The role of bright light as a protective mechanism against the development of form-deprivation myopia in chickens

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Abstract

In normal ocular development, the axial length of our eye is matched to the refractive power of the cornea and lens in a process known as emmetropisation, so that light rays emanating from objects viewed at a distance are focused clearly on the retina, rather than in front or behind it, with the accommodative system at rest. However, in the ocular disorder myopia (short-sightedness), a form of ametropia (refractive error), there is a mismatch between the axial length of the eye and the refractive power of the cornea and lens, so that now objects viewed at a distance are focused in front of the retina, instead of on it, causing the image to appear blurred.

Myopia has become a major health concern due to the rapid increase in prevalence across the world, most notably in urban East Asia, where in places up to 80% of children are now myopic. Recent epidemiological studies have reported that children who spend greater amounts of time outdoors are less likely to develop myopia. It has been postulated that the protective effects of time outdoors could be driven by higher retinal levels of the light-driven neurohormone dopamine in response to the higher light intensities experienced outside. In agreement with this hypothesis, animal studies have shown that daily exposure to high light intensities can inhibit the development of experimental myopia with work in chicks showing a reduction in the development of form-deprivation myopia (FDM) by roughly 60% in those animals exposed daily to ~15,000 lux, compared to the myopic shift seen in animals reared under 500 lux. Following on from these findings, this study examined three major questions:

1. Does a correlation exist between the intensity of light in which animals are reared and the degree to which the development of FDM is prevented?

2. Can exposure to bright light prevent the progression of FDM, or can it only affect the onset of experimental myopia?

3. What are the biochemical pathways that underlie the protective effects of high light?

The findings of aim one demonstrate a strong logarithmic correlation between light intensity and the development of FDM (F= 4.96, df= 35, 290, p< 0.0005), with the higher the light intensity to which diffuser-fitted chicks were exposed (500 lux, 10,000 lux, 20,000 lux, 30,000 lux or 40,000 lux), the less relative myopic shift in refraction that was observed and the shorter the axial length of the eye. By 40,000 lux, FDM was abolished, with no statistically significant difference in refraction or axial length seen between the high light group and those values seen in contralateral control (p=0.09) or age-matched untreated eyes (p=0.09).
The findings of aim 2 showed that exposure to elevated light intensities (~40,000 lux), which were shown in aim 1 to abolish the development of FDM, can halt the progression of myopia. Specifically, animals in which FDM was initially induced by rearing chicks, with translucent diffusers, under illumination levels of 500 lux for a period of 3 days (Δ 4.89 D over 3 days), showed no further progression when switched to daily exposure to 40,000 lux for a following 7 days (Δ0.63 D over 7 days), with the animals showing an initial relative hyperopic shift before stabilising at around emmetropia.

The findings of aim 3 indicate a possible role for immediate early gene Egr-1 in lights protective effects, with the down-regulation in Egr-1 mRNA levels normally observed in response to diffuser-wear under 500 lux abolished by exposure to 40,000 lux.

Exposure to elevated light levels has a significant effect on the development of FDM over time, with the onset of FDM abolished by 40,000 lux. Moreover, exposure to 40,000 lux can halt further progression of FDM. Finally, the down-regulation in the mRNA levels of Egr-1 are abolished by daily exposure to 40,000 lux, giving further evidence that it may be directly involved in growth modulation.
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Table of Contents

Chapter 1: Introduction .......................................................................................................................... 1
  1.1 General overview .......................................................................................................................... 1
  1.2 Eye Anatomy ............................................................................................................................... 2
    1.2.1 Fibrous layer ....................................................................................................................... 2
    1.2.2 Vascular layer ..................................................................................................................... 2
    1.2.3 Neural layer ......................................................................................................................... 2
  1.3 Emmetropisation .......................................................................................................................... 5
  1.4 Myopia ........................................................................................................................................ 7
  1.5 Experimental Paradigms of Eye Growth ....................................................................................... 7
    1.5.1 Form-deprivation myopia ................................................................................................. 7
    1.5.2 Recovery from form-deprivation myopia ......................................................................... 8
    1.5.3 Negative lens-wear ............................................................................................................. 10
    1.5.4 Positive lens-wear .............................................................................................................. 10
  1.6 Considerations related to experimental paradigms .................................................................... 12
    1.6.1 Positive and negative lenses worn alternately ................................................................. 12
    1.6.2 Effect of normal vision during lens wear ......................................................................... 12
  1.7 Eye growth .................................................................................................................................. 13
    1.7.1 Ocular growth is visually guided ....................................................................................... 13
    1.7.2 Local mechanisms .............................................................................................................. 13
  1.8 Environmental influences on ocular growth – near work ............................................................ 14
  1.9 Environmental influences on ocular growth – time outdoors ...................................................... 15
    1.9.1 High light and the development of myopia ...................................................................... 16
  1.10 Changes in gene expression during recovery from form-deprivation myopia ......................... 19
    1.10.1 Dopamine ......................................................................................................................... 19
    1.10.2 Retinoic acid ..................................................................................................................... 20
    1.10.3 Glucagon .......................................................................................................................... 21
    1.10.4 Early growth response 1 (Egr-1) ..................................................................................... 21
    1.10.5 cFos ................................................................................................................................... 22
  1.11 Aims of the study ....................................................................................................................... 23

Chapter 2: Methods ............................................................................................................................... 25
  2.1 Animal housing ............................................................................................................................ 25
  2.2 Experimental conditions .............................................................................................................. 25
    2.2.1 Induction of form-deprivation myopia (FDM) ................................................................. 25
    2.2.2 Lighting conditions ............................................................................................................ 25
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3 Measurement of ocular parameters</td>
<td>30</td>
</tr>
<tr>
<td>2.4 Tissue preparation</td>
<td>32</td>
</tr>
<tr>
<td>2.5 Preparation of total RNA and reverse transcription to cDNA</td>
<td>32</td>
</tr>
<tr>
<td>2.6 Real time RT-PCR</td>
<td>33</td>
</tr>
<tr>
<td>2.7 Primer specificity</td>
<td>33</td>
</tr>
<tr>
<td>2.8 Statistical analysis</td>
<td>39</td>
</tr>
<tr>
<td>Chapter 3: Results</td>
<td>40</td>
</tr>
<tr>
<td>3.1 Changes in refraction over time in response to exposure to different light intensities</td>
<td>40</td>
</tr>
<tr>
<td>3.1.1 Refractive changes in age-matched control eyes over seven days in response to different light intensities</td>
<td>40</td>
</tr>
<tr>
<td>3.1.2 Refractive changes in contralateral control eyes over seven days in response to different light intensities</td>
<td>40</td>
</tr>
<tr>
<td>3.1.3 Refractive changes in diffuser-treated eyes over seven days in response to different light intensities</td>
<td>41</td>
</tr>
<tr>
<td>3.2 Changes in axial length over time in response to different light intensities</td>
<td>49</td>
</tr>
<tr>
<td>3.2.1 Axial length changes in age-matched control and contralateral control eyes over six days in response to different light intensities</td>
<td>49</td>
</tr>
<tr>
<td>3.2.2 Axial length changes in diffuser-treated eyes over six days in response to different light intensities</td>
<td>49</td>
</tr>
<tr>
<td>3.3 Changes in refraction over time in response to transfer to high light</td>
<td>53</td>
</tr>
<tr>
<td>3.3.1 Refractive changes in contralateral control eyes over ten days in response to transfer to high light</td>
<td>53</td>
</tr>
<tr>
<td>3.3.2 Refractive changes in diffuser-treated eyes over ten days in response to transfer to high light</td>
<td>53</td>
</tr>
<tr>
<td>3.4 Changes in axial length over time in response to transfer to high light</td>
<td>57</td>
</tr>
<tr>
<td>3.4.1 Axial length changes in contralateral control eyes over nine days in response to transfer to high light</td>
<td>57</td>
</tr>
<tr>
<td>3.4.2 Axial length changes in diffuser-treated eyes over nine days in response to transfer to high light</td>
<td>57</td>
</tr>
<tr>
<td>3.5 Molecular changes following one day of form-deprivation myopia in 40,000 lux</td>
<td>59</td>
</tr>
<tr>
<td>Chapter 4: Discussion</td>
<td>61</td>
</tr>
<tr>
<td>4.1 Exposure to high light intensities and the development of deprivation-myopia</td>
<td>61</td>
</tr>
<tr>
<td>4.1.1 The effect of high light on lens-compensation</td>
<td>64</td>
</tr>
<tr>
<td>4.1.2 Possible mechanisms underlying the protective effects of time outdoors</td>
<td>65</td>
</tr>
<tr>
<td>4.1.3 What are the relevant light intensities required to prevent deprivation-myopia?</td>
<td>66</td>
</tr>
<tr>
<td>4.1.4 The role of light intensity in human myopia</td>
<td>67</td>
</tr>
<tr>
<td>4.2 Once myopic, can progression be halted by exposure to elevated light intensities?</td>
<td>68</td>
</tr>
</tbody>
</table>
4.3 What biochemical pathways underlie the protective effects of high light?............. 69

4.3.1 Dopamine and tyrosine hydroxylase (TH) .......................................................... 69

4.3.2 Early growth response 1 (Egr-1) .................................................................... 70

4.3.3 FBJ osteosarcoma oncogene (cFos) .............................................................. 71

4.3.4 Glucagon ........................................................................................................ 71

4.3.5 Retinoic acid (RA) ....................................................................................... 72

Conclusion ............................................................................................................... 73

References ........................................................................................................... 74
List of Tables

Table 1. Primer information.................................................................................................................. 37
Table 2. Primer efficiency ....................................................................................................................... 38
Table 3. Refraction data for age-matched untreated control eyes for all light intensities .......... 43
Table 4. Refraction data for diffuser-treated and contralateral control eyes for all light intensities.............................................................................................................................................. 43
Table 5. Axial length data for age-matched untreated control eyes for all light intensities ...... 50
Table 6. Axial length data for diffuser-treated and contralateral control eyes for all light intensities.............................................................................................................................................. 50
Table 7. Changes in refraction for form-deprived animals exposed to 500 lux, 40,000 lux or 500/40,000 lux ............................................................................................................................................... 55
Table 8. Changes in axial length for form-deprived animals exposed to 500 lux, 40,000 lux or 500/40,000 lux ............................................................................................................................................... 58
List of Figures

Figure 1. Morphology of a vertebrate retina ............................................................. 4
Figure 2. Myopic and Hyperopic defocus ................................................................. 6
Figure 3. Growth paradigms of FDM and recovery from FDM ............................... 9
Figure 4. The effect of positive and negative lenses on ocular growth .................. 11
Figure 5. Daily treatment cycle ........................................................................... 27
Figure 6. Experimental design for attachment of translucent diffusers and retinal tissue collection .................................................................................. 29
Figure 7. Image obtained by A-scan ultrasonography ........................................... 31
Figure 8. RT-PCR melt (dissociation) curves analysis for the primer pairs of TH, β-actin and HPRT .................................................................................. 35
Figure 9. RT-PCR melt (dissociation) curves analysis for the primer pairs of RAR-β, PPG, Egr-1, DRD2 and cFos ..................................................................... 36
Figure 10. Gel electrophoresis for all amplified gene products ................................ 37
Figure 11. Primer efficiencies for DRD2, Egr-1, RAR-β, cFos, PPG and TH ............. 38
Figure 12. Change in refraction, over seven days, in age-matched untreated control eyes reared under different light intensities .............................................. 44
Figure 13. Change in refraction, over seven days, in contralateral control eyes reared under different light intensities ............................................................ 45
Figure 14. Change in refraction, over seven days, in form-deprived eyes reared under different light intensities ................................................................. 46
Figure 15. Combined refractive data, over seven days, from form-deprived eyes reared under different light intensities ......................................................... 47
Figure 16. Logarithmic non-linear regression curve of light intensity versus absolute change in refraction over seven days ......................................................... 48
Figure 17. Changes in axial length for age-matched untreated (A) and contralateral control (B) eyes over six days under different light intensities ......................... 51
Figure 18. Changes in axial length for diffuser-treated eyes over six days under different light intensities ................................................................. 52
Figure 19. Changes in refraction, over time, for form-deprived animals exposed to 500 lux, 40,000 lux or 500/40,000 lux ........................................................................ 56
Figure 20. Changes in axial length, over time, for form-deprived animals exposed to 500 lux, 40,000 lux or 500/40,000 lux ................................................................. 58
Figure 21. Mean normalised expressions in fold change in mRNA levels of primers ...... 60


Chapter 1: Introduction

1.1 General overview

Myopia, or short sightedness, is a visual disorder in which a mismatch between the optical powers of the eye and its axial length, due most commonly to excessive elongation of the eye during development, causes objects viewed at a distance to appear blurred as they are focused in front of the retina rather than on it. A rapid increase in myopia prevalence has been observed, most notably in urban East Asia, where in parts greater than 80% of children are myopic (Goh and Lam 1994, Matsumura and Hirai 1999, Wu, Seet et al. 2001, Lin, Shih et al. 2004, Qian, Chu et al. 2009, Shih, Chiang et al. 2009, Morgan, Ohno-Matsui et al. 2012, Jung, Lee et al. 2012 ). Recently, a number of critical epidemiological studies have reported a strong negative correlation between time spent outdoors and the development of myopia, with those children who spend greater amounts of time outdoors less likely to become myopic (Mutti, Mitchell et al. 2002, Jones, Sinnott et al. 2007, Rose, Morgan et al. 2008, Rose, Morgan et al. 2008, Dirani, Tong et al. 2009, Jones-Jordan, Mitchell et al. 2011, French, Morgan et al. 2013). Rose et al. (2008) have postulated that this protective effect may be driven by light-stimulated dopamine release from the retina associated with the higher illumination levels experienced outdoors. In support of this hypothesis, animal studies have shown that the development of experimental myopia can be retarded by daily exposure to high illumination levels (>15,000 lux), with animals reared under high illumination levels showing a less myopic refraction and shorter eyes compared to those animals reared under normal laboratory illumination levels (~500 lux) (Ashby, Ohlendorf et al. 2009, Ashby and Schaeffel 2010, Siegwart, Ward et al. 2012). Furthermore, the protective effects of bright light against the development of form-deprivation myopia (FDM) can be abolished by the administration of a dopamine D2 receptor antagonist, spiperone, suggesting that the protective effects of bright light are, to some extent, driven by retinal dopamine (Ashby and Schaeffel 2010).

In chickens, exposure to 15,000 lux for a period of five hours per-day retards the development of FDM by roughly 60% (Ashby, Ohlendorf et al. 2009). This study will examine whether greater protection is afforded with higher light intensities (ranging from 10,000 – 40,000 lux). Furthermore, this study will determine whether high light levels are only effective at preventing/retarding the onset of experimental myopia, or whether they can affect the progression of FDM. Finally, this study will begin to elucidate the possible biochemical pathways underlying the protective effects of bright light, by investigating the role of
candidate systems previously postulated to play a role in the regulation of ocular growth, including: dopamine, Egr-1, cFos, glucagon, and retinoic acid.

1.2 Eye Anatomy

The eyeball sits within a bony socket (orbit) of the skull. The wall of the eye is composed of three main layers: outer fibrous layer (sclera), intermediate vascular layer (choroid) and inner neural layer (retina).

1.2.1 Fibrous layer

The fibrous layer is the outer-most layer of the eye and consists of the sclera and cornea. The white opaque sclera is a fibrous shell of collagenous, fibroblast maintained connective tissue, and is composed of different collagen fibrils, glycosaminoglycans (GAG) and matrix proteins (McBrien 2003). The sclera provides protection to the delicate intraocular structures beneath, and provides a stable attachment site for muscles involved in accommodation and eye movement. In addition, the sclera facilitates vascular and neural access to underlying intraocular structures, allowing the optic nerve to exit the eye and major vessels to enter (Kandel, Schwartz et al. 2000, McBrien and Gentle 2003). In other species, such as chickens, the sclera contains both a fibrous and cartilaginous layer (Kusakari, Sato et al. 1997).

Anteriorly, the sclera is attached with the cornea and forms a closed shell around the eye (McBrien and Gentle 2003). The cornea is a transparent collagenous tissue that allows light to enter the eye and forms its major refractive surface accounting for two thirds of the optical power (Kandel, Schwartz et al. 2000).

1.2.2 Vascular layer

The choroid, together with the ciliary body and the iris forms the uvea, or the intermediate vascular layer. The choroid contains an extensive vascular and lymphatic network that supplies nourishment and oxygen for the retina and retinal pigment epithelium (RPE) (De Stefano and Mugnaini 1997).

1.2.3 Neural layer

The neural layer is the inner-most layer of the eye and consists of the retina and a thin outer pigment epithelium (Bear, Connors et al. 2007). The retina contains five major neuronal cell types, the light-responsive rod and cone photoreceptors, bipolar cells, horizontal cells, amacrine cells and ganglion cells. Cones are responsible for day vision, while rods mediate night vision as they are extremely sensitive to light. Photoreceptors make direct contact with bipolar cells that in turn directly contact ganglion cells (the most direct pathway through the
Horizontal cells do not make direct contact with bipolar cells; instead, they synapse on photoreceptors and are electrically coupled with one another through gap junctions, laterally influencing bipolar cells (Bear, Connors et al. 2007). Amacrine cells are the most diverse neuronal cell type of the retina based on morphology, size, retinal coverage and neurochemical makeup, with between 30 to 40 distinct types postulated (Kandel, Schwartz et al. 2000, Bear, Connors et al. 2007). Amacrine cells receive input from bipolar cells, as well as other amacrine cells, and project laterally to influence surrounding ganglion, bipolar and amacrine cells (Kandel, Schwartz et al. 2000, Bear, Connors et al. 2007) (figure 1.1). Ganglion cells are the only source of output from the retina and fire action potentials in response to light (Bear, Connors et al. 2007). Axons of ganglion cells form the optic nerve, which projects towards the visual cortex within the brain, via the lateral geniculate nucleus (Bear, Connors et al. 2007).

The RPE lies between the photoreceptors and vascular choroid, providing nutrients and maintenance to the photoreceptors, while also acting as a barrier to ions and most molecules. The RPE contains black pigment, composed of melanin, which absorbs stray light, therefore preventing image degradation (Kandel, Schwartz et al. 2000, Bear, Connors et al. 2007).

The retina is composed of five distinct layers. Three of these layers contain the neural cell bodies (the outer nuclear layer, inner nuclear layer and ganglion cell layer), while the remaining two layers, the outer plexiform layer and the inner plexiform layer, contain the cell processes and synapses that occur between the different neuronal cells (figure 1.1). The outer most layer (outer nuclear layer), which abuts the RPE, contains the cell bodies of the photoreceptor cells. The inner nuclear layer contains cell bodies of bipolar, horizontal and amacrine cells, while the ganglion cell layer, as the name suggests, contains the ganglion cell bodies. The retina contains two intermediate layers, the outer plexiform layer (positioned between outer and inner nuclear layers) where photoreceptors make synaptic contact with the bipolar and horizontal cells, and the inner plexiform layer (located between the inner nuclear layer and ganglion cell layer), where synaptic contact is made between bipolar, amacrine and ganglion cells (Bear, Connors et al. 2007).

The most direct pathway from the eye to the brain is from the photoreceptors to the ganglion cells via bipolar cells. Light entering the neural tissue must first pass through all layers of the retina before reaching the photoreceptors.
The outer nuclear layer, inner nuclear layer and ganglion cell layer, contain the neural cell bodies while the outer plexiform layer and the inner plexiform layer, contain the cell processes and synapses that occur between the different cell types. The pigment epithelium lies between the photoreceptors and the choroid, providing nutrients to the photoreceptors. Light must pass through all layers of the retina before reaching the photoreceptors. From there the most direct pathway from the eye to the brain is from the photoreceptors to the ganglion cells, via the bipolar cells, where the signal is propagated along the optic nerve towards the higher visual centres in the brain.

(Ashby 2007)
1.3 Emmetropisation

During development, the axial length of the eye is slowly matched to the optical power of the cornea and lens in a process referred to as emmetropisation (Wallman, Adams et al. 1981). In an emmetropic eye, light rays emanating from an object viewed at a distance are focused clearly on the retina when the accommodative system is at rest, resulting in perfect vision (no refractive error, figure 1.2 A), if one discounts optical aberrations. An eye in which the focal plane and the retina are misaligned due to a mismatch between the axial length and optic power is referred to as being ametropic, with the two most common forms being hyperopia and myopia. Hyperopia (long-sightedness) refers to the refractive condition in which the axial length of the eye is too short for the optical powers, causing the focal plane to fall behind the retina. Most commonly, this is due to the rate of axial elongation being too slow, rather than an excessive decrease in optical power (figure 1.2 C). Conversely, myopia (short-sightedness) refers to a refractive condition in which the axial length of the eye is too long for the optical powers, causing the focal plane to fall in front of the retina (figure 1.2 B). Again, this is commonly associated with excessive axial elongation rather than alterations in the optical power of the eye. Humans, like most other mammals, are, for the most part, born hyperopic. However, during development, the eye slowly moves towards emmetropia (Morgan 2003, Zadnik, Mutti et al. 2004, Cohen, Belkin et al. 2011).

During development, emmetropisation is actively regulated by vision-dependent processes, so that the rate of eye growth is continuously adjusted to match the natural reduction in optical power seen during development (Brown, Koretz et al. 1999, Morgan 2003) (figure 1.2 A). Emmetropia is maintained by matching the rate of lens thinning and corneal flattening with the rate of elongation of the eye during normal development (Brown, Koretz et al. 1999, Zadnik, Mutti et al. 2004, Mutti, Mitchell et al. 2005). If however, the eye reaches a length to which the lens cannot further thin, it will result in a mismatch between the axial length of the eye and the optical powers of the eye (ametropia), therefore leading to the development of myopia (Wildsoet and Wallman 1995) (figure 1.2 B).
(A) Emmetropia. The axial length of the eye is matched to the focal length of its optical powers.

(B) Myopia. The axial length of the eye is too long compared to the optical powers and therefore images of objects viewed at a distance fall in front of the retina, instead of on it.

(C) Hyperopia. The axial length of the eye is too short for its optical powers and therefore images of objects viewed at a distance fall behind the retina, instead of on it.

(Figure adjusted from Ashby 2007)
1.4 Myopia

Myopia has emerged as a major public health concern due to the rapid increase in prevalence rates across the world, most notably in urban East Asia (Morgan, Ohno-Matsui et al. 2012), more specifically Singapore (Wu, Seet et al. 2001), China (He, Zeng et al. 2004, Qian, Chu et al. 2009), Hong-Kong (Goh and Lam 1994), Taiwan (Lin, Shih et al. 2004, Shih, Chiang et al. 2009), Japan (Matsumura and Hirai 1999) and Korea (Jung, Lee et al. 2012), where between 80 and 90% of school-leavers are myopic (Morgan, Ohno-Matsui et al. 2012). This epidemic however, is also emerging in other parts of the world, perhaps not at the same rate, including the United States (Kempen, Mitchell et al. 2004, Vitale, Ellwein et al. 2008, Vitale, Sperduto et al. 2009) and probably in Europe (Logan, Davies et al. 2005, Jobke, Kasten et al. 2008), where the prevalence of myopia in young adults is now between 30 and 50%. Even though myopia can be easily corrected by glasses, contact lenses and even refractive surgery, the rapid prevalence of myopia around the world poses a major health concern as the World Health Organisation (WHO) recognises if myopia is not fully corrected, it is a major cause of visual impairment (Resnikoff, Pascolini et al. 2008). Moreover, roughly 10-20% of the myopic children can be characterised as high myopes (≤ -6D), which increase their risk of developing chorio-retinal diseases (Leske, Chylack et al. 1991, Yongphanit, Mitchell et al. 2002, Chou, Yang et al. 2007, Resnikoff, Pascolini et al. 2008, Marcus, de Vries et al. 2011, Morgan, Ohno-Matsui et al. 2012) such as an increased risk of retinal detachment (Chou, Yang et al. 2007) and other potentially blinding pathologies that are not prevented by optical corrections (Saw, Gazzard et al. 2005).

1.5 Experimental Paradigms of Eye Growth

Four experimental paradigms have been extensively used to study eye growth in animal models by altering their visual experience:

1- Form-deprivation myopia

2- Recovery from form-deprivation myopia

3- Negative lens-wear

4- Positive lens-wear

1.5.1 Form-deprivation myopia

Normal ocular development appears to be visually guided. If a clear visual image is obscured (loss of form-vision) through the attachment of a translucent diffuser over the eye, or through suturing the eye lids closed, excessive axial elongation, as well as thinning of the choroid, will
occur resulting in form-deprivation myopia (FDM) (figure 1.3 A) in all animals studied (For review see Wallman and Winawer 2004). During the development of FDM, the eye appears to go into a “default” mode of excessive growth (Nickla 2013), in what appears to be an attempt to compensate for the visual blur by moving the retina backwards. This is achieved by rapid thinning of the choroidal tissue and a slower, but more powerful response, of increasing the rate of axial elongation. As the visual blur associated with the translucent diffusers is not compensated for by increased axial growth (i.e. no end-point, creating an opened-looped system), the eye will continue to elongate for as long as the loss of form-vision remains and/or developmental plasticity remains (Wallman and Adams 1987). Choroid thinning is observed within 1 hour of diffuser wear in chickens, while increased axial lengthening is measurable within 24 hours (Kee, Marzani et al. 2001, Nickla 2013).

1.5.2 Recovery from form-deprivation myopia

Following the induction of FDM, if the diffusers are removed, myopic blur is experienced (the image falls in front of the retina, figure 1.3 B). To regain sharp focus, the retina is displaced forward to where the new image plane falls, by rapid thickening of the choroid and cessation of axial elongation (Wallman and Winawer 2004) (figure 1.3 C). Such changes in the rate of axial elongation, along with the continual loss of optical power during development, ultimately corrects for the refractive errors experienced.
Figure 3. Growth paradigms of FDM and recovery from FDM

(A) Fitting of translucent diffuser results in loss of form-vision. The eye attempts to compensate by increasing the rate of axial elongation and choroidal thinning.

(B) Removal of diffusers results in myopic blur due to the excessive axial elongation associated with the previous diffuser-wear. The focal plane falls in front of the retina, instead of on it.

(C) Recovery from FDM occurs through rapid choroidal expansion (dark blue region) and through a reduction in the rate of axial elongation. These two processes, combined with the natural loss of optical power over time, allows the image plane to once again fall on the retina.

(Figure adjusted from Ashby 2007)
1.5.3 Negative lens-wear

The rate of eye growth can be modulated by fitting lenses over the eye of animals to create either myopic or hyperopic defocus. Negative lenses push the focal plane of viewed images behind the retina by reducing the optical power of the eye causing hyperopic defocus (Schaeffel, Troilo et al. 1990, Smith and Hung 1999)(figure 1.4 A). This induces increased axial growth and a rapid thinning of the choroid in attempt to pull the retina backwards towards the new focal plane to regain emmetropia (Kee, Marzani et al. 2001, Morgan 2003) (figure 1.4 B). This paradigm is also referred to as lens-induced myopia (LIM), and along with FDM, is used to study biochemical changes that occur during periods of increased ocular growth, although some differences exist between these two paradigms including the time course of axial length changes and the biochemical markers involved (Wallman and Winawer 2004). LIM is referred to as a closed-looped system, as a specific end-point (target refraction) exists. In contrast, FDM is referred to as an open-looped system, as there is no set end-point (target refraction) to achieve, therefore, the eye will continue to elongate for as long as the diffusers remain attached and/or developmental plasticity remains (Kee, Marzani et al. 2001). LIM induces faster changes in axial length, as well as the synthesis of proteoglycans needed for scleral growth (Kee, Marzani et al. 2001).

1.5.4 Positive lens-wear

Fitting of positive lenses suppresses eye growth similar to that seen during recovery from FDM (Wildsoet and Wallman 1995, Hung, Wallman et al. 2000), although in the positive lens paradigm the eye starts off in a normal state. As with the recovery paradigm, positive lenses cause the image plane to fall in front of the retina by increasing the optical power of the eye, therefore causing myopic defocus (Schaeffel, Glasser et al. 1988, Irving, Sivak et al. 1992) (figure 1.4 A). The eye compensates by reducing the rate of axial elongation and by pushing the retina forward through rapid choroidal thickening. These processes, combined with the natural reduction in the eyes optical power over time, allow the image plane to once again fall on the retina (Wallman, Wildsoet et al. 1995, Wildsoet and Wallman 1995) (figure 1.4 B). This paradigm is also referred to as lens-induced hyperopia (LIH) as hyperopic defocus would be experienced if the lenses were to be removed.
Negative lenses push the focal plane of viewed images behind the retina by reducing the optical power of the eye causing hyperopic defocus (A). In attempt to compensate, increased axial length and choroidal thinning occurs to push the retina back, therefore regaining sharp focus (B). Positive lenses cause the image to fall in front of the retina by increasing the optical power of the eye causing myopic defocus (A). In attempt to compensate, the rate of eye growth is decreased and choroidal thickening occurs to move the retina forward, therefore regaining sharp focus (B).

(Figure adjusted from Ashby 2007)
1.6 Considerations related to experimental paradigms

During lens-wear, the eye compensates for the imposed optical defocus. When removing the lens following full compensation, the eye experiences a refractive error directly opposite to the original defocus imposed on the eye (Kee, Marzani et al. 2001). As mentioned above, positive lenses impose a myopic defocus, leading to the suppression of eye growth (“STOP” growth signal) (Irving, Callender et al. 1991, Hung, Crawford et al. 1995, Irving, Callender et al. 1995, Backhouse and Phillips 2010). However, when the lens is removed, the eye is functionally hyperopic (Kee, Marzani et al. 2001). On the other hand, negative lenses impose a hyperopic defocus leading to eye growth (“GO” growth signal) (Shaikh, Siegwart et al. 1999, Siegwart and Norton 2001). When the lens is removed, the eye is myopic (Kee, Marzani et al. 2001). These changes affect the rate of eye growth as well as choroidal thickening to allow the retina to be moved to the new focal plane to correct the imposed defocus (Brown, Koretz et al. 1999).

1.6.1 Positive and negative lenses worn alternately

Wearing positive and negative lenses alternately do not cancel out the refractive errors imposed by either of the lenses. Instead, positive lenses have a dominant effect when equal amounts of positive and negative lenses are worn (Winawer and Wallman 2002). Even when chicks are exposed to negative lenses for five times more than positive lenses, Winawer and Wallman (2002) have shown that the responses were dominated by positive lenses. This suggests that myopic defocus, imposed by positive lenses, results in a stronger growth response than hyperopic defocus. This therefore implies that eye growth is more susceptible to stimuli producing a “STOP” growth signal rather than to “GO” growth signals.

1.6.2 Effect of normal vision during lens wear

The development of FDM can be retarded by the removal of diffusers for short periods of time (Wallman and Adams 1987, Napper, Brennan et al. 1995, Napper, Brennan et al. 1997, Ashby, Ohlendorf et al. 2009). Napper et al. (1995, 1997) have shown that the daily removal of diffusers, for as little as 15 minutes per day, significantly reduces the development of myopia. This retardation is enhanced when diffusers are removed while the animals are exposed to higher ambient illumination (Ashby, Ohlendorf et al. 2009). This suggests that eye growth is affected by periods of normal vision as it can almost entirely block the stimulation of eye growth induced by fitting of diffusers, therefore preventing the development of myopia.
1.7 Eye growth

1.7.1 Ocular growth is visually guided

As discussed above, animal studies have indicated that ocular growth is visually guided, as imposed myopic or hyperopic defocus through the use of negative and positive lenses respectively, induces changes in ocular growth that compensate the imposed refractive error (Schaefker, Glasser et al. 1988). The eye compensates for these refractive errors by rapid alterations in the rate of axial elongation of the eye and choroidal thickness, as discussed above. This suggests that the eye can detect visual defocus/blur, through an as yet unknown mechanism, and adjusts the growth rate to achieve emmetropia accordingly. The fitting of translucent diffusers, causing a loss of clear visual cues, leads to uncontrolled axial elongation of the eye (Wallman and Adams 1987). Furthermore, the fitting of diffusers or lenses over only one half of the eye, providing a defocus signal to only one half of the retina, induces compensatory growth changes in only that half of the eye. This further demonstrates that the eye is responding directly to the visual blur experienced in a very localised manner (Wallman, Gottlieb et al. 1987, Wallman and Winawer 2004).

1.7.2 Local mechanisms

Eye growth does not appear to be centrally regulated by the higher visual cortices in the brain, but rather locally driven by retinal pathways. Animal studies have demonstrated that gross compensation for lens-wear and the responses to diffuser-wear are relatively unaffected if the optic nerve is severed, action potentials of the ganglion cells are blocked, or if the Edinger-Westphal nucleus (nucleus of the oculomotor nerve supplying the eye muscles) is destroyed (Troilo, Gottlieb et al. 1987, Wildsoet and Wallman 1995, Schmid and Wildsoet 1996). This suggests higher visual centres are not required, although compensation is not as precise, indicating that accommodation and higher visual centres are required for fine tuning of the process (Wildsoet 2003). Furthermore, as described earlier, if only the nasal or temporal region of the eye is exposed to FDM or negative lens-wear, only that region shows compensatory changes in ocular growth in chicks, guinea pigs and monkeys (Wallman, Gottlieb et al. 1987, McFadden 2002, Smith, Huang et al. 2009, Smith, Hung et al. 2010, Zeng and McFadden 2010), which is indicative of a very localised response that is not easily explained by a centrally driven mechanism (Wallman, Gottlieb et al. 1987). In support of this conclusion, localised changes in ocular growth, in response to nasal or temporal defocus, still occurs following optic nerve sectioning (Wallman, Gottlieb et al. 1987). The compensation to positive lenses still occurs after optic nerve section (Wildsoet and Wallman 1995) with choroidal thickening, the initial stage of compensation, occurring in local retinal areas (Wallman,
Wildsoet et al. 1995), although the development of FDM is suppressed with lenses (Wildsoet and Wallman 1995). Together these findings suggest that visually-induced changes in the rate of ocular growth are mainly driven by local retinal mechanisms.

1.8 Environmental influences on ocular growth – near work

Epidemiological studies have reported a correlation between years of schooling, educational achievement and the development of myopia (Shulkin and Bari 1986, Teasdale, Fuchs et al. 1988, Chow, Dhillon et al. 1990, Saw, Katz et al. 1996), is believed to be associated with the accommodative demand associated with heavy levels of near-work tasks such as reading. Supporting this hypothesis are two lines of evidence. Firstly, a number of studies have demonstrated a correlation between educational achievement and myopia prevalence (Chow, Dhillon et al. 1990, Lin, Shih et al. 1996, Woo, Lim et al. 2004), with young adult populations that undertake intense study (e.g. medical school) showing a rapid increase in myopia prevalence over their study period (Midelfart, Aamo et al. 1992, Lin, Shih et al. 1996, Fledelius 2000, Onal, Toker et al. 2007). Secondly, native populations that previously lived an outdoor life style, such as the Inuit or native American Indians, have had a dramatic increase in the prevalence of myopia after the introduction of compulsory education within one generation (Bear 1991). It has been hypothesised that the high levels of near-work associated with intensive study could either: place excessive strain on the accommodative system, as the ciliary muscles must be continuously contracted during near-work tasks; or during near-work tasks the eye will receive a level of hyperopic defocus that although reduced by the accommodative system, is not abolished due to the negative feedback by which accommodation is controlled (McCarty and Taylor 2000, Mutti, Mitchell et al. 2002, Saw, Chua et al. 2002). However, such accommodative strain could be reduced through elongation of the eye (myopia development), and therefore removing the accommodative strain. This excessive accommodation theory has been supported by the finding that fitting negative lenses, which induce continuous hyperopic defocus and a large accommodative load, results in elongation of the eye (Schaeffel, Glasser et al. 1988, Irving, Callender et al. 1991, Irving, Sivak et al. 1992, Irving, Callender et al. 1995, Wildsoet and Wallman 1995, Schmid and Wildsoet 1996).

However, a consistent correlation between near-work and the development of myopia has not been seen in studies that have attempted to specifically quantify hours of near-work undertaken (Rose, Morgan et al. 2008). Findings from animal studies have also suggested the accommodative system is not required for visually-guided emmetropisation, as compensation for lens-wear, or the increased growth associated with diffuser-wear, can still occur without an active accommodative system (Wallman, Gottlieb et al. 1987, McFadden 2002, Smith, Huang et al. 2009, Smith, Hung et al. 2010, Zeng and McFadden 2010). Secondly, atropine, which
initially was believed to block the development of myopia by manipulating the accommodative system, has since been shown to work through a, as yet unknown, non-accommodative mechanism (McBrien, Moghaddam et al. 1993). Furthermore, if diffusers or lenses are fitted only on one half of the retina, only that half shows changes in growth rates (Wallman, Gottlieb et al. 1987, McFadden 2002, Smith, Hung et al. 2010, Zeng and McFadden 2010), a response difficult to explain via a system driven by accommodation.

1.9 Environmental influences on ocular growth – time outdoors

Evidence from both cross-sectional and longitudinal data suggests that children who spend more time outdoors are less likely to develop myopia (Parssinen and Lyyra 1993, Mutti, Mitchell et al. 2002, Khader, Batayha et al. 2006, Chou, Yang et al. 2007, Jones, Sinnott et al. 2007, Onal, Toker et al. 2007, Rose, Morgan et al. 2008, Rose, Morgan et al. 2008, Jones-Jordan, Mitchell et al. 2011, Marcus, de Vries et al. 2011, Guggenheim, Northstone et al. 2012, Sherwin, Hewitt et al. 2012). It was first established in 1993 in a cohort of Finnish school children that spending greater time outdoors was associated with less myopic refraction (Parssinen and Lyyra 1993). This was confirmed by the Orinda Longitudinal Study of Myopia (OLSM) in the United States, which reported that children that became myopic were significantly less involved with outdoor activities compared to the children who remained emmetropic (Mutti, Mitchell et al. 2002). A follow-up study by the authors examined two age cohorts and found a higher rate of myopia development in those children that spent less time outdoors. The authors suggested that this may be due to children who spend more time outdoors are less involved in near-work activity. A later cross-sectional study (Sydney Myopia Study, SMS), conducted in 2008, reported that total time spent outdoors, rather than time engaged in sports per se, was negatively correlated with the development of myopia (Rose, Morgan et al. 2008). Rose et al. (2008) reported that children who spent the most amount of time outdoors combined with low levels of near-work had the most hyperopic mean refraction, while those children that combined limited time outdoors with high levels of near-work had the least mean hyperopic refraction. These studies were primarily based on populations of European origin, although similar protective effects have been reported in a sample of children of East Asian origin (Dirani, Tong et al. 2009, Guo, Liu et al. 2013, Xiang, Zeng et al. 2013). In general, the negative correlation between time outdoors and the development of myopia has now been confirmed by numerous studies from various geographical locations (Parssinen and Lyyra 1993, Mutti, Mitchell et al. 2002, Khader, Batayha et al. 2006, Jones, Sinnott et al. 2007, Onal, Toker et al. 2007, Jones-Jordan, Mitchell et al. 2011, Guggenheim, Northstone et al. 2012, Sherwin, Hewitt et al. 2012), although not always (Saw, Wu et al. 2001, Saw, Shankar et al. 2006, Lu, Congdon et al. 2009, Low, Dirani et al. 2012).
Clinical trials are currently underway in China (Yi and Li 2011) and Taiwan (Wu, Tsai et al. 2013) associated with increasing the time spent outdoors by children during the school day, with preliminary findings showing promising results.

Initially, it was suggested that the protective effects associated with time outdoors may simply be a substitution effect, in which the more time children spend outdoors the less time they spend on near-work tasks such as reading, which has previously been correlated with the development of myopia. However, Rose and colleagues (2008) reported that a less myopic refraction was still observed in children who engaged in significant amounts of near-work if combined with greater amounts of time outdoors, suggesting this is not a substitution effect (Rose, Morgan et al. 2008). Instead, the authors hypothesised that the reduced prevalence of myopia seen in those children that spend greater amounts of time outdoors could be associated with light-induced increases in retinal dopamine release, a neurohormone previously postulated to be involved in the regulation of ocular growth, associated with the higher illumination levels experienced outdoors. In support of this hypothesis, seasonal differences in the progression rates of myopia have been found with higher rates in winter compared to summer (Goss and Rainey 1998, Fulk, Cyert et al. 2002, Donovan, Sankaridurg et al. 2012, Fujiwara, Hasebe et al. 2012, Cui, Trier et al. 2013, Gwiazda, Deng et al. 2014). However, it is unclear if this seasonal effect is associated with high illumination levels seen during summer, or due to more near-work being undertaken in the winter months (Rose, Morgan et al. 2008, Gwiazda, Deng et al. 2014).

1.9.1 High light and the development of myopia

Although renewed interest has been generated in the role of light in ocular development, based on the protective effects of time spent outdoors, considerable work has already demonstrated that alterations in the lighting conditions (rhythmicity, intensity and spectral composition) in which animals are reared, can significantly affect ocular development. Early work by Lauber and colleagues (1979), amongst others (Stone, Lin et al. 1995, Li and Howland 2000, Li and Howland 2003), demonstrated that rearing chicks, although not primates (Smith, Bradley et al. 2001), in continuous illumination produces vitreal chamber enlargement both in the axial and equatorial direction. Conversely, it induces a hyperopic shift in refraction due to thickening and flattening of the cornea, as well as shallowing of the anterior chamber (Stone, Lin et al. 1995, Li and Howland 2000, Li and Howland 2003). Similar ocular changes have been observed in response to constant dark-rearing, suggesting that circadian and diurnal rhythms are critical to ocular development. More recently, the illumination levels to which chicks are exposed during the light phase have been shown to be critical. For example, the unilateral
rearing of chicks under low illumination levels (<50 lux) during the light phase, using neutral density filters, can induce a myopic shift due to excessive vitreal chamber elongation, although the results are less consistent when the illumination of the entire room is dropped by a similar amount (Gottlieb, Fugate-Wentzek et al 1987). Finally, ocular development can be altered by rearing animals under monochromatic light due to compensation for longitudinal chromatic aberrations (a failure to focus all colours in a spectrum to the same convergence point due to difference in their wavelengths) (Kroger and Wagner 1996, Seidemann and Schaeffel 2002). Specifically, emmetropisation has been considered thus far with respect to a single focal plane; however, in practice, polychromatic light is not focused as a single plane on the retina; rather, it displays a point spread function created in part by chromatic aberrations (for review see Rucker 2013). This inherent property of our optical system means that shorter wavelengths (e.g. blue) are refracted (converged) to a greater extent than longer wavelengths (e.g. red), creating multiple focal planes around the retina, with the eye focused to the midpoint within that animals visible spectral range. Consequently, rearing chickens in monochromatic red light, in which the focal plane falls normally behind the retina, induces a relative myopic shift in refraction (increased axial elongation), compared to those chickens reared in monochromatic blue light, in which the focal plane would naturally fall in front of the retina (Kroger and Binder 2000, Rucker and Wallman 2008).

In response to the reported protective effects provided by time outdoors against the development of myopia, a number of animal studies have recently investigated the ability of high light levels to alter normal ocular growth and to prevent the development of experimental myopia (Cohen, Belkin et al. 2008, Ashby, Ohlendorf et al. 2009, Ashby and Schaeffel 2010, Norton, Amedo et al. 2010, Cohen, Belkin et al. 2011, Cohen, Peleg et al. 2012, Smith, Hung et al. 2013).

Ashby and colleagues (2009, 2010) have shown that exposure to elevated light levels of 15,000 lux for a period of 5 hours per day retards the development of FDM by roughly 60%, through a reduction in the rate of axial elongation, compared to those animals fitted with translucent diffusers and reared under normal laboratory lighting levels of 500 lux. Similar results have since been reported in tree shrews (Norton, Amedo et al. 2010, Siegwart, Ward et al. 2012) and rhesus monkeys (Smith, Hung et al. 2012), suggesting that this is a maintained mechanism. As with chicks, following the fitting of translucent diffusers, tree shrews exposed to illumination levels of 15,000 lux for 7.5 hours per day, over a period of 10 days, showed an approximately 60% reduction in the development of FDM compared to those animals reared with attached translucent diffusers under 500 lux (Siegwart, Ward et al. 2012). In rhesus monkeys, the protective effect was greater than that observed in chicks, with eight of the ten
monkeys that were fitted with translucent diffusers and exposed to 18-28,000 lux for five hours per day, developing no myopia and actually showing a small hyperopic shift which was associated with shorter vitreal chambers (Smith, Hung et al. 2012). The enhanced protective effects seen in the primates may be associated with the greater light intensities used, therefore, one of the primary aims of this study is to determine if, in chicks, greater protection comes with greater light intensities (e.g. do light intensities above 15,000 lux (the current maximum intensity previously studied) have a greater protective effect?). Supporting the hypothesis that the development of FDM may be retarded to a greater degree with higher light intensities, Ashby et al. (2010) have shown that the protective effects of brief periods of normal vision (15 minutes daily) against the development of FDM is more pronounced when higher light intensities are used during this diffuser-free period (15 minutes of diffuser-free vision: under 500 lux lead to a 40% reduction; under 15,000 lux resulted in 65% reduction; and under 30,000 lux lead to 80% reduction in FDM development) (Ashby, Ohlendorf et al. 2009). Furthermore, Cohen et al. (2011) have reported that the normal emmetropisation process in chicks appears to be intensity dependent. In chicks, during normal refractive development, the eye displays a postnatal hyperopic refraction which reduces significantly towards emmetropia over the first eight weeks (Wallman, Adams et al. 1981). Cohen and colleagues (2011) have reported, however, that this emmetropisation process is dependent, to some extent, on light intensity. Over a period of 90 days, the refractive development of chicks was observed when exposed to one of three light intensities (50 lux, 500 lux or 10,000 lux). Those chicks reared under 50 lux showed a myopic refraction by day 90. Those chicks raised under the medium light intensity of 500 lux were emmetropic by day 90, while those chicks reared under higher light levels of 10,000 lux remained mildly hyperopic from 60 days onwards. This suggests that light levels in which the animals are reared under can affect ocular development and emmetropisation, such that lower illumination levels can induce a myopic shift, while high levels actually hold the eye in a hyperopic state, at least over the first 90 days of life (Cohen, Belkin et al. 2011).

Ashby and colleague (2010) have also shown that exposure to elevated light levels of 15,000 lux for a period of 5 hours per day reduced the rate of compensation for negative lenses and enhances the rate of compensation for positive lenses, although in both cases full compensation for the lenses is still achieved. Similar to that observed in chicks, exposure to high light reduces the rate of compensation for negative lenses in tree shrews, with full compensation to −5 D lenses taking 17.5 days in those animals exposed to 15,000 lux, compared to 9 days for full compensation in those animals kept under 500 lux (Siegwart, Ward et al. 2012). However, high light exposure would appear not to affect the rate of negative lens
compensation in primates (Smith, Hung et al. 2013), although a subsequent much smaller study by Stell and colleagues (2013) has suggested that there may be a small effect.

1.10 Changes in gene expression during recovery from form-deprivation myopia

Animal models have significantly contributed to our understanding of the biochemical pathways that underlie the visually-guided regulation of ocular growth. As discussed above, ocular growth appears to be locally controlled by signals that originate in the retina. To begin to elucidate the pathways underlying the protective effects of light, this study examines the role of specific components of four of the major molecular pathways postulated to be critical to ocular growth regulation, including: dopamine, retinoic acid, glucagon, and nuclear transcription factors. Specifically, this study will examine possible changes in the retinal mRNA expression, in response to high light, of the dopamine components; tyrosine hydroxylase (TH) (rate-limiting enzyme) and dopamine D2 receptor (DRD2); the retinoic acid receptor isoform beta (RAR-β); pre-pro-glucagon (PPG); and the nuclear transcription factors Egr-1 and cFos.

1.10.1 Dopamine

The seminal paper by Stone et al. (1989) reported a reduction in the levels of retinal dopamine and its primary metabolite 3, 4 dihydroxyphenylacetic acid (DOPAC), with any form of visual deprivation (lit suture, translucent and transparent goggles). A reduction in the activity of the rate limiting enzyme of dopamine synthesis, TH, was observed indicating a decreased rate of dopamine synthesis (Stone, Lin et al. 1989). This finding was confirmed by more direct measures of dopamine release, that of vitreal DOPAC levels (Megaw, Morgan et al. 2001). Administration of dopamine agonists, targeting the dopamine receptor families (D1 and D2), have been shown to alter the rate of ocular growth, providing the strongest evidence for a role for the dopaminergic system in the visually guided modulation of eye growth. Initial studies demonstrated that administration of the non-selective dopamine receptor agonist, apomorphine (APO), reduces axial elongation associated with lid-suturing (Stone, Lin et al. 1989, Iuvone, Tigges et al. 1991, Rohrer, Spira et al. 1993, Schmid and Wildsoet 2004, Nickla, Totonelly et al. 2010, Dong, Zhi et al. 2011). This effect was abolished, however, by the co-administration of the dopamine antagonist haloperidol, suggesting the pharmacological effects were driven specifically by the dopaminergic system, with similar finding reported in rhesus monkeys (Iuvone, Tigges et al. 1991). Rohrer et al. (1993) demonstrated that the effects of apomorphine were blocked by the co-administration of the dopamine D2 receptor antagonist spiperone, but not by the dopamine D1 receptor antagonist SCH-23390, suggesting that apomorphine acts to prevent FDM through the D2-family of receptors. The development of both FDM and LIM can be retarded by the administration of a number of dopamine agonists
including 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide (ADTN) (Ashby, McCarthy et al. 2007, McCarthy, Megaw et al. 2007) and quinpirole (McCarthy, Megaw et al. 2007, Nickla, Totonelly et al. 2010) in chicks, and the development of FDM by administration of synthetic dopamine (Gao, Liu et al. 2006, Lin, Chen et al. 2008), or its precursor levodopa (L-DOPA) (Mao, Liu et al. 2010) in guinea pigs.

Importantly for this study, the daily administration of the dopamine D2 receptor antagonist spiperone has been shown to abolish the protective effects of bright light against the development of deprivation-myopia (Ashby and Schaeffel 2010), suggesting, as originally hypothesised by Rose and colleagues (2008), exposure to high light levels may protect against the development of myopia through light-induced increases in retinal dopamine release.

1.10.2 Retinoic acid

Retinoic acid (RA) is the metabolite of vitamin A and it plays a crucial role during early retinal development, affecting the proliferation and differentiation of several types of ocular cells, such as retinal pigment epithelial cells and corneal endothelial cells (Junquero, Modat et al. 1990, Kirschner, Ciaccia et al. 1990, Patek and Clayton 1990, Campochiaro, Hackett et al. 1991), as well as chondrocytes (Lewis, Pratt et al. 1978, Pacifici, Golden et al. 1991). In mice lacking retinoic acid receptors, the eyes are small with gross morphological defects within the choroid and sclera, and display retinal dysplasia (Grondona, Kastner et al. 1996). Furthermore, in chickens and mice, RA appears to be important in dorsal-ventral patterning of the retina (McCaffery, Lee et al. 1992, Mey, McCaffery et al. 1997). Inhibition of RA synthesis during development, when only the ventral enzyme is present, results in eyes lacking ventral retinas as well as other ocular malformations such as lens defects (Jung, Lee et al. 2012). RA acts through a family of retinoic acid receptors (RAR) which consist of three isoforms (RAR-α, RAR-β and RAR-γ) that act as ligand activated transcription factors (Marill, Idres et al. 2003).

Following the attachment of translucent diffusers, inducing deprivation-myopia, RA expression within the retina has been shown to significantly increase in the chicken sclera (Seko, Shimokawa et al. 1996, Seko, Shimizu et al. 1998, Bitzer, Feldkaemper et al. 2000). Administration of RA to guinea pigs and chicks causes a rapid increase in axial elongation (McFadden, Howlett et al. 2004, McFadden, Howlett et al. 2006) while it decreases scleral glycosaminoglycan synthesis and increases vitreous chamber length in marmosets that are monocularly form-deprived (Troilo, Nickla et al. 2006). Furthermore, the loss of form vision through the attachment of translucent diffusers leads to a significant increase in retinal-RA levels after 7 days in guinea pigs. This increase doubled after 14 days when compared to the contralateral control values (Huang, Qu et al. 2011).
Retinoic acid receptor-β (RAR-β) mRNA levels have also been reported to be up-regulated in the chicken sclera (Seko, Shimokawa et al. 1996, Seko, Shimizu et al. 1998, Bitzer, Feldkaemper et al. 2000). Following 7 days of FDM, there was no significant difference in expression in the mRNA levels or RAR-β in guinea pigs, although the difference was significant after 14 days for both FDM and negative lens-wear compared to control values (Huang, Qu et al. 2011). RAR-α has previously been shown to be strongly expressed in the retina (Mattei, Riviere et al. 1991, Mori, Ghyselinck et al. 2001). However, Veerappan and colleagues (2009) observed no association between RAR-α and myopia.

Animal models have therefore suggested a role for RA in the modulation of eye growth, although human genome-wide association studies (GWAS) have not found an association to date between components of the RA system and high myopia (Verhoeven, Hysi et al. 2013). However, due to the limitations of human genetic studies, the lack of findings from GWAS by no means precludes a role for RA in the regulation of ocular growth.

1.10.3 Glucagon

Glucagon is a neuropeptide, produced by the cleavage of its precursor molecule pre-pro glucagon (PPG), that is synthesised and released from a subset of retinal amacrine cells (glucagonergic amacrine cells) (Hasegawa, Terazono et al. 1990). The precise role of this neuropeptide is unclear, however, evidence has suggested that it plays a significant role in the regulation of ocular growth. Firstly, like Egr-1, glucagon mRNA levels show a bi-directional response to opposing growth stimuli, with transcript levels down-regulated during periods of increased ocular growth induced by translucent diffusers, and increased transcript levels are seen during periods of decreased ocular growth induced by the removal of translucent diffusers or the fitting of positive lenses (Feldkaemper, Burkhardt et al. 2002, Buck, Schaeffel et al. 2004, Feldkaemper, Burkhardt et al. 2004, Ashby, Kozulin et al. 2010). Secondly, injection of the glucagon agonist Lys$^{17,18}$, Glu$^{21}$-glucagon can retard the development of FDM or LIM in a dose dependent manner (Feldkaemper and Schaeffel 2002, Vessey, Lencses et al. 2005), while the glucagon antagonist [des-His$^{5}$, Glu$^{9}$]-glucagon-NH$_{2}$, was found to prevent LIH (Vessey, Lencses et al. 2005), suggesting that endogenous glucagon may act as a “STOP” growth signal. It should be noted, however, that glucagon expression has not been detected within the mammalian retina.

1.10.4 Early growth response 1 (Egr-1)

Egr-1, known as ZENK (ZIF268, Egr-1, NGFI-A, or Krox-24) in the chicken, is an immediate-early gene that codes for a short-lived nuclear transcription factor (Fischer, McGuire et al. 1999).
Egr-1 has been implicated in the modulation of ocular growth due to its bi-directional response to opposing growth stimuli, and is the earliest known molecular change. At the peptide level, Fischer et al. (1999) have demonstrated that Egr-1 levels within glucagonergic amacrine cells show a bi-directional response to opposing growth stimuli. That is, during periods of increased ocular growth, stimulated by the fitting of either translucent diffusers or negative lenses, co-staining for Egr-1 within glucagoneric amacrine cells is significantly reduced within one hour. Conversely, in response to the removal of diffusers, co-staining for Egr-1 within glucagoneric amacrine cells is significantly elevated, again within as little as one hour. Similar changes are seen at the mRNA level, with Ashby et al. (2007, 2010), demonstrating that Egr-1 transcript levels are rapidly down-regulated (within 30-60 minutes) during periods of increased ocular growth, and remain down-regulated as long as growth rates are elevated above control values. Once growth rates return to normal, such as when the animal has compensated for the imposed hyperopic defocus associated with negative lens-wear, Egr-1 mRNA levels also return to normal control values. Conversely, during recovery from FDM, Egr-1 mRNA levels are rapidly, but transiently, up-regulated, confirming the bi-directional response of Egr-1 to opposing growth stimuli (Fischer, McGuire et al. 1999, Ashby, McCarthy et al. 2007). Similar bi-directional changes in Egr-1 mRNA levels have been seen within guinea pigs in response to negative lens-wear (increase ocular growth), and the removal of negative lenses (recovery, reduced ocular growth) (Ashby, Zeng et al. 2011). Further, Egr-1 mRNA levels have been reported to be down-regulated in the mouse retina in response to diffuser-wear (Schippert, Burkhardt et al. 2007), and appear to display a bi-directional response at the peptide level in primate retina to opposing growth stimuli (Zhong, Ge et al. 2004), although, due to the limited animal number the results are not clear. More direct evidence for a role for Egr-1 in regulation of ocular growth comes from two studies. Firstly, Egr-1 knockout mice show a relative myopic shift compared to wild-type control animals (Schippert, Burkhardt et al. 2007). Secondly, pharmacological agents which block the development of experimental myopia, such as the dopamine D2 receptor agonist 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide (ADTN) and the muscarinic-cholinergic antagonists atropine and prinzepine, prevent the down-regulation in Egr-1 mRNA levels normally seen after one hour in response to diffuser-wear (Ashby, McCarthy et al. 2007) or negative lenses (Ashby, Kozulin et al. 2010) in chicks. Together, these results suggest that a down-regulation in Egr-1 expression is associated with an increase in the rate of ocular growth and the development of myopia.

1.10.5 cFos

FBJ osteosarcoma oncogene (cFos) is a light-driven immediate early gene that belongs to the family of nuclear transcription factors to which Egr-1 also belongs. It has been suggested that
cFos is involved in the regulation of diurnal transcriptional changes that occur during normal light/dark cycles in retinal cells (Yoshida, Kawamura et al. 1993). The expression of cFos has been found to be transiently expressed in the rat retina in the inner nuclear layer (INL) and ganglion cell layer (GCL) after the onset of light, while it was expressed in the outer nuclear layer (ONL) during the dark (Yoshida, Kawamura et al. 1993). Ohki et al. (2013) showed that the diurnal variations in cFos expression are not detected in rats in the first 10 days, but were observed at 15 days post birth. Another indication that cFos is light-induced is due to the finding that photosensitive retinal ganglion cells express cFos following light stimulation in mice lacking the photopigment melanopsin (Dong, Zhi et al. 2011).

Although the retinal expression of cFos has been found to be light-induced, and was therefore used within this study primarily as a marker of light-induced activity, evidence exists for a role for cFos in the regulation of ocular growth. Fischer and colleagues (1999) have reported that cFos peptide levels, within the chicken retina, appear to be unaffected by the onset of FDM. However, staining for cFos is rapidly enhanced during recovery from FDM within a specific subset of retinal amacrine cells not positive for glucagon, vaso intestinal peptide (VIP), tyrosine hydroxylase (TH), choline acetyltransferase or somatostatin. The authors noted that when diffusers were removed but immediately replaced with neutral density filters to match the change in luminance experienced, cFos expression was still elevated, but to a lesser extent compared to diffuser-wear, suggesting that cFos, unlike Egr-1, may be responding to a change in illumination levels rather than to a specific blur signal. Furthermore, the authors reported no change in cFos immunostaining in response to positive-lens wear. However, three later independent microarray studies have reported a significant down-regulation in cFos mRNA levels in response to both the fitting of translucent diffusers and negative lenses, suggesting that cFos may respond to defocus cues (Brand, Schaeffel et al. 2007, Schippert, Schaeffel et al. 2008, Ashby and Feldkaemper 2010).

1.11 Aims of the study

As mentioned earlier, the development of myopia can be retarded by exposing animals to elevated light levels. The development of FDM in chicks is retarded by roughly 60% when animals are exposed to 15,000 lux for 5 hours per day (Ashby and Schaeffel 2010). Current treatments of human and animal myopia can only prevent or retard the onset of myopia but do not affect the progression. Therefore this study aimed to investigate whether bright light can halt the progression of FDM in myopic animals. Moreover, molecular analysis has indicated key pathways implicated in the development of myopia and the role of this study was to
further investigate the molecular pathways that underpin the protective effects of high light. Therefore the three aims of the study were:

1. Is there a correlation between the light intensities in which animals are raised and the severity of deprivation-myopia which develops?

2. Can exposure to bright light (40,000 lux) prevent the progression of deprivation-myopia?

3. What biochemical pathways are associated with the protective effects of high light?
Chapter 2: Methods

2.1 Animal housing

One day-old male White Cockerels were obtained from Barter & Sons Hatchery (Luddenham, NSW, Australia). On arrival, chickens were housed in temperature-controlled rooms under a 12:12 hour light/dark cycle with lights on at 7 am and off at 7 pm. Chickens had access to unlimited amounts of food and water, and were given four days to become accustomed to their environment before experiments started. Authorisation to conduct experiments using animals was approved by the University of Canberra Animal Ethics Committee under the ACT Animal Welfare Act 1992 (Project Number: CEAE 13-03) and conformed to the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research.

2.2 Experimental conditions

2.2.1 Induction of form-deprivation myopia (FDM)

Myopia was induced monocularly by fitting translucent diffusers over the left eye, with the right eye left untreated and serving as a contralateral internal control. Velcro mounts were fitted around the left eye with Loctite® super glue (Henkel, Düsseldorf, Germany) on the day prior to the commencement of the experiment. On the following day, the translucent diffusers, fitted to matching Velcro rings, were placed onto the mounts.

2.2.2 Lighting conditions

Aim 1: Is there a correlation between light intensity and the development of form-deprivation myopia over time?

To investigate the correlation between light levels, and the development of FDM, chicks were raised for a period of 7 days with translucent diffusers, under one of the following five lighting conditions (figure 5):

1)  40,000 lux (n=12, group 1)
2)  30,000 lux (n=12, group 2)
3)  20,000 lux (n=12, group 3)
4)  10,000 lux (n=12, group 4)
5)  500 lux (n=12, normal indoor light, group 5)

Changes in ocular parameters were compared to contralateral control values, as well as an untreated age-matched control group (n=12, group 6) that were raised with no diffusers under
normal laboratory illumination levels (500 lux) as a reference for normal ocular development (figure 5 A). Chicks in the high light groups (groups 1-4), were exposed to these intensities for a period of six hours per day (11 am- 5 pm, figure 5 B), while being kept under normal laboratory lighting levels for the remainder of the light period (7 am-11 am and 5 pm-7 pm).

Illumination of the cages was produced by a custom-made bank of 10 LED lamps, with an illumination angle of 60°, that were adjusted by a dimmer system on a linear scale between 0-45,000 lux (Electronics Department, Research School of Biology, Australian National University). Each of the 10 LED lamp banks were composed of an equal mix of cool (400-650 nm, peaking at 450 nm) and warm (430-700 nm, peaking at 630 nm) LED modules, generating minimal to no heat load, even at maximum intensity, and giving a final spectral composition similar to that produced by quartz-halogen bulbs, which were previously used to generate high light levels (Ashby, Ohlendorf et al. 2009, Ashby and Schaeffel 2010) but which suffered from generating a significant heat load. The lighting system did not emit in either the infra-red (IR) or ultraviolet (UV) spectrum. Illumination levels were uniform across the cage area, as measured by an IL1700 Research Radiometer (International Light, Inc., Chula Vista, CA, USA), with the lighting system sitting 1.5 metres above the cage roof. Cages were placed against the wall, allowing the chicks a viewing distance of 5 metres in three directions. All reported luminance used in this study were measured at the level of the cage floor, with lighting levels continuously monitored within the cages by an automated system using HOBO data loggers (HOBO ® Data Loggers, Bourne, MA, USA).
Experimental design for those chickens fitted with translucent diffusers (group 5), or age-matched untreated control animals (group 6), and reared under 500 lux.

Experimental design for those chickens fitted with translucent diffusers (groups 1-4) and reared under one of four higher illumination levels (40,000 lux (group 1), 30,000 lux (group 2), 20,000 lux (group 3), 10,000 lux (group 4)). Translucent diffusers were fitted to all animals at 7 am on day 1.

Figure 5. Daily treatment cycle
(A) Experimental design for those chickens fitted with translucent diffusers (group 5), or age-matched untreated control animals (group 6), and reared under 500 lux.
(B) Experimental design for those chickens fitted with translucent diffusers (groups 1-4) and reared under one of four higher illumination levels (40,000 lux (group 1), 30,000 lux (group 2), 20,000 lux (group 3), 10,000 lux (group 4)).
Aim 2: Can high light prevent the progression of form-deprivation myopia?

To investigate whether the progression of FDM, through excessive axial elongation, could be prevented, chickens were raised under normal laboratory lighting levels (500 lux) for a period of three days to induce myopic refractions. Following myopia induction, chicks were switched to daily high light exposure (40,000 lux for six hours per day) for the remainder of the experimental period (7 days). More specifically, chickens were split into five groups and treated for a total of 10 days:

1) Chickens were raised under 500 lux without diffusers (control group 1, n= 12)
2) Chickens were exposed to 40,000 lux for six hours per day, without diffusers (control group 2, n=12)
3) Chickens were fitted with translucent diffusers and raised under 500 lux (FDM group, n=12)
4) Chickens were fitted with translucent diffusers and exposed to 40,000 lux for six hours per day (high light group, n=12)
5) Chickens were fitted with translucent diffusers and raised under 500 lux for three days, after which chicks were switched to daily exposure of 40,000 lux for six hours per day, for a further 7 days (FDM progression group, n=12)

Aim 3: To elucidate the biochemical pathways associated with the ability of bright light to retard the development of form-deprivation myopia

To begin elucidating the biochemical pathways underlying the ability of bright light to retard the development of FDM, chickens were split into two groups:

1) Chickens were fitted with translucent diffusers and raised under 500 lux (group A, n=5)
2) Chickens were fitted with translucent diffusers and exposed to 40,000 lux for six hours per day (group B, n=5)

Translucent diffusers were fitted at 7 am on day 1 for both groups, with all animals sacrificed at 1 pm on day 2, 2 hours into the second high light phase, with retinal samples collected from both the treated (left) eye and the contralateral control (right) eye (figure 6).
Experimental design

(A) For chickens fitted with translucent diffusers (7 am, day 1) and reared under 500 lux (group A).

(B) For chickens fitted with translucent diffusers (7 am, day 1) and reared under 40,000 lux for six hours per day (group B).

Animals from both groups were sacrificed at 1 pm on day 2, 2 hours into the second high light phase, with retinal tissue collected from both the treated (left) eye and the contralateral control (right) eye.

Figure 6. Experimental design for attachment of translucent diffusers and retinal tissue collection
2.3 Measurement of ocular parameters

Refraction was measured daily at 9 am by automated infrared photoretinoscopy (system provided courtesy of Professor Frank Schaeffel, University of Tuebingen, Germany). Measurements were taken from both the experimental (left eye), contralateral control (right eye), and age-matched untreated control eyes (both left and right). Refraction represents the mean spherical equivalent of 10 measurements per eye. For a myopic eye, the camera only detects light rays emerging from the lower part of the pupil, while for a hyperopic eye it detects light rays emerging from the upper part of the pupil. The amount of defocus of the eye can be calculated by the height of the light crescent in the pupil (Schaeffel, Hagel et al. 1994). For a correct refractive value to be obtained, the Purkinje image must be centred within the pupil (correct refractive axis), with the illumination levels within the room kept to less than 5 lux to prevent light reflections in the pupil from aberrant sources, which would cause the software to erroneously estimate the Purkinje image and therefore calculate an incorrect refractive value. To account for possible accommodation, the most consistent hyperopic value was recorded (Schaeffel, Hagel et al. 1994).

Axial length was measured on day 0, serving as a baseline, and on the second last day (day 6 (aim 1) or 9 (aim 2)) of the experimental period by A-scan ultrasonography (Biometer AL-100, Tomey Corporation, Nagoya, Japan). An immersion attachment probe (Tomey Corporation, Nagoya, Japan), filled with medical grade ultrasound gel (Conductive gel, Medical Equipment Services, Keilor Park, VIC, Australia), was used to allow optimal contact to be made with the eye. Ocular parameters measured included: anterior chamber depth (anterior surface of the cornea to the anterior surface of the lens), lens thickness (anterior to posterior surface of the lens), vitreal chamber depth (posterior surface of the lens to vitreal surface of the retina), axial length (anterior surface of the cornea to the vitreal surface of the retina), for treated, contralateral control and age-matched untreated eyes for Aims 1 and 2. Before commencing ultrasound recordings, chickens were anaesthetised using isoflurane (5% in 1L of medical grade oxygen per minute, Veterinary Companies of Australia, Kings Park, NSW, Australia), with Allergan Tears Plus eye drops placed on the eye following the completion of all measurements to prevent the eyes from drying out. Ten measurements were made from each eye which was averaged to determine the ocular parameters listed above. An example output from A-scan ultrasonography is shown in figure 7.
**Figure 7. Image obtained by A-scan ultrasonography**

Showing axial length (AXIAL), anterior chamber depth (ACD) and lens thickness (LENS)

1) Anterior surface of the cornea
2) Anterior surface of the lens
3) Posterior surface of the lens
4) Vitreal surface of the retina
2.4 Tissue preparation

At the end of the experimental period, chicks were anaesthetised using isoflurane (5% in 1 L of medical grade oxygen per minute) and sacrificed by decapitation. Each eye was rapidly removed and hemisected equatorially, with the anterior portion of the eye discarded. The posterior eye cup was floated in chilled phosphate-buffered saline (NaCl, 137mM; KCl, 2.7mM; Na₂HPO₄, 11.3mM; KH₂PO₄, 1.5mM; pH 7.4) (Sigma-Aldrich, Castle Hill, NSW, Australia), allowing removal and collection of retinal tissue, free of retinal pigment epithelium (RPE), scleral and choroidal tissue. The tissue was immediately frozen on dry ice upon collection and stored at -80˚C until use. Vitreous bodies were collected in 100 μL of 0.1% ascorbic acid (Sigma-Aldrich, Castle Hill, NSW, Australia) and immediately frozen on dry ice and stored at -80˚C until use. Five retinal and vitreal samples were collected for each light group (n=5 experimental (left) eyes, and n=5 contralateral control (right) eyes. Five control samples, from chickens that had no treatment, were also collected for each light group as described above.

Vitreal samples were collected to allow for the analysis of DOPAC levels, the strongest measure of retinal dopamine release. However, the liquid chromatography-mass spectrometry (LC-MS) system at the University required for undertaking such an analysis suffered numerous catastrophic failures over the course of the study and therefore DOPAC levels could not be measured in time for the printing of this thesis. However, the analysis of DOPAC in the vitreal samples will occur in the near-future.

2.5 Preparation of total RNA and reverse transcription to cDNA

Total RNA was extracted from retinal tissue using a Trizol® / chloroform protocol following the manufacturer’s instructions (Invitrogen (Life Technologies), Mulgrave, VIC, Australia). Briefly, retinal tissue was homogenised in 400 μL of Trizol® reagent and left at room temperature for 5 minutes. Chloroform (100 μL) was gently added and mixed, with the samples incubated at room temperature for a further 3 minutes before centrifugation at 12,000g at 4˚C for 10 minutes, using a CT 15 RT Versatile Refrigerated Centrifuge (Techcomp, Inc., Chesapeake, VA, USA). The supernatant was collected into new Eppendorf° tubes to which equal volumes of 100% chilled isopropanol was added (roughly 400 μL), briefly mixed by pipetting, and left at room temperature for 5 minutes. The samples were centrifuged under the same conditions as described above for 10 minutes, after which the supernatant was removed, leaving a fine pellet containing the total RNA. The pellet was washed with 200 μL of 75% ethanol and immediately removed leaving the pellet to briefly air dry, before being dissolved in 50 μL of nuclease free water (Qiagen, Venlo, The Netherlands) on a heat block at 70˚C for 1 minute.
Total RNA purity was quantified using a Nano drop spectrophotometer (BioLab, Mulgrave, VIC, Australia). Total RNA was reverse-transcribed to first strand cDNA in a 20µL reaction using 4µL 5X VIO™ reaction mix, 2µL 10X SuperScript® Enzyme mix (Invitrogen by Life Technologies, Carlsbad, CA, USA), and 1.5 µg of total RNA, with the final volume made to 20 µL using nuclease free water (Qiagen). Reverse transcription to cDNA was undertaken with the following conditions on a Mastercycler 5333 (Eppendorf, Hamburg, Germany) PCR machine: 10 minutes at 25˚C, 60 minutes at 55˚C, and 5 minutes at 85˚C. Samples were immediately frozen at -20˚C until use.

2.6 Real time RT-PCR

Real-time reverse transcription PCR was undertaken using Fast SYBR green kit in a reaction volume of 15 µL following the manufacturer’s protocol (Invitrogen (Life Technologies), Mulgrave, VIC, Australia). Each reaction contained 1 µL of both the forward (0.5 µM) and reverse (0.5 µM) primers, 1 µL of prepared cDNA (~ 20 ng), 7.5 µL of 2X Fast SYBR Master Mix (Life Technologies, Applied Biosystems, Mulgrave, VIC, Australia) and 5 µL of nuclease free water (Qiagen). All reactions were run on a Stratagene Mx 3005P (Agilent Technologies, Mulgrave, VIC, Australia). The PCR thermo cycling conditions included an initial denaturing phase at 95˚C for 10 minutes, followed by 35 cycles of denaturation at 95˚C for 20 seconds, annealing at 60˚C for 1 minute and finished with an extension phase at 95˚C for 1 minute.

2.7 Primer specificity

All primers used for the detection of specific gene products by RT-PCR were validated by melt-curve analysis (figure 8 and 9) and gel electrophoresis (figure 10). All primers produced a single PCR product of expected size (figure 10 and table 1).

Gel electrophoresis was carried out using a 1% agarose gel (1 g of agarose was dissolved in a final volume of 100 mL of 1x Tris-Acetate- EDTA (TAE) buffer, pH= 8). For each gene (primer pair), 4 µL of amplified PCR product was added to a lane, with the control lane containing 5 µL of Hyperladder I (200-10,000 base pairs) (Bioline, Alexandria, NSW, Australia) for size determination. The gel ran at 100V for 30 minutes. Of the initial 17 genes that were prepared for screening (figure 10), 6 were chosen for the subsequent use (table 1), which specifically included: dopamine D2 receptor (DRD2), tyrosine hydroxylase (TH), Egr-1, cFos, glucagon and retinoic acid receptor Beta (RAR-β), as well as the control genes β-Actin and hypoxanthine phosphoribosyltransferase 1 (HPRT) (figures 8, 9 and table 1). The other eleven primers were not used for the analysis due to time constraints and availability of cDNA and therefore,
priority was given to those genes that have previously shown a distinct change in expression during the development of, or recovery from, FDM.

Primer efficiency ($E$) was determined from the slope of the curve generated through cDNA dilution series (cDNA dilutions: 79.4 ng, 7.94 ng, 0.79 ng and 0.079 ng) (figure 11), using the formula $E = 10^{(-1/slope)}$ (Pfaffl 2001) (table 2).

For determination of changes in transcript (mRNA) levels for each of the six target genes, the Mean Normalised Expression (MNE) of each target gene was calculated separately for each condition (treated and contralateral control) as previously described (Simon 2003, Ashby, Kozulin et al. 2010). The MNE was calculated from the efficiency ($E$) of the target gene to the power of its average threshold cycle (Ct) value ($E^{Ct}$, target), divided by the efficiency ($E$) of the reference gene ($\beta$-Actin and HPRT) to the power of its average Ct value ($E^{Ct}$, reference) (Simon 2003), with the MNE converted into a fold change value for ease of representation (e.g. no change in gene expression is represented by a fold change of 1, a 100% increase in gene expression is represented by a fold change of 2 etc.).
Analysis of tyrosine hydroxylase (TH), beta actin (β-actin) and hypoxanthine phosphoribosyltransferase 1 (HPRT), indicating a single product was obtained.

**Figure 8.** RT-PCR melt (dissociation) curves analysis for the primer pairs of TH, β-actin and HPRT

Analysis of tyrosine hydroxylase (TH), beta actin (β-actin) and hypoxanthine phosphoribosyltransferase 1 (HPRT), indicating a single product was obtained.
Analysis of retinoic acid receptor beta (RAR-β), pre-pro glucagon (PPG), early growth response 1 (Egr-1), dopamine D2 receptor (DRD2) and FBJ osteosarcoma oncogene (cFos), indicating a single product was obtained.
Table 1. Primer information
Information regarding each primer pair, including: GeneBank accession number, forward and reverse primer sequences, as well as product size (bp). Both β-Actin and Hypoxanthine Phosphoribosyltransferase 1 (HPRT) were used initially as control genes.

<table>
<thead>
<tr>
<th>Gene product</th>
<th>GenBank Acc.</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH</td>
<td>NM_204805.1</td>
<td>CAGAATGGGATAGTCAAAGC</td>
<td>TGTAGGGCTCGTATTTCACT</td>
<td>247</td>
</tr>
<tr>
<td>DRD2</td>
<td>NM_001113290.1</td>
<td>GTGGTCTATCTGGAGGTGTT</td>
<td>CTGTGTACCTGTCAATGCTG</td>
<td>133</td>
</tr>
<tr>
<td>RAR-β</td>
<td>NM_205326.1</td>
<td>GAACTCAGAGCACCAGTTC</td>
<td>CACAAGCACTGACACCATAAG</td>
<td>133</td>
</tr>
<tr>
<td>Egr-1</td>
<td>AF026082</td>
<td>ACTAACTCGTCACATCGCA</td>
<td>TGCTGAGACCGAAGCTGCT</td>
<td>241</td>
</tr>
<tr>
<td>PPG</td>
<td>NM_205260.1</td>
<td>AGCGTCATTACAAGGCA</td>
<td>TCAGAATGAGCCTGGAAAT</td>
<td>184</td>
</tr>
<tr>
<td>cFos</td>
<td>NM_205508.1</td>
<td>AGGAGGAGAAAGAGGAG</td>
<td>CTTGACGAGGTTGCTATC</td>
<td>161</td>
</tr>
<tr>
<td>β–Actin</td>
<td>NM_205518.1</td>
<td>TAAGGATCTGTATGCAAACAGT</td>
<td>GACAATGGAGGTCGGTTATGCT</td>
<td>241</td>
</tr>
<tr>
<td>HPRT</td>
<td>NM_204848.1</td>
<td>TTACGACCTGGACTTGTCT</td>
<td>GGATTTGACTTGTCACTGTT</td>
<td>234</td>
</tr>
</tbody>
</table>

Figure 10. Gel electrophoresis for all amplified gene products
A single product was obtained for each primer of expected band size (247, 133, 133, 241, 184, 161 and 241 bp for Tyrosine Hydroxylase (TH), Dopamine D2 receptor (DRD2), Retinoic acid receptor β (RAR-β), Early growth response 1 (Egr-1), Preproglucagon (PPG), FBJ osteosarcoma oncogene (cFos)and β-Actin respectively)
Figure 11. Primer efficiencies for DRD2, Egr-1, RAR-β, cFos, PPG and TH

Primers were run on RT-PCR with different cDNA concentrations (79.4 ng, 7.9 ng, 0.79 ng and 0.079 ng). The average Ct values were taken and plotted against the log concentration (ng) of cDNA to obtain their slope to determine the efficiency shown in table 2.

Table 2. Primer efficiency

Determined slope and efficiency ($E$) for each primer pair calculated from the formula $E = 10^{(-1/slope)}$

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Slope</th>
<th>Efficiency ($E$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH</td>
<td>-3.763</td>
<td>1.84</td>
</tr>
<tr>
<td>DRD2</td>
<td>-1.163</td>
<td>2.00</td>
</tr>
<tr>
<td>RAR-β</td>
<td>-3.493</td>
<td>1.93</td>
</tr>
<tr>
<td>Egr-1</td>
<td>-3.121</td>
<td>2.00</td>
</tr>
<tr>
<td>PPG</td>
<td>-3.450</td>
<td>1.95</td>
</tr>
<tr>
<td>cFos</td>
<td>-3.409</td>
<td>1.96</td>
</tr>
<tr>
<td>β Actin</td>
<td>-3.070</td>
<td>2.00</td>
</tr>
<tr>
<td>HPRT</td>
<td>-3.058</td>
<td>2.00</td>
</tr>
</tbody>
</table>
2.8 Statistical analysis

All results are presented as the mean ± the standard error of the mean. For the analysis of changes in ocular parameters, a multivariate analysis of variance (MANOVA), with repeat measures design, was used to compare between conditions (experimental, contralateral and age-matched control) over time. A one-way ANOVA, followed by a student’s unpaired t-test, with Bonferroni correction for multiple testing, was used to analyse between group values at specific treatment times. Similarly, for analysis of changes in gene expression between experimental, contralateral control and age-matched untreated control samples, a one-way ANOVA, followed by a student’s unpaired t-test, with Bonferroni correction for multiple testing, was used. All analyses were undertaken in SPSS (IBM, Armonk, NY, USA).
Chapter 3: Results

Aim 1: Correlation between light intensity and the development of form-deprivation myopia over time

To assess whether there is a correlation between light intensity and the protective effects afforded against the development of form deprivation-myopia, chicks were fitted with translucent diffusers for a period of 7 days and exposed to one of five light intensities (500 lux (group 5, mimicking the intensity experienced indoors), 10,000 lux (group 4), 20,000 lux (group 3), 30,000 lux (group 2) and 40,000 lux (group 1)). Refraction was measured daily for both treated and untreated age-matched control animals. Axial length measurements were taken at the start of the experimental period (day 0) as well as on the second last day (day 6) for both treated and untreated age-matched control animals.

3.1 Changes in refraction over time in response to exposure to different light intensities

3.1.1 Refractive changes in age-matched control eyes over seven days in response to different light intensities

Figure 12 and table 3 illustrate the data for refraction in age-matched control eyes. There was no significant difference in the refractive development of chicks that were exposed daily, for seven days, to one of four high-light intensities for six hours per day (10,000 lux (group 4), 20,000 lux (group 3), 30,000 lux (group 2) or 40,000 lux (group 1)), or when compared to those animals reared continuously under 500 lux (F= 2.18, df= 4, 0.51, p= 0.089).

3.1.2 Refractive changes in contralateral control eyes over seven days in response to different light intensities

Figure 13 and table 4 show the data for refraction in contralateral control eyes. There was no significant change in the contralateral control eyes of chicks fitted with translucent diffusers and exposed daily, for seven days, to one of four high-light intensities for six hours per day (10,000 lux, 20,000 lux, 30,000 lux or 40,000 lux) when comparing between the different high-light groups (F= 1.53, df= 4, 152, p= 0.066), or when compared to those animals reared continuously under 500 lux (p= 0.32). Refractive development in contralateral control eyes also did not significantly change over time compared to age-matched untreated control animals under any light intensity (F= 1.406, df= 4, 0.47, p= 0.24).
3.1.3 Refractive changes in diffuser-treated eyes over seven days in response to different light intensities

Figure 15 shows that, over time, light had a significant effect on myopic refractive development in chicks fitted with translucent diffusers (F= 4.96, df= 35, 290, p< 0.0001). Changes in refraction over time, in response to diffuser-wear, regressed strongly with light intensity and were best modelled by a logarithmic relationship (figure 16) (y= -3.59 log10(x) + 17.52; r² = 0.95). The percentage variation explained by the regression was 95.0%. From this analysis it can be seen that chickens raised in normal indoor light (500 lux) developed FDM to the greatest extent (Δ8.08 D over 7 days, final refraction −4.21 ± 0.17 D), while those chickens fitted with diffusers and exposed to 40,000 lux, for 6 hours per day, displayed a hyperopic refraction value (Δ0.78 D over 7 days, final refraction +3.97± 0.11 D).

Consistent with previous studies, chicks with translucent diffusers reared continuously under 500 lux developed significant amounts of myopia (Δ8.08 D over 7 days), compared to either their contralateral control eye (Δ0.09 D over 7 days; F= 596, df= 1, 729, p<0.0001) or age-matched untreated values (Δ0.08 D over 7 days; F= 559, df= 1, 641, p<0.0001). Diffuser treated eyes displayed a final refraction of −4.21 ± 0.17 D (figure 15 table 4), on day 7, which was significantly different to that seen for the contralateral control eye (figure 13 E) (+3.78 ± 0.06 D; p<0.0001), or for age-matched untreated eyes (table 3) (+3.63 ± 0.21 D; p<0.0001).

Daily exposure to 40,000 lux for a period of 7 days, abolished the development of FDM, with the refractive change seen in the 40,000 lux diffuser-treated group (Δ 0.78 D) showing no statistical difference to the refractive changes seen in either contralateral control eyes (Δ0.05 D; F= 2.14, df= 1, 0.91, p= 0.09), or age-matched untreated eyes (Δ0.08 D; F= 2.74, df= 1, 0.61, p= 0.11). In contrast, there was a significant difference in the refractive development of diffuser treated eyes of chicks raised under 40,000 lux (Δ0.78 D) compared to myopic refraction that developed in the treated eye of chicks raised under 500 lux (Δ8.08 D; F= 529, df= 1, 591, p<0.0001). The diffuser treated eyes of chicks exposed to 40,000 lux daily displayed a hyperopic refraction at the end of the experimental period (+2.97 ± 0.11 D) (figure 14 A) that was not statistically dissimilar to the final refractive values seen in the contralateral control eye (+3.70 ± 0.04 D, p=0.09) (figure 4) or age-matched untreated eyes (+3.48 ± 0.09 D, p=0.11) (figure 12 A), but that was significantly different from the myopic refraction seen in the treated eyes of birds kept under 500 lux (−4.21 ± 0.17 D, p<0.0001).

Chickens raised under 30,000 lux showed the second highest reduction in the development of FDM over 7 days (Δ 1.82 D), showing a final mean hyperopic refractive value of +2.06 ± 0.18 D (figure 14 B) that was significantly different from the final myopic refraction of −4.21 ± 0.17 D...
in the 500 lux treated group (p<0.0005). The final refractive values for chickens exposed to 30,000 lux were significantly different to age matched untreated animals (figure 12 B; +3.63 ± 0.14 D, p<0.0001) as well as to contralateral control values (figure 13 B) (+3.83 ± 0.06 D, p<0.0001) as well as to chickens raised in 40,000 lux (+2.97 ± 0.11 D, p<0.02). The final refraction of the 30,000 lux group was, however, not significantly different to those chickens kept under 20,000 lux (+2.06 ± 0.18 D vs +1.60 ± 0.08 D, p= 0.26) (tables 3 and 4).

Daily exposure to 20,000 lux for 6 hours per day, was also protective against the development of FDM (Δ2.38 D) compared to the refractive changes seen in the 500 lux group (Δ8.08 D over 7 days; F= 124, df= 1, 188, p<0.0001). The protective effect of 20,000 lux over time (Δ2.38 D), was not as powerful as that seen under either 40,000 lux or 30,000 lux, with a greater relative myopic shift seen when compared to the refractive changes seen in either the contralateral control eye (Δ0.06 D; F= 338, df= 1, 64, p<0.001) or age-matched untreated eyes (Δ0.08 D; F= 316, df= 1, 60, p<0.001). Diffuser treated eyes, exposed to 20,000lux, displayed a final refraction of +1.60 ± 0.08 D, on day 7 (figure 14 C), compared to +3.90 ± 0.12 D for the contralateral control eye (figure 13 C), and +3.73 ± 0.32 D for age-matched untreated eyes (figure 12 C) (tables 3 and 4).

Chickens exposed to 10,000 lux for a period of 6 hours per day, also showed significant reduction in the development of FDM over time (Δ 3.41 D) compared to those animals reared under 500 lux (Δ 0.08 D; F= 84.9, df= 1, 107, p<0.001).The protective effect of 10,000 lux over time, however, was not as protective against the development of FDM as that of the other high light intensities. Diffuser treated eyes displayed a final refraction of +0.43 ± 0.05 D, on day 7 (figure 14 D), compared to +3.87 ± 0.04 D for the contralateral control eye (figure 13 D) (p<0.001), and +3.88 ± 0.06 D for age-matched untreated eyes (figure 12 D) (tables 3 and 4, p<0.001).
### Table 3. Refraction data for age-matched untreated control eyes for all light intensities

Refraction (RE, n=12) for age-matched untreated control animals for all light intensities

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LI (lux)</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Change RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40,000</td>
<td>3.73</td>
<td>3.72</td>
<td>3.66</td>
<td>3.64</td>
<td>3.70</td>
<td>3.59</td>
<td>3.61</td>
<td>3.48</td>
<td>0.25</td>
</tr>
<tr>
<td>Control</td>
<td>30,000</td>
<td>3.61</td>
<td>3.56</td>
<td>3.65</td>
<td>3.67</td>
<td>3.64</td>
<td>3.61</td>
<td>3.68</td>
<td>3.65</td>
<td>0.04</td>
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<tr>
<td>Control</td>
<td>20,000</td>
<td>3.97</td>
<td>3.97</td>
<td>4.05</td>
<td>3.78</td>
<td>3.78</td>
<td>3.68</td>
<td>3.45</td>
<td>3.73</td>
<td>0.24</td>
</tr>
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<td>3.86</td>
<td>3.82</td>
<td>3.87</td>
<td>3.88</td>
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<td>3.73</td>
<td>3.65</td>
<td>3.73</td>
<td>3.63</td>
<td>0.08</td>
</tr>
</tbody>
</table>

### Table 4. Refraction data for diffuser-treated and contralateral control eyes for all light intensities

Refraction (RE, n=12) for diffuser-treated and contralateral control eyes for all light intensities

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LI (lux)</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Change RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant Diff.</td>
<td>40,000</td>
<td>3.75</td>
<td>3.56</td>
<td>3.38</td>
<td>3.05</td>
<td>3.00</td>
<td>3.05</td>
<td>3.05</td>
<td>2.97</td>
<td>0.78</td>
</tr>
<tr>
<td>Contralateral</td>
<td>40,000</td>
<td>3.75</td>
<td>3.76</td>
<td>3.76</td>
<td>3.76</td>
<td>3.82</td>
<td>3.83</td>
<td>3.89</td>
<td>3.70</td>
<td>0.05</td>
</tr>
<tr>
<td>Constant Diff.</td>
<td>30,000</td>
<td>3.88</td>
<td>2.98</td>
<td>2.61</td>
<td>2.42</td>
<td>2.34</td>
<td>2.33</td>
<td>2.06</td>
<td>1.82</td>
<td>0.05</td>
</tr>
<tr>
<td>Contralateral</td>
<td>30,000</td>
<td>3.88</td>
<td>3.84</td>
<td>3.81</td>
<td>3.87</td>
<td>3.88</td>
<td>3.88</td>
<td>3.86</td>
<td>3.83</td>
<td>0.05</td>
</tr>
<tr>
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<td>3.98</td>
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<td>1.95</td>
<td>1.99</td>
<td>1.76</td>
<td>1.80</td>
<td>1.86</td>
<td>1.60</td>
<td>2.38</td>
</tr>
<tr>
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<td>3.79</td>
<td>3.77</td>
<td>3.69</td>
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<td>3.92</td>
<td>3.98</td>
<td>3.90</td>
<td>0.06</td>
</tr>
<tr>
<td>Constant Diff.</td>
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<td>3.83</td>
<td>2.28</td>
<td>1.40</td>
<td>1.09</td>
<td>0.52</td>
<td>0.26</td>
<td>0.29</td>
<td>0.43</td>
<td>3.41</td>
</tr>
<tr>
<td>Contralateral</td>
<td>10,000</td>
<td>3.84</td>
<td>3.83</td>
<td>3.86</td>
<td>3.85</td>
<td>3.85</td>
<td>3.85</td>
<td>3.86</td>
<td>3.87</td>
<td>0.03</td>
</tr>
<tr>
<td>Constant Diff.</td>
<td>500</td>
<td>3.87</td>
<td>2.00</td>
<td>1.28</td>
<td>-0.44</td>
<td>-1.73</td>
<td>-2.85</td>
<td>-3.86</td>
<td>-4.21</td>
<td>8.08</td>
</tr>
<tr>
<td>Contralateral</td>
<td>500</td>
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<td>3.94</td>
<td>3.95</td>
<td>3.88</td>
<td>3.94</td>
<td>3.97</td>
<td>3.88</td>
<td>3.78</td>
<td>0.09</td>
</tr>
<tr>
<td>Untreated</td>
<td>500</td>
<td>3.71</td>
<td>3.59</td>
<td>3.66</td>
<td>3.6</td>
<td>3.73</td>
<td>3.65</td>
<td>3.73</td>
<td>3.63</td>
<td>0.08</td>
</tr>
</tbody>
</table>
Figure 12. Change in refraction, over seven days, in age-matched untreated control eyes reared under different light intensities
Untreated age-matched controls (A) under 40,000 lux, (B) under 30,000 lux, (C) under 20,000 lux, (D) under 10,000 lux, and (E) for all light intensities
A. Contralateral Control - 40,000 lux

B. Contralateral Control - 30,000 lux

C. Contralateral Control - 20,000 lux

D. Contralateral Control - 10,000 lux

E. Contralateral Control - 500 lux

F. Contralateral Control - Combined

Figure 13. Change in refraction, over seven days, in contralateral control eyes reared under different light intensities
Contralateral controls (A) under 40,000 lux, (B) under 30,000 lux, (C) under 20,000 lux, (D) under 10,000 lux, (E) under 500 lux, and (F) for all light intensities
Figure 14. Change in refraction, over seven days, in form-deprived eyes reared under different light intensities.
Form-deprived chicks (A) under 40,000 lux, (B) under 30,000 lux, (C) under 20,000 lux, and (D) under 10,000 lux.
Significant myopia was developed in chickens with translucent diffusers raised in 500 lux (normal indoor light levels) with increased protection seen with increased light intensities compared to untreated chickens raised in 500 lux.

Figure 15. Combined refractive data, over seven days, from form-deprived eyes reared under different light intensities

Significant myopia was developed in chickens with translucent diffusers raised in 500 lux (normal indoor light levels) with increased protection seen with increased light intensities compared to untreated chickens raised in 500 lux.
Overall changes in refraction regressed strongly with light intensity, and are best modelled by a logarithmic relationship. The percentage variation explained by the regression was 95.0%.

Figure 16. Logarithmic non-linear regression curve of light intensity versus absolute change in refraction over seven days
3.2 Changes in axial length over time in response to different light intensities

3.2.1 Axial length changes in age-matched control and contralateral control eyes over six days in response to different light intensities

Figure 17 A and table 5 illustrate the data for age-matched untreated control eyes. There was no significant difference in the axial length for age-matched untreated control chicks that were exposed daily, for seven days, for six hours per day to one of four high-light intensities (10,000 lux (group 4), 20,000 lux (group 3), 30,000 lux (group 2) or 40,000 lux (group 1)), when comparing between the different high-light groups, or when compared to those animals reared continuously under 500 lux (group 5)\((F=3.923, \ df=4, \ 0.038 \ p=0.016)\).

Similarly, as seen in figure 17 B and table 6, there was no significant difference in the axial length for the contralateral control eyes of chicks that were exposed daily, for seven days, to one of four high-light intensities for six hours per day (10,000 lux (group 4), 20,000 lux (group 3), 30,000 lux (group 2) or 40,000 lux (group 1)), when comparing between the different high-light groups, or when compared to those animals reared continuously under 500 lux (group 5), as well as comparing to age-matched untreated control values \((F=1.988, \ df=4, \ 0.021, \ p=0.134)\).

3.2.2 Axial length changes in diffuser-treated eyes over six days in response to different light intensities

Figure 18 shows that, over time, light intensity had a significant effect on axial length for diffuser-treated animals \((F=4.832, \ df=10, \ 76, \ p<0.0001)\), with chicks kept under 500 lux displaying the largest changes in axial length \((\Delta0.85 \ mm \ over \ 7 \ days)\), while those exposed to 40,000 lux displayed the smallest change \((\Delta0.17 \ mm \ over \ 7 \ days)\). Following six days of diffuser-wear, chicks kept under 40,000 lux displayed the shortest eyes \((8.77 \pm 0.03 \ mm)\) (table 6), followed by the 30,000 lux group \((9.05 \pm 0.07 \ mm)\), 20,000 lux \((9.19 \pm 0.12 \ mm)\), 10,000 lux \((9.26 \pm 0.11 \ mm)\) and finally the 500 lux group \((9.35 \pm 0.07 \ mm)\). Only the treated eyes of animals exposed to 40,000 lux showed an axial length that was statistically similar in length to contralateral control values \((p=0.87)\), or age-matched untreated values \((p=0.78)\).
Table 5. Axial length data for age-matched untreated control eyes for all light intensities
Axial length (AL, n=12) for age-matched untreated control animals for all light intensities

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LI (lux)</th>
<th>AL Day 0</th>
<th>AL Day 6</th>
<th>Change AL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40,000</td>
<td>8.61</td>
<td>8.65</td>
<td>0.04</td>
</tr>
<tr>
<td>Control</td>
<td>30,000</td>
<td>8.58</td>
<td>8.74</td>
<td>0.16</td>
</tr>
<tr>
<td>Control</td>
<td>20,000</td>
<td>8.56</td>
<td>8.91</td>
<td>0.35</td>
</tr>
<tr>
<td>Control</td>
<td>10,000</td>
<td>8.58</td>
<td>8.87</td>
<td>0.29</td>
</tr>
<tr>
<td>Control</td>
<td>500</td>
<td>8.50</td>
<td>8.62</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Table 6. Axial length data for diffuser-treated and contralateral control eyes for all light intensities
Axial length (AL, n=12) for diffuser-treated and contralateral control eyes for all light intensities

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LI (lux)</th>
<th>AL Day 0</th>
<th>AL Day 6</th>
<th>Change AL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant Diff.</td>
<td>40,000</td>
<td>8.61</td>
<td>8.77</td>
<td>0.16</td>
</tr>
<tr>
<td>Constant Diff.</td>
<td>30,000</td>
<td>8.58</td>
<td>9.05</td>
<td>0.47</td>
</tr>
<tr>
<td>Constant Diff.</td>
<td>30,000</td>
<td>8.58</td>
<td>8.74</td>
<td>0.16</td>
</tr>
<tr>
<td>Constant Diff.</td>
<td>20,000</td>
<td>8.56</td>
<td>9.19</td>
<td>0.63</td>
</tr>
<tr>
<td>Constant Diff.</td>
<td>20,000</td>
<td>8.56</td>
<td>8.91</td>
<td>0.35</td>
</tr>
<tr>
<td>Constant Diff.</td>
<td>10,000</td>
<td>8.58</td>
<td>9.26</td>
<td>0.68</td>
</tr>
<tr>
<td>Constant Diff.</td>
<td>10,000</td>
<td>8.58</td>
<td>8.79</td>
<td>0.21</td>
</tr>
<tr>
<td>Constant Diff.</td>
<td>500</td>
<td>8.50</td>
<td>9.35</td>
<td>0.85</td>
</tr>
<tr>
<td>Constant Diff.</td>
<td>500</td>
<td>8.50</td>
<td>8.67</td>
<td>0.17</td>
</tr>
<tr>
<td>Untreated</td>
<td>500</td>
<td>8.50</td>
<td>8.62</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Figure 17. Changes in axial length for age-matched untreated (A) and contralateral control (B) eyes over six days under different light intensities.
Figure 18. Changes in axial length for diffuser-treated eyes over six days under different light intensities.
**Aim 2: Can exposure to bright light prevent the progression of deprivation-myopia?**

To investigate whether the progression of deprivation-myopia, through axial elongation, could be prevented, chicks were fitted with translucent diffusers for a period of 10 days and were split into one of three conditions: 1. fitted with translucent diffusers and raised under 500 lux (FDM group), 2. fitted with translucent diffusers and exposed to 40,000 lux for 6 hours per day (high light group), 3. fitted with translucent diffusers and initially kept in 500 lux for the first three days, before being switched to daily exposure to 40,000 lux for the remaining seven days (FDM progression group). Refraction was measured daily for both treated and untreated age-matched control animals. Axial length measurements were taken at the start of the experimental period (day 0) as well as on the second last day (day 9) for both treated and untreated age-matched control animals.

### 3.3 Changes in refraction over time in response to transfer to high light

#### 3.3.1 Refractive changes in contralateral control eyes over ten days in response to transfer to high light

Over time, there was no significant difference in the refractive development for chicks that were exposed daily, for ten days, to either 500 lux (FDM group), 40,000 lux (high light group) or 500/40,000 lux (FDM progression group) in either the contralateral control eyes or when compared to age-matched untreated eyes (F= 0.003, df= 1, 0.002, p= 0.95) (table 7).

#### 3.3.2 Refractive changes in diffuser-treated eyes over ten days in response to transfer to high light

Chicks reared continuously under 500 lux, with the attachment of translucent diffusers, developed significant amounts of myopia (Δ8.16 D over 10 days), compared to refractive changes in contralateral control (Δ0.08 D over 10 days; F= 1601, df= 1, 1644, p<0.0001) or age-matched untreated animals (Δ0.32 D; F= 1472, df= 1, 1600, p<0.0001). Diffuser treated eyes displayed a final refraction of −4.47 ± 0.21 D (figure 19 and table 7), on day 10, compared to +3.69 ± 0.03 D contralateral control eyes (table 7) and +3.39 ± 0.01 D for age-matched untreated eyes (figure 19).

As in aim 1, the development of deprivation-myopia was abolished in chickens raised under 40,000 lux for 6 hours per day during the light period (Δ0.89 D), and was statistically different compared to contralateral control values (Δ0.09 D over 10 days; F= 89, df= 1, 39, p<0.0001) or age-matched untreated values (F= 83, df= 1, 38, p<0.0001). Diffuser treated eyes displayed a final refraction of +2.86 ± 0.10 D (figure 19 and table 7), on day 10, which was not statistically
different from refractive values seen in contralateral control eyes (+3.69 ± 0.03 D, p<0.0001) or age-matched untreated controls (+3.39 ± 0.01 D, p<0.0001).

The progression group, as illustrated in figure 19 and table 7, showed a significant relative myopic shift over the initial three days of diffuser-wear under 500 lux (Δ4.89 D over three days, refraction of −1.17 ± 0.21 D on day 3). Following transfer to 40,000 lux, these animals showed an initial hyperopic shift over the first two days (Δ1.17 D, −0.00 ± 0.03 D), before refractive changes stabilized (plateaued) around emmetropia for the following five days (Δ0.16 D, final refraction of +0.16 ± 0.21 D). Chickens switched to 40,000 lux (FDM progression group) did not show as large a refractive change (Δ 3.56 D) as those kept continuously under 500 lux (Δ 8.16 D), but a larger refractive change compared to those exposed to 40,000 lux (Δ 0.89 D) for the entire experimental period.
<table>
<thead>
<tr>
<th>Treatment Type</th>
<th>Constant Diff. 40,000</th>
<th>Contralateral</th>
<th>Constant Diff. Progression</th>
<th>Contralateral Progression</th>
<th>Constant Diff. 500</th>
<th>Contralateral</th>
<th>Untreated 500</th>
<th>Change RE</th>
</tr>
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<tr>
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<td>3.71</td>
<td>3.84</td>
<td>3.69</td>
<td>3.90</td>
<td>3.71</td>
<td>0.89</td>
</tr>
<tr>
<td>Day 0</td>
<td>3.75</td>
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<td>2.44</td>
<td>3.84</td>
<td>3.69</td>
<td>3.90</td>
<td>3.71</td>
<td>0.89</td>
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<td>Day 1</td>
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<td>3.86</td>
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<td>3.97</td>
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<td>3.93</td>
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<td>0.00</td>
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<td>3.99</td>
<td>3.73</td>
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<td>Day 5</td>
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<td>-0.01</td>
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<td>-3.46</td>
<td>3.99</td>
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<td>Day 8</td>
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<td>0.19</td>
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<td>3.99</td>
<td>3.44</td>
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<td>Day 9</td>
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<td>3.84</td>
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<td>3.39</td>
<td>0.32</td>
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<tr>
<td>Day 10</td>
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<td>3.69</td>
<td>3.56</td>
<td>3.92</td>
<td>8.16</td>
<td>3.92</td>
<td>3.91</td>
<td>0.01</td>
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</tbody>
</table>

Table 7. Changes in refraction for form-deprived animals exposed to 500 lux, 40,000 lux or 500/40,000 lux.
The progression group (orange) showed a slight hyperopic shift following transfer to 40,000 lux before stabilising.

Figure 19. Changes in refraction, over time, for form-deprived animals exposed to 500 lux, 40,000 lux or 500/40,000 lux.

The progression group (orange) showed a slight hyperopic shift following transfer to 40,000 lux before...
3.4 Changes in axial length over time in response to transfer to high light

3.4.1 Axial length changes in contralateral control eyes over nine days in response to transfer to high light

Table 8 illustrate the data for axial length in contralateral control eyes. There was no significant difference in axial length for chicks that were exposed daily, for ten days, to either 500 lux (FDM group), 40,000 lux (high light group) or 500/40,000 lux (FDM progression group) compared to age-matched untreated eyes ($F= 0.122, df= 2, 0.002, p= 0.887$).

3.4.2 Axial length changes in diffuser-treated eyes over nine days in response to transfer to high light

Consistent with aim 1, chicks exposed to 500 lux, displayed the longest eyes of any of the treatment groups ($\Delta 0.99$ mm over 9 days) and was significantly different compared to contralateral control ($\Delta 0.32$ mm over 9 days, $F= 38.89, df= 1, 0.429, p<0.0001$) and age-matched untreated values ($\Delta 0.34$ mm over 9 days, $F= 19.847, df= 1, 0.580, p<0.0001$). Diffuser treated eyes displayed a final axial length of $9.54 \pm 0.13$ mm (figure 20 and table 8), on day 9, compared to $8.90 \pm 0.04$ mm for contralateral control eyes and $8.93 \pm 0.07$ mm for age-matched untreated eyes.

Chickens exposed daily to 40,000 lux displayed the smallest change in axial length of the treated groups ($\Delta 0.47$ mm over 9 days) and was not significantly different compared to contralateral control eyes ($\Delta 0.31$ mm over 9 days, $F= 0.983, df= 1, 0.035, p= 0.351$) or age-matched untreated eyes ($\Delta 0.34$ mm over 9 days, $F= 0.592, df= 1, 0.026, p= 0.456$). Diffuser treated eyes displayed a final axial length of $9.05 \pm 0.15$ mm (figure 20 and table 8), on day 9, compared to $8.89 \pm 0.09$ mm for contralateral control eyes and $8.93 \pm 0.07$ mm for age-matched untreated eyes.

The progression group, illustrated in figure 20 and table 8, showed a smaller change in axial length ($\Delta 0.74$ mm), although not statistically different, compared to chicks kept under 500 lux ($\Delta 0.99$ mm, $p=0.06$), but a larger change compared to those animals kept under 40,000 lux ($\Delta 0.47$ mm, $p= 0.003$). Chicks in the progression group displayed a final axial length of $9.35 \pm 0.13$, on day 9, compared to $8.97 \pm 0.12$ mm for their contralateral control eyes.
Table 8. Changes in axial length for form-deprived animals exposed to 500 lux, 40,000 lux or 500/40,000 lux

Changes in axial length (AL) for form-deprived animals exposed to 500 lux, 40,000 lux or 500/40,000 lux

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LI (lux)</th>
<th>AL Day 0</th>
<th>AL Day 9</th>
<th>Change AL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant Diff. 40,000</td>
<td>8.58</td>
<td>9.05</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>Contralateral 40,000</td>
<td>8.58</td>
<td>8.89</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Constant Diff. Progression 8.61</td>
<td>9.35</td>
<td>0.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contralateral Progression 8.59</td>
<td>8.97</td>
<td>0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constant Diff. 500</td>
<td>8.55</td>
<td>9.54</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Contralateral 500</td>
<td>8.58</td>
<td>8.90</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Untreated 500</td>
<td>8.58</td>
<td>8.93</td>
<td>0.35</td>
<td></td>
</tr>
</tbody>
</table>

Figure 20. Changes in axial length, over time, for form-deprived animals exposed to 500 lux, 40,000 lux or 500/40,000 lux
**Aim 3: What are the biochemical pathways that are associated with the protective effects of high light?**

To begin to assess which biochemical pathways are associated with the ability of bright light to retard the development of deprivation-myopia, chicks were fitted with translucent diffusers for a period of one day and placed under either 500 lux (mimicking the intensity experienced indoors) or 40,000 lux. Retinal samples were collected on day 2 (after one full light period) to observe early changes in genes expression.

**3.5 Molecular changes following one day of form-deprivation myopia in 40,000 lux**

One day of diffuser-wear induced a significant down-regulation in Egr-1 (p<0.01), glucagon (p<0.01) and cFos (p<0.001) mRNA levels compared to contralateral control values in animals reared under 500 lux (figure 21 A, B and E respectively). This down-regulation in mRNA levels, in response to diffuser-wear, was abolished by exposure to high light (40,000 lux) for Egr-1 (p=0.42), but was not abolished for either glucagon (p<0.01) or cFos (p<0.001) (figure 3.10 A, B and E respectively). High light exposure had a significant effect on Egr-1 expression in contralateral control eyes compared to those values seen in the control eyes of 500 lux treated animals (p<0.05). In contrast, high light exposure did not alter the retinal expression of glucagon, cFos, dopamine D2 receptor, tyrosine hydroxylase or retinoic acid receptor beta in control eyes compared to those values seen at 500 lux. There was no significant change in expression in response to diffuser-wear or high light treatments for dopamine D2 receptor (p=0.23 and p= 0.89 respectively), tyrosine hydroxylase (p= 0.54 and p= 0.15 respectively) and retinoic acid receptor beta (p= 0.59 and p= 0.29 respectively).
Mean normalised expression in fold change in mRNA levels following one day of form-deprivation myopia (FDM) under normal laboratory light (500 lux) and 40,000 lux, with contralateral internal controls for Egr-1 (A), Glucagon (B), Dopamine D2 Receptor (DRD2) (C), Tyrosine Hydroxylase (D), cFos (E) and RAR-β (F). Significant down-regulation in Egr-1 following FDM in 500 lux, while 40,000 lux retards this down-regulation. Glucagon, DRD2 and cFos are also down-regulated through FDM in 500 lux.

Figure 21. Mean normalised expressions in fold change in mRNA levels of primers
Chapter 4: Discussion

During development, ocular growth is regulated through visually-guided homeostatic mechanisms to maintain a match between the axial length of the eye and its optical power, so that the light rays from objects viewed at a distance are focused clearly on the retina. Myopia occurs through a mismatch between the optical powers of the eye and the axial length, due, for the most part, to excessive elongation of the eye during development. This causes light rays to be focused in front of the retina instead of on it, leading to images appearing blurred. In a promising finding, recent epidemiological studies have indicated that children spending greater time outdoors are less likely to develop myopia. It has been postulated that the protective effects of time outdoors may be driven by light-stimulated dopamine release associated with the higher illumination levels experienced outside. In support of this hypothesis, animal studies have shown that exposure to high light levels (~15,000 lux), for 5 hours per day, can retard the development of experimental myopia by roughly 60% (Ashby and Schaeffel 2010). This study examined whether greater protection against the development of FDM could be achieved by exposing animals to even higher light intensities (>15,000 lux). The results of this study show that there is indeed a significant correlation between the light intensity to which animals are exposed and the degree to which FDM is prevented, with full protection achievable under 40,000 lux (figure 15). This study was also concerned with whether exposure to bright light could prevent the progression of FDM, or whether light was only capable of modulating the onset of myopia. The results indicate that exposure to bright light (40,000 lux) can halt further progression of FDM (figure 19), although a longer time-course is required to determine the full extent of this effect. Finally, this thesis provided a first look at the possible molecular pathways that underlie the protective effects of high light and observed that the down-regulation in the mRNA levels of the immediate early gene Egr-1, during development of FDM under 500 lux, is abolished by daily exposure to bright light (40,000 lux) (figure 21).

4.1 Exposure to high light intensities and the development of deprivation-myopia

Due to the rapid increase in the prevalence of myopia, especially in urban East Asia (Morgan, Ohno-Matsui et al. 2012), and the chorio-retinal pathologies associated with excessive axial elongation seen in high myopes (Leske, Chylack et al. 1991, Vongphanit, Mitchell et al. 2002, Chou, Yang et al. 2007, Resnikoff, Pascolini et al. 2008, Marcus, de Vries et al. 2011, Morgan, Ohno-Matsui et al. 2012), it is crucial that treatment options are found to prevent this refractive disorder. Recent epidemiological studies have suggested that spending more time outdoors has a protective effect against the development of myopia (Mutti, Mitchell et al.
2002, Jones, Sinnott et al. 2007, Rose, Morgan et al. 2008, Dirani, Tong et al. 2009, Deng, Gwiazda et al. 2010, Guggenheim, Northstone et al. 2012), with children who spend the least amount of time on near-work activities and the greatest amount of time outdoors displaying the most mean hyperopic refraction (Rose, Morgan et al. 2008). As previously discussed, it was initially suggested that time outdoors may simply provide protection through a substitution effect, in which the more time children spend outdoors, the less time they spend on near-work tasks such as reading, which has previously been correlated with the development of myopia (Mutti, Mitchell et al. 2002, Jones, Sinnott et al. 2007). However, a later study by Rose et al. (2008) reported that a less myopic refraction was still observed in children who spent considerable time outdoors even if they also engaged in significant amounts of near-work. Instead, the authors hypothesised that rather than a substitution effect, the protective effects of time outdoors may be driven by light-stimulated dopamine release associated with the higher illumination levels experienced outside. As has been discussed, supporting this hypothesis, animal models have shown that the development of experimental myopia can be retarded by exposure to high light levels (Ashby, Ohlendorf et al. 2009, Ashby and Schaeffel 2010, Cohen, Belkin et al. 2011, Siegwart, Ward et al. 2012, Smith, Hung et al. 2012), with this protective effect abolished in chickens by the injection of the dopamine D2 receptor antagonist spiperone (Ashby and Schaeffel 2010). In chicks, the development of deprivation-myopia can be retarded by approximately 60% through daily exposure to 15,000 lux, compared to those animals reared under 500 lux (Ashby, Ohlendorf et al. 2009). The aim of this study was to determine if greater protection could be achieved with higher light intensities (>15,000 lux). Supporting this hypothesis, the protective effects afforded by brief periods of normal vision (15 minutes), against the development of FDM (Napper, Brennan et al. 1995, Napper, Brennan et al. 1997), are enhanced proportionally to the light intensity in which the animals are exposed to during the diffuser free period (~48% reduction in deprivation-myopia under 500 lux, ~62% reduction in deprivation-myopia under 15,000 lux, ~79% reduction in deprivation-myopia under 30,000 lux) (Ashby, Ohlendorf et al. 2009). Further, Cohen and colleagues (2011) have reported that the normal emmetropisation process in chicks appears to be, at least in part, intensity dependent. Specifically, the authors observed that chicks reared for 90 days under an illumination level of 50 lux, during the light phase, show a slight mean myopic refraction by the end of the experimental period (~2.41 D). In contrast, chicks reared under 500 lux were emmetropic by day 90 (+0.03 D), but were possibly heading myopic, while those reared under 10,000 lux remained hyperopic (+1.1 D). Therefore, this study investigated whether deprivation-myopia could be further retarded, above that previously reported by Ashby and colleagues (2010), by exposing chickens to higher light intensities (up to 40,000 lux). The results of this study indicate that a negative correlation existed between the development
of deprivation-myopia and the intensity of light to which the animals were exposed. The refractive changes induced by the fitment of translucent diffusers regressed strongly with the intensity of light to which the animals were exposed and was best described by a logarithmic non-linear relationship ($r^2 = 0.95$). Specifically, the development of deprivation-myopia was retarded to a greater extent with each increasing light intensity (~ 60% reduction in deprivation-myopia under 10,000 lux, ~ 70% reduction in deprivation-myopia under 20,000 lux, ~ 80% reduction in deprivation-myopia under 30,000 lux), to the point that the development of deprivation-myopia was abolished in chickens exposed to 40,000 lux when compared to the ocular development observed in either contralateral control eyes or age-matched untreated animals. In contrast to that reported recently in tree shrews (Siegwart, Ward et al. 2012) and rhesus monkeys (Smith, Hung et al. 2012), contralateral control and age-matched untreated eyes did not show a relative hyperopic shift in refraction in response to exposure to higher illumination levels. Instead, refractive changes in control eyes were relatively stable, with a mild reduction in the hyperopic values seen over time that were not dissimilar to that observed under 500 lux. This lack of a relative hyperopic shift in refraction may be due to longer time courses used in the analysis of refractive changes in tree shrews and rhesus monkeys. However, Cohen and colleagues (2011) have reported that although rearing animals under 10,000 lux for 90 days holds them in a mild hyperopic state, they show a relative myopic shift over time, as the animals head towards emmetropia, rather than a relative hyperopic shift in response to high illumination levels. This may therefore represent a mechanistic difference between chicks and that of tree shrews and rhesus monkeys.

Consistent with the changes observed in refraction, chicks fitted with translucent diffusers, and reared under 500 lux, developed the longest eyes compared to control values. The excessive axial elongation associated with deprivation-myopia was, however, retarded, to increasing degrees, relative to the light intensity to which animals were exposed, so that by 40,000 lux, axial length changes were not statistically different from control values. It should be noted, however, that although changes in axial length correlated strongly with the relative direction of refractive changes, the absolute values obtained for axial length consistently underestimated the refractive values that should be observed. This may suggest that high light exposure also induced corneal flattening and hence reduce optical power, accounting for this mismatch. However, this seems unlikely as Ashby et al. (2009) found no change in the corneal radius of curvature in chicks raised for 4 days under 15,000 lux compared to those that were raised under normal laboratory light, which would appear to exclude this hypothesis.
4.1.1 The effect of high light on lens-compensation

Exposure to elevated light intensities also affects the rate at which eyes compensate to image defocus, imposed by lenses. Fitment of negative lenses (lens-induced myopia (LIM)), similar to FDM, enhances ocular growth, leading to the development of myopia (Schaeffel, Glasser et al. 1988, Hung, Crawford et al. 1995, Shaikh, Siegwart et al. 1999, Ashby and Schaeffel 2010, Siegwart, Ward et al. 2012). In chickens, the rate of compensation for negative lenses (−10 D), and hence the rate of axial elongation, is significantly reduced under high light levels (15,000 lux), when compared to animals reared with lenses under 500 lux, although in both cases full compensation still occurs (Ashby and Schaeffel 2010). Similarly, in tree shrews, the rate of compensation is significantly reduced under high light, with the average time to full compensation to −5 D lenses being 17.5 days under 15,000 lux compared to 9 days under 500 lux, although, like with chicks, full compensation still occurs (Siegwart, Ward et al. 2012). In contrast, exposure to high light appears not to affect the rate of compensation to negative lenses in rhesus monkeys (Smith 2013, Smith, Hung et al. 2013). This result is somewhat surprising as the ability of light to retard the development of FDM appears to be highly conserved between chicks (avian model), tree shrews (mammalian model) and rhesus monkeys (primate model). Therefore, one might assume that as light retards the rate of compensation for negative lenses in chicks and tree shrews, that this response may also be conserved within the primate model, as it was for FDM. It seems strange that such a system/response is conserved between an avian (chicken) and mammalian (tree shrew) model, yet not a primate (rhesus monkey) model, as phylogenetically the tree shrew is much closer to the rhesus monkey than to the chicken. Interestingly, preliminary results from a small study presented recently at the International Myopia Conference by Stell and colleagues (2013) has suggested that 3 hours daily exposure to high light may actually be able to retard the rate of compensation for negative lenses in rhesus monkeys, while also inducing a small hyperopic shift in the untreated eyes of high light reared animals. However, at present, it is difficult to draw strong conclusions from this study, as light levels were not controlled, with animals placed outside for the high light group, where the intensity recorded ranged from 10,000-30,000 lux, while the number of animals used was quite small (n= 4 for each high and low light group).

The effect of high light on plus-lens compensation has only been analysed in chicks, with 15,000 lux reducing the rate of compensation for +10D lenses compared to those animals reared under 500 lux (Ashby and Schaeffel 2010). Therefore, high light was able to enhance the “STOP” growth signal associated with plus lens-wear, further reducing the rate of ocular growth. However, as with negative lenses, although high light treatment reduced the rate of
compensation, full compensation was still achieved. Overall, studies across three species (chickens, tree shrews and rhesus monkeys) have shown that daily exposure to high light is capable of reducing the rate of axial elongation in response to visual defocus, with the obvious exception of the effect of high light on negative lens compensation in the rhesus monkey, which requires further investigation.

4.1.2 Possible mechanisms underlying the protective effects of time outdoors

A number of possible factors have been proposed to explain the protective effects of bright light. For example, bright light will lead to pupil constriction and hence greater depth of focus, bringing about reduced image blur. However, this theory does not explain the ability of high light to retard the development of FDM, as pupil constriction will be unable to alter the image blur associated with the attached diffusers. Another possible hypothesis is increased optical flow rates, previously shown to retard the development of FDM (Schwahn and Schaeffel 1997), which could be achieved through increased movement/activity outdoors. However, chickens were found not to change their rate of activity (movement) under high lights. A role for ultraviolet (UV) light and vitamin D levels has also been proposed. However, a number of points argue against the role for UV light and vitamin D levels. Firstly, the lighting systems used by a number of groups to retard the development of experimental myopia in chickens (Ashby, Ohlendorf et al. 2009), tree shrews (Siegwart, Ward et al. 2012) and rhesus monkeys (Smith, Hung et al. 2013) did not produce light in the UV spectrum. These findings exclude the requirement of UV exposure for the inhibition of myopia development. Secondly, tree shrews fed with vitamin D3 supplements are not protected against the development of FDM or LIM (Siegwart, Herman et al. 2011). Finally, a small cohort study found no differences in vitamin D levels between myopic and non-myopic cohorts (Mutti and Marks 2011). Instead, as hypothesised by Rose and colleagues (2008), evidence from animal studies, strongly suggests that the protective effects of bright light are driven, in part, by increased retinal dopamine levels. Dopamine is a light-driven neurohormone released by amacrine and/or interplexiform cells (Djamgoz and Wagner 1992, Megaw, Morgan et al. 2001, Megaw, Boelen et al. 2006, Cohen, Peleg et al. 2012), with work in chickens indicating that retinal dopamine release increases in a log-linear fashion over the light ranges normally seen during the day (100-100,000 lux) (Morgan and Boelen 1996, Cohen, Peleg et al. 2012). Three points of evidence from animal studies supports a role for dopamine in the protective effects of bright light. Firstly, retinal dopamine levels are reduced during the development of experimental myopia, with dopamine D2 receptor agonists capable of retarding the development of experimental myopia induced by either negative lens-wear or translucent diffusers (Stone, Lin et al. 1989, Iuvone, Tigges et al. 1991, Ashby, McCarthy et al. 2007, McCarthy, Megaw et al. 2007, Mao, Liu
et al. 2010). Secondly, the ability of brief periods of normal vision (diffuser removal) (Nickla, Panos et al. 1989, Napper, Brennan et al. 1995, Napper, Brennan et al. 1997, Ashby, Ohlendorf et al. 2009) to retard the development of FDM, a process which is enhanced under bright light (Ashby, Ohlendorf et al. 2009), is abolished by the administration of the dopamine D2 receptor antagonist spiperone. Finally, and most importantly, daily injections of spiperone are capable of abolishing the protective effects afforded by 5 hours of daily exposure to 15,000 lux against the development of deprivation-myopia in chicks (Ashby and Schaeffel 2010), strongly supporting a role for the dopaminergic system.

4.1.3 What are the relevant light intensities required to prevent deprivation-myopia?

The results of this study show that chickens already had a roughly 80% retardation in the development of FDM by 30,000 lux, while near-complete protection (~ 95%) was achieved by exposure to 40,000 lux. Similar to chicks (Ashby, Ohlendorf et al. 2009), tree shrews show a roughly 60% reduction in FDM in response to 15,000 lux (Siegwart, Ward et al. 2012). Based on the similarity of these results, this may suggest, although not shown, that FDM could also be abolished in tree shrews if also exposed to 40,000 lux. Conversely, daily exposure to 28,000 lux, for 6 hours per day, abolish the development of FDM in rhesus monkeys (Smith, Hung et al. 2012), while in chicks, similar light levels (30,000 lux) were only able to retard the development of FDM by roughly 80%. This may represent a mechanistic difference between these two species, or more likely, represent slight variations in the protocols used. For example, differences in the diffuser construct used will lead to differences in the degree to which light is attenuated. The differences, however, are not due to exposure time as both rhesus monkeys and chickens were exposed to bright light for 6 hours per day. What is unclear from the results to date is what the relationship is between the intensity of light used and the length of exposure time, e.g. do short times under bright lights produce similar effects as longer times under less bright lighting. Also, what is the minimum and maximum time of exposure required for full protection (e.g. would exposure to 40,000 lux for periods of less than 6 hours per day have the same effect, and would any greater effect be gained from extending the exposure time to greater than 6 hours)? Recent work in chickens had indicated that increased exposure time to bright light of 15,000 lux, from 5 hours to 10 hours per day, does not enhance the protective effects against FDM (Backhouse, Collins et al. 2012, Lan, Feldkaemper et al. 2013). However, the protective effect of light appear to be enhanced with shorter periods of intermittent exposure (Lan, Feldkaemper et al. 2013), although further analysis is required. Finally, future studies will need to determine if animals must be exposed to high light daily to receive full protection, or whether treatment can be reduced to every second day etcetera.
4.1.4 The role of light intensity in human myopia

The fact that exposure to elevated light intensities can abolish the development of deprivation-myopia in chickens may well be promising for humans. A number of epidemiological studies (For review see French, Ashby et al. 2013) have already showed a negative correlation between spending more time outdoors and the development of myopia. Furthermore, preliminary data from current trials in China, have reported that the inclusion of an extra 40 minutes of time outdoors significantly reduced myopia progression in grade 1 children (Xiang, Zeng et al. 2013). If light is the relevant factor underlying the protective effects of time outdoors, and if findings from experimental models of myopia are directly translatable to human myopia, the findings from this current study would suggest that daily exposure to light intensities between 30,000 and 40,000 lux could significantly retard the onset and progression of myopia in children. Such light intensities are highly achievable outdoors, with light levels of around 30,000 lux seen on winter days, or in the shade, in many parts of the world, while levels up to 80,000 lux can be seen in summer, or in direct sunlight, in most part of Asia and Europe, and can exceed 120,000 lux in Australia. However, although the current findings are promising, the protective effects of time spent outdoors may be multifactorial or may not even be related to light levels. For example, the larger viewing distances afforded outside would allow for greater depth of focus, and hence a sharper image due to pupil constriction, reduced peripheral hyperopic defocus, and less accommodative demand. Taken together, this would create a more uniformed dioptic space, which has been hypothesised to remove the stimulus for myopia growth (for review see Flitcroft 2012). Although plausible, this hypothesis has not been tested in any great detail. Also, this model assumes that while outdoors, one does not interact with objects in the near vicinity; however, this is unlikely as humans will continuously engage with their friends, personal electronic devices and nearby objects. Also, although laboratory animals, housed in cages, are surrounded by nearby stimuli within a 3-5 meter radius, they do not develop myopia, and under these same conditions high light still prevents the development of experimental myopia, and even induce a hyperopic shift in untreated eyes of both tree shrews and rhesus monkeys (Siegwart, Ward et al. 2012, Smith, Hung et al. 2012). Furthermore, elevated light intensities can slow the response to induced hyperopic defocus (Ashby and Schaeffel 2010, Siegwart, Ward et al. 2012), therefore suggesting that increased viewing distances and uniformed dioptic space cannot explain, at least, the protective effect against the development of deprivation-myopia. Another possible protective mechanism is spectral composition, as the emmetropisation process has been shown to be sensitive to chromatic aberrations (Kroger and Wagner 1996, Seidemann and Schaeffel 2002, Rucker and Wallman 2008). In animal models, rearing under monochromatic
red light, in which the focal plane falls behind the retina, induces a relative myopic shift compared to animals raised in monochromatic blue light, in which the focal plane falls in front of the retina (Kroger and Binder 2000, Rucker and Wallman 2008, Qian, Liu et al. 2013). As sunlight is blue heavy compared to lighting sources experienced indoors, which are predominantly red heavy, it is plausible that differences in spectral composition could explain the protective effects of time outdoors. However, such spectral differences are unlikely to explain the protective effects of high light against the development of experimental myopia, as the same light source is used to generate 500 lux as that used to generate 40,000 lux.

4.2 Once myopic, can progression be halted by exposure to elevated light intensities?

Although it has been shown that high light is capable of retarding, or even abolishing the onset of experimental myopia, a crucial question, especially for human application, is whether exposure to high light can prevent further progression in already myopic animals. To test this, FDM was initially induced for three days under 500 lux. Once a myopic refraction was established, the animals were transferred to a high light regime, in which the chicks were exposed daily to 40,000 lux for a 6 hour period. The progress of FDM was halted on switching the animals to high light, with an initial hyperopic shift observed over the first two days, before refractive values plateaued around emmetropia. Based on the size and rapid rate of the initial hyperopic shift, this most likely represents a choroidal response.

Although this is a promising finding, a longer time-course is required to determine the longevity of this halt in progression. For example, will light maintain the diffuser treated eye at this stabilised refractive value indefinitely, which in this case was around zero, or will the “GROW” signal associated with diffuser-wear eventually overpower the protective effects of bright light? Alternatively, could exposure to high light gradually push the eye back into a hyperopic state, similar to the hyperopic shift reported in untreated eyes of both tree shrews and rhesus monkeys (Siegwart, Ward et al. 2012, Smith, Hung et al. 2013). Furthermore, it needs to be determined if it was simply luck that further progression was halted with a mean average refraction around emmetropia, or whether emmetropia was a defined end-point. To answer this, a range of initial myopic refractive values will need to be induced to determine if all animals are stabilised at, or near, emmetropia, independent of their starting refraction, once switched to high light exposure. However, the fact that the progression of deprivation-myopia could be halted by exposure to high light is already a promising finding, especially as it is crucial that further progression in children is halted as soon as possible to prevent the chorio-retinal pathologies associated with excessive axial elongation.
4.3 What biochemical pathways underlie the protective effects of high light?

As discussed earlier, ocular growth is regulated, in response to visual stimuli, by local retinal pathways that ultimately modulate the rate of scleral growth. Our understanding of these retinal pathways has been significantly enhanced by the study of animal models of experimental myopia. Using this knowledge, this thesis began to elucidate the pathways underlying lights protective effects, by examining changes in specific retinal components of four of the major molecular pathways postulated to be critical to ocular growth regulation, that of; dopamine, nuclear transcription factors, glucagon and retinoic acid.

4.3.1 Dopamine and tyrosine hydroxylase (TH)

As previously discussed (page 16 and 19 of the introduction), work in chicks has already suggested that the dopaminergic system plays a key role in the protective effects of bright light against the development of FDM, as such affects can be abolished by the daily administration of the dopamine D2 receptor antagonist spiperone (Ashby and Schaeffel 2010). Furthermore, earlier work has demonstrated that retinal dopamine release (Stone, Lin et al. 1989), as best measured by vitreal DOPAC levels (Megaw, Morgan et al. 2001), is significantly down-regulated during the development of experimental myopia. Similarly, mRNA levels of the rate limiting enzyme in the synthesis of dopamine, that of tyrosine hydroxylase (TH), are also down-regulated during the development of deprivation-myopia (Stone, Lin et al. 1989), while the administration of dopamine D2 receptor (DRD2) agonists have been shown to retard the development of experimental myopia (Stone, Lin et al. 1989, Iuvone, Tigges et al. 1991, Rohrer, Spira et al. 1993, Schmid and Wildsoet 2004, Nickla, Totonelly et al. 2010, Dong, Zhi et al. 2011). Based on the critical role both TH and DRD2 appear to play in dopamine’s effects on ocular growth, this current study investigated whether changes in the mRNA levels of both molecules could be involved in the retinal pathways underlying the effects of bright light.

The current findings indicate that no significant changes in the mRNA levels of DRD2 or TH are seen in diffuser-treated chicks exposed to either 500 lux or 40,000 lux compared to those levels seen in contralateral control eyes after 1 day of treatment. This may not be surprising, as the literature would suggest that TH is regulated, for the most part, at the post-translation level, although also regulated, to some extent at the post-transcriptional level (Ribeiro, Wang et al. 1992, Kumer and Vrana 1996, Witkovsky, Gabriel et al. 2000). Therefore, the lack of change observed in TH mRNA levels may not represent retinal peptide levels or the rate of activity of this enzyme. Similarly, the lack of change observed in DRD2 mRNA levels, does not preclude changes in the number of receptors inserted within the cell membrane, or
modulation of receptor affinity, both of which would bring about a significant change in the cellular response to dopamine.

4.3.2 Early growth response 1 (Egr-1)

As discussed earlier, Egr-1 expression shows a bidirectional response to opposing stimuli, with both mRNA and peptide levels down-regulated during periods of increased ocular growth and up-regulated during decreased ocular growth (Fischer, McGuire et al. 1999, Ashby, McCarthy et al. 2007, Ashby, Kozulin et al. 2010). Consistent with this, Egr-1 mRNA levels were down-regulated in response to 1 day of diffuser-wear under 500 lux in this current study. However, this down-regulation was abolished if diffuser-wearing chicks were exposed to high light levels (40,000 lux), with Egr-1 expression returning to those values seen in the contralateral control eyes of the 500 lux treated animals. Interestingly, Egr-1 mRNA levels were also up-regulated in the contralateral control eye of chicks exposed to 40,000 lux compared to contralateral control values seen under 500 lux. Therefore, Egr-1 mRNA levels were still down-regulated in the diffuser treated eye of chicks exposed to 40,000 lux when compared to their own contralateral control values, but not when compared to the contralateral control values seen in the 500 lux group. This suggests that Egr-1 is responding to an interplay between signals associated with defocus and light intensity. If purely responding to light levels, Egr-1 mRNA expression in the diffuser treated group under 40,000 lux should have been higher than that seen in the 500 lux contralateral control group. Conversely, if Egr-1 was responding purely to defocus, mRNA levels should not have been different between the contralateral control groups, which was not the case.

The current results fit well with the literature. Egr-1 mRNA levels have been consistently observed to be down-regulated, within 1 hour, during periods of increased ocular growth associated with both lens- or diffuser-wear. This rapid down-regulation, however, can be abolished by the administration of pharmacological agents which are known to block the development of experimental myopia, such as the dopamine D2 receptor agonist ADTN and the muscarinic-cholinergic antagonists atropine and prinzepine, in chicks (Ashby, McCarthy et al. 2007, Ashby, Kozulin et al. 2010). Similarly, this study has shown that treatment with high light, which also prevents the development of FDM, abolishes the down-regulation in Egr-1 expression, giving further evidence to the hypothesis that Egr-1 is a marker for the direction of ocular growth, and, more importantly, may be directly involved in growth modulation.
4.3.3 FBJ osteosarcoma oncogene (cFos)

The immediately early gene cFos, is a light-induced member of the nuclear transcription factor family to which Egr-1 also belongs to. Initial studies reported that cFos peptide levels, as measured by immunostaining, were unaffected by the development of FDM, but were up-regulated following diffuser removal, a response that was believed to be light driven (Fischer, McGuire et al. 1999). However, later microarray studies have observed a significant down-regulation in cFos mRNA levels in response to 24 hours of diffuser-wear (Brand, Schaeffel et al. 2007, Schippert, Schaeffel et al. 2008, Ashby and Feldkaemper 2010), which appears not to be associated with changes in retinal illumination, as these changes are still observed when light levels striking the control eye are attenuated by similar amounts to that caused by diffuser-wear (Brand, Schaeffel et al. 2007). Consistent with these findings, the current results demonstrate that cFos mRNA levels are down-regulated in response to 1 day of diffuser-wear under 500 lux. Surprisingly, as cFos is a light-induced transcription factor, this down-regulation was not abolished if diffuser-wearing chicks were exposed to 40,000 lux. Furthermore, there was no significant difference in cFos mRNA levels between the 500 lux and 40,000 lux contralateral control groups, even though there is an 80 fold difference in the light levels experienced. This would suggest that the down-regulation in cFos mRNA levels was in response to the defocus signal associated with diffuser-wear, rather than to changes in retinal illumination. This is consistent with the findings of Brand and colleagues (2007).

The inability of high light levels to prevent the down-regulation in cFos mRNA levels suggests that; a) cFos expression may be up-stream of the molecular targets of light, although, one would expect cFos to behave in a similar fashion to that of Egr-1, b) cFos is not involved in the regulation of ocular growth, or, that the protective effects of bright light do not target this pathways, or finally c) retinal samples were collected at a time point which was too late/early to observe changes in cFos, although, again one would expect cFos to behave in a similar fashion to that of Egr-1. A more rigorous time-course of changes in cFos mRNA and peptide levels must be undertaken to answer this question.

4.3.4 Glucagon

As was detailed within the introduction, glucagon has previously been implicated in growth regulation due to its bi-directional response to opposing growth stimuli, while administration of glucagon agonists can retard the development of experimental myopia (Fischer, McGuire et al. 1999, Bitzer and Schaeffel 2002, Buck, Schaeffel et al. 2004, Ashby, Kozulin et al. 2010). Consistent with previous studies (Ashby, Kozulin et al. 2010), increased rates of ocular growth, associated with the development of FDM under 500 lux, induced a significant down-regulation
in glucagon mRNA levels after 1 day of diffuser-wear in this current study. However, this
down-regulation was not abolished if diffuser-wearing chicks were exposed to 40,000 lux. This
is surprising for two reasons; firstly, administration of glucagon receptor agonists inhibit axial
elongation (Feldkaemper, Burkhardt et al. 2002, Vessey, Lencses et al. 2005), while
administration of glucagon receptor antagonist prevents compensation to positive-lenses
(Vessey, Lencses et al. 2005). Therefore, the pharmacological evidence would suggest that
retinal glucagon levels should remain at control levels during periods in which increased ocular
growth is prevented, such as that achieved by exposure to 40,000 lux. Secondly, glucagonergic
amacrine cells in the chick express Egr-1, with changes in glucagon levels normally mirroring
those changes seen in Egr-1 expression, although with a time-shift of roughly 24 hours (Ashby,
Kozulin et al. 2010). Therefore, as high light is able to abolish the down-regulation in Egr-1, one
might have assumed it would therefore abolish the down-regulation in PPG mRNA levels.

The inability of light to alter glucagon levels may be for a number of reasons. Firstly, it could be
that glucagon is not part of the pathway regulating ocular growth. This however seems
somewhat unlikely based on the pharmacological evidence. Secondly, changes in PPG mRNA
levels may be up-stream of the molecular targets associated with high light, although unlikely
based on the changes observed in Egr-1 expression. Alterations in mRNA levels may not
represent changes at the peptide level under high light; however, normally changes at the
peptide level mirror those changes seen at the transcript level (Feldkaemper and Schaeffel
2002). Finally, changes in retinal glucagon mRNA levels take approximately 24 hours to appear
in response to alterations in the visual environment (e.g. lens- or diffuser-wear) (Ashby,
Kozulin et al. 2010). Therefore, this current study may have sampled too early to see a reversal
in PPG mRNA levels, which may be more likely to be observed on day 2.

4.3.5 Retinoic acid (RA)

RA appears to be an important signalling molecule in the developing eye (Mertz and Wallman
2000, Mao, Liu et al. 2012). Retinal-RA levels, as well as mRNA levels of retinoic acid receptor-
β (RAR-β) in the sclera and retina, have been shown to be up-regulated during periods of
increased ocular growth, while retinal-RA levels are down-regulated during periods of
decreased ocular growth (Seko, Shimokawa et al. 1996, Seko, Shimizu et al. 1998, Bitzer,
Feldkaemper et al. 2000, Huang, Qu et al. 2011). Similar bi-directional changes in RA levels to
opposing growth stimuli are seen within the chick choroid, although these changes are inverse
to those seen in the retina (Mertz and Wallman 2000). Moreover, administration of RA causes
a rapid increase in axial elongation in chicks and guinea pigs (Mcfadden, Howlett et al. 2004,
Mcfadden, Howlett et al. 2006). The current results, however, indicate no significant change in
retinal mRNA levels for RAR-β after 1 day of diffuser-wear. This appears contradictory to the findings reported by Huang and colleagues (2011), in which mRNA levels for RAR-β were observed to be up-regulated in the guinea pig retina, however, this was seen after 14 days, a considerably longer time-course than that undertaken presently. Therefore, possible changes in RAR-β mRNA levels need to be examined over a longer time-course in chicks, a process that needs to be undertaken for all genes investigated within this study to truly elucidate the role of post-transcriptional regulation of these candidate molecules in the modulation of ocular growth by high light exposure. Also, as with DRD2, studying possible changes in the transcript level of RAR-β will not provide insight into any possible post-translational modifications that could alter receptor affinity and cellular localisation.

**Conclusion**

In summary, exposure to elevated light levels has a significant effect on the development of FDM over time. Specifically, the results of this study have shown that there is a negative correlation between the light intensity to which animals are exposed and the degree to which deprivation-myopia develops, with the onset of FDM abolished by 40,000 lux. Further, the current results suggest that exposure to bright light (40,000 lux) can halt further progression of deprivation-myopia, although a longer time-course is required to determine the full extent of this effect. Finally, this thesis provided a first look at the possible molecular pathways that underlie the protective effects of high light and observed that the down-regulation in the mRNA levels of the immediate early gene Egr-1, during the development of deprivation-myopia under 500 lux, are abolished by daily exposure to bright light (40,000 lux).
References


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