AN ABSTRACT OF THE THESIS OF

Rhianna A. Derenick for the degree of Master of Science in Nutrition and
Food Management presented on December 1, 2006.

Title: The Role of Lutein and Zeaxanthin in Protecting the Retina from Light Damage

Abstract approved:

_____
Emily Ho

Purpose:

The xanthophyll carotenoids lutein and zeaxanthin are concentrated in the *macula lutea* of the retina. It is thought that the two plant pigments may play a role in preventing the development of age-related macular degeneration (AMD). Exposure to damaging light plays a role in retinal deterioration and AMD. The antioxidant capabilities of lutein and zeaxanthin, combined with their ability to filter short-wavelength blue light, may serve to protect the retina from photooxidative damage and photoreceptor cell death. The goal of this thesis was to investigate whether dietary supplementation of lutein and zeaxanthin protects the retina against damaging light in the chick model. In the first study, chicks were fed diets supplemented with lutein and zeaxanthin and exposed to damaging light; functionality of the retinas was assessed with electroretinography. The second study attempted to create lutein and zeaxanthin-depleted chicks to investigate

whether carotenoid deficiency exists and the impact of low lutein and zeaxanthin status on retinal functionality.

Methods:

In the first study, 12 newly hatched White Leghorn chicks were fed a control diet or a lutein and zeaxanthin supplemented diet for 32 days. The supplemented diet was prepared by supplementing the control diet with 30μg per g diet of lutein and 15μg per g diet of zeaxanthin. All chicks were raised in cyclic light (12 hrs light/12 hrs dark). At 32 days of age, 6 were exposed to 10 hours of intermittent light (1 hr light/2 hrs dark, for a total of 10 light hrs). Baseline and post-treatment electroretinography (ERG) were performed on all chicks at 32 days and 36 days. Biochemical analysis of lutein and zeaxanthin in plasma, retina, and other tissues was assessed by HPLC at Day 40.

In the second study, lutein and zeaxanthin-depleted chicks were to be produced from White Leghorn laying hens fed a deficient diet. Hens were placed on a deficient diet and after 28-30 days mated with a rooster. The subsequent fertilized eggs were collected. The eggs were stored in an incubator for hatching. Biochemical analysis was used to confirm that egg yolks were deficient in lutein and zeaxanthin. Hatched chicks were to be raised on deficient diets for 32 days and exposed to the same methods employed in the first study.

Results:

In the first study, plasma and tissue levels of chicks significantly increased their contents of lutein with the supplemented diet ($p < 0.0001$); the same result was found for zeaxanthin except for the plasma and skin. Interestingly, total retinal zeaxanthin levels

were greater than the total retinal lutein levels in supplemented and control chicks (p <

0.005, 7.60 µg/g ± 3.73 compared to 6.41 µg/g ± 1.44 and 4.28 mg/g ± 2.0 compared to

3.62mg/g ± 1.09, respectively). In the retina, zeaxanthin also was found to exist in the *cis*

form. There were significantly greater levels of *cis* in supplemented diet groups

independent of light exposure. For both the supplemented and control diet chicks, there

were significant increases between baseline and post-treatment ERG amplitude responses

in both non-light exposed and light exposed chicks (p= 0.007,α=0.05). No significant

increases were found in the maximum amplitude or sensitivity (Kd) ERG responses for

lutein supplemented or control chicks between treatment groups.

In the second study, the lutein and zeaxanthin content of yolks from eggs laid by

laying hens fed a carotenoid-deficient diet declined from Day 2 to Day 36 (11.19 µg/g

and 18.09 µg/g to 0.19 µg/g and 0.23 µg/g, respectively). However, the laying hens

suffered dramatic weight loss, decreased egg laying and hatchability, and illness during

the course of the experiment. Even with new housing facilities, a new rooster, and

increased animal care, the hens did not produce carotenoid-depleted progeny and thus the

proposed light exposure experiments could not be accomplished.

Conclusions:

In the first study, plasma and tissue lutein and zeaxanthin concentrations in chicks

fed supplemented diets significantly increased; the retina had higher levels of zeaxanthin

even though the diet had greater lutein levels, whereas in the other tissues lutein was

greater than zeaxanthin. This suggests the retina has a preference for zeaxanthin. The *cis*

isomer of zeaxanthin, which is isomerized from the *trans* form by bright light, was only

present in the retina. Supplementation increased both *cis* and *trans* zeaxanthin, with no increase in the *cis* form as a result of light treatment. In addition, there were no significant differences between control and supplemented diet chicks in retinal photoreceptor sensitivity and maximum amplitude response with light damage. These results indicate that damage to the retinas of both supplemented and control diet chicks <u>did not occur</u> as a result of light damage treatment.

<u>In the second study</u>, assessment of retinal function in carotenoid-depleted chicks could not be accomplished due to lack of production of depleted progeny. Lutein and zeaxanthin concentrations in egg yolks dramatically decreased with subsequent deterioration of the maternal hens' healths. The conclusions drawn from our efforts were that producing carotenoid-deficient chicks from a maternal hen is difficult and perhaps physiologically impossible. Modifying the experimental design for the deficient hen and investigating other methods to produce carotenoid-depleted progeny are necessary for further studies.

In conclusion, these two studies were the first attempts at investigating retinal function, dietary intervention, and light damage within the chick model. With greater power and different experimental approaches more promising results that can be generalized for the human eye disease, AMD, may be uncovered.

The Role of Lutein and Zeaxanthin in Protecting the Retina from Light Damage

by
Rhianna A. Derenick

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented December 1, 2006
Commencement June 2007

Master of Science thesis of <u>Rhianna A. Derenick</u>
presented on <u>December 1, 2006</u>.

APPROVED:

_____

Major Professor, representing Nutrition and Food Management

_____

Chair of the Department of Nutrition and Exercise Sciences

_____

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection
of Oregon State University libraries. My signature below authorizes
release of my thesis to any reader upon request.

_____

Rhianna A. Derenick, Author

ACKNOWLEDGEMENTS

CONTRIBUTION OF AUTHORS

Dr. Yingming Wang, M.D., assisted with biochemical analyses. Dr. Brett Jeffrey, Ph.D., assisted with interpretation of electroretinography data and analyses. Dr. Emily Ho, Ph.D., assisted with the design and formatting of this document. Dr. Gita Cherian, Ph.D., gave insight to the diets and raising of laying hens in Chapter 5.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

The Role of Lutein and Zeaxanthin in Protecting the Retina from Light Damage

CHAPTER 1

INTRODUCTION

1.1 Lutein and Zeaxanthin and Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is a leading cause of irreversible vision loss in developed countries today (1). It is a degenerative condition of the region of the retina responsible for central vision (*macula lutea*). Light damage to the retina may be an important factor in the development of this eye disorder. There are currently no effective treatments for AMD, thus there has been increased interest in possible prevention strategies, especially through nutrition. Epidemiological evidence suggests that a high intake of foods rich in carotenoid plant pigments such as lutein and zeaxanthin are correlated with a lower risk for AMD (1). In addition, plasma circulating levels of lutein and zeaxanthin have inverse correlations with risk for AMD (2-4). Also, AMD progression was slowed with lutein and zeaxanthin supplements in a prospective cross-sectional study (5).

The *macula lutea* of the retina contains lutein and zeaxanthin, giving the region its characteristic yellow color. Lutein and zeaxanthin are found in abundance in colorful plant sources such spinach, corn and yellow peppers. Of the ten carotenoids found in the body, lutein and zeaxanthin are exclusively concentrated in the *macula lutea*. Patients with AMD and people with an increased risk for AMD were correlated with having low macular pigment density (5); in addition, patients with high macular pigment density showed preservation of visual cone function (6). Dietary lutein and zeaxanthin either in

the form of vegetables or supplements can increase the amount of macular pigment (7-9).

This is important because dietary intake of lutein has been shown to influence plasma

concentrations and its content in the *macula lutea* (9). It is thought that lutein and

zeaxanthin protect the retina against light damage since these compounds are antioxidants

and act as filters of blue light, the most damaging light to the retina (10). Thus, for the

aging human eye, lutein and zeaxanthin may be important in decreasing phototoxicity in

the *macula lutea* and lower the rate of AMD progression. Animal models are useful for

studying dietary interventions and potential cellular mechanisms which are otherwise

difficult to do in humans; results can be generalized to longer periods in people since we

have variability in lifestyle conditions. It would be beneficial to use an animal model

whose retinas are similar to humans to address mechanisms of AMD development, such

as the monkey. However, since monkeys were not available for this study, avians were

chosen since their retinas are cone-dominant and accumulate carotenoids like human

retinas. Also, the pathology of the chick retina as a result of light damage has *not yet*

been ascertained, providing an opportunity for our lab to investigate this area.

Therefore, utilizing the chick as a <u>novel</u> model for AMD, we can examine

biochemical, physiological and dietary mechanisms of carotenoids and light damage

at the level of the retina which is not possible in the human.

1.2 Hypotheses

Therefore, this thesis project tested *two* hypotheses:

1) Lutein and zeaxanthin will protect the retinal photoreceptors after light exposure

in the chick.

2) Lutein and zeaxanthin deficiency results in greater damage to retinal photoreceptors after light exposure in the chick.

1.3 Specific Aims

The specific aims of this project were to:

1) To determine the effects of dietary lutein and zeaxanthin *supplementation* on retina function in chicks following light exposure as assessed by the electroretinogram.

In order to investigate whether these two carotenoids indeed protect the retinal photoreceptors after light exposure, supplementation of the chicks' diets was necessary to allow for accumulation in the retina. Once the chicks were exposed to light, assessment of retinal function with the electroretinogram determined whether there was a protective effect of dietary supplementation against the light exposure.

2) Produce lutein and zeaxanthin depleted chicks from a laying hen consuming a diet deficient in these carotenoids

Direct dietary intervention was not the appropriate method for studying carotenoid deficiency in the chick. Chicks raised on carotenoid-deficient diets retain normal lutein and zeaxanthin levels in the retina; therefore, for a chick to have complete retinal lutein and zeaxanthin deficiency, progeny from deficient laying hens must be produced.

3) To determine the effects of lutein and zeaxanthin *deficiency* on retina function in the deficient chicks following light exposure as assessed by the electroretinogram

Retinal function as a result of lutein and zeaxanthin deficiency after light damage was to be assessed using the chicks produced from carotenoid-deficient hens. These chicks would have had no macular pigment and therefore had little protection from damaging light. The damage that would have incurred as a result of light exposure might have been greater than normal due to the absence of carotenoids. This was to be reflected in the analysis of the electroretinography data.

1.4 Significance of Study

AMD is the leading cause of blindness in the population aged over 65 years. Nearly 15 million people in the U.S. alone have some form of AMD (11). Unfortunately, there is no effective treatment or cure for this disease, which raises concern as the elderly population experiences tremendous growth. Health costs for AMD can cost thousands of dollars; 460 billion dollars are spent annually in the U.S today (12). There have been many clinical studies suggesting that dietary consumption of lutein and zeaxanthin may protect against AMD (1-5). There is also evidence for a more direct association between the levels of lutein and zeaxanthin in the retina and the risk of AMD (6-9). However, there has not been a clinical study that has proven that these carotenoids actually prevent AMD. Lutein and zeaxanthin show the most promising evidence; however, the mechanisms of action of the carotenoids are still unknown. Investigating the mechanisms of lutein and zeaxanthin in protecting the retina from light damage will provide valuable

information to the field of nutrition about establishing adequate dietary levels for

prevention against AMD.

CHAPTER 2

LITERATURE REVIEW

2.1 Background

Among the various natural plant pigments, carotenoids comprise an important

group of more than 600 structurally different compounds (13). Lutein and zeaxanthin

belong to the carotenoid family, which includes the carotenes and lycopene. Many

carotenoids, such as lutein and zeaxanthin, have long been known as powerful

antioxidants. Carotenoids are components of the light harvesting system in chloroplasts

and play a role in protecting plants from photooxidative damage (13). The colors of

plants and plant foods are caused by the presence of carotenoids which do not contain the

green pigment chlorophyll; however, many carotenoids are still present in the green parts

of plants yet their colors are masked by chlorophyll. Humans and animals are not capable

of carotenoid biosynthesis; therefore, the presence of this group of pigments in their

bodies is solely dependent on dietary intake of pigmented foods.

Absorption efficacy of dietary lutein and zeaxanthin in the intestine and tissues is

greater than other carotenoids, which is attributed to their orientation in epithelial cell

membranes, thereby allowing for greater uptake (10). In foods, lutein and zeaxanthin are

found in their free form, bound to transport proteins, or esterified. Most studies show that

lutein and zeaxanthin are absorbed better from foods which contain the esterified forms

(presence of lipids). There is also evidence that zeaxanthin may enhance lutein absorption

in the intestine (14). Bioavailability and metabolism of carotenoids are affected by

several factors including food matrix properties, preparation, cooking, amount and

frequency of consumption, intestinal disease status, body fat, malnutrition and Vitamin A

status (15). Carotenoids are fat-soluble and thus are more readily absorbed with fat in a

meal; while high fiber may hinder absorption. In the food matrix, carotenoids are

resistant to isomerization; the all-*trans* form is the most abundant and stable form in

nature but several *cis* isomers are present in blood and tissues (16). Once they are

released from the food matrix, the hydrophobic carotenoids are absorbed from the diet

carried on chylomicrons from the enterocyte to the liver. They are then absorbed and

resecreted on plasma lipoproteins, VLDL, LDL and HDL (17). The carotenoids are then

protein bound, held in lipid vesicles, or solubilized in membranes and transferred in

blood to target tissues (18).   Serum concentrations of lutein and zeaxanthin tend to

reflect dietary intake and tissue concentration; however, they are highly variable among

subjects due to the variation in sources and subject response.

Distribution of carotenoids to tissues correlates with tissues that express high

amounts of HDL receptors (adrenal, testes, liver); however, distribution of lutein is

determined partly by LDL-receptor density (18). Adipose tissue is a major storage organ

for carotenoids as a result of the partition of carotenoids into fat as anhydrolutein (a

dehydrated product) while isomers and ester forms of lutein and zeaxanthin are the forms

found in human serum and tissues (19). Zeaxanthin dominates in the ovary as well (19).

Interestingly, both lutein and zeaxanthin selectively accumulate in the eye.   Both LDL

and HDL carry and deliver lutein and zeaxanthin to the retina where they accumulate

(20). HDL is the major transporter of lutein and zeaxanthin, and also under conditions of

either a low or high carotenoid diet (21). The uneven distribution of carotenoids suggests tissue-specific biological roles for these compounds.

Once distributed into tissues, pro-vitamin A compounds (α and β-carotene and β-cryptoxanthin) are converted biologically into Vitamin A in mammals. This is accomplished by central cleavage into two molecules of retinal through β-oxidation by the enzyme 15, 15'-dioxygenase. Metabolism of other carotenoids, including non-provitamin A compounds (xanthophylls e.g lutein, zeaxanthin), are also be cleaved by this enzyme but at a much lower rate. Efficacy of cleavage, substrate specificity, and genetic variations impact expression of the dioxygenase. Lutein and zeaxanthin are oxidized to their 3' keto derivatives, isomerized to the 6'S forms or converted to epilutein. Some carotenoids, such as lycopene and canthaxanthin, are metabolized by poorly understood pathways and thus their metabolic products are rarely detectable. The metabolites of carotenoids are what influence the expression of certain genes and enzymes, serve as antioxidants, and play other protective roles for the cell (22).

It is difficult to examine the specific metabolic effects of lutein and zeaxanthin alone in foods since they are very similar in terms of structure (**Figure 1**), metabolism and tissue storage. However, a few differences do exist between food sources for the two carotenoids. For example, the two foods that have the highest amount of lutein are the leafy green vegetables spinach and kale (23). Lutein is the most dominant xanthophyll in all food sources (19); major dietary sources include broccoli, peas, brussel sprouts, and egg yolk. The best sources of zeaxanthin are egg yolks, corn, orange peppers and oranges. Recent data suggest that lutein and zeaxanthin from egg yolks are highly bioavailable despite their low content (23).

2.2 The Retina and Lutein and Zeaxanthin

Lutein and zeaxanthin are polar, isomeric and are found throughout the tissues of the eye; however, they are heavily concentrated in the *macula lutea* of the retina. The function of the *macula lutea* is to produce the sharp central vision needed for activities requiring focusing like sewing, reading and driving. When it becomes degenerated, a small black hole in the center of the visual field appears; however, peripheral vision is unaffected. The degeneration of the *macula lutea* and the loss of macular pigment is what inevitably progresses to AMD. The *macula lutea* is a yellow, pigmented structure containing a central *fovea*. This area contains two types of photoreceptors (PRs), cones and rods, of which only cones allow for maximal central and color vision acuity and are where light exposure is most intense. PRs are contained throughout the *macula lutea*; however, cones are denser within the *macula lutea* rather than the peripheral retina and the *fovea* has exclusively cones. Rods are more concentrated in the perifoveal region and peripheral retina (24). Rods, used only in dim light, contain the protein rhodopsin, whose primary function is to generate neural signals to the brain once exposed to light ("bleaching") at 500nm. Cones have three opsins for color acuity (blue, green and red); only blue cones absorb light in the 400-450nm range (25), the most damaging light.

Within the *macula lutea*, lutein and zeaxanthin are found predominately in the PR axons, outer and inner plexiform layers, and PR outer segments (26;27). Zeaxanthin predominates in the central *fovea* while lutein is found in the periphery; this suggests that lutein may play a role in protecting rods, while zeaxanthin protects cones (28). It has

long been known that the two compounds accumulate in the macular pigment and are even present in different ratios (29). Lutein and zeaxanthin accumulate 500-fold higher in the *macula lutea* than in other tissues (at 1mmol/L) (30;31). Of all the carotenoids, only lutein and zeaxanthin are found in significant amounts in the retina, which implies there is a high specificity for their uptake (30). *In vivo* evidence of the selective accumulation of lutein and zeaxanthin in the retina was found first in monkeys; no macular pigment was observed after many years of being fed a carotenoid deficient diet (32). Further, supplementation of lutein and zeaxanthin in the diets showed increased levels of the carotenoids in the *macula lutea* after 32 weeks (32;33). A follow up study showed increased levels of carotenoids in the retina after being depleted from birth to 7-16 years of age and then having 6 months of supplementation (34).

The uptake, transport, and metabolism of these xanthophylls in the retina are mediated by specific xanthophyll-binding proteins; glutathione S-transferase (GSTP1) is a transferring protein for glutathione, but has also been shown to be specific for zeaxanthin in the human macula (35). The retina actually preferentially absorbs zeaxanthin rather than lutein while adipose tissue prefers lutein, suggesting that adipose tissue may compete with the retina for lutein (36).

It is suggested that the carotenoids specific orientations in biological membranes might help explain their high concentration in the retina as opposed to other carotenoids (37). Lipids of disc membranes in the retinal cells consist of phospholipids, primarily docashexanoic acid (DHA) an essential poly-unsaturated omega 3-fatty acid (22:6n-3) (38). The pigments readily localize by spanning the membranes and contacting their hydrophilic groups with the polar head groups of the lipid bilayer (39). They have been

shown to act much like cholesterol does in membranes: by increasing the order of phospholipid chains, surface density, decreasing permeability, increasing mechanical strength of the bilayer and increasing membrane fluidity (40). The ability of lutein and zeaxanthin to filter out blue light on entering the retinal tissue has the effect of decreasing chromatic aberration associated with lower wavelengths of light (39). This action, in addition to their disposition in cell membranes, gives insight as to why the retina specifically accumulates these carotenoids.

2.3   Protection against Light Damage by Lutein and Zeaxanthin in the Retina

Epidemiological studies have identified antioxidants with a substantially reduced risk of developing AMD, with lutein and zeaxanthin showing the strongest effect. When dietary intake of different carotenoids and antioxidant vitamins were analyzed in patients, the sum of lutein and zeaxanthin, had the strongest correlation against development of neovascular AMD (41). There is also evidence from clinical trials to support the hypotheses that lutein and zeaxanthin play a role in reducing the risk of progression of AMD. The Age-Related Eye Disease Studies (AREDS) were large randomized clinical intervention trials of high-dose vitamin and mineral supplements for AMD. These trials demonstrated, over 6 years, up to a 25% reduction in development of advanced disease or severe visual impairment in the group taking large, combined doses of zinc and vitamins A (as β-carotene), C and E. However, there is now increasing evidence for beneficial effects from lutein and zeaxanthin, which were not incorporated in the AREDS supplement (42). As a result, the role of lutein and zeaxanthin in the treatment of AMD was further investigated in the Lutein Antioxidant Supplementation Trial (LAST). LAST

was a randomized controlled trial investigating the role of lutein supplementation on progression of AMD in patients who already had atrophic AMD. The results of the study reported improvements in glare recovery, and distance/near visual acuity with lutein alone and lutein in combination with other antioxidants (5).

In a study using flicker photometry to measure macular pigment density in humans found less macular pigment in those at high risk for AMD (living in areas exposed to high amounts of sunlight) as compared to those at lower risk (43). Similar results were observed using resonance Raman spectroscopy to detect lutein and zeaxanthin levels in the maculas of individuals. One study found that levels of lutein and zeaxanthin were 32% lower in AMD eyes versus control eyes; patients who began to consume lutein supplements after diagnosis of AMD had normal macular pigment levels and were higher than patients with AMD not taking the supplement (44). Another study using Raman spectroscopy found that people with high sunlight exposure had reduced macular carotenoid levels (45). Patients in the early stages of AMD taking lutein supplements for one year had increased amplitudes of the electroretinogram harmonic component after retinal function assessment with a focal electroretinogram. This suggests that increasing the level of retinal antioxidants might influence macular function early in the disease process (46).

The first *in vivo* evidence suggesting that lutein and zeaxanthin in the retina may protect PRs from light damage and apoptosis was shown in quails fed a carotenoid deficient diet (47). This study found that retinal zeaxanthin dose dependently reduced the number of apoptotic PRs and microglial invasion in PRs after light damage, but not elevated zeaxanthin serum levels. In these deficient quails, the total number of apoptotic

rods and cones was increased after 14 hours of intermittent light exposure, with cones yielding the most, and declined rapidly at 48 hours. Avian cones contain oil droplets where lutein and zeaxanthin are stored. The cones of the quail retinas still retained bright yellow, red, orange and pale green oil droplets, despite the depleted diet. A follow-up study using quails with carotenoid deficient and supplemented diets further confirmed that PR death is prevented dose-dependently by retinal lutein and zeaxanthin (48). The study showed that high concentrations of lutein and zeaxanthin in the retina allow the PRs to endure damaging light exposure for a longer period of time before apoptosis is initiated. High retinal zeaxanthin correlated with less PR death, while zeaxanthin deficient retinas had high PR death. Therefore, it was concluded that PR death was significantly altered by dietary lutein and zeaxanthin.

Other studies investigating light damage and apoptosis in monkeys fed carotenoid-deficient diets who had retinas without macular pigment developed abnormalities which were consistent with defects in the cones (49). Defects included fragility, prone to splitting of photoreceptor axons, deterioration of membrane integrity and damaged retinal pigment epithelial cells. Retinal cells from carotenoid-deficient monkeys were more vulnerable to cell death from blue light in the macula, in the area where macular pigment normally is present (50). In normal monkeys the macula was protected compared with an area farther from the fovea where there is normally little macular pigment.

Several *in-vitro* studies have attempted to investigate carotenoids and protection of the retina at the cellular level. One study found that irradiation of the fluorophore A2-PE (a byproduct of the visual cycle which is photooxidized to lipofuscin in the retinal

pigment epithelia) suppressed A2-PE photooxidation in the presence of lutein and
zeaxanthin; zeaxanthin had the greatest effect. Lipofuscin produces reactive oxygen
species (ROS) which are derived from photoreceptor outer segment and cause cellular
damage to the retinal pigment epithelial cells. This suggests that the oxidative
photochemical reactions against which lutein and zeaxanthin protect include those
initiated by A2-PE (51). Another study found that in retinal pigment epithelial cells
cultured in oxygen, lutein and zeaxanthin reduced lipofuscin formation (52). An in-vitro
study investigating the blue light filter efficacy of lutein and zeaxanthin utilized
liposomes loaded into a hydrophilic core space with a dye excitable by blue light.
Fluorescence emission was lower in carotenoid-containing liposomes than controls when
exposed to blue light, indicating a filter effect (10).

Absorption of blue light by lutein and zeaxanthin has been proposed as a possible
mechanism protecting the retina against cell death as a result of photooxidative damage,
much like they do in plants. Almost all short wavelength ultraviolet light (290nm-
400nm) is absorbed by the cornea and lens; slightly longer short wavelength blue light
(400-520nm) reaches the *macula lutea* and is absorbed by the macular pigment (18).
According to their structure, most carotenoids exhibit absorption maxima around 450 nm,
which is within the absorption range for short-wavelength damaging blue light (400-
460nm). The ability of carotenoids to absorb light arises from the presence of their
polyene chains (23). The wavelength maximum of the absorption band is related to the
extent of the conjugation in the polyene chain. The filtering effects of lutein and
zeaxanthin were found to be more effective than lycopene and β-carotene (10). Lutein
has an absorption maximum of 445nm; zeaxanthin has 451nm. Therefore, both reduce

the amount of blue light in the retina and lower the chances of peroxyl radical-induced

oxidative chain reactions in the *macula lutea*. Both lutein and zeaxanthin contain nearly

identical conjugated polyene chains and hydroxyl groups, suggesting similarity in

physical properties (23); however, lutein possesses more rotational freedom in its ε ring

(39). Isomers of lutein and zeaxanthin are also present in low amounts in the retina since

the macula is exposed to bright light, which isomerizes carotenoids (19). Depending on

the number of double bonds, an array of *cis/trans* isomers is possible for a given

carotenoid (53). When carotenoids isomerize they form a mixture of mono- and poly-*cis*

isomers in addition to the all-*trans* form. In addition, carotenoids exist in several

stereoisomeric forms; two steroisomers of zeaxanthin have been identified in the human

macula lutea (31).

Another possible mechanism lutein and zeaxanthin may protect the retina from

light damage is by limiting retinal oxidative damage by quenching reactive oxygen

species (ROS); especially singlet molecular oxygen and peroxyl radicals. This decreases

opportunities for propagation of oxidative chain reactions and thus inhibits lipid

peroxidation. The retina is a highly aerobic tissue with an exceptionally high rate of

metabolism. Thus, the PR outer segments and retinal pigment epithelium experience the

greatest oxidative damage due to two facts: first, the partial pressure of oxygen is much

higher there (>30mmHg) than most tissues (54) and second, both contain extremely high

concentrations of long-chain polyunsaturated fatty acids (24). However, under specific

conditions, carotenoids may also act as pro-oxidants (55). Docosahexaenoic acid (DHA),

a major dietary omega-3 fatty acid, is shown to provide protection against light and

oxidative damage (56); however, light can release DHA from rod outer segment

membranes in the retina, preventing the protective effects (57). There is strong evidence suggesting that the photosensitizing molecule lipofuscin, which produces ROS, derived from oxidatively damaged PR outer segments accumulates in the RPE causes cellular damage (58). Lutein and zeaxanthin could protect the PR cells by reducing the amount of lipofuscin formed or by inhibiting oxidation by reacting with peroxynitrite to protect the RPE (58;59).

A third possible mechanism for protecting the retina is inhibition of apoptosis. In the quail studies discussed above, it was found that retinal zeaxanthin dose dependently reduced the number of apoptotic PRs as detected by the TUNEL assay and microglial invasion in PRs after intermittent light exposure. However, these studies only looked at the protection of the retina at a *structural* and *histological* level. It would be interesting to know *functionally* if the carotenoids are providing protection against light damage. Investigating at a functional level will allow for a more thorough understanding of the physiological mechanisms of light damage and dietary modifications.

In conclusion, using light damage and dietary interventions *in vivo* provides a valuable model for studying protective mechanisms against AMD. The *rat* as a model would appear to be an easy model to use to address our questions; most of the studies examining the effects of light damage on the retina have been done in this model. However, these animals do not concentrate carotenoids in the retina (60). Also, we are interested in macular degeneration and light damage, and cones dominate the *macula lutea*. Rats have very few cones; 99% of their PRs are rods, while only 1% are cones (61).

However, the quail model proved to be a useful model for studying the effects of carotenoid supplementation and protection of the retina from light. Other avian models, such as the chick, may just as well be a good model for investigating dietary modifications and retinal light damage. Chicks have large eyes and rapid growth, rendering this animal a useful tool. Thus, the chick is an ideal model for investigating the roles of lutein and zeaxanthin in protecting the retinal PRs from blue light damage.

CHAPTER 3

METHODOLOGY

To address the *first hypothesis* and *first specific aim* of this project (to determine

whether lutein and zeaxanthin protected the retina in chicks against light damage), the

following methodology was employed:

Newly hatched Leghorn chicks were fed a control diet or a lutein and zeaxanthin

supplemented diet for 32 days. To reach maximum carotenoid tissue content in response

to a supplemented diet can take 14-35 days after feeding (62). The supplemented diet

was prepared by supplementing the control diet with 30µg per g diet of lutein and 15µg

per g diet of zeaxanthin (Roche TG/P, Lutein 5%, Zeaxanthin 5%) for approximately 32

days (**Tables 1, 2**). Note that control levels of lutein and zeaxanthin are at 5.2 and 1.7

mg/kg, respectively, and supplemented levels were equally increased five and nine-fold

(27.2 and 15.3mg/kg, respectively). Our decision to increase the supplementation five

and nine-fold for the carotenoids was decided based on personal communication that the

carotenoid concentration of the retina would double with the said increases in the diet.

Also, vitamins and minerals were added to the diet by the food company to ensure

adequate nutrition for developing chicks (**Table 3**).

All chicks were raised in cyclic light (12 hours light/12 hours dark). At 32 days

of age, they were subjected to one of two treatments: kept in cyclic (control) or exposed

to 10 hours of intermittent (experimental) light.

3.1 Electroretinography (ERG)

ERG was used to assess retinal cone photoreceptor function. The

electroretinogram is a record of electrical responses generated by the neuronal cells in the

retina and is used to assess the functionality of the retinal cells. These responses occur as

a result of light-induced changes in movements of sodium and potassium ions across

membranes. The sodium and potassium ion flux generates action potentials which dictate

the level of neuronal activity in the cell. Therefore, the ERG record represents the

intensity of action potentials generated by neurons in the retina, providing an analysis of

the overall electrical activity of cells in the retina. An advantage of this measure is that it

reflects the general physiological health of the photoreceptors in the retina; healthy cells

will generate quick robust electrical responses while damaged or altered cells will

generate slower and less neuronal activity. Specific analysis of cone cell activity can also

be accomplished because cone cells respond to light-induced changes while rods, the

other photoreceptor type, only respond to dim light in the dark. The ERG was used for

these studies because it has the advantage of being non-invasive and thus provided a

physiologically relevant measure of retinal activity without sacrificing or causing harm to

the animal. Another advantage is that we had assistance from a researcher experienced in

ERG methodology. A disadvantage however, is that it was not a means of examining the

cells at a structural level; it was exclusively a measure of activity at the cellular

membrane level. Also, it is important to mention that the chick was a novel model for the

researcher, who is an expert with rats and monkeys, and therefore the ERG equipment

was designed for these animals. Also, few protocols from other studies and little data

exists on retinal function of the chick utilizing ERG.

ERGs were obtained from chicks at baseline (Day 32), which corresponded approximately to the amount of time it takes for chicks to reach maximum carotenoid tissue content in response to a supplemented diet (62), establishing any changes recorded between control and supplemented diet groups as a dietary effect. Light exposure was performed quickly after the baseline measurements (Day 34). Post-light treatment measurements, (Day 36), for all chicks, light exposed and non-light exposed, were recorded a day after light exposure. The tight time frame between baseline measurements, light exposure, and post-light treatment measurements was established to ensure controlled comparisons in ERG activity between measurements and exposure. Centering the light exposure tightly between the measurements was an attempt to prevent any permanent recovery the retinal cells may experience after light exposure; this allowed us to assess if there was immediate damage to the retinas induced by light. To obtain the ERGs, increasing intensities of light were given to the eye of the chick. This was accomplished by a photostimulator, a light stimulus that allows variation in light intensity over a range. A corneal contact-lens electrode was placed on the left eye of the anesthetized, light-adapted chick. The eyelid was held open by a cup designed exclusively to fit the size of the standard chick eye ball; however, there were difficulties inserting and maintaining the eye lid opening since it was a new piece of equipment and a novel model. Caution had to be consistently taken to ensure the eye stayed moist with eye drops (Murocell) and that the chick did not blink. With practice, precision of placement of the electrode, and being consistent with hydrating the eye, this difficulty was overcome. The purpose of the electrode was to record the retinal responses from the increasing intensities of flashes of light given by the photostimulator.

Once the chick was anesthetized and the corneal electrode appropriately situated, the ERG recordings began by giving series of flashes of light to the eye with the photostimulator which was situated directly above the eye of the chick. Each series of light were composed of brief flashes of light in rhythmic succession, with each pulse increasing in intensity and separated by two second interval recovery times. With every flash of light, an immediate response from the retinal neuronal cells was measured by the electrode. The recorded response was amplified and displayed as a biphasic waveform onto specific ERG computer software, allowing us to view and record each neuronal response with the increasing intensities of light flashes. At the end of every series all waveforms were superimposed onto each other forming the electroretinogram for that series. The first series of light was of the lowest intensity; a background light (100 cd/m2) was used to saturate all rod photoreceptors, thus recorded ERGs were all from cone cell activity (63). Once the first series was complete, a filter was placed in the photostimulator to increase the intensity of the next series of light. All following series of light were given with increasing intensity by exchanging various filters causing a progressive increase in cone cell activity with no rod cell activity involved. The series of light used for these experiments was determined by previous experiments by the researcher. The final flash of light in the final series (1000 cd/sm2) was known to saturate the cone photoreceptor cation channels, thus giving the maximal response. ERG amplitudes do not follow a normal gaussian distribution so therefore it was necessary to convert amplitude values into log values to normally distribute the data for analysis. At the end of the experiment, all ERG waveforms from all series had been collected and

were now superimposed onto each for other further analysis of the waveform components.

The biphasic waveforms recorded from each response consisted of two components: the "A" wave and the "B" wave **(Figure 2).** The initial negative deflection ("A-wave") was followed by a positive wave with greater amplitude ("B-wave"). The A-wave represented the closing of sodium channels as a result of light in the plasma membranes of photoreceptor cell outer segments. This resulted in hyperpolarization of the cell and this electrical change was measured as the negative A-wave of the ERG. The flash intensities commonly used to elicit these responses ensured that the A-wave reflected primarily cone photoreceptor cell activity. Next, modulation of neurotransmitter release caused a depolarization of bipolar neuronal cells as sodium channels opened, causing an increase in intracellular sodium, and resulted in the positive B-wave. Therefore, the amplitudes of the A and B-waves represented the functionality of the sodium channels and activity of the cells.

The A-wave reflects the physiological health of the photoreceptors which are located in the outer retina; whereas the B-wave reflects the functionality of the inner neuronal layers of the retina. Since we were interested in photoreceptor cone cell activity, the principal outcome measure we took from the ERG waveform was the amplitude responses of the A-wave. Also, since retinas of chicks are cone dominant, this ensured that the responses seen were primarily from cones and little from rods. Upon examination of the superimposed ERG waveforms, the cone cell amplitude responses during the A-wave took on the average approximately 3 to 10 ms (**Figure 3**). Therefore, by analyzing the amplitudes only within this time frame, we were assured we were

examining exclusively cone cell activity. However, for the analysis, only the maximal

response was needed which represented the highest level of activity for the cone cell.

Upon closer examination of the time range (**Figure 4**), the maximum amplitude response

occurred approximately at 8 ms and the B-wave had not yet begun. Therefore, amplitude

responses obtained at 8 ms was considered a "pure" measure of cone photoreceptor

activity. The stimulus/response relationship, $Y=Bmax(X)/(Kd + X)$, was used to

determine the curve fit for non-linear regression analysis and to derive our parameters of

interest. These derived parameters included Bmax, the maximal amplitude response and

Kd, the intensity at half Bmax, an index of sensitivity (**Figure 5**). The equation was used

to obtain the amplitude responses at 8ms (Y) with increasing flash intensities (X). Since

we were investigating the function of a light damaged retina, we expected a loss of cells

and thus loss of sodium channels or shortening of outer segments which would reduce the

maximum amplitude response (Bmax). An increase in Kd corresponds to a decrease in

sensitivity. A decrease in sensitivity would indicate a loss of visual pigment primarily, or

shortening of outer segments or a change at the receptor level. **Figure 6** shows a

hypothetical example of the changes in parameters as a result of light damage to the

retina (63).

A multivariate repeated measures design analysis at a an $\alpha=0.05$ was performed

to determine if there were significant relationships between light exposure and diet

(Between-Subjects factors) with pre and post-measurements as Within-Subjects factors.

Tests of normality were performed with Kolmogorov-Smirnov and Shapiro-Wilk tests.

Post hoc comparisons were made with Bonferroni test for simple main effects of the

between-subjects factors.

3.2 High Performance Liquid Chromatography (HPLC)

The amount of lutein and zeaxanthin in plasma and tissues of chicks was measured by reverse phase high performance liquid chromatography (HPLC). The purpose for this analysis was to compare and contrast differences in carotenoid concentrations amongst the tissues of chicks fed diets supplemented with lutein and zeaxanthin and chicks fed control diets. Differences in tissue concentrations reflected dietary status of the chick. Of particular interest was the concentration of lutein and zeaxanthin in the retina, since adequate amounts may have ensured protection from light damage.

The tissues harvested from chicks included retina, brain, adipose, liver, skin, heart, muscle and plasma and were kept frozen immediately after removal. When ready for analysis, 30 mg of tissue were taken for two ether:hexane extractions to extract the carotenoids via hydrophobic interactions (64). 200 μl of plasma was used for carotenoid extractions with choloform:methanol and not ether:hexane because of the polar interactions within plasma. Both plasma and tissues used Echinenone as an internal standard because of its structural similar properties to carotenoids. Plasma and tissues were both dried with nitrogen. The extracted samples were then ready for analysis of lutein and zeaxanthin using HPLC.

The standards and samples were injected into a C30 hydrophobic carotenoid column in a mobile phase of methanol: methyl-tert-butyl ether: H_2O (83:15:2 by vol, solvent A) and methanol: methyl-tert-butyl ether: H_2O (8:90:2 by vol, solvent B). Therefore, the gradient from solvent A to solvent B increases in its concentration of organic solvent. As a result of the increased hydrophobic interactions between the

carotenoids and increasing levels of organic solvent, the sample flew through this gradient in steps and was eventually completely eluted out into the solvent.

When the samples flew through the spectrophotometer, light passed through was converted to an electrical current which was measured as absorbance. Using Beer's Law, the absorbance measured was directly proportional to the solute concentration in the tissue. The wavelength of light selected for a particular analysis was the absolute absorbance maximum peak. The areas under these curves were converted to $\mu V/s$, which reflected the concentration of the compound and was used for data analysis (64).

The lutein and zeaxanthin in the samples were detected by a spectrophotometer, which detects all wavelengths of light, at peaks 445 nm and 451 nm, respectively, at retention times of 6-7 minutes and 8-9 minutes, respectively (**Figure 7**) based on seven standards (**Figure 8**). These standards were run before the tissue sample for a standard curve so that concentrations of the carotenoids in the specific tissue could be calculated. The range of standard concentrations from the quickest retention time (the time it takes for the compound to elute out into the solvent) to the longest included lutein, zeaxanthin, cryptoxanthin, echinenone (internal standard), α-carotene, β-carotene, and lycopene (with retention times of 7.12, 8.62, 13.91, 14.72, 17.45, 19.36, and 35.32/35.67 minutes, respectively, **Figure 8**). The retention times of 28.48 and 31.69 minutes were from an unknown source. Since the *cis* isomer of zeaxanthin had recently been identified in the retina, it was brought to our attention that another standard needed to be used in addition to the seven standards to identify this compound and perform the analysis on retinal tissues.

However, at the time of analysis our lab did not have this standard, so a colleague, Dr. Elizabeth Johnson, an expert in chick physiology, analyzed a retina sample from a control diet chick of ours using her laboratory's *cis* standard, confirming and identifying the distinct peak at approximately 8.41 minutes **(Figure 9)** (65). Johnson determined the *cis* peak by examining the retention time relative to the *trans* isomer and the absorption spectrum **(Figure 9).** To prove this, it was previously established that the two peaks before lutein at retention times of 2.44 and 2.86 minutes **(Figure 7)** are *cis* isomers of lutein. A peak similar in characteristics and area to the lutein *cis* isomers was found between lutein and zeaxanthin **(Figures 7, 9)**. Since the lutein *cis* isomers were situated similarly to lutein as the newly identified peak was to *trans* zeaxanthin on the chromatogram, it was concluded that the new peak was a *cis* isomer of *trans* zeaxanthin. Apart from the new peak and the lutein isomers the rest of the peaks in the chromatogram represented the *trans* isomers. Therefore, it was concluded that this peak was *cis* (**Figures 7, 9**). Therefore, we continued to utilize the seven standards for the retinal tissues and with confidence were now able to identify the *cis* isomer in the analysis without the 8th standard.

3.3 Research Design

The research design and methodology discussed is illustrated in **Figure 10.**

CHAPTER 4

PROTECTION OF THE CHICK RETINA FROM LIGHT DAMAGE
WITH DIETARY LUTEIN AND ZEAXANTHIN SUPPLEMENTATION

Many studies have shown that high levels of lutein and zeaxanthin in the human macula may protect against light damage and thus may help in preventing age-related macular degeneration (AMD). A high dietary intake of lutein and zeaxanthin has shown to be correlated with a lower risk for AMD in humans (1). Also, high circulating plasma levels of these carotenoids and patients with high macular pigment density were associated with a decreased risk (4;6). Lutein and zeaxanthin may protect the macula against light damage and thus AMD by filtering blue light and decreasing phototoxicity to the retina.   However, most evidence is primarily from human epidemiological studies; more studies investigating the physiological mechanisms of protection to the retina by lutein and zeaxanthin would be useful to the nutritional science community. Animal models serve as a useful tool in studying dietary interventions and cellular mechanisms which are otherwise difficult to do in humans. Since avian retinas and human retinas have similar characteristics, it would be great scientific interest to investigate whether avians can also benefit from high levels of the carotenoids in the retina as humans. If so, avians could serve as a valuable model for studying AMD.

Avian retina is similar to the human retina in that birds have cone dominated retinas and concentrate lutein and zeaxanthin, unlike most mammals. All avians have four single cone types, one for each of the photopigments with peak sensitivities at 418nm (violet), 455nm (blue), 507nm (green) and 569m (red) (25). Avian retinas are characterized by the presence of oil droplets within the inner segment of the cones; each

cone contains a clear, red, pale-blue or yellow oil droplet, depending on carotenoids present. Colored droplets serve as long pass filters interposed between light and photopigments. Therefore, accumulation of lutein and zeaxanthin in the oil droplets in avians may serve the same purpose as the macular pigments in humans, that is, to prevent harmful short wavelength light from reaching photopigments in the cone outer segments.

However, the protective effects on the retina against light damage as a result of dietary lutein and zeaxanthin supplementation have only been investigated in one avian model, the quail. These studies showed less retinal cell death at the structural level in quails fed carotenoid-supplemented diets (47). Unlike the quail studies (47;91), our specific goal was to determine the effects of lutein and zeaxanthin supplementation on retinal function in chicks following light exposure rather than the effects at a histological level.

The first goal of this study was to test for accumulation of lutein and zeaxanthin in the chick retina with supplementation with lutein and zeaxanthin. The second goal of the study was to test the effects of supplementation on retinal function. This was done by exposing the chicks to extreme light treatment after allowing for the appropriate amount of time for retinal accumulation of lutein and zeaxanthin. Assessment of retinal function was done by the electroretinogram before and after light treatment to assess effects with lutein and zeaxanthin supplementation with and without light exposure.

4.1 Materials and Methods

Animals and Dietary Treatments

Newly hatched Leghorn chicks (Skylane Farms, Laurelwood, Or) were raised in cyclic light (12 hours light/12 hours dark) beginning at Day 1.

On Day 1, immediately post-hatch, chicks were fed either a control or lutein and zeaxanthin supplemented diet for 32 days, with n=12 per group. The supplemented diet was prepared by supplementing the control diet with 30μg per g diet of lutein and 15μg per g diet of zeaxanthin (Roche TG/P, Lutein 5%, Zeaxanthin 5%) for approximately 32 days (**Tables 1, 2**). The control dietary levels of lutein and zeaxanthin were at 5.2 and 1.7 mg/kg, respectively, and supplemented levels were equally increased five and nine-fold (27.2 and 15.3mg/kg, respectively); these supplemented levels were chosen based on previous literature showing that retinal carotenoid concentrations double at these dietary levels. Vitamins and minerals were added to the diet by the food company to ensure adequate nutrition for the developing chicks (**Table 3**).

At 32 days of age, they were subjected to one of two treatments: kept in cyclic (control) or exposed to 10 hours of intermittent (experimental) light.

Light Exposure

On Day 34, half of the chicks in each group were exposed to INTERMITTENT light (3200 cd/m^2 for one hour light/ two hours dark for a total of 10 hours of light) (66) before being returned to their normal light cycle. Intermittent light has been shown to be

more damaging to the retina than continuous light (67) and was the form of light

exposure used in the quail studies (47;91) which also demonstrated retinal damage.

Chicks were given two eye drops of 2% vecuronium bromide in five minute

intervals for pupil dilation and then placed 15 minutes later into a light exposure

chamber. This procedure was repeated four hours later to ensure dilation (68). The

chamber consisted of a particle board box (45 cm high, 60 cm deep and 43 cm wide) with

twelve 24 inch length full spectrum fluorescent tubes mounted on all four sides inside of

the box with two on each side. The light bulbs (GE Daylight tubes, 20 W, 36 in; General

Electric, Fairfield, CT) produced 3200 lux diffuse cool white light on all sides of the cage

(47). The spectrum of this light ranged from 280 to 870 nm, which included blue light.

Every measure was taken to build the light chamber with the same dimensions and to use

the same light bulbs as the previous quail studies to ensure replication of the experiment

(47). A wire mesh cage was loosely set inside so that it could be easily moved into and

out of the box. The chicks were placed into this cage outside of the box then returned to

the cage for the light experiment. The air temperature inside the chamber was kept

constant with a fan. Chicks were given food and water ad libitum during the experiment.

Non-exposed chicks were maintained on the cyclic light cycle in the animal

housing facilities. Chicks had ERGs recorded post-treatment and then sacrificed; retinas

and other tissues were harvested for biochemical analysis.

Electroretinography (ERG) Recordings

To detect a 30% difference in ERG sensitivity with a $p < 0.05$, 80% power, a mean

of 150uV, SD of 30uV and a 30% change in means (195uV), a requirement of *12*

chicks/group was needed to detect statistical significance. However, only 6 chicks per group were used due to the lengthy time involved to analyze all chicks in one day.

ERGs recordings were used to assess the function of the retina at baseline (Day 32, the length of time required to reach maximum carotenoid tissue content in response to supplementation) and post-light treatment (Day 36). Recording procedures were performed the same way for both measurements. One chick per dietary group was recorded at a time, one after another, such that all 12 chicks could be measured in one day. Each chick prep and recording lasted approximately 40 minutes. Chicks were anesthesized with ketamine: xylazine (80:8 mg/kg) administered via intramuscular injection into the calf. The chick was laid on its side with the right eye positioned at the entrance of a 14" Ganzfield. A custom made Burian-Allen contact lens electrode was inserted into the eye with drops of murocel after the cornea was anesthesized with proparacaine and pupils dilated with two drops 2% vecuronium bromide. Photopic ERGs (background = 30 cd/m^2) were recorded for flash intensities from –1.6 to 3.7 log cd-s/m^2 in 0.5 log steps and analyzed.   This method was completed with the assistance of Dr. Brett Jeffrey to measure ERG amplitude (Bmax) and sensitivity (Kd).

Tissue Preparation

Chicks were sacrificed after administration of ketamine: xylazine (50:10 mg/kg) on Day 40. Approximately 5ml of blood was taken from the left ventricle of the heart, collected into sterile clinical vials and centrifuged to separate plasma from blood cells. Plasma was then removed and stored in sterile vials and immediately stored at -80 deg C until ready for analysis. Both eyes were removed. One retina was dissected from one

eye and stored at –80deg C for biochemical analysis while the other eye was fixed in formaldehyde for histology for future studies. Liver, fat, heart, brain, muscle, and skin were harvested and stored at –80 deg C for HPLC analysis.

HPLC

Retina, brain, heart, liver, skin, muscle, and adipose tissue were harvested on Days 40 for HPLC. 30mg of tissues was added to 100uL of 12% pyrogallol in EtOH, 200 uL of 30% KOH and 1 mL of EtOH. Samples were saponified at 37 deg C for two hours. Echinenone in EtOH was added as an internal standard. A sample was extracted by 3 mL ether and hexane (2:1 by vol) twice and the extract evaporated to dryness under nitrogen. The residue was dissolved in 100 uL EtOH and a 50 uL sample used for HPLC analysis. The HPLC system (C30 carotenoid column) has a mobile phase of methanol: methyl-tert-butyl ether: H_2O (83:15:2 by vol, solvent A) and methanol: methyl-tert-butyl ether: H_2O (8:90:2 by vol, solvent B). The gradient procedure used: flow rate of one ml/min, begin 100% solvent A before going to 93% solvent A and 7% solvent B over a one min linear gradient. This was followed by a three-min hold at 93% solvent A followed by a 17 min linear gradient to 45% solvent A and a 1 min hold at 45% solvent A, an 11-min linear gradient to 95% solvent B, a four-min hold at 95% solvent B and finally a two min gradient back to 100% solvent A. The system was held at 100% solvent A for 10 min for equilibration to resolve back to initial conditions. Lutein and zeaxanthin were quantified by determining peak areas in the HPLC chromatograms calibrated against known amounts of standards. They were corrected for extraction and handling losses by monitoring the recovery of internal standards (64).

Statistics

A commercial statistics package (SPSS version 14.0) was used for analysis of
ERG data. A multivariate repeated measures design analysis at an α=0.05 was performed
to determine relationships between light damage and diet (Between-Subjects factors) with
pre and post-measurements as Within-Subjects factors. Tests of normality were
performed with Kolmogorov-Smirnov and Shapiro-Wilk tests. Post hoc comparisons
were made with Bonferroni test for simple main effects of the between-subjects factors.
Mean differences in post-treatment carotenoid concentrations determined by HPLC
between supplemented and control diet tissues and plasma were analyzed using a paired
student's t test in Excel. Mean differences between cis and trans zeaxanthin in both light
exposed and non-light exposed groups was analyzed using a one-way ANOVA with
pairwise comparisons and Bonferroni correction.

4.2 Results

**Tissue Concentrations of Lutein and Zeaxanthin in Control and Lutein and
Zeaxanthin Supplemented Diet Chicks**

Before sacrificing the chicks, all body weights were taken. At time of tissue
harvest, the average weights of supplemented chicks (530 g) were not statistically
different from control chicks (526 g), p=0.22.

Both tissues and blood were taken from the sacrificed chicks for analysis of lutein
and zeaxanthin concentrations. Plasma lutein concentration was almost 4 times higher in
lutein and zeaxanthin supplemented chicks than in chicks fed a control diet (2159 $\pm$ 433
compared with 566μmol/L $\pm$ 123, respectively, p <0.0001, **Figure 11**). However, plasma

zeaxanthin levels showed no increases in concentration (181 ± 27 compared with 186

mmol/L $\pm$ 37, respectively, p=0.611, **Figure 11**). Plasma levels of lutein higher in

plasma in both groups than zeaxanthin.

Like the plasma, lutein concentrations in the retinas of supplemented chicks were

significantly higher than in control chicks (6.41 ± 1.44 and $3.62\mu g/g \pm 1.09$, respectively,

p=0.0003, **Figure 12).** Other tissues from supplemented chicks also showed higher mean

concentrations of lutein than compared to control (p<0.0001, **Figure 12**). This was

especially notable for the liver, which was approximately 9 times higher with means of

35.8 ± 12.7 and $3.97\mu g/g \pm 0.84$, respectively (p<0.0001). Also, the supplemented chicks

all showed higher zeaxanthin levels than controls in all tissues (p <0.001) except for the

skin (p=0.25) (**Figure 14**). The light exposed and non-light exposed groups were pooled

together for tissue analysis since retinal carotenoid content showed no differences

between groups (**Figure 13**).

The total zeaxanthin concentration was greater in the retinas of supplemented

chicks than controls (p < 0.005, 7.60 ± 3.73 compared with 4.27 $\mu g/g \pm 2.0$, respectively,

Figure 15). Further, the total retinal zeaxanthin level was greater than the total retinal

lutein level in both supplemented and control chicks (p < 0.005, 7.60 ± 3.73 compared

with $6.41\mu g/g \pm 1.44$ and 4.28 ± 2.0 compared with $3.62mg/g \pm 1.09$, respectively,

Figure 16). Unlike lutein, in the retina, zeaxanthin exists in two isomeric forms, *cis* and

trans (**Figure 15**). In peripheral tissues, only the *trans* form is present whereas in the

retina, *cis* is also present because bright light isomerizes carotenoids in the macula (69).

Concentrations in the retinas of *cis* and *trans* zeaxanthin in the retinas were each greater

in supplemented chicks than compared with control diet chicks, (p=0.0001, p=0.03,

respectively, **Figure 15**). The *cis* form was 6 fold higher in supplemented chicks than control diet chicks (2.85± compared with 0.44µg/g ±, SD = 1.36 and 0.23); there was only a 1.23 fold greater concentration in the *trans* form (4.75± compared with 3.85 µg/g±, SD = 2.37 and 1.77). However, the *trans* form was found at much higher basal concentrations in the retinas than the *cis* form in both control and supplemented chicks (**Figure 15**).

Even though light exposed and non-light exposed groups were originally pooled together for tissue analysis, significant differences were found (upon further examination) in the amount of *cis* zeaxanthin in the retina when all four groups, control and supplemented diet and light and non-light exposed, were compared (p=0.000172, **Table 6A**) but not for the amount of *trans* zeaxanthin (p=0.360). Further statistical analysis uncovered that *cis* zeaxanthin was greater in the retinas of supplemented diet light exposed chicks than control diet light exposed chicks (3.02 vs 0.47µg/g, p=0.0073, **Table 6B, Figure 25**). Also, there were greater *cis* zeaxanthin concentrations in the retinas of supplemented diet non-light exposed chicks than control diet non-light exposed chicks (2.66 vs 0.41µg/g, p=0.0033, **Table 6B, Figure 25**). Further, supplemented diet non-light exposed chicks had greater levels in their retinas than control diet light exposed chicks (2.66 vs 0.47µg/g, p=0.0057, **Table 6B, Figure 25**) and supplemented diet light exposed chicks had greater levels than control diet non-light exposed chicks (3.02 vs 0.41µg/g, p=0.008, **Table 6B, Figure 25**).

However, control diet light exposed chick retinas did not have greater *cis* zeaxanthin levels than control diet non-light exposed chicks (0.47 vs 0.41µg/g, p=0.57) and supplemented diet light exposed and supplemented diet non-light exposed chick

retinas also did not differ in *cis* zeaxanthin concentration (3.02 vs 2.66μg/g, p=0.50,

Figure 25). This proves that the light exposure was not the root cause of the observed

increases in retinal *cis* zeaxanthin levels in the light exposed chicks. This also suggests

that not enough light damage to the retinas occurred as a result of the light exposure

treatment given to the chicks.

All these results showed more *cis* zeaxanthin was present in the retinas of

supplemented diet chicks than control diet chicks. However, there were no increases in

cis zeaxanthin in the retinas as a result of light exposure in control or supplemented diet

chicks, proving that the difference was solely a dietary effect as a result greater

zeaxanthin levels in the supplemented diet and not as a result of light exposure.

Effects of Diet on Light-Induced PR damage

Before performing the baseline ERG recordings, weights from all chicks were

recorded. At Day 32, the average weights of supplemented chicks (394 g) were not

statistically significant from average weights of control chicks (388 g).

On Day 32, baseline measurements were recorded in both dietary groups. Post-

treatment measurements were made on all chicks, light exposed and non-light exposed,

on Day 36. Both diet and treatment groups had unequal sample sizes, due to the

unexpected death of a supplemented, non-light exposed chick.

The ERG cone A-wave response parameters for baseline and post-treatment for

both non-light exposed and light-exposed control diet chicks are shown in **Figure 17**.

There were significant increases in the post-treatment responses as compared to baseline

in the group not exposed to light (**17A**) and those exposed to light treatment (**17B**), (p

0.000, $\alpha=0.05$). These increases were most likely due to the growing and maturation of

the chick as a result of developing outer segments and insertion of more sodium channels

in cell membranes causing greater amplitude responses.

However, there were no significant decreases or increases in the maximum

amplitude (Bmax) ERG responses or cone A-wave sensitivity (Kd) for control diet chicks

(**Figures 18A and B)** between light exposed and non-light exposed chicks, proving that

the retinas were not damaged as a result of the light exposure treatment.

The cone A-wave components for both non-light exposed and light-exposed lutein

supplemented diet chicks are displayed in **Figure 19**. Like the control diet chicks, there

were significant increases in post-treatment responses from baseline responses in both

non-light exposed (**19A**) and light exposed chicks (**19B),** (p 0.007,$\alpha=0.05$). These results

support the retinal development effect observed in the control diet chicks. Like control

chicks, no increases or decreases were found in the Bmax responses for lutein

supplemented chicks between light exposed and non-light exposed groups (**20A**).

However, there was a significant decrease in Kd, suggesting increased sensitivity in the

retina, in the non-light exposed group at baseline measurements (**20B**), (p 0.008,$\alpha=0.05$).

This maybe due to random chance or a low sample size (n=6), since all chicks should

have had identical baseline responses.

Comparison of post-treatment control and supplemented diet chicks and for light

exposed and non-light exposed chicks (**Figure 21**) support further the idea of retinal

development resulting in increases in maximum amplitude responses from the

photoreceptor cone cells. However, there were no significant decreases in Bmax

response as a result of the diet or light exposure. Also, comparison between dietary

groups of Kd emphasizes the retinal development and changes in sensitivity (**Figure 22**); a decreased Kd relates to increased sensitivity as the retinal cells develop and gain visual pigments in their membranes as well as insertion of functional proteins in the photoreceptor cell membranes. Like the Bmax responses, there were no significant decreases or increases amongst diet or treatment groups for Kd ($p = 0.253$).

The overall findings suggest that damage to the retina as a result of light exposure did not occur since there were no significant alterations in Kd or Bmax. Increased Kd or decreased Bmax values would reflect poorer retinal function as a consequence of light damage. Also, a diet supplemented with lutein and zeaxanthin did not appear to benefit the chicks' retinal development since all baseline ERG measurements for all chicks were all the same.

4.3 Discussion and Conclusions

Lutein and zeaxanthin supplementation of the chicks' diets increased lutein plasma levels four times higher than control levels; control plasma lutein levels were three fold higher than zeaxanthin levels indicating a physiological significance exists for zeaxanthin in the periphery (**Figure 11**). Interestingly, plasma zeaxanthin levels were not elevated in the supplemented diet chicks. The reason that plasma zeaxanthin did not increase despite supplementation could be due to a tight regulatory mechanism to ensure a proper plasma level that does not reflect tissue concentrations. Interestingly, there was even a slightly lower level of plasma zeaxanthin in the supplemented chicks (186 vs 181 μmol/L), indicating the possibility that excess zeaxanthin may be suppressing, triggering excretion of already existing zeaxanthin in the plasma, or it was not absorbed. The idea

of a suppressive effect was suggested by previous results from similar supplementation experiments (70);71) and supports the idea of a physiological mechanism to keep an appropriate zeaxanthin balance between plasma and tissues.

However, in all other tissues, zeaxanthin levels increased in the supplemented diet chicks **(Figure 14).** Lutein levels also increased in the tissues of supplemented chicks. The increase in tissues occurred because exposure to carotenoids early in embryonic development enables chicks to better deposit dietary carotenoids later in life (72). Therefore, by being fed a lutein and zeaxanthin supplemented diet immediately post-hatch, the chicks absorbed and deposited carotenoids rapidly and efficiently. Further, at hatch, the concentration of the chick liver far exceeds that of any other tissue; even after 4 weeks, 25% of liver carotenoids are yolk derived, suggesting active accumulation of lutein and zeaxanthin in the liver during development (62). This accumulation explains why the livers of the supplemented chicks showed much greater increases in lutein content than in any other tissues **(Figure 12)**. However, as discussed above, plasma zeaxanthin levels did not increase in the chicks fed the supplemented diet. This phenomenon further supports the idea of the existence of a specific physiological mechanism to maintain a carotenoid ratio balance which ensures tissues receive adequate zeaxanthin.

Interestingly, the total zeaxanthin concentrations in the control diet and supplemented diet chick retinas were greater than lutein concentrations (**Figure 15 and 16**). This is intriguing since the control and supplemented diets had much less zeaxanthin (1.7 mg/kg vs. 15.3 mg/kg) than lutein (5.2 mg/kg vs 27.2mg/kg). Further, the fact that zeaxanthin levels were much higher in the retina regardless of amount in the diet supports

evidence from other studies that the retina actually shows a preference for zeaxanthin over lutein (23). This retinal retention may be from a specific binding protein for zeaxanthin or the presence of zeaxanthin as esters in the oil droplets (73). Also, the high accumulation of zeaxanthin in the retina may explain the tight control over zeaxanthin plasma levels which would ensure that enough zeaxanthin is spared for the retina.

Trans zeaxanthin was the predominant form of zeaxanthin found in plasma and tissues in both supplemented and control diet chicks. Interestingly, an isomer of zeaxanthin, *cis*-zeaxanthin, was found only in the retina and in no other tissues, supporting findings from other studies (31;71). Previous experiments with fellow colleagues first identified the *cis* and *trans* isomers in the chick retina. The specific functions of the two isomers in the retina are yet to be investigated.

It is thought that the presence of *cis*-zeaxanthin in the retina results from isomerization of *trans*-zeaxanthin in the retina as a result of light exposure (34). The reason for the exclusive presence of *cis* in the retina may be due to the energetic and stereochemical aspects of zeaxanthin, which determines the isomer pattern.

The results from these experiments showed greater levels of *cis* zeaxanthin in the retinas of supplemented diet non-light exposed and supplemented diet light exposed chicks when compared to control diet non-light exposed and control diet light exposed chick retinas (**Table 6B**). The results from the analyses indicated that there was not enough light exposure to cause light damage to the chicks' retinas since *cis* zeaxanthin levels were not increased in light exposed groups. Greater dietary levels of zeaxanthin in the supplemented diet caused greater retinal accumulation and therefore resulted in greater levels of the *cis* isomer in supplemented chicks. Even though there was no light

damage to the retinas in our experiments, there were baseline levels of the *cis* isomer in all chicks as it is formed naturally as a result of being raised in cyclic light for 32 days.

Interestingly, post-treatment ERG measurements were significantly greater than baseline measurements within all groups; however, this could be due to variability in the animals or in the measurements. Retinal development of the chicks between baseline, light treatment, and post-treatment measurements could also have been a variable. Stress to the chick during this time could also have been another reason for the elevated post-treatment responses in both diet groups (**Figures 17 and 19**). Unfortunately, since there were no decreases or increases between light exposed and non-light exposed groups in maximum amplitude response and sensitivity, this indicated that damage to the retinas of both supplemented and control diet chicks did not occur as a result of light exposure treatment **(Figures 17, 19, 21, and 22).**

However, the reason for the lack of significant differences between diet and light exposed and non-light exposed groups could be due to a few other factors. Light exposure treatment and diet were not adequately powered (43%, 20%), whereas the baseline and post-measurements were (100%). Also, it may be that we did not provide enough lutein in the diet to provide substantial protective amounts to the retina; lutein supplemented retinas only had 1.7 times more lutein than control retinas (**Figure 12**). Or, just the opposite, lutein may have been acting as a pro-oxidant at levels that high and destroyed cell membranes, and thus sodium channels. There is evidence that carotenoids can act as pro-oxidants, but it has only shown in-vitro (55). Also, lutein may not be the carotenoid that could be providing the most beneficial protection. Zeaxanthin predominates in the central *fovea* while lutein is found in the periphery; this suggests that

lutein may play a role in protecting rods, while zeaxanthin protects cones (28). The presence of the *cis* and *trans* zeaxanthin isomers in the retina (**Figure 15)** were indicators that zeaxanthin may play a larger role in protection of the retina. Indeed, the lutein detected in the chick retinas may be accumulated specifically in rods, which would not respond to bright light. Therefore, future studies should focus on zeaxanthin in protection of the retinal cone photoreceptors rather than lutein.

Further, another reason for the lack of differences may be that the retinal cells were compensating for any photoreceptor death by inducing more sodium channels, which would make the ERG results appear to have no differences. Histologically, the damaged cells would be detected, but the ERG responses would not change or change very little. In the quail studies, photoreceptor damage and death were detected with histological measurements, but perhaps if the study used actual functional measurements of retinal activity the same results may not have been observed. Another reason for the lack of differences is that degeneration of the retina may still occur once light exposure has been terminated (74). Perhaps waiting for a longer period of time before executing the post-treatment ERG measurements may have revealed greater cone damage. Future directions should include histological in addition to functional measurements to gain a clearer picture of lutein and zeaxanthin supplementation and retinal physiology.

Even further, the degree of damage to the retina depends on rearing environment of the animal. It has been found that retinas of rats reared in cyclic light (like our chicks) were better protected against light induced degeneration compared with dim light reared rats and that younger rats were more resistant to damage than older rats (75;76). This suggests that a post-development response exists due to a cellular mechanism which

creates plasticity of the retina (77). Also, studies examining possible adaptive changes to the photoreceptor cells found that proteins involved in visual cell transduction (rhodopsin, S-antigen, and transducin) were observed in rat retinas in response to light rearing environment (78). Studies investigating protective cellular mechanisms against light damage showed that pigment epithelial-derived cofactor (PEDF), glial cell and brain-derived neurotrophic factors (GDNF, BDNF) and heat shock proteins Hsc/Hsp 70 may play an important role in rescuing damaged retinal cells (79-82). Finally, increased rates of glycoprotein and proteoglycan synthesis were found in rat retinas after intense light exposure, perhaps another adaptive change (83). Our chicks, which were young at the time of experimentation, were also reared in the more protective cyclic light environment. It may be that we were not able to produce enough light treatment to cause functionally damage the retinas. Future experiments should include raising the chicks in a dim light environment to exacerbate damage and also to study the chicks at a much older age.

In conclusion, there were no significant decreases between control and supplemented diet chicks as a result of light damage in retinal photoreceptor sensitivity and maximum amplitude response. This indicates that damage to the retinas of both supplemented and control diet chicks did not occur as a result of light damage, despite our cautious efforts to replicate a previously successful protocol from other studies. However, these previous studies indicated that lutein had beneficial effects to the retina against light damage *structurally.* This project was the first attempt to examine retinal *function* and in a completely *novel* model; with greater sample sizes and using different approaches to the research design, perhaps more significant results can be found.

CHAPTER 5

INTRODUCTION

5.1 Lutein and Zeaxanthin Deficiency and the Retina

Adequate nutrition is a key element to a healthy lifestyle associated with a

lowered risk for chronic illnesses. A lifelong diet of fruits and vegetables rich in

carotenoids have been proposed to sustain optimal health. Humans and animals are not

capable of carotenoid biosynthesis; therefore, the presence of this group of pigments in

their bodies is totally dependent on dietary intake. Several studies have shown inverse

correlations between food rich in carotenoids, such as lutein and zeaxanthin, and

incidence of a chronic disease affecting the *macula lutea* of the retina: age related

macular degeneration (AMD). In addition, low circulating plasma levels of lutein and

zeaxanthin have been associated with a higher risk. Patients with AMD and people with

an increased risk for AMD were correlated with having low macular pigment density.

This suggests that low lutein and zeaxanthin levels in the retina may not adequately

protect the retina against light damage. This may lead to a further increased risk for AMD

since these compounds are antioxidants and may act as blue light filters in the retina.

Whether lutein and zeaxanthin deficiency hinders protection of the retina from

light damage has not been thoroughly investigated. Thus, it is of great importance to the

nutritional science community to investigate possible dietary interventions *in vivo* to

determine if low levels do not provide adequate protection from light damage which may

exacerbate the risk for AMD. An animal model, such as the chick, would be an

appropriate model to use to address this research question since their retinas are quite

similar to humans in that they retain carotenoids and are photoreceptor cone cell

dominant. However, direct dietary intervention is not an appropriate method for studying carotenoid deficiency in the chick. Preliminary data from our lab showed that the chick displayed almost no lutein and zeaxanthin in the plasma and other tissues after being fed a carotenoid free diet for 4 weeks (70). However, retinal lutein and zeaxanthin levels were retained at the same level as compared to controls. This suggests a selective retention mechanism exists in the retina for lutein and zeaxanthin. These results are similar to those observed in other studies involving an avian model, the quail (48;84). The developing chick embryo accumulates only the oxycarotenoids (lutein, capsanthin and zeaxanthin) and only from the egg yolk (85). Coloration of egg yolk, plasma, skin and feathers, depends on accumulation and type of carotenoids. Hens accumulate these pigments in a single target tissue, the developing ova (85). Thus, for a chick to have complete retinal depletion of lutein and zeaxanthin, the laying hen must have depletion of its egg yolk deposits. Therefore, to have an appropriate chick model to study lutein and zeaxanthin deficiency, production of progeny from deficient laying hens is necessary.

5.2 Materials and Methods

Production of lutein and zeaxanthin deficient chicks

To address the *second hypothesis* (lutein and zeaxanthin deficiency results in greater damage to retinal photoreceptors after light exposure in the chick) and *second specific aim* (produce lutein and zeaxanthin depleted chicks from a laying hen consuming a diet deficient in these carotenoids) the following methods were employed:

Lutein and zeaxanthin deficient chicks were to be produced from White Leghorn laying hens fed a deficient diet. The laying hens, aged ~30 weeks old, and rooster were

housed in brooders at the Madrona Acres Farm by OHSU veterinarians, Mike and Julie

Booth, in Laurelwood, OR. Hens were placed on a lutein and zeaxanthin -deficient diet

upon arrival to the farm (Purina Mills, LLC, **Table 4**). The deficient diet was a semi-

purified diet matching the same caloric, vitamin and mineral content as the control diet

without the inclusion of lutein and zeaxanthin (**Tables 1, 3**). Soy oil was added and

mixed by hand into the deficient diet to match the same fat content as the control diet

(200 g/kg, **Tables 1, 4**). Control diet chicks were the same control chicks used for the

experiments studying the lutein and zeaxanthin supplemented diet chicks.

Depletion of egg yolk carotenoids by feeding a hen a carotenoid-free diet takes 30

days (62). After 28-30 days, the hens were mated with a rooster, and the subsequent

fertilized eggs were collected from the laying hens every day afterwards, for a total of 7-

10 days. Eggs were stored in a cooler maintained at 65 degrees F. After 7-10 days, the

eggs were picked up and brought to OHSU campus for immediate storage in an incubator

for 21-28 days for hatching. Assuming 90% fertility and 80% hatchability, if ~16 eggs

were collected, this should have yielded 12 chicks/dietary group, which satisfies

statistical requirements. Biochemical analysis was used to confirm that egg yolks were

deficient in lutein and zeaxanthin.   The newly hatched chicks were to be placed on lutein

and zeaxanthin deficient diets. Body weights and food intake were to be monitored every

other day until Day 28. All measures were taken to ensure a temperature and light-

controlled environment.

Research design and methodology for carotenoid-deficient laying hens

The research design and methodology discussed is illustrated in **Figure 23.** Briefly, three laying hens were placed in their own cages and fed carotenoid-deficient diets for 28 days. On the first day, a rooster was placed in Hen #1's cage to mate for 6 hours light/ 6 hours dark and then removed. On Days 2 and 3, the same procedures were done for Hen #2 and #3, respectively. The rooster was placed in each hen's cage every 7 days for copulation to ensure consistently fertilized eggs. Eggs were collected daily for 8 weeks. Once a week the collected batch was taken to OHSU and placed in incubators for hatching. Once the carotenoid-deficient chicks hatched, they were to be raised on carotenoid-deficent diets until Day 28 in a normal cyclic light cycle. Then, chicks were to be exposed to light treatment and ERG recordings for analysis of retinal function.

Electroretinography (ERG) and HPLC

The experimental design for determining the effects of lutein and zeaxanthin deficiency on retinal function and tissue analysis for lutein and zeaxanthin concentrations was identical to the design for lutein and zeaxanthin supplementation (see Chapter 4). The same control chicks from the supplementation experiment were to serve as controls for the lutein and zexanthin deficient model.

5.3 Results

Three White Leghorn laying hens were purchased from private vendors in Hillsboro, Or. After ~30 days, the rooster (from the Poultry Center, OSU) was placed into hens' cages according to protocol. Eggs were collected, maintained and transported as according to protocol. **Table 5** shows eggs collected and incubated weekly.

Hen #3 was euthanized after 3 weeks of being on the diet by the advice of the veterinarians due to unexpected dramatic weight loss. Hens #1 and #2 were also experiencing dramatic weight loss. A unanimous decision between the farm and our lab put forth placing the Hens #1 and #2 on a commercial diet until their healths improved. Two weeks later, the weights of the hens increased to 1.4kg, and were still laying eggs. A new hen was brought in a week later to replace Hen #3.

Approximately 3 weeks later, all three hens were placed back on the lutein and zeaxanthin deficient diet when the average weight of the hens was 1.5kg. Instead of allowing the veterinarians to continue preparing the diet, we began mixing the soy oil into the diet ourselves once the hens were healthy again and then delivering it to Madrona Farms. This was due to our concern that they were not mixing the oil well into the diet mix, causing the diet to have inadequate amounts of fat for the hens. During the entire 4 months*, no lutein and zeaxanthin deficient chicks were hatched from the incubator at OHSU*.

Concentrations of lutein and zeaxanthin in the deficient egg yolk

In the first month when the hens were first placed on the deficient diet, any subsequent eggs collected were non-fertilized and therefore used for biochemical analysis of egg yolk content. **Figure 24** shows a time scale of the lutein and zeaxanthin content of the egg yolks from the deficient hens during this time. The lutein and zeaxanthin content of the egg yolk declined from Day 2 to Day 36 (11.19 µg/g and 18.09 µg/g to 0.19 µg/g and 0.23 µg/g, respectively). All egg yolks changed color from yellow to white within this time frame. This data supports the fact that since carotenoids are only available to

birds via the diet, the consistency of yolk color is solely dependant on dietary carotenoids consumed by the laying hen (85). It is interesting to see that zeaxanthin levels were greater than lutein levels at Day 2, and then dropped to meet that of lutein at Day 10, where both carotenoid levels remained the same until both were almost completely deficient in the egg yolk. This was expected since coloring efficiency depends on carotenoid composition and zeaxanthin is more efficient than lutein (85).

Move from Madrona Farms to OHSU

After 5 months of residing at Madrona Farms, the laying hens were brought to OHSU to be housed, and therefore were under more tightly controlled supervision within the animal facility. The hens gained weight within the first three weeks. A second rooster was even brought in to OHSU after one month to establish if lack of fertility of the first rooster was the underlying cause of the lack of hatchings. There was only a small increase in hatchability with the switch in roosters and change in care. Only **10** vital chicks out of **360** eggs had been hatched over a period of **5** months, even with inclusion of the new rooster. There was a decision after two months to sacrifice one of the hens (the replacement hen for Hen #3), who laid the fewest eggs over the course and suffered from poor health.

5.4 Discussion

The embryonic chick develops within a closed system, utilizing nutrients that are pre-packaged in the egg prior to lay. Therefore, maternal nutrient input has a great impact on the physiology of the development of the chick. It is known that hens only

accumulate oxycarotenoids, and that they are only accumulated in the developing ova

(85). This suggests that lutein and zeaxanthin must play an extremely essential role in the

development and hatchability of the chick. Further, individual carotenoids are *selectively*

transferred from the yolk to specific tissues in the embryo, suggesting they serve

particular roles during development (72). Differences in concentrations of the specific

carotenoids between tissues exist, even when the diet contains equal ratios (72). This

also supports the idea that a specific binding protein or differences in transport in blood

exist for the carotenoids. These data suggest that in ovo, carotenoid exposure is essential

for absorption, metabolism and tissue deposition of dietary carotenoids. This will affect

the hatchability and vitality of the developing chick embryo.

At a cellular level, carotenoids help regulate cell proliferation, differentiation,

gene expression, signaling, immune system and gap-junction communication, all actions

important for early avian embryonic development (62). Maternal hens placed on modified

diets had decreased hatchability and chicks that did hatch had low viability due to an

association with deficient carotenoids and Vitamins A and E (86). White Leghorn laying

hens experienced decreased hatchability and egg production and hemorrhagic follicles

after 4 weeks of being fed a Vitamin A-deficient diet (87). Another study which

attempted to create carotenoid deficient chicks found low hatchability, greater body

weight losses, and immune system organ weight loss (88). High concentrations of

carotenoids are required in the bursa post-hatching which helps regulate the immune

cells' development; carotenoid deprived chicks fail to incorporate carotenoids into the

thymus. This suggests that the immune system of the chick is very susceptible to

alterations in the maternal diet and lack of carotenoid exposure affects its' proper development.

Maternally derived yolk carotenoids may serve to protect egg yolk lipids against reactive oxidative species (ROS). Egg yolk mainly consists of PUFAs which nourish the embryo, but render their tissues to vulnerable attack by ROS, due to greater risk of lipid peroxidation (as oxygen is diffused through the shell). A mother hen being fed a diet deficient in carotenoids will produce carotenoid-deficient egg yolk and thus render the developing chick to have depletion of carotenoids in tissues. This results in the chick having increased susceptibility to oxidative stress (89). This would cause damage to the embryo's development and negatively impact its hatching. All said, yolk carotenoids bestow many benefits to the embryo and induce long-lasting consequences that are relevant to the health of the bird later as it develops.

Yolks of chicken eggs produced commercially contain large amounts of lutein and zeaxanthin (~292ug/yolk and ~231ug/yolk, respectively) as compared to other dietary carotenoids (90). Yolks can contain over 20 carotenoids. Our yolks at Day 2 contained only 11ug/g of lutein and 18ug/g of zeaxanthin. Lutein and zeaxanthin are present in a nonesterified form in the lipid matrix of the yolk with fat-soluble vitamins (Vitamins A, D, E). It is most likely that the hens' weight loss resulted in loss of fat weight; this would drastically impact the health status of the egg since essential fat-soluble vitamins for development as well as carotenoid levels were extremely low. Unfortunately, there has not been much research done on carotenoid deficiency and reproduction in other animal models to give more insight to the dilemma.

Therefore, the literature encountered gives evidence that producing carotenoid-deficient chicks from a maternal hen is difficult and perhaps physiologically impossible. It has been shown that carotenoid-deficient quails have been hatched from deficient mothers, but these quails were studied at six months of age and there was no indication of length of time of maternal diet (48). Also, using the chick has never been attempted; it may be that carotenoids are more essential for their development than for quails.

5.5 Conclusion

Only epidemiological and clinical studies have shown a relationship with carotenoid deficiency and development of age-related macular degeneration (AMD). Therefore, there is a great need research focused on carotenoid depletion and impact on the physiology of the retina as well as on other tissues. Thus, it is of particular interest to our laboratory's carotenoid-based research as well as to the whole scientific community to study the retina and the impact of light damage; this will help nutrition experts understand carotenoids and their role with development of AMD.

The previous experiments with quails (36;48;91) as well as results from our research with chicks have acquired the best data on the physiology of carotenoid deficiency in avians thus far. Future directions should include modifying the experimental design for the deficient hen and investigate other methods to produce carotenoid-deficient progeny.

CHAPTER 6

OVERALL DISCUSSION AND CONCLUSIONS

In conclusion, biochemical analysis of the chicks' tissues found that supplementation of lutein and zeaxanthin of the chicks' diet resulted in increased concentrations of the carotenoids in all tissues and plasma analyzed. In the retina, zeaxanthin levels were greater than lutein, regardless of diet. This supports evidence from other studies that the retina actually shows a preference for zeaxanthin over lutein. The presence of *cis*-zeaxanthin in the retina likely results from isomerization of *trans*-zeaxanthin in the retina as a result of light exposure. Results from these experiments indicated that greater dietary levels of zeaxanthin caused greater retinal accumulation and therefore greater levels of the *cis* isomer. However, the experiment did not produce enough excess light to cause a significant increase in levels of *cis*-zeaxanthin because there were no significant differences between light exposed and non-light exposed groups within the same diet.

Analysis of the ERG data found no differences between light exposed and non-light exposed groups and control and lutein supplemented diet chicks in maximum amplitude response and sensitivity, indicating that functional damage to the retinas of both supplemented and control diet chicks did not occur as a result of light damage. However, it is possible that there were some underlying factors responsible for the lack of differences, such as lack of power and inadequate or excess dietary levels of lutein and zeaxanthin in the supplemented diet. Also, induction of sodium channels in retinal cell membranes for compensation for cell death may have occurred or the fact that rearing chicks in cyclic light causes adaptive changes to proteins involved with vision.

However, this project was the first attempt to examine retinal *function* and in a completely *novel* model; with greater sample sizes and using different approaches to the research design, perhaps more enticing results can be found.

Unfortunately, our attempts to produce lutein and zeaxanthin deficient progeny from a laying hen to investigate carotenoid deficiency and retinal function were not successful. Many underlying factors were involved that hindered our progress, such as the hens' dramatic weight loss and poor health, which most likely impacted the health status of the egg. Previous studies using other avian models gave evidence that producing carotenoid-deficient chicks from a maternal hen is difficult and perhaps physiologically impossible. In addition, using the chick has never been attempted; our lab was the first to attempt production of carotenoid deficient Leghorn chicks.

Finally, to learn more about the long term effects of lutein and zeaxanthin on preventing earlier stages of AMD, it is important to look at long-term prospective studies in primates or humans. The Age-Related Eye Disease Study II began late in 2006 which studies the inclusion of omega-3 fatty acid supplementation as well as lutein and zeaxanthin on the progression of AMD (18). This is an exciting study as it will continue to address the impact these food compounds have on retinal function on a long term basis and hopefully lead to interesting results.

Reference List

1. Mares-Perlman JA, Fisher AI, Klein R et al. Lutein and zeaxanthin in the diet and serum and their relation to age-related maculopathy in the third national health and nutrition examination survey. Am J Epidemiol 2001;153:424-32.

2. Bone RA, Landrum JT, Mayne ST, Gomez CM, Tibor SE, Twaroska EE. Macular pigment in donor eyes with and without AMD: a case-control study. Invest Ophthalmol Vis Sci 2001;42:235-40.

3. Gale CR, Hall NF, Phillips DI, Martyn CN. Lutein and zeaxanthin status and risk of age-related macular degeneration. Invest Ophthalmol Vis Sci 2003;44:2461-5.

4. The Eye Disease Case-Control Study Group. Risk factors for neovascular age-related macular degeneration. Arch Ophthalmol 1992;110:1701-8.

5. Bartlett H, Eperjesi F. Dietary analysis and patterns of nutritional supplement use in normal and age-related macular disease affected subjects: a prospective cross-sectional study. Nutr J 2004;3:16.

6. Hammond BR, Jr., Wooten BR, Snodderly DM. Preservation of visual sensitivity of older subjects: association with macular pigment density. Invest Ophthalmol Vis Sci 1998;39:397-406.

7. Berendschot TT, Goldbohm RA, Klopping WA, van de KJ, van NJ, van ND. Influence of lutein supplementation on macular pigment, assessed with two objective techniques. Invest Ophthalmol Vis Sci 2000;41:3322-6.

8. Hammond BR, Jr., Johnson EJ, Russell RM et al. Dietary modification of human macular pigment density. Invest Ophthalmol Vis Sci 1997;38:1795-801.

9. Johnson EJ, Hammond BR, Yeum KJ et al. Relation among serum and tissue concentrations of lutein and zeaxanthin and macular pigment density. Am J Clin Nutr 2000;71:1555-62.

10. Junghans A, Sies H, Stahl W. Macular pigments lutein and zeaxanthin as blue light filters studied in liposomes. Arch Biochem Biophys 2001;391:160-4.

11. www.visioncare.com. Website 2006.

12. www.nei.nih.gov. Website 2006.

13. Stahl W, Sies H. Bioactivity and protective effects of natural carotenoids. Biochim Biophys Acta 2005;1740:101-7.

14. Thomson LR, Toyoda Y, Langner A et al. Elevated retinal zeaxanthin and prevention of light-induced photoreceptor cell death in quail. Invest Ophthalmol Vis Sci 2002;43:3538-49.

15. Granado F, Olmedilla B, Blanco I. Nutritional and clinical relevance of lutein in human health. Br J Nutr 2003;90:487-502.

16. Stahl W, Sundquist AR, Hanusch M, Schwarz W, Sies H. Separation of beta-carotene and lycopene geometrical isomers in biological samples. Clin Chem 1993;39:810-4.

17. Brady WE, Mares-Perlman JA, Bowen P, Stacewicz-Sapuntzakis M. Human serum carotenoid concentrations are related to physiologic and lifestyle factors. J Nutr 1996;126:129-37.

18. Whitehead AJ, Mares JA, Danis RP. Macular pigment: a review of current knowledge. Arch Ophthalmol 2006;124:1038-45.

19. Khachik F, Bernstein PS, Garland DL. Identification of lutein and zeaxanthin oxidation products in human and monkey retinas. Invest Ophthalmol Vis Sci 1997;38:1802-11.

20. Erdman JW, Jr., Bierer TL, Gugger ET. Absorption and transport of carotenoids. Ann N Y Acad Sci 1993;691:76-85.

21. Wang, Y, Connor, W, and Wei W. 2003.
    Ref Type: Unpublished Work

22. Shils O. Modern Nutrition in health and disease. 2004.

23. Krinsky NI, Landrum JT, Bone RA. Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye. Annu Rev Nutr 2003;23:171-201.

24. Rapp LM, Maple SS, Choi JH. Lutein and zeaxanthin concentrations in rod outer segment membranes from perifoveal and peripheral human retina. Invest Ophthalmol Vis Sci 2000;41:1200-9.

25. Yoshizawa T, Kuwata O. Iodopsin, a red-sensitive cone visual pigment in the chicken retina. Photochem Photobiol 1991;54:1061-70.

26. Davies NP, Morland AB. Macular pigments: their characteristics and putative role. Prog Retin Eye Res 2004;23:533-59.

27. Snodderly DM, Brown PK, Delori FC, Auran JD. The macular pigment. I. Absorbance spectra, localization, and discrimination from other yellow pigments in primate retinas. Invest Ophthalmol Vis Sci 1984;25:660-73.

28. Bone RA, Landrum JT, Fernandez L, Tarsis SL. Analysis of the macular pigment by HPLC: retinal distribution and age study. Invest Ophthalmol Vis Sci 1988;29:843-9.

29. Wald A, Wolfowitz J. Bayes Solutions of Sequential Decision Problems. Proc Natl Acad Sci U S A 1949;35:99-102.

30. Handelman GJ, Dratz EA, Reay CC, van Kuijk JG. Carotenoids in the human macula and whole retina. Invest Ophthalmol Vis Sci 1988;29:850-5.

31. Bone RA, Landrum JT, Friedes LM et al. Distribution of lutein and zeaxanthin stereoisomers in the human retina. Exp Eye Res 1997;64:211-8.

32. Neuringer M, Sandstrom MM, Johnson EJ, Snodderly DM. Nutritional manipulation of primate retinas, I: effects of lutein or zeaxanthin supplements on serum and macular pigment in xanthophyll-free rhesus monkeys. Invest Ophthalmol Vis Sci 2004;45:3234-43.

33. Leung IY, Sandstrom MM, Zucker CL, Neuringer M, Snodderly DM. Nutritional manipulation of primate retinas, II: effects of age, n-3 fatty acids, lutein, and zeaxanthin on retinal pigment epithelium. Invest Ophthalmol Vis Sci 2004;45:3244-56.

34. Johnson EJ, Neuringer M, Russell RM, Schalch W, Snodderly DM. Nutritional manipulation of primate retinas, III: Effects of lutein or zeaxanthin supplementation on adipose tissue and retina of xanthophyll-free monkeys. Invest Ophthalmol Vis Sci 2005;46:692-702.

35. Bhosale P, Larson AJ, Frederick JM, Southwick K, Thulin CD, Bernstein PS. Identification and characterization of a Pi isoform of glutathione S-transferase (GSTP1) as a zeaxanthin-binding protein in the macula of the human eye. J Biol Chem 2004;279:49447-54.

36. Toyoda Y, Thomson LR, Langner A et al. Effect of dietary zeaxanthin on tissue distribution of zeaxanthin and lutein in quail. Invest Ophthalmol Vis Sci 2002;43:1210-21.

37. Krinsky NI. Possible biologic mechanisms for a protective role of xanthophylls. J Nutr 2002;132:540S-2S.

38. Neuringer M. Infant vision and retinal function in studies of dietary long-chain polyunsaturated fatty acids: methods, results, and implications. Am J Clin Nutr 2000;71:256S-67S.

39. Gruszecki WI, Strzalka K. Carotenoids as modulators of lipid membrane physical properties. Biochim Biophys Acta 2005;1740:108-15.

40. Strzalka K, Gruszecki WI. Effect of beta-carotene on structural and dynamic properties of model phosphatidylcholine membranes. I. An EPR spin label study. Biochim Biophys Acta 1994;1194:138-42.

41. Snodderly DM. Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. Am J Clin Nutr 1995;62:1448S-61S.

42. Schmidt-Erfurth U. Nutrition and retina. Dev Ophthalmol 2005;38:120-47.

43. Beatty S, Murray IJ, Henson DB, Carden D, Koh H, Boulton ME. Macular pigment and risk for age-related macular degeneration in subjects from a Northern European population. Invest Ophthalmol Vis Sci 2001;42:439-46.

44. Bernstein PS, Zhao DY, Wintch SW, Ermakov IV, McClane RW, Gellermann W. Resonance Raman measurement of macular carotenoids in normal subjects and in age-related macular degeneration patients. Ophthalmology 2002;109:1780-7.

45. Ermakov IV, Ermakova MR, Gellermann W. Simple Raman instrument for in vivo detection of macular pigments. Appl Spectrosc 2005;59:861-7.

46. Falsini B, Piccardi M, Iarossi G, Fadda A, Merendino E, Valentini P. Influence of short-term antioxidant supplementation on macular function in age-related maculopathy: a pilot study including electrophysiologic assessment. Ophthalmology 2003;110:51-60.

47. Thomson LR, Toyoda Y, Langner A et al. Elevated retinal zeaxanthin and prevention of light-induced photoreceptor cell death in quail. Invest Ophthalmol Vis Sci 2002;43:3538-49.

48. Thomson LR, Toyoda Y, Delori FC et al. Long term dietary supplementation with zeaxanthin reduces photoreceptor death in light-damaged Japanese quail. Exp Eye Res 2002;75:529-42.

49. Feeney-Burns L, Neuringer M, Gao CL. Macular pathology in monkeys fed semipurified diets. Prog Clin Biol Res 1989;314:601-22.

50. Neuringer, M. 2006.
Ref Type: Unpublished Work

51. Kim J, DellaPenna D. Defining the primary route for lutein synthesis in plants: the role of Arabidopsis carotenoid beta-ring hydroxylase CYP97A3. Proc Natl Acad Sci U S A 2006;103:3474-9.

52. Nilsson SE, Sundelin SP, Wihlmark U, Brunk UT. Aging of cultured retinal pigment epithelial cells: oxidative reactions, lipofuscin formation and blue light damage. Doc Ophthalmol 2003;106:13-6.

53. Liaaen-Jensen S. Developments in the carotenoid field. Experientia 1970;26:697-710.

54. Ham WT, Jr., Mueller HA, Ruffolo JJ, Jr. et al. Basic mechanisms underlying the production of photochemical lesions in the mammalian retina. Curr Eye Res 1984;3:165-74.

55. Burton GW, Ingold KU. beta-Carotene: an unusual type of lipid antioxidant. Science 1984;224:569-73.

56. SanGiovanni JP, Chew EY. The role of omega-3 long-chain polyunsaturated fatty acids in health and disease of the retina. Prog Retin Eye Res 2005;24:87-138.

57. Bush RA, Reme CE, Malnoe A. Light damage in the rat retina: the effect of dietary deprivation of N-3 fatty acids on acute structural alterations. Exp Eye Res 1991;53:741-52.

58. Kennedy CJ, Rakoczy PE, Constable IJ. Lipofuscin of the retinal pigment epithelium: a review. Eye 1995;9 (Pt 6):763-71.

59. Scheidegger R, Pande AK, Bounds PL, Koppenol WH. The reaction of peroxynitrite with zeaxanthin. Nitric Oxide 1998;2:8-16.

60. Saari JC, Huang J, sson-Batres MA et al. Evidence of retinoid metabolism within cells of inner retina. Exp Eye Res 1995;60:209-12.

61. Lavail MM, Mullen RJ. Role of the pigment epithelium in inherited retinal degeneration analyzed with experimental mouse chimeras. Exp Eye Res 1976;23:227-45.

62. Karadas F, Pappas AC, Surai PF, Speake BK. Embryonic development within carotenoid-enriched eggs influences the post-hatch carotenoid status of the chicken. Comp Biochem Physiol B Biochem Mol Biol 2005;141:244-51.

63. Fishman B. Electrophysiological Testing. 2001.

64. Perkin Elmer Instruments, et. al. 2006.
Ref Type: Catalog

65. Johnson E. 2006.
Ref Type: Unpublished Work

66. Thomson LR, Toyoda Y, Langner A et al. Elevated retinal zeaxanthin and prevention of light-induced photoreceptor cell death in quail. Invest Ophthalmol Vis Sci 2002;43:3538-49.

67. Organisciak DT, Jiang YL, Wang HM, Pickford M, Blanks JC. Retinal light damage in rats exposed to intermittent light. Comparison with continuous light exposure. Invest Ophthalmol Vis Sci 1989;30:795-805.

68. Montiani-Ferreira F, Li T, Kiupel M et al. Clinical features of the retinopathy, globe enlarged (rge) chick phenotype. Vision Res 2003;43:2009-18.

69. Milanowska J, Gruszecki WI. Heat-induced and light-induced isomerization of the xanthophyll pigment zeaxanthin. J Photochem Photobiol B 2005;80:178-86.

70. Connor W and Wang Y. 2004.
    Ref Type: Unpublished Work

71. Connor W, Wang Y, and Connor S. 2006.
    Ref Type: Unpublished Work

72. Koutsos EA, Clifford AJ, Calvert CC, Klasing KC. Maternal carotenoid status modifies the incorporation of dietary carotenoids into immune tissues of growing chickens (Gallus gallus domesticus). J Nutr 2003;133:1132-8.

73. Bhosale P, Bernstein PS. Synergistic effects of zeaxanthin and its binding protein in the prevention of lipid membrane oxidation. Biochim Biophys Acta 2005;1740:116-21.

74. SPRECHT NW. Management of the anemias; diagnosis and treatment. Ariz Med 1955;12:71-4.

75. Organisciak DT, Jiang YL, Wang HM, Pickford M, Blanks JC. Retinal light damage in rats exposed to intermittent light. Comparison with continuous light exposure. Invest Ophthalmol Vis Sci 1989;30:795-805.

76. Malik S, Cohen D, Meyer E, Perlman I. Light damage in the developing retina of the albino rat: an electroretinographic study. Invest Ophthalmol Vis Sci 1986;27:164-7.

77. Li J, Tso MO, Lam TT. Reduced amplitude and delayed latency in foveal response of multifocal electroretinogram in early age related macular degeneration. Br J Ophthalmol 2001;85:287-90.

78. Organisciak DT, Xie A, Wang HM, Jiang YL, Darrow RM, Donoso LA. Adaptive changes in visual cell transduction protein levels: effect of light. Exp Eye Res 1991;53:773-9.

79. Cao W, Li F, Steinberg RH, Lavail MM. Development of normal and injury-induced gene expression of aFGF, bFGF, CNTF, BDNF, GFAP and IGF-I in the rat retina. Exp Eye Res 2001;72:591-604.

80. Yu Q, Kent CR, Tytell M. Retinal uptake of intravitreally injected Hsc/Hsp70 and its effect on susceptibility to light damage. Mol Vis 2001;7:48-56.

81. Harada C, Harada T, Quah HM et al. Potential role of glial cell line-derived neurotrophic factor receptors in Muller glial cells during light-induced retinal degeneration. Neuroscience 2003;122:229-35.

82. Gauthier R, Joly S, Pernet V, Lachapelle P, Di PA. Brain-derived neurotrophic factor gene delivery to muller glia preserves structure and function of light-damaged photoreceptors. Invest Ophthalmol Vis Sci 2005;46:3383-92.

83. Landers RA, Rayborn ME, Myers KM, Hollyfield JG. Increased retinal synthesis of heparan sulfate proteoglycan and HNK-1 glycoproteins following photoreceptor degeneration. J Neurochem 1994;63:737-50.

84. Thomson LR, Toyoda Y, Langner A et al. Elevated retinal zeaxanthin and prevention of light-induced photoreceptor cell death in quail. Invest Ophthalmol Vis Sci 2002;43:3538-49.

85. Y Nys. Dietary carotenoids and egg yolk coloration--a review. Arch Geflugelk 2000;64:45-54.

86. Stoianov P, Zhekov R. [Causes of disorders affecting the hatching and viability of broiler chicks]. Vet Med Nauki 1982;19:47-51.

87. Bermudez AJ, Swayne DE, Squires MW, Radin MJ. Effects of vitamin A deficiency on the reproductive system of mature White Leghorn hens. Avian Dis 1993;37:274-83.

88. Koutsos EA, Garcia Lopez JC, Klasing KC. Carotenoids from in ovo or dietary sources blunt systemic indices of the inflammatory response in growing chicks (Gallus gallus domesticus). J Nutr 2006;136:1027-31.

89. Blount JD. Carotenoids and life-history evolution in animals Dietary Carotenoids and Egg Yolk Coloration--A Review. Arch Biochem Biophys 2004;430:10-5.

90. Handelman GJ, Nightingale ZD, Lichtenstein AH, Schaefer EJ, Blumberg JB. Lutein and zeaxanthin concentrations in plasma after dietary supplementation with egg yolk. Am J Clin Nutr 1999;70:247-51.

91. Thomson LR, Toyoda Y, Langner A et al. Elevated retinal zeaxanthin and prevention of light-induced photoreceptor cell death in quail. Invest Ophthalmol Vis Sci 2002;43:3538-49.

Table 1 Control diet formulation fed to control diet chicks for 36 days

Control Diet Formulation	
Diet Composition	**Control Diet** [#]
Casein (g/kg)	260
Brewer's Yeast (g/kg)	150
Sucrose (g/kg)	165
Cellulose (g/kg)	150
Soy Oil (g/kg)	200
Vitamins/Minerals (g/kg)	75
Lutein (mg/kg)	5.2
Zeaxanthin (mg/kg)	1.7

[#] From Purina Mills, LLC, St. Louis, P.O. Box 66812, MO, USA.

Table 2 Lutein and zeaxanthin supplemented diet formulation fed to supplemented chicks for 36 days.

Lutein and Zeaxanthin Supplemented Diet Formulation	
Diet Composition	**Control Diet [#]**
Casein (g/kg)	260
Brewer's Yeast (g/kg)	150
Sucrose (g/kg)	165
Cellulose (g/kg)	150
Soy Oil (g/kg)	200
Vitamins/Minerals (g/kg)	75
Lutein (mg/kg)	27.2
Zeaxanthin (mg/kg)	15.3

[#] From Purina Mills, LLC, St. Louis, P.O. Box 66812, MO, USA.

Table 3 Vitamins and minerals added to control, supplemented, and deficient diets

Vitamin or Mineral	g/kg diet
Calcium Phosphate	40.7
Calcium Carbonate	13.75
Potassium Citrate	8.25
Sodium Chloride	7.013
Magnesium Oxide	1.375
Ferric Citrate	1.1
Manganese Sulfate	0.413
Zinc Carbonate	0.165
Cupric Sulfate	0.076
Chromium Potassium Sulfate	0.041
Potassium Iodate	0.007
Sodium Selenite	0.0004
Choline Chloride	2.75
Niacin	0.041
Vitamin B12	0.069
Calcium Pantothenate	0.041
Thiamin HCl	0.014
Menadione Sodium Bisulfite Complex	0.023
Pyridoxine HCl	0.015
Riboflavin	0.015
Folic Acid	0.0041
Biotin	0.0007
Dry Vitamin E Acetate (500 U/g)	0.1375
Dry Vitamin A Palmitate	0.0275
Dry Vitamin D3	0.0083

*Diet designed for use at 80% (800 g/kg) with 20% (200 g/kg) of fats

Table 4 Lutein and zeaxanthin deficient diet formulation fed to deficient laying hens

Lutein and Zeaxanthin Deficient Diet Formulation	
Diet Composition	**Control Diet [#]**
Casein (g/kg)	260
Brewer's Yeast (g/kg)	150
Sucrose (g/kg)	165
Cellulose (g/kg)	150
Soy Oil (g/kg)	200
Vitamins/Minerals (g/kg)	75
Lutein (mg/kg)	0
Zeaxanthin (mg/kg)	0

[#] From Purina Mills, LLC, St. Louis, P.O. Box 66812, MO, USA

Table 5 Number of weekly collected and incubated lutein and zeaxanthin deficient eggs

1st batch	Weight (kg)	# eggs
Hen #1	2.2	4
Hen #2	1.6	1
Hen #3	2.2	2
2nd batch		
Hen #1	2.0	7
Hen #2	1.9	8
Hen #3	2.0	7
3rd batch		
Hen #1	1.3	2
Hen #2	1.3	2
Hen #3	1.0	3

Table 6A   There were increased levels of *cis*-zeaxanthin in the retinas of supplemented diet chicks, P < 0.005.

Cis Zeaxanthin Retinal Concentrations						
Groups	*N*	*Mean*	*Standard Deviation*			
Control Diet Light Exposed	6	0.469352	0.06703			
Control Diet non-Light Exposed	6	0.413793	0.058619			
Supplemented Diet Light Exposed	6	3.020146	2.544401			
Supplemented Diet non-Light Exposed	5	2.657582	1.376061			
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	33.80562	3	11.26854	11.3555	**0.000172**	3.127354
Within Groups	18.85449	19	0.992342			
Total	52.66011	22				

Table 6B   The increased levels of *cis*-zeaxanthin in the retina were a result of increased levels of zeaxanthin in the supplemented diet, not as a result of light exposure.

Control Diet Light Exposed vs. Supplemented Diet Light Exposed	**P=0.0073**
Control Diet non-Light Exposed vs. Supplemented Diet non-Light Exposed	**P=0.0033**
Control Diet Light Exposed vs. Supplemented Diet non-Light Exposed	**P=0.0057**
Control Diet non-Light Exposed vs. Supplemented Diet Light Exposed	**P=0.008**

Lutein
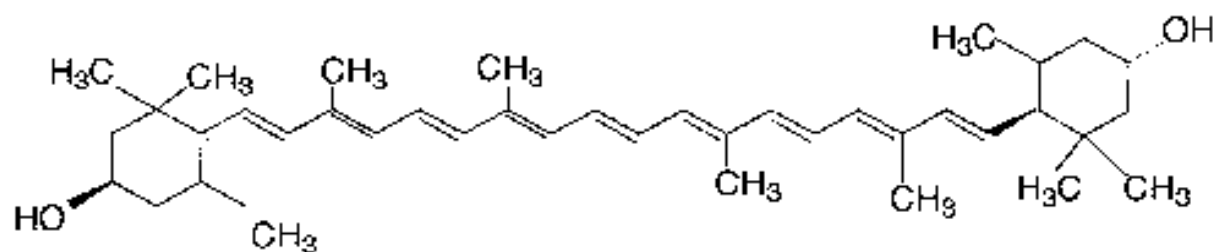
Zeaxanthin
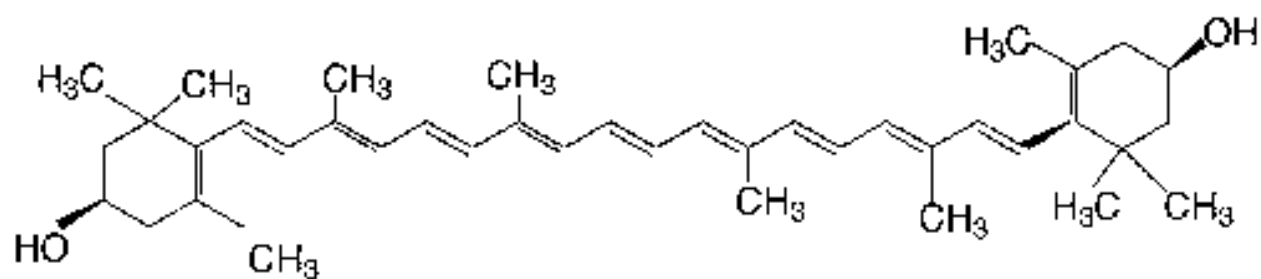
Figure 1
Chemical structures of lutein and zeaxanthin

Photopic ERG
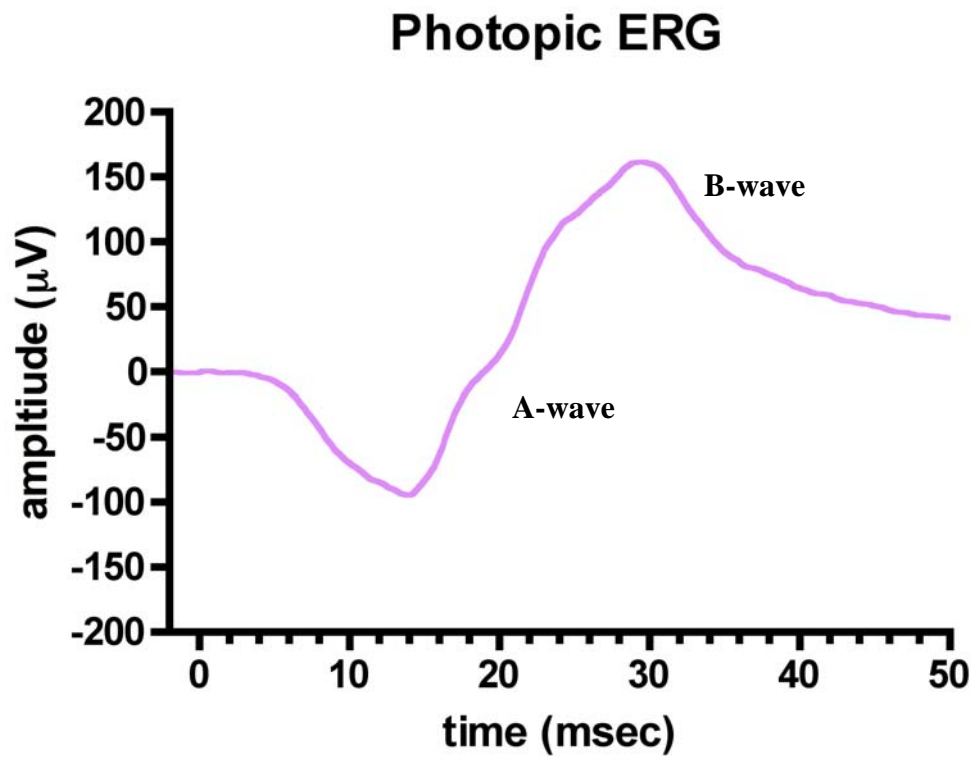
Figure 2
The ERG biphasic waveform is elicited by increasing intensities of flashes of light. The initial negative deflection (A wave) is the result of hyperpolarization of photoreceptors. The second positive deflection (B wave) is caused by depolarization of the neuronal cells.

Photopic
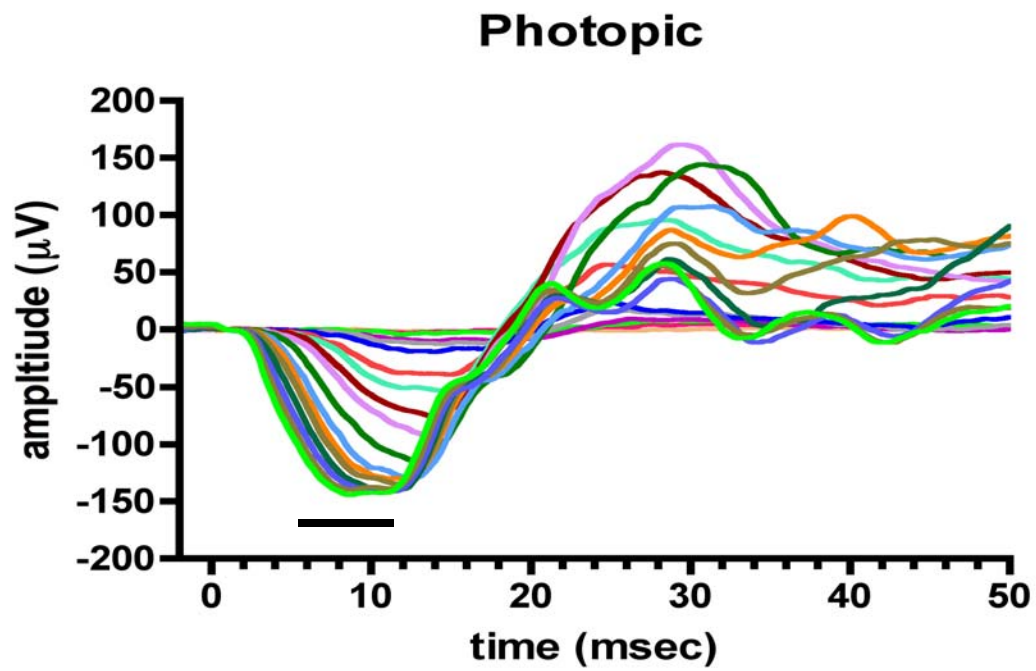
Figure 3
The superimposed ERG biphasic waveforms illustrate that the maximum cone cell amplitude response during the A-wave takes approximately 3 to 10 ms.
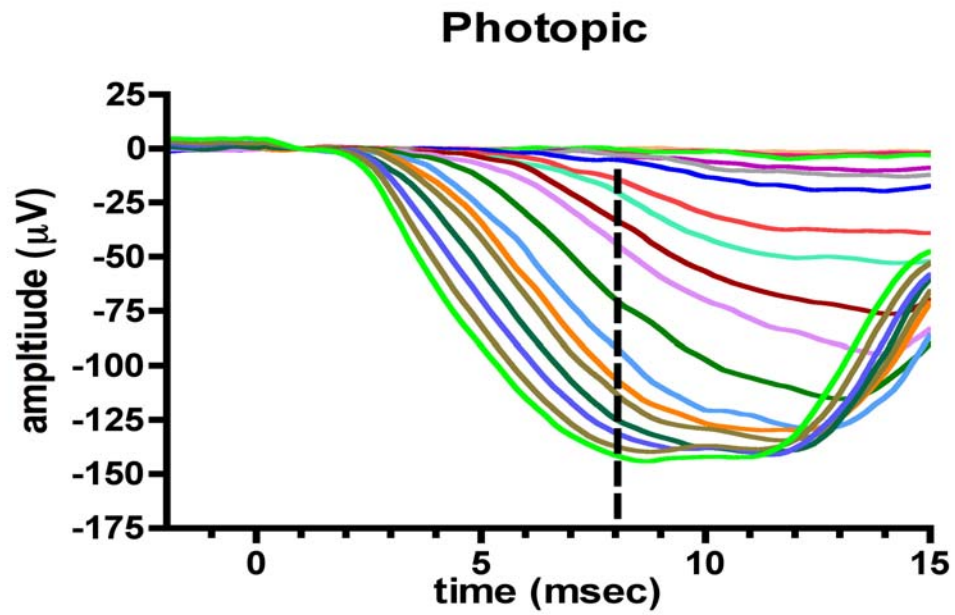
Figure 4
The 3 to 10 ms time frame indicates that the maximum amplitude cone response occurs approximately at 8 ms.
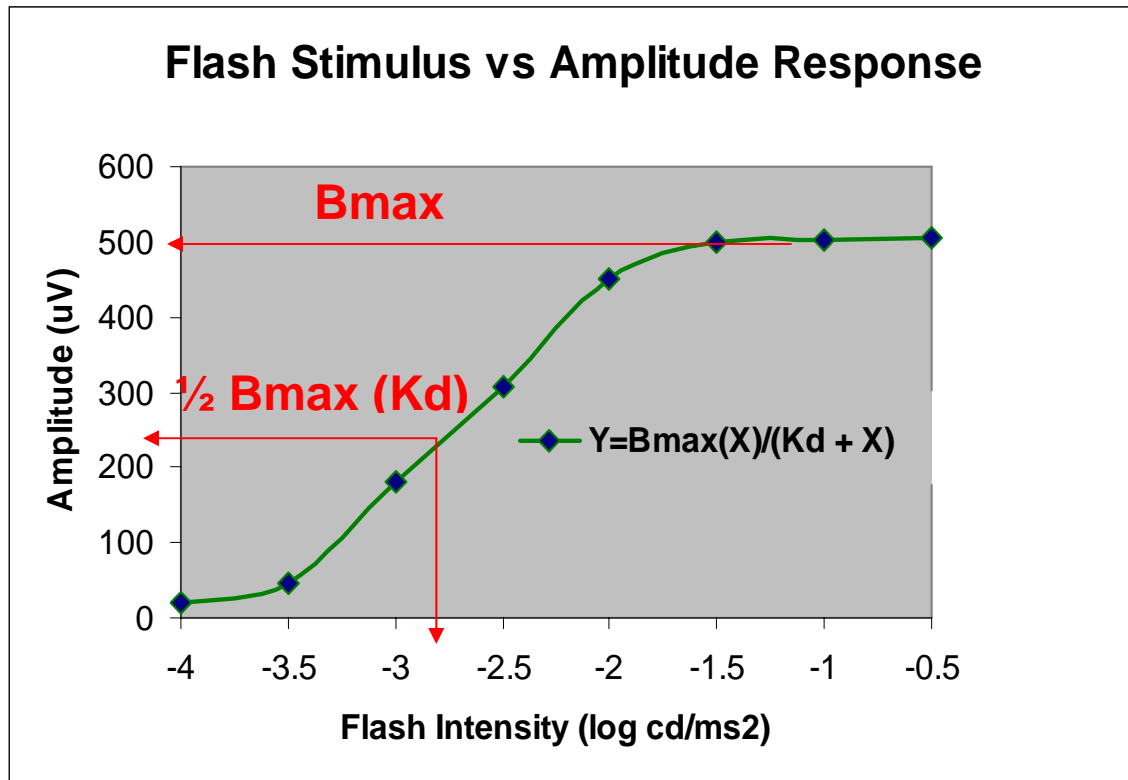
Figure 5
The ERG amplitude responses (Y) with increasing flash intensities (X) are illustrated above. The stimulus/response relationship, Y=Bmax(X)/(Kd + X), is used to derive parameters including Bmax, the maximal amplitude response and Kd, (half Bmax), an index of sensitivity
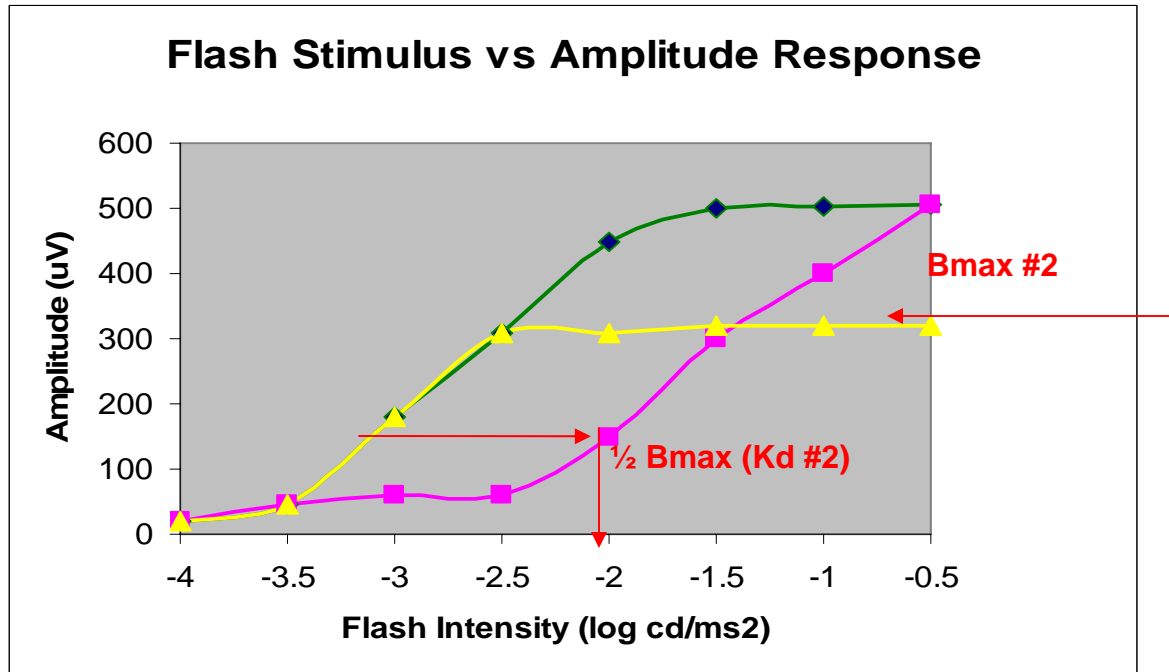
Figure 6

A hypothetical example of amplitude responses from a light damaged retina are illustrated above. When compared to the normal amplitude responses, Bmax #2 will have a decreased amplitude response. Kd #2 will be shifted to the right; an increased Kd indicates decreased sensitivity of the photoreceptors.
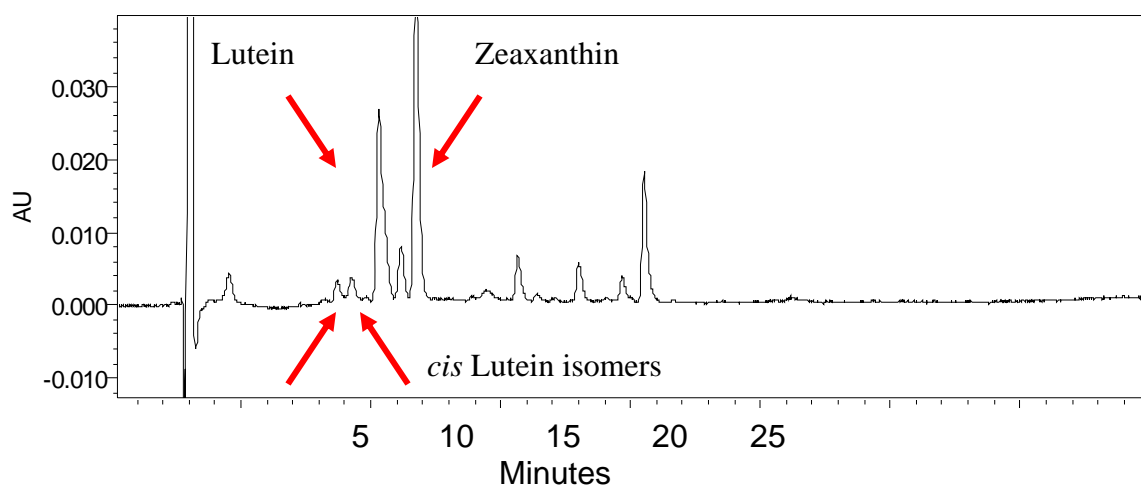
Figure 7 An HPLC chromatogram from a control diet chick retina is illustrated above. Lutein and zeaxanthin peak at retention times of 6-7 minutes and 8-9 minutes. Lutein has two isomers at retention times of approximately 2 and 3 minutes.
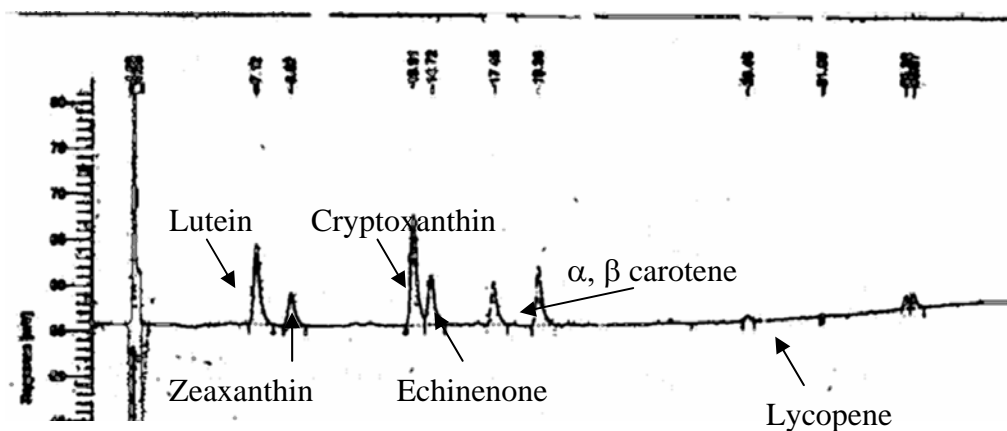
Figure 8

The 7 standards used to analyze the concentrations of lutein and zeaxanthin HPLC are illustrated above. The retention times of 7.12, 8.62, 13.91, 14.72, 17.45, 19.36, and 35.32/35.67 minutes correlate to the standard concentrations of lutein, zeaxanthin, cryptoxanthin, echinenone (internal standard), α-carotene, β-carotene, and lycopene, respectively. The retention times of 28.48 and 31.69 minutes are from unknown compounds.
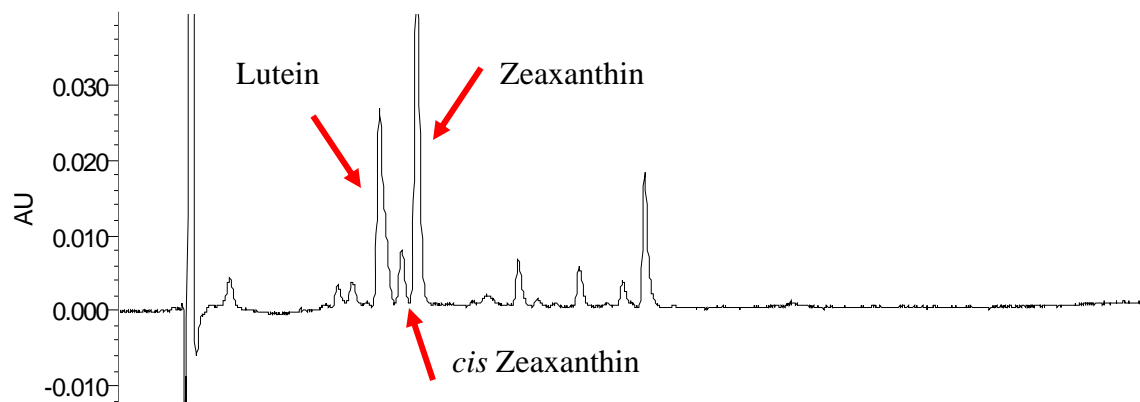
Figure 9
An HPLC chromatogram from a control diet chick retina is illustrated above. The distinct *cis* zeaxanthin isomer is situated between the peaks of lutein and zeaxanthin.

Research Design Scheme
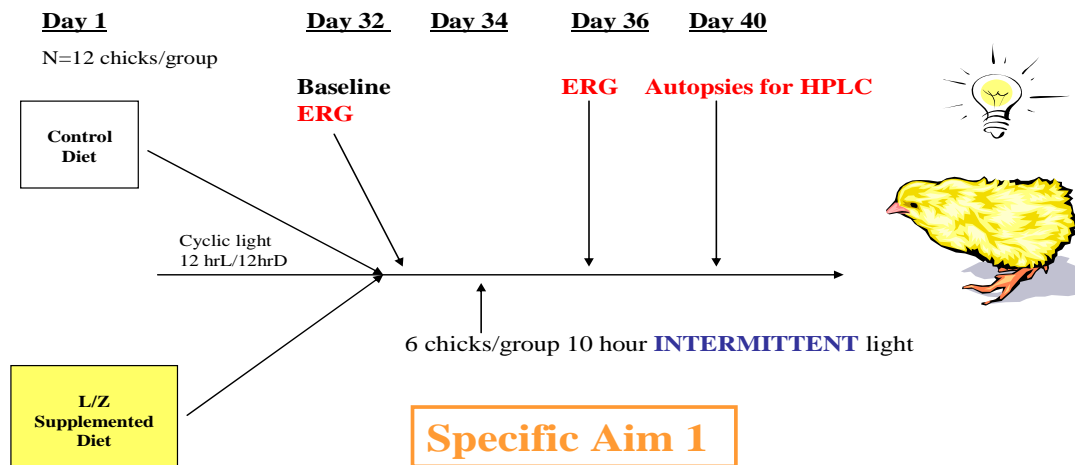
Figure 10 Research design and methodology for Specific Aim 1. Chicks were fed treatment diet on Day 1 and on Day 32 baseline ERGs were measured. On Day 34 half of each group was exposed to intermittent light for 10 hours, then measurement of post-treatment ERGs on Day 36. Autopsies for collection of tissues occurred on Day 40.
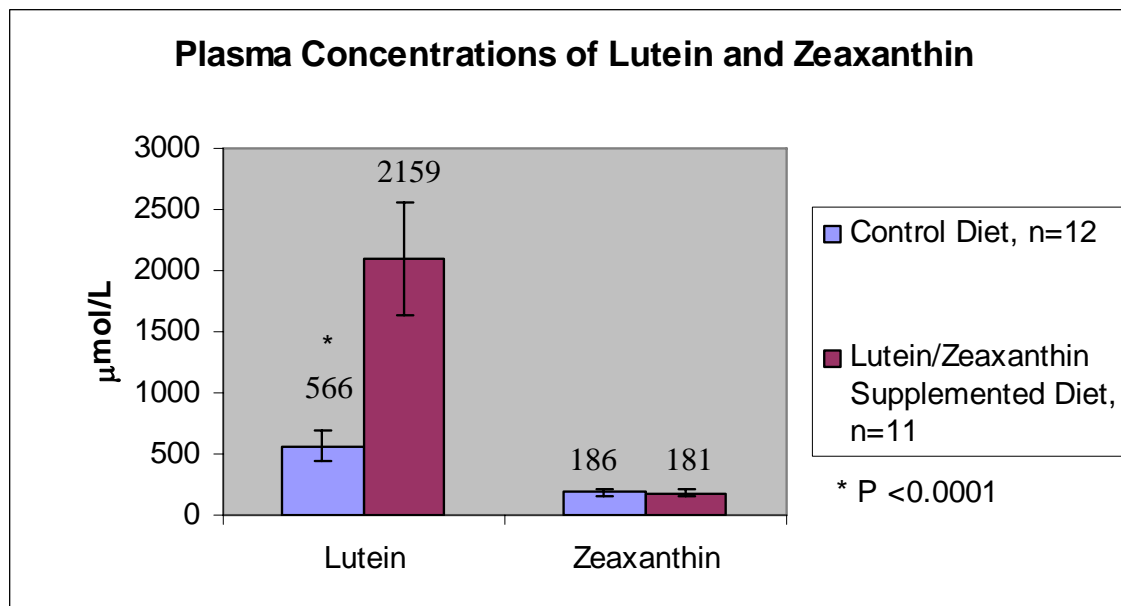
Figure 11 Plasma concentrations of lutein and zeaxanthin from chicks fed control and lutein and zeaxanthin supplemented diets. Mean values are expressed as (μmol/L $\pm$ SD). P-values from a 2 way ANOVA: Control diet lutein x Supplemented diet lutein, P <0.0001 (*); Control diet zeaxanthin x Supplemented diet zeaxanthin, P= 0.88; Control diet plasma levels of lutein were greater than zeaxanthin in plasma, (566 μmol/L $\pm$ 2.3 vs 186 μmol/L $\pm$ 3.3), P < 0.0001.
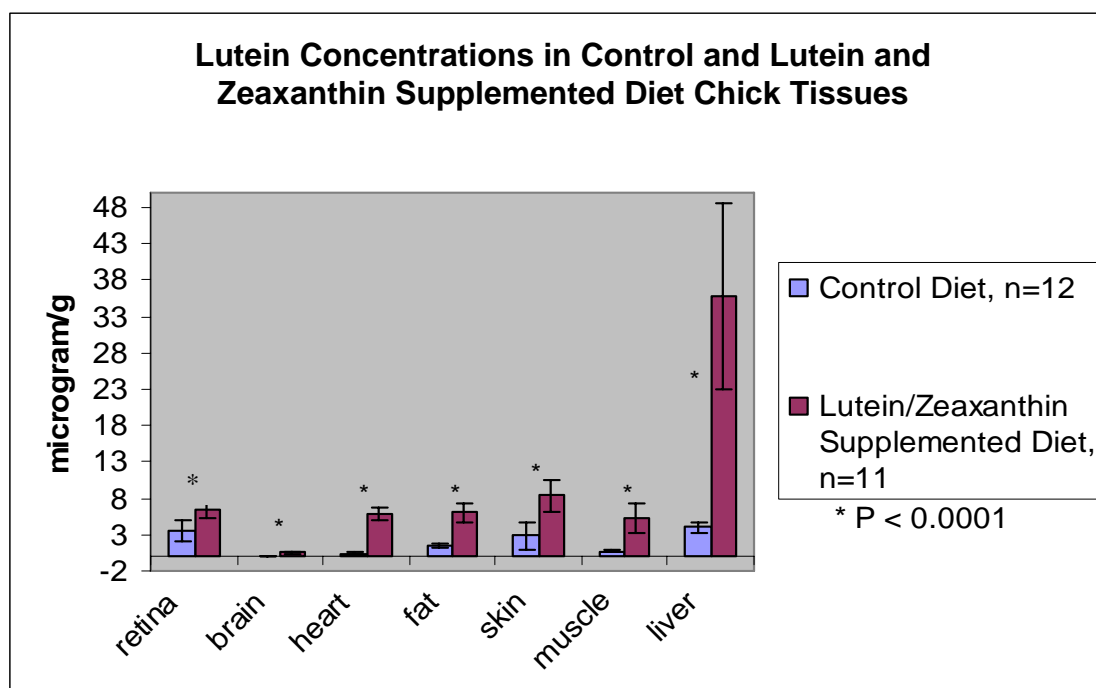
Figure 12 Lutein concentrations in retinas of supplemented were significantly greater than controls with means of 6.41$\pm$ 1.44 and 3.62 $\pm$ 1.09 μg/g, P < 0.005, respectively. The retina, brain, heart, fat, skin, muscle and liver content of supplemented chicks also had significantly greater mean concentrations of lutein than compared to controls (*), P < 0.0001. Mean values (μg/g) $\pm$ SD are given below for all tissues.

Lutein Concentrations in Tissues

Tissue	Control Diet Mean (μg/g) $\pm$ SD	Supplemented Diet Mean (μg/g) $\pm$ SD	P Value
Retina	3.62 $\pm$ 1.45	6.40 $\pm$ 1.09	0.00031
Brain	0.09 $\pm$ 0.03	0.54 $\pm$ 0.08	>0.00001
Heart	0.45 $\pm$ 0.02	5.76 $\pm$ 0.92	>0.00001
Fat	1.53 $\pm$ 0.24	6.00 $\pm$ 1.19	>0.00001
Skin	2.83 $\pm$ 1.88	8.30 $\pm$ 2.18	0.000104
Muscle	0.76 $\pm$ 0.26	5.20 $\pm$ 2.00	0.00015
Liver	3.97 $\pm$ 0.84	35.8 $\pm$ 12.8	0.000241

Supplemented Diet: Exposed vs. Non-Exposed	Exposed Mean (µg) $\pm$ SD	Non-Exposed Mean(µg) $\pm$ SD	P value
Lutein	6.49 $\pm$ 5.76	4.96 $\pm$ 0.85	0.315
Zeaxanthin	8.60 $\pm$ 4.38	6.34 $\pm$1.30	0.35
Control Diet: Exposed vs. Non-Exposed			
Lutein	3.69 $\pm$ 1.67	3.55 $\pm$ 1.48	0.89
Zeaxanthin	11.60 $\pm$ 5.86	9.46 $\pm$ 2.41	0.7

Figure 13 No differences in lutein and zeaxanthin content were found between retinal carotenoid content in light-exposed and non-light exposed groups; therefore, all data were pooled together into dietary groups for tissue analysis
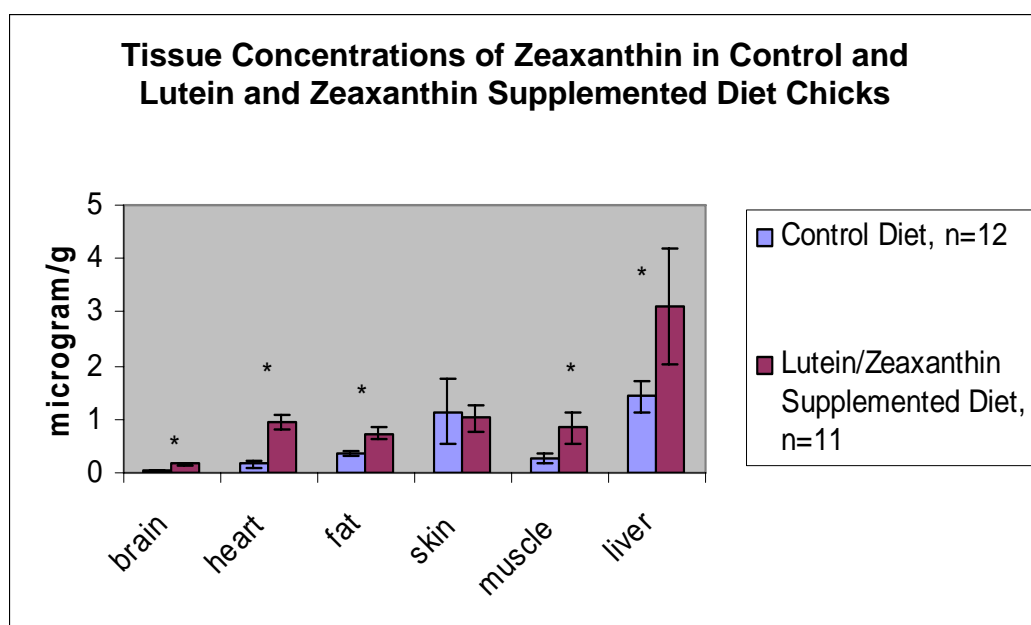
Figure 14 Supplemented chicks all showed significantly greater zeaxanthin levels than controls in all tissues, P <0.001 except for the skin. Mean values (µg/g) ± SD are given below for all tissues.

Zeaxanthin Concentrations in Tissues

Tissue	Control Diet Mean (µg/g) ± SD	Supplemented Diet Mean (µg/g) ± SD	P Value
Brain	0.052 ± 0.01	0.165 ± 0.03	>0.00001
Heart	0.16 ± 0.07	0.94 ± 0.13	>0.00001
Fat	0.36 ± 0.05	0.74 ± 0.10	>0.00001
Skin	1.13 ± 0.60	1.01 ± 0.24	0.244
Muscle	0.27 ± 0.09	0.84 ± 0.30	0.00073
Liver	1.43 ± 0.28	3.10 ± 1.08	0.00024

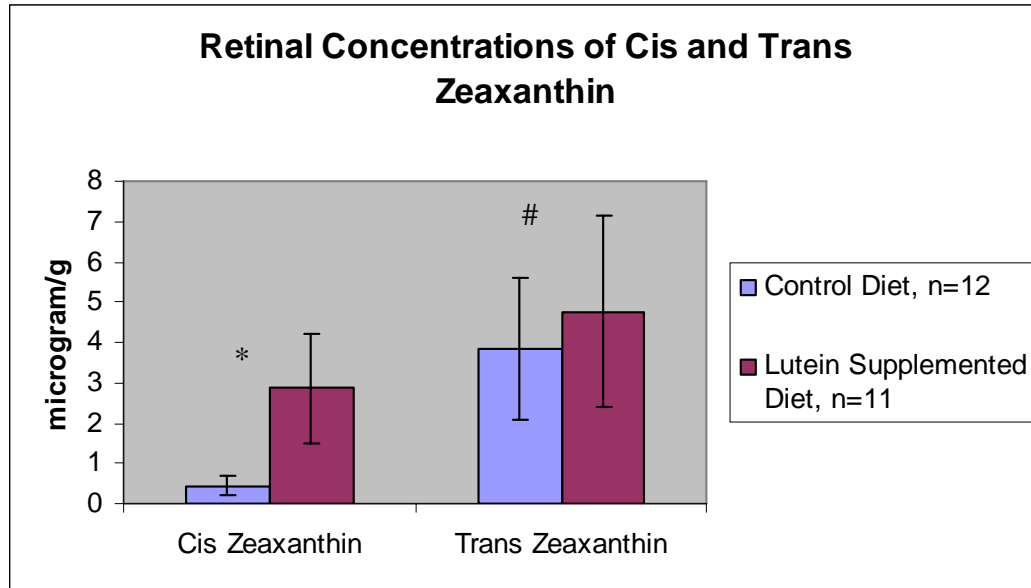**Figure 15** The *cis* form of zeaxanthin was 6 times greater in retinas of supplemented chicks than control diet chicks, 2.85 μg/g $\pm$ 1.36 vs 0.44 μg/g $\pm$ 0.23 (*), respectively, P < 0.005. There was only a 1.23 fold increase in the *trans* form in supplemented chicks as compared to control chicks, 4.75 μg/g $\pm$ 2.37 vs 3.85 μg/g $\pm$ 1.77 (#), respectively, P < 0.005. Values are expressed as mean (μg/g) $\pm$ SD.
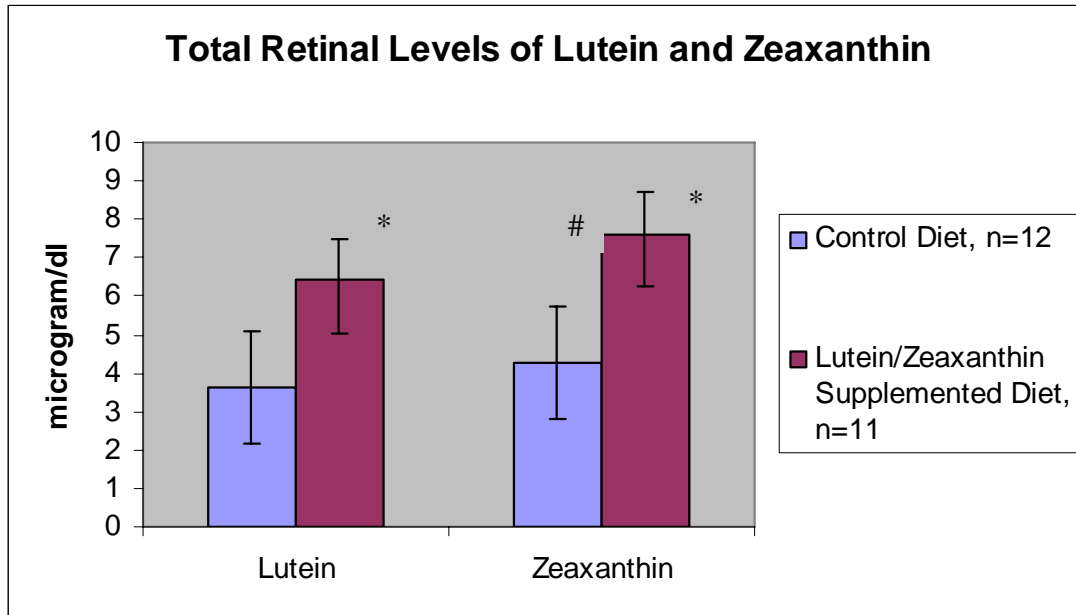
Figure 16 The total zeaxanthin levels were greater than the total lutein levels in supplemented and control chicks (*) (7.60 $\pm$ 3.73 vs 6.41 $\pm$ 1.44 µg/g, respectively) and (4.28 $\pm$ 2.0 vs 3.62 $\pm$ 1.09 µg/g, respectively), P< 0.005. The total zeaxanthin concentration was greater in the retinas of supplemented chicks than controls (#) (7.60 µg/g versus 4.27 µg/g, SD = 3.73 and 2.0, respectively), P < 0.005. Values are expressed as mean (µg/g) $\pm$ SD.
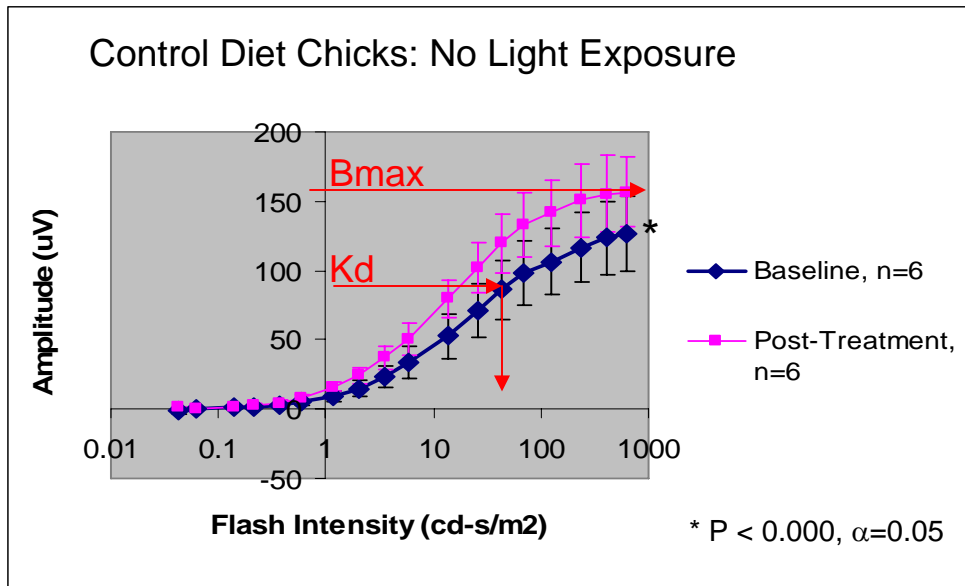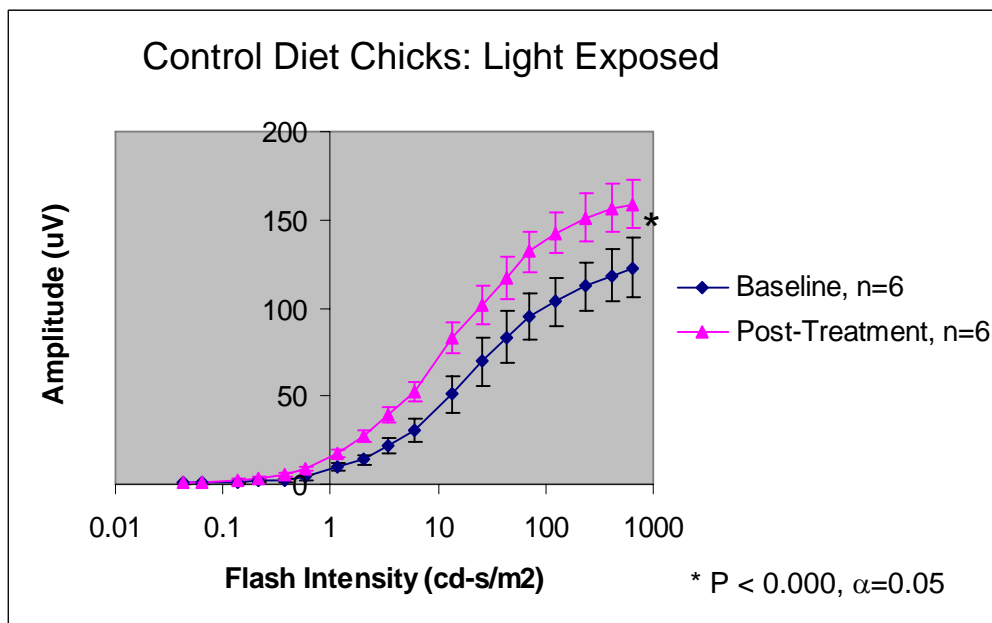
Figure 17 There was increased maximum amplitude responses post-light treatment in the chicks not exposed to light (**17A**), $p < 0.000$, and also for those exposed to light (**17B**), $p < 0.000$. This increase is not a result of the light exposure and is most likely due to retinal development of the chick causing a greater neuronal response. The parameters Bmax and Kd are illustrated in **17A** for clarity. Values are expressed as mean $(\mu V) \pm SD$.
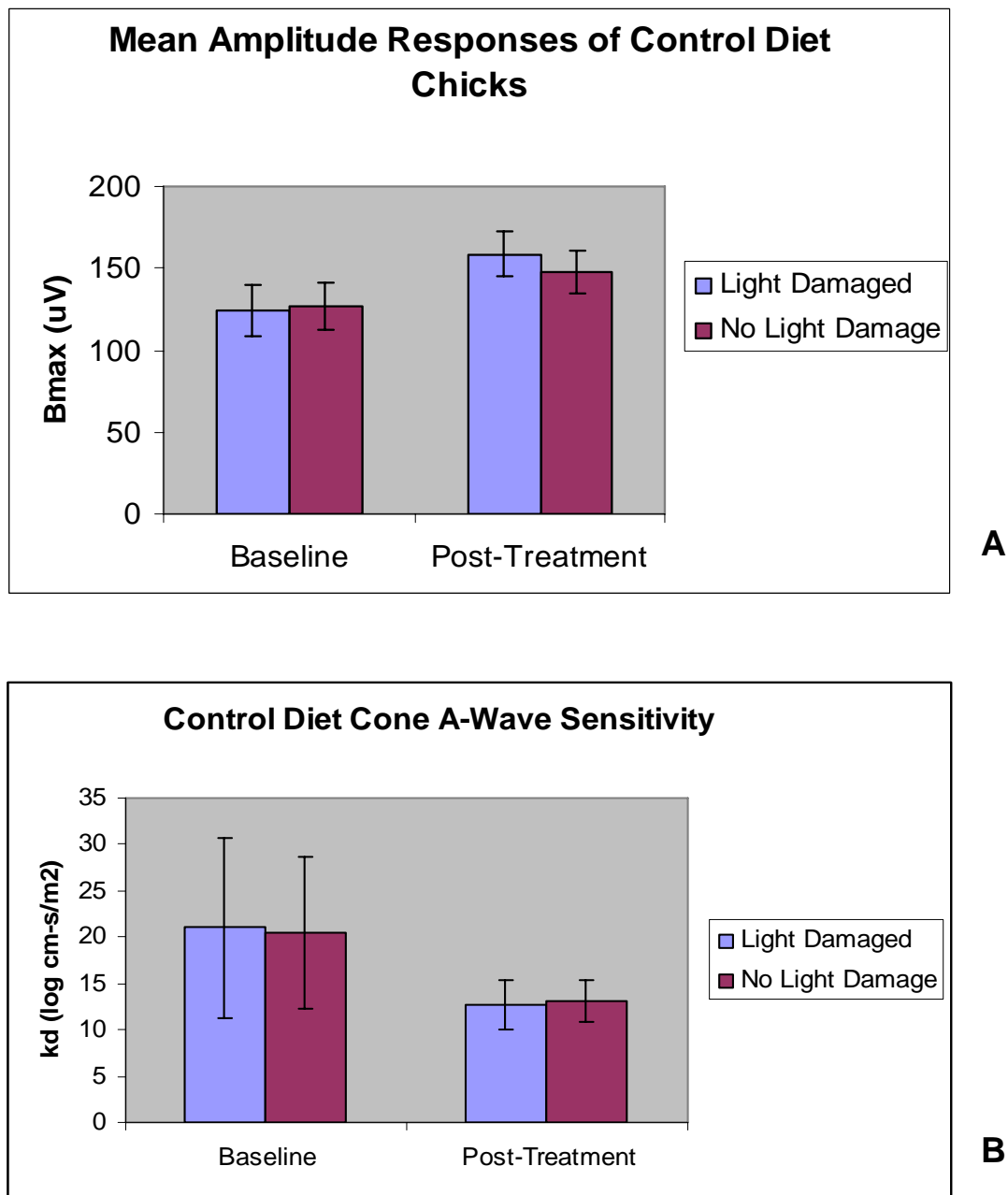
Figure 18 There were no decreases in maximum amplitude response from the photoreceptors (Bmax) (**18A)** or decreased sensitivity of the cone cells (Kd) (**18B**) as a result of light exposure. Values are expressed as mean (cm-s/m2) $\pm$ SD.
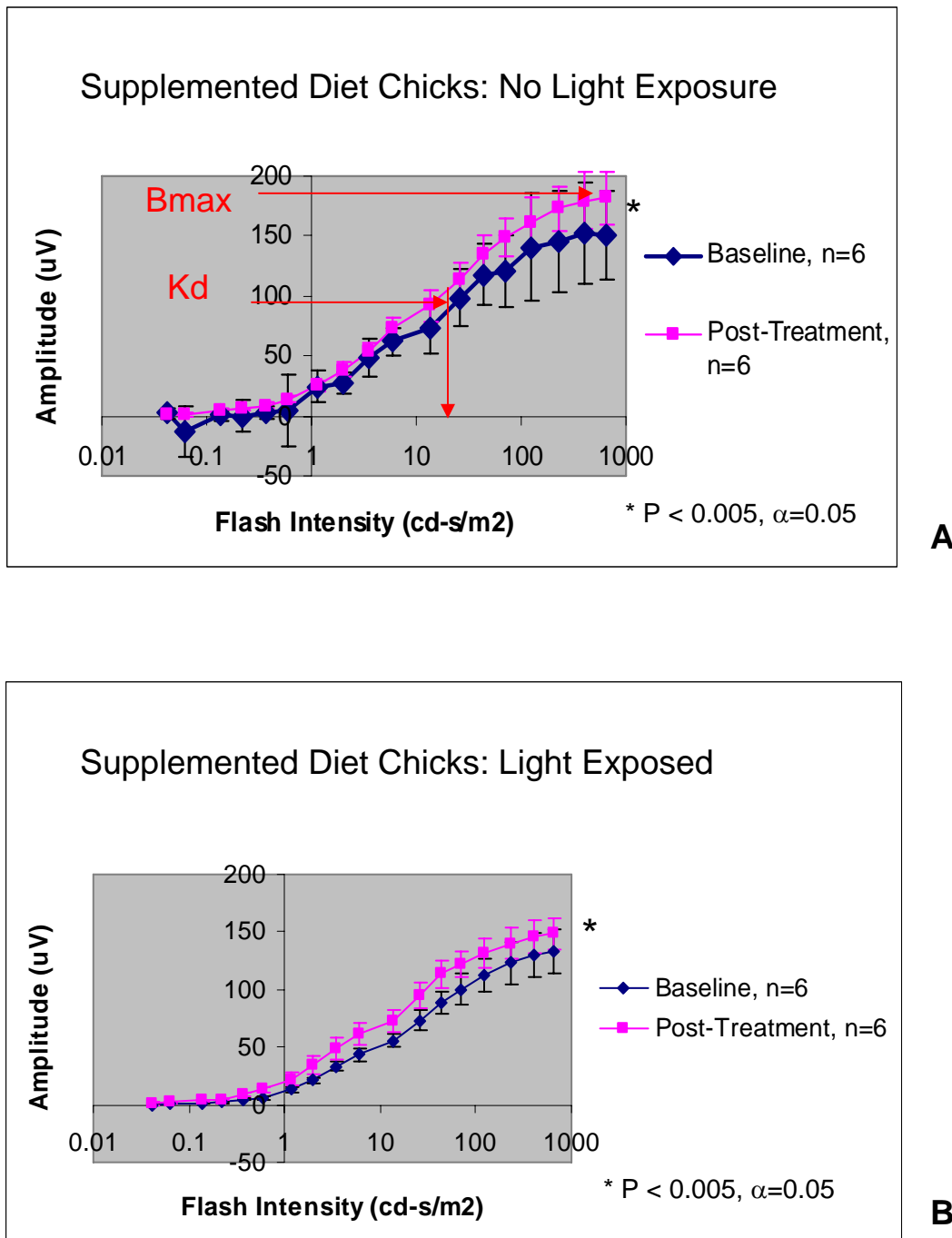
Figure 19 There was increased maximum amplitude responses post-light treatment in the chicks not exposed to light (**19A**), $p < 0.005$, and also for those exposed to light (**19B**), $p < 0.005$. This increase is not a result of the light exposure and is most likely due to retinal development of the chick causing a greater neuronal response. Values are expressed as mean (μV) $\pm$ SD.
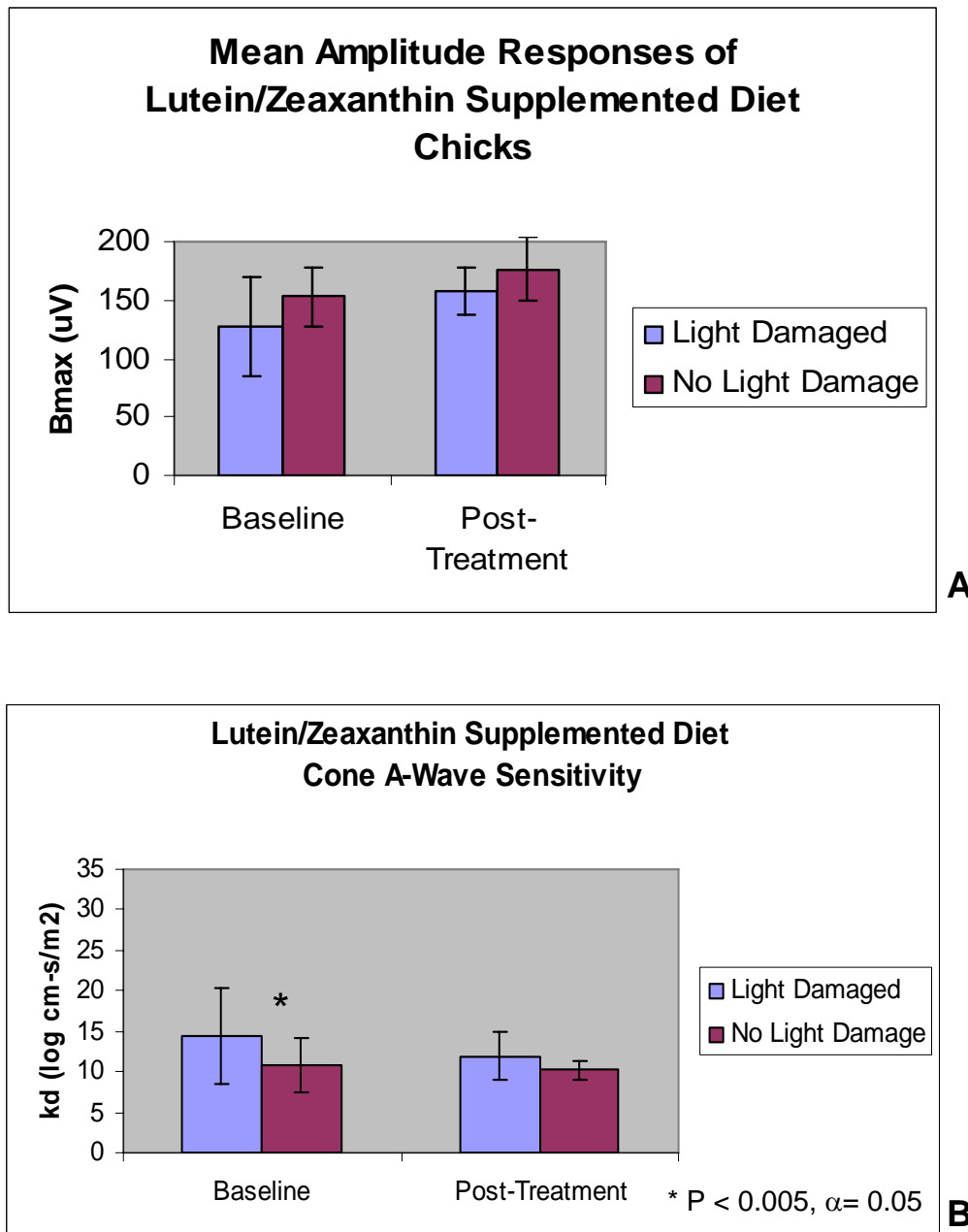
Figure 20 There were no significant changes in maximum amplitude responses (Bmax) from the cone cells in response to light exposure (**20A**). There was increased sensitivity (decreased Kd) between baseline levels for the light damaged and non-light damaged groups (**20B**), $p < 0.005$. This is due to random chance or a low sample size (n=6), since all chicks should have had identical baseline responses. Values are expressed as mean (cm-s/m2) $\pm$ SD.
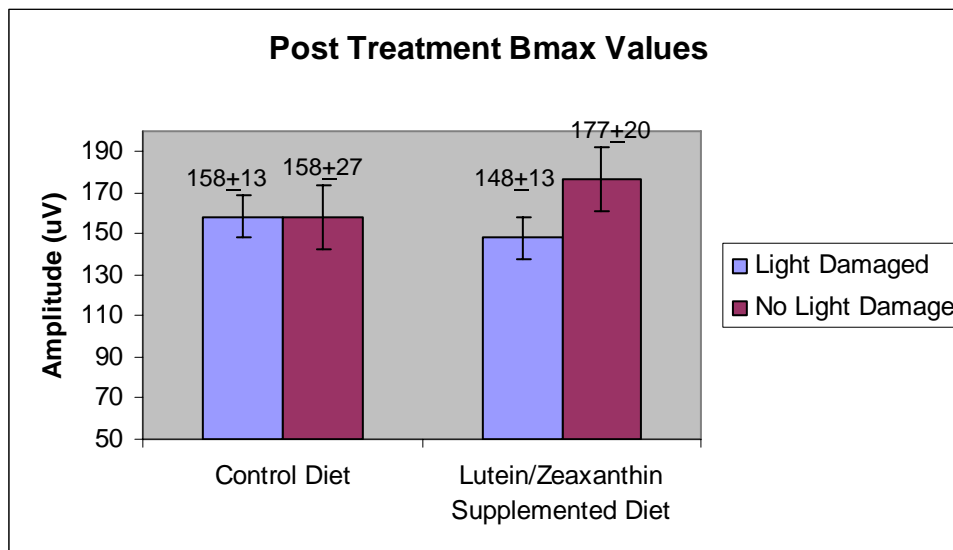
Figure 21 There were no significant effects of the supplemented diet on maximum amplitude responses (Bmax) of the cone photoreceptor cells as a result of light exposure. Values are expressed as mean (μV) $\pm$ SD.
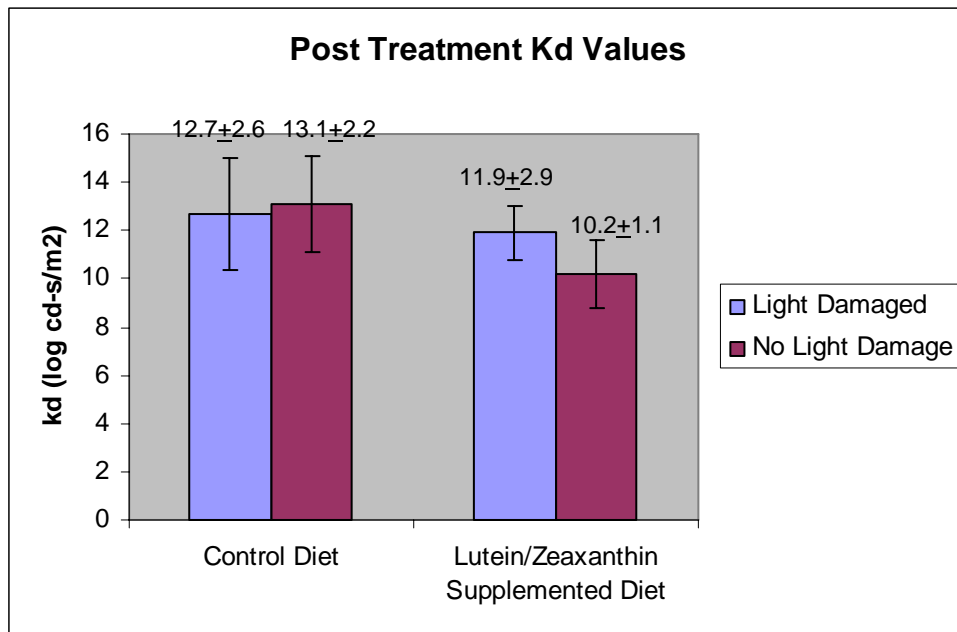
Post Treatment Kd Values

Figure 22 There were no significant effects of the supplemented diet on sensitivity (Kd) of the cone photoreceptor cells as a result of light exposure. Values are expressed as mean (cd-s/m2) $\pm$ SD.

**3 Hens in separate cages
(Hen 1, 2 and 3)**

**Rooster in
separate cage**

Fed a lutein and zeaxanthin
deficient diet for 4 weeks

**Day 1 control
chicks**

Fed commercial
rooster chow
for 4 weeks

Fed control diet at OHSU for 4 weeks
before exposure to light treatment

1) **On Day 1**, rooster was placed in cage with Hen 1 for 6 hours light/ 6 hours
    dark and then removed. Day 2, rooster was in cage with Hen 2, and Day 3
    with Hen 3. Caution was made to ensure the rooster did not eat the hens' chow.

2) Eggs were collected every day after fertilization for **8 weeks**; cages were checked at
    least 2 times/day to ensure every egg is collected.

3) Rooster was placed in cage with each hen **every 7 days** for copulation.

4) All eggs collected were kept in a **cooler maintained at 65 degrees F**, (no longer
    than 7-10 days). Each egg was labeled with Hen 1,2 or 3, and date.

5) Eggs were picked up **once a week** and transported back to OHSU.

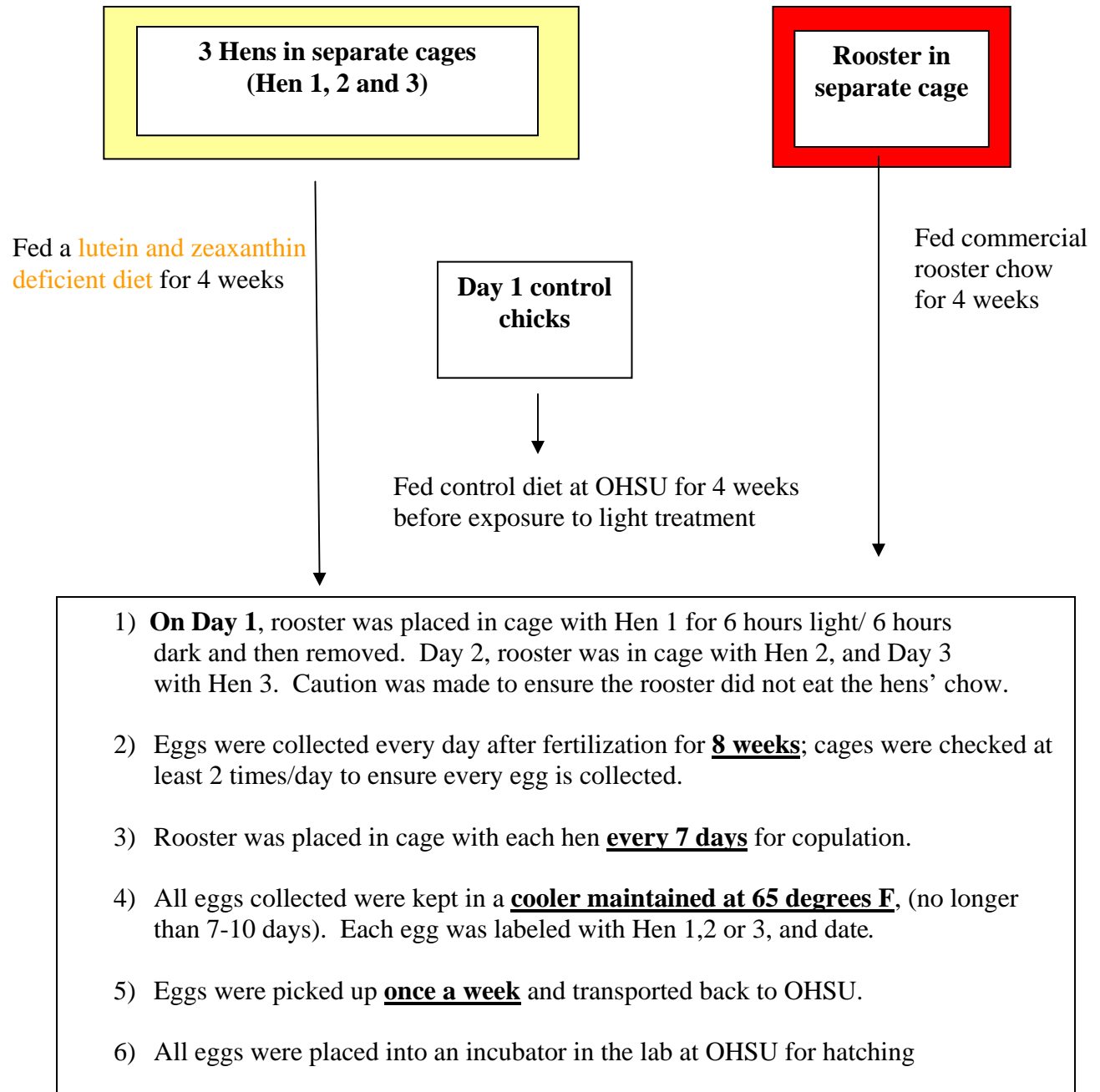6) All eggs were placed into an incubator in the lab at OHSU for hatching

Figure 23   Research design and methodology for Specific Aim 2 for raising laying
hens on a lutein and zeaxanthin deficient diet to produce deficient progeny.
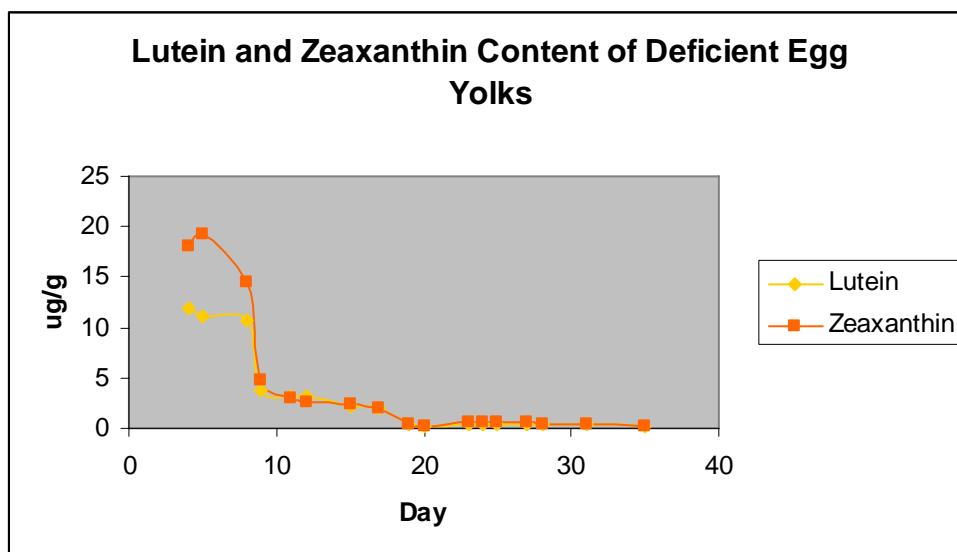
Figure 24   The lutein and zeaxanthin content of the egg yolks from the laying hens fed a carotenoid-deficient diet declined from Day 2 to Day 36 (11.19 µg/g and 18.09 µg/g to 0.19 µg/g and 0.23 µg/g, respectively, statistics not performed). One egg per day was chosen randomly amongst the three hens for analysis for a total of 35 eggs analyzed.
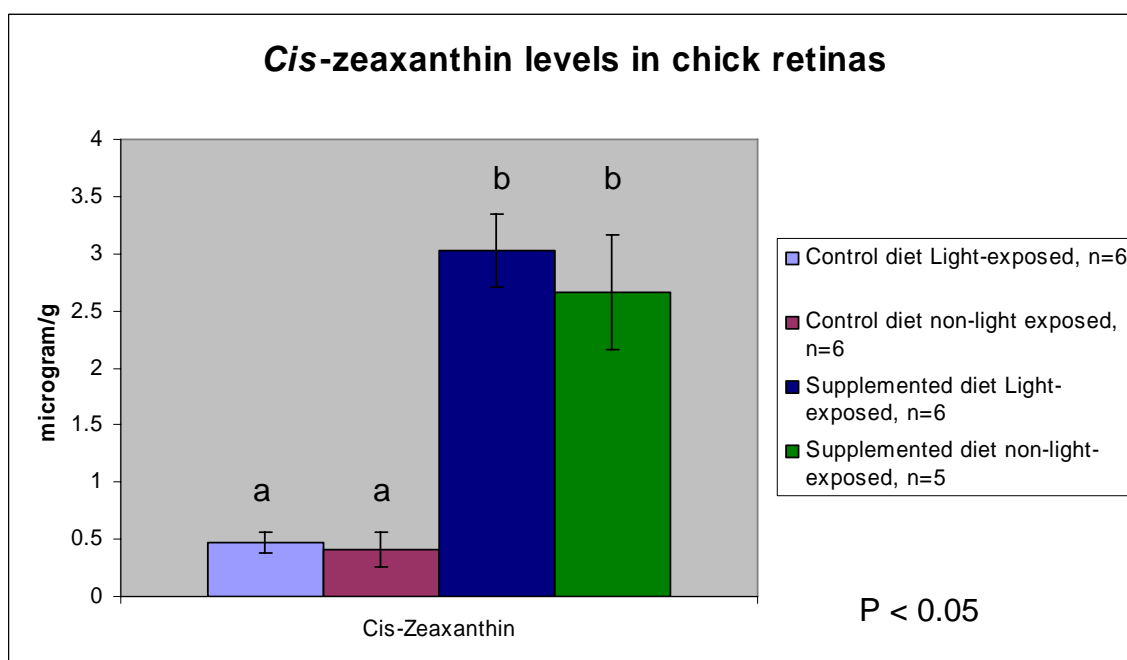
Figure 25   Greater *cis*-zeaxanthin was present in the retinas of supplemented diet chicks than control diet chicks only as a result of higher zeaxanthin levels in the diet, not as a result of light exposure. Means without a common letter differ (a versus b, p < 0.05). Values are expressed as mean (μg/g) $\pm$ SD.