids, Color evolution, Retinol

INTRODUCTION

The evolution of organisms is mediated in a significant part by their appearance. External coloration greatly determines the capacity to adapt to the environment (Manceau et al., 2011) and reinforces the differentiation of incipient species (Sætre et al., 1997; Seehausen et al., 2008). Highly diversified animal clades are indeed often associated to the evolution of conspicuous color traits, because sexual selection, which plays an important role in the generation of isolating mechanisms by adaptive radiation (Price, 1998), favors conspicuous phenotypes (Maan and Seehausen, 2011).

Many of the bright colors shown by photosynthetic organisms are produced by biosynthesizing tetraterpene compounds termed carotenoids (Alvarez et al., 2013). With the exception of few invertebrates able to synthesize these pigments (Moran and Jarvik, 2010), animals accumulate and modify carotenoids consumed with the plant products of their diet (Toews et al., 2017). Consequently, carotenoids constitute a main class of pigments responsible for the color of organisms (Alvarez et al., 2013). Another large class of biological pigments is represented by melanins, polymers derived from the oxidation of tyrosine or phenolic compounds that virtually all organisms synthesize (d'Ischia et al., 2015). The third most common class of pigments is represented by porphyrins, aromatic rings produced in virtually all cells as intermediates during the synthesis of heme, but which only certain groups of birds use as external pigments (Galván et al., 2018). Carotenoids, melanins and porphyrins derive from the metabolism of three chemical precursors: mevalonic, shikimic and levulinic acids, respectively (Gudin, 2003). Other exclusive classes of pigments are only found in particular groups of animals, such as psittacofulvins in psittaciform birds (parrots and allies; Martínez, 2009), spheniscins in penguins (Thomas et al., 2013) and ommochromes in spiders and other invertebrates (Hsiung et al., 2017).

Therefore, the synthesis, accumulation or modification of carotenoids, melanins and porphyrins, together with their interaction with specialized morphological structures (Maia et al., 2013), are mainly responsible for the observed diversity of biological colors. Here, we report the evolution of a novel class of pigments responsible for bright body coloration in a tropical species of the family Sturnidae (starlings), which comprises passerine birds widely distributed in Africa, Asia and Europe (Fig. 1).

MATERIALS AND METHODS

Reconstruction of ancestral pigmentation patterns

We investigated the pigmentation patterns of 107 species of starlings, representing the entire family Sturnidae (Lovette and Rubenstein, 2007). The 2–3 species of dark pigmented Philippine creepers (*Rhabdornis* spp.) were excluded, as their position within Sturnidae still requires additional support (Zuccon et al., 2006). By examining starling illustrations (Feare and Craig, 1998), we categorized each species as bright, if they exhibited brilliant yellow or red/pink plumage patches regardless their size or number, or dark, if they did not exhibit any plumage patch with such colors. These colors are known to be produced by the deposition of carotenoid pigments in feathers (McGraw, 2006). We did not consider iridescence in feathers nor color of skin.

We obtained 1000 probable phylogenies for the 107 starling species with branch lengths expressed as proportions of nucleotide substitutions using the phylogeny subsets tool in Birdtree (www.birdtree.org; Jetz et al., 2012). We then obtained the least-squares consensus phylogenetic tree (Fig. 2) from the mean patristic distance matrix of the set of 1000 phylogenies using the package phytools (Revell, 2012) in R environment (https://www.r-project.org/). The reconstruction of ancestral states was made using an Mk model as implemented in phytools, assuming that transitions among states follow a continuous-time Markov chain process (Pagel, 1994; Lewis, 2001). Using the set of 1000 phylogenies, we calculated the empirical Bayesian posterior probabilities and

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Fig. 1. Pigmentation patterns in Sturnidae. Despite iridescence produced by feather nanostructures, the plumage of most starlings is uniformly dark due to eumelanin pigments as exemplified by the common starling *Sturnus vulgaris* (A) and the metallic starling *Aplonis metallica* (B), or include patches of dull orange color due to pheomelanin pigments like in the superb starling *Lamprotornis superbus* (C). In contrast, some exceptional species have evolved bright plumage coloration, which is particularly notable in the large breast and ventral yellow plumage of the golden-breasted starling *Cosmopsarus regius* (D). Other starling species with bright plumage pigmentation include the rosy starling *Sturnus roseus* (E), the grosbeak starling *Scissirostrum dubium* (F), the bank myna *Acridotheres ginginianus* (G), the golden-crested myna *Ampeliceps coronatus* (H), the yellow-faced myna *Mino dumontii* (I), the fiery-browed myna *Enodes erythrophris* (J) and the Sulawesi myna *Basilornis celebensis* (K). Image credits for A–H, respectively: hedera.baltica, cuatrok77, Lip Kee, Mike, Imran Shah, Jonathan Leung, Imran Shah and Charles Lam under CC BY 2.0 license (https://creativecommons.org/licenses/by/2.0/). Image credit for I: Doug Janson under CC BY-SA 3.0 license (https://creativecommons.org/licenses/by-sa/3.0/). Images in J and K are reproduced with permission from Pete Morris/Birdquest and Pierre de Chabannes, respectively.

represented them as pie charts on the nodes of the consensus tree (Fig. 2).

Animals

We collected six yellow breast feathers from a golden-breasted starling *Cosmopsarus regius* Reichenow 1879 and six head yellow feathers from a golden-crested myna *Ampeliceps coronatus* Blyth 1842 (Fig. 1). The feathers were obtained from adult birds kept in captivity at Attica Zoological Park, Athens, Greece. Additionally, we collected pink breast feathers from an adult rosy starling *Sturnus roseus* (Linnaeus 1758) and undertail yellow feathers from an adult yellow-faced myna *Mino dumontii* Lesson 1827 (Fig. 1), using skins deposited at the Museum of Vertebrate Zoology at the University of California at Berkeley, USA (specimens MVZ 70159 and MVZ 93404). Feather samples were stored in the dark until analysis. The plumage colors of these four species thus cover the entire color diversity shown by starling species with bright plumage, and are therefore representative of the Sturnidae family (Fig. 2).

Raman spectroscopy

To obtain a molecular characterization of pigments responsible for the bright colors observed in starlings (which we hypothesized to be carotenoids), we analyzed pigmented feathers from the four species described above (golden-breasted starling, golden-crested myna, yellow-faced myna and rosy starling) by non-destructive micro-Raman spectroscopy. Raman spectra were obtained with an inVia Renishaw Microscope spectrometer (Renishaw, Gloucestershire, UK). A 532 nm laser beam was used as an excitation source, which was focused on the samples using a 100× objective. Acquisition time was 10 s, and spatial resolution was 1 µm. Laser power was fixed to a range of 1–5 mW μm^{-2}, avoiding heating damage to samples. Thirty to forty spectra were recorded for each feather, and an average spectrum was then calculated for each species. The control of the Raman system and recording of data were made using the WiRE interface (Renishaw, v4.4). The spectra were baseline corrected and normalized prior to analyses.

Vibrational signatures recorded from Raman spectra allowed the molecular identification of pigments. The main Raman bands of carotenoids are usually termed ν_1 to ν_3 and arise from stretching vibrations of C=C double bonds (ν_1), from stretching vibrations of C–C single bonds coupled with C–H in-plane bending vibration (ν_2), and from in-plane rocking vibrations of methyl groups (ν_3) (Arteni et al., 2015; Kish et al., 2015).

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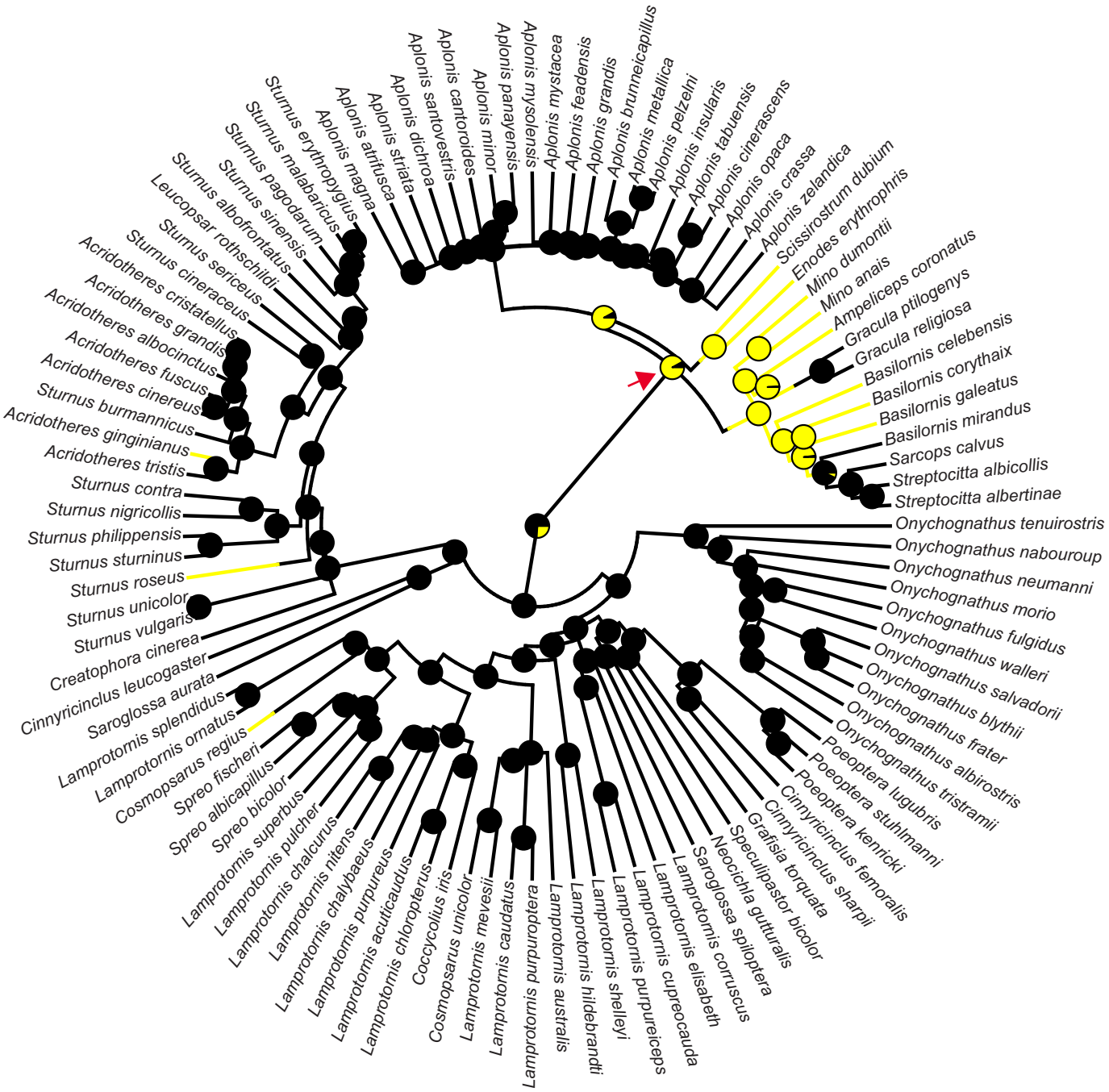

Fig. 2. Consensus phylogenetic tree for starlings. Extant species and ancestral states with bright plumage coloration are indicated in yellow. Ancestral states are reconstructed following an Mk model. Pie charts at internal nodes indicate the empirical Bayesian posterior probabilities for bright versus dark plumage coloration. The red arrow indicates the ancestor of the Oriental–Australasian clade.

RESULTS

Most starling species exhibit metallic hues due to iridescence in their plumage that is produced by interaction between specially ordered nanostructures and melanin granules in feathers (Maia et al., 2013). Despite this, the general appearance of most starlings is dark, as their plumage is colored black alone or in combination with dull brown or orange patches (Maia et al., 2013) (Fig. 1A–C) as a consequence of the deposition in feathers of two melanin forms (Galván and Wakamatsu, 2016). However, out of 107 species in the starling family, 11 (10.3%) have evolved bright (i.e. non-melanin based) yellow or red/pink plumage coloration (Fig. 1D–K). We plotted these species in a consensus phylogenetic tree for the family and reconstructed the ancestral states of their plumage coloration (dark versus bright) (see Materials and Methods). This shows that the common ancestor of starlings was most probably dark but bright plumage rapidly evolved, as the common ancestor of one of the three major lineages of starlings, the Oriental–Australasian clade (Zuccon et al., 2006), was most probably already brightly pigmented (Fig. 2). Considering 1000 probable phylogenies for the family, we calculated that, on average, 7.8 gains and losses of bright plumage have occurred during the evolution of starlings, gains (4.18) prevailing on loses (3.62) (Fig. 2). It is notable that, outside the Oriental–Australasian clade, bright plumage has independently appeared three times in species with a long evolutionary history of dark plumage (Fig. 2): the

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golden-breasted starling *Cosmopsarus regius* (Fig. 1D), the rosy starling *Sturnus roseus* (Fig. 1E) and the bank myna *Acridotheres ginginianus* (Fig. 1G).

The colors exhibited by starlings with bright plumage (yellow and red/pink; Fig. 1D–K) are known to be produced by carotenoids (McGraw, 2006). We thus hypothesized that carotenoid-pigmented feathers have independently appeared in different clades of starlings. However, although carotenoid-based plumage coloration has independently evolved in several lineages of birds (Thomas et al., 2014), all studies conducted within lineages to date indicate that extant species with carotenoid-based coloration have evolved from common ancestors that already exhibited carotenoid pigmentation (Prager and Andersson, 2010; Friedman et al., 2014). As the common starling ancestor lacked bright plumage pigmentation (Fig. 2), extant starling species with bright plumage may represent an unprecedented evolutionary gain of carotenoid-based pigmentation. We therefore investigated the chemical nature of pigments in starlings with bright plumage coloration.

The Raman spectra of golden-crested and yellow-faced myna yellow feathers showed three strong Raman bands at 1520.5–1526.6 cm^{-1}, 1151.8–1156.6 cm^{-1} and 1004.7–1006.1 cm^{-1} (Fig. 3B), coinciding with ν_1–ν_3 for lutein (Lewis, 2001; Kish et al.,

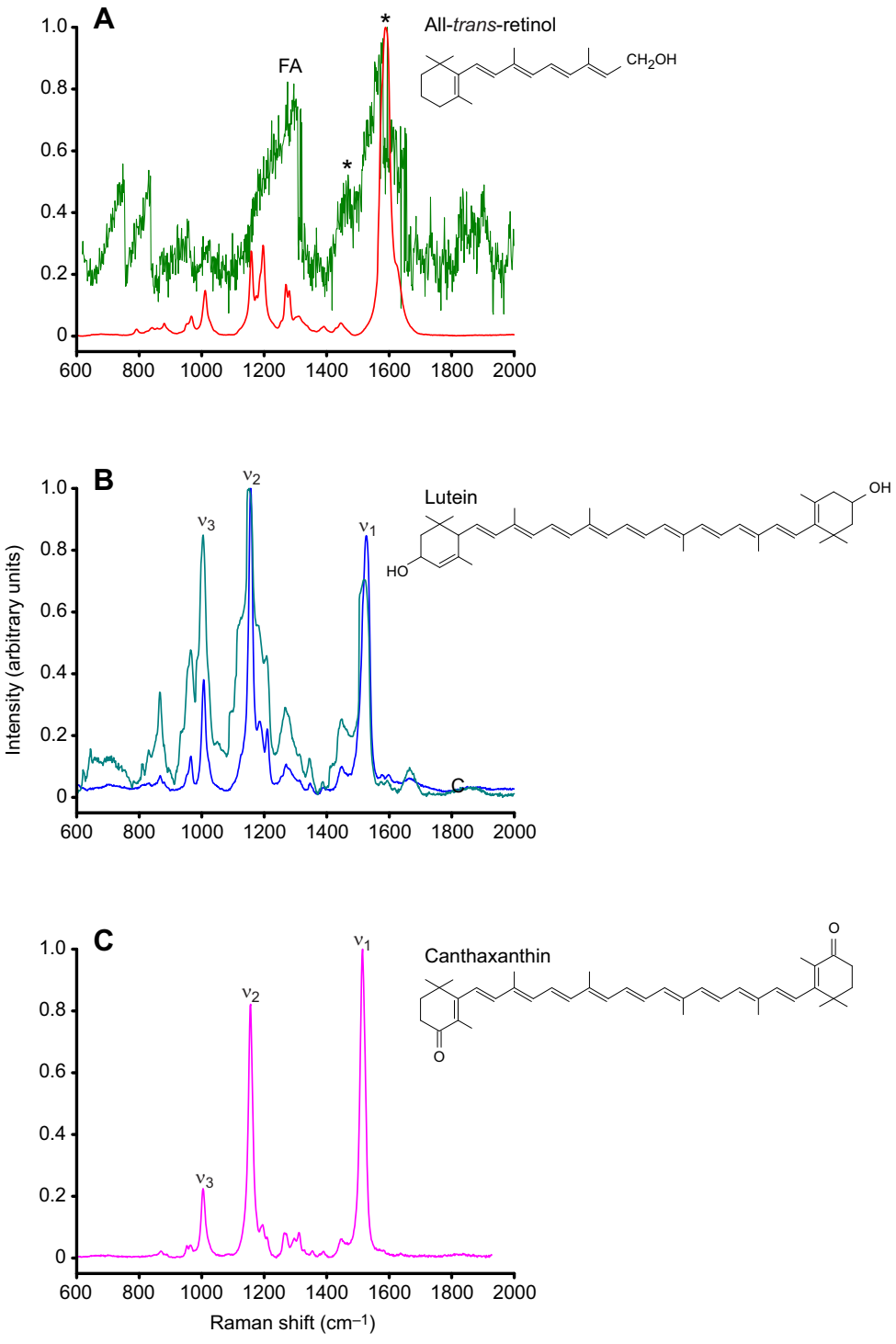

Fig. 3. Raman spectra and chemical structure of starling pigments. (A) Yellow feathers of golden-breasted starling *Cosmopsarus regius* (green) and all-*trans*-retinol standard (red). *Retinol bands; FA, fatty acid band. (B) Yellow feathers of golden-crested myna *Ampeliceps coronatus* (light blue) and yellow-faced myna *Mino dumontii* (dark blue). ν_1–ν_3 indicate characteristic Raman bands of lutein. (C) Pink feathers of rosy starling *Sturnus roseus*. ν_1–ν_3 indicate characteristic Raman bands of canthaxanthin.

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2015). The Raman spectrum of rosy starling pink feathers showed three strong Raman bands at 1514.7, 1156.7 and 1004.4 cm^{-1} (Fig. 3C), coinciding with ν_1–ν_3 for canthaxanthin (Veronelli et al., 1995; Kish et al., 2015).

On the other hand, the Raman spectrum of golden-breasted starling yellow feathers showed a strong band at 1594.4 cm^{-1} (Fig. 3A), indicative of the polyene stretching vibration of all-*trans*-retinol (Mélot et al., 2009; Saar et al., 2010). To ascertain the characterization of this pigment, we also analyzed standard all-*trans*-retinol powder purchased from Sigma-Aldrich (St Louis, MO, USA). The Raman spectrum of standard retinol showed a main band at 1594 cm^{-1} and a weaker band at 1445 cm^{-1}, both coinciding with those of golden-breasted starling yellow feathers (Fig. 3A). In addition to these bands of retinol, the Raman spectrum of golden-breasted starling feathers, but not that of standard retinol, showed a band at 1295.2 cm^{-1} (Fig. 3A) that is characteristic of fatty acids (De Gelder et al., 2007). The absorption spectrum of standard retinol shows strong absorbance in the UV spectral region and no significant absorbance in the visible region (400–700 nm) (Fig. 4), which is responsible for the reflectance of retinol in the visible region. This reflectance is perceived as an intense yellow color in both the powder and the solution (Fig. 4), which further supports the assignment of retinol as the pigment responsible for the yellow color of golden-breasted starling feathers.

DISCUSSION

The results of these analyses show that, as predicted, the pink color of feathers of the rosy starling and the yellow color of golden-crested and yellow-faced mynas are generated by two carotenoids, the xantophylls canthaxanthin and lutein, respectively (Fig. 3B,C). Surprisingly, however, the vibrational signatures of the yellow feathers of the golden-breasted starling indicate that these are not pigmented by a carotenoid. Instead, the Raman spectrum of golden-breasted starling yellow feathers shows a unique signature of all-*trans*-retinol, which is probably esterified with a fatty acid. The golden-breasted starling is thus the first organism found to be pigmented by retinol.

All-*trans*-retinol is vitamin A, which functions as a pigment only in the rod cells of the retina where the aldehyde (all-*trans*-retinal) binds to opsin to form rhodopsin, essential for the visual function (Dryja et al., 1990). Vitamin A is considered the most multifunctional vitamin, as it fulfills a diversity of physiological functions other than vision, including immunity and epithelial cell differentiation and proliferation (Alvarez et al., 2013). Vitamin A is biosynthesized by the enzymatic cleavage of β-carotene and a few other carotenoids, which generates all-*trans*-retinol and its metabolites, i.e. retinoids (Alvarez et al., 2013). Retinoids are diterpenes (C_{20}) composed of four isoprene (C_5) units, whereas carotenoids are longer molecules formed by tetraterpenes (C_{40}) composed of eight isoprene units (Alvarez et al., 2013) (Fig. 3). Carotenoids and retinoids are therefore structurally different compound classes, despite the latter being derived from the former. Mammals metabolize retinol at the skin (Roos et al., 1998), but retinol has never been found to contribute to the body pigmentation of any organism. Our results indicate that the golden-breasted starling has evolved a physiological ability to deposit retinol in the integument and create a conspicuous large yellow trait, thus representing the only organism able to use vitamin A as a body pigment.

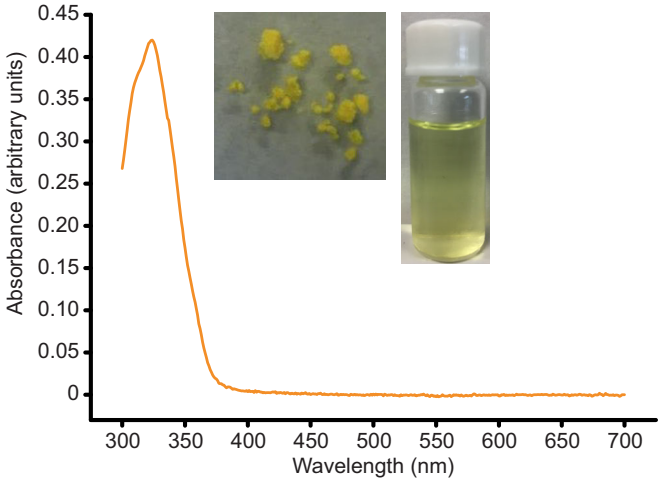

Fig. 4. UV-visible absorption spectrum of all-*trans*-retinol. The curve corresponds to the mean values of two measurements taken on a 10 ppm solution of synthetic all-*trans*-retinol in hexane. Insert photographs show the characteristic yellow color of retinol powder and of the solution.

The deposition of retinol in feathers by golden-breasted starlings appears to be an evolutionary innovation to increase conspicuousness despite phylogenetic inertia of dark pigmentation. The rosy starling and the bank myna have also independently evolved bright plumage patches from dark starling ancestors, but these species deposit carotenoids in feathers like most birds with bright plumage pigmentation (McGraw, 2006). The golden-breasted starling, in contrast, has evolved a different physiological pathway to become conspicuous. Indeed, the golden-breasted starling exhibits the brightest and largest pigmented trait in the Sturnidae family (Fig. 1). The genetic and physiological mechanisms that allow the golden-breasted starling to deposit retinol in the integument should now be elucidated, but it may be that a large number of melanin-producing cells (melanocytes) in starlings, as expected from dark organisms (McGowan et al., 2008), could have favored the evolution of retinol as a body pigment. This is because, in the skin, melanocytes show a higher capacity to uptake β-carotene and convert it to retinol than keratinocytes (Andersson et al., 2001). In birds, melanocyte progenitors located at dermal follicles transfer melanocytes vertically as feathers grow and thus create plumage pigmentation patterns (Lin et al., 2013). In golden-breasted starlings, retinol may thus enter this pathway in follicles and become incorporated in the feather surface. The ability to pump retinol out of dermal cells and deposit it in integumentary structures is unprecedented and represents a novel mechanism that determines the appearance of organisms.

Although animals obtain retinol with the diet either directly or metabolizing pro-vitamin A carotenoids (Alvarez et al., 2013), the role of retinol in vision as part of the retinal pigment rhodopsin can be unpaired by defects in genes encoding proteins involved in the retinoid cycle in the eye or by degenerative diseases that ultimately lead to blindness (Palczewski, 2010). In birds, the genes that regulate the conversion of yellow to red carotenoids are expressed in both pigmented integumentary tissues and in the retina (Mundy et al., 2016), thus it is likely that the genes involved in retinoid metabolism are expressed in both the integument and the retina of golden-breasted starlings. The physiological lability that allows these birds to permanently pigment their body surface may thus contribute to the design of novel strategies to increase the stability of retinol in the eye. Additionally, understanding how the golden-breasted starling extracts retinol from epidermal cells may help to improve the penetration of this molecule in human skin after topical application, which is inefficient without the use of synthetic enhancers but of great interest to prevent skin damage (Mélot et al., 2009).

The golden-breasted starling, which inhabits open habitats and shrublands in East Africa (Feare and Craig, 1998), may therefore represent an animal model to avoid limitations in retinol metabolism and application. Together with the recent discovery of a neotropical bat as the first mammal with the capacity to deposit significant amounts of a carotenoid (lutein) in the skin, which may help in the search for a treatment for macular degeneration (Galván et al., 2016), our findings highlight the importance of biodiversity in the tropics as reservoirs of evolutionary inventions that can provide overlooked solutions to pigmentation disorders.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: I.G.; Methodology: I.G., K.M., A.J., Á.R., M.Z.; Formal analysis: I.G., K.M., A.J., M.Z.; Investigation: I.G., K.M., M.Z.; Resources: K.M., M.Z.; Writing - original draft: I.G.; Supervision: Á.R.; Funding acquisition: I.G., Á.R., M.Z.

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