ORIGINAL ARTICLE

Estimating the distribution of carotenoid coloration in skin and integumentary structures of birds and extinct dinosaurs

Sarah N. Davis[1,2] (iD) and **Julia A. Clarke**[1,3] (iD)

[1]*Department of Geological Sciences, Jackson School of Geosciences, The University of Texas at Austin, Austin, Texas 78712*

 [2]*E-mail: sdavis6@utexas.edu*

[3]*Department of Integrative Biology, The University of Texas at Austin, Austin, Texas 78712*

Carotenoids are pigments responsible for most bright yellow, red, and orange hues in birds. Their distribution has been investigated in avian plumage, but the evolution of their expression in skin and other integumentary structures has not been approached in detail. Here, we investigate the expression of carotenoid-consistent coloration across tissue types in all extant, nonpasserine species (*n* = 4022) and archelosaur outgroups in a phylogenetic framework. We collect dietary data for a subset of birds and investigate how dietary carotenoid intake may relate to carotenoid expression in various tissues. We find that carotenoid-consistent expression in skin or nonplumage keratin has a 50% probability of being present in the most recent common ancestor of Archosauria. Skin expression has a similar probability at the base of the avian crown clade, but plumage expression is unambiguously absent in that ancestor and shows hundreds of independent gains within nonpasserine neognaths, consistent with previous studies. Although our data do not support a strict sequence of tissue expression in nonpasserine birds, we find support that expression of carotenoid-consistent color in nonplumage integument structures might evolve in a correlated manner and feathers are rarely the only region of expression. Taxa with diets high in carotenoid content also show expression in more body regions and tissue types. Our results may inform targeted assays for carotenoids in tissues other than feathers, and expectations of these pigments in nonavian dinosaurs. In extinct groups, bare-skin regions and the rhamphotheca, especially in species with diets rich in plants, may express these pigments, which are not expected in feathers or feather homologues.

KEY WORDS: Bird, carotenoids, color, dinosaurs, feather, skin.

Animal coloration can serve a range of functions, including providing camouflage, assisting in the identification of conspecifics, warning potential predators, or in attracting mates (Cott 1940; Brush 1990; Endler 2006; Renoult et al., 2011; Mann & Cummings, 2012; Vaughn et al., 2012). Color serves a variety of roles in extant birds (Aves), with impacts on life history ranging from mating success to foraging ability (Selander and Hunter 1960; Endler 2006; Stoddard & Prum, 2011). Avian pigment-based colors are most often produced by two sources: melanins and carotenoids (Stoddard & Prum, 2011). Melanins have been proposed as an ancestral color mechanism for the group (Stoddard & Prum, 2011; Eliason & Clarke, 2018), which is supported by preservation of melanosomes in the fossils of numerous avian

and nonavian dinosaurs (e.g., Zhang et al., 2010; Clarke et al., 2010, Li et al., 2010, 2012, 2014, 2018; Hu et al., 2018; Eliason & Clarke 2020) and its presence in extant outgroups. The evolutionary history of carotenoid use in color production remains much less clear. Carotenoids themselves have yet to be detected in any vertebrate fossil, although the presence of carotenoid-producing xanthophores has been suggested in a fossil snake (McNamara et al., 2016). An apparent paradox in the prevalence of carotenoid use in living birds is how quickly they degrade when exposed to environmental conditions; the pigments break down and lose color quality in the presence of light, heat, and oxygen (Woodall et al., 1997; Britton et al., 2004; Boon et al., 2010; demonstrated in Higginson et al., 2016). This combination

of instability and prevalence of use is distinct from melanins, the most common, and ancestrally present, pigments in birds, which are structurally more robust and provide mechanical support to feathers (Bonser 1995). The preferential preservation of melanins has been suggested to potentially introduce bias into the interpretation of color expression in extinct organisms, because the chemical structure of carotenoids makes them unlikely to preserve in the fossil record (see Thomas et al., 2014b, reviewed in Roy et al., 2020). However, the distribution and forms of carotenoids present across archosaurs have not been investigated using phylogenetic comparative methods. They were mistakenly reported as being present in Crocodilia (Roy et al., 2020) but are not known from that clade despite concerted attempts to locate them (see Alibardi 2011).

Living birds express combinations of 66 different described carotenoid compounds, and carotenoids have been reported from the plumage of 33% of all major extant groups (i.e., orders; Hill & McGraw, 2006; Thomas et al., 2014a; Badyaev et al., 2015). These compounds are obtained from the diet, metabolized and sometimes altered, then deposited into tissue to confer color (Brush, 1990; Hill & McGraw, 2006; see Table S1 for examples of dietary sources). Feather expression has been studied in detail across extant avian clades, and it is estimated that neognaths were expressing carotenoid-pigmented plumages by the early Cenozoic (Paleocene, 66-56 Mya; Thomas et al., 2014a). However, birds are also known to express carotenoids in other tissues, and carotenoid expression in bare parts is more common than expression in plumage (Olson & Owens, 2005; Iverson and Karubian 2017). Although most large-scale studies on bird coloration have focused on plumage (e.g., Stoddard & Prum, 2011; Thomas et al., 2014a; Nordén et al., 2018), coloration of nonplumage integument has been generally surveyed, scoring "bare part" coloration as present without reference to the exact body region or tissue in which color is expressed (e.g., Olson & Owens, 2005). Recent phylogenetic studies have also targeted the expression of melanins in the skin (e.g., Nicolaï et al., 2020). There have been neither large-scale, phylogenetic reconstructions investigating the association of pigment expression and integument structure nor reconstructions of color expression that account for the body region of color expression, leaving a gap in our understanding of the evolution of carotenoid-based color expression.

The locations and structures of pigment-bearing tissues may be an important aspect of the evolutionary pathway to carotenoid expression in birds. The role of carotenoids in physiological processes is well known in addition to coloration, and their functions in vision, immune response, and development have been studied across vertebrates (e.g., Hill & McGraw, 2006; Rhinn & Dollé, 2012; Steffen et al., 2015). In birds, they are also important in enhancing color detection and discrimination in the form of retinal oil droplets (Vorobyev, 2003; Toomey et al., 2016). Birds and turtles are the only living reptilian groups known to have these red droplets, and studies of the genes implicated in carotenoid modification suggested a duplication event associated with the gain of red retinal oil droplets (Twyman et al., 2016). This is hypothesized to have occurred at the base of Archelosauria (Testudines + Archosauria), suggesting that pterosaurs and nonavian dinosaurs may have also possessed pigmented retinal oil droplets (Twyman et al., 2016). Furthermore, it has been suggested that parts of the pathway for generating red oil droplets may have been co-opted for red integumentary coloration in birds, and that carotenoid expression may be more likely in multiple body regions or tissues given expression in one (Norden & Price, 2018).

The integumentary structures of birds have different patterns and mechanisms of growth, with different time scales for replacement. Skin can be rapidly replaced, whereas there are slow growing continuously replaced keratin structures (rhamphotheca [beak covering], podotheca [foot scales]), and seasonally or annually replaced structures (feathers; Lucas & Stettenheim, 1972). It has been shown experimentally that carotenoid colors in bare skin can change rapidly, having color quality replenished as quickly as 48 hours after eating carotenoid-rich foods (Faivre et al., 2003; Velando et al., 2006). This is different from expression in plumage, which can only have color renewed at semiannual or annual molts (Higginson et al., 2016). It therefore follows that, on evolutionary time scales, the mechanisms of carotenoid deposition may have first occurred in these more readily modified, vascularized tissues before progressing to deposition in keratinous structures.

Dietary factors may also have an impact on the extent of carotenoid expression in birds. Since expression of carotenoids is dependent on both obtaining and metabolically processing carotenoids from external sources, several studies have investigated connections between diet and color expression (reviewed in Hill & McGraw, 2006). Better access to carotenoids is generally associated with better quality of color, or with a specific coloration itself (e.g., Hill et al., 2002; Weaver et al., 2018; Peneaux et al., 2021), whereas a range of physiological processes can also impact expressed color (e.g., McGraw & Hill, 2000; Hill et al., 2019). Olson and Owens (2005) reported a strong association between dietary carotenoid intake and the presence of carotenoid-consistent colors in plumage across avian families, whereas surprisingly there was not an association between generalized "bare-part" color expression and dietary content. There may, however, be differences in how nonplumage regions of different structure relate to diet; tissues with high turnover may be less constrained by carotenoid intake, whereas more slowly replaced keratinous structures could be dependent on higher levels of dietary carotenoids at times of pigment deposition. Dietary availability of carotenoids may also constrain the number of body regions that can express pigments, as could the relative stability

of expressed compounds (Higginson et al., 2016). It is therefore necessary to more precisely consider the structures of tissues expressing color, and the extent of the body that is colorful, when considering a potential relationship with dietary availability.

Here, we investigate the occurrence of carotenoid-consistent color expression across tissue types in extant, nonpasserine birds and exemplars of archelosaur outgroups ($n = 4034$). Specifically, we test the prediction that carotenoid-consistent coloration in skin phylogenetically preceded acquisition in feathers. Continuously modified keratinous tissues such as those of the beak or foot may also be expected to precede color expression in seasonally or annually shed structures (i.e., feathers; although see the rhamphotheca in Alcidae; Lucas & Stettenheim, 1972, p. 6). For a subset of the total dataset ($n = 61$) with chemically characterized carotenoid compounds from different tissues, we investigated if there is a relationship between the stability of expressed carotenoids and the structure of the integument in which they are found. We predict that the relative stability of compounds in feathers is higher than those reported in vascularized skin and continuously replaced tissues. Finally, we assess whether dietary availability of carotenoids is correlated with the number of tissue types and body regions that express carotenoid-consistent colors, and whether different integuments are more or less associated with high levels of carotenoid intake.

Our scorings are described as "carotenoid-consistent" and not "carotenoid-produced" because chemical analyses were not undertaken for the most inclusive 4000+ species dataset (following the definition by Thomas et al., 2014a). We take a similar approach as other large-scale studies of carotenoid-consistent color expression using visual hypotheses of color source (i.e., Olson & Owens, 2005; Thomas et al., 2014a), because directly measuring the presence or absence of carotenoid pigments from avian nonplumage regions is a known challenge in color studies (discussed in Burns et al., 2017). Pigment classification is complicated not only by the copresence of a variety of compounds (Slifka et al., 1999; Ermakov et al., 2005; Hill & McGraw, 2006), but carotenoids are also present in circulating blood and plasma and are difficult to parse from those expressed as color, a factor that does not apply to feather pigments (Slifka et al., 1999; Ermakov et al., 2005; Hill & McGraw, 2006). Bare-part pigment data must also be collected from fresh specimens to avoid carotenoid degradation (McGraw et al., 2011; Burns et al., 2017), and assays typically require a relatively large tissue sample to detect compounds (Ermakov et al., 2005). Although museum specimens retain carotenoid pigments in feathers (Doucet & Hill, 2009), carotenoid stability in other tissues under similar storage conditions has not been studied. Arguably, characterizing carotenoids in these tissues for such a large sample may be infeasible; rapid color changes have been reported in skins, with skin often painted over to preserve a life-like appearance (Faivre

et al., 2003; Velando et al., 2006; McGraw et al., 2011; Burns et al., 2017). Although extensive chemical assays of nonfeather tissues are essential for definitive identification of carotenoids, our aim in the present study is to identify areas in color science that require more attention, while also providing a framework for considering the potential expression of carotenoids in deep time.

Methods

ASSESSING CAROTENOID-CONSISTENT COLOR

Colors were scored as "carotenoid-consistent" if (1) for a given species they have been shown in the literature to be produced by carotenoids or (2) are consistent with the colors produced by carotenoids (bright reds, oranges, yellows, and pinks, see Fig.1 for examples) and have not been demonstrated as coming from another coloration source (e.g., novel pigments, dust covering, blood; method adapted from Olson & Owens, 2005 and Thomas et al., 2014a). We included known co-occurrences of carotenoid pigments with other coloration mechanisms because the carotenoids themselves are integral to the final color display (e.g., Blue footed Booby feet with yellow carotenoids and structural blue coloration; Velando et al., 2006). For cases where novel pigments are known to be expressed in the plumage (i.e., penguins, parrots, turacos), we did not score plumages with carotenoid-consistent colorations as carotenoid-consistent. In penguins (Sphenisciformes), chemical data have shown that carotenoids are indeed present in bare skin parts and rhamphotheca but not in the yellow feathers (Jouventin et al., 2007; Thomas et al., 2013); because the pigments in nonplumage integuments of parrots and turacos have not been investigated, we scored those regions as carotenoid-consistent when applicable. Unique pigments are generally considered to have arisen independently in these three major clades, whereas all others have been shown to express carotenoids for coloration in at least the plumage (see References in the Supporting Information). We were also aware of instances of tissue flushing, where increased or decreased blood flow to a tissue can alter expressed color; however, because that is a short-term change to coloration it can confidently be visually distinguished from the use of pigments (see Negro et al., 2006). This also applied to iris tissue, which is highly vascularized and, in some species, has been observed to change rapidly due to flushing (e.g., pigeons [Hollander & Owen, 1939], chickens [Ball 1944], turtles [Carlson et al., 2020]). Again, because this was a short-term color change it can be visually distinguished from pigmentation.

Other pigments can confer colors similar to those of carotenoids in the skins of animals (e.g., pteridines in guppies, lizards, and turtles (Grether et al., 2001; Weiss et al., 2012; Brejcha et al., 2019); however, they are yet to be found in the skins or keratinous tissues of archosaurs (Hill & McGraw, 2006;
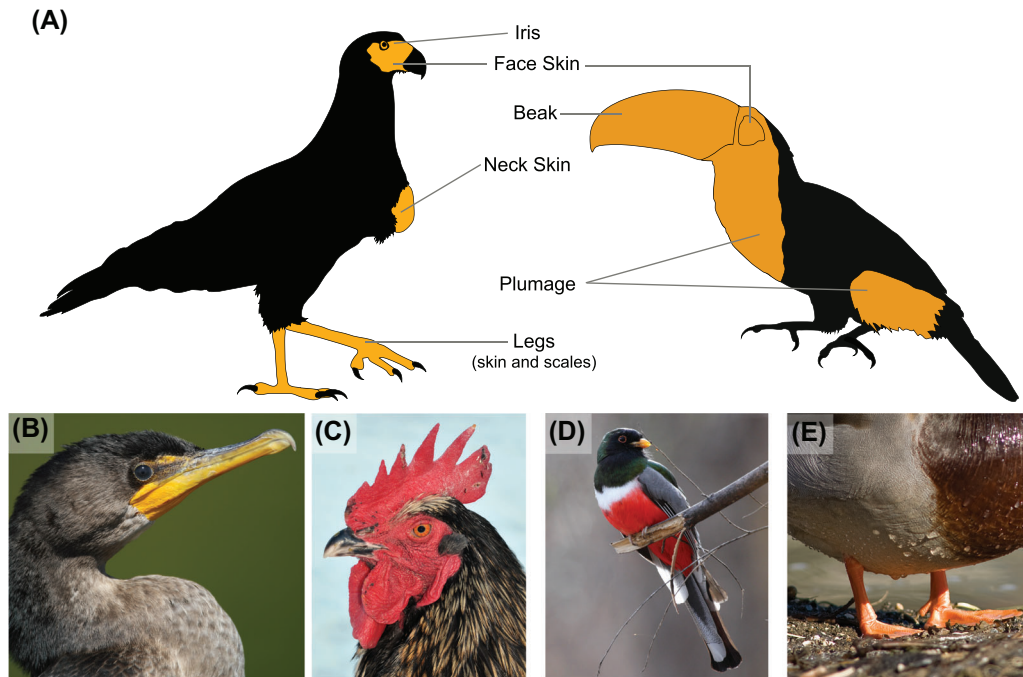
Figure 1. Body regions scored for carotenoid-consistent coloration. (A) Body regions include the iris tissue, facial skin, beak, neck skin including accessory neck integumentary structures (e.g., crop [illustrated], wattle, pouch; if present), leg skin, and plumage. (B-E) Examples of colors assessed as carotenoid-consistent, including (B) the layered rhamphotheca keratin and face skin, (C) iris tissue, face, wattle, and comb skin, (D) beak keratin and plumage (here, red regions only), and (E) leg skin and leg scales. All bird photographs available under Creative Commons licenses, and made available by Mdf (B), Michael Gäbler (C), Dominic Sherony (D), and Alain Carpentier (E).

Iverson & Karubian, 2017; Brejcha et al., 2019). Although pterins have been found in the iris tissue of birds, they are often found alongside carotenoids, and eye coloration across birds has not been widely investigated (Oliphant 1987). Pterins have been found in the yellow skins of turtles, but were present alongside carotenoids (Brejcha et al., 2019); only chemically verified instances of carotenoid expression were considered for turtles and nonavian archosaurs in this study. We investigated hypotheses that colors consistent with carotenoid expression occur first in heavily vascularized tissues such as skin or iris tissues (Lucas & Stettenheim, 1972; Hossler & Olson, 1984) before deposition in more modified, keratinous structures (e.g., rhamphotheca, feathers).

DATA COLLECTION AND COLOR SCORING
Color expression was recorded for all extant, nonpasserine bird species ($n = 4022$, based on the species list in Jetz et al., 2012) and 12 outgroup species (members of Testudines, Crocodilia; see Data in the Supporting Information). We included outgroup species from across the two major extant clades of turtles (Cryptodira and Pleurodira), and the three major extant clades within Crocodylia (Alligatoridae, Crocodylidae, and Gavialidae), and sampled a number of species proportional to the size of each outgroup clade. Passerines were not considered in this study be-

cause we focused on assessing potential carotenoid-consistent coloration in the ancestor of Aves and phylogenetically early acquisition of this trait; the highly nested position of passerines (Jetz et al., 2012; Burleigh et al., 2015; Kimball et al., 2019) means that their coloration states cannot significantly impact this ancestral avian condition. Furthermore, nonpasserines are comparatively understudied when it comes to the distribution of carotenoid-based coloration in tissues other than feathers.

For the sampled birds, the iris, beak keratin, face skin, neck skin, fleshy head and neck skin (i.e., a wattle, crop, or pouch), leg skin, leg scales, and plumage were considered in mature males and females (see Fig. 1). For seasonal color variants, color was scored as present if it was expressed by a species during some stage of the annual cycle. Data on carotenoid-consistent plumage color and other pigments assessed via Raman spectroscopy were available from Thomas et al. (2014a) and were modified only in consideration of subsequent studies (see Data in the Supporting Information for citations by species). Other nonfeather coloration was scored from open-source photographs of each species and verified using the *Handbook of the Birds of the World* (now: Birds of the World; del Hoyo et al., 2019; Billerman et al., 2020); in cases where species were split or subspecies were listed in the Handbook but not the Jetz et al. (2012) list, all subspecies were considered for scoring the Jetz species. When possible,

known pigment use was taken from the literature (see Data in the Supporting Information). Claw keratin, although a continuously growing structure, was not scored for each taxon in our analyses because we did not observe any nonpasserine species with carotenoid-consistent coloration in claws. We also did not include retinal oil droplets in this dataset because precise information on their presence across many clades is lacking, and they cannot be evaluated visually (Toomey et al., 2016). Outgroup taxa were scored for similar body regions: iris, face skin, neck skin, and leg skin for turtles with the addition of plastron keratin, and iris, face skin, neck skin, and leg skin and leg scales for crocodilians (see Data in the Supporting Information).

When available, we also collected data on the specific carotenoids present in individual tissues across species. All these species were scored as present for carotenoid-consistent coloration prior to carotenoid identity being verified from this literature search. This resulted in a small dataset ($n = 61$ species) for which specific carotenoids are known, and the tissues that they are found in. For these birds, color is considered carotenoid-produced (see Data in the Supporting Information), and in this dataset, only 30 birds have verified carotenoid use in non-plumage tissues (detailed in Data and References in the Supporting Information). We classified the expressed pigments as either being fast degrading (FD) or slow degrading (SD) based on the methods used by Higginson et al. (2016) and quantified the proportion of slow degrading versus fast degrading compounds expressed by each species.

ANCESTRAL STATE RECONSTRUCTION OF COLOR EXPRESSION

We used a Maximum Clade Credibility tree based on a set of 200 time-calibrated trees from Jetz et al. (2012) with a backbone constraint from Hackett et al. (2008) as well as the phylogenies from Burleigh et al. (2015) and Kimball et al. (2019). For the constrained Jetz et al. (2012) and Burleigh et al. (2015) trees, we used updated Palaeognathae subclade relationships (Phillips et al., 2009; Yonezawa et al., 2017; Cloutier et al., 2019) by substituting the clade with the bind.tip and drop.tip functions from phytools in R version 3.5.2 (R Development Core Team 2008; Revell 2012). The same functions were used to add taxa not represented on the Burleigh et al. (2015) and Kimball et al. (2019) phylogenies to their most inclusive clade with branch lengths from the Jetz et al. (2012) Maximum Clade Credibility tree. Outgroup taxa were added to trees in Mesquite version 3.61 (Maddison & Maddison, 2019), and relationships and branch lengths were obtained using divergence age estimates from the TimeTree database (Hedges et al., 2006; Kumar & Hedges, 2011; Hedges et al., 2015; Kumar et al., 2017) and penalized likelihood using the program treePL (Smith & O'Maera, 2012).

We reconstructed ancestral states using parsimony and maximum likelihood approaches in R (R Development Core Team, 2008). For parsimony, we used the asr_max_parsimony function from the castor package (Louca et al., 2017), and for maximum likelihood used the rerootingMethod function using the phytools and ape packages (Paradis et al., 2004; Revell 2012). For maximum likelihood, we estimated states using equal rates for all transitions (ER) and symmetric backward and forward rates (SYM). The models were assessed using log-likelihood scores. The ER and SYM models were found to perform equally well across all trees (see Tables S2-S6), so only the ER model was used to construct ancestral states of color expression for (1) each body region individually and (2) tissue types expressing carotenoid-consistent color. We also estimated the number of changes in color expression using countSimmap in phytools (Revell 2012).

To contextualize the potential traits of extinct archelosaur lineages, we also reconstructed ancestral states including extinct groups using parsimony and likelihood methods in Mesquite version 3.61 (Maddison & Maddison, 2019). We used supraspecific terminals for this analysis, including Testudines, Crocodilians, Pterosauria, Ornithischia, Sauropoda, Tyrannosauridae, Anchiornithidae, Palaeognathae, Galliformes, Anseriformes, and Neoaves (Data in the Supporting Information). Tyrannosauridae and Anchiornithidae were chosen to represent extinct parts of Paraves and Coelurosauria because their integumentary structures are fairly well known from the fossil record (e.g., Clarke 2013; Barrett et al., 2015; Xu 2020). Coloration states for extinct lineages were coded as unknown. Extant clades were scored as polymorphic or absent for color expression by integument type (based on our analyses of extant groups, see Fig. 2 and Tables S2-S7), except for crocodilians for which carotenoid-consistent coloration is completely absent (Twyman et al., 2016; Data in the Supporting Information). We also included information on retinal oil droplets in this analysis, using the framework by Twyman et al. (2016) to score retinal carotenoids as present in turtles and all extant avian lineages, absent in crocodilians, and unknown in extinct taxa.

TESTING RELATIONSHIPS BETWEEN CAROTENOID-CONSISTENT COLOR IN DISTINCT INTEGUMENTARY STRUCTURES

We used phylogenetic logistic regressions to test relationships among different integumentary categories as well as among the different body regions (Table S8). Because the characters are discrete, analyses were performed using the phylolm package (Ho & Ané, 2014). Each regression was run using the modified Jetz et al. (2012) tree and parametric bootstrapping (2000 replicates). For these analyses, we only analyzed birds that expressed carotenoid-consistent color ($n = 2183$), and adjusted for multiple testing
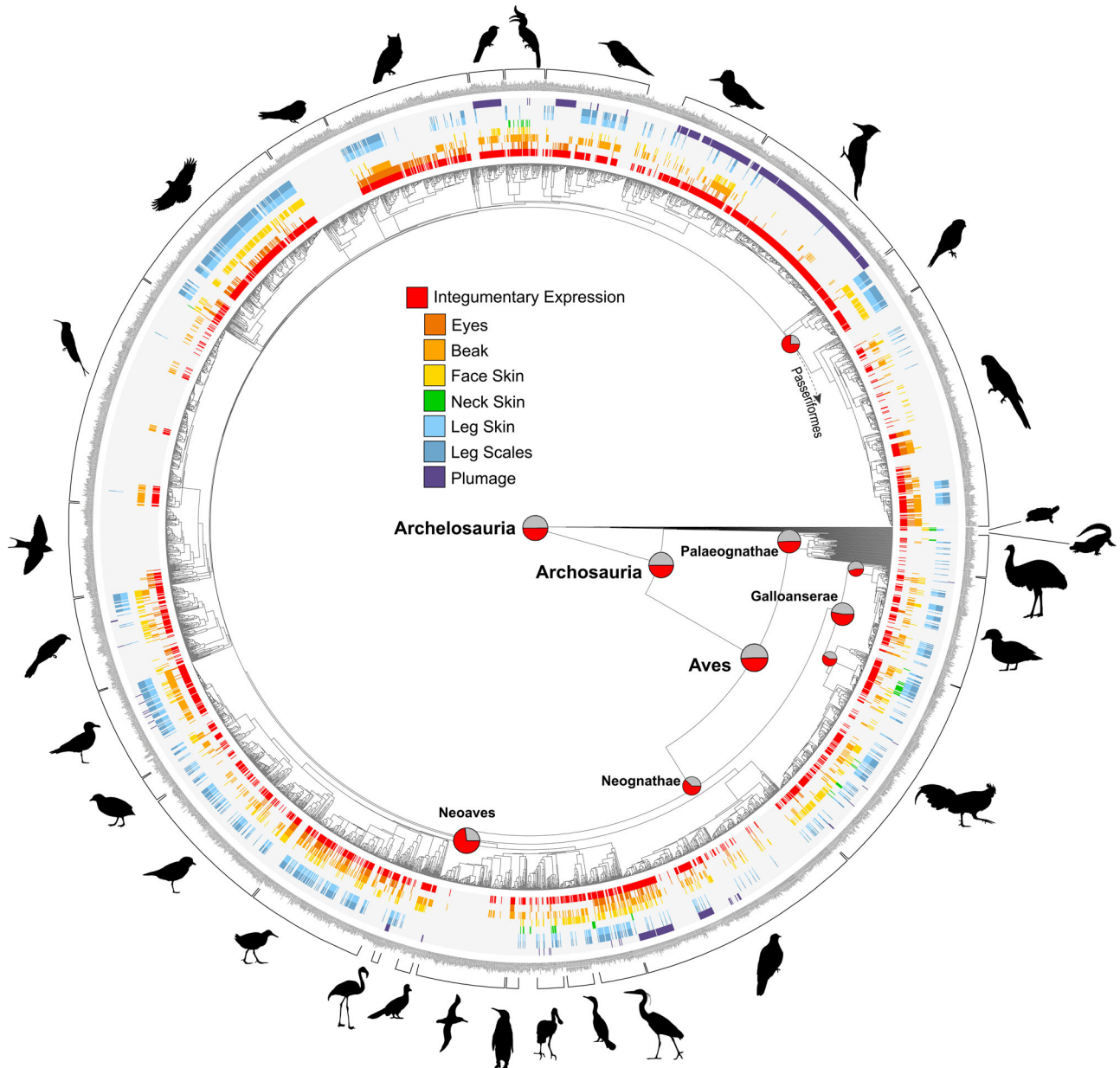
Figure 2. Ancestral state reconstructions for carotenoid-consistent color expression in Archelosauria. Bars along the outside of the phylogeny indicate where carotenoid-consistent color is present for each of the seven body regions by species (see Fig. 1), with the red bar indicating that carotenoid-consistent color is present anywhere on the body (Integumentary Expression). Pie charts at nodes indicate the reconstructed probability of expressing carotenoid-consistent color in any region, with red representing color present. Select clades are indicated by silhouettes. The phylogenetic position of passerines is indicated, but they were not surveyed for this study. High resolution version of the figure with species labels is available online.

using the p.adjust function and false discovery rate (FDR) method in R (Verhoeven et al., 2005; R Development Core Team, 2008).

We also tested if the evolution of carotenoid consistent color in different integument types was associated using the Discrete model and MCMC analysis in BayesTrait (version 3.0.2; Pagel & Meade, 2006), and compared independent and dependent models of evolution based on calculated Bayes Factors. We tested for potential dependent evolution of expression between skin and

keratin, skin and plumage, keratin and plumage, and any non-plumage expression and plumage.

ASSOCIATION OF TISSUE EXPRESSION AND DIET

Using the subset of 61 taxa with chemically characterized carotenoids, we tested whether there were relationships with the carotenoid content of bird diets, the tissues where pigments are deposited, and the stability of known expressed carotenoids (fast

degrading or slow degrading; see Higginson et al., 2016). We collected diet data from the literature for the 61 species and used the diet characterizations of Olson (2006) to assign each a minimum, maximum, and average dietary carotenoid content value based on dietary records from each species (see Data in the Supporting Information for references by species). We used Olson's coarse classification scheme, in which dietary items are quantified, placed into general categories, and then ranked from least to most carotenoid rich (Olson 2006, p. 622; summarized in Table S1). This allowed us to analyze diet as both a continuous trait based on estimated absolute carotenoid content and as a discrete category of least to most carotenoid rich (see Olson 2006). We calculated an average expected dietary carotenoid content for each species and used both that value and the log-transformed value in our analyses. To further investigate a potential relationship between the extent of coloration and diet type, we also categorized the dietary content of the 61 birds generally as either primarily vegetarian, carnivorous, or omnivorous. We then categorized birds by the total number of tissue categories that express color (skin, keratinous, or plumage) as well as by the total number of individual body regions (beak, face, neck, leg skin, leg scales, and plumage) that express color. For these analyses, eye coloration was not considered because data on carotenoid characterization in bird irises are less available. In some species, eye color is also known to change across ontogeny, with the relationship between pigment type and age-related shifts currently unknown (e.g., Rosenfield et al., 2003).

We used phylogenetic logistic regressions to test the relationships between compound stability and extent of carotenoid expression. Because these data were not binary, we used the poison_GEE method in phylolm (Ho & Ané, 2014). We investigated potential relationships between the percentage of slow degrading (SD) compounds (Higginson et al., 2016) and colorful regions, as well as total number of tissue types expressing carotenoids. For these, and the tests of dietary carotenoid content, we again adjusted for multiple testing using the FDR method and the p.adjust function in R (Verhoeven et al., 2005; R Development Core Team 2008).

Results

EXPRESSION OF CAROTENOID-CONSISTENT COLORS IN BIRDS

Out of the 4022 bird species included in the study, 2183 (54.3%) express carotenoid-consistent color in at least one integument region (bare-part skin tissues including the iris, keratinous regions, or feathers; see Fig. 2). Of those birds, 1880 (46.7% of all surveyed species; 86.1% of colorful nonpasserines) show color expression exclusively in nonfeather tissues. Plumage is the sole region of carotenoid-consistent color expression in only 303 (7.5%)

nonpasserine species. Although not included in our analyses, in nonpasserines claw keratin was not observed to have carotenoid-consistent color and was either unpigmented or black, most consistent with melanin-based coloration.

Carotenoid-consistent colors are most often seen in frequently maintained tissues (iris, face, neck, or leg skin, and fleshy features such as wattles; 1605 species), followed by nonfeather keratin structures (beaks, scales; 1493 species), and then plumage (535 species; see Fig. 2 for distribution by species). Skin and continually growing keratin structures often show the same character state (presence or absence) for carotenoid-consistent color; only 387 species express skin color without expression in these keratin structures. Similarly, only 275 species express carotenoid-consistent color in continually growing keratin integumentary structures without also expressing color in skin. Only two groups of birds (families: swifts and nightjars) were found to completely lack carotenoid-consistent coloration; all other nonpasserine clades had a few members with at least one body region expressing carotenoid-consistent coloration.

ANCESTRAL STATE RECONSTRUCTION FOR ARCHELOSAURIA

The ER and SYM likelihood models performed equally well for the maximum likelihood reconstructions (log-likelihoods for Jetz ER $= -1838$, SYM $= -1838$; for Burleigh ER $= -1100$, SYM $= -1100$; Kimball ER $= -259$, SYM $= -259$). Under both models, the ancestral condition for archelosaurs, archosaurs, and Aves has a probability of $\sim 50\%$ for expressing carotenoid-consistent color in any integumentary structure (Jetz phylogeny: P(expression) $= 0.500$ for Archelosauria, 0.500 for Archosauria, and 0.492 for Aves; Burleigh phylogeny: P(expression) $= 0.499$ for Archelosauria, 0.499 for Archosauria, and 0.494 for Aves; Kimball phylogeny: P(expression) $= 0.500$ for Archelosauria, 0.500 for Archosauria, and 0.546 for Aves; see Fig. 2 and Tables S2-S4). The ancestral condition of Palaeognathae is similarly ambiguous (P(expression) $= 0.486$ on Jetz; $= 0.426$ on Burleigh; $= 0.428$ on Kimball), but the ancestral probability for Neoaves is higher (P(expression) $= 0.730$ on Jetz; $= 0.570$ on Burleigh; $= 0.873$ on Kimball). See Tables S2-S7 for the conditions of other select nested clades. On the constrained Jetz tree, we recovered 460 gains of carotenoid-consistent color expression across all body regions, and 534 losses (see Fig. S1).

When reconstructed by tissue type, nonfeather integumentary structures had a 50% probability of expressing carotenoid-consistent colors at the ancestral archelosaur, archosaur, and avian nodes, whereas feathers were reconstructed with a 0% probability of carotenoid-consistent color at the base of Aves (Tables S5-S7). When reconstructions were made by body
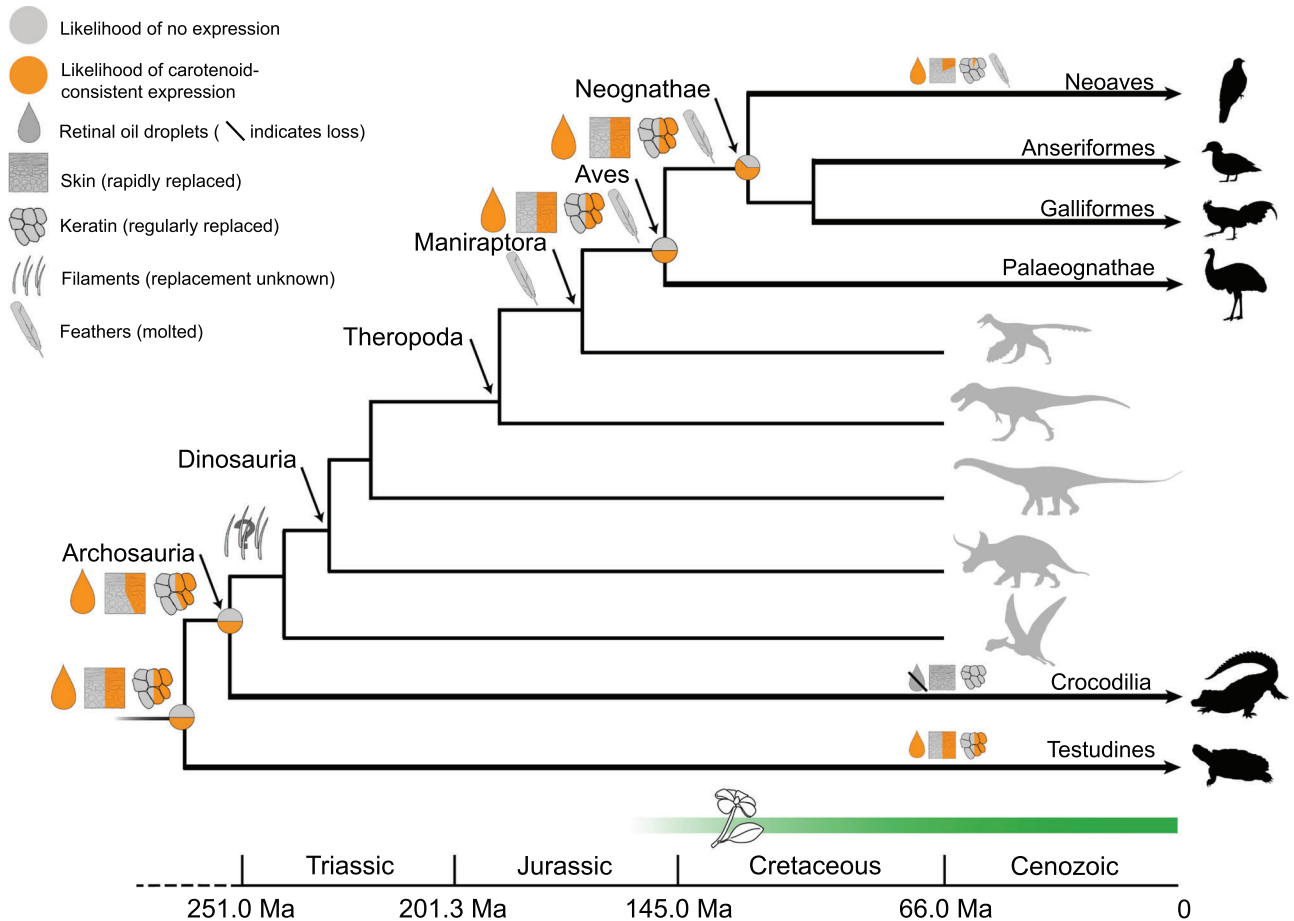
Figure 3. Ancestral state reconstructions accounting for extinct archelosaur lineages and retinal carotenoids. Indicated states represent the estimated likelihoods of carotenoid-consistent expression in each integument structure (see Tables S9A and S9B). Pie charts at nodes indicate reconstructions for expression of carotenoid-consistent colors in any integument type and are those shown in Figure 2 (see also Table S2). Carotenoid-consistent coloration is estimated to be ancestrally more likely in regularly replaced integuments, such as skin and continually growing keratin (e.g., rhamphotheca, scales), and would be less likely in seasonally growing tissues such as feathers and, potentially, filaments. Expectations for this coloration may increase during the Cretaceous when dietary sources of carotenoids shifted with early diversification in angiosperms, indicated along the time scale by the green bar. Silhouettes of extinct taxa were drawn based on skeletal reconstructions by Scott Hartman.

regions (e.g., face, beak, foot etc.; see Fig. 1 and Table S8) and by tissue type, no single body region or tissue type was reconstructed as more likely to express color than others.

The parsimony and maximum likelihood reconstructions including extinct taxa reconstruct that it is equally likely that the ancestral ornithodiran, dinosaurian, and theropod state was either expression or lack of expression of carotenoid-consistent coloration in skin and regularly replaced keratins (e.g., beak and scales) (Fig. 3; Tables S9A and S9B). These analyses also map the presence of retinal carotenoids as the ancestral state for archelosaurs in agreement with Twyman et al. (2016) (Fig. 3; Tables S9A and S9B). Finally, these analyses did not estimate that intermittently or seasonally replaced keratins (feathers, and perhaps filaments) in stem dinosaurs ex-

pressed carotenoid consistent colors (Fig. 3; Tables S9A and S9B).

RELATIONSHIPS OF COLORATION WITH TISSUE TYPE AND DIET

After analyzing the carotenoid-consistent dataset, phylogenetic logistic regressions recovered no significant relationship between expression in plumage and skin, nor between continually replaced keratin and skin (Table S10). We did recover a significant relationship between plumage and nonplumage tissues expressing carotenoid-consistent color ($p < 0.001$; Table S10). This relationship was significant after implementing FDR. Results from the independent and dependent model comparisons in BayesTraits supported correlated evolution between carotenoid-consistent color expression in keratin and skin regions (Bayes

Factor = 917.898). The other comparisons did not support a dependent model (see Table S11).

Analyses of the 61 taxa with chemically characterized carotenoids recovered a significant relationship between the number of compounds previously categorized as slow degrading (Higginson et al., 2016) and both the number of unique body regions ($p < 0.001$) and the total number of tissue types expressing carotenoids ($p < 0.001$; Table S12); these also remained significant after accounting for FDR. Nevertheless, expression in plumage specifically was not significantly related to the number of slow degrading compounds ($p = 0.08226$), nor was it associated with higher dietary carotenoid content ($p = 0.1004$; Table S12).

Dietary carotenoid content and extent of carotenoid coloration as measured showed a significant relationship in the subset of 61 taxa (Fig. S2). We found that high dietary carotenoid scores were correlated with both the number of carotenoid-colored body regions ($p < 0.001$) and a greater variety of tissue types that express color ($p < 0.001$). When discrete diet categories were used, birds with predominantly herbivorous diets expressed carotenoids in significantly more body regions than birds with either carnivorous ($p = 0.02037$) or omnivorous ($p = 0.02264$) diets. We also found that birds with a predominantly herbivorous diet expressed coloration in significantly more tissue types than birds with a carnivorous diet ($p = 0.02688$; Fig. S2). However, there was not a significant difference in number of tissue types or body regions expressing color between strict frugivores and other diet types ($p = 0.7094$; Fig. S2C).

Discussion

PATTERNS OF CAROTENOID-CONSISTENT COLOR EXPRESSION IN BIRDS

We recovered a 50% probability of carotenoid-consistent color expression in any integumentary tissues (skin or nonplumage keratin) at the base of Archelosauria, Archosauria, and Aves (Fig. 2; Table S2). Plumage had a 0% probability of being carotenoid-colored at the base of Aves, consistent with prior optimizations using supraspecific terminals (Stoddard & Prum, 2011) and large plumage-specific datasets (Thomas et al., 2014a).

Within nonpasserine birds, it was unusual to find expression of carotenoid-consistent color in only one tissue type; most often, both skin and keratin structures expressed color within the same species. Only 303 birds out of 4022 surveyed (7.5%) showed carotenoid-consistent color in just plumage. This result highlights the importance of considering all regions of color expression to fully understand how color mechanisms evolved. We find that in nonpasserines, carotenoid expression is a labile trait having been gained and lost across these lineages numerous times, consistent with work on plumage alone (Thomas et al., 2014a). Our findings

further support that carotenoid-consistent coloration most commonly occurs in the skin, beaks, and scales of birds, as observed by Olson and Owens (2005). Most nonpasserine birds expressing carotenoid-consistent color do so in tissues that are not plumage (86% of colorful nonpasserines). Of nonpasserines that do express carotenoid-consistent color in feathers, nearly half also express color in other integuments (43%). Our findings also suggest that, within nonpasserines, expression of carotenoid-consistent colors in these tissues (skin and continually replaced keratins) may be correlated (Table S11). This might suggest that expression in either of these tissues follows similar mechanisms, constraints, or selective pressures, whereas expression in intermittently, annually, or seasonally modified tissues (i.e., feathers) is potentially independent of these other integuments. These results emphasize the need for future work to include tissues other than feathers and to characterize in detail the pigments they contain to better understand how tissue-specific coloration has evolved in birds.

Based on the smaller subset of taxa with chemically verified carotenoids, we recovered an association of diet with both the number of integument types and overall total body regions expressing carotenoid color. We did not recover a significant relationship between different integument types and the stability of the carotenoids expressed in them, but did find that the percentage of stable compounds is significantly correlated with the total number of integument types (i.e., skin and keratin structures) expressing carotenoids. This result could indicate that carotenoid expression for color is primarily dependent on overall concentrations of circulating carotenoids, and not the chemical structures of the compounds themselves (Badyaev et al., 2015). Carotenoid modification to more stable forms may be more common for expression in plumage, where modification could be further linked to species-specific life history traits such as molt frequency (Higginson et al., 2016). Carotenoid expression in non-plumage tissues was previously hypothesized to potentially be less correlated with dietary carotenoid intake than expression in plumage (Olson & Owens, 2005). We did not recover support for this hypothesis (Table S12; Fig. S2). However, we do find a relationship between carotenoid intake and both the number of body regions and number of tissue types expressing them (Table S12; Fig. S2).

COLORATION IN ARCHELOSAURIA

Of the extant members of Archelosauria, only turtles and birds express carotenoids in the integument, whereas crocodilians do not; these results are despite a focused search for carotenoids in Crocodilia (e.g., Spearman & Riley, 1969; Alibardi 2011). Whether this distribution is due to loss in crocodilians or independent gains in testudines and Aves is unknown. The gene *CYP2J19* has been implicated in the metabolism of ketocarotenoids in both birds and turtles; metabolized carotenoids are not only used for

coloration, but are also deposited into retinal oil droplets of both birds and turtles (Bowmaker 2008). These retinal oil droplets act as filters that enhance color vision (Vorobyev et al., 1998; Vorobyev 2003), and this ability to better discriminate between colors may have preceded the expression of carotenoids for coloration (Twyman et al., 2016). A duplication event of this gene is thought to have occurred at the base of Archelosauria, implying that pterosaurs and extinct dinosaurs may have had red retinal oil droplets (Twyman et al., 2016). The *CYP2J19* orthologue has been lost in extant Crocodilia (Twyman et al., 2016). That loss may have implications for the physiological ability of crocodilians to express carotenoids in the integument, although this remains to be investigated.

There do appear to be dietary constraints on the number of body regions and the tissue types in which carotenoids are found in birds (Fig. S2). Although similar data were not available for turtles, whether expression in skin may precede expression in the dorsal or ventral keratinous scutes or scales should be investigated. The carnivorous diets of living crocodilians may not contain high enough concentrations of carotenoids to be used in coloration in addition to other physiological processes, although the carotenoid content of crocodile and alligator diets has not been surveyed. Some extinct Crocodyliformes had much more varied diets and included cases of herbivory (Melstrom & Irmis, 2019), raising the possibility that extinct members of the clade could have expressed carotenoids in integumentary structures, although more study into the mechanisms of and constraints on carotenoid expression in nonavian reptiles are needed. It is also possible that the absence of carotenoid-consistent color expression in skin or keratin in extant crocodilians has been driven by selection for crypsis or other environmental factors (e.g., Merchant et al., 2018; reviewed in Somaweera et al., 2019).

INTEGUMENT STRUCTURE AND EXPECTATIONS OF CAROTENOID EXPRESSION IN DINOSAURIA

Preservation of pigment molecules in fossils is rare (reviewed by Vinther 2020), and carotenoids have yet to be detected in any vertebrate fossil. Raman spectroscopy has been employed to detect carotenoid pigments in amber-preserved feathers, but the compounds had either degraded or were not originally present (Thomas et al., 2014b). Although those authors proposed that carotenoids could potentially be detected through an amber matrix, they recognized detection would depend on the compounds maintaining their chemical structure for long periods of time and through the process of fossilization (Thomas et al., 2014b). Chemical traces of carotenoids have been found in plant, plankton, and bacteria remains in rocks up to billions of years old, but only under very specific depositional conditions (Eglinton & Logan, 1991; Sinninghe Damsté & Koopmans, 1997; Brocks et al., 2005). In those cases, preservation of the compounds oc-

curred in instances of relatively high carotenoid concentration; in lower concentrations (i.e., in vertebrate skin tissue), preservation of carotenoids could be even less likely.

Although expectations for finding direct evidence of carotenoid coloration in the vertebrate fossil record remain low, a wealth of information on the integumentary structures of extinct dinosaurs has become increasingly available through the discovery and study of exceptionally preserved fossils over the past 30 years (e.g., Clarke 2013; Barrett et al., 2015; Eliason et al., 2017; McNamara et al., 2018; Benton et al., 2019; Xu 2020). We hypothesize, based on the archelosaur observations presented here, that it is unlikely for a lineage to express carotenoid-consistent color in integumentary structures that are shed and intermittently regrown (e.g., feathers) without first expressing these colors in other tissues (e.g., skin, rhamphotheca; Fig. 3). Similarly, diet is found to have a strong influence on the number of body regions and tissues expressing carotenoids in birds.

Extinct archosaur skin and integumentary structures such as scales and a rhamphotheca may have shown carotenoid coloration. However, they were likely absent in intermittently or seasonally replaced structures homologous with feathers because these are not reconstructed as present in the ancestral avian (Fig. 3). We did not score claw keratin in our analyses because we observed that carotenoid-consistent colors were not found in the claws of any nonpasserine birds or other archosaurs, although they are observed in some passerine clades (del Hoyo et al., 2019). This suggests that claw coloration is a derived condition within birds. Rhamphothecae are present in many clades, whereas foot and body scales show a heterogeneous distribution across archosaurs (Hieronymus et al., 2009; Barrett et al., 2015; Nabavizadeh & Weishampel, 2016; Arbour & Evans, 2017; McNamara et al., 2018; Benton et al., 2019; Godefroit et al., 2020; Xu 2020). However, tarsal scales are not present in many paravian fossils, which often have feathers covering the length of the legs and toes (e.g., Li et al., 2010; Li et al., 2012; Hu et al., 2018), and based on our ancestral estimation at the base of Aves scales would not be expected to be colorful even if present (Table S8). Depending on the ancestral integumentary suite of pterosaurs, it is possible that simple filament structures homologous to feathers are ancestral to at least Dinosauria (Clarke 2013; Lowe et al., 2014; Benton et al., 2019). Pterosaurs have also been found with filaments called pycnofibers (Yang et al., 2019), and it has been suggested that they had a keratinous rhamphotheca along the jaws and potential keratinous coverings on cranial crests (Kellner & Campos, 2008). Integuments such as filaments and early feather forms would likely have been seasonally molted, whereas scales and rhamphotheca are hypothesized to have grown continuously as seen in living turtles and birds (Lucas & Stettenheim, 1972; Xu 2020). Based on the ancestral state estimation for nonpasserines and other archelosaurs (Figs. 2 and 3), expression in the

former tissues is less likely than in skin, scales, or rhamphotheca keratin.

The dietary availability of carotenoids must also be taken into consideration for estimating the probability of carotenoid expression in nonplumage tissues in extinct taxa. Our analyses support that diets high in carotenoid content are associated with elaboration of color expression into more body regions and tissue types. These high-carotenoid diets are primarily herbivorous, and consist specifically of mostly vegetation, flowers, buds, and fruits (Olson 2006; see Table S1). Notably, diets characterized by many of the richest carotenoid sources would have been easier to achieve with the rise of angiosperms (discussed by Li et al., 2019 and Ramírez-Barahona et al., 2020). Plant groups that were dominant up until the Late Jurassic rise of flowering plants are notably lower in carotenoid content than angiosperms (Czeczuga 1987). A general herbivorous diet has arisen multiple times within the theropod lineage (Zanno & Makovicky, 2010) and herbivorous or mixed diets are increasingly prevalent throughout Avialae (Zhou and Zhang 2002; Zhou et al., 2004; Zheng et al., 2011; Li et al., 2014; O'Connor & Zhou, 2020). That said, it is often difficult to place species in one specific dietary category; even living birds that are primarily frugivorous have been reported to occasionally prey on vertebrates, including other birds (e.g., Mahecha et al., 2018). Therefore, diet should not be considered a straightforward indicator for carotenoid coloration, but rather a potential guide to its likelihood along with other factors.

The physiological framework for carotenoid modification and expression is present within archelosaurs (Twyman et al., 2016), meaning these same processes would likely have been available to extinct pterosaur and nonavian dinosaur groups as well. Carotenoid-consistent colors may have been present in the skin and continually growing keratinous structures, such as rhamphotheca, in more nested dinosaurian clades (Fig. 3). Shifts from a carnivorous diet to mixed feeding and herbivory (e.g., within Ornithischia, Sauropoda, Ornithomimosauria, and within Paraves; Zanno & Makovicky, 2010; Li et al., 2014) may be expected to be associated with a higher probability of carotenoid expression in these tissues in more body regions. Carotenoids are not predicted to be likely found in filaments and feathers, because the likelihood of their expression in homologous structures in the base of Aves is estimated to be zero.

USE OF NOVEL PIGMENTS WITHIN AVES

Although bright colorations are most often produced by carotenoids, notable exceptions have been found across birds. Psittacofulvins, turacin, and spheniscins have been identified as the source of plumage colors in parrots, turacos, and penguins (respectively) (Krukenberg 1882; Dyck 1992; McGraw & Nogare, 2005; Hill and McGraw 2006; Thomas et al., 2013). However, in several species of parrots, levels of carotenoids circulating in

the blood at time of feather growth were found to be comparable to those in other bird groups that do express carotenoids in their feathers, including metabolically derived compounds (McGraw & Nogare, 2005). These levels have been suggested to indicate that at least parrots have the physiological potential for carotenoid deposition in feathers, but preferentially do not. Furthermore, the presence of derived, molecularly modified carotenoids (i.e., astaxanthin) has been reported in the yolk of turaco eggs, suggesting they also possess the mechanisms to modify carotenoids into more stable forms (Lucas & Stettenheim, 1972). The pigments expressed in both parrot and turaco skin and nonfeather keratin structures have not been described, and it is possible that, similar to what is seen in penguins (Jouventin et al., 2007), those birds deposit unique pigments in the feathers while allocating carotenoids to these other tissues. If novel pigments are more chemically stable than carotenoids, it could be advantageous to deposit them in tissues that are not replenished often (i.e., feathers), whereas less stable pigments can be more frequently deposited in bare-part tissues. Birds have been shown to preferentially deposit distinct carotenoids into different tissues; somewhat surprisingly, esterified, proposed "stabilized" pigments have been found in bare-part regions, whereas nonesterified pigments are deposited into the feather matrix (reviewed in Hill & McGraw, 2006). However, it has also been shown that metabolically modified, more stable carotenoids can be selectively deposited into the feathers (Higginson et al., 2016). The expression of novel pigments may be an extension of preferential deposition. Clades with these novel pigments often molt yearly and may be under different selective pressures to maintain these colors, whereas other groups with seasonal molt cycles can restore their less stable plumage carotenoids (Higginson et al., 2016).

CONSIDERATIONS AND AREAS FOR FUTURE WORK

Our scoring of color in the 4000+ dataset is based on hypotheses of pigment expression, which in the absence of chemical data remains the best method available for investigating color expression in nonfeather tissues at this taxonomic scale. However, as with previous work that uses scorings based on visual hypotheses (Olson & Owens, 2005; Thomas et al., 2014a), there are limitations to the present study. We are confident that our scorings accurately represent colorations that are consistent with those produced by carotenoids, but recognize that perceived coloration may not reflect the presence or absence of carotenoids themselves (hence our use of "carotenoid-consistent" when referring to colors from unverified pigment sources, following Thomas et al. 2014a). However, given that previous work has not identified other similarly colored pigments in the skins or keratinous tissues of living archosaur groups (i.e., pterins; Hill & McGraw, 2006; Iverson & Karubian, 2017; Brejcha et al., 2019), the

hypothesis that these colors in birds are due to carotenoids is a reasonable one until evidence suggests otherwise. Within birds, noncarotenoid pigments of similar colors to carotenoids are thought to be exclusive to plumage (penguins, turacos, and parrots; Krukenberg 1882; Dyck 1992; McGraw & Nogare, 2005; Hill & McGraw, 2006; Thomas et al., 2013); however, it is possible that they are simply yet to be detected in other integument structures. Future investigations should focus on increased chemical analyses of pigments in nonplumage integument.

Furthermore, the patterns of color expression presented here were recovered in nonpasserine birds. It is not known if these same trends of tissue-specific coloration hold true for Passeriformes, which comprise nearly 60% of all extant bird species (Jetz et al., 2012; Burleigh et al., 2015; Kimball et al., 2019). Carotenoids are comparatively well described in passerine feathers, but characterization of pigments in other tissues is lacking for most species (see Hill & McGraw, 2006). Work by Thomas et al. (2014a) estimated more origins of carotenoid-consistent plumage expression in passerines than in nonpasserines and it would be interesting to see if this held true for nonplumage integument as well. Future work should investigate whether the same patterns of tissue-specific and body region color expression recovered here are present.

Conclusions

This study is the first to investigate carotenoid-consistent coloration across body regions while considering tissue structure in a phylogenetic context, and demonstrates the necessity of looking more precisely at regions of expression to better understand how coloration has evolved. We find that although carotenoid-consistent colors were ancestrally less likely to have been present in seasonally or annually replaced modified keratinous structures (i.e., feathers, or homologous filaments) or claws, they may have been present in skin patches or other keratin structures (i.e., rhamphotheca) depending on the dietary availability of the compounds. Expression could increase in likelihood as carotenoid-rich food items became more abundant into the Cretaceous. A more comprehensive understanding of the structure of expressed pigments by tissue type, in conjunction with the growing body of work into the physiology and genetic underpinnings of carotenoid expression in extant taxa (reviewed in Price-Waldman and Stoddard, 2021), will allow for a more comprehensive insight into how this coloration mechanism may have evolved within Dinosauria.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
SND and JAC conceived of and developed the study. SND collected and analyzed the data. SND and JAC interpreted the results and wrote the manuscript.

DATA ARCHIVING
All data used in this study are available on Dryad at https://doi.org/10.5061/dryad.4f4qrfjd3.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary material

Table S1: Table modified from Olson (2006) of common dietary items and their relative and calculated expected carotenoid content.

Figure S1. Gains and losses of carotenoid-consistent color expression in any body region, reconstructed on the constrained Jetz et al. (2012) phylogeny and the ER maximum likelihood reconstruction.

Table S2: General expression ancestral state probabilities for major clades, reconstructed using the ER and SYM likelihood models and parsimony (Pars) on the constrained Jetz et al. (2012) phylogeny.

Table S3: General expression ancestral state probabilities for major clades, reconstructed using the ER and SYM models and parsimony (Pars) on the Burleigh et al. (2015) phylogeny.

Table S4: General expression ancestral state probabilities for major clades, reconstructed using the ER and SYM models and parsimony (Pars) on the Kimball et al. (2019) phylogeny.

Table S5: Ancestral state probabilities of skin coloration for major clades, reconstructed using the ER model on all phylogenies.

Table S6: Ancestral state probabilities of keratin coloration for major clades, reconstructed using the ER model on all phylogenies.

Table S7: Ancestral state probabilities of plumage coloration for major clades, reconstructed using the ER model on all phylogenies.

Table S8: Ancestral state probabilities by individual body region (other than plumage, see Table S6) for major clades, reconstructed using the ER model on the constrained Jetz et al. (2012) phylogeny.

Tables S9A and S9B: Ancestral state estimates of carotenoid expression by tissue type from maximum parsimony (MP) (A) and maximum likelihood (ML) (B) analyses in Mesquite version 3.61 and including extinct lineages.

Table S10: Results of phylogenetic logistic regressions for the relationship of different body regions, using the constrained Jetz et al. (2012) phylogeny and only analyzing birds that express carotenoid-consistent colors.

Table S11: Summary of log marginal likelihood scores and calculated Bayes Factors from the BayesTraits Discrete model comparisons. MCMC analysis and 100 stones for 1000 iterations were used for each set of integuments after a burn-in of 20,000.

Table S12: Results of phylogenetic logistic regressions for the relationship of dietary carotenoid scores (raw score and log transformed value) and extent of carotenoid expression (total body regions and total tissue types), using the constrained Jetz et al. (2012) phylogeny.

Figure S2: Distributions of different diet categories and measures of coloration extent.