

# The Effect of Refractive Error on Melanopsin-Driven Pupillary Responses

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Received: July 2, 2019

Accepted: September 30, 2020

Published: October 22, 2020

Citation: Mutti DO, Mulvihill SP, Orr DJ, Shorter PD, Hartwick ATE. The effect of refractive error on melanopsin-driven pupillary responses. *Invest Ophthalmol Vis Sci.* 2020;61(12):22. <https://doi.org/10.1167/iov.61.12.22>

**PURPOSE.** Human and animal studies suggest that light-mediated dopamine release may underlie the protective effect of time outdoors on myopia development. Melanopsin-containing retinal ganglion cells may be involved in this process by integrating ambient light exposure and regulating retinal dopamine levels. The study evaluates this potential involvement by examining whether melanopsin-driven pupillary responses are associated with adult refractive error.

**METHODS.** Subjects were 45 young adults (73% female,  $24.1 \pm 1.8$  years) with refractive errors ranging from  $-6.33$  D to  $+1.70$  D. The RAPDx (Konan Medical) pupillometer measured normalized pupillary responses to three forms of square-wave light pulses alternating with darkness at 0.1 Hz: alternating long wavelength (red, peak at 608 nm) and short wavelength (blue, peak at 448 nm), followed by red only and then blue only.

**RESULTS.** Non-myopic subjects displayed greater pupillary constriction in the blue-only condition and slower redilation following blue light offset than subjects with myopia ( $P = 0.011$ ). Pupillary responses were not significantly different between myopic and non-myopic subjects in the red-only condition ( $P = 0.15$ ). More hyperopic/less myopic refractive error as a continuous variable was linearly related to larger increases in pupillary constriction in response to blue-only stimuli ( $r = 0.48$ ,  $P = 0.001$ ).

**CONCLUSIONS.** Repeated light exposures to blue test stimuli resulted in an adaptation in the pupillary response (more constriction and slower redilation), presumably due to increased melanopsin-mediated input in more hyperopic/less myopic adults. This adaptive property supports a possible role for these ganglion cells in the protective effects of time outdoors on myopia development.

Keywords: ipRGCs, myopia, melanopsin

An increasing prevalence of myopia in children, particularly in Asia, has sparked considerable interest in identifying environmental risk factors for myopia onset and its rate of progression.<sup>1–5</sup> Heredity is clearly important in the etiology of myopia,<sup>6–9</sup> but an increasing prevalence over time is inconsistent with a trait being solely genetic. Near work has been the variable classically associated with myopia. The balance of cross-sectional evidence supports the view that children with myopia engage in greater amounts of near work.<sup>10</sup> However, the more important question is longitudinal—namely, whether more near work results in an increase in the risk of myopia onset or the rate of myopia progression. Several studies find no substantial increase in risk of onset or rate of progression associated with more near work.<sup>9,11–15</sup> In contrast, time outdoors has been associated with a decreased risk of myopia onset more often than not in longitudinal cohort studies and clinical trials, along with inconsistent or little effect on myopia progression.<sup>12,14–19</sup>

One of the mechanisms proposed for the protective effects of time outdoors is that exposure to higher irradiance sunlight stimulates the release of more dopamine from the retina which results in inhibition of axial elongation.<sup>20–22</sup> Many animal models suggest that retinal dopamine plays an important role in the regulation of eye growth and refractive error. Induction of experimental myopia by form deprivation or minus lenses reduces levels of retinal dopamine,<sup>23,24</sup> and the application of dopamine agonists significantly inhibits the induction of myopia.<sup>23,25</sup> Exposure to high-intensity illumination in the laboratory also inhibits the induction of form-deprivation myopia in the chick, monkey, and mouse.<sup>26–28</sup> Introduction of the D2 receptor antagonist spiperone inhibits both the recovery from form-deprivation myopia<sup>25,29,30</sup> and the protective effect of high-intensity illumination in the chick.<sup>31</sup>

Given their ability to integrate high-intensity outdoor light exposure over time and to stimulate the release of retinal dopamine, it has been hypothesized that intrinsically

photosensitive retinal ganglion cells (ipRGCs) play an important role in mediating the protective effects of light exposure on myopia.<sup>21,22</sup> These melanopsin-expressing ganglion cells generate action potentials in direct response to light exposure,<sup>32</sup> but their sparse representation makes them better suited for detection of ambient illumination than for image resolution.<sup>33</sup> Like other ganglion cells, ipRGCs receive excitatory and inhibitory input from retinal bipolar and amacrine cells.<sup>34,35</sup> However, there is also evidence for a presynaptic role for ipRGCs in providing excitatory, light-evoked input to sustained-firing dopaminergic amacrine cells.<sup>36,37</sup> The central projections of ipRGC axons are diverse,<sup>38</sup> with one major target being the pretectal olivary nucleus involved in controlling the pupillary light reflex.<sup>39</sup> Clinical tests of the contribution of melanopsin to the pupillary response take advantage of two characteristics of this photopigment: its peak spectral sensitivity at 480 nm (as opposed to 420 nm, 534 nm, and 564 nm for S-, M-, and L-cones, respectively)<sup>40</sup> and the sluggish and sustained temporal firing properties of ipRGCs.<sup>33,41</sup> Sustained pupillary constriction with limited pupillary escape during the light response and a slower rate of redilation following light offset characterize melanopsin-mediated pupillary responses, whereas pupillary escape and brisk redilation are more typical of cone-mediated input.<sup>41–44</sup>

Assessment of the melanopsin-driven contribution to the pupillary response as a function of refractive error would be one test of ipRGC involvement in the protective effect of time outdoors. Longitudinal studies during the development of refractive error would be a more direct test; however, studies that have investigated ipRGC-mediated pupillary responses to small numbers (two or three pulses) of red and blue light have not found associations with refractive error in adults or children.<sup>45–47</sup> These results suggest that the subjects' refractive errors do not significantly influence the initial dark-adapted melanopsin-driven pupillary responses. However, prior work with different testing protocols has shown that ipRGC-mediated pupillary responses can be enhanced to show greater pupillary constriction and slower redilation by exposure to a larger number of repeated light pulses.<sup>48</sup> The purpose of the current study is to determine whether this adaptation in ipRGC-mediated pupillary responses that develops following repeated light and dark pulses is related to refractive error in adults.

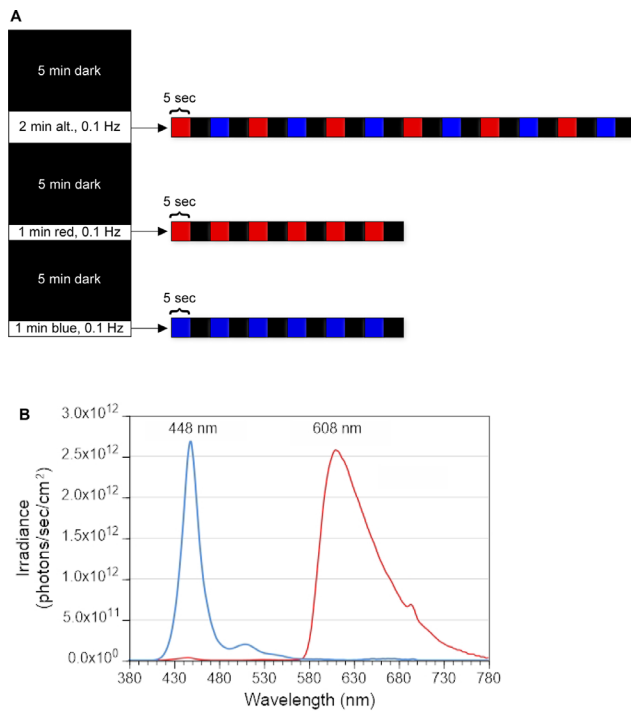
## METHODS

Subjects were 45 young adults, 33 (73.3%) of whom were female, with an average age ( $\pm$  SD) of  $24.1 \pm 1.8$  years (range, 21.4–29.7 years). Refractive error was measured using the open-view Grand Seiko WR-5100K (Grand Seiko Co., Ltd., Hiroshima, Japan; distributed by AIT Industries, Bensenville, IL, USA). Ten readings were taken on each eye without cycloplegia but with the use of a Badal lens and track to relax accommodation and provide a clear target while subjects viewed the smaller lines on an acuity card at the far point of the eye. Grand Seiko autorefraction has been reported to be comparable to subjective refraction in adults.<sup>49</sup> Myopia was defined as having a spherical equivalent (SEQ) of  $-0.50$  diopter (D) or more myopia in both eyes and an improvement in logMAR acuity by at least two lines with minus lens correction ( $n = 28$ , 62.2%). All subjects classified as myopic habitually used corrections for their refractive error. Subjects less myopic/more hyperopic than  $-0.50$  D SEQ in both eyes with distance acuity of 20/20 or better without correction were classified as non-myopic ( $n = 16$ , 35.6%). One subject was unclassified because of having a

low myopic, primarily spherical, refractive error but better than 20/20 distance acuity without correction. All subjects were included in the analysis of refractive error as a continuous variable, defined as the average SEQ of both eyes. No subject had significant anisometropia ( $\geq 1$  D). Only five subjects had astigmatism  $\geq 1$  D, and none was a case of simple or mixed astigmatism. The average refractive error ( $\pm$  SD) was  $-1.73 \pm 2.02$  D (range,  $-6.33$  to  $+1.70$  D). Most of the sample was white ( $n = 39$ , 87%); two subjects identifying as black, two as Hispanic, and two as Asian. The limited ethnic diversity did not allow for statistical analysis of this factor.

Because pupillary responses may be affected by prior activity,<sup>47</sup> environmental light exposure was measured as a covariate using the Daysimeter (Lighting Research Center, Rensselaer Polytechnic Institute, Troy, NY, USA). The device recorded average photopic lux values every minute along with a date and time stamp. It also recorded spectral data on three channels (blue, green, and red) in addition to lux. Spectral channel data were not analyzed due to the extremely high level of correlation between their output and lux. Subjects were instructed to wear the device mounted on an elastic strap on their upper arm outside their outermost layer of clothing during their normal waking hours for 7 days. Subjects were told to have the device facing outward from the torso while making no special attempt to have the device at any particular angle to the sun. When engaged in water sports such as boating or swimming, subjects were to have the device in their immediate environment but not in the water. The total lux-minutes of exposure were found for several time intervals prior to pupil testing: 1, 3, 12, 24, 72, and 120 hours. The shorter intervals included various amounts of night, morning, noon, or afternoon time, as testing was done at the convenience of the subject at any time of day during business hours. Season and time of day of testing were also recorded, as these have been shown to affect the melanopsin-mediated pupillary response in adults.<sup>50–52</sup> Birth month was noted because of a possible association with myopia prevalence.<sup>53</sup> Subjects were also asked to estimate the percent of time outdoors that they wore sunglasses. Exposure to environmental light was quantified as  $\log_{10}$  lux-minute values to create normal distributions for all intervals (Kolmogorov–Smirnov test for normality on the log-transformed data,  $P < 0.20$ ) except for 24 hours (excessive skew and kurtosis, Kolmogorov–Smirnov test for normality,  $P = 0.015$ ). Time outdoors was counted as any minute where illuminance exceeded 1000 lux.<sup>54,55</sup> A log transform of time outdoors was also used to create normal distributions.

Pupil testing was done using the RAPDx pupillometer (Konan Medical USA, Irvine, CA, USA). Pupillometry began within 10 minutes of arrival for testing after subjects' ordinary daily activity. Subjects were positioned in front of the pupillometer without refractive correction after 5 minutes of dark adaptation (0.01 lux) before each of three separate trials. The length of time chosen for dark adaptation was somewhat arbitrary but was within the range of the 2 to 10 minutes used in other studies.<sup>41,45,46</sup> The light stimuli were presented in a  $26.5^\circ$  circular field to both eyes for 5 seconds interleaved with 5 seconds of dark (0.1 Hz). The light stimuli in the three trials were (1) pulses alternating between red and blue, (2) red only, and (3) blue only. The alternating presentation lasted for 2 minutes (six presentations of red interleaved with six of blue) and each of the single-color conditions lasted for 1 minute (six presentations of either red or blue). The order of presentation was the same for all subjects: the alternating red/blue stimulus was applied



**FIGURE 1.** (A) Diagram representing the three stimulus conditions alternating between 5 seconds of light and 5 seconds of dark with 5 minutes of dark adaptation prior to each condition: alternating red/blue, red only, and blue only. The vertical scale represents the relative time taken within each step of the protocol. (B) Corneal irradiance of red and blue light stimuli used during pupil recordings. Stimuli were generated by LCD screens within the RAPDx pupilometer.

first, followed by the red-only stimulus and finally the blue-only stimulus (Fig. 1A). The dark periods between the light stimuli had an overall radiance of  $2.65 \times 10^{-3}$  W/sr/m<sup>2</sup>, with a peak at 448 nm, due to minimal residual lighting from the liquid-crystal display screen within the instrument. At this peak wavelength, the irradiance was 2.9 log units lower than it was during exposure to the blue light stimulus. The spectral distributions and the irradiances of the red and blue stimuli used in these experiments were measured using a spectroradiometer (PR-670; Photo Research, Inc.,

Chatsworth, CA, USA) (Fig. 1B). The peak intensity for the long-wavelength stimulus (red, for simplicity) was at 608 nm, and the peak for the short-wavelength stimulus (blue) was at 448 nm. This peak differs from the 480-nm maximum sensitivity of ipRGCs, but only on the order of 0.1 or less in log relative sensitivity.<sup>56</sup>

Blink artifacts were either removed from the data by the RAPDx or identified as spikes and removed manually. The RAPDx records at 40 Hz but subject data were binned into averages at 0.25-second intervals for the purposes of simplifying data handling. Using the equation below, the diameter of the pupil was normalized at each 0.25-second interval separately for each eye relative to the smallest pupil diameter (100% constriction = 1.0) and largest pupil diameter (0% constriction = 0) that occurred for that eye across all three stimulus conditions, as in previous work.<sup>48</sup> Normalized pupillary responses were then averaged for the two eyes at each 0.25-second interval. Pupillary responses are reported as normalized pupillary constriction and are dimensionless unless otherwise marked in millimeters (mm).

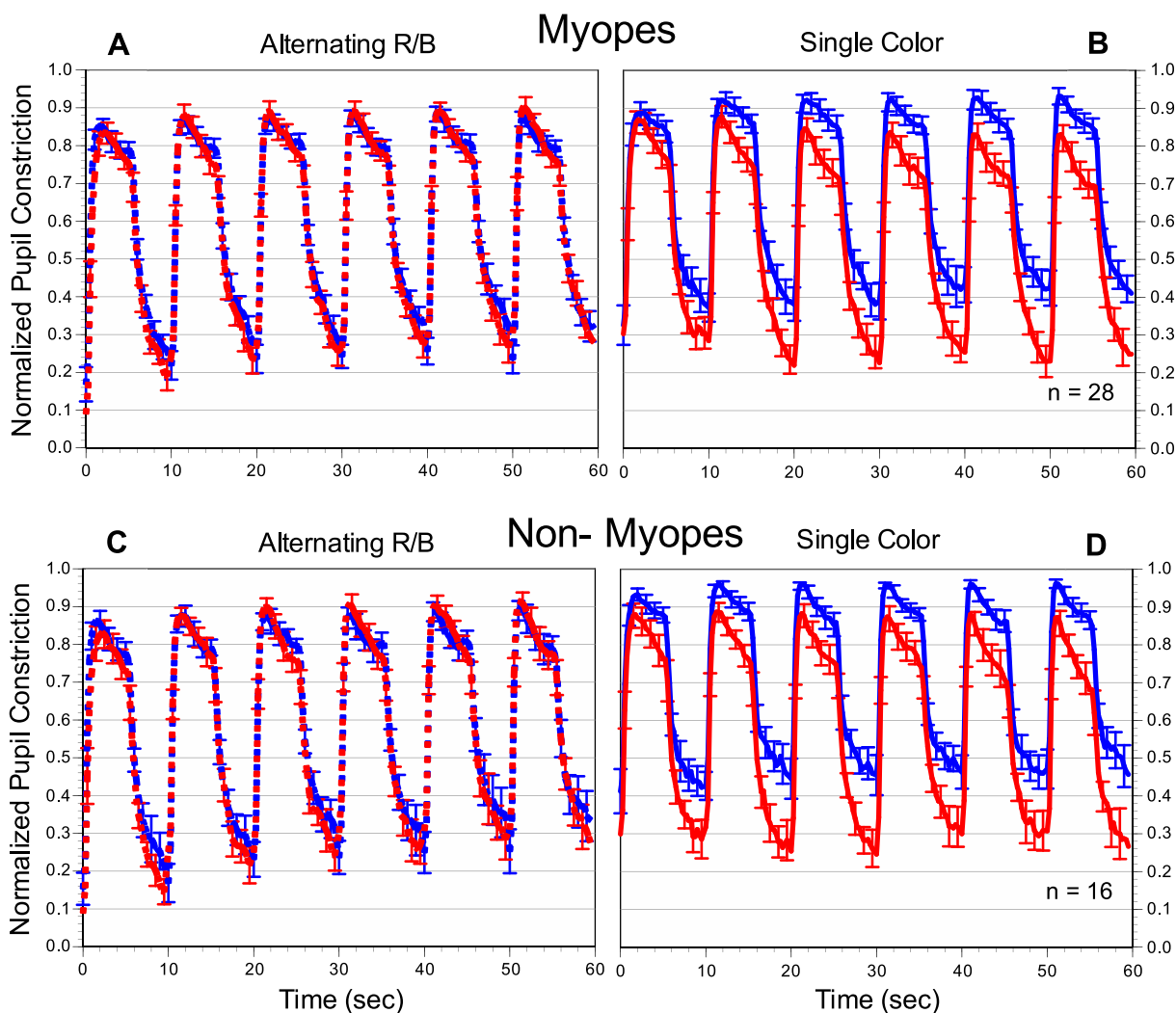
$$\text{Normalized pupillary constriction} = \frac{\text{Maximum diameter} - \text{Pupil diameter}}{\text{Maximum diameter} - \text{Minimum diameter}}$$

Four features of pupillary responses were selected for analysis. The  $\Delta$ Blue variable was the average difference at each corresponding time point throughout testing between normalized pupillary responses during the presentation of blue in the single-color condition and those in the alternating-color condition. These differences were calculated across all time points, both when the stimulus was on during constriction and when it was off during redilation. This comparison between alternating and single-color presentation was chosen in order to highlight the adaptive change in pupil size between the earlier alternating and the later single-color condition. The  $\Delta$ Red variable was the average difference at each corresponding time point throughout the testing between normalized pupillary responses during the presentation of red in the single-color condition and those in the alternating-color condition. Post-illumination pupillary redilation rates were calculated as the average of the six coefficients  $\beta$  for the exponential decay function  $e^{\beta t}$  during the final 3 seconds ( $t$ ) of each of the six pupillary redi-

**TABLE 1.** Descriptive Statistics for Cumulative Light Exposure and for Time Outdoors (Illuminance Exposure > 1000 lux) at Various Intervals for the Whole Sample and for Myopic and Non-Myopic Subjects by Group

Time Period	All Subjects $N = 45$	Myopic Subjects $n = 28$	Non-Myopic Subjects $n = 16$
Average light exposure (log <sub>10</sub> lux-min), mean $\pm$ SD			
1 h	4.60 $\pm$ 0.80	4.36 $\pm$ 0.82	4.97 $\pm$ 0.60
3 h	5.04 $\pm$ 0.66	4.93 $\pm$ 0.72	5.22 $\pm$ 0.52
12 h	5.31 $\pm$ 0.59	5.22 $\pm$ 0.66	5.44 $\pm$ 0.46
1 d	5.87 $\pm$ 0.54	5.80 $\pm$ 0.57	5.92 $\pm$ 0.43
3 d	6.45 $\pm$ 0.50	6.36 $\pm$ 0.45	6.55 $\pm$ 0.53
5 d	6.69 $\pm$ 0.47	6.59 $\pm$ 0.46	6.81 $\pm$ 0.43
Average time outdoors (log <sub>10</sub> min), mean $\pm$ SD			
1 h	1.13 $\pm$ 0.33	1.01 $\pm$ 0.32	1.25 $\pm$ 0.31
3 h	1.28 $\pm$ 0.46	1.18 $\pm$ 0.50	1.41 $\pm$ 0.39
12 h	1.35 $\pm$ 0.51	1.24 $\pm$ 0.53	1.53 $\pm$ 0.45
1 d	1.94 $\pm$ 0.37	1.87 $\pm$ 0.37	2.02 $\pm$ 0.35
3 d	2.39 $\pm$ 0.40	2.32 $\pm$ 0.38	2.47 $\pm$ 0.43
5 d	2.63 $\pm$ 0.38	2.57 $\pm$ 0.39	2.71 $\pm$ 0.35

Neither light exposure nor time outdoors was significantly different between groups ( $P = 0.055$  and  $P = 0.14$ , respectively; repeated-measures ANOVA).



**FIGURE 2.** The pattern of pupillary responses for the myopic subjects during the alternating pulses of red and blue (A) and during the presentation of red only and blue only (B). The pattern of pupillary responses for the non-myopic subjects during the alternating pulses of red and blue (C) and during the presentation as red only and blue only (D). *Solid lines* represent the single-color presentation of blue or red; *dashed lines* represent the alternating-color presentation. Pupillary constriction was greater in non-myopic subjects than in myopic subjects during the presentation of blue only compared to blue alternated with red. Pupillary constriction was not significantly different between non-myopic and myopic subjects during the presentation of red only compared to the alternating presentation of red. *Error bars* represent 95% CIs.

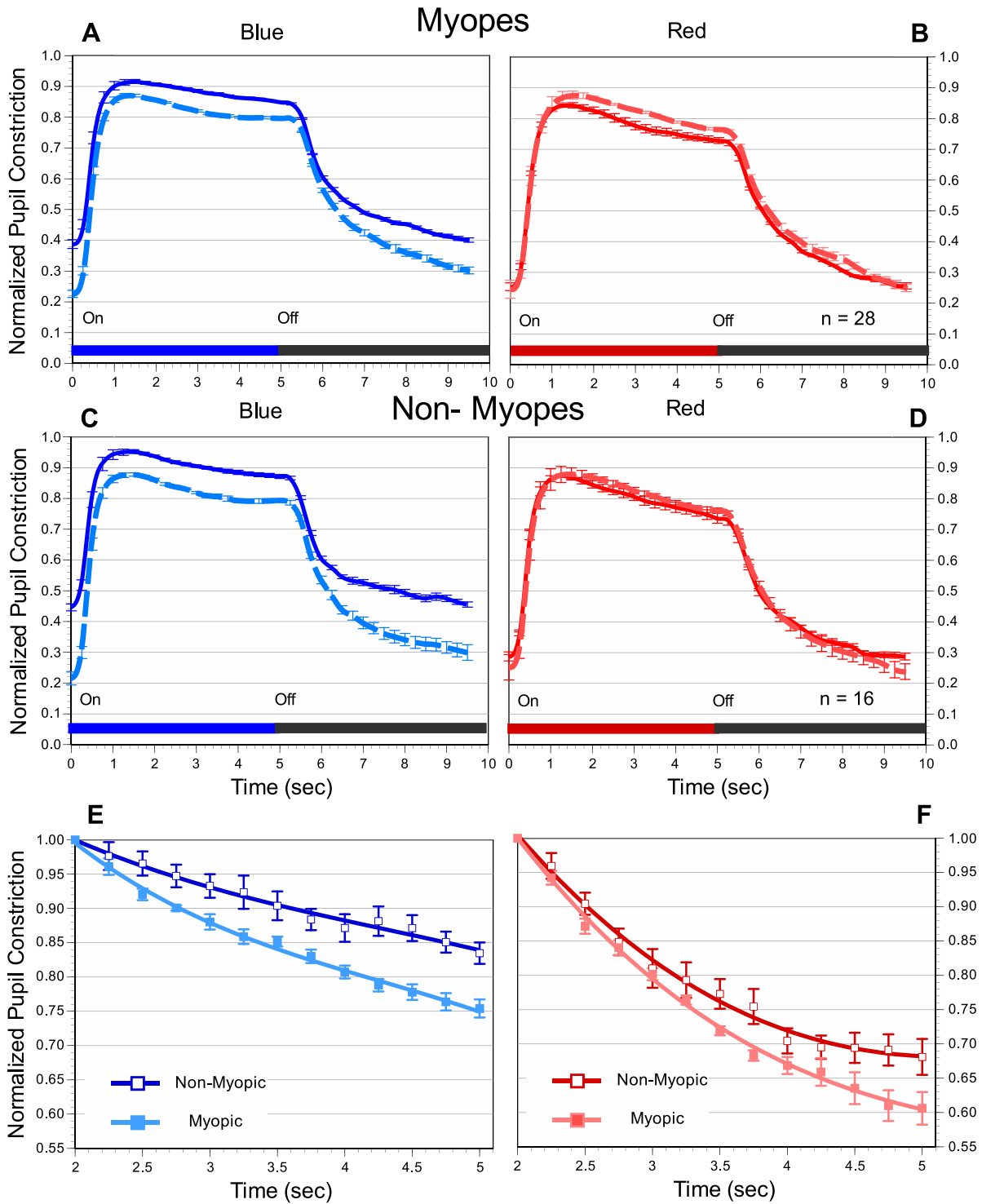
lations following presentation of blue only (ExpBlue). Only the data from the final 3 seconds were used because of the rapid rate of change during the first 2 seconds of redilation. The decay coefficient for redilation following presentation of red only was calculated in a similar manner (ExpRed).

Statistical analysis was performed using SPSS Statistics 21 (IBM, Armonk, NY, USA). Non-myopic and myopic subject characteristics were compared using independent *t*-tests or Fisher's exact test. The *t*-test *P* values were not adjusted for multiple testing. The six time periods for light exposure and time outdoors were compared between myopic and non-myopic subjects in separate repeated-measures ANOVA. The four pupillary response outcomes ( $\Delta$ Blue,  $\Delta$ Red, ExpBlue, and ExpRed) were also analyzed using repeated-measures ANOVA with color (red or blue) and outcome type ( $\Delta$  or Exp) as repeated factors. Myopic or non-myopic was a between-subject factor. Bivariate correlations were examined among the pupillary outcomes, light exposure, and SEQ

using SPSS. Significant linear relationships were fit using the orthogonal regression procedure in JMP 10 (SAS Institute, Cary, NC, USA). General linear models were then used to examine multivariate associations among SEQ, environmental light exposure, age, and sex, including all two-way interactions. *P* < 0.05 was considered significant.

## RESULTS

Myopic and non-myopic subjects were similar in average age and percent who were female (*P* = 0.46 and *P* = 0.54, respectively). There were also no significant differences between groups with respect to time of day or season of testing, birth month or season of birth, or percent time wearing sunglasses when outdoors (*P* values between 0.20 and 0.81; Supplementary Table). As shown in Table 1, subjects had an average light exposure during 5 days of 4.9 million lux-min ( $6.69 \pm 0.47 \log_{10}$  lux-min) and spent an average of 427 minutes



**FIGURE 3.** The data in Figure 2 averaged across pulses for the myopic subjects during the alternating pulses of blue and blue only (A) and during the alternating pulses of red and red only (B). The pattern of pupillary responses for the non-myopic subjects during the alternating pulses of blue and blue only (C) and during the alternating pulses of red and red only (D). *Solid lines* represent the presentation of blue only or red only; *dashed lines* represent the alternating-color presentation. The gap between the single-color and the alternating presentation of blue ( $\Delta$ Blue) is greater in non-myopic subjects than in myopic subjects (see Table 2 for quantification of effect). *Error bars* represent 95% CIs. (E, F) Normalized pupillary responses during the last 3 seconds of redilation (seconds 2–5) for each 5-second period of dark following blue-only stimulation (E) and red-only stimulation (F), averaged over the six pulses. Results are displayed by refractive error group. The rate of redilation was significantly slower for non-myopic subjects (*open symbols*) compared to myopic subjects (*closed symbols*) for the blue-only condition. *Error bars* represent 95% CIs.

TABLE 2. Descriptive Statistics for the Pupillary Response Variables for the Sample as a Whole and for Myopic and Non-Myopic Subgroups

Pupillary Response Variable	Mean $\pm$ SD			P
	All Subjects $N = 45$	Myopic Subjects $n = 28$	Non-Myopic Subjects $n = 16$	
$\Delta$ Blue	0.086 $\pm$ 0.062	0.071 $\pm$ 0.064	0.11 $\pm$ 0.050	0.038
$\Delta$ Red	-0.017 $\pm$ 0.065	-0.027 $\pm$ 0.069	0.00040 $\pm$ 0.058	0.18
ExpBlue	-0.079 $\pm$ 0.052	-0.091 $\pm$ 0.053	-0.056 $\pm$ 0.045	0.029
ExpRed	-0.16 $\pm$ 0.098	-0.17 $\pm$ 0.097	-0.14 $\pm$ 0.10	0.26

P values refer to independent *t*-tests comparing myopic and non-myopic subjects.

(2.63  $\pm$  0.38 log<sub>10</sub> minutes) outdoors. Myopic and non-myopic subjects spent similar amounts of time outdoors and had similar light-exposure histories over the sampled time intervals ( $P = 0.055$  and  $P = 0.14$ , respectively, for light exposure and time outdoors between myopic and non-myopic subjects; repeated-measures ANOVA). None of the four pupil outcome variables was related to light exposure in any of the time intervals ( $P$  values between 0.054 and 0.98).

The RAPDx recorded pupil size prior to the first pulses of alternating red and blue. These baseline pupil diameters were similar between myopic and non-myopic subjects (5.25  $\pm$  1.16 mm and 5.11  $\pm$  0.88 mm, respectively;  $P = 0.66$ ). The pupillary responses for non-myopic and myopic subjects are shown with results for each of the six pulses in Figure 2 and as the average of those six pulses by stimulus color for easier comparison in Figure 3. Pupillary constriction increased in both refractive error groups during the alternating presentation of red and blue. Pupillary responses differed, however, as a function of refractive error during the subsequent blue-only test condition. As shown in Figures 2 and 3, non-myopic subjects displayed greater pupillary constriction compared to myopic subjects during presentation of blue as a single color ( $\Delta$ Blue in Table 2,  $P = 0.038$ ). As shown in Figures 2 and 3, and in detail in Figure 3E, pupillary redilation was also slower following blue-only light offset in non-myopic subjects (ExpBlue in Table 2;  $P = 0.029$ ). Neither  $\Delta$ Red nor ExpRed was significantly different between non-myopic and myopic subjects ( $P = 0.18$  and  $P = 0.26$ , respectively) (Table 2). When  $\Delta$ Blue and ExpBlue were considered together as a repeated factor (blue), myopic subjects had greater pupillary constriction and slower redilation compared to non-myopic subjects ( $P = 0.011$ ). Pupillary responses to red as a repeated factor were not significantly different between non-myopic and myopic subjects ( $P = 0.15$ ). Stimulus outcome type ( $\Delta$  or Exp) was not a significant factor ( $P < 0.11$ ).

The four pupillary response outcomes  $\Delta$ Blue,  $\Delta$ Red, ExpBlue, and ExpRed were also analyzed with refractive error treated as a continuous variable. Linear correlations were significant between SEQ and three of the four pupil outcomes (Fig. 4). ExpRed was not associated with SEQ ( $P = 0.26$ ). The four pupil outcomes were positively correlated with each other. Correlation coefficients ranged from 0.34 to 0.79, with  $P$  values ranging from  $<0.0001$  to 0.021. Because of their intercorrelation, the three pupil outcomes associated with SEQ were evaluated in a multivariate regression model (Table 3). Only  $\Delta$ Blue retained a significant association with SEQ. Age, sex, light exposure, day of the week of testing, time of day of testing, season of testing, wearing sunglasses, or birth month were not associated with SEQ in a regression model with  $\Delta$ Blue. We also evaluated whether the positive association between more hyperopic/less myopic refractive errors and larger values for  $\Delta$ Blue was the result of the pupil

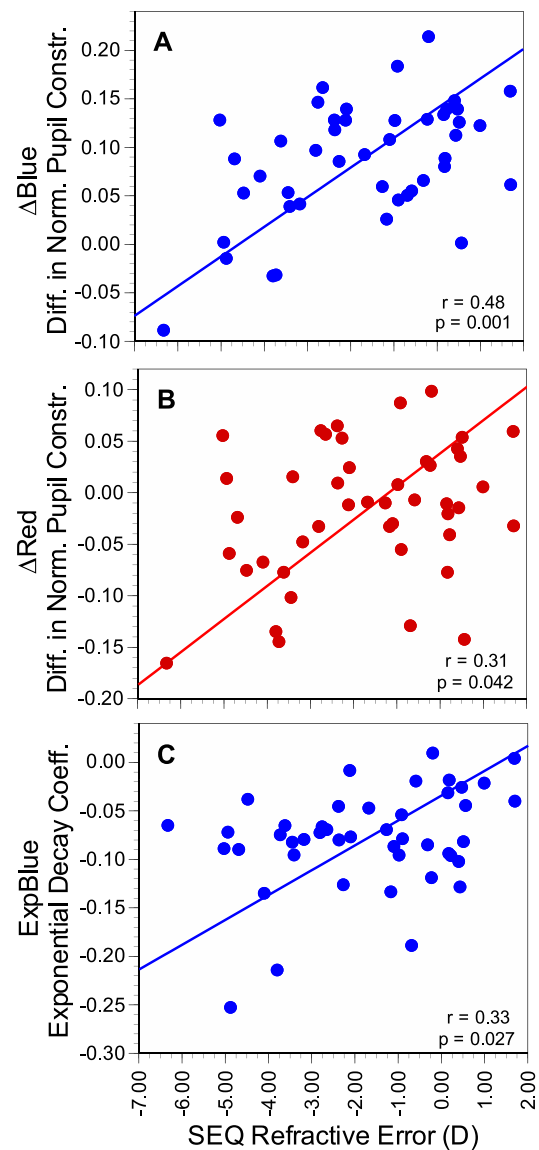


FIGURE 4. Univariate regressions between SEQ and each of the three pupil outcome variables significantly associated with refractive error: (A)  $\Delta$ Blue, (B)  $\Delta$ Red, and (C) ExpBlue.

normalization. Results for blue were similar when pupil sizes were analyzed using raw pupil diameters in millimeters. The pupil size became smaller (more constricted) during exposure to the blue-only condition compared to during the alternating red/blue sequence by  $0.30 \pm 0.20$  mm ( $P < 0.0001$ ),

TABLE 3. Univariate Regression Coefficients and Multivariate Regression Coefficients Associated with SEQ

Pupillary Response Variable	Regression Coefficients, Normalized		
	Univariate ( <i>P</i> )	Multivariate ( <i>F</i> , <i>P</i> ) <i>df</i> = 1, 41	Univariate, Pupil Diameters mm ( <i>F</i> , <i>P</i> ) <i>df</i> = 1, 43
ΔBlue	<b>15.7 (0.001)</b>	<b>18.5 (6.4; 0.015)</b>	<b>-3.7 (6.9; 0.012)</b>
ΔRed	<b>9.5 (0.042)</b>	-6.1 (0.8; 0.37)	—
ExpBlue	<b>12.9 (0.027)</b>	6.3 (1.2; 0.28)	—
Age (yr)	-0.025 (0.89)	—	—
Sex (female = 1, male = 0)	0.58 (0.40)	—	—

All three pupillary outcome variables were placed into the multivariate model for normalized pupillary response. Age and sex were not placed into the multivariate models due to their lack of significance in the univariate analysis. Coefficients with  $P < 0.05$  are indicated in bold.

and this change ( $\Delta$ Blue in mm) was associated with SEQ ( $r = -0.37$ ,  $P = 0.012$ ).  $\Delta$ Red in millimeters ( $r = -0.21$ ,  $P = 0.17$ ) and ExpBlue calculated using millimeters ( $r = -0.20$ ,  $P = 0.19$ ) were not associated with SEQ.

## DISCUSSION

Pupillary responses displayed greater constriction and slower rates of redilation following repeated stimulation by short-wavelength blue light, consistent with increasing activation of melanopsin-containing ipRGCs over the course of testing.<sup>41,44,47</sup> The largest degree of constriction and slowest redilation occurred during the final blue-only condition relative to those elicited by the same blue light in the alternating-color condition administered about 13 minutes earlier (Table 2; Figs. 2 and 3). Interestingly, this adaptive change between the alternating and single-color presentations of blue shows that the intervening 5 minutes of dark adaptation did not produce the expected return of the pupillary response to baseline. More hyperopic and less myopic subjects displayed the greatest shifts toward more constriction and slower, reduced rates of redilation during the blue-only condition. There were associations between two other pupil outcomes ( $\Delta$ Red, ExpBlue) and refractive error as a continuous variable, but these were no longer significant when adjusted for  $\Delta$ Blue in a multivariate model (Table 3).

The positive association between refractive error and these adaptive changes in pupillary responses following stimulation with blue only may seem at odds with the negative results observed in three previous studies. Abbott et al.<sup>45</sup> reported on post-illumination pupillary responses following two exposures to 1- and 5-second pulses of blue or red light in 19 emmetropic and 31 myopic adult subjects. Adhikari et al.<sup>46</sup> used the same RAPDx instrumentation as in the current study to measure peak constriction and post-illumination pupillary responses following two exposures to 1- and 10-second pulses of blue or red light in three hyperopic, 23 emmetropic, and 13 myopic adult subjects. Ostrin et al.<sup>47</sup> reported on post-illumination pupillary responses to three 1-second pulses each of red and blue in 37 children. None of these studies found an association between pupillary responses and refractive error, suggesting that the dark-adapted melanopsin-driven ipRGC responses to a small number of light pulses are not significantly associated with refractive error. The current study found that the differences between pupillary responses to red and blue light as a function of refractive error only became apparent after the subjects had undergone multiple exposures to the stimuli during the subsequent single-color conditions. Thus, our results indicate that an adaptive change in pupil-

lary responses to repeated test pulses of blue light may be more closely related to refractive error than the basal dark-adapted level of ipRGC activity that was assessed in previous studies.

The mechanism underlying this adaptive response is unknown. One possibility is that the short- and the long-wavelength light stimuli evoke a change in the light absorption properties of the melanopsin photopigment. Melanopsin shares higher homology with invertebrate photopigments than with vertebrate rhodopsin,<sup>57</sup> and certain invertebrate photopigments (such as in *Drosophila*) exhibit bistability. Short-wavelength light drives phototransduction in a bistable photopigment resulting in photobleaching of the molecule; long-wavelength light stimulates chromophore regeneration, returning the pigment to its light-sensitive state.<sup>58</sup> If melanopsin is bistable, then the repeated exposures to red light in this protocol may have increased subsequent responses to blue light because of a shift in a greater proportion of melanopsin to its light-sensitive state. Although some early studies provided evidence in support of melanopsin bistability,<sup>59-61</sup> more recent evidence refutes this hypothesis. Electrophysiological experiments on pharmacologically isolated ipRGCs in mice found that exposure to long-wavelength light provides no subsequent enhancement of ipRGC photoresponses.<sup>62</sup> Further detailed analysis of light responses from isolated rodent ipRGCs indicates that, rather than being bistable, melanopsin is a tristable molecule in which there is little spectral separation between the signaling and the silent states where photon absorption is not linked to phototransduction.<sup>63</sup> Furthermore, it is difficult to reconcile the invertebrate model of photopigment bistability with the findings of the current study that the pupil became progressively smaller in response to both red and blue stimuli during the alternating portion of the protocol.

A second possibility is that the adaptive change is mediated through the effects of retinal neuromodulators such as dopamine.<sup>23,24,31</sup> Dopamine D1 receptors are expressed by ipRGCs in rodents,<sup>64</sup> and pharmacologic activation of D1 receptors results in a rise in intracellular cyclic AMP (cAMP) levels through stimulation of adenylyl cyclase.<sup>65</sup> The application of forskolin or a cell-permeable cAMP analog increases cAMP within rodent ipRGCs, resulting in stronger and prolonged light-evoked responses.<sup>66</sup> If these results hold true for human ipRGCs, then the repeated light stimulation in this testing protocol may have had similar effects: increased levels of retinal dopamine resulting in increased cAMP within ipRGCs, more pupillary constriction, and slower post-stimulus redilation. The cAMP elevation peaks 10 to 15 minutes after D1 receptor activation in rodent striatal neurons,<sup>67</sup> consistent with the time course

of the change in the pupillary responses observed in the present study. The smaller adaptive change in the pupillary responses of myopes to multiple blue stimuli suggests that either the light-evoked rise in retinal dopamine levels is reduced in myopes or ipRGCs in this subject group exhibit less responsiveness to dopamine or to other relevant neuromodulators.

The contribution of outer retinal photoreceptors to these adaptive changes should also be considered. The outer retina certainly contributes to the pupillary response, and the peak spectral sensitivity of melanopsin is close to that of S-cones (480 nm and 420 nm, respectively).<sup>40</sup> Human and mouse data show that an intact outer retina is necessary for the pupillary response to be able to track temporally modulated stimuli, including the 0.1-Hz frequency used in the current study.<sup>43,68</sup> The outer retina can stimulate a release of retinal dopamine independent of ipRGC input by driving transient dopaminergic amacrine cell responses through connections with ON-bipolar cells.<sup>34–36</sup> Adaptive changes in pupillary response hypothesized to be due to dopamine could therefore result from stimulation of either ipRGCs or outer retinal photoreceptors, or both. The question arises whether all cone types contribute to this adaptation, S-cones in particular given the importance of short-wavelength stimulation in the results for  $\Delta$ Blue. The cone contribution seems more likely to come from L- and M-cone input, however, as S-cone input to the pupillary response appears to be inhibitory.<sup>69</sup> S-cone-driven pupillary responses to temporally modulated stimuli are substantially out of phase relative to L+M and melanopsin-driven pupillary responses.<sup>70</sup> No activity attributed to S-cones after light offset appears to be prolonged, neither spikes recorded from the olivary pretectal nucleus of mice lacking melanopsin nor human pupillary constriction using a silent substitution paradigm.<sup>68,71</sup> Therefore, the enhanced pupillary constriction and slower post-stimulus redilation resulting from repeated short-wavelength stimulation seems more likely due to changes in the melanopsin-driven pathway than in S-cone inputs. This discussion assumes that the source of the adaptation in pupillary responses to multiple short-wavelength light exposures is local at the retinal level, but adaptation at the level of the olivary pretectal nucleus cannot be ruled out. This possibility could be tested by examining whether or not the adaptation displays interocular transfer.

Previous results suggest that prior light exposure might influence post-illumination pupillary responses to blue light stimulation.<sup>45,47</sup> However, no pupil outcome was correlated with light exposure in any time interval. This inconsistency among studies may be due to the difference in stimulus protocols. The current study protocol may not be the optimal one for eliciting these adaptive changes related to refractive error. Future studies should evaluate protocol parameters such as duration and number of exposures, effects of stimulation at different wavelengths either alone or in sequence, and the effects of varying periods of dark or light adaptation. Light exposure and time outdoors were not different between refractive error groups and were not related to refractive error in any multivariate model with SEQ. These adult subjects did not follow the expected pattern for refractive error, where myopic children tend to spend less time outdoors than non-myopic children.<sup>20,72</sup> The lack of control over the time of day of testing may have limited the ability to find any significant correlation. It is not known what the subjects' habits were earlier in life or how infant or childhood exposures to light while outdoors might influence the

development of these pupillary responses. Early exposure to more time outdoors between ages 3 and 8.5 years has been shown to be effective at reducing later risk of myopia onset between 10 and 15 years of age.<sup>73</sup> The current results were cross-sectional and therefore limited to only showing associations between pupillary responses and the degree of current adult refractive error. It is unknown without longitudinal data whether any of the myopic subjects will progress or if any of the emmetropic subjects will become myopic in the future. Longitudinal data would also clarify whether differences in ipRGC-driven pupillary responses are a cause or simply a consequence of refractive error. Retinal stretching in longer, more myopic eyes could conceivably compromise the function of ipRGCs due to increased mechanical stress. Future studies could also help determine if this adaptive property of the pupillary response is an important part of the benefit of time outdoors in childhood, whether it changes with age, is altered by myopia onset, or can be enhanced by increasing early light exposure.

In summary, repeated stimulation with red and blue light resulted in adaptive changes in the pupillary response characterized by greater constriction and slower redilation in response to blue light. There was a positive association between the magnitude of these changes and refractive error, with more hyperopic/less myopic individuals exhibiting larger changes in their pupillary responses to repeated pulses of blue light. Although the following is speculative without the required longitudinal evidence, this positive association suggests that more hyperopic/less myopic individuals may have a greater ability to take advantage of light exposure, such as during time outdoors, and its accompanying protective effect on refractive error development.

### Acknowledgments

Supported by grants from the National Center for Research Resources (UL1RR025755) and the National Eye Institute, National Institutes of Health (T35-EY007151). It was also funded by the Office of the Director, National Institutes of Health, and supported by the National Institutes of Health Roadmap for Medical Research.

Disclosure: **D.O. Mutti**, None; **S.P. Mulvihill**, None; **D.J. Orr**, None; **P.D. Shorter**, None; **A.T.E. Hartwick**, None

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