TIME-OF-DAY VARIATIONS IN THE EFFECTS OF VISUAL STIMULI AND PIRENZEPINE ON OCULAR CIRCADIAN RHYTHMS IN CHICKS

A thesis presented to the graduate faculty of New England College of Optometry in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

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New England College of Optometry, 2019

Purpose. Previous research has shown that brief periods of myopic defocus at specific times of day were effective at inhibiting ocular growth. The purpose of this study was to investigate the time-of-day effects of various visual and therapeutic interventions on axial length and choroidal thickness, and the effects on the respective ocular rhythms. This study was composed of four main experiments: brief periods of hyperopic defocus, brief periods of form deprivation, brief periods of bright light exposure, and intravitreal injections of pirenzepine solution.

Methods. 12-day-old White Leghorn chicks were used for all experiments. *Hyperopic defocus*: Chicks wearing -10 D lenses were exposed to 2 hours of hyperopic defocus at morning (7:00 am to 9:00 am, n = 12), midday (12:00 pm to 2:00 pm, n = 12), and evening (7:00 pm to 9:00 pm, n = 6) for each of 5 days. *Form deprivation*: Chicks wore translucent diffusers for 2 hours at morning (7:00 am to 9:00 am, n = 12), midday (12:00 pm to 2:00 pm, n = 19), and evening (7:00 pm to 9:00 pm, n = 6) for each of 5 days. *Pirenzepine*: Chicks wearing -10 D lenses were given intravitreal injections of 10 mM pirenzepine at morning (8:00 am, n = 10), midday (2:00 pm, n = 10), and evening (8:30 pm, n = 18) or saline (n=9)

for 5 days. *Bright light*: Chicks wearing translucent diffusers were exposed to 3 or 5 hours of 20,000 lux high intensity light at morning (7:00am to 10:00am, n = 6; 7:00am to 12:00pm, n = 6), midday (1:00 pm to 4:00 pm, n =5), and evening (6:00 pm to 9:00 pm, n = 6; 5:00 pm to 10:00 pm, n=9) for each of 6 days. For all of the above experiments, ocular dimensions were measured with A-scan ultrasonography pre- and post-experiment. Refractive errors were measured using a Hartinger Refractometer at the conclusion of the experiment. A subset of chicks post-experiment were measured at 6-hour intervals over 24 hours to collect data on diurnal rhythms.

Results. *Hyperopic defocus*: 2 hours of hyperopic defocus caused growth stimulation in the morning group but growth inhibition in the evening group (change in vitreous chamber, experimental minus control or X-C: ANOVA ; < 0.0005; 120 μm vs -77 μm/5d, respectively; t-tests: p = 0.001, p = 0.01; post-hoc p = 0.002). Refractive error of the midday group was significantly hyperopic (1.4D, p < 0.0001). Diurnal rhythms of the morning group were disrupted, and the axial length rhythm of the midday group was significantly phase-delayed (p < 0.05 by circular statistics). *Form deprivation*: 2 hours of form deprivation caused growth inhibition in the morning and evening groups (changes in axial length, X-C: -122 μm and -92 μm, respectively; p = 0.006 and p = 0.016, respectively). Rhythms in axial length of the morning group were phase-advanced (p < 0.05). *Pirenzepine*: daily intravitreal injections were more effective in the midday group compared to evening group (79 μm vs 215 μm/5d; p = 0.046). There were no time-dependent effects on the ocular rhythms. *Bright light*: combined data of the 3- and 5-hours of bright light showed that evening was more effective

than morning at reducing ocular growth. There was no time-dependent effect on ocular rhythms.

Conclusions. The data suggest that there is a time-of-day effect of brief periods of hyperopic defocus, form deprivation and bright light, and pirenzepine administration on ocular growth in chick eyes. Furthermore, hyperopic defocus and form deprivation alter ocular diurnal rhythms, being more detrimental to choroidal thickness than axial length. The results imply that near work, which induces accommodative lag and subsequent hyperopic defocus, should be avoided in the morning to reduce the risk of myopia progression. The small effect of bright light at certain times of day suggest that the length of time exposed to bright light is not the only factor which elicits a protective effect against myopia, as previous research has shown. In conclusion, the data show that time-of-day should be considered when developing dose regimens for future therapies for myopia control.

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1. General Introduction 1

1.1 Myopia 1
1.2 Animal Models of Myopia 4
1.3 The Role of Circadian Rhythms 5
1.4 The Effects of Form Deprivation 8
1.5 The Effects of Lens Defocus 9
1.6 The Effects of High Intensity Light 11
1.7 The Effects of Muscarinic Antagonists 12

2. Methods 14

2.1 Subjects 14
2.2 General Protocols 14
2.3 A-scan ultrasonography 15
2.4 Refractive errors 16
2.5 Visual manipulations 16
2.6 Pharmacological manipulations 18
2.7 Data analysis 19

3. Results 21

3.1. Hyperopic Defocus 21
3.1.1. Effects on ocular dimensions and refractive error 21
3.1.2. Effects on rhythm in axial length and choroidal thickness 22
3.2. Form Deprivation 26
3.2.1. Effects on ocular dimensions and refractive error 26
3.2.2. Effects on rhythm in axial length and choroidal thickness 27
3.3. Muscarinic Antagonist – Pirenzepine 30
3.3.1. Effects on ocular dimensions and refractive error 30
3.3.2. Effects on rhythm in axial length and choroidal thickness 32
3.4. Bright Light 34
3.4.1. Effects on ocular dimensions and refractive error 34

4. Discussion and Conclusions 36

4.1. Brief periods of hyperopic defocus 36
4.1.1. Time-of-day influences on ocular growth 36
4.1.2. Implications of ocular diurnal rhythms 37
4.2. Brief periods of form deprivation 37
4.2.1. Time-of-day influences on ocular growth 38
4.2.2. Implications of ocular diurnal rhythms 39
4.3. Pirenzepine 40
4.3.1. Time-of-day influences on ocular growth 40
4.3.2. Implications of ocular diurnal rhythms 41
4.4. Brief periods of bright light exposure 41
4.4.1. Time-of-day influences on ocular growth 41
4.5. Conclusions 44

5. References 45

List of Figures

1. Diurnal rhythms of normal chick eyes. 7

2. Example of A-scan ultrasonography trace of an eye. 16

3. Effects of brief periods of hyperopic defocus on axial length, vitreous chamber depth, choroidal thickness, and refractive error. 23

4. Effects of brief periods of hyperopic defocus on ocular rhythms in axial length. 24

5. Effects of brief periods of hyperopic defocus on ocular rhythms in choroidal thickness. 25

6. Effects of brief periods of form deprivation on axial length, vitreous chamber depth, and refractive error. 27

7. Effects of brief periods of form deprivation on ocular rhythms of axial length. 28

8. Effects of brief periods of form deprivation on ocular rhythms of choroidal thickness. 29

9. Effects of low dose pirenzepine on axial length, vitreous chamber depth, choroidal thickness, and refractive error. 31

10. Effects of low dose pirenzepine on ocular rhythms in axial length. 33

11. Effects of low dose pirenzepine on ocular rhythms in choroidal thickness. 34

12. Effects of brief periods of bright light on axial length. 35

1. General Introduction

1.1 Myopia

Uncorrected myopia is the leading cause of visual impairment worldwide, affecting an estimated 1.89 billion of the global population (Holden et al. 2016). Myopia is a condition where incident light focuses anterior to the retina, resulting in image blur. This occurs when the emmetropization process fails to influence the appropriate amount of axial length growth to allow for incoming light to focus on the retina. Emmetropization is a homeostatic phenomenon where axial growth of the eye is stimulated by environmental cues in order to reach emmetropia, or a state where the eye has minimal to no refractive error (Wallman and Winawer 2004). The environment which is optimal for emmetropization has been hypothesized to involve various cues, and disruptions in this environment have been shown to lead to the development of refractive error, particularly myopia. Examples of disruptions of these visual cues include form deprivation (where visual information is severely restricted but light stimulus persists), extended amounts of light at night (indoor lighting or device screens late into the night), and lens defocus (optical stimulus that does not neutralize refractive error) (Nickla 2013). In animal models, experimental myopia can be reversed simply by removing the inducing factor, allowing the eyes to be exposed to a natural environment so that emmetropization can occur (Napper et al. 1997; Morgan, Ashby, and Nickla 2013).

In recent years, the necessity to control the onset and progression of myopia has increased drastically. The World Health Organization has reported that the myopic population is expected to increase to 2.56 billion people by 2020, and 4.76 billion people – more than 50% of the current global population – by 2050. Myopia, and in particular high myopia (defined as greater than 5 diopters (D)), is associated with an increased risk of sight-threatening comorbidities, such as cataracts, retinal detachments, primary open angle glaucoma, and choroidal neovascularization (Holden et al. 2016). The combination of increasing prevalence and associated risks of myopia raise the need to investigate different modalities to control or treat myopia.

One of the many theories which have been a focus of myopia control relates to the effects of peripheral hyperopic defocus (Berntsen, Mutti, and Zadnik 2010). This theory suggests that due to the shape of the myopic eye, even if light is focused on the foveal retina, the peripheral retina falls short of the foci of the peripheral rays leading to a visual feedback mechanism which stimulates ocular growth (Berntsen, Mutti, and Zadnik 2010). This may explain why single vision lenses are insufficient to prevent further myopic progression, as it only aligns the retina with optics for central vision while spherical optics in the periphery cause hyperopic defocus.

Several techniques have been employed to obtain a better understanding of the emmetropization process in the hopes of applying this knowledge to improve future treatment methods for myopia management. Hyperopic and myopic defocus, form

deprivation diffusers, pharmacological agents, as well as high intensity light environments have also been investigated. Newer approaches towards understanding refractive error development include genetics, sclera biosynthesis, the effects of retinal neurotransmitters, and the potential roles of the circadian rhythm (Chakraborty et al. 2018; Nickla 2013).

There are a variety of therapeutic treatments available today that aim to reduce the progression of myopia, but they are often accompanied by side effects or limited success. These treatments include optical control, such as spectacle correction or contact lenses, specialty contact lenses, such as orthokeratology and multifocal contact lenses, as well as pharmacological treatments, such as atropine (Holden et al. 2016). Optical corrective options such as bifocals have offered some success in managing myopia progression (Cheng et al. 2010, 2014), but its success may be compromised by compliance issues, often as a result of psychological and sociological factors. Orthokeratology (or ortho-k) involves a uniquely designed gas-permeable contact lens which temporarily molds the shape of the cornea to alter its refractive properties. Ortho-k has been shown to decrease myopia progression in children (Lee, Wang, and Chiu 2017) but treatment is challenged by compliance issues as well as increased risks associated with extended and overnight contact lens wear (Jun et al. 2018). Atropine is a well-known and well-studied pharmacological agent for the management of myopia progression, widely used in Asia. However, it is accompanied by side effects such as a cycloplegia, mydriasis, and rebound effect upon drug cessation (Pineles et al. 2017). Low dose atropine (0.01%) is currently used for myopia progression but it's effectiveness has not been as robust (Wu et al. 2019).

1.2 Animal Models of Myopia

Animal models play an integral role in the investigation and understanding of myopia. The major animal models for myopia include the tree shrew, guinea pig, rhesus monkey, and chicks (Nickla 2013). The eyes of chicks in particular hold numerous advantages in regards to their potential for myopia research. They require minimal time and effort to maintain, have relatively large eyes, and have sufficiently rapid developmental stages, making them most ideal for basic research (Wisely et al. 2017). The chick eye also develops postpartum in a similar fashion as the human eye – chicks are born with a hyperopic refractive error, and emmetropization occurs as the chick grows (J. Wallman, Adams, and Trachtman 1981). This process takes about 8 weeks to achieve emmetropia in chick eyes, and is equivalent to approximately the first 2 years of human life (J. Wallman, Adams, and Trachtman 1981).

Some disadvantages associated with the chick model do exist. Chick eyes do not have a fovea and the scleral composition contains more cartilage (Nickla 2013). The ciliary muscles for accommodation are striated and activated by nicotinic receptors, as opposed to humans which are composed of smooth muscle and muscarinic receptors (Nickla 2013).

Vision science research with chick eyes have led to many discoveries in uncovering the mysteries of refractive error development. Refractive error can be induced with the use of plus or minus lenses and the chick eye responds with hyperopia or myopia, respectively

(Schaeffel, Glasser, and Howland 1988). The choroidal thickness changes in response to defocus in order to move the retina towards clearer vision (J. Wallman et al. 1995). Pharmacological agents can be studied for their effects on refractive error development, such as dopaminergic and cholinergic agonists, antagonists, and retinal neurotransmitters (Nickla 2013).

1.3 The Role of Circadian Rhythms

Circadian rhythms are self-sufficient biological processes that cycle daily, synchronized to various external cues, namely the 24-hour light-dark cycle (Crnko et al. 2018; Weaver 1998). The suprachiasmatic nucleus in the hypothalamus is the master clock of the body, keeping individual circadian rhythms found throughout cells and organs in synchronicity (Mohawk and Takahashi 2011). Our bodily functions are driven by many circadian rhythms, such as core body temperature, cardiovascular activity, and gastrointestinal absorption (Hastings, Reddy, and Maywood 2003). Any disruptions in these circadian rhythms could have adverse systemic consequences, such as cardiovascular disease, obesity, psychiatric disorders, and cancer (Takahashi et al. 2008; Karatsoreos 2012; Zelinski, Deibel, and McDonald 2014).

Almost all cells in the human body contain endogenous circadian clocks of their own (Rosenwasser and Turek 2015; Yoo et al. 2004; Welsh et al. 2004). This includes the retina, such as the retinal pigment epithelium (RPE), ganglion cells, and photoreceptors where genes

that may encode the circadian rhythm have been found (Ruan et al. 2006; Tosini et al. 2007; Baba et al. 2010). Similar to other endogenous circadian rhythms that control our bodily functions, rhythms found in the retina persist even in the absence of the external cues (light) (Nickla, Wildsoet, and Troilo 2001).

Circadian rhythms which are synchronized to the light-dark cycle, meaning they oscillate with a period of 24 hours based on specific amount of light and dark cycle, are called diurnal rhythms (Rosenwasser and Turek 2015). Some circadian rhythms found in the retina are also entrained to the light-dark cycle via dopamine (Hwang et al. 2013; Baba, DeBruyne, and Tosini 2017). They govern various processes such as gene transcription and retinal structure changes (Golombek and Rosenstein 2010; Storch et al. 2007).

Diurnal patterns of axial length and choroidal thickness can be illustrated through sinusoidal wave fits of growth data of various time points within a 24-hour period (Nickla, Wildsoet, and Wallman 1998). Axial length is defined as the distance from the corneal surface to the posterior retina. Choroidal thickness is the width between the posterior retina to the anterior sclera. Since axial length and choroidal thickness exhibit diurnal rhythms, it is hypothesized that visual stimuli and pharmacological agents given at a specific time of day may disrupt these patterns.

Figure 1. Diurnal rhythms of normal chick eyes

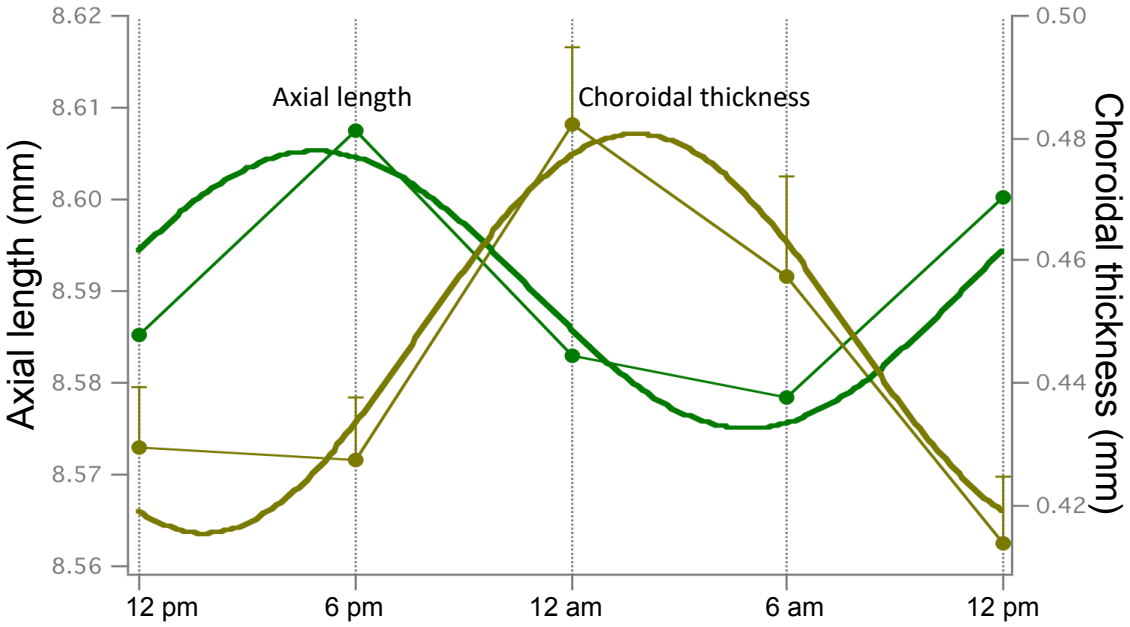


Figure 1. Illustration of normalized data of circadian rhythms in axial length (green) and choroidal thickness (brown) over a 24-hour period in normal chick eyes (image from Nickla, Wildsoet, and Wallman 1998).

Ocular dimensions such as axial length and choroidal thickness demonstrate diurnal patterns (Nickla 2006; Nickla, Wildsoet, and Wallman 1998)(Figure 1). Axial length of chick eyes were discovered to be longest in the afternoon and shortest near midnight (Nickla 2006; Weiss and Schaeffel 1993). Choroidal thickness changed in approximately the opposite pattern – thickest near midnight and thinnest in the afternoon ((Nickla 2006; Weiss and Schaeffel 1993; Nickla, Wildsoet, and Troilo 2001). Axial length and choroidal thickness of the human eye also exhibits similar patterns (Chakraborty, Read, and Collins 2011). The axial length of the human eye oscillates approximately 24-45 μm or 0.06-0.11 D (Chakraborty, Read, and Collins 2011; Wilson et al. 2006). The choroidal thickness of the

human eye oscillates approximately 30 μm or 0.18D (Chakraborty, Read, and Collins 2011; Usui et al. 2012).

The emmetropization process requires a normal light-dark cycle (Nickla 2013). When chick eyes were placed in a constant light or constant dark environment, thereby eliminating the normal cycle, excessive ocular growth and high amounts of experimental myopia were observed (Nickla, Wildsoet, and Wallman 1998; Jensen and Matson 1957). Furthermore, disruption of the light-dark cycle with a brief period of light during the dark period significantly altered the axial length diurnal rhythm with subsequent shift towards myopic refractive error (Nickla and Totonelly 2016). Other studies have also shown the effects on ocular dimensions and the development of myopia due to the shifts in phase of circadian rhythms (Nickla 2006; Nickla, Wildsoet, and Wallman 1998; Nickla et al. 2017; Papastergiou et al. 1998). With a better understanding of the effects of diurnal circadian rhythms on various ocular processes, we can use these rhythms to target specific times of day and contribute to myopia control.

1.4 The Effects of Form Deprivation

Form deprivation myopia is one of the earliest forms of experimental myopia. It involves placing a translucent diffuse lens over the eye, where the eye continues to receive light stimulus but spatial and temporal frequencies are severely reduced. Clinically, form deprivation myopia in children occurs due to conditions which degrade visual input, such as

congenital cataracts, congenital ptosis, or edematous cornea from congenital glaucoma (Meyer et al. 1999). Chick eyes subjected to form deprivation exhibit excessive ocular growth due to a loss of visual cues for growth inhibition at night – the eye normally shrinks at night in the light-dark cycle – suggesting an alteration in diurnal rhythms (Nickla 2006; Weiss and Schaeffel 1993). Here we hypothesize that the effects of brief periods of form deprivation will elicit different responses when given at specific times of day that is otherwise masked by constant periods of form deprivation.

1.5 The Effects of Lens Defocus

There are two types of lens defocus: myopic defocus and hyperopic defocus. In chicks, hyperopic defocus involves placing minus lenses over the eye, shifting the plane of focus posterior to the retina. This creates an image blur and the eye compensates by lengthening axially to bring the retina to the plane of focus. This effectively leads to a myopic shift in refractive error. In the case of myopic defocus, the plane of focus falls short of the retina, eliciting an inhibitory signal on ocular growth. This effectively leads to a hyperopic shift in refractive error.

Clinically, hyperopic defocus is found in daily near activities such as reading and studying and has been associated with myopic children (Mutti et al. 2002). Peripheral hyperopic defocus is induced with the use of single vision minus lenses in the myopic eye due to the oblate shape of the eye. Defocus lenses that effectively induce myopic defocus

have been used to counteract this effect to prevent myopia progression. Bifocal lenses (Cheng et al. 2010) and progressive lenses (Gwiazda et al. 2003) have been shown to be mildly effective against myopia progression (Huang et al. 2016). Contact lens methods such as orthokeratology (Cho and Cheung 2012; Chen, Cheung, and Cho 2013) and multifocal contact lenses (Walline et al. 2013) which induce 360 degrees of peripheral myopic defocus have also shown to be effective in the reduction of myopia.

Myopic defocus is generally understood as a robust form of stimulus to ocular dimension changes compared to hyperopic defocus. A brief period of exposure is enough to elicit a significant reduction in ocular growth (Schmid and Wildsoet 1996). In contrast, a full day of hyperopic defocus is required to elicit significant ocular growth (Zhu et al. 2005). Looking at specific time of day effects, brief periods of myopic defocus in the evening showed significant growth inhibition of the eye compared to exposures in the morning (Nickla et al. 2017). Furthermore, myopic defocus causes a phase delay in axial length rhythm and a phase advance in choroidal thickness rhythm, effectively bringing the two rhythms in phase (Nickla 2006). Hyperopic defocus on the other hand causes a shift in phase so the two rhythms are almost anti-phase (Nickla 2006). Here we hypothesize that a brief exposure to hyperopic defocus may also elicit different growth patterns when given at specific times of day compared to constant exposure.

1.6 The Effects of High Intensity Light

Since research suggests that more time outdoors can act as a preventative measure for myopia development, the effects of different amounts of daytime light intensity have been studied in children (Rose, Morgan, Ip, et al. 2008). Furthermore, time spent outdoors may be more protective than reducing near work (Jones et al. 2007; Rose, Morgan, Ip, et al. 2008; Rose, Morgan, Smith, et al. 2008), and it seems to be independent to physical activity (Rose, Morgan, Ip, et al. 2008; Dirani et al. 2009). It is generally accepted that increase in light exposure with increased outdoor activity is what leads to its protective effect against myopia (Rose, Morgan, Ip, et al. 2008). In form-deprived chick eyes, studies have shown that lower levels of light intensity reduced the rate of emmetropization compared to higher levels of light intensity, and reduced the amount of experimental myopia by eliciting a hyperopic shift in refractive error (Cohen et al. 2011; Smith, Hung, and Huang 2012). Another study suggested that higher levels of light intensity for a longer period of exposure time (greater than 2 hours) may provide more beneficial effects on myopia reduction (Backhouse, Collins, and Phillips 2013). One studied mechanism suggests that high intensity light has protective effects against myopia by acting on light-sensitive cells, increasing the release of dopamine (Chen et al. 2017). Although light appears to have a protective effect, constant light leads to hyperopia in chicks (Li et al. 1995) and constant dark leads to myopia (Gottlieb, Fugate-Wentzek, and Wallman 1987) further suggesting that duration of light exposure is critical. Here we hypothesize that longer periods of high intensity light at specific times of day will elicit a greater protective effect against myopia progression than shorter durations.

1.7 The Effects of Muscarinic Antagonists

Pharmacological methods have also been researched as potential therapies to reduce myopia progression. Dopamine is a neurotransmitter with many systemic functions, but it also plays an integral role in the diurnal circadian rhythm in the retina (Bloomfield and Völgyi 2009; Korshunov, Blakemore, and Trombley 2017). In both form-deprived eyes and hyperopic defocus-induced lens wear, it has been found in increased concentrations during periods of slow ocular growth and decreased concentrations in fast growing eyes (Stone et al. 1989; Pendrak et al. 1997). Dopaminergic agonists and antagonists have been researched, indicating a supportive role of D2 receptors and D1 receptors in ocular growth (Nickla, Totonelly, and Dhillon 2010).

Similar to dopamine, acetylcholine is also a retinal neurotransmitter and binds to 5 subtypes of muscarinic receptors found in different parts of the eye (Abrams et al. 2006). Data have suggested that muscarinic receptor subtypes, M1 and M4, play a significant role in experimentally-induced myopia (Arumugam and McBrien 2012).

It was previously thought that accommodation was involved in myopia development due to the correlation between increased near work and myopia. However, many studies in various animal models have shown that experimental myopia is induced without stimulating accommodation. For example, the ciliary muscle of chick eyes is controlled by nicotinic

receptors while the use of muscarinic antagonists can reduce myopia progression (McBrien, Moghaddam, and Reeder 1993).

Atropine, a nonselective muscarinic antagonist, has commonly been used as a pharmacological treatment for myopia control in school children in Asia. Low doses of topical atropine in humans have been shown to be most effective against myopia progression, however inconsistently, with minimal side effects and limited rebound after drug cessation (Tong et al. 2009; Chia et al. 2012; Wu et al. 2019). In experimental myopia of mammals, atropine has been shown to work against form-deprivation and lens-induced myopia (Ganesan and Wildsoet 2010).

Pirenzepine is a selective M1/M4 muscarinic antagonist and has been shown to be effective at reducing excessive ocular growth (Stone, Lin, and Laties 1991). Humans do not have M1R. Pirenzepine is an attractive pharmacological alternative to atropine due to the lack of side effects such as cycloplegia, dilation, and photophobia. Common side effects from topical pirenzepine use are papillae and follicles, mild dilation, and accommodative dysfunction, but for the most part was not a cause for discontinuation of pirenzepine (Siatkowski et al. 2008). Clinical trials have also shown that the use of 2% pirenzepine gel was effective at reducing myopia progression in children by 40- 50% (Siatkowski et al. 2008; Tan et al. 2005). The efficacy of pirenzepine has been variable in animal models. Here we hypothesize that pirenzepine administration at specific times of day may elicit a unique effect against excessive ocular growth.

2. Methods

2.1 Subjects

White Leghorn chickens (*Gallus gallus domesticus*) were hatched in an incubator and raised in temperature-controlled brooders. The light cycle was 12L/12D (hours of light and dark) for the pirenzepine and hyperopic defocus experiments. The light cycle was 14L/10D for the form deprivation and bright light experiment. Food and water were supplied *ad libitum*. Care and use of the animals conformed to the ARVO resolution for the Care and Use of Animals in Research.

2.2 General Protocols

In all experiments, the right eye of 12-day-old chicks was treated as the experimental eye and left eye was untreated as the control eye. -10 D lenses or diffusers were mounted on Velcro rings and attached to the matching ring which was glued onto the chicken's feathers around the right eye for all experiments. The fellow eye of each chick was used as a control and data was collected as interocular differences (experimental eye minus control fellow eye, X-C). Chicks were anesthetized by the inhalation of isoflurane to complete injections, refractions, and ultrasound measures (details on isoflurane preparation and administration were previously described by Nickla et al. 1998).

Ocular dimensions were measured with high frequency A-scan ultrasound at 12:00 on day 1, and again at 12:00 on the last day of the experiment. Subsets of chicks from each

experiment were measured at 6-hour intervals over 24 hours from day 5 to day 6 (12:00, 18:00, 0:00, 06:00, 12:00) to obtain parameters of diurnal rhythms in axial length and choroidal thickness (see below for group sizes).

2.3 A-scan ultrasonography

The ocular dimensions measured were defined as the following: i) axial length: anterior of cornea to posterior of retina (C1-R); ii) choroidal thickness: posterior of retina to anterior of sclera; iii) vitreous chamber: posterior of lens to anterior of retina. Chicks were anesthetized with inhalation of isoflurane and positioned such that the optical axis of the eye was perpendicular to the attached probe of the A-scan device in order to obtain optimal ultrasound peaks. A minimum of 8 measurements were collected per eye and the average allotted time to complete measurements was 5 minutes per bird (both eyes). The distance between peaks was analyzed and averaged to obtain the ocular dimensions for axial length, vitreous chamber depth, and choroidal thickness. These distances were recorded in micrometers (μm). An example of measured peaks can be seen in Figure 2.

For the subset of chicks involved in 6-hour interval measurements over 24 hours, overnight A-scan ultrasound measurements (0:00 and 06:00) were performed under photographic safe lights (red light which does not affect ocular growth).

Figure 2. Example of A-scan ultrasonography trace of an eye.

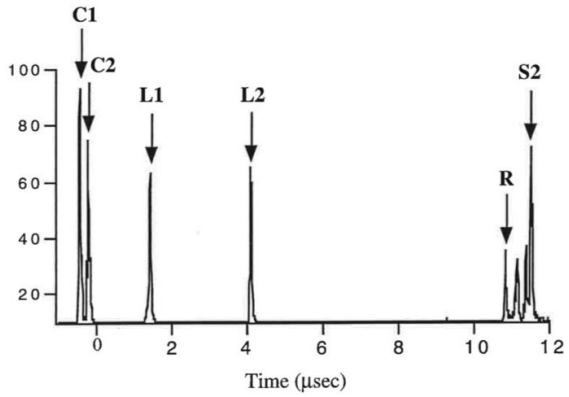


Figure 2. Example of an A-scan ultrasound trace with arrows indicating the detectable structures of the eye: C1, anterior cornea; C2, posterior cornea; L1, anterior lens; L2, posterior lens; R, retina; S2, sclera.

2.4 Refractive errors

Refractions were measured at the end of each experiment using a Hartinger refractometer. Chicks were anesthetized with inhalation of isoflurane and positioned such that the optical axis of the eye was perpendicular to the refractometer. The refractometer projects a series of horizontal and vertical mires which were adjusted until alignment was achieved. The resulting adjustment was recorded as the refractive error, in diopters (D). A minimum of 4 measurements were recorded at each axis (horizontal and vertical) and measurements were averaged to determine the refractive error of each eye.

2.5 Visual manipulations

2.5.1 Time-of-day effects of brief periods of hyperopic defocus on eye growth and rhythms

-10 D lenses were mounted on 12-day-old chicks for 2 hours of hyperopic defocus at morning (07:00-09:00, n=12), midday (12:00-14:00, n=12), or evening (19:00-21:00, n=6), for each of 5 days. Subsets of chicks were measured at 6-hour intervals (times noted above) from morning (n=8), midday (n=8), and evening (n=6) experimental groups.

2.5.2 Time-of-day effects of brief periods of form deprivation on eye growth and rhythms

Diffusers were mounted on 12-day-old chicks for 2 hours of form deprivation at morning (07:00-09:00, n=12), midday (12:00-14:00, n=19), or evening (19:00-21:00, n=6), for each of 5 days. Subsets of chicks were measured at 6-hour intervals (times noted above) from morning (n=6), midday (n=6), and evening (n=6) experimental groups.

2.5.3 Time-of-day effects of bright light on form deprived chick eyes

Diffusers were mounted on 12-day old chicks for the entire duration of the experiment of 6 days. For 3 or 5 hours a day, chicks were exposed to 24,000 lux of bright light at morning (07:00-10:00 n=6; 07:00-12:00 n=6), midday (13:00-16:00 n=5), or evening (18:00-21:00 n=6, 17:00-22;00 n=9). For control measures (n=12), chicks wore diffusers for 6 days without any bright light exposure. A subset of the chicks was then measured for diurnal rhythms: 3-hour midday (n=5). A-scan ultrasonography of this subset was measured on day 1 at 12pm, and five measures 6 hours apart starting at 12:00 on day 6 to 12:00 on day 7.

2.6 Pharmacological manipulations

2.6.1 Time-of-day effects of the muscarinic receptor antagonists, pirenzepine and atropine, on negative lens-induced myopia

-10 D lenses were mounted on 12-day-old chicks for the entire duration of the experiment. For each of 4 days, the lenses were temporarily removed and each bird received a single intravitreal injection of either pirenzepine or saline into the right eye, at morning (08:30), midday (14:00), or evening (20:30), under isoflurane inhalation anesthesia. Injections were administered using a Hamilton syringe with a 30G needle. Injections went through the skin of the lids over the superior temporal sclera, after removal of feathers and cleaning the skin with alcohol. The needle remained in place for 30 seconds before withdrawal while the skin around the site was held tightly together with small forceps to minimize leakage or bleeding. The -10 D lenses were then placed back onto the eye.

2.6.1.1 Pirenzepine

Two concentrations of pirenzepine were studied: 100mM (dose = 2 μmol; morning n=8, midday n=6, evening n=6, saline n=10) and 10mM (dose = 0.2 μmol; morning n=10, midday n=10, evening n=18). Saline injections (20 μL, 0.75%, n=9) were used as controls. Axial dimensions were measured using A-scan ultrasonography on day 1 at 08:30, and on day 5 at 12:00. Subsets of chicks were measured at 6-hour intervals (times noted above) from morning, midday (n=6), and evening (n=8).

2.7 Data analysis

2.7.1 Ocular dimensions and refractive errors

Ocular dimensions and refractive errors at each time-of-day were expressed as interocular differences, experimental minus control eyes, and paired t-tests were performed to analyze the interocular differences. One-way ANOVAs were performed to assess the between-group effects on experimental groups with post-hoc Bonferroni tests when ANOVAs demonstrated significant between-group differences.

2.7.2 Diurnal Rhythms

2.7.2.1 Axial length diurnal rhythms

Ocular growth rate was defined as the change in axial length over 6-hour intervals from 12:00 to 12:00 on the last day of each experiment. The diurnal rhythm of axial length, determined by the 6-hour interval ultrasonography measurements, was assessed in two ways. First, the change over each of the four 6-hour intervals was calculated ("interval" data). This included the "steady state" ocular growth rate which occurs over the 24-hour period measured – this is the slope of the longitudinal data, each 6-hour interval for each individual eye. Second, the "steady state" ocular growth was subtracted from the data for each individual chick eye, as described in Nickla et al. (Nickla et al. 1998). Data was normalized to the mean to control for large between-animal variability. These means were plotted as line graphs with standard error bars. These means were also fit to a sinusoidal curve within a fixed 24-hour period (Nickla et al. 1998).

Two-way ANOVAs were used to assess the time-of-day effects between groups for both methods of data analysis of diurnal rhythm of axial length. Post-hoc tests were completed between groups when the ANOVAs showed significant between-group differences.

2.7.2.2 Choroidal thickness diurnal rhythms

The diurnal rhythm of choroidal thickness was assessed by normalizing the data to the mean, to be consistent with axial length assessments. Because choroidal thickness does not demonstrate statistically significant change over time, and thus over the 24-hour period measured, regression data need not be subtracted from the data (Nickla et. al 1998). One- and two-way ANOVAs were utilized to assess time-of-day effects. Post-hoc tests were completed between groups when the ANOVAs showed significant between-group differences.

3. Results

3.1. Brief periods of hyperopic defocus

3.1.1. Effects on ocular dimensions and refractive error

Brief periods of hyperopic defocus had varying effects on ocular growth, stimulating growth in the morning and inhibiting growth at midday. 2 hours of hyperopic defocus using -10 D lenses stimulated axial length growth when given in the morning but inhibited growth when given at midday (Figure 3A, black bars; ANOVA $p = 0.003$; paired t-test: morning $p = 0.1$, midday $p = 0.0002$). Vitreous chamber growth was also stimulated in the morning but inhibited after midday exposure (Figure 3A, white bars; ANOVA $p < 0.0005$; morning X-C: 120 μm/5d, $p = 0.001$; midday X-C: -77 μm/5d, $p = 0.01$). Interocular differences (X-C) were statistically significant between the morning and midday groups in axial length and vitreous chamber changes (post-hoc Bonferroni $p < 0.001$ and $p = 0.0006$, respectively). There was no significant growth rate effect of 2 hours of hyperopic defocus on the evening group. Vitreous chamber growth was significantly reduced across the three experimental groups when compared to the full-lens control (Figure 3A, white bars; ANOVA $p = 0.00001$; control versus experimental: 278 μm versus 120 μm, -77 μm, and 42 μm/5d, respectively; paired t-test $p < 0.01$ for each). As for axial length, only the morning group was not statistically significant from the full-lens control.

There was an absence of choroid thinning exhibited by the midday group, which can be associated with the growth inhibition seen with axial length and vitreous chamber depth. Between-group differences were found from midday and evening groups (Figure 3B;

midday: 8 μm/5d, evening: – 100 μm/5d; $p < 0.005$), but there was no significant difference in choroidal thickness when comparing the morning and evening groups to the full lens control (morning: -46μm/5d, evening: -100μm/5d, control -158μm/5d; $p > 0.1$ for both).

The midday group exhibited a significant increase in hyperopia, attributed to the growth inhibition mentioned previously. (Figure 3C, X-C: 1.4 D, $p < 0.00001$); the full-lens control was significantly myopic. All three experimental groups were significantly more hyperopic when compared to the full-lens control, but a one-way ANOVA did not result in any between-group differences ($p > 0.1$).

3.1.2. Effects on rhythm in axial length and choroidal thickness

Ocular rhythms were studied by measuring ocular dimensions of axial length and choroidal thickness in 6-hour intervals over 24 hours on the last day of the experiment. The changes in axial length at each 6-hour interval did not show between-group differences across any experimental group (Figure 4A).

The mean values of axial length at each interval for each experimental group were fit into a sinusoidal function with a period of 24 hours (Figure 4B, red – morning, green – midday, blue – evening). Both midday and evening were well-fit into a sine wave. The mean rhythm phase of the midday group exhibited a significant phase-delay compared with the evening group, such that the acrophase (peak) of the midday group occurred later in time (8:45 pm versus 5:20 pm; $p < 0.05$). The mean phase data of the morning group could not be fit into a sinusoidal function.

Figure 3. Effects of brief periods of hyperopic defocus on axial length, vitreous chamber depth, choroidal thickness, and refractive error.

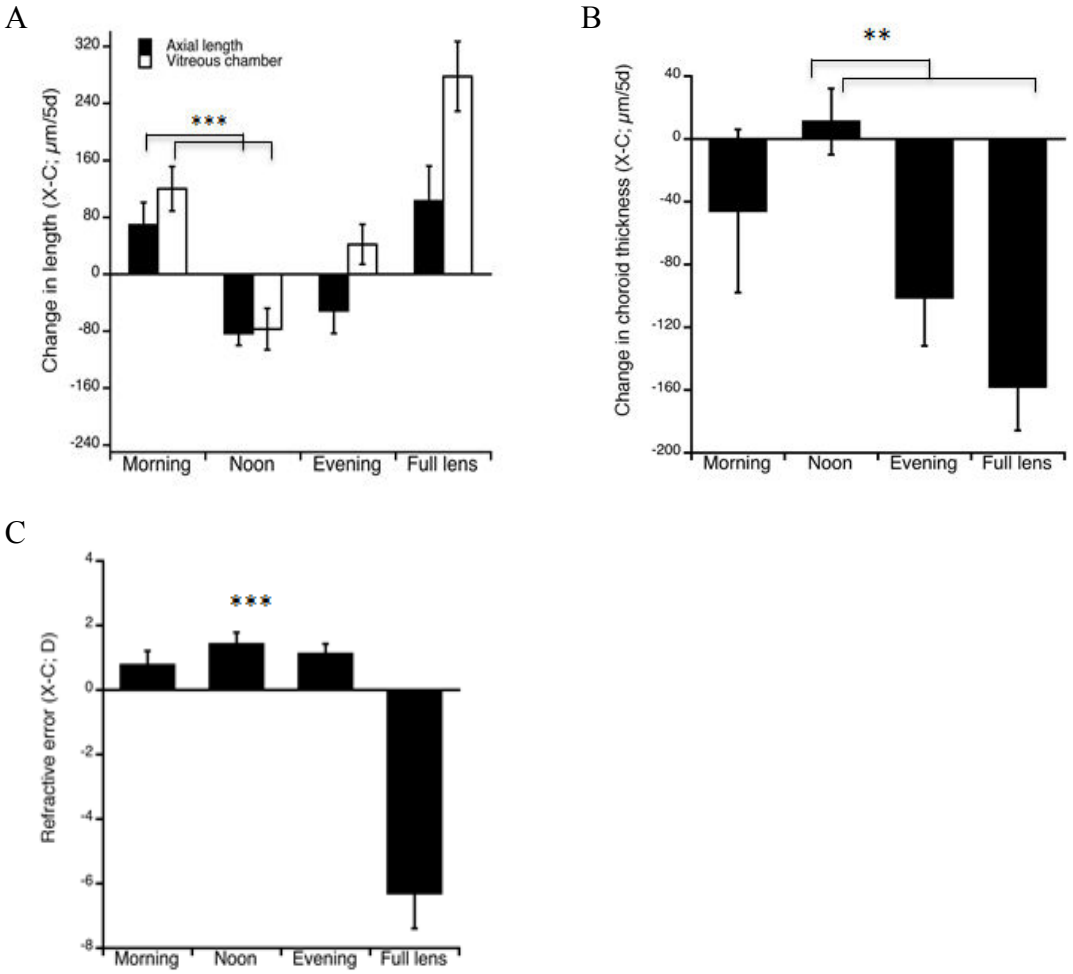


Figure 3. The effects of 2-hours of hyperopic defocus on ocular dimensions at different times of day. All data are interocular (X-C) differences. A) Effects on axial length (black bars) and vitreous chamber depth (white bars). B) Effects on choroidal thickness. C) End refractive error. Error bars in all graphs are sems. *$p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

An analysis of the amplitudes of each sine wave function for axial length changes did not yield any significant difference (Figure 4C, midday: 67 μm, evening: 91 μm; $p = 0.3$). Amplitudes derived from the maximum-minus-minimum method were not statistically significant (morning: 186 μm, midday: 155 μm, evening: 126 μm; ANOVA $p = 0.2$), nor

were there significant differences between the amplitudes of the experimental and control fellow eyes.

Figure 4. Effects of brief periods of hyperopic defocus on ocular rhythms in axial length.

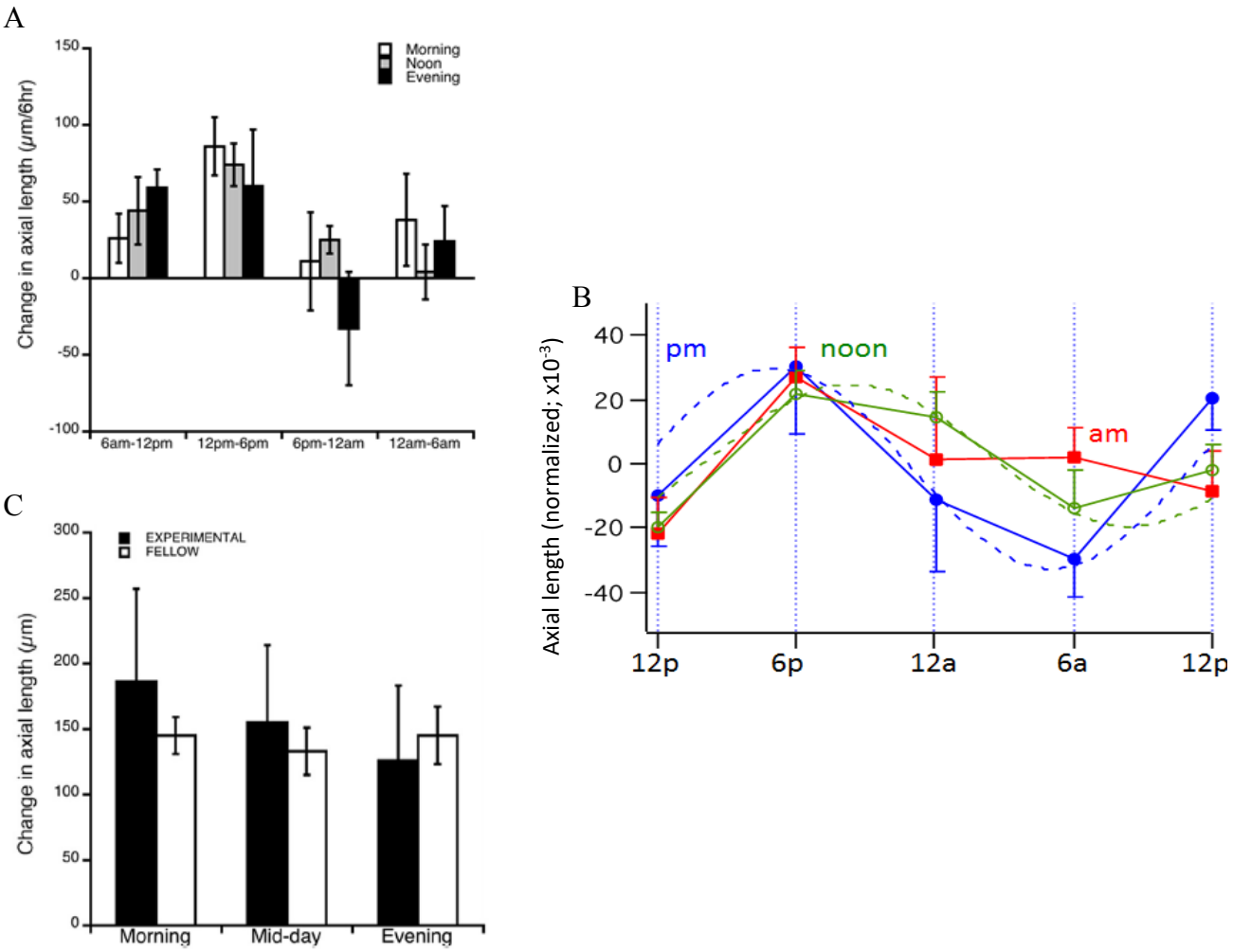


Figure 4. Effects of 2-hour hyperopic defocus on rhythms in axial length. A) Change in axial length at 6-hour intervals over a 24-hour period for all three groups. There are no significant between-group differences by ANOVA. B) Longitudinal data (length as function of time) normalized to the mean for all three groups (solid lines), and 24-hour sine wave fits to data (dotted lines). Note that the morning group ('am') cannot be well-fit to a sine function. C) Amplitude of the rhythms derived by maximum vs minimum for the experimental (black bars) and fellow control (white bars) eyes. Error bars in all graphs are sems.

Figure 5. Effects of brief periods of hyperopic defocus on ocular rhythms in choroidal thickness.

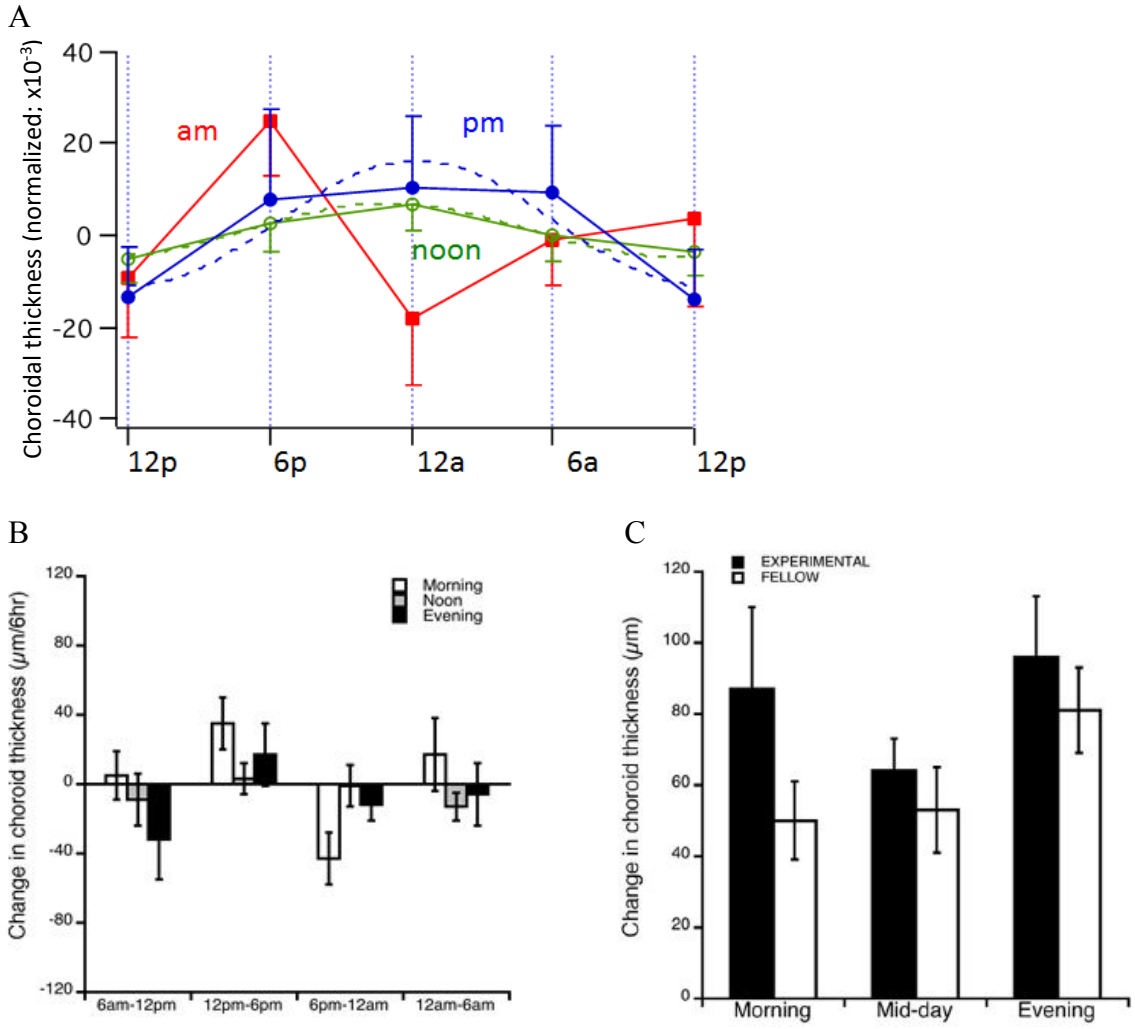


Figure 5. Effects of 2-hour hyperopic defocus on rhythms in choroidal thickness. A) Longitudinal data (length as function of time) normalized to the mean for all three groups (solid lines), and 24-hour sine wave fits to data (dotted lines). Note that the morning group ('am') cannot be well-fit to a sine function. B) Change in axial length at 6-hour intervals over a 24-hour period for all three groups. There are no significant between-group differences by ANOVA. C) Amplitude of the rhythms derived by maximum vs minimum for the experimental (black bars) and fellow control (white bars) eyes. Error bars in all graphs are sems.

Choroidal rhythm data were fit in a sinusoidal fashion (Figure 5A), however only the evening group exhibited a normal rhythm pattern (Figure 4B, mean peak: 12:12 am, mean amplitude: 27 μm). Mean choroidal rhythms for midday was well-fit into a sine wave function, but with minimal mean amplitude (11 μm). Mean wave function amplitudes were not statistically different across all groups (ANOVA $p > 0.1$), nor was there a difference between the amplitudes of the experimental and control fellow eyes (Figure 5C).

3.2. Brief periods of form deprivation

3.2.1. Effects on ocular dimensions and refractive error

Brief periods of form deprivation in the morning and midday led to ocular growth inhibition. 2 hours of form deprivation using diffusers inhibited the growth of axial length of the morning (X-C: -122 μm, paired t-test $p = 0.006$) and midday groups (X-C: -92 μm, $p = 0.016$) (Figure 6A, black bars). In addition, there was significant between-group differences of the morning and midday groups when compared to the evening group (ANOVA $p = 0.006$; post-hoc Bonferroni: morning versus evening $p = 0.006$, midday versus evening $p = 0.014$). There was not significant effect of form deprivation on the evening group. The change in vitreous chamber depth exhibited a similar trend, though without statistical significance (Figure 6A, white bars).

There was a significant increase in hyperopic refractive error from the morning and midday groups (Figure 6B, morning X-C: 1.8 D, midday X-C: 1.0 D; $p < 0.05$ for both) – this is attributed to the inhibition of axial length as mentioned. There were no statistical between-group differences in choroidal thickness (data not shown).

Figure 6. Effects of brief periods of form deprivation on axial length, vitreous chamber depth, and refractive error.

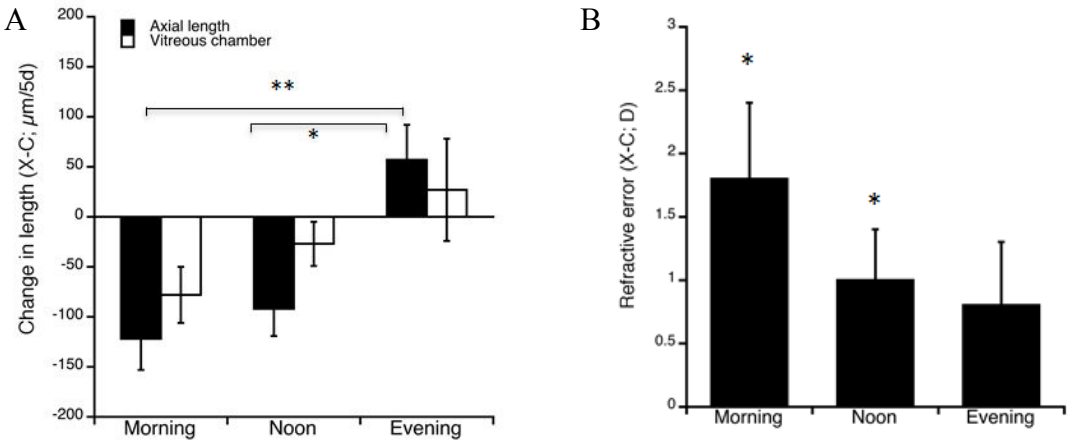


Figure 6. The effects of 2-hours of form deprivation on ocular dimensions at different times of day. All data are interocular (X-C) differences. A) Effects on axial length (black bars) and vitreous chamber depth (white bars). B) End refractive error. Error bars in all graphs are sems. *$p < 0.05$, ** $p < 0.01$.

3.2.2. Effects on rhythm in axial length and choroidal thickness

The mean values of axial length at each interval for each experimental group were fit into a sinusoidal function with a period of 24 hours (Figure 7A). There was no significant difference between the midday and evening group phase (midday peak: 2:35pm, evening peak: 3:35pm; $p > 0.1$). The morning mean rhythm was significantly phase-advanced compared to the midday and evening groups (morning peak: 10:00am, midday: 2:35pm, evening: 3:35pm; $p < 0.05$ for each). A 2-way ANOVA showed that time-of-day accounted for the differences in axial length rhythm between experimental groups (ANOVA $p = 0.0002$; interaction $p < 0.00001$). However, there were no significant differences between the 6-hour intervals (data not shown).

The morning group had a significantly greater amplitude compared to the evening group (Figure 6B, morning: 144 µm, evening: 90 µm; ANOVA p = 0.04; paired t-test p = 0.04). There was no significant difference between individual sine wave fits (ANOVA p = 0.4).

Figure 7. Effects of brief periods of form deprivation on ocular rhythms in axial length.

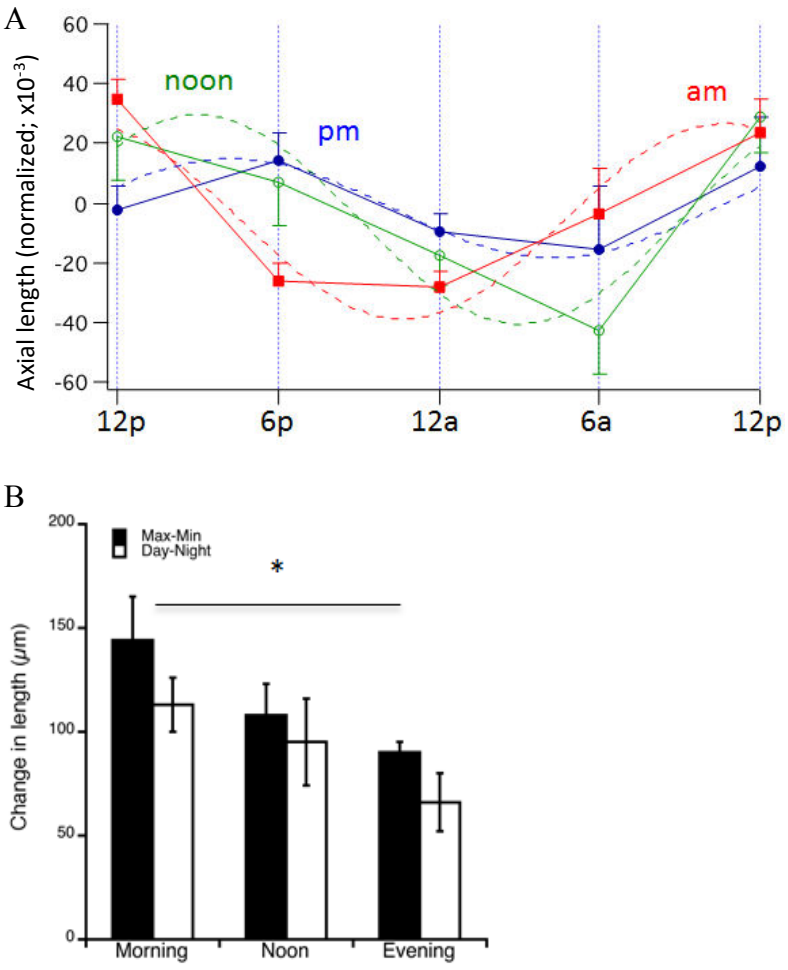


Figure 7. Effects of 2-hour form deprivation on rhythms in axial length. A) Longitudinal data (length as function of time) normalized to the mean for all three groups (solid lines), and 24-hour sine wave fits to data (dotted lines). B) Amplitude of the rhythms derived by maximum vs minimum for the experimental (black bars) and fellow control (white bars) eyes. Error bars in all graphs are sems. * $p < 0.05$.

Figure 8. Effects of brief periods of form deprivation on ocular rhythms in choroidal thickness.

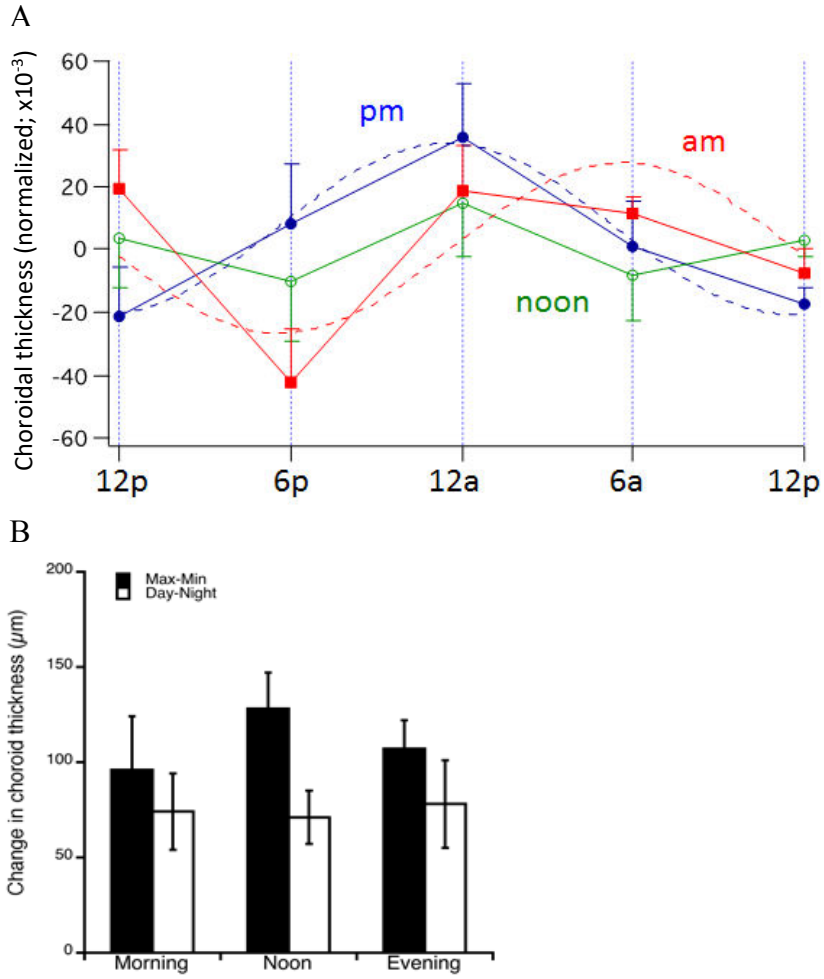


Figure 8. Effects of 2-hour form deprivation on rhythms in choroidal thickness. A) Longitudinal data (length as function of time) normalized to the mean for all three groups (solid lines), and 24-hour sine wave fits to data (dotted lines). B) Amplitude of the rhythms derived by maximum vs minimum for the experimental (black bars) and fellow control (white bars) eyes. Note that the midday group ('noon') cannot be well-fit to a sine function. Error bars in all graphs are sems.

Analysis of mean choroidal thickness rhythm showed that the evening group exhibited normal phase patterns, which is similar to the evening group exposed to 2 hours of hyperopic defocus (mean peak: 11:30pm, mean amplitude: 55 μm)(Figure 8A). The sine

wave of the morning group was not as well fit (mean peak: 6:00am, mean amplitude: 54 μm). The midday group mean rhythm could not be fit into a sinusoidal function, but rather it appeared to oscillate at 6-hour intervals.

There were no significant differences of amplitude between experimental groups or 6-hour intervals (2-way ANOVA $p > 0.1$). Experimental groups did not have any significant effect on the amplitude of the choroid rhythms (Figure 8B, morning, midday, evening: max vs min, black bars: 96 mm, 128 mm, 107 mm; ANOVA $p = 0.5$; day vs night, white bars: 74 mm, 71 mm, 78 mm; ANOVA $p = 0.9$).

3.3. Low dose muscarinic antagonist – pirenzepine

3.3.1. Effects on ocular dimensions and refractive error

Low dose of the partially selective muscarinic antagonist, pirenzepine, elicited a greater inhibitory effect on ocular growth at midday. With the dose of 100mM pirenzepine solution, the growth of the experimental eye was inhibited significantly compared to the control fellow eye (X-C: -83 μm; paired t-test $p = 0.027$). As such, we decided to continue the experiment with a lower dose of pirenzepine. A 10mM solution of pirenzepine elicited a partial effect when injected at either of the three times-of-day tested. Interocular differences were significantly different within each experimental group (experimental minus control, $p < 0.05$ for each); however, there were no statistical interocular differences when compared to the saline control group ($p > 0.05$ for each)(Figure 9A). Axial length growth rate of the midday group was significantly reduced compared to the evening group (Figure 9A, ANOVA $p = 0.032$; 79 μm versus 215 μm; post-hoc Bonferroni $p = 0.046$).

Figure 9. Effects of low dose pirenzepine on axial length, vitreous chamber depth, choroidal thickness, and refractive error.

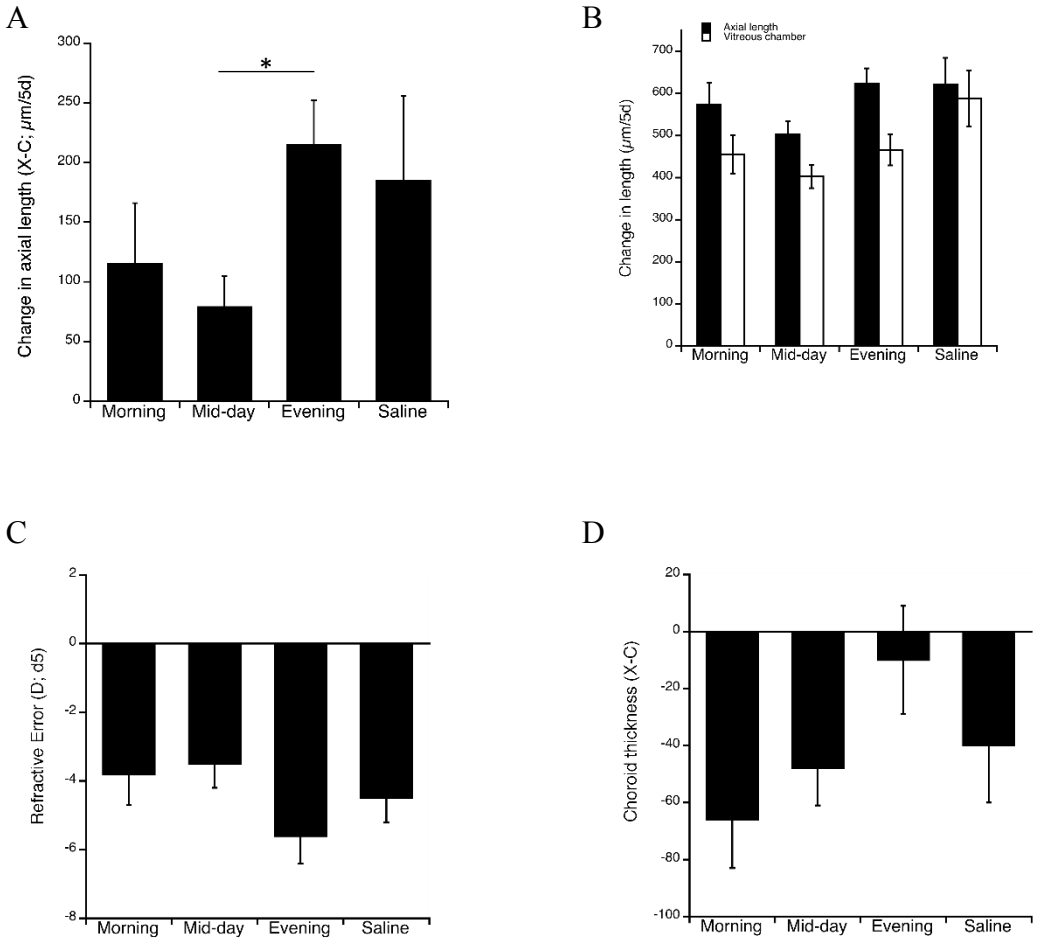


Figure 9. The effects of 10mM pirenzepine solution on ocular dimensions at different times of day. All data are interocular (X-C) differences. A) Effects on axial length (X-C) and saline controls. B) Change in axial length (black bars) and vitreous chamber depth (white bars). There were no between-group differences by ANOVA. C) End refractive error. There were no between-group differences by ANOVA. D) Effects on choroidal thickness (X-C) (ANOVA for experimental groups $p = 0.01$). Error bars in all graphs are sems. * $p < 0.05$.

Vitreous chamber depth was not significantly affected. There were no significant interocular differences (morning: 210 μm, midday: 210 μm, evening: 296 μm), nor between-group differences in growth rate (Figure 9B, white bars; ANOVA $p = 0.1$).

Refractive error was not significantly different across experimental and saline control groups (Figure 9C, ANOVA $p > 0.1$). There were no significant changes to choroidal thickness in any experimental group (Figure 9D, ANOVA $p > 0.1$).

3.3.2. Effects on rhythm in axial length and choroidal thickness

Axial length changes were measured at 6-hour intervals over a 24-hour period for the midday and evening groups. There were no significant effects between the 6-hour intervals or between experimental groups. The mean axial length rhythm was well-fit in a sinusoidal function for the midday and evening groups (Figure 10A). Data from a saline control group was also well-fit. There were no significant interactions between midday and evening groups (ANOVA $p > 0.1$). The morning group could not be fit into a sine wave model. The mean peak values were not statistically significant (midday: 4:10 pm, evening: 3:35pm, saline control: 2:45pm).

The axial length rhythm amplitudes were found to be lower than that of the saline control, however there were no significant differences of amplitude between midday, evening, and saline control groups (Figure 10B, 74μm, 67μm, and 110μm; ANOVA $p = 0.4$).

An analysis of choroidal thickness rhythms revealed no significant between-group effects at any 6-hour interval nor midday or evening groups. Longitudinal data of the mean of each group could not be well-fit into a sine wave function (Figure 11). There was also no

statistically significant between experimental groups and the saline control group (data not shown). Analysis of the mean amplitudes did not show significant differences (ANOVA $p > 0.5$, data not shown).

Figure 10. Effects of low dose pirenzepine on ocular rhythms in axial length.

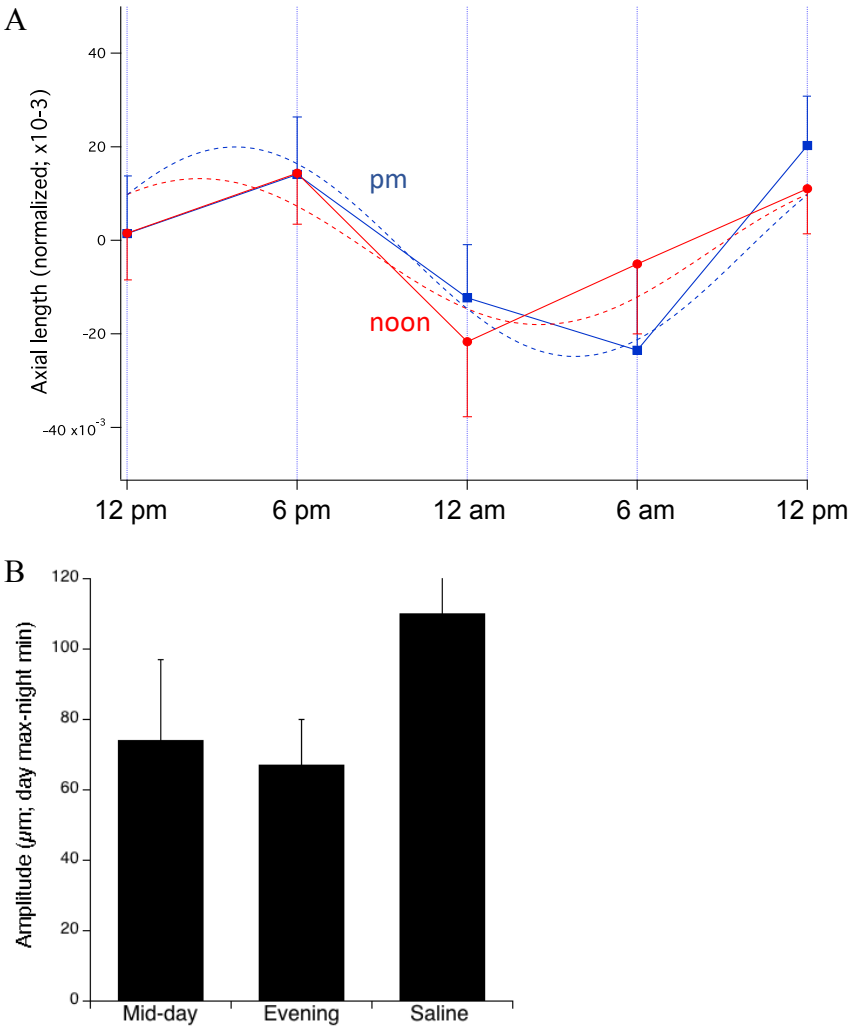


Figure 10. Effects of 10mM pirenzepine solution on rhythms in axial length. A) Longitudinal data (length as function of time) normalized to the mean for midday (red) and evening (blue) groups (solid lines), and 24-hour sine wave fits to data (dotted lines). B) Amplitude of the rhyhms derived by maximum vs minimum. Error bars in all graphs are sems. * $p < 0.05$

Figure 11. Effects of low dose pirenzepine on ocular rhythms in choroidal thickness.

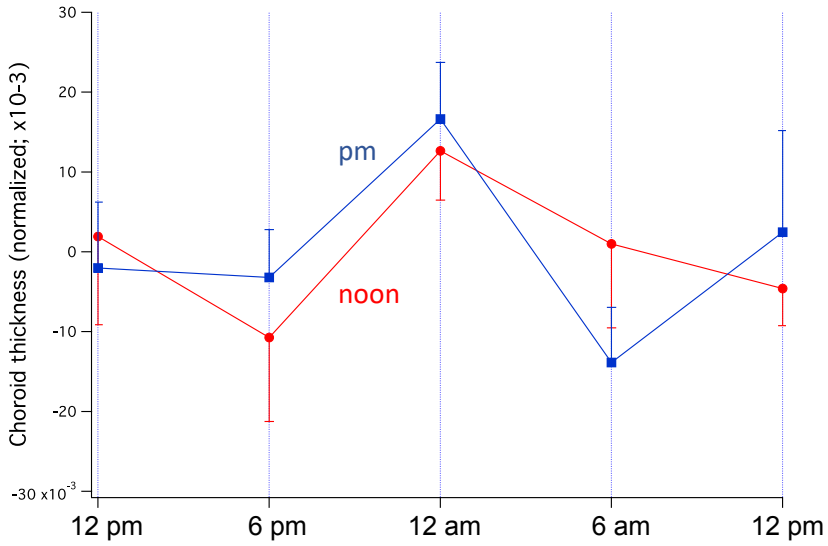


Figure 11. Effects of 10mM pirenzepine solution on rhythms in choroidal thickness. A) Longitudinal data (length as function of time) normalized to the mean for midday (red) and evening (blue) groups (solid lines), and 24-hour sine wave fits to data (dotted lines). Error bars in all graphs are sems.

3.4. Brief periods of bright light

3.4.1. Effects on ocular dimensions and refractive error

Brief periods of bright light in the evening were more effective at inhibiting ocular growth than in the morning. Data from both the 3-hour and 5-hour of bright light (20,000 lux) exposure showed no significant difference between each other. As such, morning group and evening group data were combined for further analysis. All experimental group and control groups were significantly different from the control fellow eye (experimental versus control, $p < 0.01$ for all). Axial length of the evening group was significantly different

compared to the morning group (X-C, morning: 519 µm, evening: 359 µm, control: 455 µm; ANOVA p = 0.039, post-hoc Bonferroni p = 0.036)(Figure 12).

Ocular rhythms were measured in 6-hour intervals over 24-hours, however data did not reveal any statistical significance between groups (data not shown).

Figure 12. Effects of brief periods of bright light on axial length.

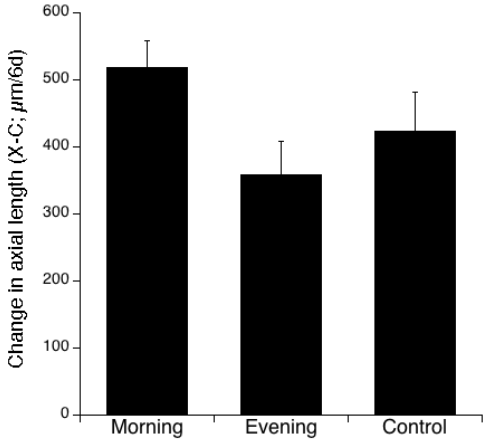


Figure 12. The effects of 3 and 5 hours (combined data) of bright light. A) Effects on axial length (X-C) and control. There were no between-group differences. B) Effects on axial length of the experimental (black bars) and fellow eye (white bars). Interocular differences were statistically significant. Error bars in all graphs are sems.

4. Discussion and Conclusions

4.1. Brief periods of hyperopic defocus

4.1.1. Time-of-day influences on ocular growth

By looking at the effects of hyperopic defocus at specific times of day, this study showed that ocular growth was stimulated in the morning, while midday exposure led to ocular growth inhibition. The midday group exhibited a lack of choroid thinning, and significant hyperopic change. It is generally understood that the growth response to full-time hyperopic defocus is both slow and less effective in reaching refractive compensation, while the effects of myopic defocus are faster and more effective (Schmid and Wildsoet 1996). However, in this study, by providing brief periods of hyperopic defocus, we reveal different effects on ocular growth depending on the time of day. Our data suggest that while long periods of hyperopic defocus leads to excessive ocular growth and myopia, brief periods have a protective effect specifically when exposed at midday. Schmid and Wildsoet (1996) found that while 9 or 12 hours of -10 D lens wear led to increase in axial length and vitreous chamber depth, with a subsequent myopic shift, 1 or 3 hours of -10 D lens wear led to a decrease in ocular dimensions. In their study, lens defocus was given in equal groups of the morning (7am) and evening (before 7pm). Their combined data of the effects of lens defocus showed a trend towards growth inhibition without statistical significance, while our study revealed the unique effects of time of day, with significant growth stimulation in the morning and significant growth inhibition at midday. Potential discrepancies between the studies could be associated with the different ages of chicks used (their chicks were younger with

lens-wear after hatching), different hours of lens wear, or less sensitivity in their ocular measurements.

4.1.2. Implications of ocular diurnal rhythms

Brief periods of hyperopic defocus appear to elicit unique responses in the axial length diurnal rhythm: a phase-delay in the midday group and an abnormal oscillation pattern in the morning group. Finally, evening exposure, which showed no significant growth changes to hyperopic defocus, did not elicit a significant difference in phase. Interesting patterns can be elucidated when these results are compared to brief periods of myopic defocus (Nickla et al. 2017). Both morning exposures of either 2-hours of hyperopic or myopic defocus showed an oscillating pattern to the wave-fit of their respective ocular rhythms, and ocular growth stimulation. Midday exposures of either lens defocus showed a phase-delay and a decrease in ocular growth. Lastly, the rhythms of either evening groups were not significantly affected, however, there was no effect on growth with hyperopic defocus, and there was growth inhibition with myopic defocus. 2 hours of light at midnight also exhibited a similar abnormal oscillation pattern in axial diurnal rhythms, along with growth stimulation (Nickla and Totonelly 2016).

4.2. Brief periods of form deprivation

Similar to hyperopic lens defocus, progression of form deprivation-induced myopia is susceptible to short periods of normal vision. While constant form deprivation over 2 weeks results in a refractive error of - 24 D, 30 minutes of normal vision (11.5 hours of diffuser

occlusion) is enough to significantly reduce ocular growth and myopia by more than half of the myopia with constant form deprivation (Napper et al. 1997). However, 2 hours of form deprivation in this study in the morning and midday resulted in growth inhibition, with an associated hyperopic shift seen in both groups.

4.2.1. Time-of-day influences on ocular growth

Brief periods of form deprivation reveal ocular growth inhibition when exposed in the morning or midday, which is contrary to the significant ocular growth produced by constant form deprivation. It has been generally accepted that form deprivation and hyperopic defocus operate around the same mechanisms, therefore the cause for the growth inhibition seen in both cases may be the same. Here we suggest that after -10 D lenses or diffuser are removed at the end of the midday treatment, it causes a brief period of myopic defocus effect as light foci are now anterior to the retina. The myopic defocus effect now at midday in our study may be the cause of the growth inhibition exhibited in the midday group. Nickla et al. (2017) found that brief periods myopic defocus at midday and in the evening were significantly more effective at inhibiting ocular growth compared to exposures in the morning. Myopic defocus has also to be shown to be effective at short periods of time (Schmid and Wildsoet 1996). Though myopic defocus is also effective in the evening, the chicks in this experiment were subjected to the dark cycle as soon as the lens or diffuser were removed for the evening groups. This would nullify the myopic defocus effect due to lens or diffuser removal. This, however, does not explain why the morning group of form deprivation also exhibited growth

inhibition. We suggest that the effect of myopic defocus experienced after morning form deprivation to be more significant compared to midday.

4.2.2. Implications of ocular diurnal rhythms

Brief periods of form deprivation produced a unique diurnal rhythm response in axial length of the morning group, but inconsistent responses in choroidal thickness diurnal rhythm. The morning axial length rhythm showed a phase-advance compared to the other two groups. The results gathered from the rhythms from both hyperopic defocus and form deprivation do not show any consistent effects associated with growth inhibition. Interestingly enough, midday hyperopic defocus showed a phase-delay and morning form deprivation showed a phase-advance while both groups exhibited ocular growth inhibition.

Only the choroidal diurnal rhythm of the evening group of form deprivation and hyperopic defocus appeared to have a normal rhythm pattern, with no effect on ocular growth. The morning and midday groups of form deprivation, both of which resulted in changes in ocular growth, exhibited a phase-delay and oscillation pattern in choroidal thickness rhythm, respectively. The morning and midday groups of hyperopic defocus showed an oscillation pattern as well. Conversely, myopic defocus did not significantly alter the choroid rhythms in any experimental group (Nickla et al. 2017). 2-hours of midnight light exposure also exhibited an oscillation pattern in choroid rhythm (Nickla and Totonelly 2016). These results suggest that hyperopic defocus or form deprivation is more detrimental to choroid growth than axial length.

These abnormal oscillation patterns and phase-delays seen in both choroidal thickness and axial length of visual penalizations and light cycle disruptions may be a consequence due to disruptions of other circadian rhythms (dopamine, acetylcholine, IOP to name a few). This further suggests that these ocular dimensions are affected by a complex system of other rhythms, and this will require further biochemical and even genetic studies to understand the mechanism behind ocular growth.

4.3. Pirenzepine

4.3.1. Time-of-day influences on ocular growth

Improving the effectiveness of low dose pirenzepine in ocular growth inhibition can be targeted when injections are administered at a specific time of day. So far, we have shown that the time-of-day of visual manipulations have an on ocular dimensions as well as their diurnal rhythms. Here, we found that pirenzepine injections were more effective administered at midday, and showed no effect in the evening. The evening group was comparable to the saline controls. A possible explanation for this is that the midday group was exposed to light before and after injection, while the evening group was exposed to 12 hours of darkness immediately after the injection. This could suggest that in order for pirenzepine to elicit an effect, it requires some amount of involvement of light. Similar findings were shown in Nickla et al. (2019) with quinpirole. However, a separate study showed that quinpirole was effective at inhibiting ocular growth when given during a dark period of 3 hours (McCarthy et al. 2007), but perhaps 3 hours was not long enough to counter the effects compared to 12 hours of darkness in the current study after evening injections. If

the mechanism for our results is unrelated to the presence or absence of light, it may be associated to the concurrent administration of the ligand and a phase of a particular ocular rhythm.

4.3.2. Implications of ocular diurnal rhythms

As the other arms of this study have shown, we expected time of day to have an influence on the ocular rhythms of injections of low dose pirenzepine, however, this was not the case. There was no significant effect on phase in any group nor a significant difference between the amplitudes of the midday (which was effective on ocular growth) and evening (not effective) groups. There was a lack of association between ocular growth rate and axial rhythm amplitude. This is consistent with previous lens defocus studies (Nickla et al. 2017) and in the hyperopic defocus experiments reported here.

Siatkowski et al. (2008) found that a 2% pirenzepine gel was effective in reducing myopia compared to placebo in humans, and with an acceptable adverse reaction profile. We suggest that further studies need to be conducted in order to understand the mechanism behind the growth inhibition effect seen at midday, as it may provide important clinical implications for the dose regimen of pharmacologic therapies against myopia progression.

4.4. Brief periods of bright light exposure

4.4.1. Time-of-day influences on ocular growth

Though the literature generally show that duration of light exposure is most important for myopia prevention, our data revealed a significant ocular growth inhibition with high

intensity light exposure in the midday (Rose et al. 2008). Time spent outdoors has for a long time been associated with myopia prevention. Ashby et al. (2009) showed that bright light from sunlight (30,000 lux) or laboratory settings (15,000 lux) was able to reduce the development of form deprivation myopia. Another study, they also found that 5 hours daily of high ambient illuminance (15,000 lux) from 10am to 3 pm (similar to our midday group) reduced the myopic shift from negative lens wear (-7 D), as well as a reduction in axial length (R. S. Ashby and Schaeffel 2010). This study, which had similar parameters of brightness and time-of-day, however showed a significant inhibitory effect with evening exposure of bright light. Although there is a discrepancy between the cited study's use of lens defocus while our study used diffusers, hyperopic defocus and form deprivation are thought to exhibit similar characteristics in time-of-day effects.

Backhouse et al. (2013) showed that 12 hours of bright light (2,000 lux) was more effective to reduce form deprivation myopia than 2 hours of 10,000 lux bright light at either morning, midday, or evening. Their study however did find a weak effect of myopia reduction in the midday group compared to evening group, as well as an increased amount of myopia of the morning and evening group compared to constant 2,000 lux. Though their data suggest a time-of-day effect unique from overall ambient light levels, our results did not find any significant between-group differences even though our illuminance was higher and exposure time was longer. The evening group in this study showed a significant decrease in axial length growth compared to morning, while their data showed an increased amount of myopia in the evening group. Ocular rhythms did not show any significant effects by time-

of-day. However, it is interesting to note that an increase in duration was more effective than increase in brightness for entraining the circadian rhythm in humans (Dewan et al. 2011).

Our data show that bright light exposure at a specific time of day elicits an interesting effect against axial length growth, while the conclusions of Backhouse et al. (2013) suggested that long periods of moderate light exposure are more beneficial to myopia prevention compared to brief periods (5 hours or less) of bright light at a specific time of day. Cohen et al. (2011) have also suggested that there is a minimum level of bright light exposure required to have a protective effect against myopia. It may be that the effects of bright light are seen with long term daily exposures as Chen et al. (2017) found that 6 hours of daily bright light exposure over 4 weeks did inhibit myopia induced by form deprivation in mice.

It has long been known that there is an association between light and increased dopamine. It has been shown that blood plasma levels of dopamine increase during the day and are lowest at night (Sowers and Vlachakis 1984; Megaw et al. 2006). Furthermore, the introduction of light at night leads to an increase of dopamine levels (Adachi, Nogi, and Ebihara 1998). Chen et al. (2017) have further shown that the effects of myopia reduction from bright light can be nullified by injecting dopamine agonist. If dopamine in the evening leads to myopia prevention, and light triggers release to dopamine, then that would suggest that light in the evening would also reduce myopia. The fact that dopamine and ocular dimensions follow a circadian rhythm, yet we have found inconclusive time-of-day effects of bright light, suggest a more complex mechanism between light exposure and subsequent dopamine release.

4.5. Conclusions

Our results from visual manipulations show that chick eyes are more susceptible to the effects of hyperopic blur in the morning compared to the midday. Interestingly, chick eyes were not only susceptible to form deprivation at midday, but in the morning as well. This suggests that periods of hyperopic blur, such as classroom or homework, would best be completed in the afternoon to reduce the risk of myopia progression. Our results also suggest that if environmental changes are used as potential therapy for myopia control, then it would be imperative to consider the effects of the time-of-day of such therapies as a contributing factor to their efficacies. However, although this may be the case for lens defocus or form deprivation, it is less convincingly so with regards to increased light exposure. While increased duration of ambient light appears to have a more protective effect than brief periods of bright light irrespective to time of day of exposure, our findings suggest otherwise. Further studies should be conducted in order to understand the mechanisms at a molecular level of the effect of time-of-day on ocular growth.

5. References

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