

# Effect of Dietary Zeaxanthin on Tissue Distribution of Zeaxanthin and Lutein in Quail

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**PURPOSE.** The xanthophyll carotenoids (lutein and zeaxanthin) are hypothesized to delay progression of age-related macular degeneration. The quail has a cone-dominant retina that accumulates carotenoids. The purpose of these experiments was to characterize the carotenoid composition of retina, serum, liver, and fat in quail and to determine whether dietary enrichment with zeaxanthin alters zeaxanthin or lutein concentrations in these tissues.

**METHODS.** Quail were fed for 6 months with a commercial turkey diet (T group;  $n = 8$ ), carotenoid-deficient diet (C- group;  $n = 8$ ), or a carotenoid-deficient diet supplemented with 35 mg 3*R*,3'*R*-zeaxanthin per kilogram of food, (Z+ group;  $n = 8$ ). Zeaxanthin was derived from *Sphingobacterium multivorum* (basonym *Flavobacterium*). Carotenoids in serum, retina, liver, and fat were analyzed by HPLC.

**RESULTS.** As in the primate fovea, the retina accumulated zeaxanthin, lutein, and cryptoxanthin, and preferentially absorbed zeaxanthin ( $P < 0.005$ ). In contrast, lutein was preferentially absorbed by liver ( $P < 0.01$ ) and fat ( $P < 0.0001$ ). In supplemented females, zeaxanthin increased approximately 4-fold in retina, and 74-, 63- and 22-fold in serum, liver, and fat, respectively. In males, zeaxanthin was elevated approximately 3-fold in retina, and 42-, 17-, and 12-fold in serum, liver, and fat, respectively. Birds fed the Z+ diet absorbed a higher fraction of dietary lutein into serum, but lutein was reduced in the retina ( $P < 0.05$ ).

**CONCLUSIONS.** Xanthophyll profiles in quail mimic those in primates. Dietary supplements of zeaxanthin effectively increased zeaxanthin concentrations in serum, retina, liver, and fat. The robust response to zeaxanthin supplementation identifies the quail as an animal model for exploration of factors regulating delivery of dietary carotenoids to the retina. (*Invest Ophthalmol Vis Sci.* 2002;43:1210-1221)

From the numerous dietary carotenoids found in plasma, the primate retina selectively concentrates two xanthophyll carotenoids, lutein and zeaxanthin, in macular pigment. Trace quantities of  $\alpha$ -cryptoxanthin and oxidized derivatives of lutein and zeaxanthin, are also present.<sup>1-4</sup> Two stereoisomers of zeaxanthin are found in the human retina: 3*R*,3'*R*-zeaxanthin (the form most common in the diet) and 3*R*,3'*S*-mesozeaxanthin.<sup>5</sup> Concentrations of lutein, and both forms of zeaxanthin peak in the fovea and decline rapidly with increasing eccentricity. Zeaxanthin predominates at the fovea and the immediate surrounding region.<sup>3,5-7</sup> Several lines of evidence support the concept that macular carotenoids may decrease the risk of age-related macular degeneration (AMD).<sup>8,9</sup> Risk for AMD, a major cause of blindness, has been strongly linked to greater age,<sup>10</sup> smoking,<sup>11,12</sup> and family history of AMD.<sup>13</sup> Increased risk for exudative AMD was associated with greater exposure to shorter wavelengths of visible light,<sup>14</sup> which is absorbed by macular pigment. Results in case-control studies indicated that risk for exudative AMD was reduced in those with elevated levels of lutein and zeaxanthin in diet<sup>15</sup> or serum,<sup>16</sup> but this correlation has not been observed in some population-based studies.<sup>17</sup> Reduced risk for drusen and pigmentary abnormalities in age-related maculopathy has also been associated with increased dietary and serum carotenoids.<sup>18</sup> In donor eyes with bull's eye macular degeneration, we found that the area of preserved central retina corresponds to the area containing macular pigment.<sup>19</sup> Bone et al.<sup>20</sup> found that lutein and zeaxanthin concentrations are significantly lower in the central and rod-rich peripheral regions of donor eyes from subjects who had AMD. Both observations could be the consequence of preferential loss of rods in AMD.<sup>21</sup> However, using flicker rates detected preferentially by cones, Beatty et al.<sup>22</sup> also detected a significant reduction in macular pigment density in eyes at high risk for AMD.

These observations led to the hypothesis that dietary supplementation with lutein and/or zeaxanthin might protect the retina and/or delay the progression of AMD.<sup>8,9,23-25</sup> Supplementation with foods rich in lutein and zeaxanthin<sup>26-28</sup> or with lutein-rich supplements<sup>29,30</sup> (derived from marigold and containing 88% lutein and 5% zeaxanthin<sup>31</sup>) increases macular pigment density in most, but not all, human subjects. Despite the possible importance of retinal carotenoids in modulating the course of AMD, critical evidence of beneficial effect has not been found, and crucial information for the most effective design of clinical trials is needed.<sup>32</sup>

Little is known about the mechanisms leading to and/or regulating xanthophyll accumulation in the retina. The human retina responds to dietary xanthophyll enrichment more slowly than does the serum.<sup>26,29,30</sup> Rapid absorption of dietary xanthophylls into liver in mice,<sup>33</sup> into liver and spleen in monkeys,<sup>34</sup> and into fat in humans<sup>28</sup> raises the possibility that these may compete with, or serve as carotenoid reservoirs for, the retina. In fact, Johnson et al.<sup>28</sup> found temporal changes suggesting that fat and retina interact during absorption of dietary lutein. Whether dietary supplementation with one xanthophyll influences the retinal concentration and/or tissue distribution of the other and why increases in plasma xanthophylls do not correlate directly with increases in retinal

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TABLE 1. Average Quantities of Zeaxanthin and Lutein Consumed by Three Groups of Male and Female Quail Fed Different Diets

Diet	Average Quantities of Zeaxanthin and Lutein Consumed									
	Diet Composition (mg/kg Food)				Females			Males		
	Zeaxanthin*	Lutein*	Total Xanthophylls†	Total Tocopherols‡	Food/Day (g)	Zeaxanthin (μg/kg/d)	Lutein (μg/kg/d)	Food/Day (g)	Zeaxanthin (μg/kg/d)	Lutein (μg/kg/d)
C-	0.2	0.34	0.54	61.6	27.2	40	60	13.7	20	30
Z+	35.7	0.24	36.8	65.5	21.6	5040	30	13.2	3500	20
T	1.0	2.84	4.0	53.5	24.3	160	450	14.0	100	290

Females ate more, weighed more, and laid eggs with high levels of lutein and zeaxanthin in the yolks. Mean weights: female  $153 \pm 4$  g; male  $135 \pm 2$  g. Food/day, average grams of food consumed per brooder (avian housing structure) per day divided by the number of birds in each. Data averaged for birds between 3 and 6 months old.  $\mu\text{g}/\text{kg}/\text{d}$ , daily intake based on average weights of all 20-week-old birds.

\* Includes the *cis*-isomers.

† Includes zeaxanthin, lutein,  $\alpha$ -cryptoxanthin,  $\beta$ -cryptoxanthin.

‡ Includes  $\alpha$ ,  $\delta$ , and  $\gamma$  tocopherols.

xanthophylls remain unresolved questions that are best addressed in an animal model.

Although carotenoids are also accumulated in cone-dominant avian retinas,<sup>35-38</sup> the potential use of birds as an animal model for retinal carotenoids has not been explored. Carotenoids in the avian retina are concentrated in brightly colored oil droplets in the cone inner segments close to the outer segment,<sup>37,39</sup> whereas those in primate cones are concentrated in the axons.<sup>6</sup> It is not known whether rod outer segments in birds accumulate lutein and zeaxanthin, as reported in human retinas.<sup>40,41</sup> Early studies of avian cone oil droplets by thin-layer chromatography (TLC) indicated they contain zeaxanthin and a number of other carotenoids,<sup>36,42</sup> but analysis by HPLC has not been reported. Available evidence on use of quail retina suggests it could be an excellent animal model in which to explore the functional significance of retinal carotenoids. Oil droplets in quail raised on carotenoid-deficient diets have been reported to have little color and to regain color with dietary replenishment.<sup>35,39</sup> Moreover, retinas of aged quail exhibit characteristics of the aging primate retina, including age-related loss of photoreceptors<sup>43</sup> and contrast sensitivity,<sup>44</sup> accumulation of RPE lipofuscin,<sup>43,45</sup> and formation of drusen-like deposits in the Bruch membrane.<sup>46</sup>

The purpose of these experiments was to characterize carotenoid profiles in serum, retina, liver, and fat of quail and to determine how the carotenoid profiles in the tissues were affected by long-term supplementation with naturally occurring 3*R*,3'*R*-zeaxanthin. Evidence that light-induced photoreceptor apoptosis is significantly and inversely related to retinal zeaxanthin concentration in quail<sup>47</sup> will be reported separately.

## METHODS

### Nutritional History of the Birds

Adult Japanese quail (*Coturnix japonica*) from the same parental stock at the University of British Columbia were raised in cyclic light (12 hours on-12 hours off). Three groups were fed one of three diets: T group, commercial turkey diet containing a full complement of carotenoids, C- group, a basal diet deficient in carotenoids (Purina Test Diet 5636C-1; Purina Mills; Richmond, IN), and Z+ group, a basal diet supplemented with natural 3*R*,3'*R*-zeaxanthin (35 mg/kg food, biosynthesized by *Sphingobacterium multivorum* [basonym *Flavobacterium*]) at Applied Food Biotechnologies, Inc., O'Fallon, MO), added as an emulsion in olive oil containing  $\alpha$ -tocopherol as an antioxidant. The composition of each diet was determined by HPLC analyses of multiple 5-g samples (Table 1). Smaller samples did not give reproducible results because of nonuniform distributions of carotenoids in the feed. However, because the birds ate 14 to 22 g of food per day (3-4

times the sample size needed for consistent results), we assume that the daily dose of carotenoid consumed by each quail was also reasonably stable.

The diets were designed to produce C- and Z+ diets that differed only in zeaxanthin content. When manufactured, the Z+ diet had less lutein than the C- diet (Table 1). Although unintended, this offered an opportunity to examine correlations of lutein in different tissues and the effects of zeaxanthin on lutein distribution. That females consumed more food than did the males and that the birds fed C- diets ate more than birds fed other diets introduced further variation in the dietary history.

### Experimental Design

The egg yolk provides the retina of the developing quail chick with a rich source of lutein and zeaxanthin. As a result, cones in the newly hatched chick have colored oil droplets. To establish carotenoid-deficient chicks, it is necessary to start with carotenoid-deficient eggs.<sup>48</sup> Groups of adult birds were fed each of the three diets. Eggs for this experiment were collected when carotenoids were depleted in the eggs of the C- birds—that is, when the yolks had a minimum score (1 on the Roche Carotenoid Fan<sup>49</sup>) on four successive days. Eggs from each of the dietary groups were incubated and hatched. Forty birds (20 male and 20 female) were given color-coded leg bands and raised in dim light with the diets of their parents (T, C-, or Z+) from birth to age 6 months. To avoid any errors in feeding, the cages, feeding trays, food, and food scoops were also color coded. At 6 months, half of the birds in each dietary group were killed for this study. The remaining birds were exposed to retina-damaging light to determine whether light damage correlates with retinal carotenoid concentrations. Evidence that light-induced photoreceptor apoptosis significantly and inversely correlates with levels of retinal zeaxanthin<sup>47</sup> will be reported separately.

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and under protocols approved by the Animal Care and Use Committees of Schepens Eye Research Institute and the University of British Columbia.

### Tissue Preparation

The serum, retina, liver, and fat were collected from birds that had been fed each of the three diets. After birds were killed by decapitation, eyes were enucleated within 30 seconds and placed on ice. A circumferential incision was made 2 to 3 mm anterior to the equator, the anterior segment and lens were removed, and ice-cold phosphate buffer containing 50  $\mu\text{M}$  EDTA was placed in the eyecup. Curved iridectomy scissors and tweezers were used to cut and remove vitreous close to the inner limiting membrane, and the retina was peeled from the RPE and released by intersecting cuts lateral to the pecten. Any RPE remaining adherent to the retina was noted by visual inspection. Samples of serum, fat, liver, and one retina from each bird were frozen

in liquid nitrogen for subsequent HPLC analysis. The average time from death to freezing of all samples was less than 4 minutes. Samples were shipped on dry ice and stored at  $-70^{\circ}\text{C}$  before analysis. After excluding retinas with patches of adherent RPE, retinas from four male and four female birds with little or no adherent RPE were selected from each dietary group for HPLC analysis. The retinas, sera, fat, and liver of these birds were analyzed.

## HPLC Analysis

Carotenoids in diet and all tissues were analyzed at Craft Technologies, Inc. (Wilson, NC) according to their standard methods.<sup>50</sup> In general, samples were homogenized, deproteinated with ethanol containing butylated hydroxytoluene (BHT) as an antioxidant, twice extracted with organic solvents, and evaporated under nitrogen. The residue was dissolved in ethyl acetate, diluted in mobile phase, vortexed, and sonicated before injection. Before extraction, tocol (2-methyl-2-(4,8,12-trimethyltridecyl)-6-chromanol; a gift from Hoffman La Roche, Basel, Switzerland, also available from Matreya, Inc., Pleasant Gap, PA) was added to all tissues to serve as an internal standard. However, it was subsequently discovered that tocol does not extract well under alkaline conditions necessary for saponification. Carotenoids in retina and other tissues were measured using external standards. Preparation details specific to other tissues follow the description of analysis of retina.

**Retinas.** After homogenization of the retinas in 500  $\mu\text{L}$  PBS (50 mM PBS containing 2 mM EDTA) a 50- $\mu\text{L}$  aliquot was removed for protein analysis by the bicinchoninic acid procedure (Sigma Chemical Co., St. Louis, MO), a modified Lowry method less sensitive to buffer salts and detergents.<sup>51</sup> The remaining sample was precipitated with 500  $\mu\text{L}$  ethanol containing BHT, vortexed 15 seconds, and transferred to a  $13 \times 100\text{-mm}$  borosilicate tube. The containers and homogenizers were rinsed with an equal volume of PBS, which was added to the tube along with 200  $\mu\text{L}$  10% pyrogallol (an antioxidant) and 400  $\mu\text{L}$  40% KOH. To release esterified carotenoids, the homogenates were saponified during 15 minutes of ultrasonic agitation, then the contents were diluted with 200  $\mu\text{L}$  water and extracted twice with 2 mL 90% hexane-10% ethyl acetate. (Incorporating ethyl acetate helps to extract tocopherols and polar carotenoids more efficiently from soaps formed during saponification.) The total extract was washed with 1 mL water, and the organic extract was evaporated under nitrogen. The residue was dissolved in 15  $\mu\text{L}$  ethyl acetate, diluted with 45  $\mu\text{L}$  mobile phase, vortexed for 20 seconds, and sonicated for 15 seconds before injection. A 25- $\mu\text{L}$  sample was injected.

The HPLC system (all components from Thermo Separation Products, San Jose, CA) consisted of a solvent degasser, an autosampler (AS3000) maintaining samples at  $20^{\circ}\text{C}$ , a 3- $\mu\text{m}$  column (Spherisorb ODS2;  $4.0 \times 250$  mm with titanium frits), a guard column containing the same stationary phase, a column heater at  $31^{\circ}\text{C}$ , a programmable UV/visible detector (UV2000), a programmable fluorescence detector (FL2000), and a computer data system (PC1000). The separation was performed isocratically using a mobile phase of 80% acetonitrile-15% dioxane-5% methanol/isopropyl alcohol containing 150 mM ammonium acetate-0.1% triethylamine at a flow rate of 1.2 mL/min. Carotenoids were detected by absorbance at 450 nm, whereas fluorescence was used to measure retinol (326 nm excitation, 460 nm emission) and tocol and tocopherols (296 nm excitation, 340 nm emission).

Linear calibration curves were prepared consisting of multiple concentrations of analytes that spanned the physiological levels of micronutrients in retina. The calibrants included lutein, zeaxanthin,  $\beta$ -cryptoxanthin, lycopene,  $\alpha$ -carotene,  $\beta$ -carotene, retinol, retinyl palmitate, and  $\delta$ -,  $\gamma$ -, and  $\alpha$ -tocopherols.

In-house quality control samples were analyzed at the beginning and end of each day, and at 24 sample intervals to validate the HPLC system. The relative SD of analytes in the quality control samples ranged from 3% to 10%, with the larger variability associated with analytes near the limits of detection.

**Serum.** A modification of the procedures described by Nomura et al.<sup>50</sup> was used for serum extraction and HPLC methods. Briefly, 150- $\mu\text{L}$

aliquots of thawed serum were diluted with 150  $\mu\text{L}$  water and deproteinated by vortexing with 300  $\mu\text{L}$  ethanol containing tocol and *trans*- $\beta$ -apo-10'-carotenal oxime as internal standards and BHT. The carotenal oxime was synthesized from  $\beta$ -apo-10'-carotenol (a kind gift from Hoffman La Roche) and hydroxylamine (Sigma Chemical Co.). Serum quantitation was performed by internal standard calibration using peak area ratios; the internal standards were incorporated at levels to produce peak heights in proportion to those of the analytes being measured. The carotenal oxime was not added to other tissues, because we were concerned about the possibility that the tissues might contain unusual carotenoids that would coelute with the carotenal oxime. Each sample was extracted twice with 1 mL hexane, and combined organic supernatants were evaporated under nitrogen. The residue was dissolved with vortexing in 35  $\mu\text{L}$  ethyl acetate, diluted with 100  $\mu\text{L}$  mobile phase, and sonicated for 15 seconds, before placement in the autosampler. The injection volume was 15  $\mu\text{L}$ .

**Fat and Liver.** Samples with wet weights of approximately 0.2 g were homogenized in PBS and deproteinated with an equal volume of ethanol. The carotenoids were saponified and extracted with 90% hexane-10% ethyl acetate, as described for retina. After washing and evaporation under nitrogen, the residue was dissolved in ethyl acetate, diluted in mobile phase, vortexed, and sonicated before injection.

**Diets.** The method used to analyze carotenoids in the diet was similar to that used for tissues. Five-gram samples were necessary to obtain reproducible results. Samples were mixed with water and homogenized with methanol-tetrahydrofuran (THF). THF, a strong solvent for xanthophylls and hydrocarbon carotenoids, was stabilized with BHT. Homogenates were either saponified first and partitioned into hexane-ethyl acetate or partitioned directly. The hexane-ethyl acetate extracts were evaporated under a stream of nitrogen gas, redissolved in ethyl acetate, and diluted with mobile phase before injection. The data in Table 1 are derived from these chemical analyses.

## Statistical Procedures

All data were analyzed by computer (Statview; SAS, Cary, NC). Because the standard deviations for tissue zeaxanthin levels were much larger in birds fed the Z+ diet, the standard Student's *t*-test, correlation analysis, or analysis of variance (ANOVA)—procedures that assume normal distributions and equal variances—were invalid. Therefore, nonparametric procedures were used to determine significance. The Spearman rank correlation analyses and Mann-Whitney tests were used to compare tissues with each other, whereas Wilcoxon paired analyses were used for paired comparisons of measurements in one tissue with those in another.  $P < 0.05$  was considered significant.

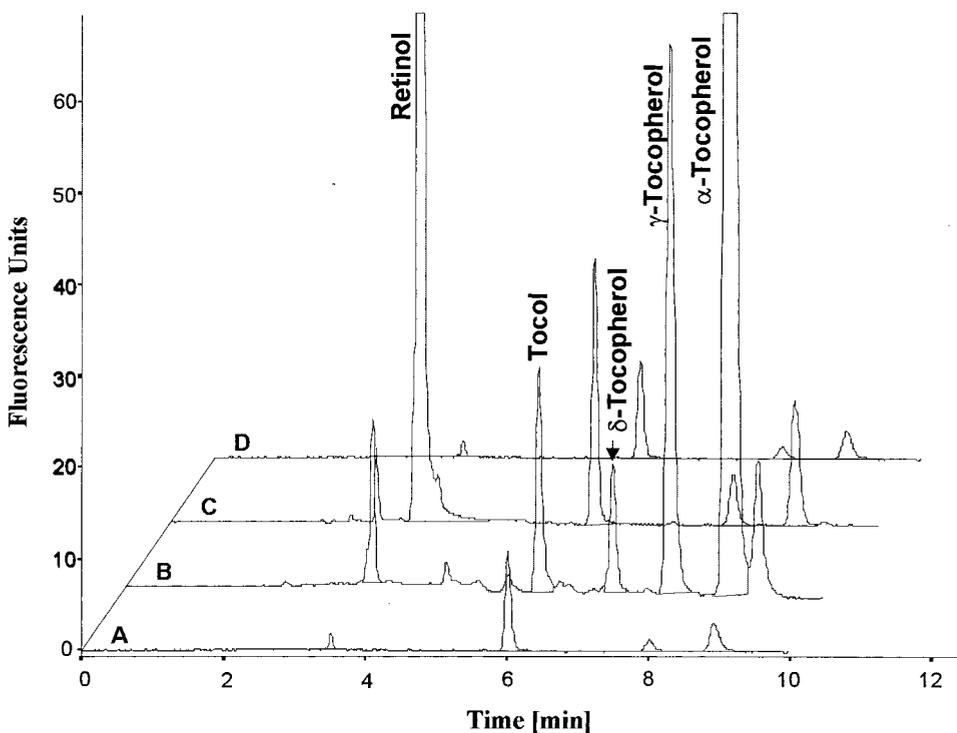
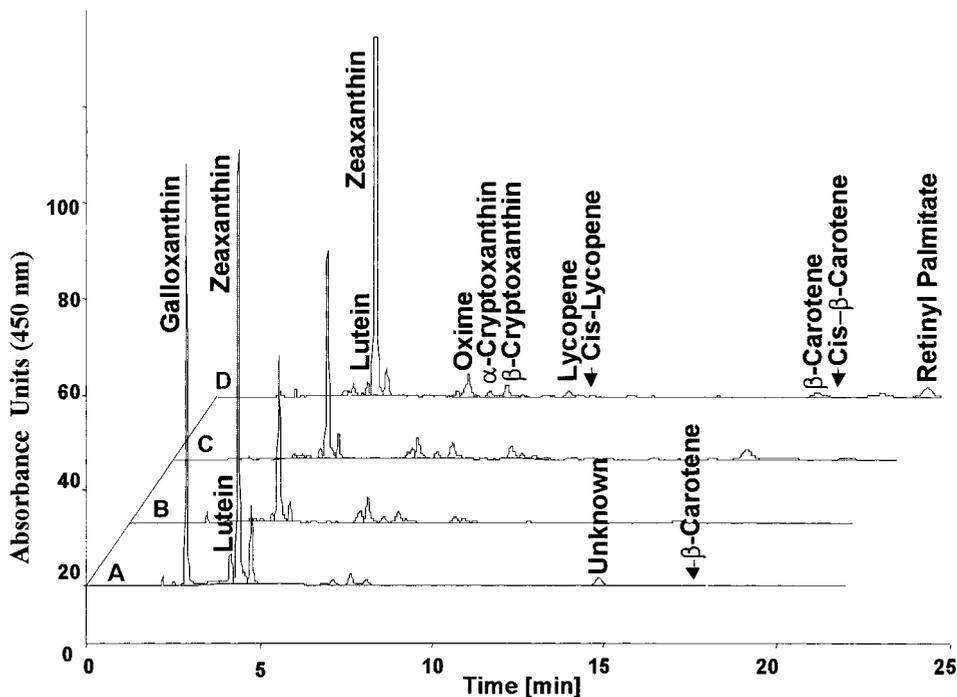
## RESULTS

The diets introduced a 179-fold variation in dietary zeaxanthin, a 12-fold variation in lutein, and a 68-fold variation in total xanthophyll (Table 1). The amounts of lutein and zeaxanthin in the C- diet were reduced to 20% and 11%, respectively, of those in the T diet. The supplemented diet had 179 times more zeaxanthin and 71% of the lutein in the C- diet. In comparison to the T diet, the supplemented diet had 36 times as much zeaxanthin and only 8% of the lutein.

As seen in Table 1, females ate more food than males. Weight gain was the same for all dietary groups (data not shown) but females weighed more than males. Even when the greater weight of the females was taken into account, they still consumed 1.4 to 1.8 times more of each of the nutrients than the males (see Fig. 2, left panels).

## Tissue Composition

Representative chromatograms of the carotenoids and tocopherols observed in quail serum, liver, fat, and retina are presented in Figure 1. An overview of the lutein and zeaxanthin concentrations in diet and tissues (Fig. 2) emphasizes that increased dietary consumption in females resulted in consistently higher tissue levels of lutein and zeaxanthin. The 250-

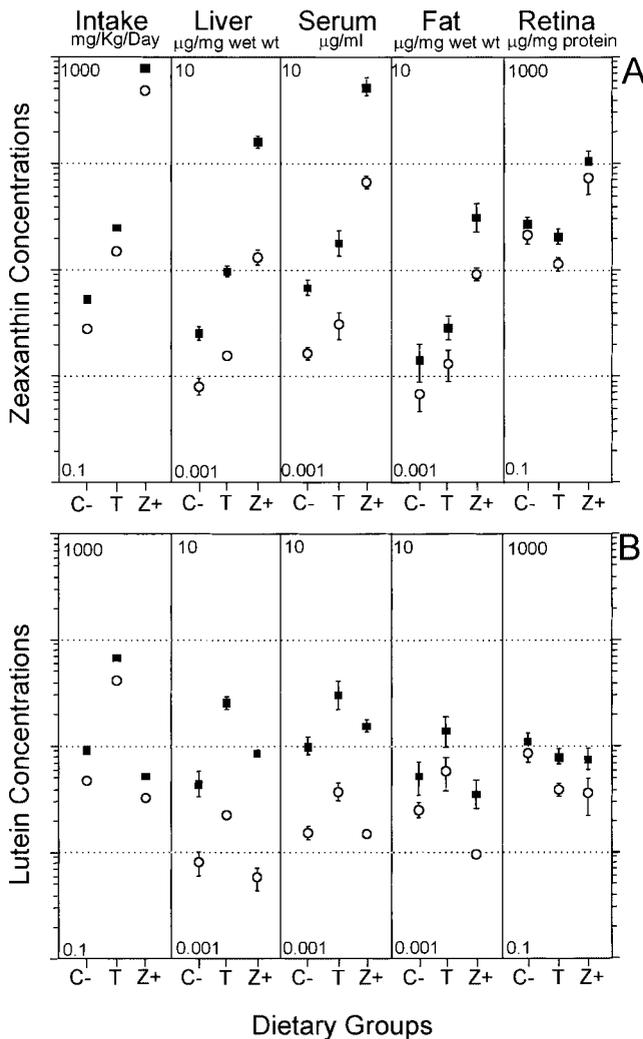


**FIGURE 1.** Representative chromatograms of the carotenoids (*top traces*) and tocopherols (*bottom traces*) observed in retina (A), fat (B), liver (C), and serum (D) of a zeaxanthin-supplemented quail. Note the absence of  $\beta$ -carotene in retina and fat. Quantities in serum were determined by peak area ratios, using tocol and carotenol oxime as internal standards. The peak eluting just before the oxime appeared in the chromatogram. Quantitation in other tissues was performed using external standards. Neither diet nor serum contained detectable galloxanthin (tentatively identified on the basis of its absorption spectrum and chromatographic behavior). It was beyond the scope of this project to identify every peak.

fold range in daily zeaxanthin consumption (from C- males to Z+ females) was reflected in the 200- and 325-fold range of zeaxanthin concentrations in liver and serum (Fig. 2A, three left panels). The concentrations of zeaxanthin found in fat and in retina varied over more narrow ranges (42- and 10-fold, respectively; Fig. 2A, two right panels). Compared with birds fed the T diet, birds fed the C- diet had less zeaxanthin in serum, liver, and fat ( $P < 0.15$ ;  $P < 0.06$ ,  $P < 0.001$ , respectively by Mann-Whitney tests) but the concentration of zeaxanthin in the retina tended to be higher ( $P < 0.07$ ). The 22.5-fold variation in dietary lutein resulted in a 70-fold varia-

tion in serum, which was reduced to 8-fold in liver, 16-fold in fat, and only 3-fold in retina (Fig. 2B). The carotenoid profiles of each of the tissues are presented in Table 2.

**Liver.** The pattern of lutein and zeaxanthin concentrations in birds fed different diets was very similar to the pattern in the diet (Fig. 2), reflecting the fact that, as is true of all nutrients, carotenoids absorbed in the gut pass through the portal vein into the liver before reaching the serum. The T diet contained approximately five times more zeaxanthin and approximately nine times more lutein than the C- diet (Table 1). The livers of birds fed T diet had approximately 4 times more zeaxanthin



**FIGURE 2.** Mean concentrations ( $\pm$ SE) of zeaxanthin (A) and lutein (B) in (left to right) diet, liver, serum, fat, and retina of male ( $\circ$ ) and female ( $\blacksquare$ ) birds raised on C-, T, or Z+ diets. Each vertical panel includes 4 log units, with the upper and lower limits of this range given at the top and bottom left corners of each panel. The units for the y-axis for each vertical panel are given beneath the label at the top of the figure. For example, the y-axis for retinal zeaxanthin (A, right) extends from 0.1 to 1000  $\mu\text{g}/\text{mg}$  protein. Females had higher zeaxanthin in serum, fat, and liver and significantly higher levels of lutein in retina ( $P < 0.01$ ), serum ( $P < 0.0001$ ), liver ( $P < 0.0001$ ), and fat ( $P < 0.02$ ).

and 4.3 times more lutein than those of birds fed C- diets (Table 2).

**Serum.** The carotenoid profiles in serum (Table 2) were most similar to those in liver, except that  $\alpha$ -cryptoxanthin was found in liver but not in serum, whereas  $\beta$ -carotene was present in serum but not in liver. Serum levels of lutein and zeaxanthin were more than two times higher in birds fed T diets than in those fed C- diets (Fig. 2) but did not reach significance unless sera from light-exposed birds were also included (the means and standard deviations were the same, whether or not birds were exposed to light). Dietary supplementation increased the serum zeaxanthin level 75-fold over that in the C- birds ( $P < 0.001$ ; Table 2). Serum zeaxanthin and serum lutein were directly correlated with the amount of each consumed ( $\rho = 0.92$ ;  $P < 0.0001$  and  $0.74$ ;  $P < 0.004$ , respectively;  $\rho = 1$  indicates perfect correlation). Serum lutein tended to correlate with dietary zeaxanthin ( $\rho = 0.36$ ;  $P < 0.05$ ), but serum zeaxanthin did not correlate with lutein consumption ( $P > 0.7$ ).

**Retina.** The carotenoid profile in the quails raised on C- or T diets was similar to that in the primate.<sup>1,2,4,52</sup> The dominant retinal xanthophylls in C- and T diets were lutein and zeaxanthin; zeaxanthin concentrations were more than twice those of lutein in birds fed the C- and T diets and approximately 20 times higher in those fed the Z+ diet ( $P < 0.0001$ ; Wilcoxon signed rank test comparing retinal lutein and zeaxanthin). Retinas contained another xanthophyll tentatively identified as galloxanthin ([3R]-10'Apo- $\beta$ -carotene-3,10'diol) based on elution characteristics and absorption spectrum, and previous reports of its presence in TLC of avian retina.<sup>36,42</sup> The amount of galloxanthin was proportional to the amount of zeaxanthin and significantly higher in the Z+ retinas (Fig. 1; Table 2). Galloxanthin was not detected in diet or serum. Its concentration correlated highly with that of zeaxanthin ( $\rho = 0.93$ ;  $P < 0.0001$ ; Spearman rank correlation analysis) but not lutein ( $P > 0.43$ ).  $\alpha$ -Cryptoxanthin,  $\beta$ -cryptoxanthin, and an unknown carotenoid (identified as xanthophyll by its elution characteristics and absorption spectra) were also present, but at concentrations that did not exceed 15% of the zeaxanthin concentration in birds fed any diet (Table 2). The retinas contained no  $\alpha$ - or  $\beta$ -carotene (Fig. 1). Traces of lycopene were detected in a few birds fed C- diets.

The concentrations of lutein and zeaxanthin in retinas of birds fed different diets varied far less than the differences in the concentrations in serum or in diet (Fig. 2). As in the case of the liver and serum, retinas of birds fed the C- diet had zeaxanthin concentrations equivalent to (but somewhat higher) than those of T-fed birds, and the lutein concentrations were significantly lower ( $P < 0.01$ ; Table 2). In Z+ retinas, zeaxanthin was elevated substantially,  $\beta$ -cryptoxanthin increased, and lutein decreased; however, the net result was that the total of retinal xanthophylls was 2.5 times higher than in C- retinas ( $P < 0.005$ ).  $\beta$ -Cryptoxanthin concentrations were less than 1% of the zeaxanthin concentration. All carotenoids occurred at higher concentrations in female retinas.

**Fat.** Only the xanthophyll carotenoids (zeaxanthin, lutein, and  $\beta$ -cryptoxanthin) were detected in fat (Table 2), and lutein predominated on both the C- and T diets ( $P < 0.01$  and  $P < 0.01$ ; Wilcoxon analysis). Concentrations of lutein and zeaxanthin were significantly higher in females than in males.

These observations demonstrate that, as in the human,<sup>53</sup> different tissues in the quail have distinct carotenoid profiles.

### Comparison of C- and T Diets

The differences in the lutein and zeaxanthin contents in tissues of birds fed C- and T diets are summarized in Figure 2. The reduced dietary zeaxanthin in the C- diets was associated with 40% to 60% reductions in zeaxanthin content in the serum, fat, and liver of C- birds and slight elevation of zeaxanthin in the retina, but none of these differences achieved statistical significance (even when comparisons were made within each gender). (The inability to detect significant differences was related to the small sample sizes,  $n = 8$  for each tissue; all were significantly different if the number of samples was doubled by including the tissues from light-exposed birds.) More severe depletion of lutein in the C- diet resulted in significantly reduced lutein in liver, retina, and fat (Table 2); lutein also tended to be lower in serum. Lutein exceeded zeaxanthin in liver ( $P < 0.002$ ), serum ( $P < 0.003$ ), and fat ( $P < 0.0004$ ), but zeaxanthin was predominant in retina ( $P < 0.0004$ ).

### Effects of Zeaxanthin Supplementation

As seen in Figure 3A, supplementation significantly increased mean zeaxanthin in each tissue ( $P < 0.001$  for each). The increase in retina was significantly less than that in serum, liver, or fat ( $P < 0.01$ ,  $P < 0.02$ , and  $P < 0.02$ , respectively, Wilcoxon analysis). Increases observed in female serum and

TABLE 2. Concentration of Carotenoids in Quail Tissues

Carotenoid	Dietary Groups*			Gender
	C-	T	Z+	
Liver ( $\mu\text{g}/\text{mg}$ wet weight)				
Zeaxanthin	0.02 $\pm$ 0.004	0.06 $\pm$ 0.02 <sup>  a</sup>	0.88 $\pm$ 0.30 <sup>‡</sup>	F>M <sup>  </sup>
Lutein	0.03 $\pm$ 0.01	0.13 $\pm$ 0.05 <sup>§</sup>	0.05 $\pm$ 0.02	F>M <sup>‡</sup>
$\alpha$ -Cryptoxanthin	0.04 $\pm$ 0.04	0.005 $\pm$ 0.002 <sup>  a</sup>	0.03 $\pm$ 0.01 <sup>§</sup>	NS
$\beta$ -Cryptoxanthin	ND	0.005 $\pm$ 0.002 <sup>§</sup>	0.07 $\pm$ 0.03 <sup>‡</sup>	NS
$\beta$ -Carotene	0.003 $\pm$ 0.002	0.004 $\pm$ 0.002 <sup>  a</sup>	0.09 $\pm$ 0.03 <sup>‡</sup>	NS
Lycopene	0.70 $\pm$ 0.45	ND <sup>‡</sup>	0.19 $\pm$ 0.05	NS
Serum ( $\mu\text{g}/\text{mL}$ serum)				
Zeaxanthin	0.04 $\pm$ 0.01	0.11 $\pm$ 0.04 <sup>  b</sup>	3.0 $\pm$ 0.96 <sup>‡</sup>	F>M <sup>  </sup>
Lutein	0.06 $\pm$ 0.02	0.17 $\pm$ 0.07 <sup>  b</sup>	0.08 $\pm$ 0.03	F>M <sup>‡</sup>
$\beta$ -Cryptoxanthin	ND	0.39 $\pm$ 0.02 <sup>§</sup>	0.74 $\pm$ 0.26 <sup>‡</sup>	NS
$\beta$ -Carotene	ND	ND	0.048 $\pm$ 0.001 <sup>†</sup>	NS
Lycopene	ND	0.01 $\pm$ 0.001 <sup>‡</sup>	0.05 $\pm$ 0.02 <sup>§</sup>	F>M <sup>§</sup>
Retina ( $\mu\text{g}/\text{mg}$ protein)				
Zeaxanthin	23.3 $\pm$ 2.4	15.5 $\pm$ 2.4 <sup>  </sup>	85.7 $\pm$ 15.5 <sup>‡</sup>	NS
Females	26.2 $\pm$ 3.5	20.1 $\pm$ 3.3	102.3 $\pm$ 23.1	
Males	20.3 $\pm$ 3.0	10.7 $\pm$ 1.2	69.1 $\pm$ 20.2	
Lutein	9.7 $\pm$ 1.2	5.9 $\pm$ 1.0 <sup>§</sup>	5.5 $\pm$ 1.2 <sup>§</sup>	F>M <sup>§</sup>
Females	11.1 $\pm$ 1.8	7.9 $\pm$ 1.3	7.5 $\pm$ 1.6	
Males	8.2 $\pm$ 1.3	3.9 $\pm$ 0.54	3.5 $\pm$ 1.3	
$\alpha$ -Cryptoxanthin	3.5 $\pm$ 0.5	1.4 $\pm$ 0.2 <sup>†</sup>	3.3 $\pm$ 0.8	F>M
$\beta$ -Cryptoxanthin	ND	0.4 $\pm$ 0.04 <sup>§</sup>	0.74 $\pm$ 0.26 <sup>‡</sup>	F>M
Lycopene	0.17 $\pm$ 0.02	ND <sup>‡</sup>	ND <sup>‡</sup>	NS
Galloxanthin	6.2 $\pm$ 0.53	5.0 $\pm$ 0.56	30.2 $\pm$ 3.1 <sup>‡</sup>	F>M <sup>§</sup>
Unknown	2.9 $\pm$ 0.21	0.8 $\pm$ 0.12 <sup>†</sup>	6.9 $\pm$ 0.89 <sup>  </sup>	F>M
Fat ( $\mu\text{g}/\text{mg}$ wet weight)				
Zeaxanthin	0.01 $\pm$ 0.003	0.02 $\pm$ 0.005 <sup>  </sup>	0.34 $\pm$ 0.17 <sup>‡</sup>	F>M
Lutein	0.04 $\pm$ 0.01	0.10 $\pm$ 0.03 <sup>†</sup>	0.02 $\pm$ 0.007 <sup>§</sup>	F>M <sup>§</sup>
$\beta$ -Cryptoxanthin	ND	ND	0.31 $\pm$ 0.02 <sup>‡</sup>	—

Data from 24 quail are expressed as the mean  $\pm$  SE. ND, not detected. Galloxanthin the Unknown (both xanthophylls) were identified based on absorption spectrum and HPLC retention times. Statistical Comparisons with birds fed the C- diet by Mann-Whitney test.

\* Effects of diet and sex were determined using Mann-Whitney Test. <sup>a</sup> Effect was significant at  $P < 0.01$  when light-exposed birds were also included ( $n = 16$ ). <sup>b</sup> Effect was significant at  $P < 0.05$  when light-exposed birds were also included ( $n = 16$ ).

<sup>†</sup> Significant at  $P < 0.01$ .

<sup>‡</sup> Significant at  $P \leq 0.001$ .

<sup>§</sup> Significant at  $P \leq 0.05$ .

<sup>||</sup> Trend,  $P < 0.1$ .

liver were significantly greater than in males ( $P < 0.05$  and  $P < 0.0001$ , respectively). Zeaxanthin concentration in each tissue correlated significantly and positively with serum zeaxanthin concentrations and with those in other tissues (Table 3). Similarly, the serum concentrations of lutein correlated with those in other tissues. Serum lutein and zeaxanthin correlated positively but not as strongly as other correlations involving a single xanthophyll in different locations. Serum zeaxanthin did not correlate with tissue lutein, nor did serum lutein correlate with tissue zeaxanthin (data not shown).

**Serum.** Dietary supplementation with native zeaxanthin resulted in striking increases in zeaxanthin in serum (Fig. 3). In fact, supplemented females and males had mean serum zeaxanthin concentrations that were elevated 74- and 42-fold, respectively (Fig. 3A). The Z+ diet had no significant effect on serum lutein. Serum from Z+-fed birds had significantly higher levels of  $\beta$ -cryptoxanthin, lycopene, and  $\alpha$ - and  $\beta$ -carotene (Table 2).

**Retina.** Supplementation increased the retinal zeaxanthin concentrations 3.9-fold in females and 3.4-fold in males (Table 2; Fig. 3A). The retinal concentrations in Z+-fed birds were five times greater than those in birds fed the T diet. Dietary zeaxanthin was not the only factor determining retinal zeaxanthin. The retinas from the birds fed C- diets tended to have more lutein and zeaxanthin than those fed T diets (Table 2). In contrast, and despite the absence of effect of supplementation on lutein concentration in serum, the lutein concentration in supplemented retinas was 25% to 50% lower than in birds fed

TABLE 3. Correlations between Serum and Tissue Lutein and Zeaxanthin

Tissue/Xanthophyll	Spearman Rank Correlation Coefficient
Serum-Tissue Correlations	
Serum zeaxanthin to retina zeaxanthin	0.84*
Serum zeaxanthin to fat zeaxanthin	0.90 <sup>†</sup>
Serum zeaxanthin to liver zeaxanthin	0.98 <sup>‡</sup>
Serum lutein to retina lutein	0.55 <sup>§</sup>
Serum lutein to fat lutein	0.58 <sup>§</sup>
Serum lutein to liver lutein	0.83*
Tissue-Tissue Correlations	
Retina zeaxanthin to fat zeaxanthin	0.72*
Retina zeaxanthin to liver zeaxanthin	0.76*
Fat zeaxanthin to liver zeaxanthin	0.86 <sup>‡</sup>
Retina lutein to fat lutein	0.46 <sup>§</sup>
Retina lutein to liver lutein	0.34 <sup>  </sup>
Fat lutein to liver lutein	0.67*
Serum-Serum Correlation	
Serum zeaxanthin to serum lutein	0.42 <sup>§</sup>

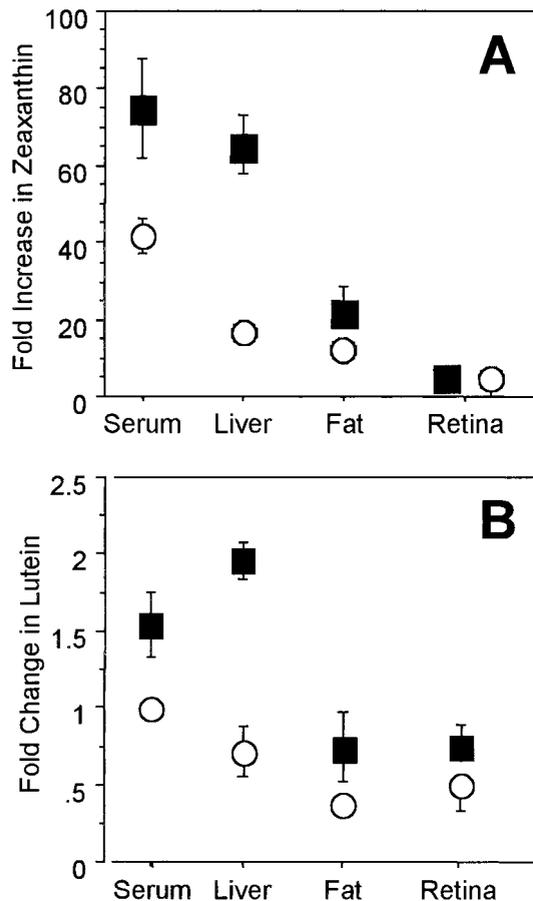
\* Significant at  $P \leq 0.001$ .

<sup>†</sup> Significant at  $P \leq 0.01$ .

<sup>‡</sup> Significant at  $P \leq 0.0001$ .

<sup>§</sup> Significant at  $P \leq 0.05$ .

<sup>||</sup> Trend, with  $P < 0.1$ .



**FIGURE 3.** (A) Z+ diet increased zeaxanthin in females (■) and males (○). Each observation was divided by the mean in birds of the same gender fed a C- diet, and the means ( $\pm$ SE) of those ratios were plotted. Some SEs are smaller than the symbol. Increases were significant in every tissue ( $P < 0.001$ ; nonparametric Mann-Whitney comparisons of Z+ and C- groups). (B) Reductions in lutein content of zeaxanthin-supplemented birds were significant in retina and fat ( $P < 0.04$  and  $P < 0.03$ , respectively; Mann-Whitney comparisons). Changes in livers of Z+-fed females were significantly different from those in males ( $P < 0.05$ ).

the C- diet (Fig. 3B). Possible reasons for this difference are explored later. Lutein and zeaxanthin concentrations in the retina correlated positively with those in serum, fat, and liver, but levels in retina correlated more strongly with those in serum than with those in liver or fat (Table 3). Supplemented females had significantly higher retinal concentrations of zeaxanthin, lutein, and  $\beta$ -cryptoxanthin than did males (Fig. 3, Table 2). Supplementation did not influence  $\alpha$ -cryptoxanthin in the retina, but increased the concentrations of  $\beta$ -cryptoxanthin, galloxanthin, and the unknown (Table 2). In fact, the concentration of galloxanthin correlated strongly with zeaxanthin concentrations in serum ( $\rho = 0.73$ ;  $P < 0.001$ ), retina ( $\rho = 0.93$ ;  $P < 0.0001$ ), liver, and fat ( $\rho > 0.68$ ;  $P < 0.001$  in each case). The concentration of unknown correlated more strongly with zeaxanthin in retina ( $\rho = 0.75$ ;  $P < 0.0003$ ) than with zeaxanthin in serum ( $\rho = 0.43$ ;  $P < 0.04$ ), liver, or fat ( $\rho = 0.34$ ;  $P < 0.1$ ), and it correlated strongly with the level of galloxanthin ( $\rho = 0.68$ ;  $P < 0.001$ ). These observations suggest that galloxanthin was derived from zeaxanthin.

**Fat and Liver.** Zeaxanthin concentrations in the liver and fat of supplemented females were 63 and 22 times (respectively) higher than those in the C- group (Fig. 3A). Zeaxanthin concentrations in male liver and fat were 16.5 and 12 times those in birds fed the C- diet. Liver zeaxanthin concentrations

correlated strongly with those in serum, retina, and fat (Table 3). In comparison with fat (by Wilcoxon signed rank test), liver accumulated approximately three times more zeaxanthin ( $P < 0.0005$ ) and comparable levels of lutein.

Lutein concentration was not significantly altered in liver of supplemented birds, but the mean concentration declined in fat (Table 2). Comparisons of birds of the same gender fed Z+ and C- diets revealed that the lutein level was significantly reduced in liver of Z+ males ( $P < 0.02$ ; Fig. 3B) and almost doubled in liver of Z+ females ( $P < 0.05$ ; Fig. 3B).

Tocopherols were analyzed in all tissues (Fig. 1). There was no significant effect of diet or sex on serum tocopherols, but  $\alpha$ -tocopherol was elevated in liver of Z+-fed birds, and in retina of birds fed the C- diet ( $P < 0.0001$ ). Males accumulated more  $\alpha$ -tocopherol in the retina than did females ( $P < 0.0003$ ; ANOVA), but there were no significant gender differences in the concentrations found in the other tissues.

## DISCUSSION

### The Quail Model

The quail retina actively accumulated xanthophyll carotenoids (zeaxanthin, lutein, galloxanthin, and cryptoxanthins) and excluded carotenes (except for lycopene found in retinas of some birds on severely deficient diets). The preferential accumulation of xanthophylls and exclusion of carotenes is also characteristic of human and primate retinas, but galloxanthin is never found in human retinas.<sup>2,4,5,7,52,54,55</sup> However, it was beyond the scope of this study to identify in quail the oxidized metabolites and geometric isomers of lutein and zeaxanthin found in human retinas (e.g., 3R,3'S-mesozeaxanthin, 3'-epilutein, and 3-hydroxy- $\beta$ , $\epsilon$ -carotene-3'-1).<sup>4,5</sup> Earlier examination of both chick and quail retinas by TLC suggested that the carotenoids in avian retinas include astaxanthin, zeaxanthin, and galloxanthin (3R-10'apo- $\beta$ -carotene-3,10'diol).<sup>36</sup> TLC in another study identified the predominant carotenoids as lutein and zeaxanthin, accompanied by astacene, phenocoxanthin, galloxanthin, neochrome, and four unidentified carotenoids.<sup>42</sup> In HPLC analyses we found no trace of astaxanthin in the quail retinas or diet. One peak had chromatographic and absorption characteristics of galloxanthin, but further definitive characterization by mass spectrophotometry was not undertaken. The high correlation between zeaxanthin and galloxanthin suggests that galloxanthin may derive from zeaxanthin. The absence of other carotenoids in retinas examined in this study may reflect dietary differences. Birds reported in the current study had no access to grasses, grains, or insects that would enrich the dietary, and probably the serum, carotenoids. For example, the presence of astaxanthin in tissues and feathers of storks has been tied to whether they eat crayfish.<sup>56</sup> Whether these carotenoids could accumulate in the retina is not known.

The concentrations of lutein and zeaxanthin in the quail retinas (23–85 ng zeaxanthin and 20–69 ng lutein per milligram protein) are of the same magnitude as the zeaxanthin (50.6 ng/mg protein) and lutein (54.8 ng/mg) found in membrane-rich fractions of human retinas (combining data for rod outer segments and residual membranes).<sup>40</sup> The levels observed cannot be compared with levels obtained from microdissected primate retinas (expressed in nanograms per square millimeter).<sup>5–7</sup> The concentrations of xanthophylls in sera of birds raised on T and C- diets were comparable to those reported in primates and other birds. Sera of quail fed C- and T diets from birth had mean concentrations of 40 to 110 ng zeaxanthin/mL and 60 to 170 ng lutein/mL. The total lutein plus zeaxanthin in quail fed T diets (280 ng/mL) was well within the ranges reported by Slifka et al.<sup>57</sup> in 13 primate species (49–1791 ng/mL). The total found in supplemented birds (3080 ng/mL) was comparable to levels reported in gray

gulls and Mandarin ducks, but was only 10% of the sera concentrations in two species of flamingo.<sup>57</sup> The maximum concentration of lutein in quail fat (0.1  $\mu\text{g}/\text{mg}$  wet weight;  $\sim 1$  nmol/mg dry weight) was well below the basal levels found in human adipose tissue (0.23  $\mu\text{mol}/\text{mg}$  dry weight).

Gender was a significant influence in absorption and distribution of lutein and zeaxanthin. The mean concentrations of lutein and zeaxanthin in female serum and liver exceeded those in males by 3- to 10-fold, but the mean concentrations of lutein and zeaxanthin in female retina and fat were at most two and four times, respectively, those in males. This difference is explained by the deposition of lutein and zeaxanthin in the eggs, which the females laid continuously.

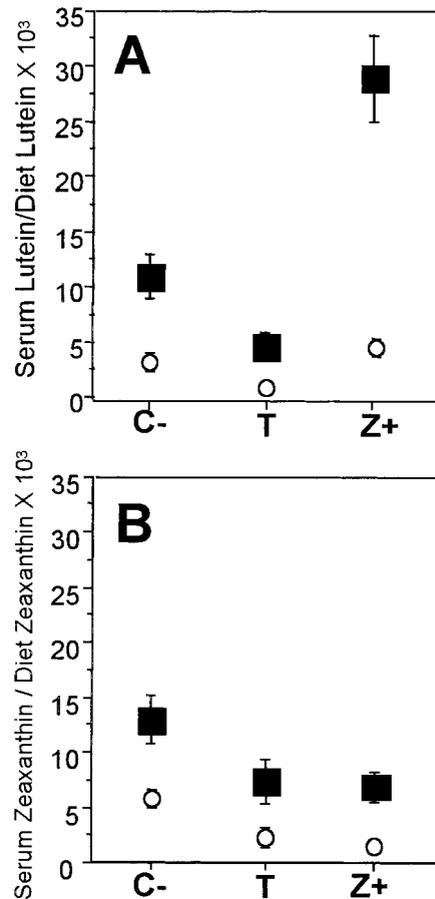
### Response to Dietary Zeaxanthin Supplementation

Zeaxanthin levels in serum, liver, retina, and fat were robustly elevated in quail fed a carotenoid-deficient diet supplemented with 3*R*,3'*R*-zeaxanthin. Several groups have previously determined that dietary modification with foods or dietary supplements rich in lutein (but containing 2% zeaxanthin<sup>31</sup>) increase serum lutein concentrations in humans and primates<sup>26,27,29,58</sup> and optical density of macular pigment in humans.<sup>26,29</sup> Zeaxanthin supplementation has been shown to increase serum zeaxanthin in squirrel monkeys<sup>58</sup> and in Rhesus monkeys.<sup>34</sup> After 6 weeks of daily dietary supplementation with a zeaxanthin-rich extract of *Fructus lycii* berries, zeaxanthin concentrations in serum of Rhesus monkeys had approximately doubled, and zeaxanthin concentrations in liver and spleen were approximately double those in unsupplemented control subjects. A doubling in retinal zeaxanthin was estimated after mathematical correction for the fact that retinal samples from control monkeys were more than triple in area<sup>34</sup> and included retinal regions that generally contain lower concentrations of carotenoids than the more central fovea.<sup>3,7</sup> In human subjects, daily dietary zeaxanthin supplements caused an increase in serum zeaxanthin, followed in 30 to 40 days by increases in macular pigment density.<sup>59</sup>

The quail results confirm that zeaxanthin supplementation elevates zeaxanthin concentrations in serum, liver, and retina and provide the first report that zeaxanthin is also elevated in fat. Female quail given 5 mg zeaxanthin/kg for 6 months had 74-, 63-, and 3.9-fold increases in zeaxanthin concentrations in the serum, liver, and retina, respectively. Although these were far higher than the increases in serum, liver, and retina of the monkeys, the supplemented quails were given a higher concentration of zeaxanthin for a longer period and their tissues compared with those in birds raised on deficient diets, whereas control monkeys had consumed a normal diet. In addition, there may have been differences in the bioavailability of the zeaxanthin and/or the biology of the two species.

### Effect of Zeaxanthin on Lutein Absorption into Serum

Carotenoid interactions may influence tissue deposition. Tang et al.<sup>60</sup> found that long-term canthaxanthin supplementation in ferrets reduces lutein-zeaxanthin in fat, whereas data from Golier et al.<sup>61</sup> indicate that canthaxanthin interferes with absorption or processing of  $\beta$ -carotene. In contrast, zeaxanthin supplementation did not influence plasma lutein of spider monkeys<sup>58</sup> and appeared to enhance it in Rhesus monkeys.<sup>34</sup> Although the Z+ diet had less lutein than the C- diet, the Z+-fed birds had as much or more lutein in serum and liver, and serum lutein weakly but positively correlated with serum zeaxanthin. The possibility that zeaxanthin influences absorption of dietary lutein was probed by examining the effect of zeaxanthin on the fraction of dietary lutein absorbed, which is estimated by dividing the serum lutein concentration by the quantity of lutein consumed daily.



**FIGURE 4.** Absorption of dietary lutein (A) and zeaxanthin (B) into serum of males (■) and females (○) raised on C-, T, or Z+ diets. A greater fraction of dietary lutein was absorbed into the serum from the C- diet than from the T diet ( $P < 0.03$ ), but a greater fraction of lutein was absorbed from the Z+ diet than from either the C- or the T diets ( $P < 0.0001$  for both comparisons). (B) The fractions of zeaxanthin absorbed from the T and Z+ diets (with high and low lutein) were comparable, and both were significantly lower than the fraction absorbed by birds fed the C- diet ( $P < 0.0001$  for both). Higher fractions of dietary lutein and zeaxanthin were absorbed by females than males ( $P < 0.0001$ ).

This approach is reasonable, considering that each bird ate a constant diet. As seen in Figure 4, dietary lutein was more effectively absorbed from the Z+ diet than from the C- diet (Fig. 4A;  $P < 0.0001$ ), suggesting that lutein absorption was facilitated by elevated zeaxanthin. A higher fraction of dietary lutein was also absorbed from the Z+ diet than from the T diet, but it was unclear whether increased absorption reflected a positive effect of elevated zeaxanthin or simply differences in the composition of the diet. The texture of the T diet was coarser than the C- or Z+ diets and contained a different mixture of grains, including dried corn, which may have reduced the bioavailability of lutein. In human subjects, the plasma response to dietary supplementation was determined not only by the amount of carotenoid consumed, but also by its source, and form. For example, a greater percentage of dietary lutein consumed was absorbed from peas and broccoli than from spinach.<sup>62</sup> Disruption of the matrix also appears to increase bioavailability of carotenoids. Mean serum lutein rose 14% more in human subjects given chopped spinach than it did in those given whole leaf spinach.<sup>62</sup> Similarly, serum  $\beta$ -carotene in subjects whose diet was supplemented with homogenized, cooked carrots and spinach was three times that observed in subjects given raw spinach and carrots, and lycopene

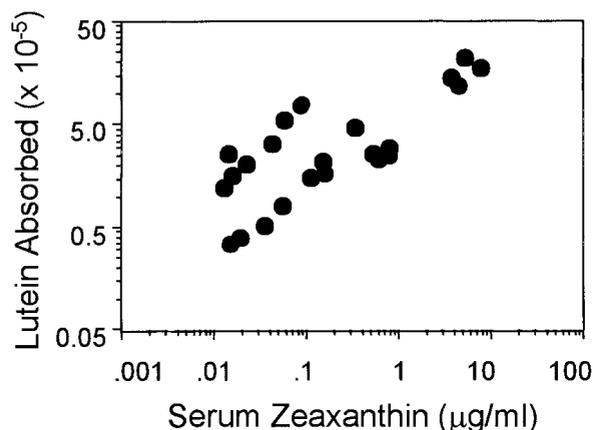


FIGURE 5. The fraction of dietary lutein absorbed into serum (estimated as serum lutein divided by dietary lutein) was positively correlated with serum zeaxanthin ( $\rho = 0.69$ ;  $P < 0.001$ ;  $n = 24$ ; Spearman rank correlation).

responses were greater after consumption of tomato paste than after consumption of the same amount of lycopene in fresh tomatoes.<sup>65</sup>

The possibility that zeaxanthin enhances lutein absorption was further supported by the direct correlation between lutein absorption and serum zeaxanthin concentration (Fig. 5). This correlation was not dependent on any dietary group: the correlation coefficient ( $\rho$  increased when the C- or T groups were excluded and decreased only 30% when the Z+ group was excluded:  $\rho = 0.95, 0.73,$  and  $0.48,$  respectively). The zeaxanthin enhancement of lutein absorption is sufficient to explain why serum and liver lutein were not reduced in supplemented birds whose diets contained 30% less lutein than the C- diet. The recent report that serum and retinal lutein were doubled in zeaxanthin-supplemented monkeys<sup>34</sup> may reflect a parallel positive influence of supplemental zeaxanthin on the absorption of lutein in the gut. However, those investigators raised the possibility that the lutein fraction included some zeaxanthin.

Dietary lutein did not influence zeaxanthin absorption (Fig. 4B). We speculate that elevated serum zeaxanthin may reduce oxidative loss of lutein<sup>4,55</sup> by scavenging peroxy radicals in serum lipoproteins.<sup>64</sup> Although both lutein and zeaxanthin scavenge peroxy radicals,<sup>65,66</sup> zeaxanthin is reported to be better at protecting biological membranes from oxidation.<sup>67,68</sup> If true, this could help explain why elevated levels of zeaxanthin appeared to enhance lutein absorption, but lutein did not enhance zeaxanthin absorption.

### Factors Influencing Tissue Carotenoid Composition

Although the concentrations of tissue lutein and zeaxanthin correlated directly with the serum concentration, tissues differed in the relative amounts of uptake of lutein and zeaxanthin. Kaplan et al.<sup>53</sup> determined that each of 10 different human tissues had a distinct carotenoid profile but did not distinguish lutein and zeaxanthin. In quails fed C- or T diets, the mean lutein-to-zeaxanthin (L-Z) ratios in serum and liver ranged between approximately 1.0 and 2.5, whereas those in the retinas were significantly below 1.0 ( $P < 0.0001$  for each, nonparametric one sample sign tests) and those in the fat were above 4.0 (Fig. 6). The L-Z ratios in all tissues of Z+-fed birds were below 1.0 (Fig. 6;  $P < 0.0001$  for each tissue), but the mean in fat (0.1) was still significantly higher than in serum (0.027;  $P < 0.02$ ; Wilcoxon analysis). However, the L-Z ratio in fat of Z+-fed birds could have been increased (relative to the C- birds) by capturing more lutein and/or less zeaxanthin

from the serum than the C- birds captured. Elevation in the L-Z ratio does not necessarily require competition between lutein and zeaxanthin, and increasing capture of one xanthophyll does not automatically mean that the other is decreased.

To better understand the basis of these differences in the L-Z ratio, we estimated the tissue's capacity to concentrate xanthophyll from the serum by determining the ratio of tissue xanthophyll to serum xanthophyll, which we call "capture efficiency," because it reflects both uptake and stabilization in the tissue. The capture efficiencies (Fig. 7) identified differences in the xanthophyll preferences of the tissues. Fat captured lutein far more efficiently than zeaxanthin, whereas liver was only slightly more effective in capturing lutein than zeaxanthin. In birds fed all but the Z+ diets, retina had capture efficiencies for zeaxanthin that were more than twice those for lutein (Fig. 7). Capture efficiencies for zeaxanthin in retina and fat were significantly reduced in the Z+-fed birds, suggesting that serum zeaxanthin concentration in the Z+-fed birds was sufficient to saturate the proteins that mediated zeaxanthin capture in these tissues. A preparation containing xanthophyll-binding proteins from human retina appeared to have binding capacities in the concentration range<sup>69</sup> found in the serum of Z+-fed birds.

Capture efficiencies for lutein in the retina and fat were also significantly reduced in Z+-fed birds. This observation suggests that elevated zeaxanthin in the serum interfered with the capture of lutein by these tissues. The different xanthophyll preferences of retina and fat suggest that different proteins mediate xanthophyll capture: a binding protein in fat that preferentially binds lutein and another in retina that preferentially binds zeaxanthin. If so, it is reasonable to consider that high levels of one xanthophyll would inhibit the binding of the other. Differential concentration of lutein and zeaxanthin in tissues could also occur through a shared uptake mechanism

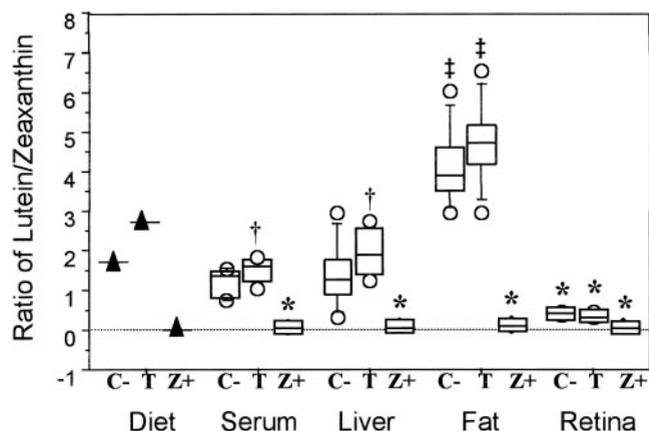


FIGURE 6. L-Z ratios in 24 birds raised on C-, T, or Z+ diets. The values between the 25th and 75th percentiles of individually calculated L-Z ratios are enclosed by the boxes, the median value is marked by the central line, error bars mark the 10th and 90th percentile, and circles identify each value beyond the error bars. (▲) Mean L-Z ratios in the diets. The Z+ diet produced L-Z ratios that were significantly below 1.0 in all tissues ( $P < 0.0001$  for each) and significantly lower than those in tissues of birds fed basal or T diets ( $P < 0.0001$  for all comparisons). L-Z ratios in fat were significantly higher than those in serum, liver, or retina ( $P < 0.0001$  for each comparison). Diet effects and differences between tissues were evaluated with the nonparametric Mann-Whitney test and Wilcoxon signed rank test, respectively. †Ratio significantly  $> 1$ :  $P < 0.003$ ; ‡ratio significantly  $> 1$ :  $P < 0.0001$ ; \*ratio significantly  $< 1$ :  $P < 0.0001$ .

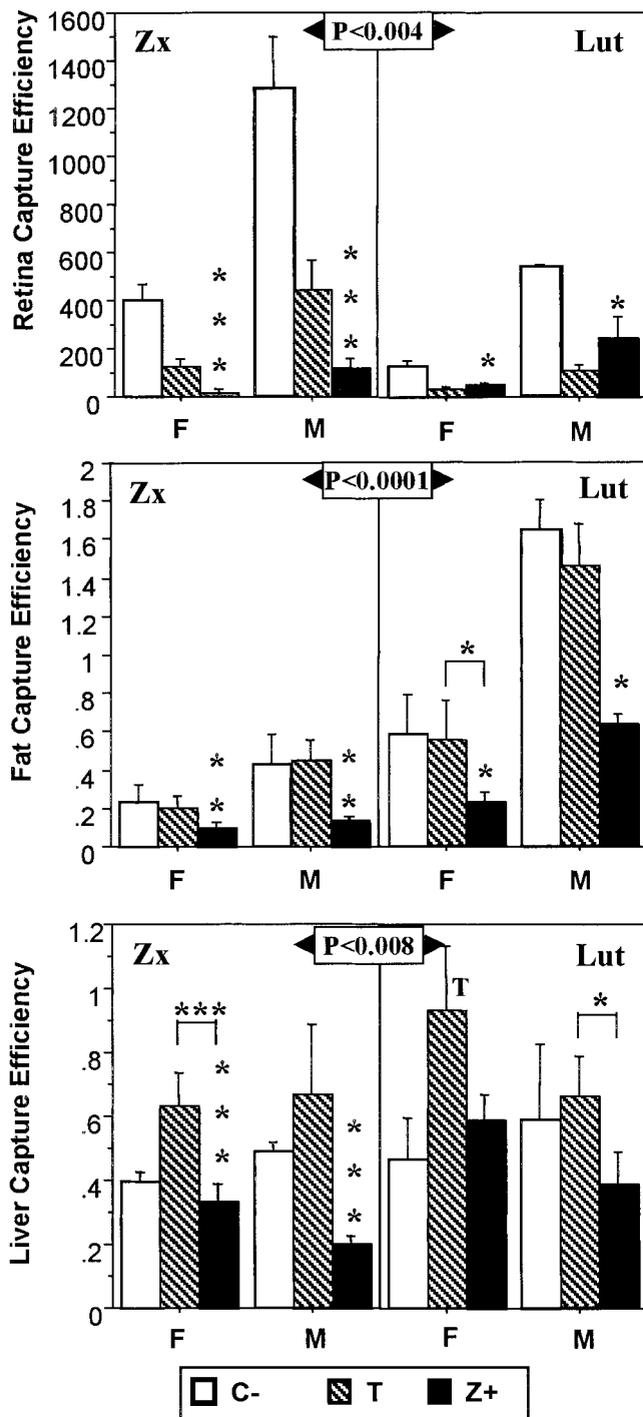


FIGURE 7. Means ( $\pm$ SE) of capture efficiencies for zeaxanthin (Zx, left) and lutein (Lut, right) in retina (top), fat (middle), and liver (bottom). Comparisons of capture efficiencies in birds fed C- diets with those fed other diets were completed with the Mann-Whitney test; significant differences indicated by vertical asterisks above the bars; significant differences in T- and Z+-fed birds indicated by brackets that apply to both genders but are represented only once. Significant differences in the tissue capture efficiencies for lutein and zeaxanthin in each tissue are presented above the centered arrow; for example, retina captured zeaxanthin more efficiently than lutein ( $P < 0.004$ ). Males had higher capture efficiencies in retina and in fat.  $T = 0.06 \leq P \leq 0.9$ ;  $0.03 \leq P \leq 0.05$ ;  $0.01 \leq P \leq 0.02$ ;  $0.001 \leq P \leq 0.009$ .

and differential stabilization in the tissue. Alternatively, preferential accumulation of zeaxanthin could be accomplished by preferential oxidative loss of lutein.<sup>4,67,68</sup>

**Tissue Interactions**

Johnson et al.<sup>28</sup> observed that retinal lutein correlated positively with adipose lutein in men and correlated negatively in women, who had higher lutein concentrations. They further observed that changes in adipose tissue were inversely related to the changes in retina and suggested that these tissues may interact. Retinal lutein also correlated positively with fat lutein in quail of both sexes ( $\rho = 0.46$ ;  $P < 0.03$ ). Zeaxanthin in retina and fat also correlated positively ( $\rho = 0.72$ ;  $P < 0.0007$ ). The preferential capture of lutein in fat (indicated by high L:Z ratios and high lutein capture efficiencies) and fat's greater mass and highly vascular character support the concept that fat may physiologically compete with retina for lutein; however, lutein concentrations in these tissues correlated positively. A similar preference for lutein in primate fat could explain slow retinal response to dietary supplementation with lutein-rich foods<sup>26,28</sup> or supplements.<sup>29,30</sup>

Reduced xanthophyll capture efficiencies in retinas and fat of female quail may derive from competition with the oviduct, which continuously produces eggs with yolks high in lutein and zeaxanthin.<sup>27</sup>

**Implications for AMD**

The specific role(s) of lutein and zeaxanthin sequestered by avian, primate, and human retinas is not known. In birds, absorption of shorter-wavelength light by carotenoids in oil droplets clearly shifts the photoreceptor responses toward longer wavelengths.<sup>70</sup> Evidence that light-induced photoreceptor apoptosis in quail was inversely related to retinal zeaxanthin<sup>47</sup> will be reported separately. In primates, absorption of shorter-wavelength light may improve resolution by reducing light scatter and chromatic aberration.<sup>71</sup> Elevated consumption of dietary carotenoids has been associated with reduced risk for, or delayed progression of, age-related maculopathy and/or AMD,<sup>12,15,16,18</sup> and reduced macular pigment levels have been found in eyes with AMD.<sup>20,22</sup> If reduced macular pigment increases risk for progression to AMD, then it seems logical to consider that elevating macular pigment may be an effective means of preventing or slowing the disease. Although it may be desirable to increase macular pigment, it is not clear which xanthophyll would be the more desirable supplement. The possibility that they have different functions in the retina is supported by the fact that they have different retinal distributions<sup>7</sup> and different distributions within biological membranes.<sup>72</sup> Zeaxanthin is preferentially accumulated in the foveal region of the retina,<sup>3,7</sup> which is initially less likely to exhibit degenerative features of late age-related maculopathy.<sup>73</sup> The preservation of the central retina could derive from the high concentrations of both carotenoids found there,<sup>19</sup> from the higher concentration of zeaxanthin in this region,<sup>7</sup> or from the low number of rod photoreceptors, which are preferentially lost during aging and in AMD.<sup>21,74</sup>

Preferential loss of rods, which accumulate lutein and zeaxanthin<sup>40,41</sup> could, in turn, relate to the location of rods in retinal regions with lower zeaxanthin content. We have found that light-induced photoreceptor loss in the quail was inversely related to retinal zeaxanthin concentration in quails whose dietary zeaxanthin was manipulated.<sup>47</sup> Although preservation of both rod and cone sensitivity is correlated with higher levels of macular pigment in human subjects,<sup>75</sup> it is not clear whether the macular pigment is higher because the photoreceptors containing lutein and zeaxanthin were preserved, or whether higher macular pigment preserved the photoreceptors, or vice versa. From our data demonstrating that zeaxanthin can protect photoreceptors from lethal levels of light, it is appealing to speculate that lutein would also be protective. However, to our knowledge, there are no data directly demonstrating that lutein reduces photoreceptor death in vivo.

The mechanism driving the distributions of lutein and zeaxanthin in the primate retina is not known, nor is it known whether lutein or zeaxanthin would be more effective in raising the macular pigment concentration in the regions of increased vulnerability. If adipose tissue and liver compete with the retina for dietary lutein, as suggested by observations in human subjects,<sup>28</sup> data presented herein would suggest that macular pigment may be more effectively increased through supplementation with zeaxanthin (preferentially absorbed by the retina) than with lutein (preferentially absorbed by fat). It has been proposed that selective enrichment of zeaxanthin in the central retina is the result of retinal conversion of lutein to mesozeaxanthin.<sup>52</sup> Whether mesozeaxanthin is generated in the retina or derived from dietary sources such as shrimp and fish,<sup>76</sup> the data presented herein and recent experiments in monkeys have unequivocally demonstrated that dietary zeaxanthin is effectively delivered to avian retina and the primate macula.<sup>34</sup>

In summary, the data obtained in the current study demonstrate that dietary supplementation with the natural isomer of zeaxanthin is effective in increasing the zeaxanthin content of serum, retina, liver, and fat in quail, and suggest that both preferential absorption and xanthophyll interactions influence the sequestration of specific xanthophylls in specific tissues. The robust response of the quail tissues to dietary zeaxanthin indicates that it is a useful model in which to explore the factors regulating xanthophyll concentrations in retina and their influence on retinal integrity, function, and/or aging.

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