

## Astaxanthin is responsible for the pink plumage flush in Franklin's and Ring-billed gulls

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Received 12 October 2004; accepted 18 May 2005

**ABSTRACT.** Carotenoid pigments produce the red, orange, and yellow plumage of many birds. Carotenoid-containing feathers are typically rich in color and displayed by all adult members of the species. In many gulls and terns, however, an unusual light pink coloring (or flush) to the normally white plumage can be found in highly variable proportions within and across populations. The carotenoid basis of plumage flush was determined in an Elegant Tern (*Sterna elegans*; Hudon and Brush 1990), but it is not clear if all larids use this same mechanism for pink plumage coloration. We examined the carotenoid content of pink feathers in Franklin's (*Larus pipixcan*) and Ring-billed (*Larus delawarensis*) gulls and found that a single carotenoid—astaxanthin—was present. Astaxanthin was primarily responsible for the flush in Elegant Terns as well, but was accompanied by other carotenoids (e.g., canthaxanthin and zeaxanthin), as is typical of most astaxanthin-containing bird feathers. In both gull and tern species, carotenoids were contained within feathers and did not occur on the plumage surface in preen oil, as some have previously speculated. We hypothesize that some gulls turn pink because they acquire unusually high amounts of astaxanthin in their diets at the time of feather growth. It is tempting to link the increase in sightings of pink Ring-billed Gulls since the late 1990s with the introduction of pure, synthetic astaxanthin to the diets of hatchery-raised salmon.

**SINOPSIS.** Astaxantina, responsable del plumaje Rosado en *Larus pipixcan* y *Larus delawarensis*

Los pigmentos carotenoides son los responsables de la coloración roja, anaranjada y amarilla en el plumaje de aves. Plumajes que contienen carotenoides, generalmente son ricos en color y son mostrados en variados patrones de conducta por los adultos con dicha coloración. No obstante, en muchas gaviotas se puede observar una coloración rosada, en vez del típico plumaje blanco y su proporción puede ser variable en una o entre poblaciones. La presencia de carotenoides, y plumaje rosado, fue documentado en *Sterna elegans*, pero no quedó claro, si el mismo tipo de mecanismo aplica a otros laridos. Examinamos el contenido carotenoides en plumas rosadas de las gaviotas, *Larus pipixcan* y *L. delawarensis*, y encontramos que la presencia de astaxantina. Este mismo carotenoides fue el principal responsable de la coloración rosada en *Sterna elegans*, sin embargo estuvo acompañado de otros carotenoides (ej. cantaxantina y seaxantina). En las diferentes gaviotas estudiadas se encontró que los carotenoides se encontraban dentro de la pluma y no presentes en la superficie de estas o en el aceite de la glándula uropigial, como se había previamente especulado. Nuestra hipótesis es que algunas gaviotas adquieren un plumaje rosado a consecuencia de la dieta ingerida durante el periodo de crecimiento de las plumas. Es tentador tratar de atar el color rosado en gaviotas como *L. delawarensis* a partir del 1990 con la introducción de astaxantina sintética en la dieta de salmones criados en cautiverio.

*Key words:* carotenoid pigmentation, Laridae, *Larus delawarensis*, *Larus pipixcan*, plumage coloration

Brilliant red, orange, and yellow colors in birds are often due to the presence of carotenoid pigments (Fox 1976). Several species of songbirds incorporate high concentrations of carotenoids into feathers or bare parts like the beak and legs to indicate their potential worth as a mate (Hill 1999) or progeny (Fenoglio et al. 2002).

In most of the familiar and well-characterized examples of carotenoid coloration in birds, such

as in male House Finches (*Carpodacus mexicanus*; Hill 2002), all individuals of a species show at least some measurable amount of pigment and color. The challenge is to accumulate enough pigments to display the degree of vibrant coloration that is most sexually attractive. In other species, like many gulls and terns, however, forms of carotenoid-based plumage coloration are much less intense and not necessarily characteristic of the entire population or species. Worldwide, at least 13 species of gulls and five species of terns are capable of developing a light pink wash (or 'flush') of color across some or all

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of their white body plumage (Hardy 2003). In Franklin's Gulls (*Larus pipixcan*), for example, most or all adults show a pink flush in fresh plumage, though the intensity of the flush can vary widely. Only a few Ring-billed Gulls (*Larus delawarensis*) exhibit the flush, and these individuals have largely been restricted to the Pacific Northwest (e.g., the California-Oregon border; Hardy 2003). Interestingly, this range has expanded in recent years. Pink Ring-billed Gulls have been reported from the state of Washington since 1998, where they now make up some of the highest proportions (up to 30%) in western populations (Hardy 2003).

Hudon and Brush (1990) characterized the carotenoid pigments responsible for the pink plumage flush in an Elegant Tern (*Sterna elegans*). Aside from this, the mechanisms and functions of the pink plumage coloration in larids have largely been unexplored. We obtained pink feathers from two species of gulls (Franklin's Gull and Ring-billed Gull) and used traditional biochemical techniques to determine whether (a) carotenoids also produce plumage flush in these species, (b) similar types of carotenoids were found in gull and tern feathers, (c) plumage pigments match those present in internal tissues like liver and fat, and (d) pigments were deposited into or on the surface of feathers. Stegmann (1956) previously hypothesized that, because preen oil can be red in some birds, larids apply carotenoids to feathers externally to acquire their flush.

## METHODS

**Feather collection.** Pink feathers from the carcass of an adult male Franklin's Gull were obtained from the Bear River Migratory Bird Refuge in Utah in June 2003. Two adult male Ring-billed Gulls were collected in the spring of 2003 from Wells Dam on the Columbia River in Douglas County, Washington, under animal control activities of the U.S. Department of Agriculture. One bird was faintly flushed with pink, and the other showed no flush. All feathers were plucked from the breast and stored in envelopes at room temperature until analyzed. Liver and fat tissue from the pink Ring-billed Gull were collected and stored at  $-80^{\circ}\text{C}$ .

**Carotenoid extraction.** Feathers were washed in ethanol and hexane sequentially for 15 min to remove surface lipids, including

carotenoids if they were present. They were then subjected to thermochemical extraction that recovers plumage carotenoids in a host of birds (Hudon and Brush 1992, McGraw et al. 2002a, 2003a, 2004). Feathers (0.5 g) were immersed in 5 mL acidified pyridine in a 20-mL glass tube. We filled the headspace with argon to prevent pigment oxidation. The tube was held at  $95^{\circ}\text{C}$  for 3 h. After cooling the tube to room temperature, we added 5 mL distilled water to the pyridine, inverted the tube a few times, and added 5 mL hexane:MTBE (1:1, v:v). We shook the tube vigorously for 2 min, centrifuged at 3000 RPM for 5 min, then transferred the supernatant to a fresh tube and evaporated the solvent to dryness under a stream of nitrogen.

Three 0.5 g samples of liver and adipose tissue were separately ground in the presence of 3 mL tetrahydrofuran with a mortar and pestle (*sensu* McGraw et al. 2002b). The three solvent fractions for each tissue were removed, pooled, and centrifuged at 3000 RPM for 5 min. We transferred the supernatant to a fresh tube and evaporated the solvent to dryness under a stream of nitrogen.

**Carotenoid analysis.** Dried pigment residues were redissolved in 200  $\mu\text{L}$  HPLC mobile phase and 50  $\mu\text{L}$  were injected into a Waters<sup>TM</sup> 717plus Autosampler HPLC (Millipore Corp., Bedford, MA, USA) fitted with a Develosil RPAqueous RP-30 column (250  $\times$  4.6 mm; Nomura Chemical Co. Ltd., Aichi, Japan) and a column heater (Eppendorf TC-50, Hamburg, Germany) set at  $27^{\circ}\text{C}$ . We used two different isocratic systems (Hewlett-Packard 1050 Series Isocratic Pump, Houston, TX, USA), both at a constant flow rate of 1.2 mL per min, to analyze xanthophylls and carotenes separately, if they were present. For xanthophylls, we used acetonitrile:methanol:chloroform (46:46:8, v:v:v) as the mobile phase. For carotenes, we used methanol:dichloromethane (50:50, v:v) as the mobile phase. Data were collected from 250 to 600 nm using a Waters<sup>TM</sup> 996 photodiode array detector (Waters Corporation, Milford, MA, USA). The minimum detection limit of our PDA detector was 0.0001 AU (absorbance units), which amounts to approximately 1 ng of carotenoid per 50  $\mu\text{L}$  injection, or 8 ng carotenoid per g of feather for this analytical system. All samples were run in duplicate. We report values as means  $\pm$  1 standard error.

## RESULTS

Ethanol and hexane solvent washes from all feather samples yielded no carotenoids. In pyridine extracts of feathers, we detected only a single carotenoid. In our xanthophyll procedure, this carotenoid eluted from the column at 13.0 min and absorbed light maximally ( $\lambda_{\max}$ ) at 485 nm. Based on comparisons with authentic reference carotenoids donated by Roche Vitamins (Parshippany, NJ, USA) and Riccardo Stradi (University of Milan, Italy), we identified this pigment as all-*trans* (*E*) astaxanthin (3,3'-dihydroxy- $\beta$ -carotene-4,4'-dione). As determined from our external standard curve, astaxanthin was present at a concentration of  $12.3 \pm 0.5 \mu\text{g/g}$  in the feathers of the Franklin's Gull and  $7.4 \pm 0.9 \mu\text{g/g}$  in the feathers of the Ring-billed Gull. Franklin's Gull feathers appeared redder to the naked eye than those of the Ring-billed Gull. Liver and body fat of the pink Ring-billed Gull also contained only astaxanthin (at concentrations of  $31.8 \pm 1.3$  and  $12.6 \pm 0.8 \mu\text{g/g}$ , respectively).

## DISCUSSION

Hudon and Brush (1990) previously identified astaxanthin and other minor carotenoid components such as canthaxanthin and zeaxanthin as the colorants of pink plumage in an Elegant Tern from California. We found that astaxanthin was the only carotenoid in pink feathers of Franklin's and Ring-billed gulls from the western United States. Astaxanthin was not extractable with organic solvents alone, suggesting that, as in terns, carotenoids were not deposited on the feather surface in preen oil. Instead, pigments were bound in feather tissue and released into the solution only when hydrogen bonds between carotenoids and keratin were destroyed by thermochemical treatment.

Astaxanthin is a widespread colorant of bird feathers, and is found in flamingos (*Phoenicopterus* spp.; Fox et al. 1967, Stradi 1999), Northern Flickers (*Colaptes auratus*; Stradi 1998), Northern Cardinals (*Cardinalis cardinalis*; McGraw et al. 2001), and several other species (Stradi 1999). It is commonly acquired from food sources like fish and crustaceans (Goodwin 1984), but can be metabolically derived from xanthophyll precursors like zeaxanthin by some birds (Stradi 1998, McGraw et al.

2001). However, the fact that only astaxanthin was present in gull plumage was unusual. Astaxanthin, the predominant component of several red-colored avian tissues, is nearly always accompanied by other 4-oxo-carotenoids like adonirubin,  $\alpha$ -doradexanthin, and canthaxanthin (Stradi 1999). This suggests that, compared to pink-colored terns, gulls either feed on a unique source of astaxanthin-rich food or that their physiological systems are geared specifically to accumulate or produce astaxanthin only. Moreover, the fact that only some Ring-billed Gulls acquire this coloration indicates that only these birds have sufficient access to or sufficiently utilize carotenoids for deposition in plumage.

At present, we are unsure which of these factors plays a more important role in creating plumage flush in gulls. However, a few observations point to a dietary mechanism. First, although birds are known to physiologically favor certain carotenoid types (e.g., xanthophylls over carotenes; Scheidt 1998), they rarely accumulate only one xanthophyll when several are ingested (due to the general hydrophobic characteristics of lipoprotein transporters; Parker 1996, Slifka et al. 1999) or metabolically derive just one xanthophyll (due to the generalized activity of carotenoidogenic enzymes; McGraw et al. 2003b). Second, the only other bird species known to deposit pure astaxanthin in the integument is the White Stork (*Ciconia ciconia*), and this occurs in a Spanish population because of a recent change in their diet—the introduction of the red swamp crayfish (*Procambarus clarkii*) from North America (Negro and Garrido-Fernandez 2000). This situation is similar to the appearance of orange-tipped tails in Cedar Waxwings (*Bombycilla cedrorum*) since the middle of the 20th century. Waxwings have added berries from the recently introduced Morrow's honeysuckle (*Lonicera morrowi*) to their diet, and these berries contain the red pigment, rhodoxanthin, that is now deposited in the tail feathers (Hudon and Brush 1989, Brush 1990a, Mulvihill et al. 1992, Witmer 1996). Last, and perhaps most intriguingly, an increasing number of pink gulls have been reported in the Pacific Northwest since 1998 (Hardy 2003). About this time, in 1996, salmon hatcheries began providing supplements of purified, synthetic astaxanthin in the diets of juvenile fish to improve their health and flesh pigmentation (Torrissen and Christiansen 1995, Hardy

2003). Because these salmon are prey items of Ring-billed Gulls throughout the year (Hardy 2003), we speculate that the increased levels of astaxanthin in juvenile salmon are being directly transferred to the feathers of gulls that consume these fish. Franklin's Gulls, on the other hand, consume mostly insects (which lack astaxanthin) on their North American breeding grounds (Burger and Gochfeld 1994), so they might obtain astaxanthin from fish and crustaceans ingested during the winter in South America. A synthetic source of this pigment is less obvious.

It would also be worthwhile to investigate the physiological machinery responsible for plumage flush in gulls and other larids. Our hypothesis predicts that, in birds that develop a pink color, carotenoid concentrations in blood are so high that they flood feather follicles and accumulate in plumage. Follicles from normal white feathers likely exclude carotenoids not only because they are in lower concentrations, but also due to the low abundance or affinity of carotenoid-binding proteins (Brush 1990b, McGraw et al. 2003a). It would be interesting to sample blood from several larid species, manipulate dietary-carotenoid content experimentally during molt, and conduct studies on carotenoid uptake by feather follicles to examine the best determinants of pink plumage coloration. An alternative hypothesis is that there is a carotenoid-independent environmental or physiological cue that triggers pigment uptake by follicles in some individuals. Sex hormones like testosterone and estrogen are known to influence follicular development (Kovacs et al. 1986) and carotenoid pigmentation in songbirds (McGraw 2003), and it is conceivable that certain gulls develop pink plumage because they experience hormonal perturbations (caused by environmental hormone mimics, for example) that improve feather uptake of carotenoids. This would not, however, explain why all individuals of some larid species display pink color, as in Ross's Gulls (*Rhodostethia rosea*). In *R. rosea*, all individuals must either obtain high dietary carotenoid levels compared to other species or have improved physiological means of incorporating carotenoids into feathers.

Finally, there remain both evolutionary and applied questions about the pink plumage flush in gulls. Because carotenoid colors often are sexually selected indicators of mate quality in adult birds (Hill 1999), there may be signaling

benefits to producing pink plumage in these gull species. Moreover, if our proposed link between gull pigmentation and salmon consumption is correct, over time western Ring-billed Gulls may begin to preferentially forage on salmon for these pigments, which would present a challenge to aquaculturists seeking to restock diminishing populations of Pacific salmon. Identifying the dietary and functional basis of plumage flush will be important first steps toward solving the mystery behind these unusually reduced and variable forms of carotenoid-based coloration.

#### ACKNOWLEDGMENTS

We thank R. Parker for providing access to lab equipment, C. Thompson for donating Ring-billed Gull specimens, B. Olson for donating Franklin's Gull feathers, and C. Brown, A. Brush, and an anonymous referee for helpful comments on the paper. During manuscript preparation, KJM was supported by the United States Department of Agriculture (grant to K. C. Klasing) and by the School of Life Sciences and College of Liberal Arts and Sciences at Arizona State University.

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