

Nutritional Manipulation of Primate Retinas, II: Effects of Age, n-3 Fatty Acids, Lutein, and Zeaxanthin on Retinal Pigment Epithelium

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PURPOSE. To study the effects of age and of n-3 fatty acids, lutein, and zeaxanthin on the retinal pigment epithelium (RPE).

METHODS. Rhesus monkeys (age range, 7-17 years; $n = 18$) were fed xanthophyll-free semipurified diets from birth. The diets had either low or adequate amounts of n-3 fatty acids. Six monkeys remained xanthophyll-free until death. Six received supplements of pure lutein and six of pure zeaxanthin for 6 to 24 months. The central retina was serially sectioned, and the number of RPE cells were counted in an 8- μ m strip along the vertical meridian. Cell counts were compared with data from control monkeys ($n = 15$) fed a standard laboratory diet.

RESULTS. Foveal and parafoveal RPE cell densities increased with age. Xanthophyll-free monkeys had a dip in the RPE cell density profile at the foveal center, rather than the normal peak. After supplementation with xanthophylls, the RPE profile of animals low in n-3 fatty acids no longer had a dip at the foveal center but became asymmetric, with higher densities in the inferior retina. In animals with adequate n-3 fatty acid levels, xanthophyll supplementation did not restore the foveal peak, and resulted in an asymmetric profile with higher densities in the superior retina.

CONCLUSIONS. RPE cells are sensitive to the absence of macular pigment. Supplemental xanthophylls interact with n-3 fatty acid levels to produce asymmetries in the RPE profile. Xanthophylls and n-3 fatty acids are essential for the development and/or maintenance of a normal distribution of RPE cells. (*Invest Ophthalmol Vis Sci.* 2004;45:3244-3256) DOI:10.1167/iops.02-1233

The retinal pigment epithelium (RPE) serves many functions. Its roles include modifying and recycling retinoids in the visual cycle, phagocytosis and recycling of photoreceptor

disc materials, and transport of nutrients from the choroidal capillaries to the photoreceptors.^{1,2} Pathologic changes in RPE cells may lead to photoreceptor death and eventually result in irreversible visual loss. Loss of central vision occurs in macular diseases that involve interactions between RPE cells and photoreceptors, such as age-related macular degeneration, Best's disease, and Stargardt's disease. Although morphologic changes of RPE cells have been documented in macular diseases³⁻⁶ and in aging,^{7,8} there is considerable controversy about the effects of aging on RPE cell density.⁹⁻¹⁴

Recently, we measured the RPE cell density distribution in the central retina of normal rhesus monkeys.¹⁵ The distribution peaks in the foveal center and declines gradually to a plateau in the parafovea. In the present study, we investigated the effects of age and of nutritional manipulations on the RPE cell distribution in the central retina. We examined the effects of life-long diets lacking all xanthophylls, combined with low or adequate levels of n-3 fatty acids (polyunsaturated fatty acids with a double bond at the third carbon from the methyl end of the chain). These xanthophyll-free diets result in absence of macular pigment,¹⁶ allowing us to determine the effect of introduction of the individual pure macular carotenoids lutein and zeaxanthin.

Although earlier studies using rats,¹⁷ dogs,¹⁸ and monkeys¹⁹ have shown that the RPE is sensitive to long-term vitamin E deficiency, there appears to be little work on effects of chronic deficiency of other nutrients. Morphologic changes in RPE of pigmented rats caused by vitamin E deficiency include increased lipofuscin production and an increased number of phagosomes. These changes also occur at a slower rate in normal aging,^{17,20} and they are reduced by restriction of caloric intake.²¹

Diets low in n-3 fatty acids impair retinal development in humans and monkeys,^{22,23} but they also reduce vulnerability to acute light-induced damage.^{24,25} RPE cells in n-3 fatty acid-deficient rats show an increased accumulation of large lipid droplets and a decreased number of phagosomes, particularly after exposure to bright light.^{24,26} Based on experiments with monkeys and guinea pigs,^{23,27} these effects are thought to be related to reduced levels of docosahexaenoic acid (DHA, 22:6n-3). DHA is a major component of photoreceptor membranes in the retina and appears to optimize the efficiency of phototransduction²⁸ and of retinoid transport.²⁹

The xanthophylls lutein and zeaxanthin are concentrated in the central retina,^{30,31} where they are the major components of the macular pigment. Although dietary modification or supplementation is known to alter the levels of macular pigment in humans³²⁻³⁴ (Bone RA, et al. *IOVS* 2001;42:ARVO Abstract 1254) and in rhesus monkeys,³⁵ there is limited knowledge about the effects on the retina of long-term xanthophyll deficiency.

The experimental monkeys in this study were fed semipurified diets with no xanthophylls for their entire lives (7-17 years). They are termed xanthophyll-free monkeys, because they had no detectable lutein or zeaxanthin in their blood and no macular pigment in their retina³⁶ (Johnson EJ, et al., manu-

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script submitted, 2002). The experimental diets had two different levels of α -linolenic acid (18:3n-3), the precursor of DHA. The animals had been fed these diets to study the effects of n-3 fatty acid status on the development and function of the retina and the brain.^{23,37} Therefore, these animals provided the opportunity to examine the interaction between n-3 fatty acids and xanthophylls. Although the gross appearance of the retinas of these xanthophyll-free monkeys was normal, fluorescein angiography showed a significant increase in macular RPE transmission defects compared with age-matched control monkeys (Neuringer M, et al. *IOVS* 1999;41:ARVO Abstract 882).

To quantify the effects of the dietary manipulations, we measured the profile of RPE cell density along the vertical meridian. We chose to sample along the vertical meridian because of evidence that photoreceptors in the superior and inferior retina differ in their vulnerability to light damage, nutritional manipulation, and deleterious mutation.^{25,38-40} We show that the xanthophyll-free monkeys had an abnormal RPE profile that was modified by adding pure lutein and pure zeaxanthin supplements to the diet. A separate paper reports complementary measurements of photoreceptor cell density (Leung IY-F, et al., submitted, 2004). Other papers from this project describe additional data from the same animals, including the effects of dietary xanthophyll supplementation on serum carotenoids and macular pigment density in vivo,³⁶ the chemical composition of the xanthophylls accumulated in the retina (Johnson EJ et al., manuscript submitted, 2002), and the effects of xanthophyll exclusion and supplementation on the susceptibility to blue-light-induced damage (Barker FM et al., manuscript in preparation, 2004).

MATERIALS AND METHODS

Animals and Diets

All procedures were approved by the Institutional Animal Care and Use Committee of the Oregon National Primate Research Center (ONPRC) and The Schepens Eye Research Institute (SERI), and they conformed to NIH guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The experimental subjects included 18 rhesus monkeys (*Macaca mulatta*) born at the ONPRC. They were fed one of two semipurified diets, both of which contained adequate levels of all known essential nutrients including vitamin A and α -tocopherol, but no detectable carotenoids. The semipurified diets were fed to the mothers of the subjects throughout pregnancy^{23,37} and to the subjects from the day of birth, continuously until the completion of the study. The two diets differed only in their fat sources and therefore in their content of n-3 and n-6 fatty acids. We refer to the monkeys fed low levels (0.3% of total fatty acids) of α -linolenic acid as low n-3 animals. This dietary manipulation has been shown to reduce retinal docosahexaenoic acid (DHA) by 80% compared with levels in control monkeys fed stock diets.^{23,41} The other treatment group, the adequate n-3 animals, received 8% of total dietary fatty acids as α -linolenic acid, which has been shown to produce normal concentrations of retinal DHA.^{23,41} For more details see the companion paper.³⁶

At the beginning of this study, all experimental animals had undetectable levels of lutein and zeaxanthin in serum and adipose tissue and no measurable macular pigment³⁶ (Johnson EJ, et al., manuscript submitted, 2002). Beginning at 8 to 16 years of age, six monkeys were fed supplements of pure lutein (100% all-*trans*-lutein) and six were fed pure supplemental zeaxanthin (90% all-*trans* zeaxanthin and 10% 13-*cis* zeaxanthin). The lutein and zeaxanthin supplements were purified or synthesized by Roche Vitamins, Ltd., now DSM Nutritional Products Ltd. (Basel, Switzerland) and formulated into gelatin beadlets containing 4% to 9% of the purified xanthophylls, as confirmed by reversed-phase HPLC analysis.³⁶ Supplements were given at a dose of 3.9 μ mol/kg per day (2.2 mg/kg per day), initially for 7 days a week. After

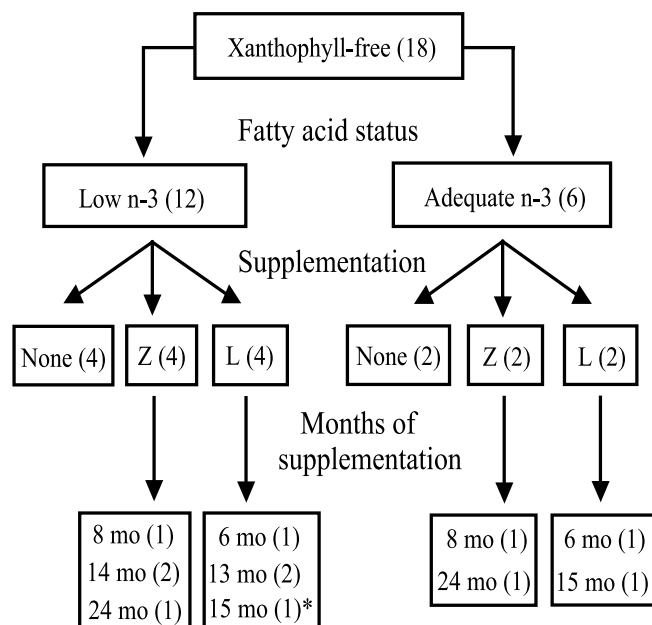


FIGURE 1. Treatment outline of the xanthophyll-free monkeys. The supplementation history of the monkeys fed a diet low in n-3 fatty acids is shown on the *left* (Low n-3). The supplementation history of the animals fed a diet producing normal tissue concentrations of n-3 fatty acids is shown on the *right* (Adequate n-3). Low n-3 and adequate n-3 animals are further subdivided into those that received no supplement (No supp), zeaxanthin (Z-supp), or lutein (L-supp) for specific periods of time (months). Histologic data were not obtained for one animal (*asterisk*) because of a failed embedding of the retinal specimen.

15 to 52 weeks of supplementation, supplements were fed 4 days a week. Six animals received no supplement (Fig. 1).

The daily doses of xanthophylls were prepared for each animal based on body weight, which was measured at least monthly. The beadlets were inserted into food treats, such as marshmallows or small pieces of fruit and fed just before the animals' midday meal of semipurified diet. The lutein and zeaxanthin groups were balanced to the extent possible based on sex, n-3 diet group, and body weight. Four of the 12 low n-3 animals were male, whereas three of the six adequate n-3 animals were male. Characteristics of individual animals are listed in Table 1, with animals in the normal control groups ordered according to age.

Fifteen rhesus monkeys from the ONPRC colony served as the controls. The control animals were fed a standard stock diet (Purina 5047 Monkey Chow; Ralston Purina, Richmond, IN) that provided 0.26 μ mol/kg per day (150 μ g/kg per day) lutein, 0.24 μ mol/kg per day (135 μ g/kg per day) zeaxanthin, and 0.035 μ mol/kg per day (19 μ g/kg per day) β -carotene. They were also given supplemental fruits and vegetables (primarily apples and carrots) providing less than 1% of the xanthophyll intake of the stock diet. They were housed under the same general conditions as the experimental groups. All animals were housed at the ONPRC, except for one control monkey, M43, which was obtained from the ONPRC colony but housed at SERI and fed the same stock diet for 3 years before death. The monkeys were maintained on a 12-hour light-dark cycle with an ambient light level of 50 to 90 lux produced by full-spectrum fluorescent lights (F32-T8-TL850; Philips, Eindhoven, The Netherlands).

Tissue Preparation

Experimental monkeys and one control monkey (M43) were deeply anesthetized with either pentobarbital or isoflurane and then systemi-

TABLE 1. Dietary Treatment Groups

Animal ID	Sex	Age at Death (y)	Body Weight (kg)*	Supplement Duration (mo)†	Eye	n-3 Fatty Acid Status
Xanthophyll-free animals without supplementation						
776	M	7.0	11.5	0	R	Low
566	M	12.3	7.1	0	L	Low
481	F	12.9	6.5	0	L	Adequate
411	F	15.0	6.1	0	L	Low
300	F	17.3	9.6	0	R	Low
275	F	17.7	7.9	0	R	Adequate
Mean age \pm SD: 13.7 \pm 4.0, <i>n</i> = 6						
Xanthophyll-free, then zeaxanthin supplemented						
642	F	9.0	9.7	14 (12, 2)	L	Low
567	F	10.9	9.6	14 (12, 2)	L	Low
224	F	18.3	9.1	24 (10, 14)	L	Low
217	F	18.4	6.0	24 (10, 14)	L	Adequate
586	M	11.6	12.1	8 (4, 4)	L	Low
398	M	15.4	11.3	8 (4, 4)	L	Adequate
Mean age \pm SD: 13.9 \pm 4.0, <i>n</i> = 6						
Xanthophyll-free, then lutein supplemented						
602	F	10.0	8.5	13 (12, 1)	L	Low
585	F	10.5	6.1	13 (12, 1)	L	Low
362‡	F	15.0	6.1	15 (10, 5)	L	Low
397	M	14.6	8.3	15 (10, 5)	L	Adequate
636	M	10.2	10.9	6 (4, 2)	L	Low
463	M	13.8	11.7	6 (4, 2)	L	Adequate
Mean age \pm SD: 11.8 \pm 2.2, <i>n</i> = 5 successful embedment						
Normal control (serial sections)						
653	F	7.8	5.4	NA	L	Normal
651	F	7.9	5.7	NA	L	Normal
M43	F	14.0	5.6	NA	R	Normal
449	F	14.0	6.4	NA	L	Normal
009	M	14.2	11.7	NA	L	Normal
011	M	15.8	9.3	NA	L	Normal
840	F	18.8	6.0	NA	R	Normal
04§	F	30.0	7.4	NA	L	Normal
Normal control (wholemounds)						
668	F	7.6	5.5	NA	R	Normal
653	F	7.8	5.4	NA	L	Normal
007	F	14.0	7.2	NA	R	Normal
331	F	15.0	5.6	NA	R	Normal
269	M	16.0	9.6	NA	R	Normal
113	F	20.5	8.4	NA	L	Normal
35§	F	31.7	7.4	NA	R, L	Normal
47§	M	33.0	6.4	NA	R, L	Normal
Mean age \pm SD: 13.8 \pm 4.2, <i>n</i> = 12 in same age range as experimental animals						

NA, Control animals were fed stock diet with carotenoids.

* Body weight at start of study for xanthophyll-free unsupplemented animals, at start of supplementation for supplemented groups and at death for normal control animals.

† Total duration of lutein or zeaxanthin supplementation. Values in parentheses indicate months of daily xanthophyll supplementation, followed by months of four times/week supplementation.

‡ Excluded because of failed embedding.

§ Excluded from control group-experimental group comparisons to match ages.

cally perfused with fixative through the ascending aorta under physiological pressure (120 mm Hg) as measured by a blood pressure monitor (Marquette, Milwaukee, WI). Perfusion was initiated within 3 minutes of opening the chest, without prior exsanguination. One liter of one-tenth strength fixative (0.4% paraformaldehyde plus 0.05% glutaraldehyde at 37°C) was followed by 5 L 4% paraformaldehyde plus 0.5% glutaraldehyde, the first liter at 37°C and the remainder at room temperature. Both fixatives were in 0.1 M sodium phosphate buffer (pH 7.4) with 0.15 mM calcium chloride. All except the last liter also contained 4% polyvinyl-pyrrolidone (PVP; average molecular weight 40,000; Sigma-Aldrich) which simulated the osmolarity of plasma, thereby helping to preserve the structure of the choroid and RPE during perfusion.⁴² Inclusion of PVP resulted in a marked reduction of artifactual vacuoles that were present in the RPE cells when fixatives of lower osmolarity were used. PVP was omitted from the last liter to wash it from the tissue.

Thirty to 60 minutes after the end of the perfusion, the eyes were enucleated, the globes were hemisected at the equator, and the posterior half of one eye was immersed in the same full-strength fixative without PVP. After 1 hour, this eyecup was rinsed twice with 4% paraformaldehyde in the same buffer and stored in 4% paraformaldehyde overnight. It was then rinsed three times in 0.1 M sodium phosphate buffer (pH 7.4) with 0.15 mM calcium chloride and 5% sucrose and stored in the same buffer with 0.04% sodium azide until embedding for morphometric study (1 week to 12 months). For all xanthophyll-supplemented animals, the left eye was used in these studies. The other eye was processed for biochemistry as described in another paper from this project (Johnson EJ, et al., manuscript submitted, 2002). No eyes that were studied morphometrically had been subjected to blue-light-induced damage.

Retinas from 13 of 14 control animals were obtained through the tissue-distribution program of the ONPRC. These animals were primar-

ily assigned to other projects that determined their perfusion protocol (the exception was M43). They were deeply anesthetized with pentobarbital and then perfused with saline (1–2 L) followed by 5 to 6 L 4% paraformaldehyde. After perfusion, the eyes were processed in the same way as those of the experimental monkeys.

A 5 × 5-mm square of tissue centered on the fovea was cut from the posterior retina using a transparent template aligned horizontally with the fovea and optic disc. Two notches were removed from the superior and inferior corners of the temporal region of this 5-mm square. These notches indicated temporal–nasal orientation during embedding and facilitated proper alignment for vertical sectioning. Although other regions of the retina were also preserved, they were not analyzed in this portion of the study. Except for one control retina (monkey 653), choroidal tissue was attached to the RPE in 25 of 26 retinas used for serial sectioning, which helped to maintain the structural integrity of the retina and the RPE. In preparation for embedding in methacrylate, the natural curvature of the retinal tissue was maintained by encapsulation in agarose gel at 42°C (8% wt/wt in distilled water, type VII low-gelling-temperature agarose, Sigma-Aldrich) while draped over a stainless steel ball 19 mm in diameter. The encapsulated tissue, together with the metal ball, was then rapidly put into an ice bath for less than 30 seconds to harden the agarose and facilitate removal of the encapsulated retina from the ball. The agarose embedment was trimmed with a cylindrical trephine with a 10-mm diameter so that the edge of the agarose formed a circle on which the specimen rested to maintain its orientation and curvature during embedding in plastic. After dehydration in 50%, 75%, and 95% alcohol for 30 minutes each, the specimen was immersed in resin (Histo-resin Plus; Leica, Heidelberg, Germany) overnight at 4°C in a covered container undergoing gentle agitation. This resin was used to maintain immunoreactivity of the tissue.

A small portion of resin was mixed with hardener, poured into the mold, and allowed to harden for 3 hours in a dry nitrogen atmosphere to form a base. Then the tissue was placed on top of this base, resting flat on its circular rim, along with fresh resin and hardener for an additional 3 hours in nitrogen. The agarose and fovea were embedded off-center within the rectangular methacrylate block to indicate the orientation of the superior and inferior borders of the tissue. When the block was fully hardened, the retina was well supported and precisely oriented. This preparation facilitated positioning of the block in the microtome so that sections could be cut perpendicular to the surface of the retina and parallel to the axis of the photoreceptors. After the embedding procedure, the block was trimmed to orient the sections along the vertical meridian, using the edge of the tissue as a guide.

During embedding of one of the retinas (monkey 362), bubbles formed inside the block, and the tissue could not be sectioned for anatomic analysis. However, this animal is included in Table 1 for completeness, because data from it are reported in other papers from this project³⁶ (Johnson EJ, et al., manuscript submitted, 2002).

It would have been much easier to count the RPE cells in whole-mounts for the present study. However, preparation of useful whole-mounts requires a clean separation of the RPE from the choroid. Attempts to separate the choroid from the RPE often result in RPE or photoreceptor cell loss in the foveal region, or adherent patches of choroid that obscure RPE and retinal features.¹⁵ To avoid the risk of failed dissections in the valuable xanthophyll-free monkeys, we prepared sections of intact retina/RPE/choroid, with the result that foveal regions were well preserved in 17 of 18 experimental animals. Preparation of sections also enabled us to count S-cones and rods in the same eyes; these data are reported in a separate paper (Leung IY-F, et al., manuscript submitted, 2004).

Sections of 2- μ m thickness oriented along the vertical meridian were collected serially when the depression of the fovea was first noted as the block was cut. The superior retinal border in the block was indicated with a red mark to aid in orientation of sections during mounting. The center of the fovea was identified as the thinnest region by comparing sections 30 μ m apart. Slides with five serial sections through the center of the fovea were selected for analysis. Cell nuclei were labeled by incubating the sections with 0.83% 4'-diamidino-2-

phenylindole dihydrochloride (DAPI; Roche Diagnostic Corp., Mannheim, Germany) in PBS for 15 minutes, followed by rinsing three times with PBS. A drop of mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) was placed on the slide, and the slide was coverslipped.

Image Capture, Alignment, Counting

Each 2- μ m section was divided into calibrated sampling frames referenced to the center of the fovea, which was identified as the thinnest point on the section where only the photoreceptor layers were present. The sampling frames were recorded by an epifluorescence microscope (Ellipse E800; Nikon, Melville, NY) connected to a color digital camera (Spot-RT; Diagnostic Instruments, Sterling Heights, MD). The UV-2A filter set (Nikon) was used to capture the images of the DAPI-stained nuclei. This filter set has an excitation bandpass from 330 to 380 nm (peak wavelength 360 nm) and accepts emitted light from 400 nm upward (50% transmission at 420 nm, high-pass filter). We exposed the images with high blue values in the RGB color space when the UV-2A filter set was in place. All the DAPI-stained nuclei appeared blue. Images were also captured with a Texas red filter set (excitation 540 to 580 nm, emission 600 to 660 nm) for the recognition of immunochemical labeling of photoreceptors (Leung IY-F, et al., manuscript submitted, 2004). For purposes of the present study, the Texas red images were used only to demonstrate the difference between images that visualize lipofuscin and those that do not (illustrated in Figs. 2A–D). Comparison of the images taken with the two filter sets in Figures 2A to 2D clearly shows that the conditions used to count the nuclei virtually eliminate potential confounding contributions by other cellular components.

In the fovea, five central sampling frames, each 0.365 mm in length, were captured at 400 \times magnification. There was a 20- μ m overlap from one frame to another. The full extent of foveal coverage at 400 \times was 1.75 mm. On each side of the fovea, two to three additional sampling frames were captured at 200 \times , extending the sampling coverage to a total length of 5.3 mm. Data are referred to the center of each sampling frame and plotted at 0 (the foveal center), ± 0.35 -, ± 0.69 -, ± 1.22 -, ± 1.93 -, and ± 2.64 -mm eccentricity.

An identification code was assigned to each monkey before cells were counted. The observer who counted the cells did not know the treatment group of each monkey until the completion of counting. The serial color images were converted to grayscale bitmap images at 300-dpi, 8 bits per pixel, for use with the public domain software SEM Align and IGL Trace (available at: <http://synapses.mcg.edu/tools/index.stm>).⁴³ Images taken from corresponding regions in the five serial sections were aligned by the corresponding point method,⁴⁴ using cell nuclei in various layers of the retina as the corresponding points.

Colored markers were used to identify every slice of a given RPE cell nucleus in each serial set of images. The color of the marker indicated the section within which the nucleus first appeared. The first section in our reconstructed volume was an exclusion zone, or reference section. To avoid overcounting, we did not count the RPE cell nuclei encountered in this section.⁴⁵ Starting from the second section, emerging RPE cell nuclei were counted from the second to the fifth section. Nuclei touching the edges of adjacent frames were also not counted, which resulted in a slight undercount of less than 2%. The counting methods for RPE nuclei are illustrated in Figures 2E to 2H. The number of RPE nuclei in the four serial sections at each eccentricity were tabulated and expressed in number per square millimeter, assuming that the four sections summed to a total thickness of 8 μ m. No corrections were made for shrinkage because there was no detectable difference in the size of fixed tissue measured before and after methacrylate embedding.⁴⁶

Statistical Analyses

As a measure of the peak of the cell density profile, the difference (D) between the cell density at the foveal center (zero eccentricity) and the average of the 2 points at ± 0.35 -mm eccentricity was computed. For the asymmetry (A) of the profiles, four points at 0.35-, 0.69-, 1.22-,

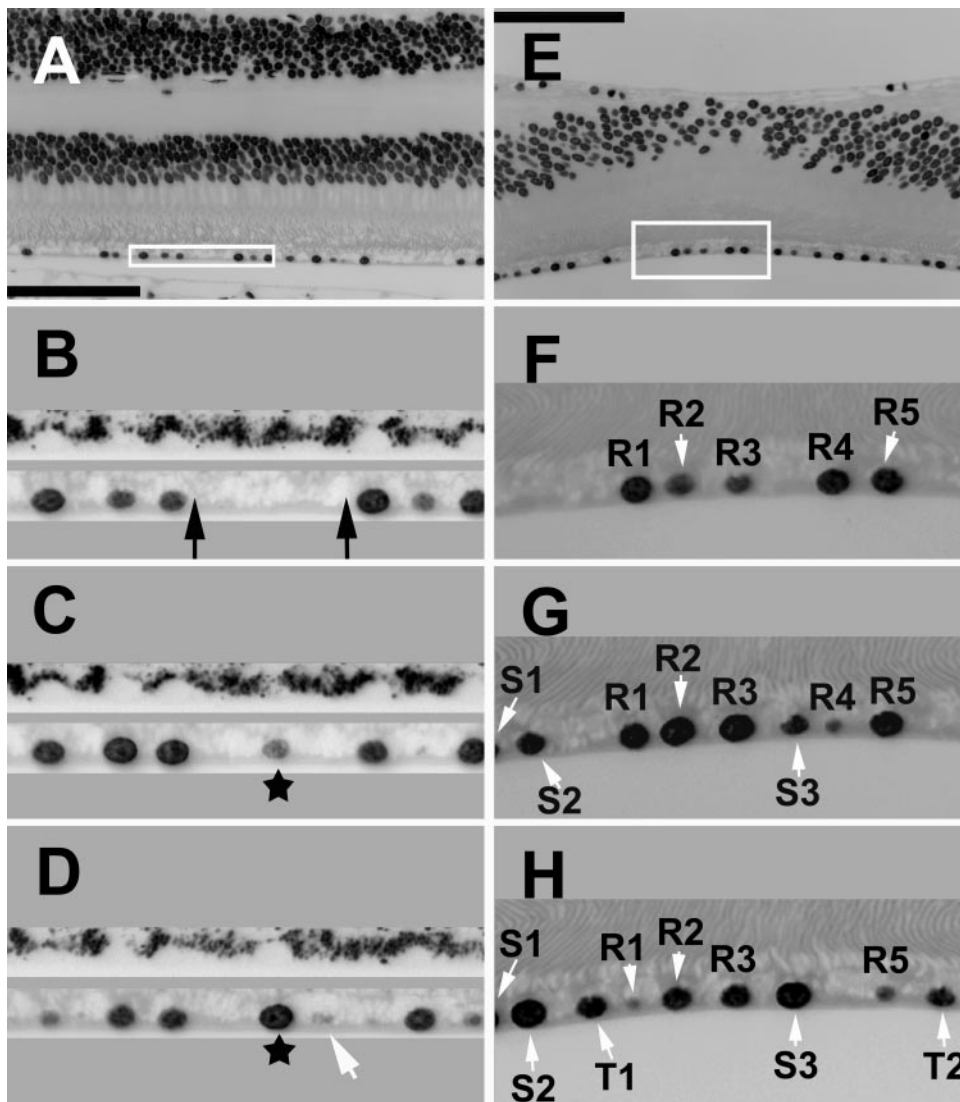


FIGURE 2. Fluorescence micrographs of 2- μm -thick sections in the foveal region transformed to grayscale. The *left* column illustrates the clarity with which DAPI-stained fluorescent nuclei are distinguished from other cellular components. (A) Retinal region corresponding to a counting frame 0.7 mm superior to the foveal center of animal 275 (400 \times) photographed with the UV-2A filter set to visualize the DAPI. The *white* rectangle (108 μm width) in (A), magnified in the *bottom* strip in (B), outlines a strip of retina encompassing the RPE. The *top* strip in (B) is the same rectangular region imaged with a Texas red filter that visualizes lipofuscin in the RPE. Lipofuscin granules fill most of the space between the *arrows*, where no nuclei were found. (C, D) Corresponding images are shown from the next two serial sections. (C) Slice of a nucleus appears (\star) and the lipofuscin granules are reduced in number. (D) An empty space appeared in the strip of lipofuscin as the nucleus became prominent (\star). A dark spot in the *bottom* strip in (D, *arrow*) may be a nucleus starting to appear. To be counted it must also have appeared in the next section. Scale bars: (A, *bottom left corner*, E, *top left corner*) 100 μm long. (E-H) The counting method used for the DAPI-stained nuclei. (E) The central counting frame in the fovea (400 \times) of the same animal. (F) Magnified image of the region (102 μm width) within the *white* rectangle in (E). Aligned corresponding regions of two additional serial sections with stained nuclei are shown in (G) and (H). Five positively stained nuclear slices labeled R1 to R5 are visible in (F). In (G) and (H), the corresponding slices are similarly labeled. In (G), two new nuclei (S1 and S2) appeared at the

left and the *center* (S3) of the frame. In (H), two more nuclei (T1 and T2) emerged and R4 disappeared. Because (F) was the reference section, R1 to R5 were not counted. S1, at the edge of the adjacent frame was not counted, whereas S2, S3, T1 and T2 were counted.

and 1.93-mm eccentricity in the superior region were averaged and compared to the points at corresponding eccentricities in the inferior region. Cell density in the foveal center (at 0 eccentricity) was not included in the symmetry calculation. The sum (S) of cell densities at the 5 measured loci at 0, ± 0.35 , and ± 0.69 mm eccentricities was also computed for each retina as a measure of overall cell density. D, A, and S in different groups are expressed as the mean \pm SEM. Error bars in all figures denote the SEM. Correlations between variables were examined with the Pearson r .

Because there were small numbers of animals in the six individual treatment groups, we could not easily use a standard statistical design based on analysis of variance. We relied instead on careful examination of graphs and use of two-tailed t -tests for combined groups with $n \geq 4$. In many cases, we present individual data to buttress the plausibility of this approach. When analyzing D and S, where multiple comparisons were made, we considered differences between groups to be significant only at $P \leq 0.01$. For analyzing A, only one comparison was made, and the results were considered significant at $P < 0.05$.

RESULTS

We first describe the effects of age on the RPE cell density of the control animals and then consider the effects of dietary

manipulation on age-matched experimental animals. Previously published data¹⁵ on the RPE density profile measured in retinal wholemounts from normal monkeys are included to increase the sample size.

Density Profile of RPE Cells in Control Animals: Wholemount Versus Serial Sections

Mean RPE cell density profiles along the vertical meridian of retinas from control animals obtained by counting cells in wholemounts and in serial sections are shown in Figure 3. To match the two groups for age, we excluded wholemount data from one old monkey (no. 47, 33 years). Measurements made on serial sections from retinas of eight monkeys (mean age \pm SD, 15.3 \pm 7.0 years) were therefore compared with data from wholemounts of retinas from seven monkeys (mean age \pm SD, 16.1 \pm 8.3 years). One animal was studied by both methods, and so a total of 14 normal animals contributed to these graphs. The results obtained by the two methods were remarkably consistent. The percentage difference between the mean values obtained by the two methods for the three locations sampling the center of the fovea and the two adjacent points was only 5% to 11%. Because the two methods produced such

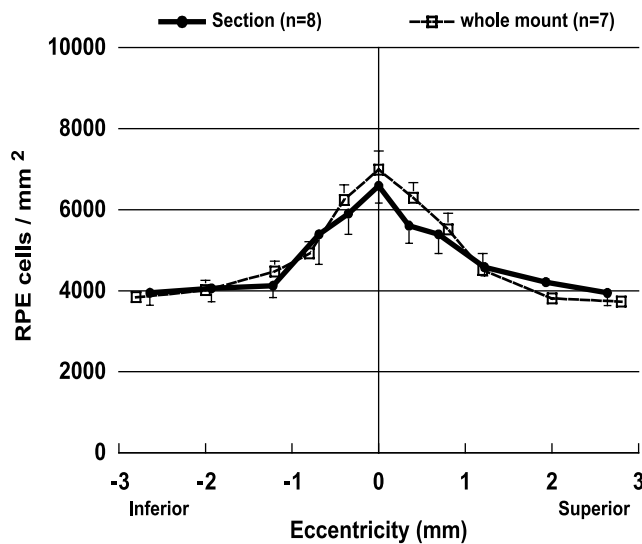


FIGURE 3. Mean RPE profiles in the central retina of normal monkeys derived by two different counting methods. (●, thick line) The profile determined by counting nuclei in 5 serial sections ($n = 8$). (□, thin line) The profile derived by counting nuclei in retinal wholemounts ($n = 7$).¹⁵ Error bars, SEM.

similar results, we combined the data to increase the statistical power of our analyses.

Although there were large individual variations in the RPE cell density in the foveal region, the shapes of individual profiles were generally similar. Ten of 14 animals had maximum RPE cell density at the foveal center, three had maximum density at the immediately adjacent locus, and 13 of 14 had higher RPE cell density at the foveal center than the mean of the densities of the two loci immediately adjacent to the center. This consistently high density at the foveal center is an important feature that will be used for comparison with the dietary treatment groups.

Age and RPE Cell Density

The relationship between the ages of the control monkeys and their RPE cell densities is illustrated in Figure 4. Foveal RPE cell density, represented by the mean for the three counting frames with centers within 0.5 mm eccentricity, increased with age (Fig. 4A, $r = 0.72$, $P < 0.005$). Parafoveal RPE cell density, represented by the average values of counting frames with centers between 1- and 2-mm eccentricities, also correlated strongly with age (Fig. 4B, $r = 0.84$, $P < 0.0001$).

RPE Cell Density Profiles of the Dietary Treatment Groups

Because age had such a strong effect on RPE cell density, we excluded the three oldest animals (monkeys 35, 47, and 04) when comparing the control group with the experimental animals so that all groups had similar mean ages and age ranges (Table 1). Consequently, the mean combined data for the age-matched control group was derived from retinas of 12 monkeys. For the one control monkey studied by both counting methods, the data from the wholemount determination was used for the grand average.

Results from each of the dietary treatment groups are described with the help of the flow chart in Figure 1. The effects of dietary manipulation were clearest for the low n-3 group (left column), which began the study with low n-3 fatty acid status as well as the absence of macular xanthophylls. The other six animals were fed a synthetic diet that was adequate to

produce normal tissue levels of n-3 fatty acids.²³ Thus, the adequate n-3 group (right column) was only deficient in xanthophylls.

We carefully examined the effect of supplementation duration on the RPE density profile. We did not find any obvious differences in the density profiles of individual animals when the duration of xanthophyll supplementation varied from 6 to 24 months. We also analyzed the number of months of lower frequency of supplementation (4 d/wk) in the lutein- and zeaxanthin-supplemented animals by *t*-test. There is no statistically significant difference between the 2 groups ($P > 0.1$, Mann-Whitney test). Thus, the reduction of dosage from daily supplementation to 4 days per week did not significantly affect the comparison of lutein- and zeaxanthin-supplemented animals. Inspection of individual curves suggests that even the shortest supplementation (6-8 months) of the low n-3 animals was sufficient to produce a strong effect. The individual profiles also showed no obvious effect of gender. Therefore, the groups defined by Figure 1 will form the basis for our analyses.

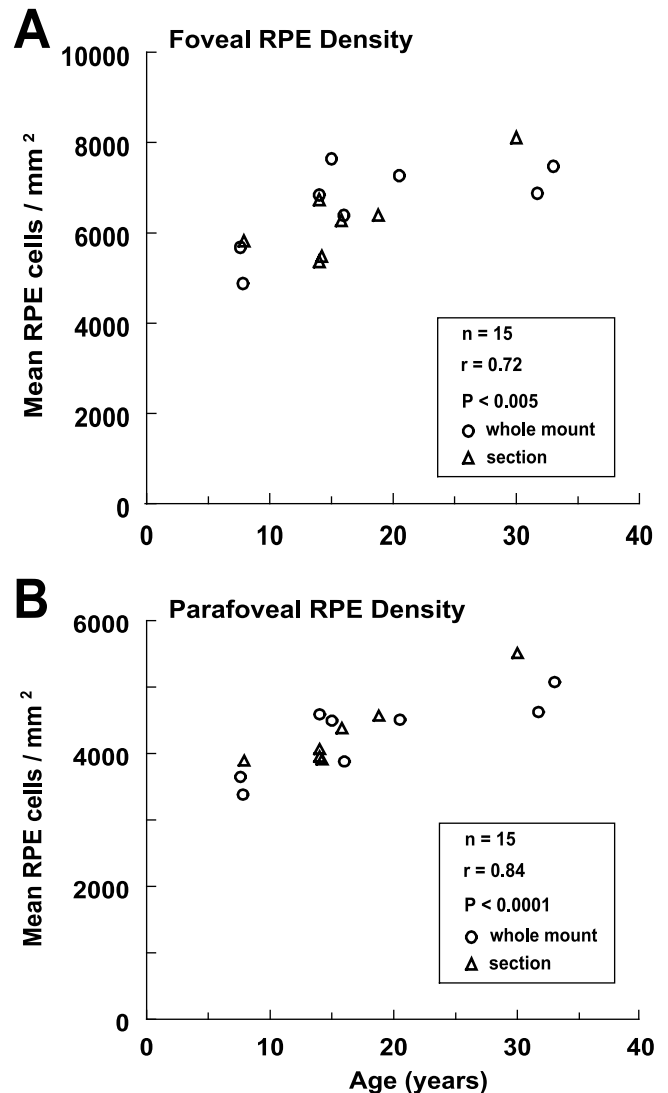


FIGURE 4. Correlation between RPE cell density and age. (A) Mean results for the three loci within 0.5 mm of the foveal center. (B) Mean results between 1- and 2-mm eccentricity.

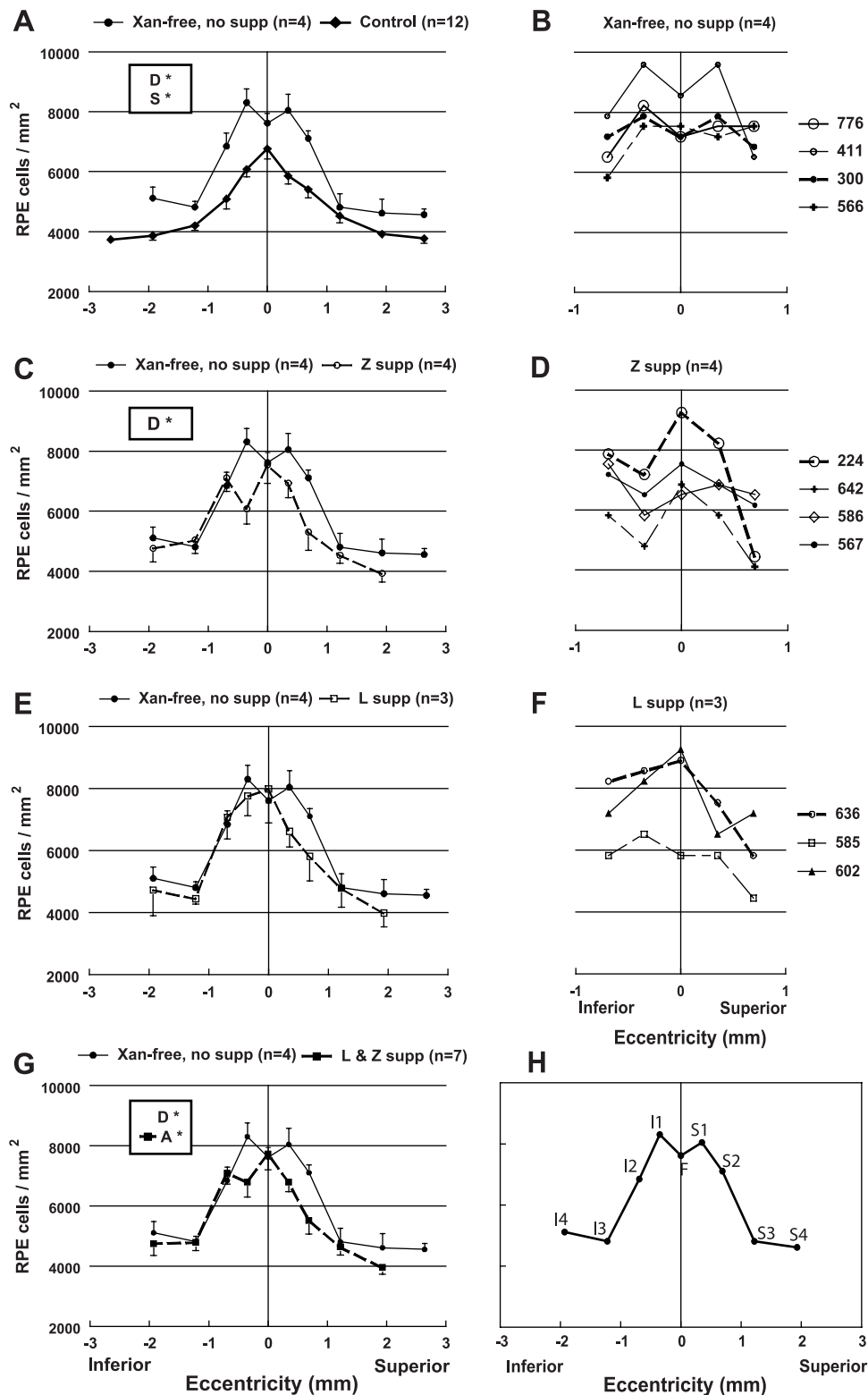


FIGURE 5. Effects of xanthophyll supplementation on RPE cell density profiles of xanthophyll-free low n-3 monkeys. (A) Mean RPE density profiles of xanthophyll-free ($n = 4$) and control monkeys. In this and subsequent figures, the mean profile of the control monkeys is based on the 12 animals with a mean age similar to the mean ages of the experimental groups (Table 1). (B) Individual foveal RPE density profiles of xanthophyll-free low n-3 monkeys, plotted with an expanded spatial scale. (C) Mean RPE density profile of xanthophyll-free, low n-3 animals supplemented with zeaxanthin ($n = 4$) compared with unsupplemented low n-3 animals ($n = 4$). (D) Individual foveal RPE density profiles of low n-3 monkeys supplemented with zeaxanthin, plotted with an expanded spatial scale. (E) Mean RPE density profiles of xanthophyll-free, low n-3 monkeys fed supplemental lutein ($n = 3$) compared with unsupplemented low n-3 animals ($n = 4$). (F) Individual foveal RPE density profiles of low n-3 monkeys supplemented with lutein. (G) Mean RPE density profile of the xanthophyll-free low n-3 animals supplemented with either lutein or zeaxanthin ($n = 7$), compared with the unsupplemented group ($n = 4$). (H) Profile of RPE density of unsupplemented xanthophyll-free, low n-3 monkeys showing the points used to calculate parameters A, D, and S for assessing changes in the density profile. The numerical values at the particular loci are labeled as: F in the foveal center; S1 as the superior locus (0.35 mm) closest to the foveal center; and S2, S3, and S4 at 0.69, 1.22, and 1.93 mm eccentricity. In the inferior region, the symmetrical corresponding values are labeled as I1, I2, I3, and I4.

Xanthophyll-Free, Low n-3 Animals with No Xanthophyll Supplement

The mean RPE cell density profile of the unsupplemented, xanthophyll-free, low n-3 animals is compared with the profile of normal control monkeys in Figure 5A. The mean curve for the xanthophyll-free monkeys is reproduced in Figure 5H with labels for the data points. To compare the density in the foveal center with the immediately adjacent points, we computed the

difference, D, between the RPE cell density in the center of the fovea, F, and the average of the two adjacent points I1 and S1 at ± 0.35 mm eccentricity for each retina. This parameter was positive when the RPE profile had a peak at the foveal center and negative when the profile showed a dip at the center similar to that in the xanthophyll-free, low n-3 animals.

To compare the total number of RPE cells in the different groups, the sum, S, of the RPE cell densities of the five central

TABLE 2. D (mean ± SEM) of Control Group and Selected Combinations of Experimental Groups

Control (n = 12)	Low n-3	Comparison with . . .			Comparison with . . .			
		Control	No Supp.	Adequate n-3	Control	Low n-3		
797 ± 238	No supp. (4)	-558 ± 257	P = 0.009	NA	No supp. (2)	-938 ± 431	—	—
797 ± 238	Z supp. (4)	1028 ± 327	P = 0.620	P = 0.009	Z supp. (2)	-1116 ± 78	—	—
797 ± 238	L supp. and Z supp. (7)	929 ± 303	P = 0.740	P = 0.009	L supp. and Z supp. (4)	-686 ± 403	P = 0.007	P = 0.010
797 ± 238	Supp. and no supp. (11)	NA	NA	NA	Supp. and no supp. (6)	-770 ± 283	P = 0.001	NA

D = F - (I1 + S1)/2 (variables defined in Figure 5H) and is a measure of the degree of the central foveal peak or depression. The number of animals in each group is enclosed in parentheses. P is based on two-tailed t-tests with no correction for multiple comparisons. Normalization of D by dividing by S reduces the P for the control versus the low n-3, no-supplement group slightly to P = 0.007, but does not change other probabilities shown. NA, Either obvious differences in profiles made combining groups inappropriate, or the cell implied comparison of a group with itself; —, one of the comparison groups had n < 4.

points within 1.8-mm diameter of the foveal center (I2+I1+F+S1+S2) was tabulated for each monkey. Finally, the symmetry of the profile was evaluated by comparing the mean density superior to the fovea, As, [(S1+S2+S3+S4)/4] to the mean density inferior to the fovea, Ai, [(I1+I2+I3+I4)/4]. Comparisons of the parameters are summarized in Table 2 for D, Table 3 for S, and Table 4 for Ai and As. We will refer to these tabular summaries as we describe the graphs for each of the treatment groups. In addition, when parameters are significantly different for a particular comparison that is illustrated in a graph (P < 0.01), the parameters are displayed with an asterisk in a box in the top left corner of the graphs.

When comparing the control animals with the xanthophyll-free monkeys, we cannot exclude the possibility that differences in the perfusion technique could influence the results. However, one of the control monkeys (M43) whose retina was serially sectioned was perfused in exactly the same manner as the experimental animals. The RPE profile for M43 had a peak at the foveal center, and it was symmetric as in the other control monkeys. M43's S, however, was 32,888, which is approximately 12% higher than the mean of the other age-matched control monkeys. These results suggest that the improved perfusion procedure used for the experimental animals may have improved recognition of nuclear profiles and resulted in a slightly higher overall cell density (S), but the procedure did not change the shape and symmetry of the profile. The effect on the estimates of cell density did not appear to be large, because the mean S of the adequate n-3 group (n = 6) with the improved perfusion was only slightly higher than that of the control group (8%, Table 3). Note that comparisons between dietary treatment groups of the experimental monkeys are not affected by this reservation, because all experimental monkeys were perfused in the same manner. Thus, the fact that the low n-3, xanthophyll-free animals (n = 4) had higher Ss than the adequate n-3 animals (n = 6, P < 0.001) strongly supports the idea that the number of RPE cells is modulated by our dietary manipulations.

The RPE profile of the xanthophyll-free, low n-3 animals differed from control animals in having a prominent dip, rather than the normal peak, at the foveal center (Fig. 5A). The change in D was significant, whether the raw densities were used (P = 0.009, Table 2), or the Ds were normalized by

dividing them by the Ss to adjust for the difference in overall RPE cell density (P = 0.007). The groups also differed in the overall RPE cell density, S. The mean of the low n-3 group was 29% higher than the mean of the control group (P < 0.001). We believe this is a real effect, although slightly exaggerated in magnitude by the differences in perfusion procedure discussed earlier. The profile of both the xanthophyll-free, low n-3 group and the control animals was symmetric along the vertical meridian (Table 4).

Figure 5B illustrates the consistency of the shape of the foveal profile in the xanthophyll-free, low n-3 animals by plotting the profiles of individual animals at an expanded scale. Three of four animals had a dip at the foveal center and one (monkey 566) had a relatively flat profile, but none of these animals had the clear central peak that is characteristic of normal RPE profiles (Fig. 3; see also Ref. 15).

Effects of Xanthophyll Supplementation of Low n-3 Animals

Zeaxanthin supplementation altered the RPE density profile of the low n-3 animals (Fig. 5C). The mean RPE profile of the zeaxanthin-supplemented animals peaked at the foveal center and the dip in the profile was offset inferiorly. When examined individually (Fig. 5D), the profiles of the three animals with the longest duration of supplementation (14–24 months) had maximum RPE density at the center of the fovea. The animal with the shortest period of supplementation (monkey 586, 8 months) was the only one that did not have a sharp foveal peak. All four animals in this group had a dip in the profile shifted inferiorly relative to the fovea.

Lutein supplementation also eliminated the central dip in the RPE cell density profile of the low n-3 animals (Fig. 5E). Inspection of the graphs for the mean of the group (Fig. 5E) and the individual animals (Fig. 5F) suggests in addition that the number of RPE cells in the superior retina was reduced. As a result, the curve shifted inferiorly, as also occurred after zeaxanthin supplementation (Fig. 5C). However, because of the small number of animals in the lutein group, the results should be considered tentative.

To confirm the overall effects of xanthophyll supplementation of the low n-3 animals, we combined data from the

TABLE 3. S (mean ± SEM) of Selected Combinations of Experimental Groups with n ≥ 4

Control (n = 12)	Low n-3	Comparison with . . .			Comparison with . . .			
		Control	No Supp.	Adequate n-3	Control	Low n-3		
29,294 ± 11,082	No supp. (4)	37,931 ± 1,434	P < 0.001	NA	No supp. (2)	30,994 ± 856	—	—
	Z supp. (4)	32,972 ± 2,015	P = 0.12	P = 0.09	Z supp. (2)	31,519 ± 681	—	—
	L supp. and Z supp. (7)	33,963 ± 1,749	P = 0.03	P = 0.16	L supp. and Z supp. (4)	31,853 ± 437	P = 0.21	P = 0.40
	Supp. and no supp. (11)	35,406 ± 1,325	NA	NA	Supp. and no supp. (6)	31,567 ± 397	P = 0.17	NA

S = I1 + I2 + F + S1 + S2—that is, the sum of cell densities at the five central loci defined in Figure 5H. The number of animals in each group is enclosed in parentheses.

TABLE 4. Assessment of Symmetry and Asymmetry of RPE Profiles

	As	Ai	Ai - As	P	
No supplement					
Controls (12)	4927 ± 175	4853 ± 153	-74 ± 167	0.67	Symmetric
Xan-free low n-3 (4)	6146 ± 167	6259 ± 304	113 ± 421	0.81	Symmetric
After supplement					
Low n-3 + L and Z (7)	5235 ± 193	5863 ± 319	627 ± 201	0.02	Asymmetric
Adequate n-3 + L and Z (4)	5535 ± 96	5287 ± 79	-248 ± 48	0.01	Asymmetric

The mean cell density superior to the fovea, $As = [(S1 + S2 + S3 + S4)/4]$ is compared with the mean density inferior to the fovea; $Ai = [(I1 + I2 + I3 + I4)/4]$. Data are expressed as the mean ± SEM. Variables are defined in Figure 5H. The number of animals in each group is enclosed in parentheses. Because of the limited number of xanthophyll-free adequate n-3 animals, their profiles could not be tested statistically for symmetry, but the appearance of the curves did not suggest a substantial departure from symmetry. P by two tailed t -test.

lutein-supplemented animals and the zeaxanthin-supplemented animals. Figure 5G compares the mean RPE density profiles of low n-3 monkeys with and without xanthophyll supplements. RPE profiles of the supplemented group had Ds that differed from those of the unsupplemented group ($P = 0.009$, Table 2). Supplementation also reduced the density of RPE cells in the superior retina, resulting in a significant asymmetry in the distribution ($P = 0.02$, Table 4).

Results in the supplemented low n-3 animals are compared with those in control animals in Figure 6. The most striking effects of supplementation were the presence of a central peak in the fovea and an asymmetry along the vertical meridian in both the zeaxanthin- (Fig. 6A) and the lutein- (Fig. 6B) supplemented animals. When these two supplemented groups were combined (Fig. 6C), the Ds for the peaks did not differ from the control (Table 2), but the curves were asymmetric compared with the control ($P = 0.02$, Table 4). Overall, there was a clear asymmetry in the RPE profile resulting from xanthophyll supplementation. In addition, the overall density of RPE cells, S , was reduced after xanthophyll supplementation (Table 3). We will return to this point in the Discussion.

Effects of Xanthophyll Supplementation on Xanthophyll-Free, Adequate n-3 Animals

Because only two xanthophyll-free, adequate n-3 animals did not receive a supplement, we relied on inspection of the graphs to judge the characteristics of their retinas. The unsupplemented, adequate n-3 animals (Figs. 7A, B) had a dip in the RPE profile at the foveal center similar to the dip observed in the low n-3 animals. This similarity suggests that the central dip is related to the absence of xanthophylls, whether n-3 fatty acid levels are low or normal.

The effects of supplementation with lutein or zeaxanthin on adequate n-3 animals were similar (Fig. 7D), and so data from the two lutein-supplemented and the two zeaxanthin-supplemented monkeys were averaged (Fig. 7C). Xanthophyll supplementation did not eliminate the abnormal foveal dip in the RPE profile. Thus xanthophyll supplementation of the adequate n-3 animals had less effect on the central fovea than supplementation of the low n-3 animals ($P = 0.01$, Table 2). Indeed, the density profiles of the xanthophyll-free, adequate n-3 animals were very similar, with or without supplementation (Fig. 7C).

The lack of a foveal peak in the adequate n-3 animals was a significant difference from the control whether the control was compared only with the four supplemented animals (Fig. 7E, Table 2) or with the combined group of six supplemented and unsupplemented animals (Fig. 7F, Table 2). For the adequate n-3 animals, the overall density of RPE cells, as measured by S , was about the same for supplemented or unsupplemented animals. RPE density of the adequate n-3 animals was lower than the RPE density of the low n-3 animals, but there

were too few animals to derive a statistically significant comparison between the adequate n-3 and low n-3 groups. A clearer outcome of xanthophyll supplementation of the adequate n-3 group was an asymmetry in the RPE profile, with higher RPE densities in the superior retina ($P = 0.01$, Table 4).

Comparison of the Effects of Xanthophyll Supplementation on Low n-3 and Adequate n-3 Animals

When we compared the RPE density profiles of the low and the adequate n-3 animals that received supplemental zeaxanthin or lutein, both fatty acid groups had a dip in the profile, but the position of the dip was shifted inferiorly in low n-3 animals (Fig. 8). This shift caused a difference in D between the xanthophyll-supplemented fatty acid groups (Table 2) and it contributed to a difference in the measure of asymmetry $Ai - As$ (Table 4). It is striking that after supplementation (Table 4) RPE cell densities were higher in the *inferior* retina of the low n-3 animals but the asymmetry was reversed so that cell densities were higher in the *superior* retina of the adequate n-3 animals. These results imply an intricate interaction between the xanthophylls of the macular pigment and the n-3 fatty acids of the retina.

DISCUSSION

Age and RPE Cell Density

The effect of age on RPE cell density in human eyes has been studied numerous times, with contradictory results. Our data from monkey retinas are consistent with the study by Harman et al.¹⁵ of RPE cells in wholemounts of human retinas. RPE cell density increased with age in the central retina. In contrast, earlier studies have reported a reduction with age in foveal RPE cell density^{9,12} or no change.^{10,11,14} The possible reasons for these conflicting results include the large variability in the normal number of RPE cells,^{9,15} small sampling areas,¹⁰ and variations in sampling location.^{11,12,14} Precise location of the samples is very important when they are taken within 2 mm of the foveal center, where cell density varies by a factor of 2.¹⁵ In our study, we were able to locate the foveal center very accurately in both wholemounts and serial sections, to establish a secure reference for the sample locations. Furthermore, the use of serial sections avoided potential sampling biases that can occur with analyses based on single histologic sections⁴⁷ and enabled us to achieve good agreement with data from wholemounts. We found an increase in foveal RPE cell density from 7 years (young adulthood) to 33 years (advanced age). Age-matching of animals assigned to subgroups enabled us to avoid confounding by age in the experimental comparisons.

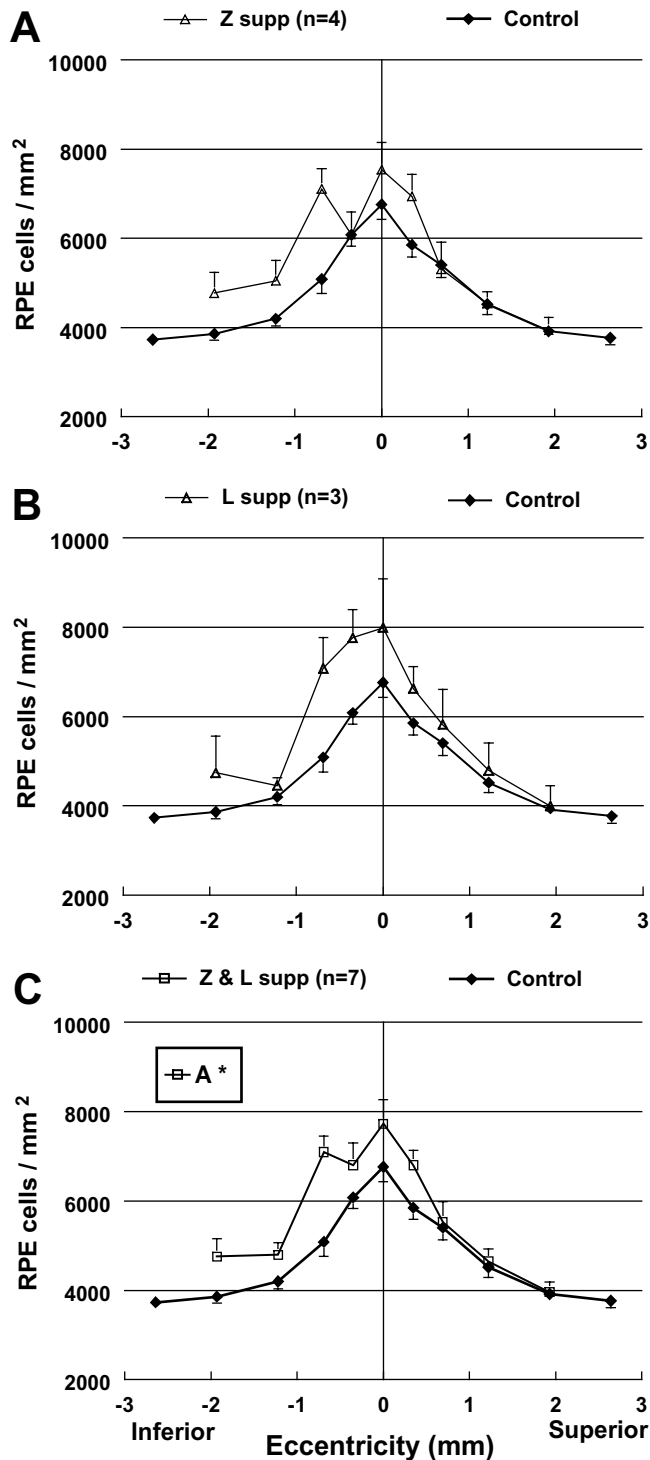


FIGURE 6. RPE density profiles of supplemented xanthophyll-free, low n-3 animals compared with control animals. (A) Mean RPE density profiles of zeaxanthin-supplemented ($n = 4$) and control animals. (B) Mean RPE density profiles of lutein-supplemented ($n = 3$) and control animals. (C) Combined data from lutein- and zeaxanthin-supplemented animals ($n = 7$) compared with control animals.

Response of the RPE to Lack of Dietary Xanthophylls and n-3 Fatty Acids

In contrast to control animals, monkeys reared without dietary xanthophylls consistently showed a dip in the RPE cell density profile at the center of the fovea, regardless of the fatty acid status of the animal. However, there appeared to be a second

effect of low n-3 fatty acid status, which was associated with an increase in the overall RPE cell density in the central retina. Indeed, S increased in an orderly fashion from adequate n-3, xanthophyll-supplemented animals to low n-3, xanthophyll-supplemented animals to low n-3, unsupplemented animals (Table 3). This new finding suggests that increased foveal RPE cell density may be a compensatory response to reduced ability of RPE cells to digest and recycle materials and to participate in the retinoid cycle. Increased RPE density may be induced by stress on the photoreceptor-RPE complex due to altered metabolism or other unfavorable factors. Similar mechanisms may underlie the increased density of RPE cells with normal aging.

Effects of Xanthophyll Supplementation

When xanthophyll supplements were given to the experimental animals, there was a complex interaction between the fatty acid status and xanthophyll status. One of the more tantalizing findings was the asymmetry in RPE profiles. Eyes of supplemented low n-3 animals had lower cell density than unsupplemented animals in the superior retina, and the profile shifted inferiorly so that the fovea had a central peak as in control animals, but a dip was still present inferior to the fovea. In contrast, the RPE profile of supplemented, adequate n-3 animals shifted superiorly, but the dip remained in the foveal center.

Because of the low number of xanthophyll-free, adequate n-3 animals, it is difficult to be certain whether the xanthophylls by themselves can modulate the overall number of RPE cells in the central retina. However, the induction of asymmetries in the RPE profile by supplementation for as little as 6 months implies to us that the xanthophylls could stimulate migration of RPE cells that would alter the RPE profile. Migrations of RPE cells relative to the photoreceptor layer are believed to be an integral part of the development of the eye,^{48,49} and the capability for migration may continue into adulthood.^{13,14} These cellular movements could be a response to the modified pattern of blue light impinging on the retina as macular pigment accumulates. Alternatively, the macular xanthophylls or the distribution of light may affect the distribution and diffusion of cellular metabolites that act as chemotactic agents or trophic factors that could stimulate migration of RPE cells.⁵⁰ Differential distribution of those factors related to gravity or to differences between superior and inferior retina in light intensity⁵¹ could contribute to the emergence of asymmetries in the RPE profile. Our results suggest that macular pigment plays a vital role in shaping and maintaining the RPE cell distribution in the foveal region. Further work is needed to determine the factors establishing the asymmetric distribution of the RPE cells in response to dietary manipulation of lutein and zeaxanthin.

Cell proliferation and cell death may also contribute to the observed changes in cell density profiles. Proliferation of RPE cells in monkeys and humans has been reported in pathologic conditions⁵² such as ARMD⁵³ and retinal detachment,⁵⁴ but cell division is rare in adulthood^{7,48,49,55} and may occur primarily in the periphery.¹⁴ In our study, we found no mitotic figures in the foveal region, but evidence of cell division may be difficult to detect when only a small region is examined at a single time point.⁵⁶ A local loss of RPE cells causing the foveal dip would be consistent with increased damage due to increased exposure to short-wavelength light in the absence of macular pigment, plus the loss of any antioxidant actions of xanthophylls. It would also be consistent with the window defects in fluorescein angiograms of these and previous groups of xanthophyll-free monkeys¹⁶ (Neuringer M, et al. *IOVS* 1999;40:ARVO Abstract 882). These defects were shown to correspond to lipoidal degeneration in individual RPE cells, together with patches of RPE cells with high lipofuscin and low melanin content.⁵⁷

Effects of Fatty Acid Status

α -Linolenic acid is essential as a dietary precursor for DHA, a major component of photoreceptor outer segment mem-

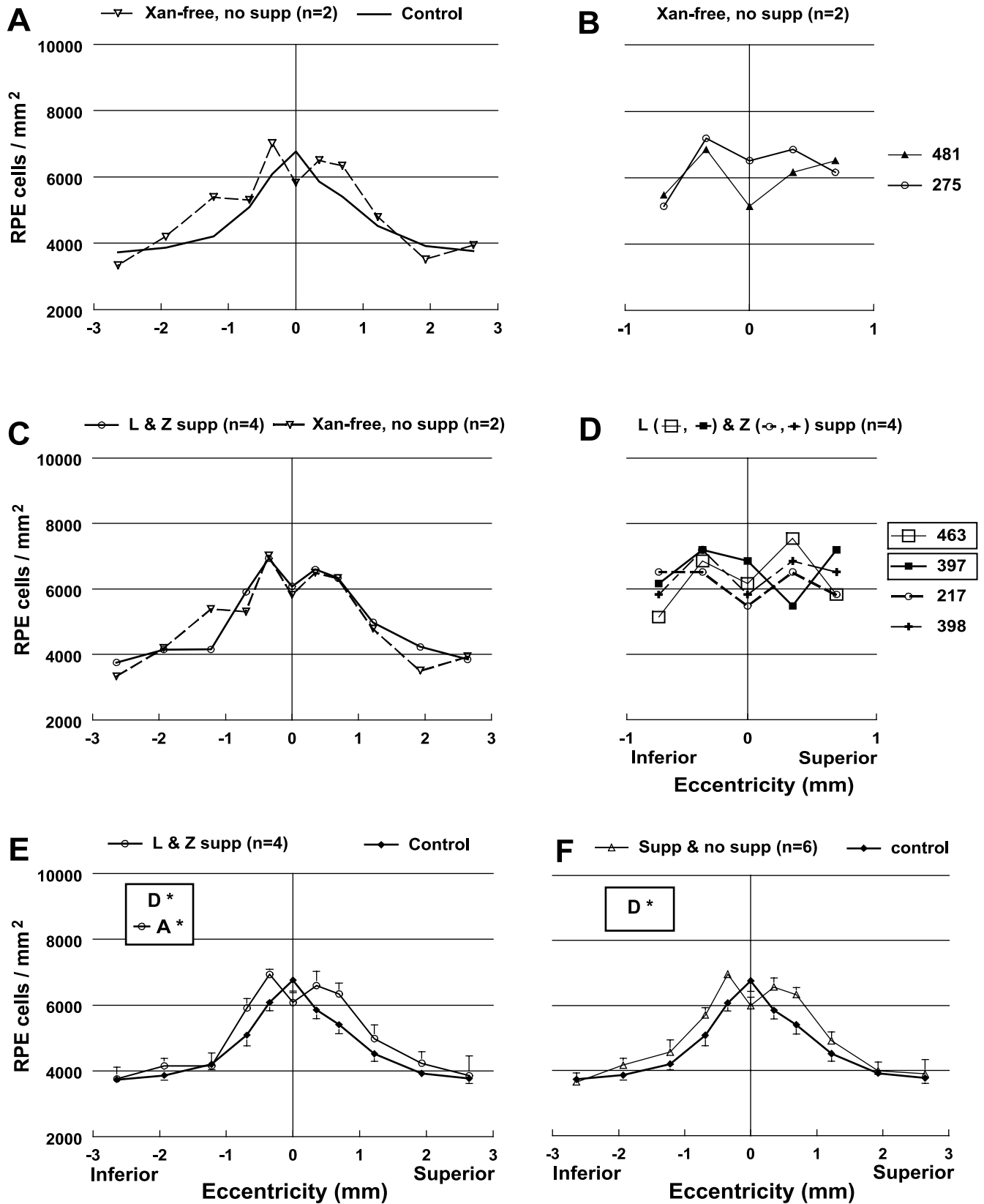


FIGURE 7. Effects of xanthophyll supplementation on RPE cell density profiles of xanthophyll-free, adequate n-3 monkeys. (A) Mean RPE density profile of two xanthophyll-free animals compared with the control animals. (B) Magnified RPE density profiles of the two xanthophyll-free, adequate n-3 monkeys. (C) Mean RPE density profile of the adequate n-3, xanthophyll-free monkeys that received supplemental zeaxanthin or lutein ($n = 4$), compared with the unsupplemented group ($n = 2$). (D) Magnified RPE density profile of individual supplemented animals. IDs of those that received supplemental lutein are enclosed by rectangles in the figure key. (E) Mean RPE density profile of the xanthophyll-free, adequate n-3 monkeys receiving supplemental zeaxanthin or lutein ($n = 4$) compared with control animals. (F) Mean RPE density profile in all the xanthophyll-free, adequate n-3 monkeys, with or without carotenoid supplementation, ($n = 6$) compared with control animals.

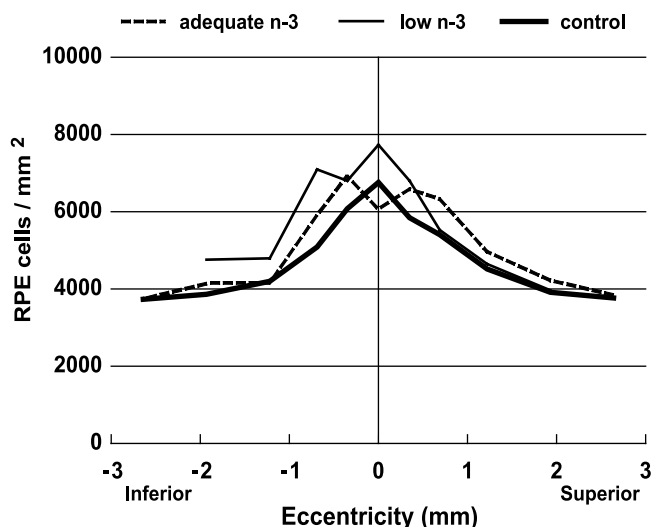


FIGURE 8. Mean RPE density profiles of control and xanthophyll-free monkeys after supplementation with xanthophylls. Data from groups that received supplemental zeaxanthin and lutein were combined to compare the effects of xanthophyll supplementation on animals with different fatty acid status.

branes.⁵⁸ In human and monkey infants, delayed development of visual acuity and abnormalities of retinal function result from low dietary levels of α -linolenic acid.^{22,23,59} A lack of dietary n-3 fatty acids during development affects the composition and function of the photoreceptor outer segments.⁶⁰⁻⁶² It may also affect the RPE cells directly, or through interactions with *cis*-retinal and interphotoreceptor retinoid binding protein (IRBP), which associate selectively with DHA.²⁹ The RPE has a much lower content of DHA than the photoreceptors. Chen et al.²⁹ proposed that the outer segment-RPE gradient of DHA drives the transfer of regenerated 11-*cis* retinal from the RPE back to the outer segments. In animals deficient in n-3 fatty acids, including monkeys fed the same low n-3 fatty acid diet used in this study, the DHA in the outer segments is largely replaced by 22:5n-6, a fatty acid with one less double bond than DHA.^{23,60,63} It is not known how this compositional change might affect either the process of photoreceptor disc degradation by the RPE or the transport of retinoids between the outer segments and the RPE. At the least, our results suggest that tissue levels of DHA may modulate the RPE cell distribution in the central retina, which may be linked to a homeostatic regulation to maintain high recycling rates of retinoids.

Epidemiologic studies regarding dietary fat and risk for age-related macular degeneration (AMD) have produced conflicting results, but several studies have found that reduced risk of advanced AMD is related to increased intake of DHA, total long-chain n-3 fatty acids, and/or fish, the richest dietary source of these fatty acids⁶⁴⁻⁶⁷ (SanGiovanni JP, et al. *IOVS* 2003;44:ARVO Abstract 2112). The outcome may depend on the balance of different fatty acids in the diet as well as the amount of n-3 fatty acids consumed.⁶⁵ Our results add evidence that further investigations of the effects of dietary fats on RPE function would be worthwhile, and these effects may be related to risk for AMD.

In conclusion, we have demonstrated that the density profiles of RPE cells are altered in xanthophyll-free monkeys. Lack of xanthophylls led to a dip, rather than a peak, of cell density in the foveal center. Dietary levels of n-3 fatty acids affected both the cell density and the response to xanthophyll supplementation. Thus, both macular pigments and n-3 fatty acids appear to be essential for the development and/or maintenance of a normal density profile of RPE cells in the central

retina. Changes in the foveal RPE density profile may be a response to physiological stresses that could render the macular region vulnerable to degenerative disease.

Acknowledgments

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