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The effects of dietary carotenoid intake on carotenoid accumulation in the retina of a wild bird, the house finch (*Carpodacus mexicanus*)

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ABSTRACT

Carotenoid pigments accumulate in the retinas of many animals, including humans, where they play an important role in visual health and performance. Recently, birds have emerged as a model system for studying the mechanisms and functions of carotenoid accumulation in the retina. However, these studies have been limited to a small number of domesticated species, and the effects of dietary carotenoid access on retinal carotenoid accumulation have not been investigated in any wild animal species. The purpose of our studies was to examine how variation in dietary carotenoid types and levels affect retinal accumulation in house finches (*Carpodacus mexicanus*), a common and colorful North American songbird. We carried out three 8-week studies with wild-caught captive birds: (1) we tracked the rate of retinal carotenoid depletion, compared to other body tissues, on a very low-carotenoid diet, (2) we supplemented birds with two common dietary carotenoids (lutein + zeaxanthin) and measured the effect on retinal accumulation, and (3) we separately supplemented birds with high levels of zeaxanthin – an important dietary precursor for retinal carotenoids – or astaxanthin – a dominant retinal carotenoid not commonly found in the diet (i.e. a metabolic derivative). We found that carotenoids depleted slowly from the retina compared to other tissues, with a significant (~50%) decline observed only after 8 weeks on a very low-carotenoid diet. Supplementation with lutein + zeaxanthin or zeaxanthin alone significantly increased only retinal galloxanthin and ε-carotene levels, while other carotenoid types in the retina remained unaffected. Concentrations of retinal astaxanthin were unaffected by direct dietary supplementation with astaxanthin. These results suggest highly specific mechanisms of retinal carotenoid metabolism and accumulation, as well as differential rates of turnover among retinal carotenoid types, all of which have important implications for visual health maintenance and interventions.

Introduction

Diet-derived carotenoids acting as provitamins, immunomodulators, antioxidants, and/or photoprotectants play an important role in the health and normal physiological function of humans and a diversity of animals [1,2]. Carotenoids are particularly important for visual health, and increased retinal carotenoid accumulation is associated with a reduced risk of age-related macular degeneration (AMD)[1] and cataract in humans [3,4]. Carotenoid accumulation in the retina is influenced by carotenoid intake [5,6], raising the possibility of dietary interventions to promote visual health. However, testing such interventions in humans is difficult, and thus birds have been proposed as a model system for the study of the controls and functions of retinal carotenoids because they accumulate levels of retinal carotenoids comparable to humans [7–9]. In young quail (*Coturnix japonica*), for example, dietary carotenoid supplementation elevates retinal carotenoid concentrations, enhances photoprotection, and reduces the accumulation of A2E – a marker of eye disease [8–10]. However, the mechanisms of accumulation and functions of carotenoids in the avian retina differ from humans in several important aspects. The avian retina contains metabolically derived apo- and keto-carotenoids not found in the human retina, and the majority of carotenoids in the avian retina accumulate in an esterified form [11]. Therefore carotenoid accumulation in the avian retina may be subject to constraints that are not shared by humans.

In the avian retina, carotenoids accumulate within cone oil droplets that are located between the inner and outer segments of the cone photoreceptors. In this position the light absorbance of carotenoids modifies the spectrum and intensity of light reaching the visual pigment. The types and concentrations of carotenoids in the oil droplets are specific to the type of photoreceptor. For example, the long-wavelength-sensitive cone contains a red oil droplet pigmented with astaxanthin, while the short-wavelength sensitive cone oil droplets contain galloxanthin that

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[1] *Abbreviations used:* AMD, age-related macular degeneration; HPLC, high-performance liquid chromatography; MANOVA, multiple analyses of variance; rmANOVA, repeated-measures analyses of covariance; zea, zeaxanthin; asta, astaxanthin; gal, galloxanthin; lut, lutein; ε-car, ε-carotene.

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absorbs light at shorter wavelengths [12]. Thus carotenoid-pigmented oil droplets act as matched filters, enhancing color discrimination and improving color constancy [13]. This specific and matched carotenoid accumulation is achieved through the metabolism and selective accumulation of dietary carotenoids. Avian retinal carotenoids, like galloxanthin, are found only in the eye, suggesting highly localized carotenoid metabolism [11,14,15].

Because of their essential role in color vision and photoprotection in the avian eye, variation in retinal carotenoid accumulation could impact color vision, retinal health, and have consequences for an individual's survival and fitness. However, the influence of diet on retinal carotenoid accumulation is not known for any wild bird or other wild animal species. There have been a number of comparative microspectrophotometric studies of the carotenoid-pigmented cone oil droplets of wild birds [16], and the majority of experimental studies of retinal carotenoid dynamics have been limited to young birds of domesticated species like the chicken (*Gallus domesticus*) and quail. Studying these selectively bred domesticated species makes it difficult to place these results in a natural context and limits our ability to examine the evolutionary forces shaping retinal carotenoid accumulation.

To expand these investigations to wild birds, we have initiated a series of studies on retinal carotenoids in the house finch (*Carpodacus mexicanus*), a common North American passerine bird. This species is sexually dimorphic, with sexually selected carotenoid-based male plumage coloration, and has become a model species for the study of carotenoid physiology in wild birds [17]. We have found that carotenoid levels in the retinas of wild house finches vary among seasons, and positively correlate with body condition and male coloration [18]. The purpose of the current study is to examine how dietary carotenoid intake affects accumulation of specific retinal carotenoids and may shape the variation we have seen in the wild population. We carried out three separate experiments with captive wild-caught house finches to determine: (1) the time course of retinal carotenoid depletion, as animals are being fed a very low-carotenoid diet, (2) how supplemental levels of two common dietary carotenoids (lutein and zeaxanthin) affect retinal carotenoid accumulation, and (3) the effect of separate high doses of two abundant carotenoids in the avian retina – zeaxanthin (the main carotenoid in medium-wavelength-sensitive cones and precursor of other metabolically derived carotenoids in the quail retina [11]) or astaxanthin (the metabolic derivative that pigments the oil droplets associated with the long-wave-sensitive cones) – on retinal carotenoid accumulation.

Methods

Chemicals

Solvents for carotenoid extraction and high-performance liquid chromatography (HPLC) analyses were purchased from Fisher Scientific (Waltham, MA). Purified standards of lutein and β-carotene were acquired from CaroteNature (Lupsingen, Switzerland), astaxanthin from Sigma–Aldrich (St. Louis, MO), and zeaxanthin from DSM (Heerlen, Netherlands). A diet supplement of combined lutein and zeaxanthin (OroGlo-15® containing 15 g/kg of 8.2:1, lutein:zeaxanthin) was donated by Kemin Inc. (Des Moines, IA). Zeaxanthin (OptiSharp® 5% by weight) and astaxanthin (Carophyll® Pink 10% by weight) dietary supplements were donated by DSM Inc. (Heerlen, Netherlands).

Experimental animals

Wild house finches were captured on the campus of Arizona State University in Tempe, Arizona, USA in basket traps at baited feeding stations following the methods of Toomey and McGraw [18]. The birds were housed in small wire cages ($0.6 \times 0.4 \times 0.3$ m) in two greenhouse rooms with *ad libitum* access to tap water and food (diets detailed below).

Experiment 1 – Depletion

To examine the response of retinal carotenoid accumulation to dietary carotenoid deprivation, we maintained 25 males, 10 females, and 15 juveniles (of unknown sex) in captivity on a very low-carotenoid diet of black oil sunflower seeds (0.078 ± 0.031 μg/g, lutein:zeaxanthin, 3.2:1, methods see Carotenoid extraction) for up to eight weeks. On weeks 0, 2, 4, 6, and 8, we randomly selected and euthanized five males, two females, and three juveniles to collect the left retina and liver tissue for carotenoid analysis. To track circulating carotenoid levels, we collected a blood sample (~100 μl) from the ulnar vein of all remaining birds every 2 weeks throughout the study and measured plasma carotenoid levels with HPLC (see Methods: Carotenoid extraction and HPLC analyses).

Experiment 2 – Lutein and zeaxanthin supplementation

Lutein and zeaxanthin are the most common carotenoids in the plasma of house finches [19], and the purpose of this experiment was to examine how varying levels of these two carotenoids in the diet influenced retinal carotenoid accumulation. In September 2008, we brought 28 adult male and 26 adult female house finches into captivity and maintained them on a low-carotenoid diet (Careline rice diet, Roudybush Inc. Woodland, CA; 0.31 ± 0.03 μg/g, lutein:zeaxanthin, 4:1, methods see Carotenoid extraction) for five weeks to wash out any individual differences that may have stemmed from variation in their diet in the wild. We then randomly assigned the birds to one of three diet treatments: (1) low – consisting of the low-carotenoid base diet (n = 9 males and 8 females), (2) medium – consisting of the base diet supplemented with OroGlo® to a level of 10 μg/g lutein:zeaxanthin (8.2:1) (n = 10 males and 9 females), and (3) high – consisting of the base diet supplemented with OroGlo® to a level of 30 μg/g lutein:zeaxanthin (8.2:1) (n = 9 males and 9 females). This carotenoid manipulation falls within the range of variation in carotenoid concentrations of the gut contents from wild house finches reported by Hill et al. [20] (mean ca. = 8 μg/g, range = <1–>70 μg/g). The birds were given *ad libitum* access to these diets for eight weeks, then all birds were returned to the base diet for a period of 2 weeks for behavioral experiments (not presented here), at which point they were euthanized so that the left retina of each bird could be collected for carotenoid analyses. Results of Experiment 1 indicated that this two-week return to the base diet was unlikely to affect retinal carotenoid levels. Blood samples were collected from all birds in weeks 0, 4, and 8 of the diet manipulation to track changes in plasma carotenoid levels (see below).

Experiment 3 – Zeaxanthin or astaxanthin supplementation

Because the effects of the dietary treatments in Experiments 1 and 2 were relatively limited (see Results), we designed the third experiment to maximize our likelihood of detecting a dietary effect by supplementing the birds with high levels of zeaxanthin, the putative dietary precursor for many of the carotenoids in the avian retina [11,14,15], and astaxanthin, which is not found in circulation, but is metabolically derived and the dominant carotenoid in the house finch retina [18]. In June 2009, we brought 14 adult male and 14 adult female house finches into captivity and fed them a low-carotenoid sunflower seed diet for 8 weeks. We then randomly assigned birds to one of three carotenoid treatments: (1) control – four males and four females continued to receive the

sunflower seed diet and tap water with vitamin supplement (Vita-Sol®, United Pet Group EIO, Tampa, FL), (2) zeaxanthin – five males and five females received a supplement of zeaxanthin beadlets (35 μg/ml of OptiSharp®) suspended in their drinking water along with the vitamin supplement, and (3) astaxanthin – five males and five females received a supplement of astaxanthin beadlets (35 μg/ml of Carophyll Pink®) suspended in their drinking water along with the vitamin supplement. These supplements were given for 8 weeks *ad libitum* each weekday and plain tap water was given on weekends. Then all birds were fed the control diet for two weeks during behavioral experiments (not presented here), at which point they were euthanized so that the left retina of each bird could be collected for carotenoid analyses. Blood samples were collected on weeks 0, 4, and 8 of the diet manipulation to track changes in plasma carotenoid levels.

Carotenoid extraction

One whole retina from each individual was extracted three times in hexane:methyl tert-butyl ether (MTBE) 1:1 (v:v) by homogenization in a ball mill (MM200, Retsch GmbH & Co. KG, Haan, Germany) for 3 min at 30 Hz and then evaporated to dryness under a stream of nitrogen. Because avian retinal carotenoids are esterified, we saponified the extracts following methods we previously developed to maximize the recovery of avian retinal carotenoids [21]. We split each sample from an individual into two separate tubes and then resuspended the extract in 0.2 or 0.02 M KOH in methanol, capped them under nitrogen, and incubated for 6 h at room temperature in the dark. To complete the saponification, we added 1 ml of a saturated solution of NaCl in water, 2 ml of distilled water, then extracted the free carotenoids with 3 ml of hexane:MTBE and dried the split extracts separately under a stream of nitrogen and prepared them for HPLC analyses by resuspending them in 200 μl mobile phase (methanol:acetonitrile:dichloromethane, 44:44:12, v:v:v).

Plasma and liver carotenoids were extracted following McGraw et al. [19]. Briefly, 10 μl plasma were combined with 100 μl ethanol and 100 μl hexane:MTBE, vortexed, then centrifuged for 3 min at 9000*g*. The supernatant was collected, evaporated to dryness under a stream of nitrogen, and then resuspended in mobile phase for HPLC analysis. The right lobe of the liver was excised (0.11–0.51 g) and ground three times using a mortar and pestle in the presence of 3 mL hexane:MTBE. The extract was saponified with 0.2 M KOH in methanol for 6 h at room temperature in the dark. We then extracted the free carotenoids and prepared them for HPLC as described for the retina.

Carotenoids were extracted from the sunflower seed and Careline rice diet by separately grinding three samples of each (approximately 0.5 g) in a mortar and pestle in the presence of 2 ml tetrahydrofuran. This procedure was repeated three times, at which point we collected the solvent, transferred it to a fresh tube, and centrifuged for 5 min at 3000 rpm. The supernatant was then collected, evaporated to dryness under a stream of nitrogen, and resuspended in mobile phase for HPLC analyses.

HPLC analyses

Carotenoid analyses were carried out on a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, MA) with a Waters YMC Carotenoid 5 μm column (4.6 × 250 mm) heated to 30 °C, and a Waters 2996 photodiode array detector. Retinal carotenoids were separated using a flow rate of 1.2 ml/min and a mobile phase of 44:44:12 (v:v:v) methanol:acetonitrile:dichloromethane run isocratically for the first 11 min, then a linear gradient up to 42:23:35 (v:v:v) through 21 min, holding at this condition until 25 min, followed by a return to the initial isocratic conditions from 25 to 30 min. HPLC conditions for plasma, liver, and diet carotenoid analyses were similar to those for the retina, but the initial mobile phase was 42:42:16 (v:v:v). External standards of lutein, zeaxanthin, astaxanthin, and β-carotene were also run on the HPLC for comparison to our samples; for other carotenoids where standards were not available, we used published absorbance and relative retention time values for identification (Table 1).

Statistical analyses

Reported values are mean ± standard error (SE), and statistical analyses were carried out in SPSS13 (Chicago, IL). We compared concentrations of different retinal carotenoids between the sexes and among treatments or times with separate multiple analyses of variance (MANOVA) for each experiment and Tukey post hoc tests for between-treatment comparisons. Non-significant interaction

Table 1
Putative identity, retention times (R_t), absorbance maxima (λ_{max}), and percentage of total carotenoids in house finch retina, plasma, and liver measured in week zero of Experiment 1 (n = 10). The λ_{max} values in parentheses denote the location of shoulders in the absorbance spectra. Retention times differ between tissue types because of differences in chromatographic conditions (see Methods).

Tissue	Carotenoid	R_t (min)	λ_{max} (nm)		% of total
Retina	Galloxanthin [a,b]	3.55	381.3	397.1	41.9 ± 0.3
	Astaxanthin[c]	7.94	478.2		35.3 ± 0.3
	Lutein[c]	8.87 (421.3)	446.7	474.6	10.0 ± 0.4
	Zeaxanthin[c]	11.05	453.9	479.4	7.8 ± 0.4
	Unknown[b]	14.40 (422.5)	447.9	477.0	3.4 ± 0.3
	ε-Carotene[d,e]	18.21 (420.0)	443.0	472.1	1.6 ± 0.1
Plasma	Lutein[c]	6.61 (421.3)	446.7	474.6	82.0 ± 0.5
	Zeaxanthin[c]	7.94	453.9	479.4	12.4 ± 0.2
	β-Cryptoxanthin[b]	14.05 (424.0)	453.9	480.6	2.7 ± 0.3
	β-Carotene[c]	21.04 (426.5)	456.4	483.1	2.8 ± 0.2
Liver	Lutein[c]	6.61 (421.3)	446.7	474.6	55.8 ± 3.5
	Zeaxanthin[c]	7.94	453.9	479.4	9.6 ± 0.5
	β-Cryptoxanthin[b]	14.05 (424.0)	453.9	480.6	4.6 ± 0.6
	β-Carotene[c]	21.04 (426.5)	456.4	483.1	23.6 ± 0.7
	α-Carotene[d]	21.80 (427.8)	450.9	477.0	4.5 ± 0.3

[a] Identified based on the descriptions of Goldsmith et al. [11] and Toyoda et al. [32].
[b] Quantified based on the calibration curve for lutein.
[c] Identified and quantified by comparison to purified standards.
[d] Identified based on the descriptions of Goldsmith et al. [11] and Goldsmith and Butler [39].
[e] Quantified based on calibration curve for β-carotene.

terms were removed from the models. Concentrations of many of the carotenoids in plasma and liver of house finches are significantly intercorrelated [19]; therefore we analyzed total concentrations in Experiments 1 and 2. Because we had multiple plasma samples over time from each individual, we used repeated-measures analyses of covariance (rmANOVA) to examine the effects of sex, treatment, and time on the plasma concentrations of specific carotenoids. Two-way interactions were included in all of the models but were removed when non-significant. Significance level for all tests was set at $\alpha = 0.05$.

Results

Retina and plasma carotenoid types

Consistent with our previous studies [18,22], we observed six major carotenoid types in house finch retinas from all three experiments (Table 1). The retinal carotenoid profile was dominated by astaxanthin and galloxanthin, together making up on average >75% of total retinal carotenoids. The plasma carotenoid profile of wild house finches was dominated by lutein and zeaxanthin, with small amounts of β-cryptoxanthin and β-carotene making up <5% of total at the time of capture (Table 1). On the captive diets, the birds circulated only lutein and zeaxanthin; however, when we supplemented the birds with astaxanthin in Experiment 3, we found significant amounts of astaxanthin in circulation (see Experiment 2 – lutein and zeaxanthin supplementation). The liver predominantly contained lutein, β-carotene, zeaxanthin and small amounts of β-cryptoxanthin and α-carotene (Table 1).

Experiment 1 – Depletion

Retinal carotenoid levels declined significantly over the course of the 8 weeks that birds were fed a very low-carotenoid diet (MANOVA week: Wilks' $\lambda = 0.188$, $F_{24,133.8} = 3.43$, $p < 0.0001$, Fig. 1A). Retinal astaxanthin concentrations were significantly lower in birds sampled at weeks 4 and 8 compared to those at week 0 (Tukey's post hoc, $p \leqslant 0.025$). Retinal galloxanthin and ε-carotene levels were significantly lower in birds collected at week 8 than birds collected at week 0 (Tukey's post hoc, $p \leqslant 0.012$). However, there was not a consistent temporal decline in retinal carotenoid levels, and birds collected on week 6 did not differ significantly from those in week 0 (Tukey's post hoc, $p \geqslant 0.574$, Fig. 1A). There was a significant difference in overall retinal carotenoid accumulation among juvenile, adult male, and adult female house finches (Wilks' $\lambda = 0.217$, $F_{12,76} = 7.27$, $p < 0.0001$). Regardless of time-point of sampled, juvenile finches had significantly higher levels of galloxanthin and ε-carotene than adult males (Juvenile: Gal – 0.393 ± 0.031 μg/retina, ε-car – 0.0154 ± 0.0001 μg/retina, Adult Male: Gal – 0.190 ± 0.024 μg/retina, ε-car – 0.0147 ± 0.0002 μg/retina; Tukey's post hoc, $p \leqslant 0.032$). Retinal carotenoid levels, of any type, did not differ significantly between juveniles and adult females or between adult females and adult males (Tukey's post hoc, $p \geqslant 0.140$).

Plasma carotenoid levels declined rapidly and significantly in captive birds fed a very low-carotenoid diet (ANOVA week: $F_{4,43} = 34.04$, $p < 0.0001$, Fig. 1B). Birds collected in weeks 2–8 had significantly lower total plasma carotenoid levels than birds collected at week 0 (Tukey's post hoc, $p < 0.0001$). There were no significant differences in total plasma carotenoid concentrations among birds collected in weeks 2–8 (Tukey's post hoc, $p > 0.812$). Liver carotenoid concentrations followed a pattern similar to plasma and declined significantly in response to dietary carotenoid depletion (ANOVA week: $F_{4,43} = 13.00$, $p < 0.0001$, Fig. 1C). Finches collected in weeks 2–8 had significantly lower total liver carotenoid concentrations than birds collected at week 0 (Tukey's post hoc, $p < 0.0001$). There were no significant differences in total liver carotenoid concentrations among birds collected in weeks 2–8 (Tukey's post hoc, $p > 0.99$).

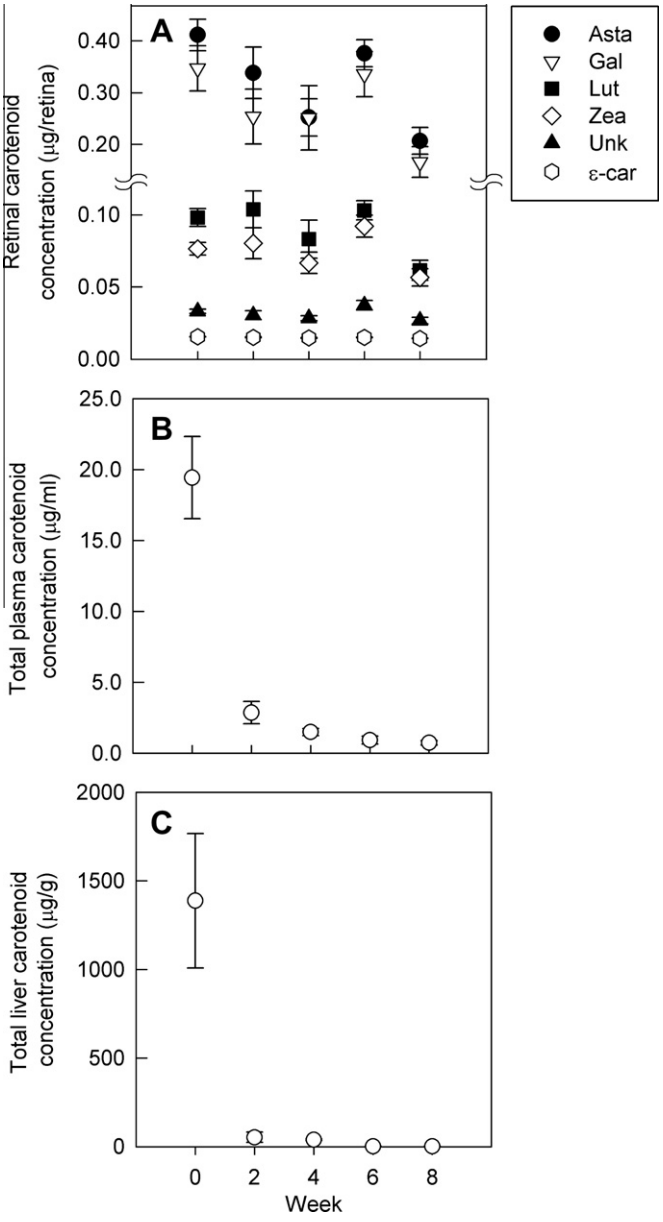

Fig. 1. Mean ± S.E. (A) retinal, (B) total plasma, and (C) total liver carotenoid concentrations of house finches maintained in captivity on a very low-carotenoid sunflower seed diet for 0, 2, 4, 6, or 8 weeks. At each time-point, 10 different individuals were collected and measured.

Experiment 2 – Lutein and zeaxanthin supplementation

Dietary supplementation with lutein + zeaxanthin for eight weeks significantly affected retinal carotenoid levels of captive house finches (MANOVA treatment: Wilks' $\lambda = 0.290$, $F_{12,90} = 6.44$, $p < 0.0001$, Fig. 2A). Birds receiving the highest dietary carotenoid level had significantly higher retinal galloxanthin concentrations than birds on the medium diet, and both high- and medium-diet birds had significantly higher levels than birds receiving the low diet (Tukey's post hoc, $p < 0.001$, Fig. 2A). Birds on the high-carotenoid diet had significantly higher levels of ε-carotene than

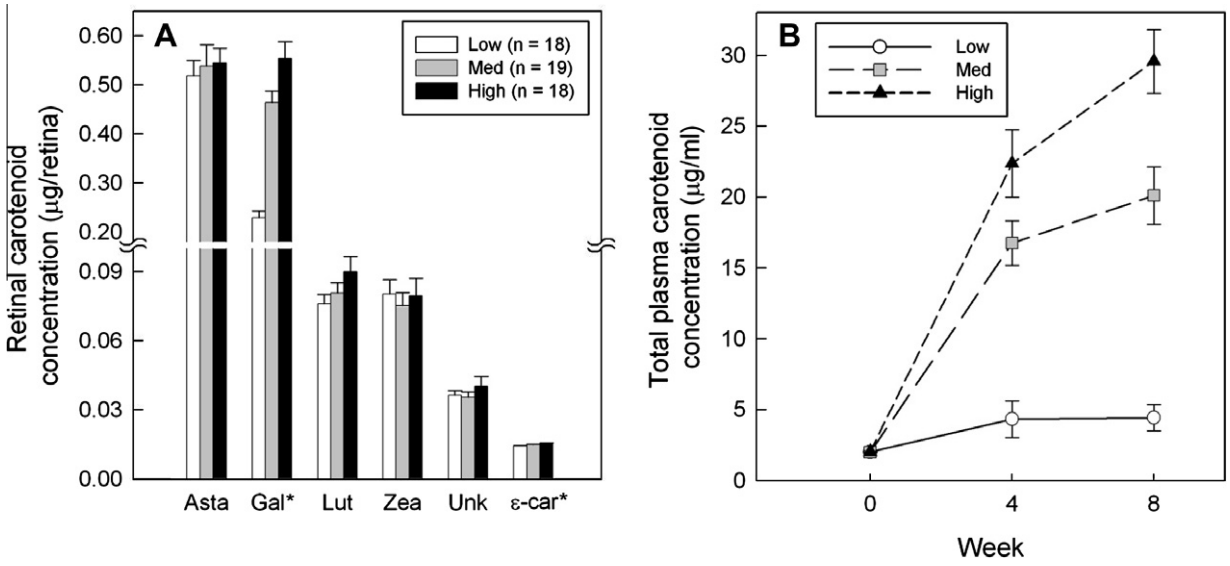

Fig. 2. (A) Mean ± S.E. retinal carotenoid concentrations of adult house finches supplemented with low (0.31 μg/g), medium (10 μg/g), or high (30 μg/g) levels of lutein:zeaxanthin for eight weeks. Asterisks indicate significant pairwise differences between the low-carotenoid diet and other diet treatments for that particular retinal carotenoid type (see text). (B) Total plasma carotenoid concentrations of house finches across the 8 weeks of supplementation.

birds on the low-carotenoid diet (Tukey's post hoc, $p < 0.001$, Fig. 2A). There were no significant differences in retinal carotenoid accumulation between male and female finches (Wilks' $\lambda = 0.933$, $F_{6,45} = 0.537$, $p = 0.777$).

Plasma carotenoid levels changed over the course of the experiment and as a function of dietary carotenoid treatment (rmANOVA time × treatment: $F_{2.56,76.86} = 37.75$, $p < 0.0001$, Fig. 2B). Treatment groups did not differ significantly at the beginning of the experiment (week 0 – prior to diet manipulation; Tukey's post hoc, $p \leqslant 0.782$, Fig. 2A). In week 4, high- and medium-diet birds had significantly higher plasma carotenoid levels than low-diet birds (Tukey's post hoc, $p < 0.001$, Fig. 2B). In week 8, plasma carotenoid levels of high-, medium-, and low-diet groups all differed significantly, with the highest levels in the high-diet group (Tukey's post hoc, $p \leqslant 0.004$, Fig. 2B).

Experiment 3 – Zeaxanthin or astaxanthin supplementation

Dietary supplementation with zeaxanthin significantly affected retinal carotenoid levels of captive house finches (MANOVA treatment: Wilks' $\lambda = 0.290$, $F_{12,90} = 6.44$, $p < 0.0001$, Fig. 3). Zeaxanthin-supplemented birds had significantly higher levels of retinal galloxanthin and ε-carotene than unsupplemented birds (Tukey's post hoc, $p \leqslant 0.028$, Fig. 3). Supplementation with dietary astaxanthin had no significant effect on the accumulation of any retinal carotenoid types, and retinal levels of astaxanthin-supplemented birds did not differ significantly from those of the low-carotenoid (control) group (Tukey's post hoc, $p > 0.332$, Fig. 3). There were no significant differences in retinal carotenoid accumulation between male and female finches (Wilks' $\lambda = 0.795$, $F_{6,19} = 0.815$, $p = 0.572$).

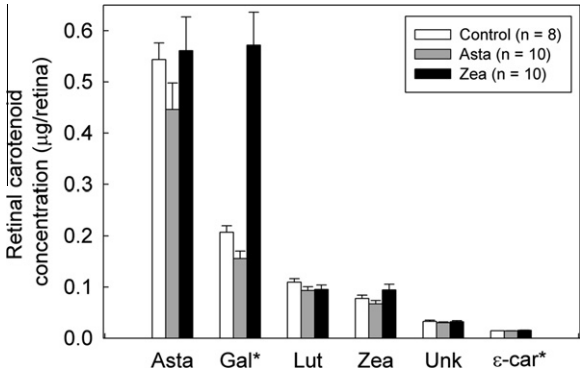

Fig. 3. Mean ± S.E. retinal carotenoid concentrations of adult house finches supplemented with 35 μg/ml of zeaxanthin (Zea) or astaxanthin (Asta) for eight weeks and an unsupplemented (Control) group. Asterisks indicate significant pairwise differences between the control and other treatment groups for that particular retinal carotenoid type (see text).

Because we administered specific purified carotenoid supplements in Experiment 3, we used separate statistical tests to analyze the three plasma carotenoids (lutein, zeaxanthin, and astaxanthin) and determine whether and how these purified supplements were taken up into circulation. Plasma levels of all three carotenoids were significantly affected by carotenoid supplementation (rmANOVA time × treatment: $F_{3.23,37.18} \geqslant 17.69$, $p < 0.0001$, Fig. 4). In week 0, prior to supplementation, plasma lutein levels were low (mean 0.26 ± 0.044 μg/ml) for all treatment groups, but levels increased significantly in zeaxanthin-supplemented birds compared to controls in weeks 4 and 8 (Tukey's post hoc, $p < 0.0001$, Fig. 4A). Plasma lutein levels of astaxathin-supplemented birds did not differ from controls (Tukey's post hoc, $p > 0.9$). Prior to supplementation, all birds had low levels of zeaxanthin in circulation (mean 0.29 ± 0.056 μg/ml). As expected, supplementation with zeaxanthin significantly increased circulating zeaxanthin levels in weeks 4 and 8 of the study (Tukey's post hoc, $p < 0.0001$, Fig. 4B). Plasma zeaxanthin levels of astaxathin-supplemented birds did not differ from those in controls (Tukey's post hoc, $p > 0.9$). Consistent with observations from wild house finches [18,19], none of the birds were circulating astaxanthin in their plasma at the beginning of the study. However, birds supplemented with astaxanthin did circulate significant astaxanthin levels through plasma in weeks 4 and 8 of the study (Tukey's post hoc, $p < 0.0001$, Fig. 4C).

Discussion

Carotenoid types and concentration

The carotenoid profile of the wild house finch retina was dominated by astaxanthin and galloxanthin, making up 42% and 35% of

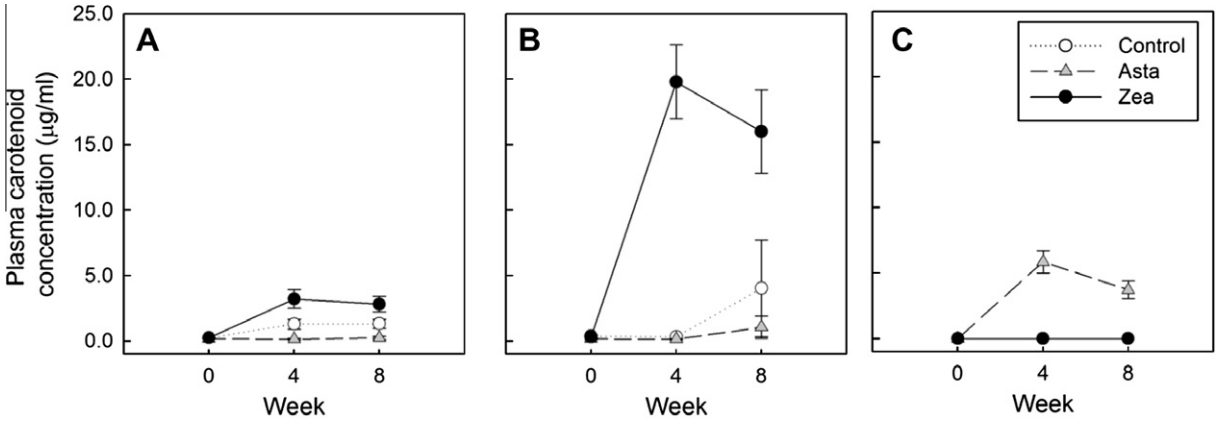

Fig. 4. Mean ± S.E. plasma concentrations of (A) lutein, (B) zeaxanthin, and (C) astaxanthin in adult house finches supplemented with 35 μg/ml of zeaxanthin (Zea) or astaxanthin (Asta) for eight weeks and an unsupplemented (Control) group.

total, respectively. This is roughly similar to the turkey (*Meleagris gallopavo*), consisting of 23% astaxanthin and 28% galloxanthin [14], but contrasts with results from domesticated chickens (*Gallus domesticus*) with 71% and 7%, respectively and quail with 29% and 13%, respectively. These species-specific differences could stem from: (1) variation in the frequency of retinal cone types, (2) differences in rearing and dietary conditions among studies, and/or (3) species-specific patterns of retinal carotenoid accumulation within cone types. The frequency of astaxanthin pigmented R-type and galloxanthin pigmented P-type oil droplets are unlikely to explain differences among these species, as they are relatively similar: chickens have an R to P-type ratio of 1:2.5 [23], quail 1:3.1 [24], and house finches 1:3.0 [unpublished data]. Recent studies of chickens, zebra finches (*Taeniopygia guttata*), and crimson rosellas (*Platycercus elegans*) suggest that galloxanthin pigmentation of the P-type oil droplet is particularly sensitive to environmental light conditions and dietary carotenoid access [25,26]. The results of our three experiments and another recent study on house finches confirm that galloxanthin is particularly sensitive to dietary carotenoid input and influenced by immune system activity [22]. Therefore, the differences among studies and species in the relative accumulation of astaxanthin and galloxanthin may be influenced by lighting, diet, and the general health of the experimental animals. However, we cannot rule out genetically based species differences in the uptake, metabolism, and deposition of carotenoids into the retina. Qualitative comparisons indicate that interspecific variation in oil droplet pigmentation variation may be functionally relate to differences in spectral environment and foraging ecology [16]. For example, nocturnal species, like owls, tend to have relatively pale oil droplets, with presumably lower carotenoid concentrations, which should improve sensitivity in low light conditions [16].

Carotenoid depletion

Maintaining house finches on a very low-carotenoid diet depleted retinal levels of astaxanthin, galloxanthin, and ε-carotene. This contrasts with the reported stability of retinal carotenoids to dietary depletion, ranging from 4 weeks in young chickens to several months in adult quail [27,28]. However, Wang et al. [28] only measured retinal lutein and zeaxanthin in chickens, which we also found to be unaffected by diet depletion, and Meyer et al. [27] only qualitatively examined the coloration of cone oil droplets of quail and were unlikely to detect subtle variation in concentration. Despite depletion, retinal carotenoid levels in house finches were much more stable than plasma and liver carotenoid levels. Significant depletion of retinal carotenoids was detected only after 4 weeks for astaxanthin and 8 weeks for galloxanthin and ε-carotene, while total plasma carotenoid levels dropped by >85% and liver carotenoid levels declined >95% in the first two weeks on the very low-carotenoid diet. Moreover, the final magnitude of the decline in retinal carotenoid concentration was much less than that of plasma and liver. By week 8, retinal levels had declined to ca. 50% of initial levels, while circulating and liver carotenoids had dropped to 4% and 2% of initial values, respectively. Although we observed a decline in retinal carotenoid levels over the course of the depletion study, there was considerable variation among weeks that was not attributable to dietary depletion. For example, the retinal carotenoid concentrations of birds collected in week six did not differ significantly from birds collected in week zero and deviates from the trend of decline seen in other weeks. This deviation is unlikely to have resulted from systematic error, because when we repeated our analyses on extracts from the other whole eye of each individual (data not reported) we found the same patterns as we present from the retina. Because the measurement of retinal carotenoids required destructive sampling of retinal tissue, we were limited to a cross-sectional analysis of different birds over time. We expect that this unexplained variation would diminish if we were able to measure retinal carotenoid accumulation longitudinal in the same individuals.

The relatively limited effects of dietary depletion suggest that inter-individual differences other than diet can influence carotenoid accumulation and retention in the avian retina. Retinal carotenoid accumulation may be influenced by an individual's health and condition. In wild house finches, we found retinal carotenoid accumulation to be positively correlated with body condition [18], and recently we showed that long-term immune system challenges deplete retinal carotenoids in finches [22]. Retinal carotenoid accumulation may also reflect an individual's genetic predisposition to accumulate retinal carotenoids. Among humans there is up to an 85% heritability of retinal carotenoid accumulation (macular pigment density) [29]. In birds, the heritability of retinal carotenoid accumulation has not been examined; however, the carotenoid-based plumage coloration of male house finches is positively correlated among fathers and sons [30], and the carotenoid-based bill color of male zebra finches is heritable (h^2 = 0.33) [31].

Effects of dietary carotenoid supplementation

Adult house finches supplemented with lutein + zeaxanthin in Experiment 2 or with zeaxanthin alone in Experiment 3 had significantly higher levels of retinal galloxanthin and ε-carotene than birds maintained on the low-carotenoid diet. The strong response of galloxanthin to supplementation is consistent with the results

of Knott et al. [26], who found that dietary supplementation of crimson rosella and zebra finches with lutein and zeaxanthin caused shifts in the absorbance properties of cone oil droplets, with the largest effects occurring in the galloxanthin-pigmented, P-type oil droplets. We found no significant effect of carotenoid supplementation on the accumulation of other retinal carotenoids, including lutein and zeaxanthin, which have been shown to respond positively to supplementation in quail and chickens [8,9,11,32]. However, these studies involved dietary manipulation of young growing birds, which, in some cases [8,9,32], began life entirely devoid of carotenoids through multi-generational carotenoid depletion. The maturing retina may be particularly sensitive to dietary manipulations, as the development and pigmentation of cone oil droplets, initiated *in ovo*, is completed [33]. The adult retinas we examined may be less sensitive to diet manipulation because they are fully developed and only requiring dietary inputs to replace degraded carotenoids.

Changes in retinal carotenoid accumulation were seen only in house finches supplemented with zeaxanthin, indicating that there is selective uptake into the retina and specific precursor–product relationships. Astaxanthin is the dominant carotenoid in the house finch retina; however, direct supplementation with dietary astaxanthin had no effect on retinal carotenoid accumulation in adult house finches, despite the fact that they circulated this pigment in plasma and accumulated it in liver and feathers [unpublished data]. Zeaxanthin has been suggested as the primary dietary precursor for many of the metabolically derived retinal carotenoids in birds, like astaxanthin and galloxanthin [14,15]. This product–precursor relationship was recently confirmed in a study of retinal carotenoid accumulation in quail utilizing labeled (deuterated) dietary carotenoids. Bhosale et al. [11] found that feeding young quail with labeled zeaxanthin for 16 weeks led to significant labeling of zeaxanthin, astaxanthin, galloxanthin, and ε-carotene in the retina.

Although zeaxanthin is the likely precursor for many of the carotenoids found in the retina, our supplementation experiments only influenced the accumulation of two retinal carotenoids – galloxanthin and ε-carotene. There are several possible explanations for this specificity: (1) Galloxanthin and ε-carotene may turn over more rapidly than other retinal carotenoid types. This could occur if these carotenoids were more prone to photodegradation than the other retinal types, but the relative stability of these carotenoids *in vivo* is not known. (2) The potential for increased accumulation may be limited if cone oil droplets are already saturated with carotenoids. Across a number of bird species, R-type oil droplets contain very high concentrations of astaxanthin and have absorbance values >20. Y-type oil droplets contain lutein and zeaxanthin and range up to 10 absorbance units, while P-type oil droplets containing galloxanthin and ε-carotene tend to have absorbance values less than two [12]. Therefore, P-type oil droplets, with their presumably lower concentration of carotenoids, have the greatest potential to accumulate carotenoids, while the R-type oil droplets may be saturated with astaxanthin, limiting the potential for further increase.

On a functional level, the carotenoid-specific responses to dietary change may be related to their role in color vision. Knott et al. [26] suggested that birds shunt excess carotenoids into P-type oil droplets to preserve spectral tuning and sensitivity of the single cone photoreceptors. The P-type oil droplet is part of the double-cone photoreceptor that affects motion and luminance detection, but does not play a role in color vision [34]. In the P-type oil droplet, there is relatively little overlap between the carotenoid absorbance and the visual pigment, whereas the absorbance spectra of the single cone visual pigments overlap considerably with the carotenoid absorbance of their respective oil droplets. Thus, increased carotenoid accumulation in the single cones would result in reduced sensitivity, while the double-cone sensitivity may be less affected by the increased carotenoid accumulation.

Conclusion

Carotenoid accumulation in the retina varies significantly among adult wild house finches and correlates with condition and male carotenoid-based plumage coloration [18]. The studies presented here suggest that, although dietary carotenoid access does affect retinal accumulation, diet is not the primary source of variation observed among wild birds. Compared to other body tissues, retinal accumulation in adult house finches is relatively stable to dietary changes over time, and carotenoid supplementation only affects the accumulation of a small subset of retinal carotenoid types. In some sense, this stability is not surprising, because retinal carotenoids play an essential role in color vision [13] and photoprotection [8,9]. Rapid changes in retinal carotenoids could compromise visual health and function and thus drive selection for greater stability and a decoupling of retinal accumulation away from current dietary intake. However, we are still left to account for the variation we see amongst wild house finches. In contrast to our results, studies of young growing domesticated chickens and quail indicate that dietary access can have profound effects on retinal accumulation [8,9,11,32]. In zebra finches, neonatal diet quality has been shown to affect the ability of adult birds to assimilate carotenoids into circulation [35]. Therefore, adult retinal carotenoid accumulation could be shaped by conditions during early life and reflect carotenoid access during key developmental periods. The maternal transfer of carotenoids to eggs may be particularly important because oil-droplet development and pigmentation occurs *in ovo*. Similarly, in humans, early-life carotenoid access may also be important for visual health and function. Among human infants, retinal carotenoid levels are highly variable [36] and recently it has been suggested that dietary carotenoid intake during development may be important for retinal development and life-long visual health [37,38]. Although there are limitations of birds as model organisms [15,21], because of their high levels of retinal carotenoid accumulation and relatively short life-spans, birds still provide an excellent opportunity to examine the life-long effects of carotenoid availability during the developmental period. Dietary carotenoid manipulations at various stages of development in a rapidly maturing species, like quail, could provide clues to the organizational effects of dietary carotenoid intake on adult retinal accumulation and visual health.

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References

[1] N.I. Krinsky, J.T. Landrum, R.A. Bone, Annu. Rev. Nutr. 23 (2003) 171–201.
[2] B.P. Chew, J.S. Park, J. Nutr. 134 (2004) 257S–261.
[3] J.A. Mares-Perlman, A.E. Millen, T.L. Ficek, S.E. Hankinson, J. Nutr. 132 (2002) 518S–524.

[4] C.R. Gale, N.F. Hall, D.I.W. Phillips, C.N. Martyn, Invest. Ophthalmol. Vis. Sci. 44 (2003) 2461–2465.
[5] W. Schalch, W. Cohn, F.M. Barker, W. Köpcke, J. Mellerio, A.C. Bird, A.G. Robson, F.F. Fitzke, F.J.G.M. van Kuijk, Arch. Biochem. Biophys. 458 (2007) 128–135.
[6] R. Bone, J. Landrum, Y. Cao, A. Howard, F. Alvarez-Calderon, Nutr. Metab. 4 (2007) 12.
[7] F. Khachik, F.F. de Moura, D.Y. Zhao, C.P. Aebischer, P.S. Bernstein, Invest. Ophthalmol. Vis. Sci. 43 (2002) 3383–3392.
[8] L.R. Thomson, Y. Toyoda, F.C. Delori, K.M. Garnett, Z.Y. Wong, C.R. Nichols, K.M. Cheng, N.E. Craft, C.K. Dorey, Exp. Eye Res. 75 (2002) 529–542.
[9] L.R. Thomson, Y. Toyoda, A. Langner, F.C. Delori, K.M. Garnett, N. Craft, C.R. Nichols, K.M. Cheng, C.K. Dorey, Invest. Ophthalmol. Vis. Sci. 43 (2002) 3538–3549.
[10] P. Bhosale, B. Serban, P.S. Bernstein, Arch. Biochem. Biophys. 483 (2009) 175–181.
[11] P. Bhosale, B. Serban, D.Y. Zhao, P.S. Bernstein, Biochemistry 46 (2007) 9050–9057.
[12] T.H. Goldsmith, J.S. Collins, S. Licht, Vision Res. 24 (1984) 1661–1671.
[13] M. Vorobyev, Proc. R. Soc. B 270 (2003) 1255–1261.
[14] K. Schiedt, in: N.I. Krinsky, M.M. Mathews-Roth, R.F. Taylor (Eds.), Carotenoids: Chemistry and Biology, Plenum Press, New York, NY, 1989, pp. 247–268.
[15] K. Schiedt, S. Bischof, E. Glinz, Pure Appl. Chem. 63 (1991) 89–100.
[16] N.S. Hart, Prog. Retin. Eye Res. 20 (2001) 675–703.
[17] G.E. Hill, A Red Bird in a Brown Bag: The Function and Evolution of Colorful Plumage in the House Finch, Oxford University Press, 2002.
[18] M.B. Toomey, K.J. McGraw, Funct. Ecol. 23 (2009) 321–329.
[19] K.J. McGraw, P.M. Nolan, O.L. Crino, Funct. Ecol. 20 (2006) 678–688.
[20] G.E. Hill, C.Y. Inouye, R. Montgomerie, Proc. R. Soc. B 269 (2002) 1119–1124.
[21] M.B. Toomey, K.J. McGraw, Invest. Ophthalmol. Vis. Sci. 48 (2007) 3976–3982.
[22] M.B. Toomey, M.W. Butler, K.J. McGraw, J. Exp. Biol. 213 (2010) 1709–1716.
[23] J.K. Bowmaker, A. Knowles, Vision Res. 17 (1977) 755–764.
[24] J.K. Bowmaker, J.K. Kovach, A.V. Whitmore, E.R. Loew, Vision Res. 33 (1993) 571–578.
[25] N.S. Hart, T.J. Lisney, S.P. Collin, J. Exp. Biol. 209 (2006) 4776–4787.
[26] B. Knott, M.L. Berg, E.R. Morgan, K.L. Buchanan, J.K. Bowmaker, A.T.D. Bennett, Proc. R. Soc. B 277 (2010) 953–962.
[27] D.B. Meyer, S.R. Stuckey, R.A. Hudson, Comp. Biochem. Physiol. 40 (1971) 61–70.
[28] Y. Wang, S.L. Connor, W. Wang, E.J. Johnson, W.E. Connor, Exp. Eye Res. 84 (2007) 591–598.
[29] S.H.M. Liew, C.E. Gilbert, T.D. Spector, J. Mellerio, J. Marshall, F.J. van Kuijk, S. Beatty, F. Fitzke, C.J. Hammond, Invest. Ophthalmol. Vis. Sci. 46 (2005) 4430–4436.
[30] G.E. Hill, Nature 350 (1991) 337–339.
[31] T.R. Birkhead, E.J. Pellatt, I.M. Matthews, N.J. Roddis, F.M. Hunter, F. McPhie, H. Castillo-Juarez, Evolution 60 (2006) 2389–2398.
[32] Y. Toyoda, L.R. Thomson, A. Langner, N.E. Craft, K.M. Garnett, C.R. Nichols, K.M. Cheng, C.K. Dorey, Invest. Ophthalmol. Vis. Sci. 43 (2002) 1210–1221.
[33] S.M. Wai, D.T. Yew, Cell. Mol. Neurobiol. 22 (2002) 57–85.
[34] A.T.D. Bennett, M. Thery, Am. Nat. 169 (2007) S1–S6.
[35] J.D. Blount, N.B. Metcalfe, K.E. Arnold, P.F. Surai, G.L. Devevey, P. Monaghan, Proc. R. Soc. B 270 (2003) 1691–1696.
[36] R.A. Bone, J.T. Landrum, L.M. Friedes, C.M. Gomez, M.D. Kilburn, E. Menendez, I. Vidal, W. Wang, Exp. Eye Res. 64 (1997) 211–218.
[37] J.P. Zimmer, B.R. Hammond, Clin. Ophthalmol. 1 (2007) 25–35.
[38] B.R. Hammond Jr., Nutr. Rev. 66 (2008) 695–702.
[39] T.H. Goldsmith, B.K. Butler, J. Comp. Physiol. A 189 (2003) 135–142.