A description of unique fluorescent yellow pigments in penguin feathers

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Dear Sir,

Animals generate their colors using a diversity of mechanisms, involving the interaction between pigment biochemicals and structural features of the tissue. Animal pigments come in several classes that vary in molecular structure, source (e.g. exogenously acquired or endogenously synthesized), and coloration. Birds have served as model systems for the study of pigmentary colors in animals, and to date five major pigment classes have been described from bird feathers—carotenoids, melanins, porphyrins, psittacofulvins, and iron oxides (Hill and McGraw, 2006). However, we have recently gathered preliminary data on yellow feathers from several species of penguin that indicate the presence of a new class of pigment in bird plumage (McGraw, 2004; McGraw et al., 2004). Here, we present a detailed biochemical and microscopic evaluation of these autofluorescent, base-soluble, nitrogen-rich pigments from yellow feathers of six penguin species (Figure S1A).

We obtained feathers from penguins in the wild (Electronic Appendix S1) and first measured full-spectrum color of yellow feather portions using UV-VIS reflectance spectrophotometry (*sensu* Siefferman and Hill, 2005). Color was scored from 1–10 plumes or feathers, from each of five individuals per species, that we mounted on a black card in an overlapping manner to mimic the natural arrangement on the bird. Reflectance from all feathers lacked a peak in the ultraviolet (UV) (Jouventin et al., 2005) and steadily increased from short to long wavelengths before plateauing above 600 nm (Figure 1A). This is unlike carotenoid- (Shawkey and Hill, 2005) and psittacofulvin-based (Pearn et al., 2003) yellow feathers in birds, which exhibit a UV reflectance peak. We also visually inspected feathers under a UV lamp (λ = 366 nm) and detected that all fluoresced a yellow-green color.

We then employed chemical extraction methods to attempt to remove yellow pigments from feathers. Carotenoid and psittacofulvin extractions (*sensu* Hudon and Brush, 1992) failed to recover any pigments, but aqueous basic solutions successfully recovered yellow pigments from all species. Pigments were best extracted in a zirconia mixer mill, so we ground 3 mg of pigmented feather barbs from each of four emperor, four king, four rockhopper, five macaroni, five Snares, and six yellow-eyed penguins for 10 min at 30 Hz in the presence of 2 ml 0.5 M NaOH or of 0.5 M KOH (for yellow-eyed and Snares penguins only, because NaOH extracts were cloudy). We removed feather residue via centrifugation and used the yellow-colored supernatants for two subsequent analyses: (1) determining spectral absorbance and estimating pigment concentration with an absorbance spectrophotometer; and (2) quantifying fluorescence emission with a scanning spectrofluorometer.

Absorbance values, as determined in a quartz cuvette at 1 nm intervals from 300–520 nm for each sample, were generally consistent for all samples (peaking in the UV-A region, from 370–400 nm; Figure 1B); however, peak absorbance was slightly short-wave-shifted in extracts from rockhopper, Snares, and yellow-eyed penguins compared to extracts from the other species. Feathers from the six species differed in estimated yellow-pigment concentration (macaroni = king > emperor > rockhopper = yellow-eyed = Snares; ANOVA, $F_{5,23}$ = 73.5, P < 0.0001; using *post-hoc* Scheffe's tests with α = 0.05; Figure 1C). Fluorescence emission (from 415–700 nm) was determined for 1 ml of each sample in a 1 × 1 cm quartz cuvette at a fixed excitation wavelength of 398 nm (the mean peak absorbance wavelength for all samples). We found that all extracts of feather pigments fluoresced at consistent wavelengths as well, approximately 90–100 nm longer than their absorbance wavelengths (Figure 1B).

The autofluorescent and base-soluble characteristics of these penguin pigments led us to investigate two additional molecular characteristics of these feather

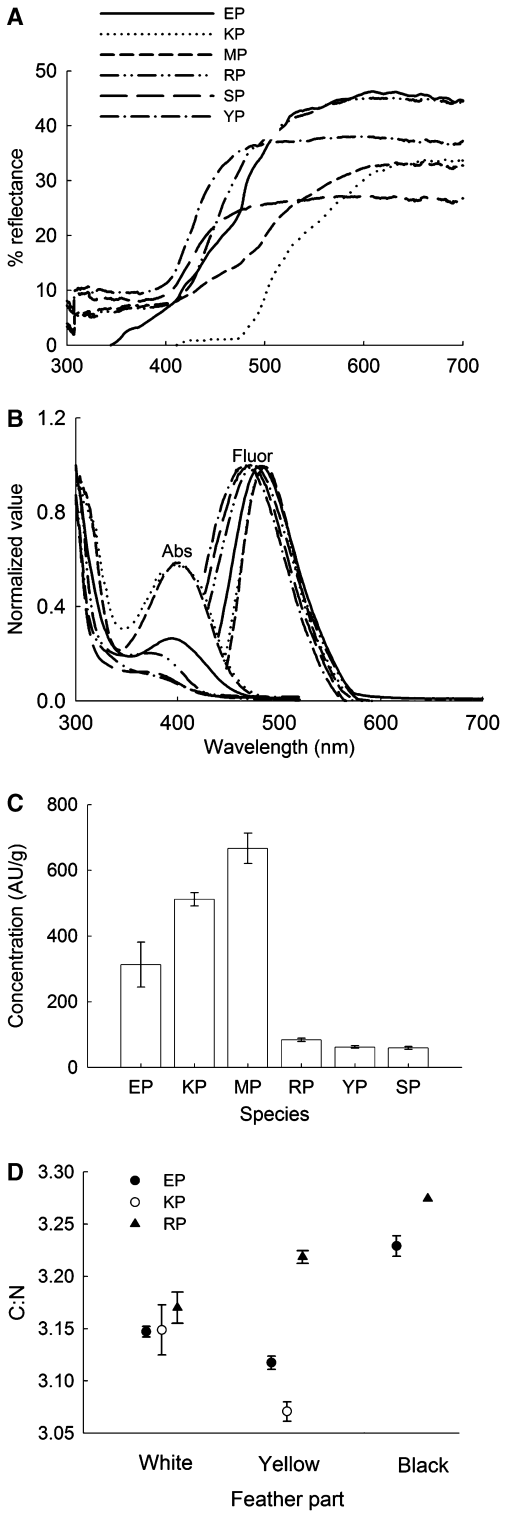

Figure 1. (A) Light reflectance values of yellow penguin feathers (species means shown); (B) Light absorbance (Abs) and fluorescence (Fluor) spectra of pigment extracts from yellow penguin feathers (data normalized to peak values); (C) Pigment concentrations of yellow feather pigments from all six study species, estimated as absorbance units (AU) at the wavelength of maximum absorbance (398 nm) per g of feather analyzed, which, in the absence of a known compound with which we could compute absolute concentration, was the only quantitative approach available to us; and (D) Differences in carbon:nitrogen (C:N) content of yellow, white, and black feather regions in three penguin species. Means +/− SE shown in (C) and (D).

compounds. First, we were interested in the elemental composition of these pigments, based on the fact that different pigment classes contain different carbon-to-nitrogen (C:N) ratios (Needham, 1974). We used a standard elemental analyzer to determine C and N content of feather barbs from eight emperor, 10 king, and 10 rockhopper penguins (we were not able to measure C:N for macaroni, yellow-eyed, or Snares penguins for logistical and financial reasons). We separately trimmed off and analyzed yellow-pigmented and white feather barbs from the same feather, so that we could explicitly understand elemental differences between pigmented and unpigmented regions. Melanin-pigmented black feathers were also available for analysis from three emperor and one rockhopper penguin. Approximately 2 mg of feather barbs were packed in clean tin capsules and weighed to the nearest 0.001 mg. Encapsulated samples were then flash-combusted at 1760°C, the resulting gases were chemically scrubbed to remove halogens and sulfur, and separated in a gas chromatography column. C and N were detected with a thermal conductivity detector and total content was calculated with a precision of 0.1 μg. We found that yellow-pigmented feather tips of emperor and king penguins were significantly more enriched with nitrogen (had a lower C:N ratio) than were white feather bases from the same feather (paired *t*-tests, emperor: $t_7 = 3.715$, $P = 0.008$; king: $t_9 = 3.103$, $P = 0.013$; Figure 1D). However, yellow plumes from rockhopper penguins had significantly less nitrogen (higher C:N ratio) than the white bases ($t_9 = -3.721$, $P = 0.005$; Figure 1D). We presume that this was due both to the low concentration of yellow pigments in these feathers (see above) and to the presence of substantial amounts of melanin in these feathers, which remained a light brown color after yellow-pigment extraction. In fact, melanized black feather barbs had a greater C:N ratio than any other feather parts measured (Figure 1D). Murphy et al. (1990) noted similar reduced nitrogen content in the melanin-pigmented feathers of *Pygoscelis* penguin species (e.g. Adelie, chinstrap, gentoo). Our second piece of evidence that yellow pigments themselves are nitrogen-rich is the significant negative correlation that existed between yellow pigment concentration (as above) and C:N ratio in yellow feather barbs from emperor, king, and rockhopper penguins (Spearman's rank correlation, $r_s = -0.80$, $n = 12$, $P = 0.008$).

Second, we used the autofluorescence of these pigments to image their location within feather barbs using laser scanning confocal microscopy. We mounted

feather barbs on microscope slides using high viscosity microscope immersion oil and a coverslip, laser-illuminated the samples ($\lambda_{excitation}$ = 488 nm), and imaged fluorescent regions by collecting emitted light from 500–700 nm. These images revealed the presence of concentrated regions of fluorescence in each species (Figure S1B). These occurred as angular, plate-like structures of approximately 2–5 μm in thickness, much like pterin pigments (termed 'reflecting platelets') in avian irises (Oliphant and Hudon, 1993). Light emission spectra for these platelets matched those in Figure 1B, indicating that these platelets are responsible for the fluorescence of feather-pigment extracts.

Based on chemical characteristics determined to this point, we hypothesized that pterin pigments were responsible for the production of yellow color in penguin feathers. We sought to test this using HPLC, by attempting to match the pigment(s) in penguin feathers to known pterins, as well as other candidate molecules, like purines, flavins, and papiliochromes, that can give yellow color to animal integuments. Methods followed those in McGraw et al. (2004), except that a mobile phase of 0.3% methanol in water was used to maintain system pressure at ca. 465 psi using a C-18 reverse-phase column (Varian Microsorb-MV 100, Lake Forest, CA, USA; 5 μm particle size, 250 mm long x 4.6 mm inner diameter). Penguin pigments were compared against external purified standards of three yellow pterins, two red pterins, five colorless pterins, four colorless purines, riboflavin, as well as papiliochrome (a unique yellow butterfly pigment; Umebachi, 1985) and its yellow precursor, kynurenine (Electronic Appendix S2).

We detected one major compound in extracts from yellow feathers of emperor, king, and macaroni penguins, which eluted at the same time in all species and exhibited maximal light absorbance (λ_{max}) at 298 nm and at 385 nm, the latter of which approximates the λ_{max} values of whole-feather extracts (in basic solution, which often shifts molecular conformation and hence light absorbance by ca. 15–20 nm compared to neutral-pH solvents; Needham, 1974). In contrast, yellow-eyed and Snares penguin feathers harbored a different compound—one that absorbed at shorter wavelengths (ca. 285 and 344 nm). Rockhopper penguin feathers contained both pigment types, at roughly equal proportions. The presence of the shorter-wavelength-absorbing pigment in the feathers of yellow-eyed, Snares, and rockhopper penguins likely explains the short-wavelength-shifted absorbance we observed in raw pigment extracts from these three penguin species compared to the others (see above). We compared absorbance spectra and retention times of these pigments in penguin feathers to 17 different pigments from four pigment classes and found no clear match with any of them (Electronic Appendix S2).

Collectively, our data indicate that penguins use a class of pigment never before described from bird feathers to color their feathers yellow. These pigments are not carotenoids or psittacofulvins because they are not lipid-soluble, which refutes previous claims about the carotenoid basis of yellow plumage color in yellow-eyed penguins (Massaro et al., 2003). Moreover, melanins, porphyrins, and iron oxides, though sharing some solubility properties with the penguin pigments (McGraw et al., 2005), do not fluoresce under UV light; phaeomelanins, eumelanins, and iron oxides do not fluoresce at all, and porphyrins fluoresce only under long, visible-light wavelengths (Needham, 1974). We can also rule out, based on light-absorbance profiles, other rare (to vertebrates) yellow colorants in animals, such as papiliochromes, riboflavin, and flavonoids (which absorb light in the visible range; Needham, 1974).

Ultimately, the attributes we have described for yellow colorants of penguin feathers are most consistent with those of pterin (also known as pteridine) pigments. Pterins are common yellow (as well as orange and red), UV absorbent and fluorescent colorants in insect wings and eyes (Pfleiderer, 1994) as well as in the skin of fishes, amphibians, and reptiles and the irises of various birds (reviewed in Oliphant et al., 1992). They have the highest nitrogen content (lowest C:N ratio) of any pigment described from animals (Kayser, 1985) and exist as crystalline 'reflecting platelets' in many tissues in which they give color (Hudon and Muir, 1996). The reasons we cannot unequivocally confirm that these are pterins are because: (1) we could not match penguin pigments to any known pterin, (2) we failed to determine a molecular weight using mass spectrometry, perhaps due to insufficient material available, and (3) pterins have few diagnostic chemical characteristics and tests (Needham, 1974). The murexide test has been used previously for identification of pterins in insects (Ford, 1947), but it is also currently in use for detecting uric acid (Stone and Simmonds, 1991) and our attempts to use it on the penguin pigments, as well as with pterin standards, proved unsuccessful.

In addition to encouraging future work on the exact chemical identity of these pigments, we are anxious to determine: (1) the source of such pigments, either dietary (from pterin-pigmented fish) or metabolically derived (as is the case for unique pterin-producing xanthophores in the avian eye; Oliphant et al., 1992); and (2) how their presence contributes to variation in yellow coloration within penguin species, as they can co-occur with melanin (which may photoprotect these feather pigments that can fade to white over the course of a year; McGraw et al., 2004; pers. obs.) to generate an orange appearance and as these colors can serve as honest signals of health and mate quality (Massaro et al., 2003; Nolan et al., 2006). We have found, however, that other colorful structures in penguins (e.g. orange beaks in king, emperor, and gentoo penguins; unpubl. data) contain carotenoids, not pterins, so more research is also needed to determine how and why these unusual pigments only color their plumage.

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Supplementary material

The following supplementary material is available for this article online from http://www.blackwell-synergy.com/doi/full/10.1111/j.1600-0749.2007.00386.x

Appendix S1. Penguin feathers collected for study. Prior to analysis, all feathers were stored in envelopes or plastic bags in the dark at room temperature.

Appendix S2. Light absorbance (λ_{max}, or wavelengths at peak absorbance) and retention times (R_t), as determined by high-performance liquid chromatography, for authentic pterin, purine, flavin, and papiliochrome standards as well as the yellow penguin pigments.

Figure S1. (A) Color photographs of the colorful plumage regions from all six studied penguin species; (B) Confocal microscopy images revealing the presence of small crystals (or "reflecting platelets") in the yellow feathers of all study species. Other feather regions subtly glow in these images because white keratin also fluoresces dimly. For comparison, we imaged red, carotenoid-based house finch (*Carpodacus mexicanus*) feathers, which also exhibited background, keratin-generated fluorescence but lacked crystalline structures (not shown). Interimage differences in fluorescence intensity are not biologically relevant and are due to alterations made to microscope sensitivity to maximize contrast of imaged structures.

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Electronic Appendix I. Penguin feathers collected for study. Prior to analysis, all feathers were stored in envelopes or plastic bags in the dark at room temperature.

Species	Feathers	Source	Date
Emperor penguin (*Aptenodytes forsteri*)	auricular	Terre Adelie, Antarctica	January 2003
King penguin (*A. patagonicus*)	auricular	Cape Ratmanoff, Kerguelen Islands	April 2003
Rockhopper penguin (*Eudyptes chrysocome*)	crest	Possession Island, Crozet Archipelago	July 2005
Macaroni penguin (*E. chrysolophus*)	crest	Possession Island, Crozet Archipelago	November 2005
Snares penguin (*E. robustus*)	crest	Northeast Island, Snares Island group	Oct.-Nov. 2002 and 2003
Yellow-eyed penguin (*Megadyptes antipodes*)	crest	Penguin Bay, Otago Peninsula, New Zealand	July 2004

Electronic Appendix II. Light absorbance (λ_{max}, or wavelengths at peak absorbance) and retention times (R_t), as determined by high-performance liquid chromatography, for authentic pterin, purine, flavin, and papiliochrome standards as well as the yellow penguin pigments. Many such molecules have a dual-peaked absorbance spectrum, so we report both wavebands here (λ_{max} 1 and 2). Samples for which no retention time is listed ('n/a') were run previously using a different HPLC (C-30) column and thus retention times are not comparable to the rest listed here.

		λ_{max} (nm)		
Class of molecule	Compound/Taxon	1	2	R_t (min)
Pterin	Pterin	251	358	12.3
	Pterin-6-carboxylic acid	262	362	8.5
	Biopterin	274	346	n/a
	Leucopterin	285	337	8.0
	Sepiapterin	266	432	17.6
	Xanthopterin	255	393	7.9
	Isoxanthopterin	281	334	11.6
	Dihydroxanthopterin	277	307	12.2
	Erythropterin	314	469	7.8
	(Iso)drosopterin	336	504	19.8
Purine	Uric acid	235	291	8.2
	Guanine	246	274	n/a
	Xanthine		267	n/a
	Hypoxanthine		249	n/a
Flavin	Riboflavin	375	475	n/a
Papiliochrome	Papiliochrome	292	339	9.3
	Kynurenine	256	357	23.3
Penguin pigments	King penguin	298	385	8.3
	Emperor penguin	same as king penguin		
	Macaroni penguin	same as king penguin		
	Yellow-eyed penguin	285	344	8.7
	Snares penguin	same as yellow-eyed penguin		
	Rockhopper penguin	both penguin pigments present		

(a)

King Penguin

Emperor Penguin

Macaroni penguin

Rockhopper penguin

Yellow-eyed penguin

Snares penguin

(b)