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## Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colorful songbird

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**Abstract** In egg-laying animals, mothers can influence the development of their offspring via the suite of biochemicals they incorporate into the nourishing yolk (e.g. lipids, hormones). However, the long-lasting fitness consequences of this early nutritional environment have often proved elusive. Here, we show that the colorful carotenoid pigments that female zebra finches (*Taeniopygia guttata*) deposit into egg yolks influence embryonic and nestling survival, the sex ratio of fledged offspring, and the eventual ornamental coloration displayed by their offspring as adults. Mothers experimentally supplemented with dietary carotenoids prior to egg-laying incorporated more carotenoids into eggs, which, due to the antioxidant activity of carotenoids, rendered their embryos less susceptible to free-radical attack during development. These eggs were subsequently more likely to hatch, fledge offspring, produce more sons than daughters, and produce sons who exhibited more brightly colored carotenoid-based beak pigmentation. Provisioned mothers also acquired more colorful beaks, which directly predicted levels of carotenoids found in eggs, thus indicating that these pigments may function not only as physiological ‘damage-protectants’ in adults and offspring but also as morphological signals of maternal reproductive capabilities.

### Introduction

Although parental effort is sometimes narrowly viewed as a behavioral strategy adopted by animals only after offspring are born or hatch (Clutton-Brock 1991), mothers can also more subtly devote a host of nutrients and other biochemicals to developing embryos (Mousseau and Fox 1998). Birds, like many other oviparous animals, provision their eggs with an energy-packed yolk reserve that embryos draw from as they grow. Among the lipid-soluble compounds found in avian egg yolks are carotenoid pigments, which are responsible for their characteristic bright-yellow coloration (Blount et al. 2000). In many animals, carotenoids are touted as potent antioxidant and immunostimulatory molecules, scavenging up potentially damaging reactive oxygen species (e.g. free radicals) that are produced by normal physiological processes (Surai et al. 1996; Krinsky 2001). Thus, by allocating high concentrations of carotenoids to eggs, female birds may afford considerable chemical protection to the cells and tissues of their developing young and dramatically influence offspring fitness.

In this study, we investigated the fitness consequences of egg yolk carotenoid investment in female zebra finches (*Taeniopygia guttata*). Because vertebrates cannot synthesize carotenoid pigments *de novo*, birds must obtain these compounds from a diet rich in plant matter (e.g. seeds, fruits, berries) (Olson and Owens 1998; Møller et al. 2000). These native Australian finches consume weed and grass seeds throughout the year (Zann 1996), with the yellow xanthophylls lutein and zeaxanthin comprising 95% of seed carotenoids (McGraw et al. 2002). We provided captive zebra finch pairs with either a carotenoid-enriched or control diet for 3 weeks prior to egg-laying and allowed birds to complete two breeding attempts, one on each diet (in random order). We then examined the effect of pigment supplementation on yolk-carotenoid concentration, reactive-oxygen-species (lipid peroxide) content in eggs, and the subsequent development and survival of young. Male and female zebra finches also display colorful red beaks that are derived from carotenoid pigments (McGraw

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et al. 2002) and are used as mating signals of sexual attractiveness (Burley and Coopersmith 1987; Houtman 1992; but see Collins and ten Cate 1996). Thus, we tested the relationship between maternal beak coloration, the concentration of carotenoids found in eggs, and the subsequent beak coloration developed by their offspring once sexually mature, to determine if mothers signal reproductive investment with their pigmented beaks as well as influence the eventual mating chances of their sons based on embryonic exposure to antioxidants.

## Methods

12 pairs of wild-type finches were housed individually in small wire cages (0.6 m long  $\times$  0.4 m wide  $\times$  0.4 m tall) under full-spectrum indoor lighting (14:10 h light/dark cycle) at 25°C and 70% humidity and were provided with *ad libitum* access to water, oystershells, cuttlebone, and a Kaytee® Forti-Finch™ food mix that was supplemented with egg whites twice per week (see McGraw et al. 2002 for additional details about the colony). These birds had similar reproductive histories, having raised two broods 12 and 9 months prior.

Carotenoid-enriched birds were fed a carotenoid supplement consisting of 9  $\mu$ g lutein beadlets and 2  $\mu$ g zeaxanthin beadlets per ml drinking water, while control birds received an equal dose of placebo beadlets in their drinking water (all beadlets kindly donated by Roche Vitamins Inc., Parsippany, N.J.). We calculated the appropriate dietary-carotenoid dose by determining average levels of food and water intake (3 g and 2 ml, respectively) along with the concentration of lutein and zeaxanthin in seeds (McGraw et al. 2001), ultimately raising average consumption levels to the upper limit of their natural range (McGraw et al. 2004). Baseline seed diets contained sufficient carotenoid pigments for control females to accumulate yolk carotenoids at levels comparable to those of wild songbirds (e.g. Saino et al. 2002).

After the 3-week diet treatment, we provided each cage with nesting material (shredded burlap) and a nest cup (Rubbermaid container with an empty top). Nests were checked on a daily basis for the presence of eggs and nestlings. We collected, weighed, and replaced (with dummy clay eggs) the second- and fourth-laid egg in each clutch for biochemical analysis. Final clutches of eggs were exchanged systematically between treatment groups, matched for laying-date within 3 days, to randomize any parental effects (e.g. incubation, nestling provisioning) that may have been influenced by carotenoid consumption. Incubation and nestling periods were calculated relative to the appearance/disappearance of the first egg or chick in/from the nest. During both breeding rounds (initiated on 9 July and 18 October 2001), we weighed adults (before and after supplementation) and offspring (at hatching and fledging) to the nearest 0.001 g on an electronic balance.

For HPLC analysis of yolk carotenoids (*sensu* McGraw et al. 2002), we homogenized egg yolks in 1 ml water and extracted lipids from 100  $\mu$ l of the homogenate

with 200  $\mu$ l of both ethanol (containing canthaxanthin as an internal standard) and *tert*-butyl methyl ether. The solution was vortexed, centrifuged, and the supernatant evaporated before redissolving the residue in 200  $\mu$ l HPLC mobile phase (methanol-acetonitrile-chloroform, 46:46:8, v/v/v). We injected 50  $\mu$ l into a Waters™ 717plus Autosampler HPLC (Millipore Corp., Milford, MA) fitted with a Develosil RPAqueous RP-30 HPLC column (250  $\times$  4.6 mm I.D.; Nomura Chemical Co., Ltd., Japan), and ran an isocratic system (HP 1050 Series Isocratic Pump) of the aforementioned mobile phase for 25 min at a constant flow rate of 1.2 ml min<sup>-1</sup>. We confirmed the identity of yolk pigments by comparing retention times to those for authentic reference carotenoids. In vivo presence of yolk-lipid peroxides was measured by spectrophotometric determination of thiobarbituric acid reacting substances (TBARS) (*sensu* Ohkawa et al. 1979; Surai et al. 1996).

Nested repeated-measures analyses-of-variance (females within order, clutches within females, eggs within clutches) were used to examine the effect of breeding round (1st or 2nd), diet treatment, and laying order (when appropriate) on yolk carotenoid content, yolk lipid peroxides, offspring survival, sex ratio, and bill coloration in mothers and offspring. Simple linear regressions were used to compare lipid peroxides, yolk-carotenoid concentration, and mother or son beak pigmentation. The significance level was set a priori at  $\alpha = 0.05$ .

## Results

### Egg yolk biochemicals

When provisioned with dietary carotenoid pigments before egg-laying, female zebra finches incorporated a higher concentration of carotenoids into egg yolks (nested repeated-measures ANOVA; treatment:  $F_{1,44} = 8.5$ ,  $P = 0.006$ ; order:  $P > 0.90$ ; treatment\*order interaction:  $F_{1,44} = 8.9$ ,  $P = 0.003$ ; Fig. 1a). These yolks contained significantly fewer lipid peroxides than those produced by control females (treatment:  $F_{1,44} = 7.8$ ,  $P = 0.01$ ; order:  $P > 0.89$ ; treatment\*order interaction:  $F_{1,44} = 4.4$ ,  $P = 0.04$ ; Fig. 1b), and levels of yolk carotenoids in eggs predicted lipid-peroxide concentration (simple linear regression:  $r^2 = 0.35$ ,  $n = 12$ ,  $P = 0.03$ ; Fig. 2a), suggesting that maternally derived yolk pigments reduced the concentration of this reactive oxygen species that could damage developing embryos.

### Carotenoids and the production of offspring

Cross-fostered eggs from carotenoid-supplemented females were more likely to hatch (treatment:  $F_{1,21} = 4.8$ ,  $P = 0.04$ ; order:  $P > 0.2$ ; treatment\*order interaction:  $F_{1,21} = 5.9$ ,  $P = 0.01$ ; Fig. 1c), and young hatched from these eggs were more likely to survive to independence (treatment:  $F_{1,15} = 5.6$ ,  $P = 0.04$ ; order:  $P > 0.35$ ; treatment\*order interaction:  $F_{1,15} = 7.3$ ,  $P = 0.02$ ;

**Fig. 1** Effects of dietary-carotenoid supplementation on egg yolk pigment and peroxide content, offspring survival and sex ratio, and ornamental coloration in captive zebra finches (mean  $\pm$  SEM illustrated). Two sets of birds (1 and 2) were fed either a carotenoid-enriched diet (solid bars) or a control diet (open bars) during successive breeding rounds (Experiments 1 and 2). Group 1 was fed the supplemented diet during Experiment 1 and the control diet during Experiment 2; Group 2 was provisioned in the reverse order

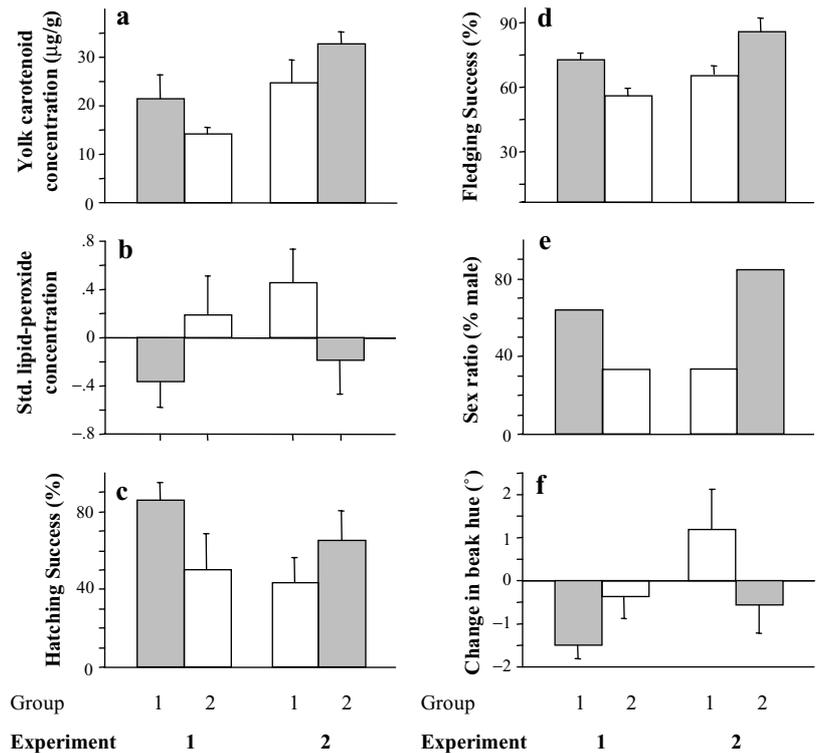


Fig 1d). At maturity, we determined the sex of all fledglings using standard morphological traits (Zann 1996) and interestingly found that clutches laid by provisioned females produced strongly male-biased offspring sex ratios (treatment:  $F_{1,15} = 5.7$ ,  $P = 0.04$ ; order:  $P > 0.35$ ; treatment\*order interaction:  $F_{1,15} = 7.8$ ,  $P = 0.01$ ; Fig. 1e). Diet treatment did not affect timing of breeding, egg mass, female mass, or the rate of egg-laying, nor did the length of incubation and nestling periods or the mass of newly hatched and newly fledged chicks differ between foster- and genetic-parents (all  $P > 0.15$ ).

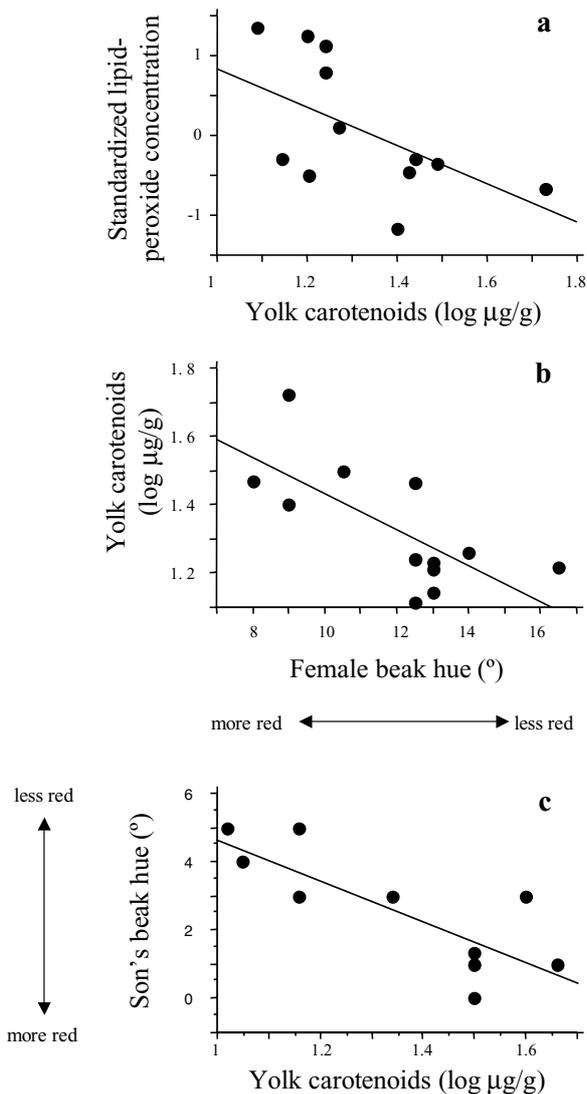
#### Carotenoids and ornamental coloration

Using a hand-held reflectance spectrophotometer (Hill 1998), we scored bill hue (assigned in degrees around a 360° color wheel) before and after dietary carotenoid supplementation and found that provisioned females developed significantly more colorful beaks (treatment:  $F_{1,21} = 4.7$ ,  $P = 0.04$ ; order:  $P > 0.5$ ; treatment\*order interaction:  $F_{1,21} = 6.2$ ,  $P = 0.02$ ; Fig. 1f). Mothers with redder bills also deposited a higher concentration of carotenoids into egg yolks ( $r^2 = 0.49$ ,  $n = 12$ ,  $P = 0.01$ ; Fig. 2b). To examine whether embryonic exposure to high levels of antioxidants could shape the colorfulness of offspring, we directly compared maternal carotenoid levels in egg yolks to the development of sexual coloration in the beaks of their fully mature (100-day-old) sons. We found that mothers who incorporated high concentrations of pigments into eggs produced sons who exhibited more sexually attractive beaks ( $r^2 = 0.61$ ,  $n = 10$ ,  $P = 0.005$ ; Fig. 2c).

#### Discussion

We show that levels of maternally derived carotenoid pigments in egg yolk are sensitive to changes in dietary access to carotenoids and may lower the activity of potentially harmful reactive-oxygen species during embryonic development. We also detected significant fitness consequences for this form of maternal investment. Carotenoid-enriched eggs were more likely to hatch and fledge offspring than controls. Blount et al. (2002, 2004) similarly found that female lesser black-backed gulls (*Larus fuscus*) deposit more carotenoids in egg yolk and raise more offspring when supplemented with dietary carotenoids (but see Bortolotti et al. 2003 for the lack of an effect on reproductive output in red-legged partridges, *Alectoris rufa*). Certain fish (Torrissen and Christiansen 1995; Shahidi et al. 1998), sea urchin (Tsushima et al. 1997; George et al. 2001), and mammal (Chew and Archer 1983; Brief and Chew 1985) species also gain reproductive benefits by consuming large amounts of dietary carotenoids.

Female animals might also be expected to maximize fitness by biasing the sex ratio of their offspring, favoring the production of sons when breeding conditions are favorable and when variance in male reproductive success is higher than that of females (Trivers and Willard 1973). In this study, we found that carotenoid-provisioned females fledged proportionally more sons than control mothers, which supports a fundamental prediction of the Trivers-Willard sex-allocation theory and offers a distinct physiological mechanism by which mothers may influence the sex of their offspring. Enriching yolks with carotenoids may be a direct biochemical means by which female animals inhibit



**Fig. 2** Relationships between yolk-carotenoid content (log-transformed  $\mu\text{g}$  pigment/g yolk) of mothers (mean of all values for each female) and **a** yolk-lipid peroxide concentration (standardized, as above), **b** beak hue of mothers at the time of egg-laying, and **c** average carotenoid-based beak coloration of sons (lower hue values are redder; too few mothers ( $n = 6$ ) fledged daughters that survived to sexual maturity to perform a comparable analysis)

the survival of female embryos or promote that of males. Prior studies of sex allocation in birds, some of which were performed using zebra finches (e.g. Bradbury and Blakey 1998; Kilner 1998; Rutkowska and Cichón 2002), have manipulated food availability or quality to test for sex ratio biases, while ignoring carotenoid levels. In fact, it may have been carotenoid pigments *per se* that influenced offspring survival and sex ratio in these experiments. Because we could not determine the sex of undeveloped eggs in our study, additional work is needed to determine whether carotenoids alter the primary and/or secondary sex ratios of *T. guttata*.

The extended effects of egg yolk carotenoids on the survival of 1-month-old fledglings suggested to us that other aspects of offspring fitness (e.g. reproduction) may be in-

fluenced by the yolk environment early in life. Few studies have examined the ontogenetic control-agents underlying the expression of sexually selected traits in animals (e.g. Nowicki et al. 1998, Ohlsson et al. 2002), and here we elucidate a relationship between embryonic antioxidant exposure and the acquisition of male sexual coloration. Sons hatched from eggs containing high carotenoid levels developed sexually attractive red beaks as adults. This likely occurred not because the pigment molecules provided by mothers in yolk served directly as colorants of the beak (they should have been processed fully within the first few months of post-hatching development; Koutsos et al. 2003), but instead because maternal antioxidant protection offered to offspring organized and improved their health early in life and allowed sons to acquire and sequester more carotenoids for beak pigmentation as adults. To this effect, supplemental carotenoids have been shown to boost immunity in male zebra finches (Blount et al. 2003a; McGraw and Ardia 2003; also see Saino et al. 2003 for carotenoid modulation of nestling immunity in barn swallows, *Hirundo rustica*). Moreover, (Koutsos et al. 2003) and (Blount et al. 2003b) found in chickens and zebra finches, respectively, that early-life exposure to carotenoids (either in yolk or the diet) has long-lasting effects on carotenoid accumulation in internal tissues and fluids. Previous studies of the heritability of beak coloration in zebra finches (Price and Burley 1993; Price 1996) did not take into account the relative contribution of maternal egg yolk carotenoids, and our results highlight the general importance of considering maternal effects when assessing the environmental and genetic determinants of naturally and sexually selected traits in offspring. It will also be valuable to consider how other yolk biochemicals (e.g. hormones, antibodies, lipids), or carotenoid-driven mechanisms other than antioxidant action (e.g. regulation of immune-response genes; Sharoni et al. 2002), affect immune-system organization or beak-color development.

Certain female birds, including zebra finches, also don colorful sexual ornaments; however, the function of female coloration is unclear in these instances (Amundsen 2000). We tested the ideas that the colorful beak of females is sensitive to dietary carotenoid access and is correlated with their reproductive potential (e.g. yolk carotenoid levels). Because female beaks reddened in response to a change in carotenoid availability and predicted the concentration of carotenoids deposited in eggs, it seems that female ornamentation may indeed serve as a reliable signal of maternal investment. In fact, male zebra finches selectively pair with the most fecund females (Monaghan et al. 1996), but this sexual selection pressure appears to be opposed by the high survival costs of beak redness (Price and Burley 1993; Price 1996) that may limit the degree of carotenoid-based color expression in females and maintain sexual dichromatism in this species.

Overall, these results illustrate the powerful, mechanistic action of dietary and maternally derived egg yolk carotenoids on offspring survival, sex ratio, and sexual attractiveness in a passerine bird. Incorporating carotenoid pigments into the nutritive egg yolk is a common feature

of many oviparous organisms (e.g. insects, fishes, reptiles), and thus these reported maternal effects may be widespread in the animal kingdom. Mammalian mothers, which shunt carotenoid pigments to the developing corpus luteum and uterine endometrium (Weng et al. 2000), and to their dependent offspring via breast milk (Khachik et al. 1997), may attempt to maximize offspring fitness in a similar fashion.

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