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A simple and inexpensive chemical test for behavioral ecologists to determine the presence of carotenoid pigments in animal tissues

Received: 28 April 2004 / Revised: 20 August 2004 / Accepted: 5 September 2004 / Published online: 4 November 2004
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Abstract Animals use several different types of pigments to acquire their colorful ornaments. Knowing the types of pigments that generate animal colors often provides valuable information about the costs of developing bright coloration as well as the benefits of using these signals in social or sexual contexts. It is often assumed that red, orange, and yellow colors in animals are derived from carotenoid pigments, when in fact there are other pigments that confer similar colors on animals. These include the pteridine pigments in a wide range of organisms, hemoglobin in blood-filled sinuses, the psittacofulvins of parrot feathers, and the phaeomelanin pigments in rufous or yellow feathers and fur. In this paper, we describe a quick and easy, two-step chemical method for field biologists to determine if their study species uses carotenoid pigments as integumentary colorants. This laboratory procedure first employs a thermochemical extraction technique, in which acidified pyridine is used under high

temperature to free carotenoid pigments from tissue to produce a colorful, pigmented solution. Red, orange, or yellow tissues containing pteridines, hemoglobin, or eumelanins do not release colored pigments into heated pyridine. However, psittacofulvins, and occasionally phaeomelanins, will also solubilize using this method. Thus, a follow-up test is needed, using solvent transfer, to confirm the presence of carotenoids in animal tissues. The use of absorbance spectrophotometry on the colorful solution may also provide information about the predominant carotenoids that bestow color on your study animal.

Keywords Carotenoids · Coloration · Pigments · Sexual selection

Introduction

Many animals use carotenoid pigments to color their integument red, orange, or yellow (Fox 1976). These carotenoid-based colors are often more pronounced in males and serve as important sexually selected traits that signal mate quality (Olson and Owens 1998; Hill 1999; Møller et al. 2000).

Evolutionary biologists have expressed recent interest in identifying the carotenoid-derived nature of animals colors, as knowing the specific type of ornamental color is important for determining the signaling information contained within showy sexual displays (Owens and Hartley 1999; McGraw and Hill 2000; Grether et al. 2001). However, the presence of carotenoids in animal tissues cannot be determined solely on the basis of color. There are several other types of pigments that also bestow red, orange, or yellow colors on animals. These include: (1) the red, orange, and yellow pteridine pigments found in the wings and eyes of insects, the skin of fishes, amphibians, and reptiles, and the irises of birds (Ziegler 1965; Oehme 1969; Oliphant et al. 1992); (2) the red hemoglobin in blood-filled tissues (Laruelle et al. 1951; Oehme 1969); (3) the psittacofulvins that give parrots their rainbow of colors (Völker 1937; Stradi et al. 2001);

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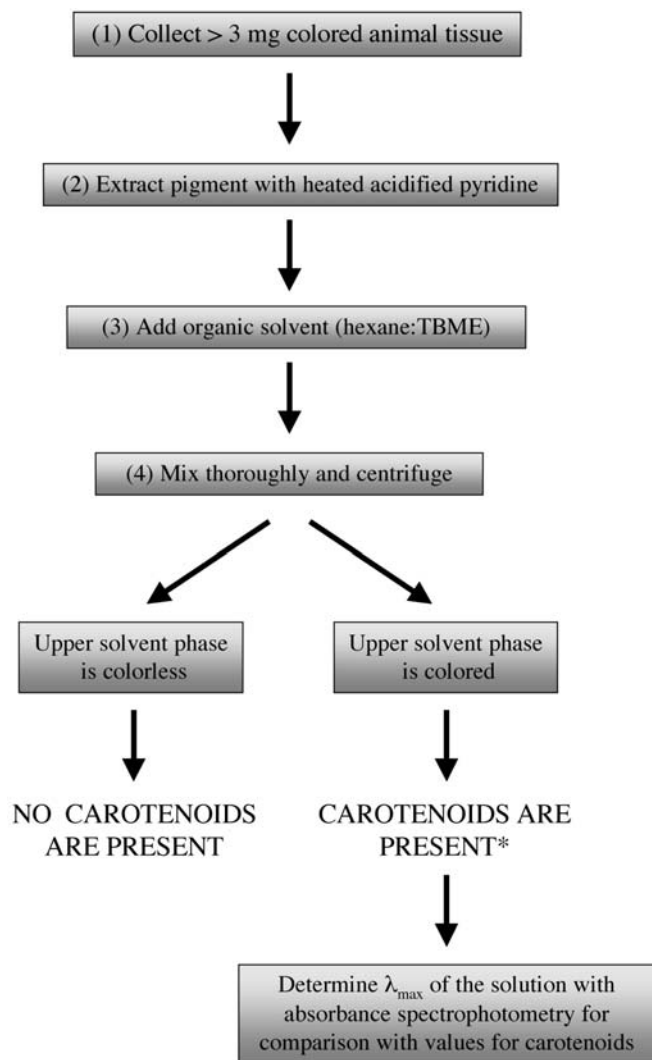


Fig. 1 Flow diagram of biochemical procedures for identifying the presence of carotenoid pigments in animal tissues. The *asterisk* indicates that lipid-soluble psittacofulvins from parrot feathers can also be transferred to the upper solvent phase and give it color; carotenoids and psittacofulvins must then be distinguished using absorbance spectrophotometry (see text footnote no. 2)

(4) the phaeomelanin pigments in rufous-, chestnut-, and yellow-colored bird feathers (Frank 1939; Lubnow 1960; Brush 1978) and mammalian hair (Cone et al. 1996; Miltenberger et al. 1999); and (5) the colorants acquired from the environment, such as iron-oxide deposits, and applied to the integument (Berthold 1967; Negro et al. 1999). Thus, biochemical tests are needed to unequivocally identify pigments as carotenoids in animal tissues.

Here, we describe a quick and easy chemical method for determining the presence of carotenoid pigments in animal tissues (Fig. 1). This should prove ideal for biologists who are simply interested in classifying their study ornament as carotenoid-based or not and who do not need to characterize the precise types and amounts of tissue carotenoids, which requires expensive chromatographic equipment (e.g., high-performance liquid-chromatogra-

phy; Britton et al. 1995). It should be made clear that we in no way see this method as a replacement for these sophisticated chromatographic techniques, but rather as an inexpensive, straightforward, and handy 'litmus test' for behavioral ecologists to perform on their colorful tissue(s) of interest.

This two-step procedure first involves a thermochemical extraction technique that was developed by Völker (1936) and later refined by Hudon and Brush (1992) to isolate lipid-soluble pigments from bird feathers. We have found that it can also be used to extract carotenoids from other animal tissues, from insect wings to fish skin to bird beaks. With this procedure, a simple color change in the chemical solution can reveal the presence of carotenoid pigments in animal tissue. However, other animal pigments will occasionally be solubilized in heated acidified pyridine. Thus, a second, follow-up step is needed, using organic solvent transfer, to further characterize the study compounds as carotenoids.

Methods and results

First, 3–5 mg of the colored tissue of interest should be collected for analysis. The amount of material needed for analysis will vary across taxa and depend on the intensity of color in the tissue and the density of pigment within. We recommend this minimum amount to detect even the most dilute quantities of carotenoids (e.g., in the pale plumage blush of elegant terns, *Sterna elegans*; Hudon and Brush 1990). In richly colored tissues, however, as in the lemon-yellow plumage of wild American goldfinches (*Carduelis tristis*), even a single feather can provide sufficient pigment for detection of feather carotenoids using this method (McGraw et al. 2001, 2002a). For best results, samples should be analyzed soon after collection or stored at ultra-cold temperatures, as tissue that is stored for long periods of time may leach pigments into preservatives (Blair and Graham 1954) or harbor degraded pigments due to exposure to light or oxygen (Jørgensen and Skibsted 1990; Chen et al. 1994). We have found, however, that carotenoid-pigmented bird feathers (from Evening Grosbeaks, *Coccothraustes vespertinus*) stored as museum specimens for nearly 40 years yield carotenoid profiles that match those of feathers from recently captured individuals (McGraw et al. 2003b). Tissues that are surgically removed from animals should also be rinsed free of any spilled blood with water, as the carotenoids that animals circulate through their blood would contaminate the samples.

Tissue should be placed in a glass tube or vial that can hold no less than 5 ml and can be capped tightly. At this stage, we also recommend preparing a positive control tube, in which similar biological material that is known to contain carotenoid pigments is used (see lists of carotenoid-colored species in Goodwin 1984; Stradi 1998; and Table 1 in this paper). To the tubes containing the tissues (and to a third, blank, negative-control tube), add 1 ml of acidified pyridine. Pyridine is a chemical solvent that can be obtained from the chemistry stockrooms at most universities or by ordering from chemical supply companies (e.g., Sigma Chemical, St. Louis, Mo.) for around US\$15.00 per 100 ml. It should be purchased and used fresh, as pyridine shelved for many years acquires a yellowish coloration. It also has a very nasty smell (like rotting corn) and can be a potent mutagen/carcinogen, so exercise caution and handle this chemical using laboratory gloves and only in a fume hood. Before use with animal tissue, pyridine should be acidified by adding four drops of concentrated (12 N) hydrochloric acid for every 100 ml of pyridine. The tubes of tissue and pyridine should then be capped tightly and placed in a 95-°C water bath for 4 h (Hudon and Brush 1992). This extraction technique works by weakening the non-covalent hydrogen bonds that bind the pigments

Table 1. Identification of carotenoid pigments in various animal tissues using the acidified-pyridine and solvent-transfer tests. High-performance liquid chromatography (HPLC) methods of McGraw et al. 2003a) was later used to confirm the presence of carotenoids in these samples (following the

Class	Common name	Scientific name	n ^a	Tissue	Carotenoids present? ^b	Related citations
Insecta	Multicolored Asian lady beetle	<i>Harmonia axyridis</i>	20	Red elytra	Yes (BCAR, ACAR)	Valadon and Mummery 1973
	Ornate moth ^c	<i>Utetheisa ornatrix</i>	2	Red/orange wings	No	Collins and Kalmns 1970
Crustacea	Pea aphid ^c	<i>Acyrtosiphon pisum</i>	12	Green body	Yes (LUT, ZEA)	Czezuga 1980a
	Fruit fly ^c	<i>Drosophila melanogaster</i>	30	Red eyes	No	Wolf et al. 1987
	California spiny lobster ^c	<i>Penaeus interruptus</i>	1	Orange carapace, eggs	Yes (AST)	Katayama et al. 1973
	Shrimp ^c	<i>Penaeus</i> sp.	3	Orange tail	Yes (AST)	Yamada et al. 1990
	Krill ^c	<i>Euphausia</i> sp.	2	Orange tail	Yes (AST)	Yamaguchi et al. 1983
	Plainfin midshipman ^c	<i>Porichthys notatus</i>	1	Yellow belly skin, eggs	Yes (TUNA)	none
	Goldfish ^c	<i>Carassius auratus</i>	2	Orange scales/fins	Yes (TUNA)	Hata and Hata 1970
	Guppy ^c	<i>Poecilia reticulata</i>	1	Orange scales/fins	Yes (TUNA)	Grether et al. 2001
	Common platy ^c	<i>Xiphophorus maculatus</i>	1	Orange scales	Yes (TUNA)	Rempeters et al. 1981
	California newt ^c	<i>Taricha torosa</i>	1	Orange venter	Yes (BCAR, CAN, LUT)	Matsui et al. 2002
Amphibia	Green treefrog ^c	<i>Hyla cinerea</i>	1	Yellow/green skin	Yes (LUT, ZEA)	Czezuga 1980b
	Green anole ^c	<i>Anolis carolinensis</i>	1	Green dorsum/red dewlap	Yes (LUT, ZEA)	Macedonia et al. 2000
Reptilia	Common merganser ^d	<i>Mergus merganser</i>	1	Yellow feather blush	Yes (LUT, ZEA)	Stresemann 1927–1934
	Common eider ^d	<i>Somateria mollissima</i>	1	Green head feathers	No	Dyck 1992
Aves	Tundra swan ^d	<i>Cygnus columbianus</i>	1	Rust-tipped feathers	No	Kennard 1918; Höhn 1955
	Domestic chicken ^c	<i>Gallus domesticus</i>	9	Yellow downy feathers	No	Völker 1934
	Wild turkey ^c	<i>Meleagris gallopavo</i>	2	Brown hackle feathers	No	Lucas and Stettenheim 1972
	Scarlet macaw ^c	<i>Ara macao</i>	2	Red caruncles	No	Lanuelle et al. 1951
	Knysna turaco ^d	<i>Tauraco corythaix</i>	1	Red feathers	No	Stradi et al. 2001
	American crow ^d	<i>Corvus brachyrhynchos</i>	1	Green feathers	No	Moreau 1958; Dyck 1992
	Eastern bluebird	<i>Sialia sialis</i>	6	Dark eggshells	No	With 1973; Miksik et al. 1996
	Barn swallow	<i>Hirundo rustica</i>	9	Brown feathers	No	McGraw et al. 2004a
	House finch	<i>Carpodacus mexicanus</i>	3	Red/orange feathers	No	McGraw et al. 2004a
	American goldfinch	<i>Carduelis tristis</i>	42	Yellow feathers, beak	Yes (3HE and 13 others)	Inouye et al. 2001
	Evening grosbeak ^d	<i>Coccothraustes vespertinus</i>	7	Yellow feathers	Yes (CXA, CXB)	McGraw et al. 2001, 2002a
	Northern cardinal	<i>Cardinalis cardinalis</i>	8	Red feathers, beak	Yes (LUT)	McGraw et al. 2003b
	Zebra finch ^f	<i>Taeniopygia guttata</i>	12	Red beak	Yes (AST, ADOR, ADON, CAN)	McGraw et al. 2001
	Red-billed quelea	<i>Quelea quelea</i>	5	Red feathers, beak	Yes (AST, ADOR, ADON, CAN)	McGraw et al. 2002b
	Red-winged blackbird	<i>Agelaius phoeniceus</i>	4	Red feathers	CAN	Dale 2000
	Yellow warbler	<i>Dendroica petechia</i>	1	Yellow feathers	Yes (AST, CAN, LUT, ZEA)	McGraw et al. 2004c
	Common yellowthroat	<i>Geothlypis trichas</i>	1	Yellow feathers	Yes (LUT)	McGraw et al. 2003b
	American redstart	<i>Setophaga ruticilla</i>	14	Yellow/orange feathers	Yes (LUT)	McGraw et al. 2003b
	Yellow-breasted chat	<i>Icteria virens</i>	21	Yellow feathers	Yes (CAN, CXA, CXB)	None
					Yes (LUT)	Mays et al. 2004

^a Number of individuals tested in each species

^b Key to carotenoids detected with HPLC (in parentheses): ACAR = α -carotene, ADON = adonirubin, ADOR = α -doradexanthin, AST = astaxanthin, BCAR = β -carotene, CAN = canthaxanthin, CXA = canary xanthophyll A, CXB = canary xanthophyll B, LUT = lutein, TUNA = tunaxanthin, ZEA = zeaxanthin, 3HE = 3-hydroxy-echinenone

^c Animals obtained from captivity

^d Samples collected from museum specimens

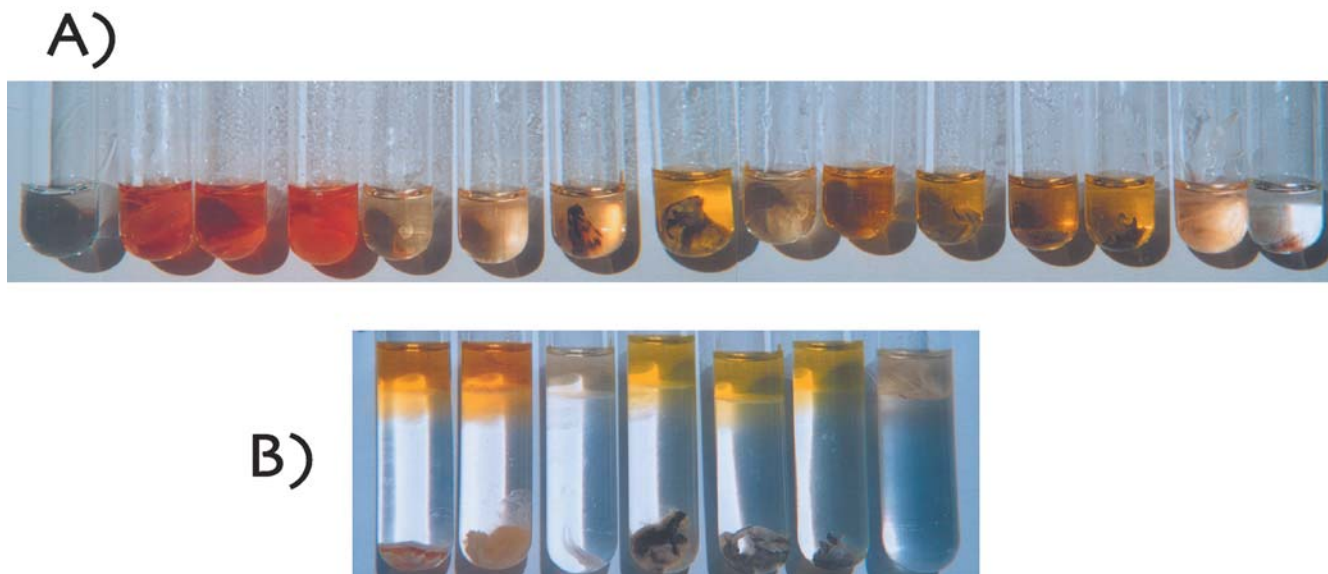


Fig. 2A, B Representative photographs of the color changes evident during animal tissue extraction with acidified pyridine. **A** Carotenoids are released into pyridine to generate a red, orange, or yellow pigmented solution. Test tube contents are as follows, from *left to right*: control (no tissue), krill tail, lobster carapace, lobster eggs, platy scales, goldfish fin, guppy tail, midshipman belly,

midshipman eggs, newt skin, treefrog skin, anole dewlap, anole dorsum, cardinal feathers, and swallow feathers. **B** Carotenoids are transferred to hexane TBME (upper, colored phase) from pyridine that is neutralized with water (from *left to right*, lobster carapace, lobster eggs, goldfish fin, midshipman belly, treefrog skin, anole dorsum, and cardinal feathers)

to proteins and subsequently releasing pigments into solution, so it is important that this high temperature is reached¹. Allow the tubes to cool to room temperature before handling.

If carotenoids are present in the tissue of interest, you will find that the once-pigmented tissue has lost color and that the heated pyridine solution is now colorful after the treatment (Fig. 2a, 3b). Tissue remnants can be removed from the solution at this point if there is any doubt whether the solution contains the yellow/orange/red color. Using this technique, we have confirmed the presence of carotenoids in insect wings, crustacean exoskeletons, the scales and fins of fishes, amphibian and reptile skin, and the feathers and beaks of birds (Table 1). It should also prove useful for detecting carotenoids in other body tissues and fluids (e.g., blood, liver, adipose tissue, egg-yolk).

We have also analyzed other pigmented animal tissues that we believed did not contain carotenoids and thus should not have leached pigment into pyridine. Specifically, we examined the rufous- and chestnut-colored feathers of various bird species (e.g., eastern bluebirds, *Sialia sialis*; and barn swallows, *Hirundo rustica*), which contain melanin pigments (McGraw et al. 2004a). We also tested the red eyes of wild-type *Drosophila melanogaster* and the brilliantly colored red-orange wings of an adult arctiid moth (*Utetheisa ornatrix*), which presumably derive their color from pteridine pigments (Collins and Kalnins 1970; Wolf et al. 1987; authors' unpublished data). Lastly, we investigated red caruncle tissue from wild turkeys (*Meleagris gallopavo*), which circulate blood through this tissue and derive their color from hemoglobin (Laruelle et al. 1951). None of these tissues released any pigment into heated-pyridine; all samples retained their color and yielded colorless solutions (e.g., see swallow sample in Fig. 2 and 3). Thus, the acidified-pyridine test provides a reliable chemical means by

which carotenoid pigments can be distinguished from other red, orange, or yellow pigments in animal tissues.

There are a few important exceptions to this carotenoid-specific, chemical-detection method, however. For example, the reddish-brown feathers of domestic, red-leghorn chickens (*Gallus domesticus*), the rich red plumage of scarlet macaws (*Ara macao*), and the rust-tipped feathers of tundra swans (*Cygnus columbianus*) all depigment during pyridine treatment and produce colorful solutions, yet do not contain carotenoid pigments (Table 1). Instead, chicken feathers contain phaeomelanin pigments (Lucas and Stettenheim 1972), macaws enrich their feathers with psittacofulvins, which are pigments found to date only in parrots (Völker 1936, 1937; Stradi et al. 2001), and swans, like other waterfowl and cranes, adventitiously stain their plumage with iron-oxide (Kennard 1918; Höhn 1955; Berthold 1967). Although not yellow, orange, or red in color, we also found that porphyrin pigments in the green feathers of Knysna turacos (*Tauraco corythaix*) and the dark eggshells of American crows (*Corvus brachyrhynchos*) (Völker 1938; With 1973; Dyck 1992), and an unknown pigment in the green head feathers of common eiders (*Somateria mollissima*; Dyck 1992) are also soluble in acidified pyridine (Table 1). Thus, a follow-up analysis is required to confirm the presence of carotenoids in animal tissues.

For this, we offer a straightforward, supplementary chemical procedure. As lipid-soluble compounds, carotenoid pigments will transfer readily to strong, non-polar organic solvents, such as hexane (for the non-polar carotenoids) and tert-butyl methyl ether (TBME) (for polar carotenoids). In contrast, other chemical colorants of animals (e.g., pteridines, melanins, porphyrins) are soluble in aqueous solutions, either under acidic or basic conditions (Fox and Vevers 1960). This difference in pigment solubility can be taken advantage of here, by partitioning water- and lipid-soluble components between aqueous pyridine and hexane:TBME, respectively. To the colored acidified pyridine solution, first add 2 ml distilled water. Invert the capped tube a few times to homogenize the mixture. Next, add 1 ml hexane: TBME(1:1), each of which can again be purchased from a chemical stockroom or distributor for around US \$20.00 per 100 ml. After capping, shake the tube vigorously for 2 min, ensuring to mix the two phases well. Then, to

¹ For certain carotenoid-enriched tissues (e.g., fruits, fish skin), other solvents, such as acetone (Grether et al. 2001) or tetrahydrofuran (McGraw et al. 2001), will also solubilize carotenoids. However, we recommend a standardized procedure that ensures that even the most tightly bound animal tissue matrices will release pigments into solution.

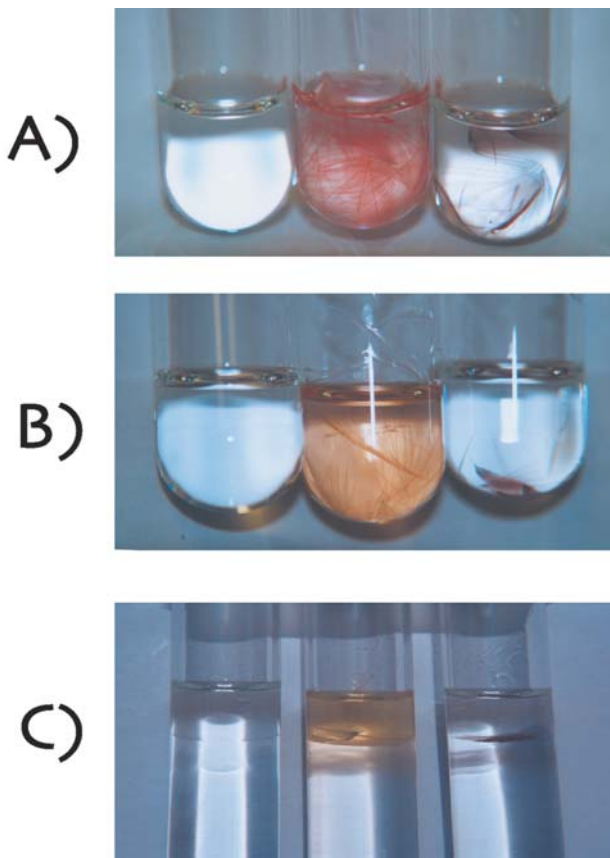


Fig. 3A–C Change in solution color resulting from acidified-pyridine extraction of carotenoids in bird feathers. Tubes in each photo are (from left to right): control (no feathers), northern cardinal feathers, and barn swallow feathers. **A** Feathers in solution prior to extraction, **B** leaching of pigments into pyridine, for the cardinal feathers only, and **C** transfer of cardinal carotenoids to hexane TBME (*upper*, colored phase)

fully separate the hexane:TBME from the now-aqueous pyridine, either centrifuge the solution at 3,000 RPM for 5 min or let the solution stand overnight at room temperature in the dark (all done in a fume hood). If your tissue contains carotenoid pigments, the upper, hexane:TBME phase should now contain the color, whereas the lower, pyridine–water phase should be colorless (Fig. 2b, 3c). If other pigments are responsible for the color, the bottom phase should retain the color². If the mixture was shaken well, yet both fractions retain some color, try re-extracting the lower (aqueous) phase with more hexane:TBME; if the lower phase still is colored after multiple hexane:TBME extractions, it is possible that two types of pigment confer color on your study tissue (like the carotenoids and melanins in crown feathers of western tanagers, *Piranga ludoviciana*; Hudon 1991), which would then require additional biochemical tests for these other pigments (e.g., Ito and Fujita 1985).

For researchers that are interested in acquiring even more information about the nature of their tissue carotenoids, without using detailed chromatographic procedures, absorbance spectrophotometry may offer an idea of the predominant carotenoids that give

color to their study animal. Carotenoids exhibit characteristic spectral-absorbance profiles based on differences in molecular structure, and a determination of the light-absorbance properties for carotenoids in this hexane:TBME solution may help identify carotenoids as either dietary in origin (e.g., lutein or β -carotene) or as products of metabolism (e.g., ketocarotenoids, canary xanthophylls, tunaxanthins; Goodwin 1984; Brush 1990; Stradi 1998). Most general biology labs at universities use these rather inexpensive, portable spectrophotometers, and these can be borrowed to characterize spectral absorbance at critical light wavelengths. Transfer your colored hexane TBME solution to a cuvette for analysis in the spectrophotometer, and prepare a control (or blank) cuvette containing pure 1:1 hexane:TBME. Take absorbance readings every 5 nm from 400–500 nm after calibrating the instrument at each interval with your blank. Carotenoids exhibit distinct absorption maxima (λ_{\max} values) within this wavelength range; for example, in comparable solvents like hexane, lutein absorbs maximally at 445 nm, β -carotene at 450 nm, and astaxanthin at 470 nm (see Britton 1985; Appendix in Bauernfeind 1981; or Table 8 in Rodriguez-Amaya 1999 for λ_{\max} values for different carotenoids).³ Unfortunately, because many animals deposit a host of carotenoid pigments in tissue (Goodwin 1984), this profile will not unequivocally identify each of the different types of carotenoids present, but instead will suggest predominant tissue pigment(s) based on λ_{\max} . In the end, properly dispose of all chemicals and tubes in solvent jars and waste bins following hazardous waste regulations.

Discussion

The aim of this study was to communicate to behavioral ecologists the set of laboratory methods needed to diagnose the simple presence of carotenoid pigments in animal tissues. Recently, some researchers have assumed that certain red, orange, or yellow colors in animals are carotenoid-derived and have drawn conclusions about the information content and signaling value of these colorful traits based on the nutritional (e.g., diet-derived) and physiological (e.g., immunomodulatory) properties of carotenoids (e.g., Camplani et al. 1999, Massaro et al. 2003). However, biochemical evidence for carotenoid coloration in these studies has been weak or absent, and in two of these cases subsequent chemical tests demonstrated the absence of carotenoids and the presence of other colorants in tissues. Barn swallows (*Hirundo rustica*), for example, color their brown feathers with melanins (McGraw et al. 2004a, 2004b), and penguins like king (*Aptenodytes patagonicus*) and yellow-eyed (*Megadyptes antipodes*) penguins use as of yet undescribed fluorescent compounds to acquire yellow plumage (McGraw et al. 2004a, McGraw 2005). We hope, by exposing this simple chemical method, in which we couple a time-tested pyridine extraction technique with solvent transfer and spectrophotometry to identify carotenoid pigments in animal tissues based on solution color and absorption characteristics, that behavioral ecologists will avoid future misclassifications of pigments and color types and be better able to characterize the types of color signals they

² This was the case for non-carotenoid pigments extracted from chicken, turaco, swan, and eider feathers and crow eggshells. However, macaw psittacofulvins did transfer to hexane:TBME, but have never been found in animals other than parrots, and carotenoids have never been found in parrot feathers.

³ It is also worth noting here that lipophilic psittacofulvins and carotenoids have distinct λ_{\max} values (Hudon and Brush 1992), such that absorbance spectrophotometry can be used if necessary as a third diagnostic test to confirm the presence of carotenoids in animal tissues.

are studying so that they may acquire a more detailed understanding of the costs, signal content, and function of these ornaments.

Acknowledgements This paper evolved from several inquiries and discussions at the 9th International Behavioral Ecology Congress in Montreal, Quebec, Canada. The Institutional Animal Care and Use Committee at Cornell University approved all procedures reported in our study (protocol no. 99–89). We thank T. Czeschlik and two anonymous referees for helpful comments on the manuscript. This research was funded by the Environmental Protection Agency (STAR fellowship to K.J.M.); during manuscript preparation, K.J.M. was supported by the United States Department of Agriculture (grant to K. Klasing) and by the College of Liberal Arts and Sciences and the School of Life Sciences at Arizona State University.

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