PLUMAGE PIGMENT DIFFERENCES IN MANAKINS OF THE *PIPRA ERYTHROCEPHALA* SUPERSPECIES

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ABSTRACT.—We investigated the carotenoids found in the head feathers of three members of the *Pipra erythrocephala* superspecies by chromatographic, spectrophotometric, and chemical means. The Golden-headed Manakin (*P. erythrocephala*) primarily deposited yellow hydroxy-carotenoids in its head feathers, predominately lutein. Red keto-carotenoids were also deposited locally. Canthaxanthin, for instance, was identified in the tips of nape feathers. In contrast, the Red-headed Manakin (*P. rubrocapilla*) deposited mostly orange and red keto-carotenoids. An orange pigment identified as α-doradexanthin was the most abundant. The common red pigments astaxanthin and canthaxanthin were also present. Finally, feathers of the red-headed Round-tailed Manakin (*P. chloromeros*) yielded a complement of carotenoids very similar to the Red-headed Manakin's. In addition, the Round-tailed Manakin deposited moderate amounts of rhodoxanthin, a plant keto-carotenoid of pronounced red hue.

Within individual Red-headed Manakins, we observed differences in total carotenoid content and composition among head regions that differed slightly in color. The distal and proximal portions of individual feathers also differed markedly. We discuss possible physiological and biochemical mechanisms for these conditions, and suggest their relationship to the mechanisms responsible for the species-specific differences in manakin coloration. We reveal the probable origin of the Latin misnomer for the Golden-headed Manakin. *Received 2 August 1988, accepted 2 August 1988.*

THE four species of Neotropical manakins that compose the *Pipra erythrocephala* superspecies (Snow 1979) differ conspicuously in the combination of their head, thigh, and underwing colors (Table 1). The difference in head color is particularly striking between the Golden-headed Manakin (*Pipra erythrocephala*) and its close relatives. The golden top and sides of the head of *P. erythrocephala*, belying its scientific name, contrast sharply with the bright red head of the other three allospecies. The colors involved are generally bright, and presumably implicate carotenoid pigments (Brush 1981). However, the biochemical and genetic basis of the color differences remains unexplored.

P. erythrocephala is closely related to the Red-headed Manakin (*P. rubrocapilla*), with which it has been grouped in a single species (*P. e. erythrocephala* and *P. e. rubrocapilla* of Hellmayr 1929). The distribution of *P. erythrocephala* and *P. rubrocapilla* at their area of closest contact is delimited largely by the Amazon River. The occurrence of morphological differentiation associated with rivers is often found among understory Amazonian forest birds, a pattern largely unique to Amazonia (e.g. Hellmayr 1910, Snethlage 1913). One interpretation is that rivers act as barriers to gene flow and serve as geographical isolating mechanisms (Sick 1967). Capparella (1987, in press) reported large genetic distances between the two manakins, in excess of the mean for avian species [Nei's (1978) $D = 0.101$; avian mean 0.0440 ± 0.0221 SD, Barrowclough 1980] based on allozyme studies. Moreover, fixed differences were detected at three loci, a condition often lacking between undisputed avian species.

Carotenoid pigments, responsible for most of the bright colors in birds, are relatively well-known chemically. Furthermore, considerable work has been concerned with their biochemical modification and processing in birds (reviewed in Brush 1981). Differences in feather carotenoids are determined by both the processes responsible for their absorption and transport, and the metabolic capacities of the

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birds to modify the pigments. Hence, carotenoid analysis can be used to infer physiological, and the underlying genetic, differences. In addition to providing specific plumage color and patterns (Fox and Hopkins 1966, Fox et al. 1967, Brush 1970, Brush and Johnson 1976, Troy and Brush 1983), carotenoid differences account for color polymorphisms (Völker 1964, Brush and Seifried 1968, Johnson and Brush 1972), subspecific plumage variation (Test 1942, Ford and Simpson 1987), and plumage variants (Völker 1964, Brush 1970, Hudon and Brush in press). To elucidate the nature of the physiological differences that confer species specificity of coloration in the manakins, we determined the pigment constitutions responsible for the difference in head color between *P. erythrocephala* and *P. rubrocapilla.* The Round-tailed Manakin (*P. chloromeros*), another member of the *P. erythrocephala* superspecies, was also examined.

TABLE 1. Color differences among members of the *erythrocephala* superspecies complex of *Pipra* manakins.

Species	Head	Thigh	Under-wing
P. mentalis	red	yellow[a]	yellow
P. erythrocephala	yellow	red	black
P. rubrocapilla	red	red	white
P. chloromeros	red	yellow[a]	black

[a] Many specimens show a red streak on the predominantly yellow thigh.

MATERIAL AND METHODS

We examined head feathers from one specimen of *P. erythrocephala* (collected in Colombia early in the twentieth century, specific locality unknown); two specimens of *P. rubrocapilla,* one from Peru (collected in 1983, Loreto Department, south of the Amazon River, ca. 10 km SSW of the Napo River mouth, east bank of the Quebrada Vainilla) and one from Bolivia (collected in 1986, Pando Department, ca. 12 km south of Cobija, ca. 8 km west on the road to Mucden); and two specimens of *P. chloromeros* from Peru (collected in 1987, Ucayali Department, east of the Ucayali River, ca. 65 km ENE of Pucallpa, west bank of the Shesha River). Crown colors were evaluated using color chips (Smithe 1975) under daylight illumination in early afternoon. The reflectance spectra of head feathers were scanned in a Perkin-Elmer 552 recording spectrophotometer equipped with an integrating sphere. A 6 × 8 mm sample window was used. Feathers from each sample were mounted on glass slides and examined microscopically for structural modification.

We extracted carotenoid pigments from whole feathers in warm, acidified (HCl) pyridine (Völker 1936) under dim light. The pigments were transferred to hexane in a separatory funnel, and the organic epiphase washed repeatedly with water and dried with anhydrous sodium sulfate. The carotenoids were then stored in hexane under nitrogen in the dark. Ultraviolet-visible absorption spectra for each extract in hexane were obtained on a Perkin-Elmer 552 recording spectrophotometer equipped with cuvette holders.

Each extract was subjected to thin-layered chromatography (TLC) on silica gel IB and aluminum oxide IB plates (Baker-Flex, J. T. Baker Chem. Co., Phillipsburg, NJ). We used various solvent systems to separate the pigments (usually different mixtures of hexane and acetone). Pigment identification was based on relative mobility (R_f) on TLC, color, and several chemical tests. The latter included sodium borohydride reduction of carbonyl groups in methanol (Andrewes et al. 1974), acetylation of hydroxyl groups with acetic anhydride in dry pyridine (Andrewes et al. 1974), and oxidation of acidogenic carotenoids and canary-xanthophylls in alkaline methanol (Partali et al. 1987, Hudon and Brush in prep.). We determined R_f values for the carotenoid pigments in the solvent system hexane : acetone (3:1). We evaluated pigment colors on silica gel by subjective assignment to yellow, orange or red. Standards of canthaxanthin, echinenone, lutein and zeaxanthin obtained from Hoffman LaRoche (Basel) were used for identification. Standard astaxanthin and rhodoxanthin pigments were prepared from lobster (*Homarus americanus*) shell and yew (*Taxus baccata*) arils, respectively. Lobster carotenoids were extracted as described for feather pigments. The yew arils were mixed with sand (J. T. Baker Chem. Co.), homogenized in a mortar, and the carotenoids extracted repeatedly with a mixture of ethanol : acetone (1:1). The extracted carotenoids were transferred to hexane. We co-chromatographed extracts from feathers of the Scarlet (*Piranga olivacea*) and Western (*P. ludoviciana*) tanagers, whose pigments have been thoroughly characterized (Brush 1967, Hudon and Brush in prep.).

Some pigments, notably unidentified yellow xanthophylls from *P. erythrocephala,* were isolated by preparative TLC on precoated plates of Kieselgel 60 (E. Merck, Darmstadt, Germany). The pigments were separated with a mixture of hexane : acetone (2:1), eluted from the gel with acetone, and transferred to hexane.

Quantitative determinations.—The feathers were weighed prior to extraction. We calculated carotenoid content from the optical density of the whole extracts in hexane at 450 nm ($A_{450\ nm}$). We used 2,500 as the extinction coefficient ($E^{1\%}_{1\ cm}$) for carotenoid mixtures (Britton 1985). Content was determined from the formula:

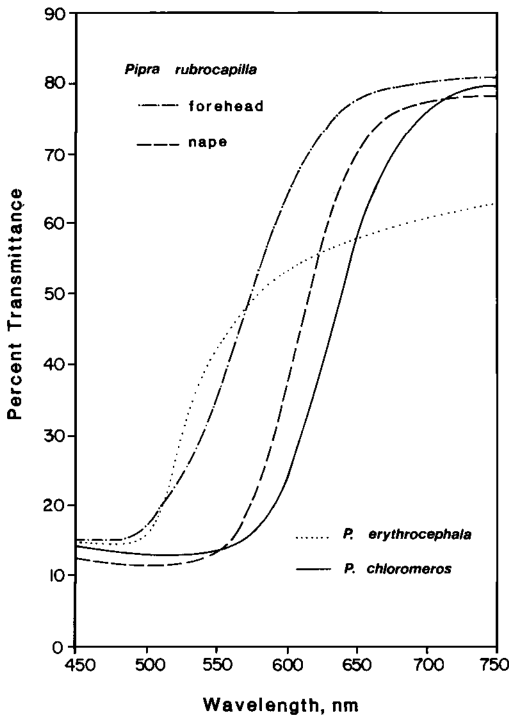

Fig. 1. Reflectance spectra of samples of head feathers from three species of manakins. The spectra were determined in the transmittance mode.

$$\frac{\text{mg carotenoid}}{\text{1 g feather}} = \frac{\text{optical density } (A_{450\text{ nm}}) \times \text{volume (ml)} \times 10}{\text{extinction coefficient } (E^{1\%}_{1\text{ cm}}) \times \text{feather mass (g)}}$$

The relative concentration of several extracted carotenoids was determined by High-Performance Liquid Chromatography (HPLC). We used a Waters (Millipore Corp., Milford, MA) liquid chromatograph equipped with a model 501 pump and a Lambda Max model 481 LC variable wavelength detector. A Zorbax ODS (Dupont Company, Wilmington, DE) reverse-phase column (4.6 mm i.d. × 25.0 cm) was used for pigment separation. Pigments were eluted at 1.0 ml/min with an acetonitrile : dichloromethane : methanol (7:2:1) solvent mixture (Nelis and De Leenheer 1983). The pigments were detected at 450 nm. Prior to injection, the samples in hexane were taken to dryness under nitrogen, and dissolved in the HPLC mobile phase. The elution was isocratic and did not require a gradient. The chromatographic conditions separated the acidogenic carotenoids poorly. Therefore, we pooled their abundance in the quantitative work. Because of overlap of some visually distinct peaks, we determined the area under the curves (a measure of concentration) by printing the tracings, manually cutting and weighing the different peaks on an analytical balance.

To investigate the distribution of carotenoids across individual scalps, one full hemi-scalp (cut along the mid-sagittal plane) of a Red-headed Manakin was divided into three regions that differed slightly in color. The areas were the forehead (Spectrum Orange, #17, color names and numbers from Smithe 1975), the crown and nape (Scarlet, #14), and the auriculars (Pratt's Ruby, #210). Each was extracted separately, and the pigments separated on HPLC. In a different experiment, loose head feathers from a different specimen of Red-headed Manakin were individually cut at their distal third, and the distal and proximal portions were pooled and extracted separately.

RESULTS

Color and structural modifications.—Crown feathers of the 3 species examined had reflectance spectra of broadly similar shape (Fig. 1). All largely absorbed light of short and middle visible wavelengths (to 500 nm). The specimens examined varied greatly in color and this was reflected in the position of the absorption cutoff. In the golden-colored *P. erythrocephala* (Orange Yellow, #18), half-maximal absorption occurred at about 540 nm. As the color became redder, the position of the absorption cutoff shifted to longer wavelengths, from about 570 nm (Spectrum Orange, #17) to 610 nm (Scarlet, #14) in *P. rubrocapilla* to 635 nm (Crimson, #210) in *P. chloromeros*. The gradual absorption of the middle visible wavelengths can account for the change in color.

The terminal parts of the colored head feathers in all 3 species were modified as is common in small, carotenoid-containing feathers (Desselberger 1930, Brush and Seifried 1968, Brush 1969, Olson 1970). These modifications are characterized by a flattening of the barbs and the absence of barbules.

Pigments.—At least 5 carotenoid pigments were detected in extracts from the crown of *P. erythrocephala*. All were yellow. Two of these matched lutein, a major constituent (29%), and zeaxanthin (undetermined concentration), on TLC and HPLC. We then tested whether some pigments might be canary-xanthophylls of endogenous origin (Brockmann and Völker 1934). Two pigments ($R_f = 0.47$, and $R_f = 0.32$), present in amounts too small to characterize extensively, were isolated on preparative TLC and incubated in alkaline methanol. This treatment converts putative canary-xanthophylls to more polar, orange-appearing compounds (Hudon and Brush in prep.). None changed in chro-

TABLE 2. Carotenoid content and composition of head feathers of the Red-headed Manakin (*P. rubrocapilla*). Individual scalps were examined.

	Carotenoid content (mg/g feather)	Composition (%)			
		Lutein	Canthaxanthin	Acidogenic carotenoids[a]	Others
Peru specimen					
Base	0.86	5	7	47	41
Tip	1.70	2	13	44	41
Bolivia specimen					
Forehead	0.85	11	3	36	50
Crown and nape	2.23	7	2	56	35
Auriculars	3.16	6	3	65	26

[a] Excluding hydroxyechinenone, which was included in the "others" category.

matographic behavior as is characteristic of the canary-xanthophylls.

The red tips of feathers from the nape of *P. erythrocephala* yielded a pigment that corresponded to canthaxanthin on HPLC. We also suspected 3-hydroxy, 4-keto-carotenoids, (e.g. α-doradexanthin, astaxanthin, and phoenicoxanthin) in this preparation, but could not resolve them chromatographically.

The red head feathers of *P. rubrocapilla* yielded at least 9 carotenoid pigments. In contrast to *erythrocephala*, practically all of these pigments ranged from orange to red when separated on TLC. The only exceptions were lutein and some unidentified yellow xanthophylls (data not shown) that constituted only a small fraction of the total (Table 2). We identified α-doradexanthin (4-keto-lutein) ($R_f = 0.24$, yellow-orange, 1 keto and 2 hydroxyl groups, acidogenic) as the major pigment in *P. rubrocapilla*. The keto-carotenoids canthaxanthin ($R_f \doteq 0.49$, orange, 2 keto and no hydroxyl groups), phoenicoxanthin ($R_f = 0.36$, orange, 2 keto and 1 hydroxyl groups, acidogenic), astaxanthin ($R_f = 0.27$, orange, 2 keto and 2 hydroxyl groups, acidogenic), and echinenone ($R_f = 0.83$, orange-yellow, 1 keto and no hydroxyl groups) were present in smaller amounts. We found chromatographic evidence of small amounts of hydroxyechinenone ($R_f = 0.65$, yellow-orange, acidogenic), and 1 unidentified yellow-orange pigment ($R_f = 0.32$).

The red crown of *P. chloromeros*, another member of the *erythrocephala* superspecies, had carotenoids very similar to those of *P. rubrocapilla*. Pigments observed included α-doradexanthin, astaxanthin, phoenicoxanthin, canthaxanthin, and echinenone. In addition, there were moderate amounts of rhodoxanthin (3 isomers), a plant chromophore of deep red hue. The amounts of rhodoxanthin present differed more than twofold between the 2 individuals from the same locality. The deposition of rhodoxanthin presumably reflects its concentration in the diet. The carotenoid pigments that composed a red streak on the yellow thigh feathers of *P. chloromeros* were similar to those found in the head feathers, and consisted mostly of doradexanthin, astaxanthin, and phoenicoxanthin. The yellow thigh feathers contained a mixture of lutein and unidentified yellow pigments.

Distribution.—We determined carotenoid concentration and composition for the distal (tip) and proximal (base) portions of single feathers of *P. rubrocapilla* (Table 2). The distal third of the head feathers contained about twice as much carotenoids per gram of feather as the proximal portion. Carotenoid composition differed slightly in that the distal portion of head feathers had relatively more of the identified keto-carotenoids (58% compared with 54%) and less lutein (2% vs. 5%), a potential keto-carotenoid precursor, than the proximal portion.

Three head regions of a single specimen of *P. rubrocapilla* that differed slightly in color differed dramatically in their carotenoid content and composition (Table 2). We observed an almost fourfold difference in carotenoid concentration between the forehead and auricular feathers. Profound differences in relative composition of carotenoids were also observed among these head regions. For example, the content of acidogenic carotenoids varied from 36% of the total on the forehead to 65% on the auricular feathers. There was a concomitant slight decrease of lutein. Carotenoid content was expressed relative to total feather mass, but

individual feathers from each region differed in the proportion that contained pigment. Nape feathers were the longest and least pigmented overall, while the auriculars were the shortest and most pigmented. This leads to a distortion of the difference in carotenoid concentration of the colored parts among the regions. However, this could only partly explain the differences, as the pigment proportion varied by a factor of 1.5–2 times as great across the head. Unidentified peaks were also quantified but their origin, natural or artifactual, was not determined.

The relative concentration of canthaxanthin differed between the 2 *P. rubrocapilla* (Table 2). The birds originated from 2 different localities (Peru and Bolivia) and the basis for the difference is unknown.

DISCUSSION

Nomenclature.—Because of the marked difference in head color between the Golden-headed and Red-headed manakins, we were puzzled by the fact that the scientific names for the two manakins both refer to *red*-headed birds (*erythro*—from the Greek *ερυθρος*, and *rubro*—from the Latin *ruber*). The name *erythrocephala* has been applied to a species that clearly has a yellow or golden crown, with only a red tint at the tips of the crown feathers along the nape. This is even more puzzling when the nomenclatural history reveals the use of *auricapillus* by Klein (1750) upon which Linnaeus (1758) partially based his description. Although it is not possible to determine with certainty why Linnaeus did not retain the root *auri-* (from the Latin *aurum* = gold), one possibility is that he relied primarily on the plate in Edwards (1743), which clearly depicts a manakin with an orange or red head (two copies examined). Edwards refers to the bird as the Golden-headed Black Tit-mouse and uses the terms "orange and golden" to describe the crown color; the plate clearly shows a bird with a red head. Therefore, we conjecture that Linnaeus, who reported that he examined Edwards' work (Linnaeus 1758), altered the name of this form to reflect the redness in the plate. Subsequently, when it was discovered that a red-headed manakin resided south of the Amazon, the name *rubrocapilla* was applied by Temminck (1821). Therefore, the use of *erythrocephala* for the Golden-headed Manakin is a misnomer, although it is the valid name for this taxon.

Pigmentation.—The head feathers of *P. erythrocephala*, and *P. rubrocapilla* and *P. chloromeros* contained pigments that differed conspicuously in their chemical identities. Specifically, *P. erythrocephala* deposited considerable amounts of hydroxy-carotenoids, with lutein predominating, while *P. rubrocapilla* and *P. chloromeros* feathers contained a mixture of 4-keto-carotenoids. These pigments are closely related biochemically. Four-keto-carotenoids are formed by the addition of oxo (=O) groups at the carbon 4 of one or both end-rings of xanthophylls and carotenes (Thommen 1971, Davies 1985).

Xanthophylls (hydroxy-carotenoids) and carotenes in birds are obtained directly from the diet (Giersberg and Stadie 1933, Brockmann and Völker 1934, Völker 1962). These pigments (especially lutein) are abundant in green leaves and found in an array of fruits (Goodwin 1980). In some birds, unmodified xanthophylls are important feather pigmentors (Völker 1951, Partali et al. 1987). *P. erythrocephala* is an example. In other birds, the dietary pigments are modified metabolically (Brockmann and Völker 1934, Völker 1962). This changes the physical properties of the yellow dietary precursors, which frequently become orange or red. *P. rubrocapilla* and *P. chloromeros* deposited such endogenously modified carotenoids.

The variety of keto-carotenoids we observed in *P. rubrocapilla* and *P. chloromeros* presumably result from the modification of dietary precursors by an enzyme with a specificity for β-end-rings. Both end-ring structure and the degree of modification determine the variety of compounds produced. Until recently, however, rather "pure"deposits of carotenoids were reported in bird feathers (Völker 1961, Brush 1981). Major improvements in separation and detection techniques, in particular HPLC, now permit the resolution of complex mixtures of pigments like those found in the manakins (see also Rüedi 1985).

In spite of marked pigment differences in the head feathers between *P. erythrocephala*, and *P. rubrocapilla* or *P. chloromeros*, all species deposited the same pigments (except for rhodoxanthin, an exogenous carotenoid of restricted distribution; Hudon and Brush in press). All species deposited unmodified xanthophylls. All also deposited keto-carotenoids, although this was expressed to different degrees. *P. rubrocapilla* and *P. chloromeros* deposited keto-carotenoids over

the entire head, whereas *P. erythrocephala* deposited keto-carotenoids only at the tip of nape feathers. The presence of red thigh feathers in *P. erythrocephala* further suggests that this manakin deposits keto-carotenoids, but feathers were not available. In *P. chloromeros*, a red streak on the yellow thigh feathers contained a mixture of keto-carotenoids similar to that found in the red head feathers. Thus all species shared the same capabilities to process carotenoids in various ways. It is not necessary to postulate the evolution of new enzymatic activities or transport systems to account for the observed color differences. Rather, our observations suggest that *P. erythrocephala* differs from *P. rubrocapilla* and *P. chloromeros* in the spatial and temporal expression of those processes responsible for the fractionation, modification, and deposition of pigmentary carotenoids.

The color of individual barbs of the nape feathers in *P. erythrocephala* changes rapidly from red to golden from the tip to the proximal section. This represents a temporal change in the processing of carotenoid pigments during feather growth. We suspect this represents a change in the expression and activity of an enzyme involved in the production of 4-keto-carotenoids in follicular cells or in the selectivity of pigment uptake by the follicular cells. In the European Goldfinch (*Carduelis carduelis*) carotenoid modification follows the uptake of circulating carotenoids by the integument (Völker 1958). Indeed, birds fed exogenous red keto-carotenoids deposited them in all areas pigmented by carotenoids, including those that would normally have been pigmented yellow (Völker 1958, Reuter 1964). The uptake characteristics of the follicular cells simply determine those areas that will be pigmented (Giersberg and Stadie 1933), and only partly the nature of the pigments deposited.

In contrast, *P. rubrocapilla* head feathers were red along their entire pigmented length. Still, considerably more pigment was deposited in the exposed feather parts than in the remainder. The distal third contained almost twice as much pigment per gram of feather as the proximal two-thirds. This also reflects a difference in pigment deposition. Higher carotenoid concentrations in feathers lead to a shift toward a purer red (Frank 1939, Desselberger 1930). It was precisely these highly pigmented barbs that were most modified morphologically. In addition, the distal portion contained slightly more keto-carotenoids and less lutein than the proximal portion.

We found dramatic differences in content and composition across a single scalp of *P. rubrocapilla*, an all red-headed bird. When compared to the differences observed within individual feathers, the spatial pattern was dominated by variation in the composition of dietary and modified carotenoids. This implies differences in the processing, particularly end-ring modification, and deposition of carotenoids among the various regions. The differences in concentration and composition can explain the slight changes in color among regions of individual *P. rubrocapilla* scalps (Desselberger 1930).

The unique, species-specific color combinations in the manakins probably arose from genetic changes that altered the temporal and spatial expression of the pigmentary processes among species. Changes in genes that control the expression of structural genes ("regulatory genes") would thus have been of greater importance in producing the species differences than changes in the structural genes (Wilson 1976).

Three congeners not part of the *P. erythrocephala* superspecies (*P. cornuta, P. fasciicauda*, and *P. aureola*) exhibit red heads; none has a yellow head (Meyer de Schauensee 1966). In one species, the Crimson-hooded Manakin, (*P. aureola*), the head feathers contain a mixture of 4-keto-carotenoids (Brush 1969), and resemble *P. rubrocapilla* and *P. chloromeros*. Based on outgroup analysis and parsimony (Maddison et al. 1984), red-headedness and the correlated deposition of keto-carotenoids would assume an ancestral position (plesiomorphic) within the *P. erythrocephala* superspecies. Restriction of the red pigmentation to a few nape feathers in contemporary *P. erythrocephala* would then represent a uniquely derived (autapomorphic) condition in that lineage. Because shared derived conditions (synapomorphies) were not identified within the *P. erythrocephala* complex, our data cannot resolve the branching order of the various clades within the superspecies (Hennig 1966).

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