EFFECT OF DURATION AND TEMPORAL MODULATION OF MONOCHROMATIC LIGHT ON EMMETROPIZATION IN CHICKS

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ABSTRACT

Introduction: Previous experiments have shown some discrepancy in the effects of monochromatic light on different animal species. Some species become more myopic in monochromatic red light and more hyperopic in monochromatic blue light, while the opposite occurs for other species. This study aims to look at the role of age of onset, duration, modes of lighting, and circadian interruption in emmetropization in monochromatic light.

Methods: The thesis reports two experiments: the first experiment exposed birds to monochromatic red, blue, or white light. The light was either steady or flickering. We measured refractive status and ocular biometry, starting with 1 week old chicks, over the course of 10 days of light exposure. Activity was measured via a motion detection algorithm and an IR camera. In Experiment two, chicks were exposed to red and blue steady light at hatch. The duration of exposure was extended by 7 days to see how age of onset and duration affected eye growth.

Results: When 1 week old chicks were placed in the lighting conditions, there was a significant increase in axial length and vitreous chamber depth in chicks reared in red or white light compared to blue light in both steady and flickering light. However, this increase in axial length and vitreous chamber depth was not seen when chicks were placed in the lighting conditions for a longer duration. Chicks were also more active during the day in red light compared to blue light, indicating an effect of lighting on circadian activity rhythms.

Conclusion: Our results indicate that with short duration monochromatic light exposure, chicks rely on wavelength defocus to guide emmetropization. However, with longer exposure duration other factors affect the outcomes. Our results support the notion that the difference between species may be due to differences in age, experimental duration, and the resulting interference with circadian activity rhythms.

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INTRODUCTION

Emmetropization is the process that refers to the growth of an eye so that its length matches the power of its optic. At birth, most, but not all, animals tend to be hyperopic as a result of being born with eyes that are too short for their optics (Mayer et al, 2001). Then as the animal grows, the emmetropization process ensures the eye grows toward emmetropia. A failure in the emmetropization process results in an ametropic eye. If the eye grows too long, or the cornea too steep, the eye will become myopic, too little, or too flat and the eye will be hyperopic.

The emmetropization process uses a number of feedback signals to determine growth (Raviola & Wiesel, 1985; Wallman & Winawer, 2004). One signal is defocus. If an image is blurry or lacks contrast as a result of defocus, the eye will adjust its growth in order to make the image clearer. Past studies have shown that inducing blur using positive or negative lenses can induce ametropia in animals and cause the eye to grow in such a way as to compensate for the lens induced defocus (Irving et al, 1992).

The intensity and spectral content of light also plays an important role in emmetropization. Observational studies have shown that children who spend more time outdoors are less likely to become myopic (Jones-Jordan et al, 2012; Mehdizadeh et al, 2009; Rose et al, 2008), leading to the idea that brighter lighting could guide emmetropization and protect against myopia. Chicks reared in brighter lighting were shown to be less myopic than those reared in dim lighting (Cohen et al, 2008). However, other studies showed that brighter lighting alone could not prevent myopia induced by negative lens wear (Ashby 2010; Smith 2013). Another study showed that the spectral content of light also plays a role in emmetropization. Children who spent more time under fluorescent lighting were more hyperopic than those under tungsten lighting (Czepita et al, 2004). Both outdoor lighting and fluorescent lighting share a similar

characteristic in that they both contain a greater proportion of blue and green light than tungsten lighting, but the spectral distribution of tungsten and fluorescent lighting differs from sunlight and intensity is several orders of magnitude brighter in the outdoor environment.

Longitudinal chromatic aberration (LCA) has been implicated as a significant signal for emmetropization. LCA principally means that light of different wavelengths will converge at different focal planes as seen in Figure 1 as a result of differences in dispersion. Longer wavelength light will converge farther from the lens and shorter wavelength light will converge closer. There are several different ways in which the eye can use a signal from LCA to guide emmetropization that arise from wavelength defocus and the resulting chromatic signals.

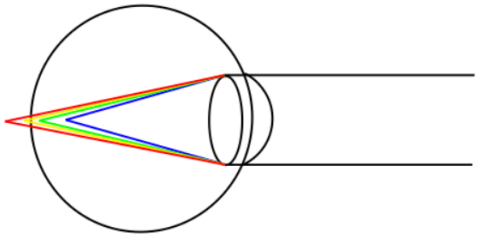


Figure 1- Longitudinal chromatic aberration in the eye

According to the wavelength defocus theory, the eye will use the focal plane of light as a target and adjust its growth to bring the retina closer to focal plane of that light. In the presence of predominantly long wavelength light, the eye will grow longer to stay within the plane of focus of the incident light. In the presence of predominantly short wavelength light, the eye will grow less. This will allow the eye to keep its sharpest focus in whichever light the subject is in. In chicks, it has been shown that there is about a 1.25D difference between the focal planes of 470nm and 680nm wavelengths of light (Mandelman & Sivak, 1983) and over 1.95 diopters of LCA over the visible spectrum (Wildsoet 2003, Schmid 1994)

Evidence for the wavelength defocus theory has been demonstrated in the past in accommodation and in emmetropization. In accommodation, Seidemann & Schaeffel (2002), and Kroger & Binder (2002) showed that there is a shift in accommodation due to LCA; humans and chicks accommodate more in red light than in blue light to bring the more distant focal plane on to the retina. Also, with regards to emmetropization, they found that chicks, after a short period, become more hyperopic after being reared in blue light (430nm), but become myopic when reared in red light (615nm). Rucker & Wallman (2008) found similar results in chicks in dim light that was designed to isolate the individual cone types by reducing the stimulation of the unwanted cones below the threshold for emmetropization. Interestingly, Foulds et al (2013) demonstrated that long exposure to red light caused chicks to become even more myopic than LCA would predict, and switching lighting conditions from red to blue, or vice versa, has been shown to cause a reversal in refractive error (Foulds et al 2013, Seidemann and Schaeffel 2002). Myopic chicks became hyperopic after switching from red lighting to blue lighting, and hyperopic chicks became myopic after switching from blue lighting to red lighting. Liu et al (2011) demonstrated a similar reduction in growth in guinea pigs in blue light to that seen in the above experiments.

Another theory for how light guides the emmetropization process is from a chromatic cue from LCA via a relative cone contrast signal. The chromatic cue theory suggests that the eye compares contrast using long- and short-wavelength sensitive photoreceptors. When the retinal image has high contrast for red light and low contrast for blue, the eye interprets this as a signal that the eye needs to grow less. When the retinal image has high contrast for blue light and low contrast for red light the eye interprets this as a signal that the eye needs to grow more. The chromatic cue from LCA is only detectable in broadband light when there is sufficient cone

excitation at both ends of the spectrum to produce a detectable contrast difference between the longer and shorter wavelength components of the retinal image. Support for this theory came from experiments in human accommodation (Kruger et al 1993, 1995, 1997; Rucker et al. 2004a, 2004b, 2006) and in chick emmetropization (Rucker & Wallman 2009) that demonstrated that high contrast in red and low contrast in blue led to a relaxation in accommodation and hyperopic changes in growth, respectively. The reverse conditions led to myopic changes in growth.

Monochromatic light can only provide a wavelength defocus signal for emmetropization because the light is focused at a single plane, thus, there is no difference in focus or contrast between the cone types. However, it is possible that in, for example, long-wavelength monochromatic light, the excitation of long-wavelength cones (L-cones) and the lack of excitation of short-wavelength cones (S-cones) could be used as an open-loop chromatic cue that the eye has grown too long and should slow its growth. The lack of excitation of the S-cones in the eye could indicate that the eye is myopically defocused due to blue light being blurred beyond detection. Similarly, in the presence of short-wavelength light, the eye could use the lack of excitation in L-cones as a cue that the eye is too short and should continue to grow.

In support of this hypothesis, Smith et al (2015) demonstrated that when rhesus monkeys wore red filters lenses for 120 days, they became more hyperopic than controls which were raised in broadband white light. Gawne et al (2016) also found that tree shrews reared in red light for 13 days remained hyperopic while tree shrews that were raised in blue light became slightly more myopic. It should be noted that they became more hyperopic initially and only became myopic after around 35 days (Gawne et al. 2018). When the tree shrews were brought back to normal colony lighting, all groups recovered towards emmetropia showing that the retinas had not been damaged. Contrary to the monochromatic light experiments performed in other species,

these studies would indicate that long-wavelength narrowband light provides a signal to slow eye growth while short-wavelength light provides a signal to increase eye growth.

Gawne et al (2016, 2018) also included a flicker condition in the blue and red lighting conditions. S-cones are sparsely and not evenly distributed in the retina compared to M- and L-cones (Hunt 2013), so resolution is poor and focus would be difficult to compare between the different cone types. However, S-cones respond to temporal stimulation (Smithson 2014; Stockman 1991), so adding a flicker condition could alter the sensitivity of the S-cones to changes in defocus. Gawne found that tree shrews exposed to blue flickering light became more myopic than the much larger group of untreated tree shrews (but not compared to those in steady blue light). The flickering light will induce temporal stimulation of the retina and was designed using a pseudorandom pattern of 4-5 Hz with varied changes in luminance. The authors suggest that flicker produced an enhanced signal that the eye was too short, causing the eye to grow and become more myopic.

The disagreement among the experiments shows that the eye's interpretation of LCA is still unclear. The disparity could be the result of a species difference and whether they use LCA as a wavelength defocus target or a chromatic cue to guide emmetropization. Chicks and guinea pigs may be more sensitive to detecting LCA as a wavelength defocus target while tree shrews and rhesus monkeys may rely more on LCA as a cue to guide emmetropization. This is possibly due to differences in the animals color vision; chicks are tetrachromats (Osorio, Vorobyev, and Jones 1999) whereas tree shrews are dichromats (Petry and Kelly 1991), meaning that they are only able to detect a blue/yellow color signal. However, both animals possess the older blue/yellow color vision system that would be required for this type of signal. However, other differences exist; for example, dichromats may have an advantage at detecting fast modulating

targets compared to trichromats (Sharpe et al, 2006). Indeed, tree shrews have a temporal frequency sensitivity function that is skewed towards high temporal frequencies (Callahan & Petry, 1999).

It should also be considered that the prolonged exposure to monochromatic red light may have affected the circadian rhythms and behavior of the affected animals via the lack of stimulation of intrinsically photosensitive retinal ganglion cells (iPRGCs). Research has shown that interference with the circadian rhythms affects emmetropization (Nickla et al, 1998). A differentiating factor between the experiments could be whether the lighting condition stimulated iPRGCs, which have a peak spectral sensitivity around 480nm (Panda et al, 2005). It is notable that Foulds (2013) had a red light condition with a spectrum of 550-680nm, whereas Gawne (2016) used a red light with a spectrum of 616-636nm, meaning that Foulds' red light condition would have stimulated iPRGCs whereas Gawne's red light condition would not. It is also interesting to note that Rohrer (1992) demonstrated no refractive differences between rearing chicks in short-wavelength light (<400nm) and long wavelength light (>665nm). The spectral output of both monochromatic lights is well outside the spectral sensitivity of iPRGCs, which may partially account for this finding, though another explanation may be that dim UV light does not provide a signal for emmetropization.

Two other differences between the studies is the age of onset when the animals were exposed to the lighting conditions and the duration of the exposure. Foulds (2013) introduced 1-day old chicks to the lighting conditions and they were kept in the same lighting condition for 28 days. Gawne (2016)'s tree shrews were exposed to lighting conditions around 1 month-old after having 11 days of visual experience (eyes typically open 3 weeks after birth) for 13 days. Smith (2015)'s rhesus monkeys were introduced to their lighting conditions at 25 days of age to about

146 days of age. These longer duration experiments may expose the eye to confounding effects on eye growth due to the release of growth hormones. Tree shrews typically reach puberty around 2 months and puberty is often a milestone for the end of critical periods for the development of visual mechanism (Daw 2006). Short duration experiments in immature animals are required to isolate the effects of wavelength on these animals.

The goal of the present study is to determine the role of monochromatic light in guiding emmetropization in chicks. The study will also attempt to resolve some of the differences between the previous studies by examining the temporal aspects of light, the duration of light exposure, and the age of onset when the animals are exposed to the light. The study consists of two experiments. The first experiment looks at how different monochromatic lights and flicker affect refractive state and growth of the eye, as well as seeing if circadian activity rhythms are affected by the lighting conditions. As a follow up, the second phase of the study looks at how the age of onset and duration affect the responses to the monochromatic light conditions.

METHODS

Methods common to the two experiments will be described first, and then subsections for Experiment 1 and Experiment 2 will describe methods specific to each experiment.

Animals

Chicks (White leghorn, K strain, Cornell University, Ithaca, NY) were chosen as the model organism because chick eyes grow rapidly and are easily manipulated in experimental conditions (Feldkaemper & Schaeffel 2015; Wisely et al 2017). The chick temporal sensitivity function is very similar to humans but of lower amplitude (Jarvis et al 2002).

Chicks had access to food and water *ad libitum*. Use of animals in this study was in compliance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the NECO Institutional Animal Care and Use Committee.

Lighting and Condition Cages

Chicks were housed in 48x79x40 cm (width x length x height) wired cages with 10 chicks maximum in each cage. 8 strips of RGB LEDS (Superbright NFLS-RGBX2) were attached to the roof of the cage. The LED strips were 0.5 meters long and were paired together. Each pair was evenly spaced 10cm from each other as seen in Figure 3. LEDs were controlled via Raspberry PI and could be set to red (628nm), blue (464nm) or white light and produced steady or flickering light depending on the condition. Figure 2 shows the spectral emission curve of the LED lights that were predominately red and blue.

Lighting conditions were matched closely to Gawne (2016)'s conditions both spectrally and temporally. For the flicker conditions, lights were set to flicker from 4-5Hz over a range of

random luminances (4-96% of the maximum luminance) during the day time. Average illuminance was 424 lux for all conditions and was measured with Dr.Meter LX1330B Digital Illuminance/Light Meter. The illumination settings differed from Gawne et al (2016) whose average illuminance varied across time during the wavelength and flicker conditions. Mean light intensity measures were also measured with a Newport Power Meter and were found to be 266 μW for Blue and 116.2 μW for Red. Luminance was also measured with a Spectroscan PR670 and was found to be 84cd/m^2 for Blue and 114 cd/m^2 for Red.

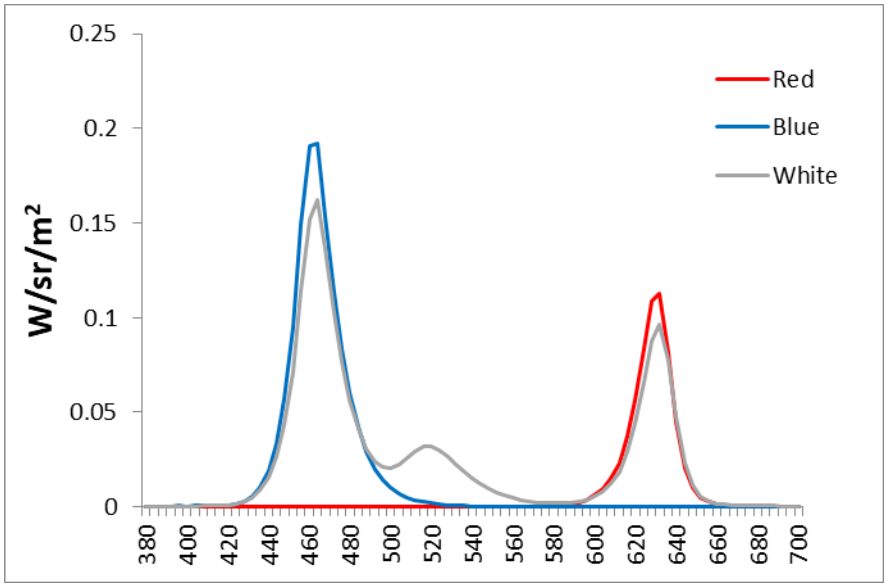


Figure 2- Spectral emission curve of LED lights used in the blue, red, and white conditions Blue LEDs had a peak at 464 nm. Red LEDs had a peak at 628nm. White LEDs had peaks at 464, 520 and 628nm.

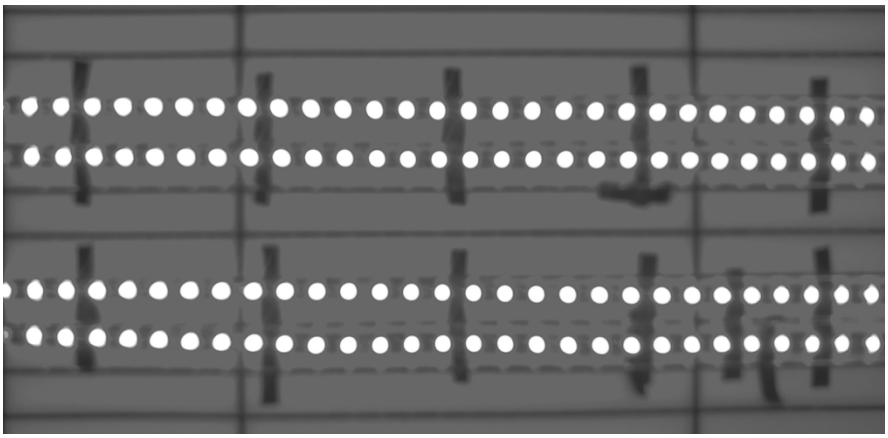


Figure 3- Arrangement of LEDs. Pairs of LED strips were glued and taped to a white poster board that was placed on top of the cage, 40 cm above the floor.

Eye Measurements

On measurement days, refraction and eye measurements were performed sequentially on each eye at the same time of day. During measurements, chicks were anesthetized with 1.5% isoflurane gas in 2% oxygen and had minimal exposure to light outside of their condition cages. The conditions were protected from extraneous light by a double door system and measurements were performed in the dark.

Refraction was performed using an infrared photorefractometer as described by Choi et al (2000). Refraction is measured at a distance of about 1m with a sampling frequency of 62 Hz. The infrared refractometer setup consisted of a camera (IC Imaging Control, Charlotte, NC) with a Pentax 75mm f/1.4 lens and IR LEDs mounted on the bottom half of the front of the lens. The refraction and standard deviation are calculated from the average of 650 measurements. Refraction software, developed by Dr. Frank Schaeffel, took measurements of the light gradient in the pupil, in the 90 and 180 degree meridians, and converted them to dioptric values using a pre-determined calibration.

Axial length, choroidal thickness, vitreous chamber depth, lens thickness and anterior chamber depth were measured with the Lenstar LS-900 (Haag-Streit, USA). Five measurements

were made at each measurement time point. The peaks in the traces that corresponded to each of the ocular components were set automatically and then manually refined by a masked researcher.

Methods Experiment 1 (Shorter Duration)

The first experiment looked at how monochromatic light and flicker affects eye growth in the chick eye. One-week-old chicks were placed into one of three illumination conditions, Blue, Red, or White, and two temporal conditions, Steady or Flicker (Hz) to give a total of six conditions; see Table 1: (1) Blue Steady (n=6), (2) Blue Flicker (n=6), (3) Red Steady (n=5), (4) Red Flicker (n=5), (5) White Steady (n=4), and (6) White Flicker (n=6). Chicks were exposed to the illumination conditions for 10 days, with a light cycle of 12 hours (on/off). The White Steady condition served as the control condition. Measurements took place on Days 1, 3, 6, 8 and 10 of the experiment between 11am and 1pm. Day 1 in Figure 5 represents the baseline measurement at 7 days old, and took place prior to placing the chicks in the condition cages.

To get a sense of whether circadian rhythms were affected by the lighting conditions, activity of the chicks was recorded throughout the experiment. An infrared video camera was set up to monitor the chicks throughout the experiment during the day and night. The activity monitoring system captured degree and size of movement and output a value proportional to the amount of motion in the scene (see Figure 4). The monitoring system was paused during measurements and cage cleaning until chicks were placed back in the cage.

Refractions and eye size measurements of both eyes were averaged at each time point. Since there were multiple time points, a linear regression was plotted for every chick at every measurement time point spanning from Day 1 to Day 10. The primary outcome was the slope of

the linear regression multiplied by 10 to represent the total change over 10 days of light exposure. This analysis has the advantage that data from all measurement points is included. A two-way ANOVA, with main effects of color and temporal frequency, and Tukey post-hoc *t*-tests were performed on the total amount of change in the ocular measurements over 10 days using SPSS (version 20, IBM) statistical software.

Activity was recorded via automatic motion capture software that computed the area of motion (in pixels/second). The units were transformed and scaled to give a dimensionless number that represents the amount of motion and observed in the scene during each one-minute period. The camera had a night vision mode (using IR) that could capture motion during the night. Overall very little activity was captured during this period (8PM to 8AM) in all the groups. To avoid the effect of mixing the activity of day and night time periods, only the daytime period (8AM to 8PM) was used for analysis. A one-way ANOVA of the activity metrics was performed. SPSS (version 20, IBM) statistical software was used for analysis.

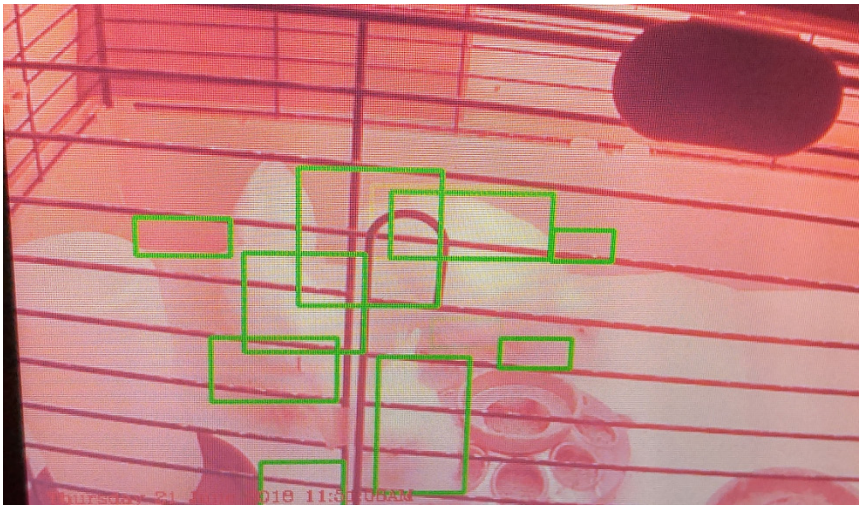


Figure 4- Activity is noted by movement of the chicks. A frame of motion capture is shown above. The green boxes indicate that motion was detected and the size of the box denotes the area where motion was detected. A dimensionless number denoting amount of activity is measured every minute. This arbitrary number includes the area of movement (i.e. more chicks moving would show a wider area of movement) as well as speed.

Experiment 2

Experiment 2 investigated how duration and age of onset of exposure to the lighting conditions plays a role in the growth and refractive state of the chicks' eyes. 16 chicks were hatched in incubators (which were kept under dim broadband white light) and then immediately transferred to the lighting condition cages. Chicks were either placed under the Steady Blue (n=10) or Steady Red (n=6) lighting conditions.

Chicks were kept in the lighting condition from hatching and ocular measurements and refractive errors were performed in the dark on days 10 and 17 of light exposure (birds are too small to measure on Day 1). For measurement purposes, chicks were taken out of their lighting conditions and exposed to minimal amounts of light during measurements. Measurements were averaged between both eyes for each chick at each time point. The difference between Day 17 and Day 10 measurements was calculated and represents the change in each component over 7 days. *T*-tests were performed using SPSS statistical software (version 20, IBM). The timeline for the measurements can be seen in Figure 5.

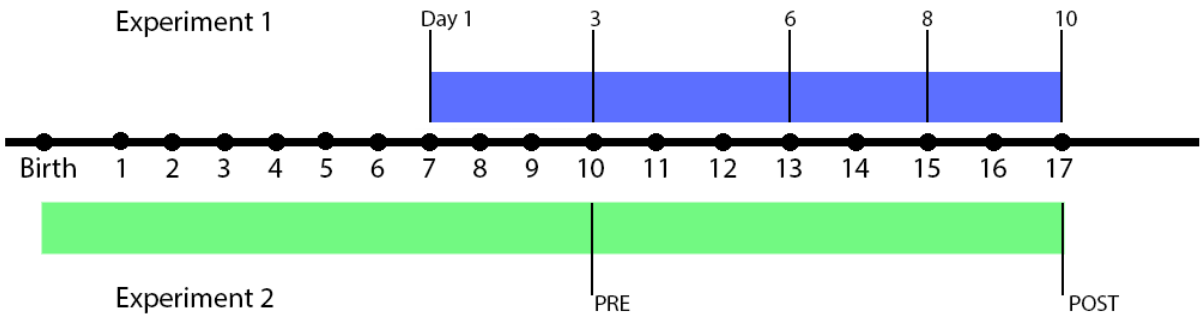


Figure 5- Timeline of the light exposure and measurements for both experiments. The blue colored bar represents when chicks in Experiment 1 were placed into the lighting conditions with the days of light exposure and measurement times noted above. The green colored bar below represents when chicks in Experiment 2 were placed into the lighting condition with PRE and POST measurements measured on days 10 and 17.

RESULTS

Experiment 1: Shorter duration experiment

The change data for each treatment group is summarized in Table 2, for refractive error, axial length, choroidal thickness, vitreous chamber depth, anterior chamber depth and lens thickness. Data represents the change over 10 days of treatment from age 7 days to 17 days with light exposure starting at age 7 days.

Refractive Error

Among all the illumination groups, there were only small changes in refractive error (Figure 6).There was no significant effect of illumination condition on refractive error (Figure 6A: ANOVA; F-value=1.74, p=0.196). Birds exposed to blue light showed a mean change in refractive error of -0.82±0.81D, while birds in red light showed a mean change of -0.31±0.95D, and birds in white light showed a change of +0.44D±0.99D over 10 days.

There was no significant effect of flicker on refractive error (Figure 6B: ANOVA; F-value=0.697, p-value=0.411). On average, refractive error changed by only -0.08±0.98D with flicker, while in steady light conditions, refraction changed by only +0.12±0.90D over 10 days. The 10-day exposure to the illumination and frequency conditions did not affect refractions (Figure 6C).

Axial Length

Mean changes in axial length by condition are shown in Figure 7. There was a significant inhibition in axial growth when chicks were raised in blue light compared to red light and white light (Figure 7A: ANOVA; F-value= 10.18, p=0.001**:** Blue v Red: p=0.004; blue v white: p=0.001). On average, the axial length of the chicks raised in blue light increased by only 0.945±0.055mm, while axial length increased 1.089±0.075mm in red light, and 1.114±0.011mm in white light.

There was a significant inhibition in axial growth in chicks reared in flickering light compared to steady light (Figure 7B: ANOVA; F-value= 6.176; p=0.020). In steady light, axial length increased by 1.086±0.108mm, while in flicker, axial length increased by only 1.00±0.120mm over 10 days. There were no significant interactions between the groups (Figure 7C: ANOVA; F-value=1.45; p=0.255).

Choroid

Mean choroidal changes in each illumination and flicker condition are shown in Figure 8. The color of the illuminant had no effect on choroidal thickness (Figure 8A: ANOVA; F-value=0.266, p=0.77). On average, choroidal thickness increased by only +0.018±0.039mm in blue light, +0.012±0.029mm in red light, and +0.028±0.036mm in white light over 10 days.

There was no significant effect of flicker on choroidal thickness (Figure 8B: ANOVA; F-value=0.469, p=0.50). On average, choroidal thickness increased by 0.024±0.033mm in flickering light, and by 0.015±0.033mm in steady light. Choroidal thickness was unaffected by interactions between the color of the illuminant and flicker (Figure 8C).

Vitreous

Mean changes in vitreous chamber depth are shown in Figure 9. Chicks reared in blue light showed a significant inhibition of vitreous chamber growth compared to those reared in red light or white light (Figure 9A: ANOVA; F-value=14.47; $p<0.001$; Red v Blue: Tukey post-hoc; p=0.02; Blue v White: Tukey post-hoc; $p<0.001$) over the 10 days of exposure. On average, chicks showed a +0.286±0.009mm change in vitreous depth over 10 days when raised in blue light, +0.401±0.058mm in red light, and +0.482±0.109mm change in white light.

Chicks reared in flickering light had slower overall growth of the vitreous chamber (Figure 9B: ANOVA; F-value=5.74: p=0.025). The vitreous chamber depth in chicks in steady

light grew an average of 0.426±0.120mm, while those in flickering light grew only 0.357±0.112mm in vitreous chamber depth over 10 days. No interaction was seen between the color of light and temporal properties (Figure 9C: ANOVA; $F=1.663$; $p=0.211$).

	n	Refractive Error (D)		Axial Length (mm)		Choroidal Thickness(mm)		Vitreous Chamber (mm)		Anterior Chamber (mm)		Lens Thickness (mm)	
		Day 1	Day 10	Day 1	Day 10	Day 1	Day 10	Day 1	Day 10	Day 1	Day 10	Day 1	Day 10
Blue Steady	6	+0.87±0.83	+1.50±0.65	8.922±0.134	9.826±0.156	0.158±0.356	0.202±0.016	5.220±0.144	5.536±0.144	1.381±0.028	1.549±0.034	1.838±0.042	2.196±0.056
Red Steady	5	+0.67±0.65	+0.72±1.01	8.970±0.083	9.934±0.124	0.226±0.362	0.226±0.031	5.180±0.178	5.542±0.142	1.427±0.094	1.613±0.103	1.839±0.066	2.208±0.087
Blue Flicker	6	+0.57±0.54	+0.45±0.56	8.772±0.091	9.580±0.092	0.198±0.041	0.210±0.021	5.078±0.078	5.308±0.096	1.346±0.045	1.550±0.024	1.823±0.069	2.181±0.029
Red Flicker	5	+1.10±0.72	+0.47±0.36	8.635±0.194	9.610±0.187	0.158±0.033	0.185±0.037	5.000±0.155	5.378±0.258	1.339±0.028	1.540±0.028	1.814±0.029	2.159±0.038
White Steady	4	+0.75±0.48	+0.01±0.10	8.992+0.200	10.022±0.297	0.196±0.031	0.225±0.039	5.078±0.170	5.536±0.068	1.423±0.059	1.650±0.067	1.956±0.012	2.257±0.050
White Flicker	6	+0.99±0.48	+0.99±0.68	8.673±0.132	9.702±0.079	0.162±0.025	0.194±0.034	5.005±0.130	5.464±0.273	1.356±0.023	1.509±0.041	1.829±0.077	2.181±0.039

Table 1- Mean measurement values for refractive error, axial length, choroidal thickness and vitreous chamber depth on Day 1 (baseline) and Day 10.

	n	ΔRefractive Error (D)	ΔAxial Length (mm)	ΔChoroidal Thickness (mm)	ΔVitreous Chamber Depth (mm)	ΔAnterior Chamber(mm)	ΔLens Thickness(mm)
Blue Steady	6	-0.08±0.10	+1.032±0.051	+0.030±0.044	+0.331±0.0952	+0.194±0.046	+0.402±0.066
Red Steady	5	+0.03±0.87	+1.093±0.071	+0.002±0.034	+0.398±0.0787	+0.213±0.149	+0.417±0.034
Blue Flicker	6	-0.08±0.90	+0.877±0.065	+0.010±0.041	+0.242±0.0504	+0.210±0.052	+0.402±0.061
Red Flicker	5	-0.66±0.98	+1.078±0.079	+0.024±0.018	+0.405±0.0278	+0.232±0.028	+0.383±0.014
White Steady	4	+0.38±0.96	+1.153±0.137	+0.013±0.025	+0.547±0.109	+0.276±0.118	+0.319±0.064
White Flicker	6	+0.53±1.19	+1.075±0.073	+0.038±0.040	+0.55±0.0930	+0.177±0.058	+0.402±0.039

Table 2- Mean change in ocular measurements over 10 days of exposure for each treatment group.

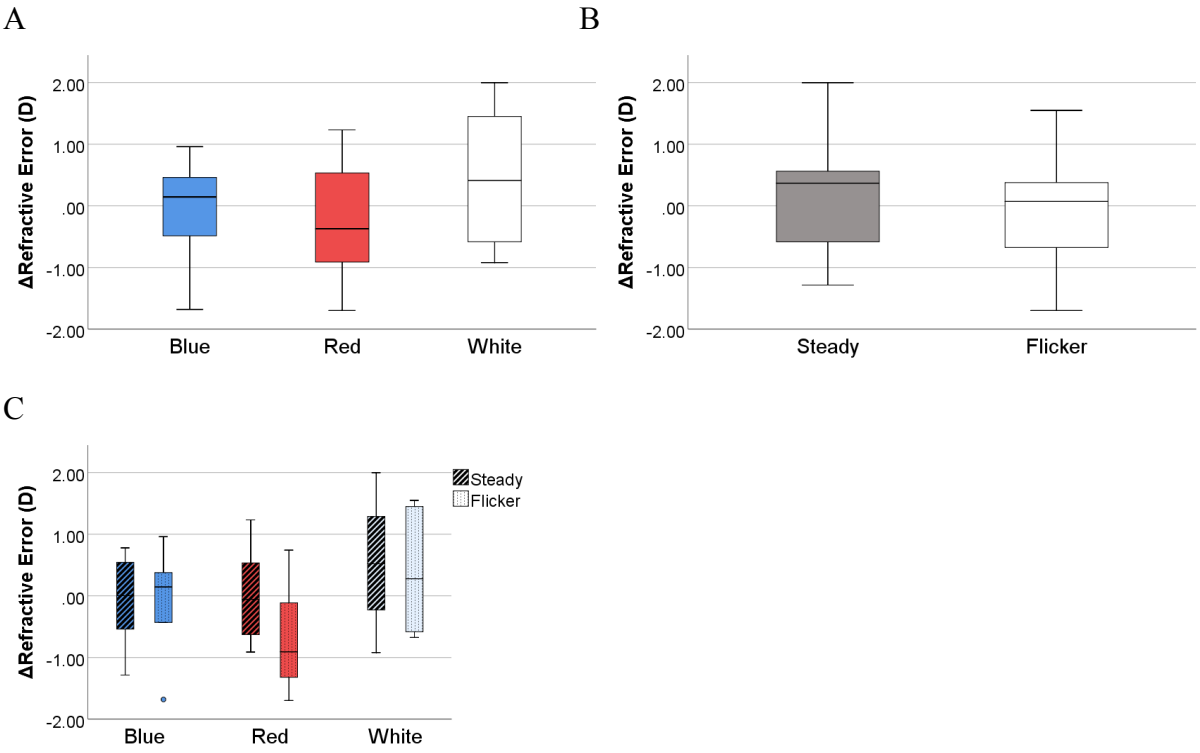


Figure 6- Experiment 1 (Short duration) Mean change in refractive error over 10 days with treatment starting at 7 days old. **A.** Pooled and grouped by color. **B.** Pooled and grouped by temporal condition. **C.** Grouped by color and temporal condition. Horizontal line in the box indicates the median. Boundaries of the box indicate the 25th and 75th percentile. The whiskers represent the high and low values 1.5x the interquartile range. Dots represent outliers. No significant differences or interaction seen in groups.

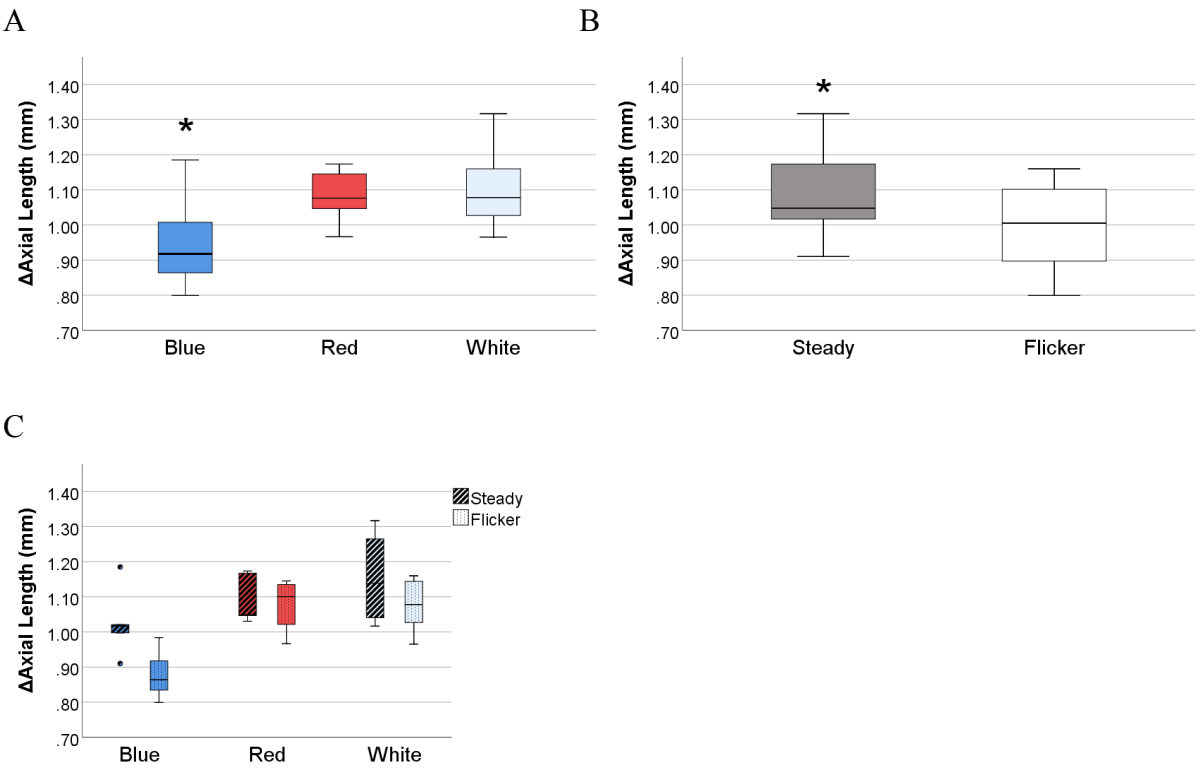
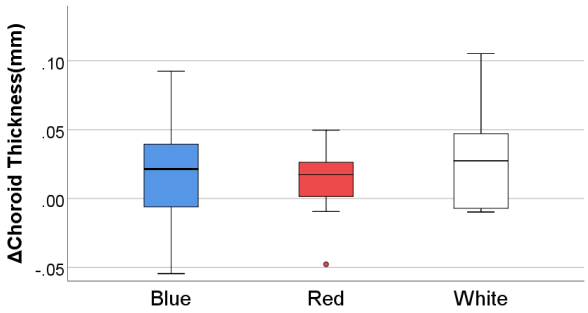
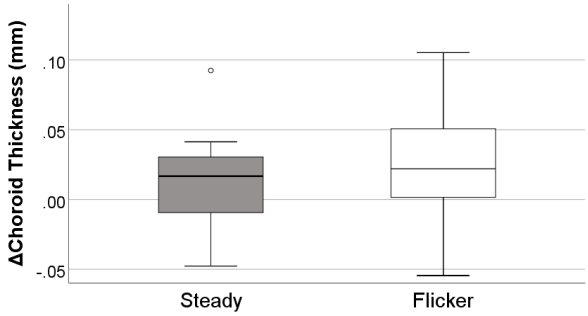


Figure 7- Experiment 1 (Short duration) Mean change in axial length over 10 days with treatment starting at 7 days old. **A.** Pooled and grouped by color. **B.** Pooled and grouped by temporal condition. **C.** Grouped by color and temporal condition. Horizontal line in the box indicates the median. Boundaries of the box indicate the 25th and 75th percentile. The whiskers represent the high and low values 1.5x the interquartile range. Dots represent outliers. No significant differences seen in groups. White and Red light produced greater axial length growth compared to Blue Light. No difference seen between temporal frequency conditions. No interaction was noted.

A



B



C

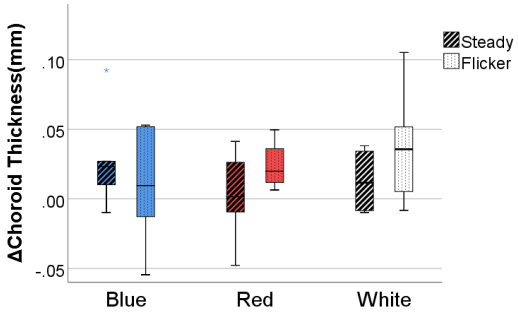


Figure 8- Experiment 1 (Short duration) Mean change in choroid thickness over 10 days with treatment starting at 7 days old. **A.** Pooled and grouped by color. **B.** Pooled and grouped by temporal condition. **C.** Grouped by color and temporal condition. Horizontal line in the box indicates the median. Boundaries of the box indicate the 25th and 75th percentile. The whiskers represent the high and low values 1.5x the interquartile range. Dots represent outliers. No significant differences or interactions seen in groups.

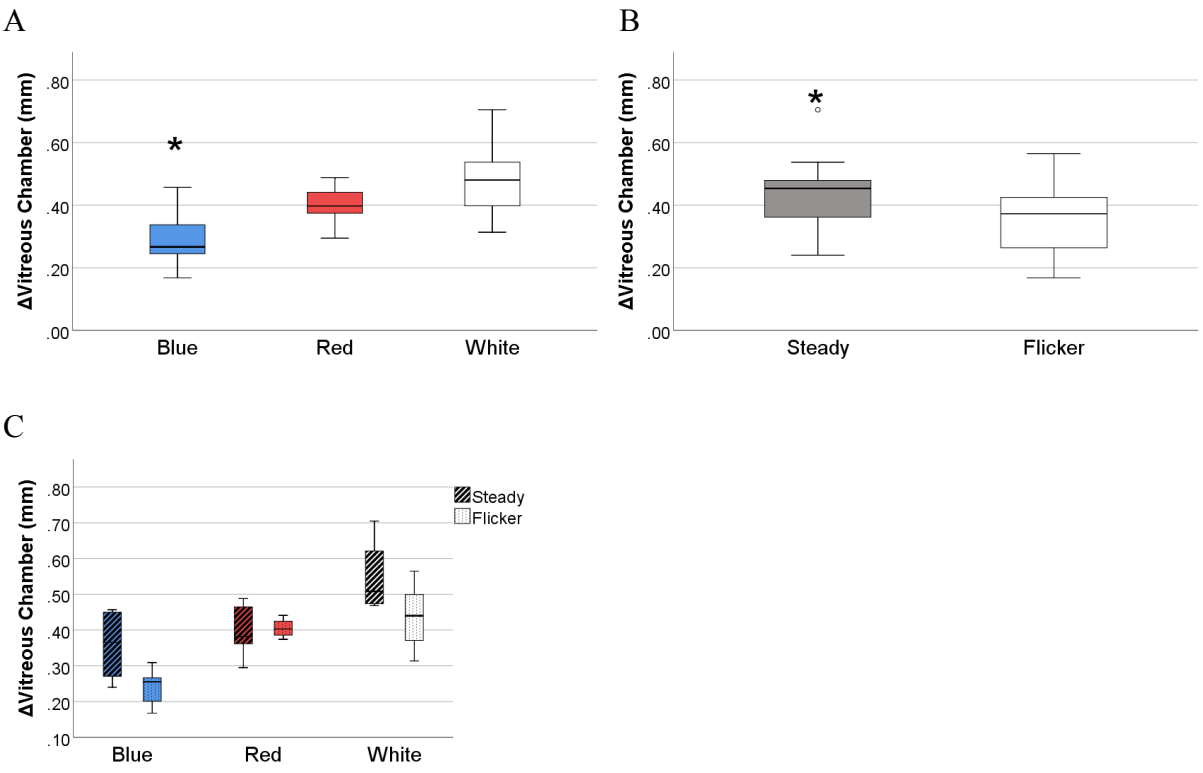


Figure 9- Experiment 1 (Short duration): Mean change in vitreous chamber depth over 10 days with treatment starting at 7 days old. **A.** Pooled and grouped by color. **B.** Pooled and grouped by temporal condition. **C.** Grouped by color and temporal condition. Horizontal line in the box indicates the median. Boundaries of the box indicate the 25th and 75th percentile. The whiskers represent the high and low values 1.5x the interquartile range. Dots represent outliers.

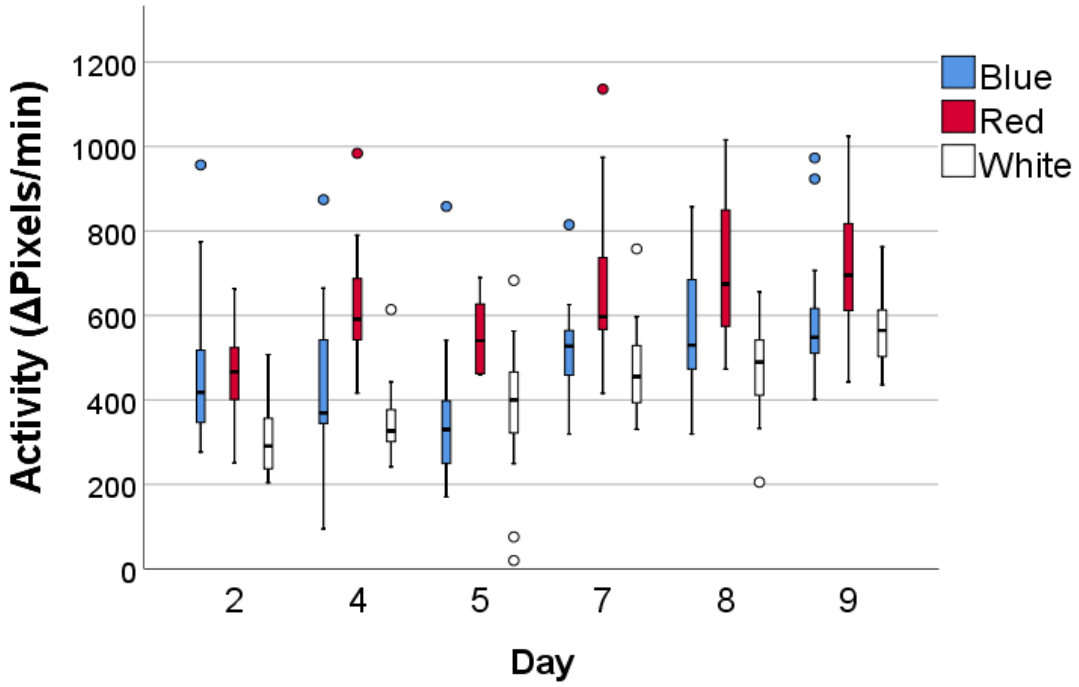


Figure 10. Box plot of Activity. Horizontal line in the box indicates the median. Boundaries of the box indicate the 25th and 75th percentile. The whiskers represent the high and low values 1.5x the interquartile range. Dots represent outliers. Asterisks represent the extreme outliers (points greater than 3 times the interquartile range), respectively. The box plot represents activity measured during day time only. Activity was quantified by the detected change in pixels per minute throughout the recording. On Day 2, there was greater activity in Red and Blue Light than White Light, but no difference between Red and Blue Light. On Days 4, 5, 7, 8, and 9, there was greater activity measured in Red Light than Blue or White Light with no difference between Blue and White Light.

Activity of the chicks was measured in the Blue Flicker, Red Flicker, and White Flicker conditions only. Activity was continuously measured while the chicks were in their condition cages. There was very minimal activity at night with no significant differences between the groups (ANOVA; $F=1.91$; $p=0.565$), so only daytime is represented in Figure 10. To ensure equal recording time across days, only the data from days 2, 4, 5, 7, 8, and 9 was analyzed, since there were no other measurements made on these days.

Because greater activity is expected as the chick grows (i.e., more pixels will change per frame as a larger bird moves), analysis was done separately for each day. On Day 2, increased

activity was seen in chicks reared in Blue Flicker and Red Flicker light compared to White Flicker (ANOVA; F-value=11.96; $p<0.001$). Then on Days 4, 5, 7, 8 9, increased activity was seen in chicks reared in Red Flicker compared to Blue and White Flicker (ANOVA; F-value=52.9, $p<0.001$). For all days, activity was measured to be 482.8±170 Δpixels/min for chicks raised in Blue Flicker, 622.4±165 Δpixels/min for Red Flicker, and 426.8±135 Δpixels/min for White Flicker.

There was no difference in activity when comparing different times of the day. We saw no difference between morning and afternoon activity in Blue Flicker (ANOVA; F-value=0.63, p=0.438), Red Flicker (ANOVA; F-value=0.263, p=0.613), or White Flicker (ANOVA; F-value=2.95, p=0.100)

Experiment 2: Longer duration experiment

Even though chicks were exposed to the lighting conditions for a longer duration, we did not see as many salient changes in ocular measurements compared to the shorter duration experiment. Data values can be seen in Table 3 and 4 and in Figure 11.

Refraction

After 17 days of exposure, there was no significant difference in change in refractive error between the two illumination conditions (Figure 11A: *t*-test; T-value=2.48, p=0.141). In blue light, the average change in refractive error was -0.69±1.10D from Day 10 to Day 17, while in red light, they became +0.12±0.39D more hyperopic.

Axial Length

After 17 days of exposure, there was no difference in the change in axial length between the two illumination conditions (Figure 11B: *t*-test; T-value=0.259, p=0.620). In blue light, the

average change in axial length was +0.898±0.089mm from Day 10 to Day 17, while in red light, the average change in axial length was +0.871±0.103mm.

Choroid

After 17 days of exposure, choroidal thickness was significantly increased in red light compared to in blue light (t-test; T-value=13.62, p=0.003). Choroidal thickness increased on average of only +0.003±0.268mm in blue light, but by +0.054±0.021mm in red light from Day 10 to Day 17.

Vitreous

After 17 days of exposure, there was no difference in vitreous chamber growth (Figure 9C: *t*-test; T-value=0.020; p=0.891). Vitreous chamber depth increased on average by 0.384±0.021mm in blue light and 0.389±0.099 in red light from Day 10 to Day 17.

Lens and Anterior Chamber Depth

After 17 days of exposure, there was no difference in the change in lens thickness in the two conditions *(t*-test; T-value=0.736, p=0.408). Lens thickness increased on average by 0.219±0.034mm in blue light and 0.224±0.046mm in red light from Day 10 to Day 17.

After 17 days of exposure, there was no difference in the change in anterior chamber depth (*t*-test; T-value=0.047, p=0.832) from Day 10 to Day 17. Anterior chamber depth increased on average by 0.267±0.083mm in blue light and 0.224±0.102 in red light from Day 10 to Day 17.

Weight

There was no significant difference in weight gain in the chicks between the lighting conditions (*t*-test; T-value=0.224, p=0.644). Chicks raised in blue light weighed on average

51.92±2.6g on Day 10 and 132.0±9.2g on Day 17. Chicks raised in red light weighed on average 53.6±1.96g on Day 10 and 135.6±7.3g on Day 17.

Comparing Experiments 1 and 2: Duration (age matched)

The axial, vitreous and choroidal changes with the color of the illuminant that were seen in Experiment 1 were not seen in Experiment 2. To determine if this was because of transient ocular changes that occur early on and then diminishes by Day 17, we looked at the mean change in ocular measurements for age-matched chicks after different durations of light exposure. For this analysis, we used chicks in "Day 3" of Experiment 1 and "Day 10" of Experiment 2, which were both 10 days old. While the chicks were the same age, chicks in Experiment 2 had an extra week of light exposure compared to those in Experiment 1.

Refractive Error

When the data from Experiment 1 and 2 was pooled, the results showed that chicks were overall more hyperopic in blue light (+2.24±0.96D) than in red light (+0.18±0.49D); (ANOVA; F-value=47.08, $P<0.05$) at 10 days of age. When the lighting conditions were pooled, there was no difference in refractive errors between Experiment 1 and 2 (ANOVA; F-value=0.023, P=0.880). Additionally, the difference in refractive errors between red and blue lighting was significantly larger in Experiment 1 (Blue-Red=2.84D) than in Experiment 2 (Blue-Red=1.42D) (ANOVA; F-value=5.21: p-value=0.033). These results indicate that refractive effects of the different lighting conditions are transient, existing after 3 days but diminishing by 10 days of continued light exposure.

Axial Length

Pooling data from Experiment 1 and 2, the 10 day old chicks had shorter axial lengths in blue light (9.112±0.112mm) than in red light conditions (9.311±0.221mm) (ANOVA; F-value=14.7, P=0.001). Pooling the data from the color conditions, chicks in Experiment 1 (3 days of colored light exposure) had a shorter mean axial length (9.105±0.114mm) than those in Experiment 2 (10 days of exposure) (9.260±0.210mm) (ANOVA; F-value=12.9, P=0.002). However, we did not see a significant interaction between Experiment and color of illuminant (ANOVA; F=3.173, p=0.09). These results indicate that at 10 days old an increase in axial length is associated with a longer duration of exposure to monochromatic light regardless of the color.

Choroid Thickness

Pooling the data from the two experiments, there was no difference in choroidal thickness in blue light (0.207±0.024mm) and red light (0.196±0.031mm) (ANOVA; F-value=0.001, p=0.993) and no difference between Experiments 1 (0.207±0.02mm) and Experiment 2 (0.196±0.03mm). Overall, there was no effect of the color of lighting conditions on choroidal thickness with duration of exposure (ANOVA; F-value=0.222, P=0.643).

Vitreous Chamber Depth

When pooling the data from the two experiments, no difference was found in the vitreous chamber depth between blue (5.195±0.11mm) and red lighting (5.381±0.184mm) (ANOVA; F=3.48, p=0.077) in 10 day old chicks. Pooling the data from the color of the lighting, there was no difference in vitreous chamber depth between Experiment 1 (5.246±0.137mm) and Experiment 2 (5.246±0.174mm) (ANOVA; F= 0.212, P=0.650). Overall, there was no interaction between Experiment and color of the lighting condition (ANOVA; F=1.82, p=0.192).

The results of this analysis indicate that at 10 days old, there is a lack of an effect of the duration of exposure on vitreous chamber depth.

Comparing Experiments 1 and 2: Age of onset (duration matched)

To examine the effect of age of onset on the growth of the eye, we looked for differences between Experiments 1 and 2 after the same duration of exposure to the lighting conditions. For this comparison, ocular components from chicks from Experiment 1 (Age: 17 days) and Experiment 2 (Age: 10 days) were compared.

Refractive Error

We found that on the 10^{th} day of colored light exposure, chicks in both experiments were only marginally more hyperopic in blue light (+1.76±0.92D) conditions than in red light conditions (+0.62±0.82D) (ANOVA; F-value=8.74, P=0.08). There was no interaction between illumination condition and age (ANOVA; F-value=0.746, p=0.397).These results indicate that age of onset did not affect refractive errors induced by the color of the illuminant.

Axial length

After 10 days of exposure to the relevant color of light, axial growth in chicks was inhibited in blue light (Mean: 9.391±0.355mm) compared to red light (Mean: 9.696±0.301mm) (ANOVA; F=11.617, p=0.003). Also, as expected, chicks showed a greater change in axial lengths in Experiment 1 than 2 (ANOVA; F=88.8, P<0.05) due to age differences. These results indicate that age of onset is a factor when considering the effects of the color of the illuminant on changes in axial length.

Choroidal thickness

After 10 days of exposure to the lighting conditions, there were no significant changes in the chicks' choroidal thickness associated with the color of the light (ANOVA: $F=0.539$, $P=0.471$), nor between experiments (ANOVA; $F=2.131$, $P=0.16$). These results indicate that age of onset does not affect changes in choroidal thickening.

Vitreous chamber depth

After 10 days of exposure there was no difference in vitreous chamber depth between red and blue light (AN OVA; $F=2.78$, $P=0.110$). Again, on average, chicks in Experiment 1 had larger changes in vitreous camber depth than those in Experiment 2 due to age differences (ANOVA; $F=18.5$, $P<0.05$). These results indicate that age of onset is a factor when considering the effects of the color of the illuminant on changes in vitreous chamber depth.

	n	Refractive Error (D)		Axial Length (mm)		Choroidal Thickness (mm)		Vitreous Chamber (mm)		Lens thickness (mm)		Anterior Chamber (mm)	
		Day 10	Day 17	Day 10	Day 17	Day 10	Day 17	Day 10	Day 17	Day 10	Day 17	Day 10	Day 17
Blue Steady	9	+1.94±1.06	+1.24±0.55	9.148±0.097	10.047±0.161	0.198 ±0.038	0.201±0.017	5.175±0.102	5.558±0.145	2.036±0.019	2.256±0.05	1.410±0.02	1.677±0.085
Red Steady	5	+0.51±0.47	+0.64±0.24	9.461±0.206	10.332±0.256	0.193±0.019	0.247±0.041	5.374±0.207	5.736±0.132	2.067±0.026	2.291±0.059	1.489±0.03	1.713±0.104

Table 3– Mean measurement values for Experiment 2 for refraction, axial length, choroidal thickness, vitreous chamber depth, lens thickness and anterior chamber depth on Days 10 and 17.

	n	ΔRefractive Error (D)	ΔAxial Length (mm)	ΔChoroidal Thickness (mm)	ΔVitreous Chamber (mm)	ΔLens thickness (mm)	ΔAnterior Chamber (mm)
Blue Steady	9	-0.69±1.10	+0.898±0.089	+0.003±0.268	+0.384±0.021	+0.219±0.034	+0.267±0.083
Red Steady	5	+0.12±0.39	+0.871±0.103	+0.054±0.021	+0.389±0.099	+0.224±0.046	+0.224±0.102

Table 4– Mean change in ocular measurements values for Experiment 2 for refraction, axial length, choroidal thickness, vitreous chamber depth, lens thickness and anterior chamber depth on Days 10 and 17.

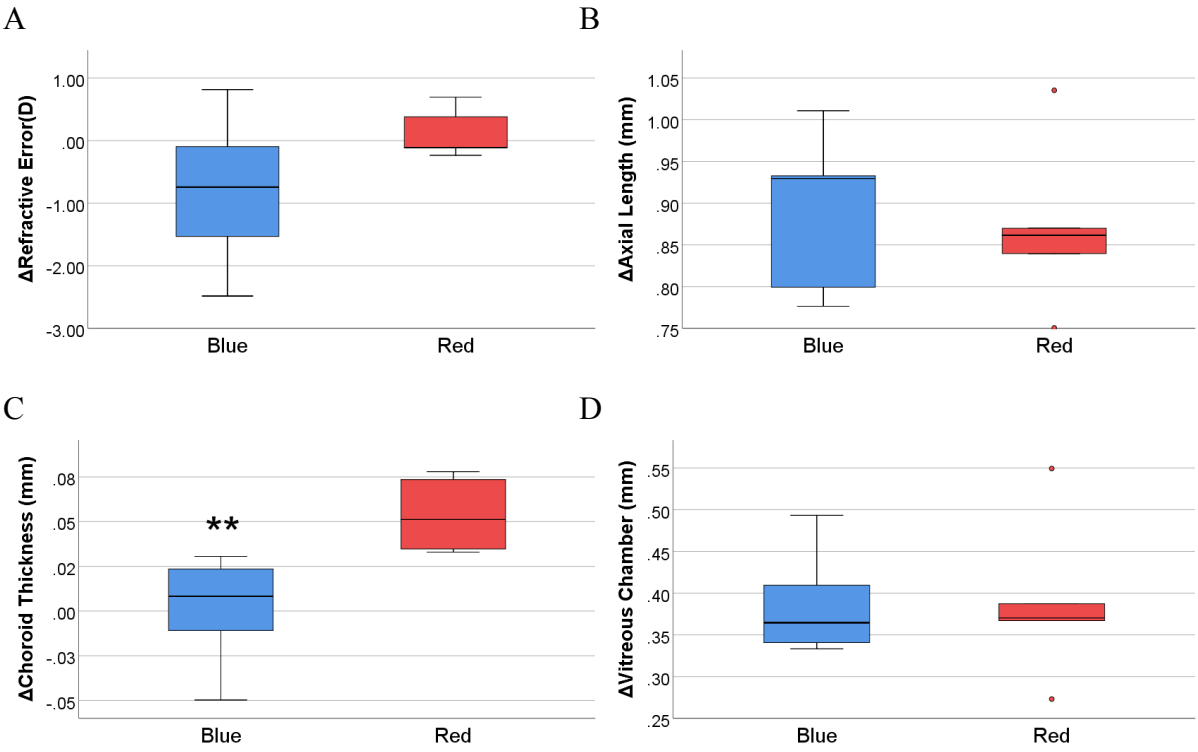


Figure 11- Experiment 2 (Long duration): Change in ocular measurements after 17 days of exposure to the illumination condition from hatching. **A.** Change in refractive error. **B**. Change in axial length. **C.** Change in choroidal thickness. Red light produced greater choroidal thickening than blue light. **D.** Vitreous chamber depth. Horizontal line in the box indicates the median. Boundaries of the box indicate the 25th and 75th percentile. The whiskers represent the high and low values 1.5x the interquartile range. Dots represent outliers. Asterisks represent a p-value < 0.01.

DISCUSSION

Duration effects

We performed two experiments to look at the effects of duration and flicker on eye growth and refractive status in chicks reared in monochromatic light. In the first experiment, which had a shorter duration of 10 days, we found that exposure to either narrowband red light, or white broadband light, led to greater changes in axial length and vitreous chamber depth, while exposure to narrowband blue light led to growth inhibition. Overall, flicker decreased eye growth compared to steady light but the effect was not specific to the wavelength.

In the second experiment, the lighting conditions were modified so that chicks were exposed to narrowband light for 17 days instead of 10, and with exposure starting at hatch. However, this time we were unable to reproduce the differences in axial length seen between red and blue narrowband light that were found in the short duration experiment. It should be noted that there was a shift towards hyperopia in red light in the longer duration experiment as opposed to the myopic shift seen with the shorter duration experiment. This hyperopic trend may be due to the difference in choroidal thickness in the two conditions, since red light produced thicker choroids than blue light with longer duration exposure, an observation that has been seen in other experiments (Gawne et al 2017).

The lack of a salient effect on the ocular components after 17 days of exposure, in the second experiment, was surprising as we expected to see a larger effect of color on axial growth given the results of Foulds (2013). The lack of axial effect in the second experiment suggests that it was due to the longer duration of exposure and introduces the possibility that illuminant related eye growth is transient, or nonlinear.

To examine the possibility that there were transient growth effects, we compared the changes in the ocular components for age matched 10-day old chicks. At the same age, the chicks in Experiment 1 had only 3 days of light exposure, compared to the chicks in Experiment 2 who had 10 days of light exposure. The results showed that chicks raised for a shorter duration in blue light became more hyperopic than those with longer duration exposure. This results indicates that the most salient changes in refractive error related to the color of the illuminant are transient, decreasing as the duration of the light exposure continues. The results also indicated that eyes exposed for a longer duration in monochromatic light grow more. This result is in line with conclusions drawn by Gawne et al. (2018) that the signals for emmetropization are confused in the absence of feedback from LCA.

To test the hypothesis that the ocular effects of colored lighting were affected by age, we compared the ocular measurements after 10 days of light exposure (with chicks being older in Experiment 1). We found that chicks showed no refractive differences with age but that the axial and vitreous changes were greater in the older chicks. This comparison indicated that age related differences affect axial and vitreal responses in monochromatic light experiments.

Comparison with long duration monochromatic light experiments in chick

With a similar lighting setup, and with animals exposed from hatch, Foulds (2013) was able to demonstrate a significant -1.62±0.54D myopic shift in red lighting and a +3.06±0.29D hyperopic shift in blue lighting after 14 days of light exposure in newborn chicks. However, in this study, we failed to show a change in refractive error or eye growth after 17 days of light exposure. Axial length did not change in our long duration experiment but changed in the predicted direction in our shorter duration experiment and in the Foulds study. The discrepancy

may have arisen for several reasons including wavelengths selected, light intensity and breed of chick.

The wavelengths used in the Foulds' experiment and in our current experiments differed in that we used narrow band 460nm blue light and 620nm red light compared to Fould's 470 and 640nm wavelengths. The peak spectral sensitivity of the chick long-wavelength sensitive cone is 560nm, making the eye around 50% less sensitive to 620nm and 90% less sensitive to 640nm. This lack of sensitivity may have meant that the condition was dimmer for the chick than the human lux measure might imply.

Light intensity is likely to have been a major factor in the disparity in results. The luminance at the center of the enclosure in Foulds' (2013) experiment was 33.37cd/m^2 for red light, 34.44 cd/m^2 for blue light, and 117.32 cd/m^2 for white light LEDs. Mean light intensities for the current experiments were 424 lux as described in the Methods section, these lux values are equivalent to human luminance values 84 cd/m^2 for blue and 114 cd/m^2 for red. The dimmer lighting conditions in the Foulds' experiment are likely to produce greater eye growth (Feldkaemper et al 1999). Since birds have different spectral sensitivity than humans, the lux or luminance measurements, which are based on the sensitivity of the human cone system, are not the ideal measurement for these experiments and power measurements can provide a better description of the light source.

Another possible explanation for the differences between Foulds (2013) experiments and ours may be due to breed differences. Foulds (2013) found an unusually large difference (0.82mm) in vitreous chamber depth when comparing the difference between the two lighting conditions over 14 days of exposure. By comparison, in the short duration experiment, we only observed 0.48 mm change in vitreous chamber depth between the two conditions after 10 days in

C strain White Leghorn chicks. Despite these differences, our results had the same pattern of agreement with the effects of colored light on eye growth.

Comparison with monochromatic light experiments in tree shrew

We are particularly interested in how this study compares to Gawne et al (2016, 2017)'s study in tree shrews, since these experiments showed hyperopic refractive changes in red light and myopic refractive changes in blue light. While lighting conditions were very similar in both studies, Gawne (2017) showed that exposure to blue flickering light for 25 days led to a larger myopic shift and increase in eye growth than was seen in red flickering or steady light. However, in a later experiment (Gawne et al 2018), it was noted that the animals became hyperopic during the early phase at around 27 days of visual experience (DVE) before becoming myopic in both steady and flickering light during the late phase. The authors suggest that the animals lose their ability to detect the sign of defocus through feedback mechanisms in narrowband blue light, a result that suggests that LCA is important in emmetropization. However, repeated experiments in red light (Gawne 2016, 2017) have continued to show hyperopic shifts in refraction in juvenile and adolescent tree shrews that may arise partially from choroidal thickening in red light. These blue light results resemble the findings from our current study in chick, in that while hyperopic changes were initially found in blue light with short duration exposures, no difference in refraction or eye length was found with longer duration exposures. The current study also found choroidal thickening in red light with longer duration exposure.

The daily rhythm of locomotor activity is a well-recognized indicator of circadian rhythms (Moore-Ede, Czeisler & Richardson1983a, Moore-Ede, Czeisler & Richardson 1983b)**.** The increase in activity in red light compared to blue light indicates that there was a possible

effect of the lighting conditions on activity levels and by association on the circadian rhythm. This is likely because red light does not stimulate the photopigment melanopsin in iPRGCs which has a peak spectral sensitivity around 476-484nm (Torii et al, 2007) while blue lighting does. We suspect that the differences observed in the outcomes in the longer duration experiments in monkey and tree shrew (Gawne 2018, Smith 2015) and the shorter duration monochromatic light experiments in chick, guinea pig and fish may be partially due to lack of stimulation of the circadian rhythms in red light.

In addition to the interference with normal circadian rhythms, the effects of flicker need to be taken into consideration. Gawne manipulated temporal frequency of the lighting conditions by introducing a pseudo-random flicker. However, short wavelength cones are more sensitive at low temporal frequencies than high temporal frequencies (Smithson, 2014) and the lower temporal frequencies in Gawne's flicker stimulus may have biased the blue light response towards the development of myopia (Rucker 2013, Rucker & Wallman 2012, Rucker 2015, 2018). A bias towards high temporal frequencies in red light may have biased the response towards hyperopia. Gawne's flicker stimuli also had a RMS contrast of only 65% making the stimulus more likely to drive the eye towards myopia, at least in chick (Rucker et al 2017).

Hormonal influences

Hormone release may play a role in tree shrew experiments since tree shrews reach puberty around 2 months old (~30 DVE), the age at which there was a reversal in refractions in blue light reared animals (Gawne 2018). However, in chicks, puberty is generally not reached until 18-21 weeks of age. As puberty can lead to increased eye growth, we need to consider the

role of testosterone in older pre and post-pubertal animals as that may help to explain the differences between our study with chicks and Gawnes' studies with tree shrews.

Lewis and Morris (2000) reviewed the effect of colored light on growth in poultry. They found that body weight increased more in short-wavelength light (415-560nm) than long wavelength light (>635nm) or broadband light, and there was a negative regression in body weight, such that there was a 50g decrease in body weight for each 100nm increase in wavelength. It was suggested that this may be because birds were more active and aggressive under red light, a finding which was attributed to greater testosterone release. This notion can also be seen when we measured activity where chicks raised in red light were more active throughout the experiment than those in blue light.

Critical development period

Our results also suggest that there may be a critical period where cones are becoming sensitized and that lack of exposure to specific wavelengths may interfere with this process. There was less of an effect on eye growth in the longer duration experiment and it is possible that this may be due to underdevelopment of cones due to lack of stimulation (Zou et al. 2018). If this were the case, it would mean that the normal eye relies on a mechanism that compares the stimulation of long- and short-wavelength sensitive cones, and that these cones need to undergo normal visual experience to normalize growth and development.

Our study shows that the duration of different monochromatic lighting conditions and age of onset has an effect on eye growth under monochromatic illumination conditions. The color of monochromatic light also has an effect on circadian activity rhythms. These effects may contribute to the species differences noted between chick, tree shrew and monkey. Further

studies would need to be done to explore the possibility of differences in hormone release as a result of circadian interference and onset of puberty to determine how this affects emmetropization.

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