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DIURNAL RHYTHMS AND EYE GROWTH IN CHICKS

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

Diurnal Rhythms and Eye Growth in Chicks

by

Debora L. Nickla

Adviser: Professor Josh Wallman

To explore the possibility that ocular diurnal rhythms are involved in the regulation of ocular growth and its control by the retina, I examined several ocular rhythms (axial length, choroid thickness, proteoglycan synthesis and intraocular pressure) in normal eyes, form-deprived eyes (which are elongating twice as rapidly as normal), and eyes recovering from deprivation myopia (which have slowed elongation). In addition, I examined the efficacy of visual manipulations on deprivation myopia as a function of time of day.

The principal finding are: (1) Visual manipulations that decrease eye growth are most effective during the night. (2) Eyes in all three conditions show diurnal fluctuations in axial length. In normal and form-deprived eyes, elongation is greatest in the morning and least in the evening. (3) Eyes in all three conditions show diurnal fluctuations in choroidal thickness. In normal and form-deprived eyes, the choroid thins in the morning and thickens in the evening, hence the axial and choroidal rhythms are approximately anti-phase to one another. (4) In recovering eyes, the axial and choroidal rhythms are shifted, so that they are now in phase. (5) Intraocular pressure shows a diurnal rhythm in normal eyes, being high during the day and low at night. This rhythm persists in constant darkness.

However, in form-deprived eyes the rhythm becomes desynchronized with respect to the light/dark cycle. (6) The phase difference between the rhythms in axial length and intraocular pressure predicts the rate of ocular growth. (7) There is a diurnal rhythm in proteoglycan synthesis in the sclera, being higher during the day than during the night. This rhythm persists in culture for at least 3 cycles.

We propose that the phase relationships between these rhythms determine the rate of ocular growth. Specifically, we argue first, that the synthesis of scleral extracellular matrix, as reflected by proteoglycan synthesis, is an important correlate of ocular elongation. Second, that it is stimulated by the daily intraocular pressure changes, especially at certain times of day; third, that proteoglycan synthesis is inhibited by daily increases in choroidal thickness, again in a phase-dependent manner.

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CHAPTER 1

Introduction and General Overview

Neonates of most species are born ametropic (generally hyperopic) and gradually adjust the growth of the components of the eye to attain emmetropia, such that the physical length of the eye matches the focal length of the optics. Of long-standing controversy is how this match is attained; that is, whether the growth is a pre-programmed developmental feature or whether the growth is "guided" by visual cues. An understanding of the factors involved in emmetropization and the control of eye growth is a necessary prerequisite for any hope of understanding what goes awry in the development of refractive anomalies such as myopia.

Much evidence from animal studies that used lid suture or translucent diffusers to deprive the eye of form vision support the view that emmetropization is guided by visual cues in a negative feedback control system, and that the absence of visual cues constitute a loss of an error signal. It is thought that the absence of form vision results in a reversion to a default state of growth in which the eye grows abnormally fast. When the diffuser is removed and vision restored (or the lids opened), the eye slows its growth until emmetropia is re-established. This "recovery" has been cited as evidence for visual modulation of eye growth. The strongest evidence, however, is that eyes of chicks and monkeys grow to compensate for the refractive error induced by spectacle lenses (chicks: Schaeffel et al., 1988; Irving et al., 1992; Wildsoet and Wallman, 1992; Wallman et al., 1995; monkeys: Hung et al., 1995). These two separate visual

manipulations (form-deprivation defocus and spectacle lens-induced defocus), that have in common a "bi-directional" growth response in chickens, appear to differ in crucial ways from one another, suggesting different underlying mechanisms. Specifically, it has been reported that the induction of myopia via form deprivation is suppressed by the neurotoxin 6-hydroxy-dopamine and by continuous light rearing, but its induction via negative spectacle lenses is not affected by these treatments (Bartmann et al., 1994; Schaeffel et al., 1994). (It should be noted, however, that because the effect of these treatments were assessed by the difference in response between the two eyes wearing opposite sign lenses, it was not possible to distinguish the effects of the compensation for negative and positive lenses, weakening the evidence for a difference between the mechanisms underlying deprivation and lens-induced myopia (Wallman, 1995)). In addition, optic nerve section does not affect the growth response of the eye to form deprivation, while the response to negative lenses is reduced (Wildsoet and Wallman, 1995). Although evidence from these studies and other studies using pharmacological manipulations hint at various possible mechanisms by which the eye may regulate its growth, we are still far from understanding the processes at the cellular or molecular basis.

One commonly cited hypothesis regarding the growth-inducing effects of form deprivation is that an impoverished visual environment causes the eye to adopt a default growth pattern in which the growth system runs "open loop" in the absence of an error signal. What the retinal signal for this is, how this signal reaches and influences the sclera, and what other ocular physiological processes are affected are largely unknown. Several recent investigations have hinted at an association between form deprivation myopia and changes in ocular diurnal rhythms. First, the eye

growth system is altered under conditions of constant light and constant dark, showing a general increase in eye growth and flattening of the cornea (constant dark: Gottlieb et al., 1987; constant light: Lauber et al., 1961; Lauber and McGinnis, 1966; Gottlieb et al., 1991; Li et al., 1995). The corneal flattening that occurs in constant light may be the result of the dampening of the corneal mitotic rhythm in constant light (Sasaki et al., 1995). The fact that these effects are associated with the absence of a *Zeitgeber* (light and dark) suggests that the abnormal eye growth might be a result of alterations in diurnal rhythms. Second, form-deprived eyes of both chicks and monkeys show a decrease in the levels of retinal dopamine, a retinal neuromodulator whose levels are normally high during the day and low during the night (Iuvone et al., 1989; Stone et al., 1989). Restoring dopamine levels by administering dopamine agonists to the eye results in a more emmetropic eye (Stone et al., 1989; Iuvone et al., 1990; Iuvone et al., 1991). Therefore, dopamine might be one of the "growth" signals, and the fact that the daytime levels in myopic eyes are lower than normal may indicate that the rhythm in dopamine is damped. Finally, it was shown that the elongation of the eye is diurnally rhythmic (Weiss and Schaeffel, 1993; Nickla and Wallman, 1995a), and that this rhythm is altered by constant light, and by various visual manipulations (Weiss and Schaeffel, 1993; Bartmann et al., 1994).

An underlying assumption for the hypothesis that visual manipulations such as deprivation of form vision will have an effect on a rhythm (be it on the amplitude or phase), is that form vision (and not just light *per se*) must be a signal for the circadian ocular system. Specifically, form vision must be an essential stimulus for the normal expression of these rhythms. Because the essential function of the retina is to detect patterns, it is not

implausible that the absence of form vision resulting from translucent diffusers may have an effect on the ocular clock, perhaps by a desynchronization of the individual oscillators, or abolishing the oscillation itself, and that the abnormal eye growth is a result of this. These alterations in ocular rhythms might occur at any of a number of levels in the pathway of the signal from retina to sclera. That is, they could be expressed in the retina, as indicated by the decreased levels of dopamine, and/or they may also be expressed in rhythms in the other ocular tissues such as RPE, choroid or sclera.

This thesis examines five rhythms related to ocular growth: rhythms in axial length, choroid thickness, anterior chamber depth, intraocular pressure, and scleral proteoglycan synthesis. These rhythms were studied in normal eyes and in eyes that were deprived of form vision with the aim of elucidating how these rhythms might interact in regulating the growth of the eye. Because this body of work lies at the intersection of two quite large and separate fields, circadian rhythms and ocular development, the following will be a review of the literature pertinent to the work in this thesis: the evidence for visual modulation of ocular development, and a synopsis of various ocular diurnal rhythms.

The Visual Regulation of Eye Growth

Form deprivation myopia

The first studies showing that the deprivation of form vision produces ocular elongation were by Wiesel and Raviola (1977), who found that lid-sutured eyes of young macaques became elongated and myopic. Lid suture has been found to produce myopia in a variety of species (tree shrews:

Sherman et al., 1977; McKanna and Casagrande, 1978; cats: Wilson and Sherman, 1977; Gollender et al., 1979; Kirby et al., 1982; chickens: Yinon et al., 1980; Lauber and Oishi, 1987). A less invasive means of depriving the retina of high-spatial frequency components is the use of plastic translucent diffusers that are glued to the skin or feathers around the eye. Myopia produced in this manner was found in chicks (Wallman et al., 1978; Hodos and Kuenzel, 1984; Hayes et al., 1986) tree shrews (Norton, 1990), and guinea pigs (personal communication, Sally McFadden). In chicks, severe myopia (up to -30D) is produced in a very short amount of time (2 weeks). The "critical period" for the induction of form deprivation myopia is presumably during the period of post natal development during which the eye is still elongating. In chicks, for example, the degree of myopia produced is dependent on the age at which the deprivation is begun; 4 week old birds deprived of form vision for two weeks become significantly less myopic than those deprived at hatching or at two weeks of age (Wallman and Adams, 1987). In monkeys, form deprivation can be produced during the first year of life. The implication of the discovery that form deprivation produces ocular elongation and myopia, and the impetus for all subsequent research in the field, is the notion that the growth of the eye can be changed by visual manipulations that alter the retinal input, and therefore that emmetropization requires normal visual experience. Support for this "visual modulation" comes from three lines of evidence: one, that the myopia produced by form deprivation is the result of an absence of vision *per se*, and not due to a non-specific mechanism (for example, decreased light levels), two, that recovery from deprivation myopia occurs upon removing the diffusers and restoring vision, and conversely, that if the myopia is corrected by lenses the eye remains myopic, and three, that

eyes grow to compensate for defocus produced by spectacle lenses. These three lines of evidence will be discussed below.

Evidence for the role of vision in emmetropization

1. Form deprivation myopia is due to the absence of form vision *per se*

Three lines of evidence support the notion that the myopia and axial elongation produced by visual deprivation are the result of a visually-mediated mechanism (and not, for example, due to mechanical or thermal effects, nor to a reduction in light intensity). The first stemmed from the original study on macaques by Wiesel and Raviola (1977); in a subsequent paper Raviola and Wiesel (1978) reported that a lid-sutured monkey reared in darkness did not develop myopia. These authors argued that the myopia was not the result of the attenuated light *per se*, nor of a mechanical pressure exerted by suturing the lids, but of the "distorted representation of the visual world", that is, the loss of high frequency visual information. Similarly, dark rearing alone (without depriving the eye) does not produce myopia. Although indicative that the myopia produced by deprivation was a result of the absence of "patterns", this evidence must be viewed with caution because constant conditions of both dark and light are known to affect eye growth. In most species studied, both constant dark and constant light produce enlarged, but hyperopic eyes (macaques, Regal et al., 1976; cats: Yinon and Koslowe, 1984; Yinon et al., 1984; chickens: Gottlieb et al., 1987; Gottlieb et al., 1991; Lauber et al., 1961), because of flattening of the cornea.

The second line of evidence that the absence of patterned vision is the crucial factor in form deprivation myopia is that in chicks, depriving half the retina of form vision by using diffusers that obscure half the visual

field results in myopia and axial elongation confined to the deprived half of the retina (Wallman et al., 1987). This result demonstrates two important features of deprivation myopia: that the retina can control the growth of the eye locally, and that the effect of deprivation cannot be a non-specific effect (for instance, thermal or lower levels of light).

The third line of evidence is that myopia can be produced by less severe types of image-degradations; ones that do not involve a total absence of patterned visual input. For example, diffusers that reduce the contrast of the image by varying degrees (quantified by measuring their modulation transfer functions using a video system) resulted in varying degrees of myopia in chicks (Bartmann and Schaeffel, 1994). Specifically, "slightly frosted" diffusers resulted in significantly less myopia than "heavily frosted" diffusers, and both produced more myopia than "clear" ones (these clear diffusers produce a small amount of image degradation). Furthermore, myopia can be produced without the use of any physical barrier over the eye by creating "deprivation-like conditions" in the visual environment. We found that when a form-deprived eye is exposed to 2 hours of visual stimulation in a "feature-less" (blank) environment, the amount of myopia was greater than eyes exposed to 2 hours in a "rich" visual environment (Nickla et al., 1989).

2. Restoring vision results in the return to emmetropia from myopia.

When the diffuser is removed from a form-deprived eye so that vision is restored, the eye eventually returns to emmetropia. This "recovery" from myopia has two components: a fast choroidal component, whereby the choroid thickens, moving the retina forward, and a slower scleral component, whereby there is a decrease in axial elongation. The

initiation of the scleral response (i.e. the cessation in axial elongation) has been shown to take several days in 2-4 week old chicks (Wallman et al., 1995). This anatomical response has a biochemical correlate; the reduction in scleral proteoglycan synthesis (scleral matrix molecules) shows a lag of several days (Nickla et al., 1992; Rada et al., 1992). (The time lag for the reduction in scleral proteoglycan synthesis may be dependent on the age of the chick; Rada et al. (1992) used younger chicks than we did and found a shorter response delay). The initiation of the choroidal response, on the other hand, is very rapid and occurs within hours (unpublished observations). The increase in choroidal thickness makes the eye functionally emmetropic before it is possible for the scleral response (cessation of elongation) to do so (Wallman et al., 1995). This rapid return to emmetropia is evidence that the eye can sense the amount and direction of the defocus and respond to compensate for it.

The compensatory response of the previously deprived eye to normal vision also occurs in eyes that had had their optic nerve sectioned. Both the cessation of the scleral growth as well as the increase in choroidal thickness occurred in eyes that had been disconnected from the brain. Furthermore, in eyes in which only half of the retina had been deprived of form vision this "recovery" was restricted to the deprived half of the eye. These findings indicate that both scleral and choroidal responses are under local retinal control (Xu, 1992).

Recovery from form deprivation myopia is not a non-specific response to removing the diffuser but instead requires the presence of a refractive error: if the deprivation-induced myopic refractive error is corrected by the appropriate power spectacle lens, the eye remains at the length and refractive error corrected for by the spectacle lens (Wildsoet

and Schmid, 1996; Schaeffel and Howland, 1991). Similarly, if the myopia is under-corrected, the elongation of the eye ceases until the refractive error of the eye matches the refractive power of the lens (Wildsoet and Schmid, 1996). The fact that the eye returns to emmetropia from myopia when a myopic refractive error is present and not if the refractive error induced by the deprivation is corrected, is strong evidence that vision guides emmetropization.

3. The eye returns to emmetropia from dark-induced hyperopia.

Rearing chickens in the dark for 4 weeks produced hyperopic eyes that were abnormally elongated, and had flatter than normal corneas (Gottlieb et al., 1987; Troilo, 1989). After 1 week in a normal diurnal cycle of light and dark, the refractive error was reduced and the eye had returned to emmetropia (Troilo, 1989; Troilo and Wallman, 1991). This emmetropization was due mainly to continued elongation of the vitreous chamber (even though the eyes were longer than normal at the start as a consequence of dark rearing). This result argues that the vitreous chamber grew in order to compensate for the hyperopic refractive error, and suggests that it was the refractive error of the eye, and not its size *per se*, that dictated the growth response of the eye towards emmetropia.

4. The eye shows bi-directional compensatory responses to defocus induced by positive and negative spectacle lenses.

By far the most compelling evidence for the visual modulation of eye growth is that the eye compensates for the defocus induced by various powers of spectacle lenses. When negative spectacle lenses (which impose hyperopic defocus) are worn, the eye elongates and the choroid thins to

bring the image into focus on the retina. Conversely, when positive spectacle lenses are worn (myopic defocus), the eye stops growing and the choroid thickens to bring the retina toward the image plane (Wallman et al., 1995; Wildsoet and Wallman, 1995). These anatomical changes have a biochemical correlate in the sclera: the scleras of eyes wearing negative lenses increase proteoglycan synthesis, while the scleras of eyes wearing positive lenses decrease proteoglycan synthesis (Nickla et al., 1992). In addition, when the lenses are removed from the eyes, the eyes that wore negative lenses will find themselves functionally myopic, and will stop their growth and thicken their choroids to compensate. Eyes that wore positive lenses will find themselves functionally hyperopic and will increase their growth and thin their choroids to compensate (Wallman et al., 1995). These effects are very strong in chickens, who show almost complete compensation to lenses of different powers (Irving et al., 1991; Wallman et al., 1995), but are also present in guinea pigs (McFadden and Wallman, 1995) and monkeys (Hung et al., 1995). These studies provide the most definitive evidence to date for the existence of visual control of eye growth.

Ocular circadian rhythms

Many ocular processes show diurnal oscillations which optimize retinal function under the different conditions of ambient illumination that the animal encounters over the course of the 24 hour diurnal cycle. These rhythms have been found across phyla, and include changes in retinal circuitry, photomechanical movements of photoreceptors, synthesis and release of neurotransmitters, and changes in the phototransduction pathway (reviewed in: Cahill and Besharse, 1995). Some of these rhythms have been

shown to be endogenous, that is, they are controlled by a clock within the organism, and are not solely dependent on changes in illumination. These rhythms are circadian rhythms, which, by definition, are rhythms that have an approximate 24 hour period, are entrainable by a *Zeitgeber* (usually light) and will free-run in constant dark. The major evolutionary advantage of circadian clocks is to enable the organism to anticipate changes in ambient illumination, and so maximize the efficiency of its physiological and behavioral processes.

In vertebrates, self-sustaining oscillators (clocks) have been shown to be located in the pineal gland, retina and suprachiasmatic nucleus. It has recently been shown that several of the endogenous retinal rhythms are controlled by oscillators located within the eye itself, probably within the photoreceptor layer, although the identity of the cells involved is still unknown (Cahill and Besharse, 1993). We do know that individual cells can act as independent oscillators; dissociated chick pineal cells (Deguchi, 1979; Robertson and Takahashi, 1988; Bolliet et al., 1994) and isolated basal retinal neurons of *Bulla* (Block and McMahon, 1984; Michel et al., 1993) and *Aplysia* (Jacklet and Barnes, 1995) show circadian rhythms. It is possible therefore, that the ocular clock is composed of single oscillators that are coupled. Ocular rhythms have been demonstrated at many levels of organization, from the molecular (i.e. rhythms in dopamine, melatonin, opsin mRNA, and transducin) through the cellular (retinomotor movements) up to the organ levels (rhythms in intraocular pressure and axial elongation). It is becoming apparent that a complete understanding of ocular development depends on understanding how these rhythms interact and possibly influence cell metabolism and growth.

Retinal and ocular rhythms

Retinomotor Movements

In some vertebrates, one of the retinal adaptations to light and dark involves movement of the outer segments of the photoreceptors and migration of pigment granules in the retinal pigment epithelium (RPE) to maximize sensitivity during the night. In diurnal animals, cone myoids contract during the day, positioning the outer segments and the transduction machinery closest to the incoming photons of light. Concurrently, there is a movement of the melanin granules from the cell body of the RPE into the apical processes, which shields the rods from the incoming light. At night the opposite movements occur; the cone myoids elongate, positioning the outer segments further from the light, and the rods contract, thereby moving towards the vitreous. Concurrently, the pigment granules migrate back into the cell bodies of the RPE cells, allowing for maximum capture of photons by the more sensitive rods (reviewed in: Burnside and Nagle, 1983; Burnside and Dearry, 1986; Besharse et al., 1988).

The cone retinomotor movements have been found to be circadian in most species studied, that is, they persist in constant darkness. In fact, in *Xenopus* (Besharse et al., 1982), green sunfish (Dearry and Burnside, 1986), goldfish, neon tetras, and *Midas cichlid* (McCormack and Burnside, 1991) the rhythms are retained *in vitro*, and can be phase-shifted by light. This system is presumably under control of the dopamine/melatonin system. Dopamine agonists of the D2 receptor result in cone myoid contraction and rod elongation, the same effects as light (Dearry and Burnside, 1986), while melatonin mimics darkness by causing cone

elongation (Pierce and Besharse, 1985). There is some inter-species variation in the degree of control by the circadian clock in these retinomotor rhythms. In green sunfish, for example, although the cones showed rhythmic movements when the animal was kept in darkness, the rods did not, nor did the pigment migration continue to oscillate (Dearry and Barlow, 1987). Various aspects of these rhythms are controlled by the ocular circadian clock; this too is species-dependent. In green sunfish, sectioning the optic nerve does not abolish the cone retinomotor rhythm, although it reduces the night-adaptive elongation, and hence the amplitude (Dearry and Barlow, 1987). These authors conclude that input from the brain may participate in some aspects of the rhythm in these animals.

In summary, the circadian nature of retinomotor movements of the rods and cones is species-dependent. In some species, the rhythm in cones is clearly driven by an ocular clock while the rhythm in rods is driven by light and dark, other species show evidence that both rhythms are circadian. The chemical signal mediating these effects appears to involve the D2 dopamine system, with "light" being mediated by dopamine and "dark" by melatonin. Finally, the location of the oscillator for the rhythms in cones appears to be species dependent, with some being completely regulated by the ocular clock, and others (i.e. the sunfish) showing a combination of retinal and extra-retinal control.

Disk shedding

In many species, the photoreceptors continually renew their outer segment disk membranes. This is accomplished by a shedding process whereby the photoreceptors discard old membrane from their tips and synthesize new membrane in the inner segments. The discarded membranes

are ingested and degraded by the adjacent RPE cells (Young and Bok, 1969). This renewal process occurs in both rods and cones, and can be quantified by counting the number of phagosomes in the RPE. In this way it was found that the process of disc shedding by the rods and cones in chicken eyes exhibited a daily rhythm, with the rods shedding immediately after lights on, and the cones after lights off (Young, 1978).

The process of disc shedding of outer segments is coupled to the cycle of light and dark in several species, however, only the rod's rhythm appears to be under the control of the circadian clock; the cone rhythm being driven by light. In rods, there is usually a burst of shedding at dawn, while for cones there is variability between species in the time of peak shedding (reviewed in: Besharse et al., 1988). A persistent rhythm of rod disc shedding in constant dark has been found in rats (LaVail, 1976; Goldman et al., 1980; LaVail, 1980), *Xenopus* (Besharse et al., 1977; Besharse, 1982; Flannery and Fisher, 1984), goldfish (O'Day and Young, 1978), and chickens (Young; 1978), with a burst of shedding occurring at "dawn" (preceding or right after lights on). Even in some of these species, however, the amplitude of the rhythm is reduced in darkness (Besharse et al., 1977), indicating a role for the light signal in the regulation of the rhythm. In addition, the rhythm is abolished in constant light (Besharse et al., 1977; Hollyfield and Basinger, 1978), but if returned to a short period of darkness, will be re-initiated (Currie et al., 1978). For still other species, such as *Rana pipiens*, the shedding by rods appears to be completely driven by the light/dark cycle, and does not cycle in constant darkness (Basinger et al., 1976). For cones, there is no evidence that the rhythm persists in darkness, and so is not under circadian control.

There is evidence that the rhythm in disc shedding is influenced by the neuromodulators melatonin and dopamine. In *Xenopus* eye cups, disc shedding can be activated by melatonin in constant light (Besharse and Dunis, 1983). This result suggests that melatonin is a signal for the "dark process" required for disc shedding. Conversely, dopamine (and D2 receptor agonists) inhibits rod disc shedding, and is therefore a candidate for the "day" signal (Besharse, 1987).

Visual sensitivity

In many species, the sensitivity of the retina can be modified over the diurnal cycle by a variety of mechanisms, so that sensitivity increases during the night, when it is needed. This phenomenon was first discovered in invertebrates: the lateral eye of the horseshoe crab *Limulus* was found to increase its sensitivity by about a million-fold during the night and decrease its sensitivity during the day (Barlow et al., 1977; Barlow, 1988). The rhythm persisted in constant darkness for up to a year. The site of the pacemaker driving this rhythm was found to be in the brain, because sectioning the optic nerve abolished the rhythm. Furthermore, stimulating the fibers in the cut optic nerve resulted in an increase in retinal sensitivity, although not of the same magnitude as seen in the intact eye (Barlow et al., 1977). The mechanisms by which these changes are accomplished involve efferent signals from the brain that cause increased responsiveness to light, and changes in the morphology of the ommatidia that make the eye more effective at photon capture (Barlow et al., 1977; Barlow et al., 1980).

Since these early studies in invertebrates, changes in retinal sensitivity have been found in several vertebrates, including humans. For example, Dearry and Barlow (1987) recorded ERGs in sunfish and found a 10-fold

increase in sensitivity to dim flashes of light at night. Similarly, behavioral measurements show a 3-fold increase in sensitivity during subjective night in goldfish (Bassi and Powers, 1987) and a increase of about 20% in humans (Bassi and Powers, 1986). In both chickens and lizards, the amplitude of the ERG b-wave shows a diurnal rhythm, increasing during subjective night in chickens (Schaeffel et al., 1991) and subjective day in lizards (Fowlkes et al., 1984). In fact, in chickens, rod function is apparent only during subjective night, as determined by spectral sensitivity and ERGs (Schaeffel et al., 1991).

Melatonin and Dopamine

Melatonin is a neuromodulator found in vertebrate retinas, as well as in the pineal gland, which is the source of the serum melatonin. It is synthesized from serotonin by the enzymes tryptophan hydroxylase, N-acetyltransferase (NAT) and hydroxyindole-O-methyl-transferase (HIOMT); the rate limiting enzyme being NAT, the penultimate one in the pathway (Cahill et al., 1991). In the retina, much evidence indicates that the cells that synthesize melatonin are the photoreceptors, based on the finding of melatonin-like immunoreactivity in the outer nuclear layer (Bubenik et al., 1976), and that the mRNA encoding HIOMT is localized to photoreceptors in chickens (Wiechmann and Craft, 1993). Furthermore, isolated layers of photoreceptors *in vitro* continue to synthesize melatonin (Cahill and Besharse, 1993). In rat retina, the enzyme HIOMT was found in some bipolar cells (Wiechmann and O'Steen, 1990).

A role for melatonin in the functioning of the circadian clock has been speculated upon since the finding of a rhythmic synthesis of melatonin in the pineal gland, which in non-mammalian vertebrates is photoreceptive

and constitutes one of the pacemakers of the circadian system. In birds, lizards, and amphibians, both the pineal gland and the retina show large diurnal fluctuations in melatonin and its enzyme NAT, with melatonin synthesis and NAT activity increasing nearly 4-fold during the night (reviewed in: Underwood and Groos, 1982). In most non-mammalian species examined (and one mammal, the golden hamster, Tosini and Menaker, 1996), the rhythm in retinal melatonin is circadian; it continues to oscillate in constant darkness, and can be phase-shifted by light during the night (chicken: Hamm and Menaker, 1980; quail: Underwood and Siopes, 1985; Underwood et al., 1988; *Xenopus*: Cahill and Besharse, 1989; 1990). Light at night also results in the acute suppression of NAT activity and melatonin synthesis. In these species, retinal melatonin is controlled both by the circadian clock and by light, with light acting as an entraining agent. Furthermore, the rhythm in melatonin synthesis persists in isolated eyecups in some species (teleosts and amphibians), and so is driven by a local (ocular) pacemaker. Because of its temporal association with night, and because exposure to melatonin in *in vitro* preparations has been demonstrated to cause "dark-adaptive" changes, melatonin has been postulated to be a mediator of the "night" signal for several ocular rhythms. For instance, in *Xenopus* eye cups, exogenous melatonin promotes the dark-adaptive elongation of cones and the aggregation of melanin pigment granules in RPE cells (Pierce and Besharse, 1985). In addition, melatonin has been shown to activate disc shedding in rods *in vitro* (Besharse and Dunis, 1983).

The fact that the melatonin rhythm persists in isolated eye cups, and can be phase-shifted by light and dopamine agonists, indicates that the rhythm is under local control in some species; specifically that there is an

independent ocular clock that drives the rhythm. Where is this clock located? Using *Xenopus* eyecups, which continue a robust rhythm in melatonin synthesis, it was possible to begin to answer this question. Cahill and Besharse (1993) used specific agents to lesion the inner layers of the retina of isolated *Xenopus* eye cups, leaving intact only the photoreceptors and retinal pigment epithelium. The rhythm in melatonin produced by these "reduced" eyecups was similar to that in intact eyecups; there was a sustained rhythm that was phase shifted by light, having normal nighttime levels. The absolute levels of melatonin were decreased, however. Furthermore, when the RPE was separated from the photoreceptors, the photoreceptors continued to synthesize melatonin rhythmically. This important study provides strong evidence that the photoreceptor layer is the site of the circadian pacemaker that drives the melatonin rhythm (although the intact retina seems to be required for the full expression of the rhythm).

Xenopus are not unique in possessing an independent ocular pacemaker. The eyes of birds also comprise an independent clock (independent of the brain and each other). Underwood and co-workers (Underwood et al., 1990) found that in the Japanese quail, the melatonin rhythm persisted in eyes in which the optic nerve was sectioned. In an elegant experiment, Underwood showed that alternating opaque patches over the two eyes in constant light in optic nerve sectioned quails resulted in a rhythm in melatonin synthesis in each eye that was 180 degrees out of phase with the rhythm in the fellow eye. Therefore, not only is the clock located within the eye, but separate and independent ones exist in the two eyes (Underwood et al., 1990).

To summarize, retinal melatonin in many vertebrates plays a role in the circadian regulation of certain retinal rhythmic processes, mediating the "nighttime" signal. Whether it is an integral part of the ocular circadian clock, or merely an output of the ocular clock has not been resolved to date. In birds, *Xenopus*, and most recently hamsters (Tosini and Menaker, 1996), there is a circadian rhythm in melatonin synthesis that is driven by a clock located within the eye, probably in the photoreceptor layer.

Dopamine is a neurotransmitter found in amacrine and interplexiform cells in vertebrate retinas (reviewed in: Witkovsky and Dearry, 1992). Its synthesis and release are also regulated by the light/dark cycle, with higher levels occurring during the day than during the night, the inverse of the rhythm in melatonin. The rate limiting enzyme for dopamine synthesis is tyrosine hydroxylase; levels of this enzyme are also diurnally rhythmic (Iuvone et al., 1978). Retinal dopamine synthesis is increased by continuous light, and by flickering light (Iuvone, 1984; Besharse et al., 1988). Dopamine and melatonin therefore form reciprocal rhythms in the retina, regulating one another via an inhibitory feedback system. Melatonin inhibits the calcium-dependent release of dopamine (Dubocovich, 1983), and dopamine suppresses retinal NAT activity in *Xenopus* eyecup preparations during subjective night (Iuvone and Besharse, 1986).

Light-evoked modulation of dopamine release can be demonstrated in isolated retinas of rabbit (Godley and Wurtman, 1988), *Xenopus* (Boatright et al., 1994), and teleosts (Dearry and Burnside, 1989), therefore control by light is locally mediated. However, unlike melatonin, the rhythm does not persist under constant conditions, nor in isolation from the organism, and so is not controlled by the ocular clock, and probably does not constitute an endogenous circadian rhythm in most species (Iuvone, 1986).

There are reports, however, of a circadian rhythm in dopamine in some species; for example, in the rat retina dopamine continued to fluctuate in constant darkness (Wirz-Justice et al., 1984), and in the cichlid fish, tyrosine hydroxylase activity showed diurnal fluctuations for 2 cycles in constant darkness (McCormack and Burnside, 1993). These conflicting results can be taken to indicate, not lack of control by a clock, but perhaps a dual influence of light and a circadian clock whose relative influences are species dependent.

It is generally agreed that dopamine and melatonin constitute mutually inhibitory reciprocal rhythms, in which melatonin is the signal for "night" and dopamine the signal for "day" (reviewed in: Besharse et al., 1988; Witkovski and Dearry, 1992). The nighttime rise in melatonin synthesis causes a reduction in dopamine synthesis and release; conversely, the light-evoked increase in dopamine synthesis inhibits melatonin synthesis. Dopamine D2 receptor agonists decrease NAT and melatonin at night, and can mimic the phase shifts caused by pulses of light. (However, dopamine antagonists do not block the effects of light, hence, dopamine is not the sole mediator of the light signal). Dopamine and its agonists cause similar changes in the retina as those induced by light. For example, injecting dopamine agonists into the quail eye during subjective night (in constant dark) shifts the retina to being cone-dominated, which decreases visual sensitivity. By the same token, injections of the dopamine antagonist haloperidol shifts the (dark-adapted) retina from cone- to rod-domination and increases the visual sensitivity during subjective day (Buelow et al., 1992). In addition, dopamine induces light adaptive movements of the photoreceptor outer segments (cone contraction and rod elongation) and pigment migration in the RPE in *in vitro* preparations in green sunfish

(Dearry and Burnside, 1986; Dearry and Burnside, 1988) and in *Xenopus* and bullfrog retinas (Pierce and Besharse, 1985; Besharse et al., 1988). Several other diurnally rhythmic phenomena in the retina have been linked to dopamine; for example, the electrical coupling between horizontal cells is reduced by injecting dopamine agonists (Gerschenfeld et al., 1982; Laufer, 1982). To summarize, retinal dopamine levels fluctuate in a diurnal rhythm, being increased by light and decreased by dark (and melatonin). Dopamine is an output of the circadian ocular clock that has a role in mediating the effects of light on various retinal processes. Evidence indicates however, that the effects of light are not solely mediated via this pathway.

I have reviewed rhythms in melatonin and dopamine in some detail because of recent evidence implicating dopamine (and perhaps melatonin) in ocular growth. It was found that form-deprived myopic eyes showed lower daytime levels of dopamine than normal eyes of both chickens (Stone et al., 1989) and monkeys (Iuvone et al., 1989). In addition, injections of the dopamine agonist apomorphine into deprived eyes in both species suppresses the myopia (Stone et al., 1989; Iuvone et al., 1991; Rohrer et al., 1993). On the other hand, manipulations that are purported to *reduce* dopamine levels in form-deprived eyes (such as constant light, 6-hydroxy-dopamine and reserpine) also *reduce* the degree of myopia (Schaeffel et al., 1995). Therefore, there is no conclusive evidence for a specific role for dopamine in reducing eye growth. A role for melatonin in eye growth is even more speculative as no changes in retinal melatonin in form-deprived eyes have been reported. Furthermore, administering melatonin either via intraocular injection (Schaeffel et al., 1995) or continuous release via

silastic implanted capsules (unpublished observations) had no effect on the growth of the eye (except at very high doses: Schaeffel et al., 1995).

Intraocular Pressure

Intraocular pressure (IOP) results from the continued secretion of aqueous humour from the ciliary epithelium of the ciliary body into the posterior and anterior chambers of the eye. The mechanism of fluid production is believed to be an active secretion as opposed to ultrafiltration, as evidenced by the difference in ion concentrations in aqueous and plasma. The aqueous humour is continually being drained via a combination of 2 pathways: the trabecular meshwork in the Canal of Schlemm and then to the aqueous veins, or via the uveoscleral pathway where fluid passes into the uveal tissues and finally through the sclera. Hence, intraocular pressure is governed by the rate of secretion and the resistance to drainage (reviewed in: Bill, 1977). Because both of these factors are subject to variations dependent on time of day, as well as other physiological events, IOP can vary throughout the diurnal cycle. Diurnal rhythms in intraocular pressure have been documented in humans, rats, and rabbits.

In humans, intraocular pressure undergoes diurnal fluctuations of about 5 mm Hg, being lowest during the night and highest during the early morning (Drance, 1960; Henkind et al., 1973; Frampton et al., 1987). In glaucomatous eyes, there is a large amount of variability in the amplitude of the IOP rhythm, and in some of these eyes the amplitude is much larger than normal. In rabbits, IOP is low during the day and high at night, and the amplitude of the rhythm is quite large (about 10 mm Hg) (Rowland et al., 1981; Gregory et al., 1985). In rabbits, the rhythm persists in constant

darkness, and is therefore circadian (Rowland et al., 1981). In rats, the IOP is high in the evening and lowest at 6 am (Krishna et al., 1995). We find that there is a circadian rhythm in IOP in the eyes of chickens, that is of opposite phase to that in the rat and rabbit; IOP is low during the night and increases in the early morning (Nickla and Wallman, 1995).

The mechanisms underlying the rhythm in intraocular pressure have been most extensively studied in the rabbit. Changes in IOP could be the result either of changes in the rate of secretion from the ciliary body (inflow) or of changes in the outflow resistance. It has been shown that an increase in proteoglycan synthesis by cells in the trabecular meshwork (outflow pathway) increases the resistance to the outflow, hence a rhythmic production of these molecules could underlie the rhythm in IOP (review: Acott, 1992). There is also evidence that aqueous inflow (secretion) is circadian, and that changes in inflow determine the rhythm in IOP in some species (Smith and Gregory, 1989; Kiuchi and Gregory, 1992). In rabbits, for example, the rhythm in aqueous inflow is in phase with the rhythm in IOP, being high during the night and low during the day (Smith and Gregory, 1989). This inflow rhythm persisted in constant darkness and was phase shifted by light, so it too, is driven by the clock. In a separate study it was shown that the increase in aqueous inflow was sufficient to account for the observed concomitant increase in IOP (Rowland et al., 1986).

The neural circuitry involved in the diurnal changes in IOP has not been definitively worked out, however, there is evidence for the involvement of both melatonin and the sympathetic nervous system. Lesions of the superior cervical ganglion (Gregory et al., 1985) or section of its preganglionic input (de-efferentation) (Braslow and Gregory, 1987) both caused a decrease in the dark-induced increase in IOP in rabbits. Also,

an antagonist of beta-adrenergic receptors (timolol) reduced the dark-phase increase in IOP and aqueous flow (Gregory, 1990). These authors concluded that the catacholamine norepinephrine is the primary transmitter controlling the diurnal fluctuations in IOP.

The fact that the rhythm in ocular melatonin is in phase with the rhythm in IOP in rabbit eyes suggested to some that melatonin might be involved in the diurnal fluctuations in IOP. Because melatonin does not have any known antagonists, this is a difficult hypothesis to test. Thus, the evidence for the involvement of melatonin in the rhythm in IOP is indirect: for instance, intraocular injections of melatonin increased IOP in a dose dependent manner (Chiou et al., 1985). However, while this result shows that melatonin affects an underlying process controlling the increase in IOP, it does not definitively show that it is part of the clock-controlled mechanism. Other evidence implicating melatonin in intraocular pressure fluctuations is a study showing that chickens with light-induced glaucoma have significantly higher levels of N-acetyl-transferase, the rate limiting enzyme for melatonin (Aimoto et al., 1985). These studies suggest, but do not prove that melatonin may be a modulator of the rhythm in IOP.

Rhythms in Growth

Rhythm in corneal mitosis

One of the first ocular growth rhythms to be described was the diurnal rhythm in mitosis in the corneal epithelium, demonstrated by counting mitotic figures at intervals over the 24 hour cycle. A diurnal rhythm in the cornea has been found in several species: rats (Cardoso et al., 1972), rabbits (Fogle et al., 1980), chickens and quails (Oishi, 1984; Oishi and Matsumoto, 1985). In rats, the peak in both DNA synthesis

(incorporation of tritiated thymidine) and mitotic index (cell division) occurred in the morning, and the trough near midnight (Scheving and Pauly, 1967). In chickens, the peak in mitosis occurred early in the dark phase (2 hours after lights off), and decreased again 8 hours after lights off. This rhythm persisted in constant darkness for several cycles, but dampened out in constant light (Oishi, 1984). In quail (Oishi and Matsumoto, 1985), the rhythm was shown to exist even in embryos under a light/dark cycle, however, in darkness the rhythm disappeared.

In birds, the rhythm in mitosis in the corneal epithelium is driven by an intraocular clock, similar to the rhythms in melatonin and retinomotor movements. The rhythm is independent of the pineal gland, as it persisted after pinealectomy (Oishi and Matsumoto, 1985). In addition, when eyes were alternately patched using opaque and clear patches in constant light, and so received alternating periods of light and dark, the rhythm in the two eyes became 180 degrees out of phase with one another (Oishi and Matsumoto, 1985), similar to what was found in the melatonin rhythm (Underwood et al., 1988). These alternating rhythms persisted upon release into constant darkness (Oishi and Matsumoto, 1985). This finding constitutes further evidence that a clock is located in the eyes in birds. It is likely that the entrainment of this rhythm is influenced by the melatonin rhythm, as intraocular injections of melatonin in a normal light/dark cycle resulted in a phase advance of 8 hours (Sasaki et al., 1995).

The implications of the existence of this rhythm in the context of eye growth are several fold. First, it was the first study showing that some aspect of ocular growth is diurnally rhythmic. Furthermore, these studies showed that alterations in the light/dark cycle (for example, constant light) resulted in the dampening of this rhythm, in addition to flattening the

cornea. It follows that alterations in ocular rhythms might cause alterations in growth, the premise of this thesis. That this is unlikely to be the whole story is indicated by the fact that although the mitotic rhythm is unaffected by constant darkness, the cornea still flattens. Regardless of the uncertainty regarding the involvement of the mitosis rhythm in corneal growth, the evidence is suggestive of a link between ocular rhythms and ocular growth.

Rhythms in cell cycles

There is abundant evidence that cell division in unicellular organisms shows a circadian rhythm, usually occurring during subjective night under constant conditions of dark or light. These rhythms have been shown to be entrained by cycles of light and dark, persist under constant conditions with a period around 24 hours, can be phase shifted by pulses of light, and are temperature compensated (reviewed in: Edmunds and Laval-Martin, 1984). The cell division cycle and mitosis are believed by most investigators not to be an essential part of the oscillator, but to lie downstream from the clock, and so be an output, or a "hand", of the clock. Because cell division is believed to be an output of the clock, and because it occurs at the level of the single cell (some even believe it can occur in the absence of a nucleus), the components and organization of a fundamental cellular clock mechanism must exist at the level of the single cell.

The growth of many tissues in vertebrates, too, have been found to be synchronized to the light/dark cycle, and many show a prominent 24 hour periodicity in growth parameters such as cell division and protein synthesis (Edmunds and Adams, 1981; review: Thorud et al., 1984). In vertebrates, prechondroblasts, the progenitors of chondrocytes, show generation times (intermitotic intervals) close to 24 hours in isolated single cells, as well as

in cell culture (in human and rat tissue) (Petrovic et al., 1984). Therefore, it is possible that even in vertebrates, circadian organization can be found at the level of a single cell that is not of neural origin. These rhythms in cell division presumably influence the temporal pattern of many other cellular metabolic processes, including protein and DNA synthesis.

Diurnal rhythms in bone and cartilage growth

The metabolic activities of many, if not all, tissues in the vertebrate body are under the control of the endocrine system, which in turn is influenced by the circadian clock. Both cartilage and bone tissues are targets of calciotrophic hormones (parathyroid hormone, calcitonin), which are themselves regulated by the circadian clock, therefore, it follows that many parameters of the growth activities in these tissues would show circadian rhythms (reviewed in: Simmons, 1992). These diurnal activities include proteoglycan synthesis, mitosis, protein synthesis, DNA synthesis, osteoclast activity and calcification. In general, the events involved in growth and cell division in bone are tightly coordinated, with DNA synthesis occurring at the onset of dark, mitosis during the latter phase of the dark period, and collagen synthesis during the early part of the light period.

There is ample evidence that cartilage growth *in vivo* is controlled by the circadian clock (Oudet and Petrovic, 1982). Studies done in the 1960s by David Simmons (1962; 1964; 1968) show peaks in DNA synthesis, mitosis and proteoglycan synthesis to occur at specific times in the diurnal cycle, with DNA synthesis occurring in the morning (or late night), proteoglycan synthesis in the afternoon, and mitosis about 3 hours later in the day (Simmons, 1964; Simmons, 1968). Because these rhythmic events

are temporally correlated with changing levels of serum calcitonin, parathyroid hormone, growth hormone, cAMP and a myriad of other messengers (reviewed in: Buchanan and Preece, 1992), their oscillations are presumably driven by a master oscillator, that is, they would not oscillate in the absence of these endogenous factors. However, an intriguing study indicates that this might not be so, and that the rhythmicity might be at the level of the tissue itself. Stutzmann and Petrovic (1978) showed that a circadian rhythm in DNA synthesis in rat cartilage persisted in culture for up to 72 hours. They removed tissue from rats at intervals of 2 hours and put it into culture for 48-72 hours, or 86-120 hours, so that start times and end times occurred at all the times of day sampled. Tritiated thymidine was introduced into the medium one hour prior to the end of the experiment, and the labeled cells were assayed using autoradiography. DNA synthesis was found to be maximal at 7:00-9:00 hours (clock time) and minimal at 17:00 and 23:00 hours. Furthermore, when the culture medium was enriched with growth hormone and somatomedins (insulin-like growth factors), the growth rate increased and there was a phase shift of 2 hours. To control for possible undetected exogenous time cues, some rats were exposed to a reversed light/dark cycle for several cycles prior to being killed; the rhythms in this tissue were shifted by 180 degrees relative to the animals in a "normal" cycle. Hence, the rhythm in DNA synthesis in cartilage continued in the absence of external cues (both exogenous time cues as well as physiological endocrine cues). In conclusion, this study is unique in vertebrates for showing that a diurnal growth rhythm persisted for several cycles in the absence of extrinsic phase signals in non-neuronal tissue, and by implication, that there is a time-keeper (clock) within this tissue. (In invertebrates, rhythms in non-neural tissues have been shown to

persist in culture; Giebultowicz et al., 1989; Vafopoulou and Steel, 1991; Giebultowicz and Foster, 1995). In Chapter 4, we present evidence that a diurnal rhythm in proteoglycan synthesis exists in the cartilaginous chick sclera, and that it too, shows a persistent circadian rhythm in the absence of extrinsic phase signals.

Recap: Ocular rhythms and ocular growth: is there a connection?

Is the abnormal growth concomitant with form deprivation the result of alterations in ocular rhythms? Put more generally, is the regulation of ocular growth dependent in some way on ocular rhythms so that any visually-induced change is the result of changes in some underlying rhythmic process? There is intriguing evidence implicating diurnal rhythms in ocular growth, however, many mysteries remain.

As discussed earlier in this chapter, constant conditions of light and darkness result in abnormal axial elongation. In addition, varying the amount of light and dark across the 24 hour cycle influences the amount of myopia caused by form deprivation (Gottlieb et al., 1992). This evidence has been cited as support for the notion that ocular diurnal rhythms may be involved in the regulation of eye growth, and that altering these rhythms alters the growth. More recent evidence implicating diurnal rhythms in eye growth is that the daytime levels of the retinal neuromodulator dopamine are reduced under three visual conditions that cause in common an increase in the axial elongation of the eye: form deprivation (Stone et al., 1989), constant light (Bartmann et al., 1994) and negative spectacle lens wear (Guo et al., 1995). A simple hypothesis based on this evidence is that dopamine is a "growth inhibitor". Two lines of evidence suggest that this is

false. First, injections into a deprived eye of two compounds that are presumed to decrease retinal dopamine, 6-hydroxydopamine and reserpine, both decrease the amount of myopia (Schaeffel et al., 1995). (The 6-OH-dopamine result must be viewed with caution because retinal dopamine levels were not measured in that study (Schaeffel et al., 1994). Furthermore, it is possible that compounds that deplete neuromodulators also cause a supersensitivity to the molecule due to an increase in receptor number). Second, during the nighttime when dopamine levels are lowest (Hamm and Menaker, 1980), the elongation of the eye is slowest (Weiss and Schaeffel, 1993; Nickla and Wallman, 1995). Therefore, although the dopamine system may be involved in eye growth, it is not via a simple mechanism whereby there is a direct correlation between growth and retinal dopamine levels. It is possible, though, that the important aspect is not the absolute level of retinal dopamine, but the changes in these levels.

If it is true that deprivation of form vision comprises a type of "constant condition", then form deprivation would be an inadequate paradigm to study emmetropization, as emmetropization implies the use of visual signals to guide eye growth. In fact, there is evidence suggesting that the mechanisms underlying deprivation-induced changes in eye growth differ from those underlying lens-induced changes in eye growth, which might support the notion of essential differences in the regulatory mechanisms for these two manipulations. First, 6-hydroxy-dopamine suppresses form deprivation myopia, but does not affect the compensatory responses to spectacle lenses (neither negative or positive) (Schaeffel et al., 1994). Second, constant light is reported to reduce deprivation induced myopia, but have no effect on the negative-lens-induced myopia (Bartmann et al., 1994). Third, optic nerve section has no effect on form deprivation

myopia (the eyes still elongate), but optic nerve sectioned eyes wearing negative lenses do not become myopic (although the response to positive lenses is unaffected) (Wildsoet and Wallman, 1995). It is plausible therefore, that a primary effect of form deprivation is on ocular rhythms, and that the compensatory growth responses to spectacle lenses are via a completely different mechanism. However, it must be emphasized here that an effect of form deprivation on ocular rhythms does not preclude ocular rhythms being involved in normal growth regulation, including the compensatory response to lens wear. In fact, we have evidence showing that lens-induced defocus causes immediate shifts in the axial length and choroid rhythms, suggesting that diurnal ocular rhythms may be involved in growth regulation and emmetropization, and hence, that deprivation-induced elongation and lens compensation do not represent essentially separate mechanisms.

The aim of this dissertation is to elucidate the influence of ocular diurnal rhythms on the growth of the eye in chicks, and explore the notion that altered growth is a direct result of altered ocular rhythms. To this end, I examined the rhythms in ocular dimensions in normal, form-deprived and "recovering" eyes to characterize their parameters (frequency, phase and amplitude) and to ascertain which dimensions of the eye showed rhythmic changes. Second, I looked for differences between these rhythms in these three types of eyes. Third, I looked for other possible growth-related rhythms and examined them in normal and form-deprived eyes. Because the underlying theme of this dissertation is the role of form vision in ocular diurnal rhythms and ocular growth, an important distinction must be made between two aspects of vision that are not usually thought of as comprising separate attributes, light and spatial and/or temporal transients.

Throughout this work, the use of the term "vision" will be used in the context of "patterned" vision, specifically, visual transients.

There are rhythms in axial length, choroidal thickness and anterior chamber depth in normal, form-deprived and recovering eyes; the phase relationships between the axial length and choroidal rhythms depend on the type of treatment. There is also a rhythm in intraocular pressure in normal eyes. The phase of this rhythm is more variable in form-deprived myopic eyes. Finally, there is a rhythm in the synthesis of matrix proteoglycans in the sclera of normal and form-deprived eyes. In the "Discussion" sections of the three main chapters (Chapters 3, 4 and 5) I speculate on possible functions for these different rhythms in the regulation of eye growth based on the differences found in eyes growing at different rates. Finally, I attempt to explain how the difference in the rate of growth between normal and form-deprived eyes may be accounted for by the deprivation-induced alteration in the phase of the rhythm in intraocular pressure.

CHAPTER 2

Three visual manipulations have phase-dependent effects on eye growth

Introduction

Depriving the eyes of patterned vision by translucent diffusers results in excessive ocular elongation and myopia in chicks and other species. Several quite dissimilar procedures ameliorate this myopia: first, stroboscopic stimulation given during the light part of the diurnal cycle results in a significant reduction in the refractive error of the form-deprived eye (Gottlieb and Wallman, 1987; Brennan et al., 1993). Second, daily brief periods of vision (as little as 15 minutes per day) given to an otherwise form-deprived eye results in an almost emmetropic eye (Nickla et al., 1989; Napper et al., 1995). Third, eliminating the normal diurnal cycle of light and dark either by constant light or constant darkness reduces the amount of deprivation-induced myopia. By the same token, altering the normal diurnal light/dark cycle by exposing the eye to alternating 15 minute periods of light and dark significantly reduces the deprivation-induced myopia (although alternating 2 hour periods are ineffective) (Gottlieb et al., 1992). These procedures can be seen as supportive of any one of 3 hypotheses regarding the mechanisms whereby deprivation produces abnormal elongation: (1) Deprivation myopia is a consequence of a reduction in the amount of daily retinal activity and can be ameliorated by artificially increasing it. This hypothesis is supported by the facts that both stroboscopic stimulation and brief periods of vision, both of which

presumably increase the amount of retinal activity, reduce the myopia. (2) Deprivation myopia requires a critical duration of darkness for its expression. This hypothesis is supported by the findings that constant light, as well as alternating 15 minutes of light and dark both reduce myopia but 2 hour alternating periods of light and dark do not. (3) Normal eye growth is dependent on the normal expression of ocular diurnal rhythms, as both constant light and constant darkness induce abnormal ocular elongation. It is plausible then that the induction of deprivation myopia is also dependent on some aspect of ocular diurnal rhythms, as constant light, constant darkness, and alternating periods of light and darkness all reduce the severity of deprivation myopia. In addition, we know that the elongation of the chick eye occurs in a diurnally rhythmic manner (Weiss and Schaeffel, 1993; Nickla and Wallman, 1995a).

In order to explore the notion that circadian rhythms are critical for normal ocular elongation, we used 3 different visual manipulations known to reduce deprivation myopia: strobe stimulation, brief periods of vision, and light/dark transitions, and asked the question, are the efficacies of these manipulations dependent on the time of day at which they are given? If so, it would indicate that the mechanisms underlying the control of eye growth are phase dependent, and hence that alterations in diurnal ocular rhythms might alter eye growth.

We found that all three visual manipulations were more effective at reducing deprivation myopia when given during the night than when given during the day. In addition, strobe stimulation at transition times of "dawn" and "dusk" are more effective than strobe at other times of the day. Two possible explanations for why visual stimulation is most effective at preventing deprivation-induced ocular elongation when given during the

night (or transition times) are first, because they "re-align" the ocular circadian rhythms more effectively and hence "normalize" the growth of the eye, and alternatively, stimulation at night impacts on some aspect of the growth system that has a phase-dependent sensitivity to the stimulus. In conjunction with data from prior experiments, our results support the notion that the mechanisms underlying normal ocular growth are influenced by diurnal rhythms and may themselves be rhythmic.

Methods

Experiments

We studied the effects of 3 visual manipulations that reduce form deprivation myopia to determine whether the time of day at which they are given is important in their efficacy. We used White Leghorn chickens (*Gallus gallus*) that were hatched in our laboratory. They were housed in temperature-controlled chambers, and food and water were available *ad libitum*. The light/dark cycle was 14L/10D for all experiments: lights went on at 8 am, off at 10 pm.

1. Brief periods of vision during day versus night

Twenty-five birds, 4 days of age, were deprived of form vision by using white translucent diffusers mounted on Velcro rings; the mating ring of Velcro was glued onto the feathers around one eye. This method permitted easy removal and replacement of the diffusers. On day 5 through day 9, the diffusers were removed for 20 minutes at either 2 pm (n=15) or 2 am (n=10). On day 10, refractive error was measured using a Hartinger refractometer, and ocular dimensions were measured using A-scan ultrasonography (described in detail in Methods section of Chapter 3).

2. Stroboscopic stimulation and phase

We tested two different phase-related hypotheses in these experiments. First, that visual stimulation that keeps circadian rhythms entrained with the light/dark cycle will reduce form deprivation myopia. It is known that free-running circadian rhythms can be entrained by a "skeleton photoperiod": brief pulses of light at transition times of dawn and dusk. This phenomenon is presumably the result of light falling on the phase of the rhythm during which it can cause a phase shift, and so result in entrainment to external time cues. With this in mind, we tested whether strobe stimulation would be more effective in reducing deprivation myopia if given at times of "dawn" and "dusk" than if given at other times of the day. In this paradigm, birds were deprived of form vision on day 12 and divided into 3 groups: one group ("transition", n=19) was given half-hour trains of 15 Hz stroboscopic stimulation at times of "dawn" and "dusk" (8 am and 9:30 pm) for 9 days. The second group ("random", n=13) was given 2 half-hour trains of 15 Hz strobe daily at random times over the 24 hour period for 9 days, with the requirement that trains be spaced at least 10 hours apart to resemble the spacing of the stimuli in the first group. The third group of form-deprived birds were the controls, and received no strobe stimulation ("no strobe", n=20). The fellow normal eyes of 14 birds from this group served as controls (n=14). Refractive error was measured in all groups on the 10th day of the experiment. Ocular dimensions and keratometry were measured in the "transition", "no strobe" and "normal" groups only.

In the second paradigm, we tested the hypothesis that strobe stimulation has a phase-dependent efficacy. Twelve birds were deprived of form vision via diffusers on day 3. One group was given 15 Hz stroboscopic stimulation

for 1 hour at 2 pm ("day", n=6); the second group was given 1 hour of strobe at 2 am ("night", n=6) for 6 days. The fellow normal eyes served as the controls. Refractive error was measured on the last day.

3. "Interrupted Day" versus "Interrupted Night"

This experiment examined the phase dependent effects of light/dark transitions in reducing form deprivation myopia. Birds wearing diffusers over one eye starting on day 2 were divided into two groups. One group ("interrupted night", n=7) was subjected to 5 minute pulses of light every 20 minutes (5 minutes light/15 minutes dark) during the 10 hours of night (total light: 17.3 hours). The second group ("interrupted day", n=9) was subjected to alternating 15 minutes of light and dark during their 14 hours of day (total light: 14 hours). In this way, both groups received intermittent stimulation either during the day, or during the night, (although the total amount of light and dark were not equal).

Refractometry and ocular dimensions

For measurement of refractive error, birds were anaesthetized with chloropent, a mixture of chloral hydrate and sodium pentobarbitol. The eyes were cyclopleged using 6 drops/eye of (10 mg/ml) vecuronium bromide (Norcuron, Organon, West Orange, N.J.) and benzalkonium chloride (0.26mg/ml) in saline. Refractive error was measured using a Hartinger refractometer (Jena Coincidence Refractometer).

The pupillary axis of the eye was aligned with the refractometer by centering the corneal reflection of a ring of light coaxial with the instrument. Two measurements each were made on the horizontal and vertical axes (by rotating the barrel of the instrument 90 degrees after each

measurement). The eye was realigned twice more, and two more measurements for each axis were taken per alignment. The median of these 6 measurements per eye (2 measurements per 3 re-alignments) yielded the spherical equivalent refractive error of the eye. Refractive errors were not corrected for the artifact of retinoscopy.

To measure ocular dimensions, two different systems of A-scan ultrasonography were used. For experiments 1 and 3 (20 minutes of vision and "interrupted day and night"), a 7.5 MHz transducer was used and the signal was recorded on diskettes by a Nicolet digital oscilloscope. For experiment 2 (strobe), we used a higher frequency transducer (30 MHz), sampled at a higher frequency. This high frequency system allowed for finer resolution of the various peaks, yielding more accurate measurements of the choroid and retinal layers. The axial length is defined as the distance between the cornea and the sclera. Vitreous chamber depth is defined as the distance between the back of the lens and the retina. The details of this high frequency system and the reproducibility and assignment of the ultrasound peaks corresponding to the various ocular interfaces are provided in Chapter 3.

Corneal curvature was measured using a keratometer (Topcon OM-3). A supplemental lens of +8D was attached to the instrument to adapt it for the highly curved corneas of chicken eyes. All statistical comparisons used a 2-tailed t-test unless otherwise indicated.

Results

I. Brief periods of vision are most effective during the night.

It has previously been shown that exposure to daily brief periods of normal vision greatly reduces the degree of form deprivation myopia (Nickla et al., 1989; Wallman, 1993; Napper et al., 1995). We here show that birds given vision during the night were less myopic and had smaller vitreous chamber depths than those given vision during the day (figure 2.1a, compare black circles to white squares) (mean refractive errors: -2D vs -4.4D, $p=0.08$). The refractive error is significantly correlated with vitreous chamber depth across all three types of eye (figure 2.1a, slope = 8D/mm, $r=-0.75$, $p<0.001$). Hence, the decrease in myopic refractive error in birds given vision during the night is due, at least in part, to a reduction in the length of the vitreous chamber. The mean interocular difference in vitreous chamber depth is significantly greater in the group given vision during the day than in the group given vision at night (figure 2.1b, 0.52 vs 0.25 mm, $p<.05$).

II. Strobe stimulation is most effective at transition times, or at night.

It has previously been shown that the addition of stroboscopic illumination throughout the light phase of the diurnal cycle is effective in reducing the degree of form deprivation myopia (Gottlieb and Wallman, 1987; Brennan et al., 1993). We find that merely two daily half-hour trains of 15 Hz stroboscopic stimulation significantly decrease the deprivation induced myopia (figure 2.2a: "transition" vs "no strobe": -5.2D vs -18.4D, $p<0.0001$; "random" vs "no strobe": -9.8D vs -18.4D, $p<0.0005$). This effect is dependent on the time of day at which they are given; strobe given

at the "transition" times of dawn and dusk are more effective than the same duration pulses given at other randomized times (figure 2.2a: "transition" vs "random", -5.2D vs -9.8D, $p<0.05$). Furthermore, 1 hour of strobe stimulation during the night tended to be more effective than 1 hour of strobe during the day (figure 2.2b: -8.3D vs -14.2D), although the difference is not significant. Two half-hour pulses of strobe at transition times is more effective than one hour given during the day ($p=0.0001$); the difference between "transition" strobe and strobe during the night is not significant.

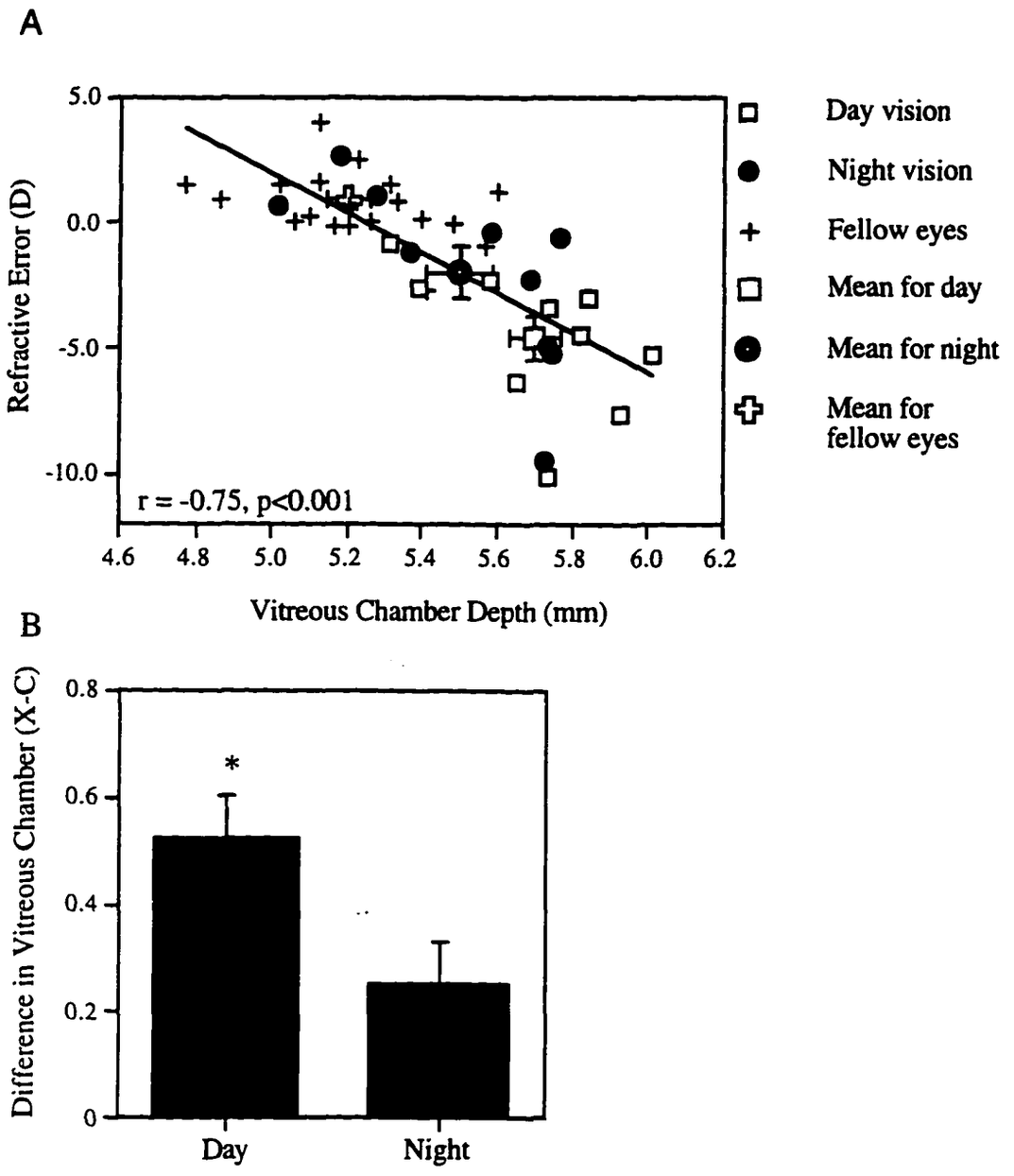


Figure 2.1. The effect of 20 minutes of vision on refractive error and vitreous chamber depth of form-deprived eyes. A. Refractive error plotted as a function of vitreous chamber depth for deprived eyes given vision during the day (squares) or night (black circles), and fellow normal eyes (crosses). The means and standard errors are also show (symbols with error bars). B. Mean interocular difference in vitreous chamber depth (deprived minus fellow eyes) for day vs night vision ($p<0.05$, two-tailed t-test).

The strobe-induced reduction in refractive error in deprived eyes is due to three effects (figure 2.3): First, stroboscopic stimulation reduces the deprivation-induced ocular elongation; deprived eyes exposed to strobe are shorter than untreated deprived eyes (figure 2.3a, "strobe" vs "no strobe" mean axial length: 10.0 mm vs 11.25 mm, $p=0.001$) and do not differ from normal eyes (10 vs 9.8 mm). Second, strobe reduces the choroidal thinning that is a result of form deprivation (figure 2.3b). The choroids in form-deprived eyes exposed to strobe stimulation are significantly thicker than the choroids in the "no strobe" control eyes (figure 2.3b, 0.18 mm vs 0.13, $p<0.05$), but are also significantly thinner than choroids from normal eyes (0.18 mm vs 0.26 mm, $p<0.005$). Finally, strobe stimulation causes a significant flattening of the cornea compared to both normal and "no strobe" control eyes (figure 2.3c, mean radius of corneal curvature = 3.55 vs 3.34 and 3.19 mm, respectively, $p<0.01$, $p<0.005$). Corneal flattening reduces the myopia by increasing the focal length of the eye. We conclude that merely 1 hour of daily stroboscopic stimulation is effective in reducing myopia; furthermore, the efficacy of the effect is dependent on the phase of the diurnal cycle during which it is given: stimulation given at transition times of dawn and dusk appears to be the most effective at reducing deprivation myopia.

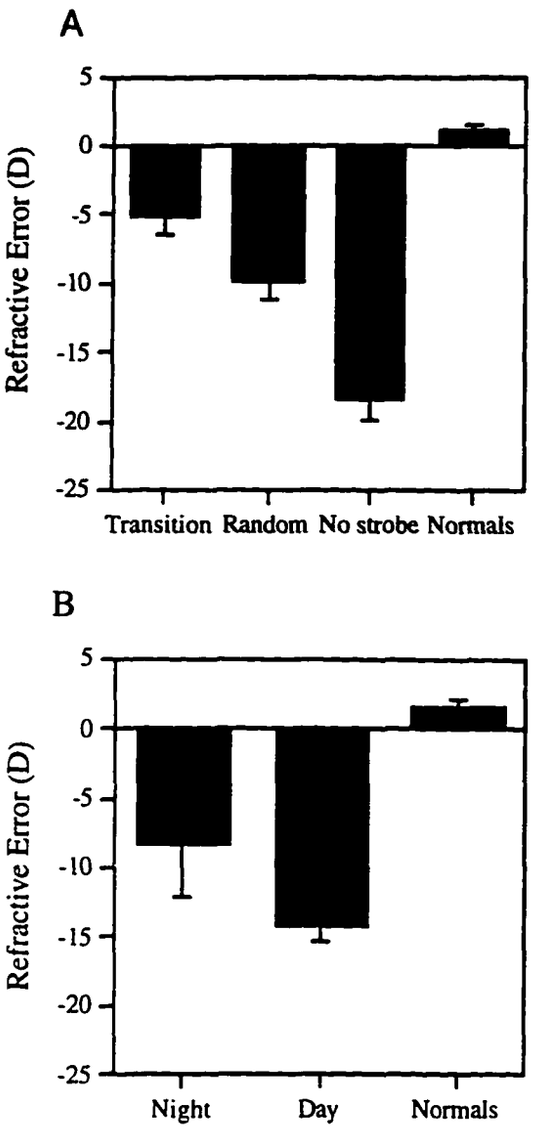


Figure 2.2. A. Mean refractive errors (and standard error bars) of form deprived eyes given 15 Hz strobe stimulation for one half hour at "dawn" and "dusk" (8 am and 9:30 pm= "transition"), at random times ("random"), and untreated control birds ("no strobe" = form deprived eyes; "normals" = fellow untreated eyes). B. The effect on refractive error of one hour of strobe given during the day (2 pm) or during the night (2 am).

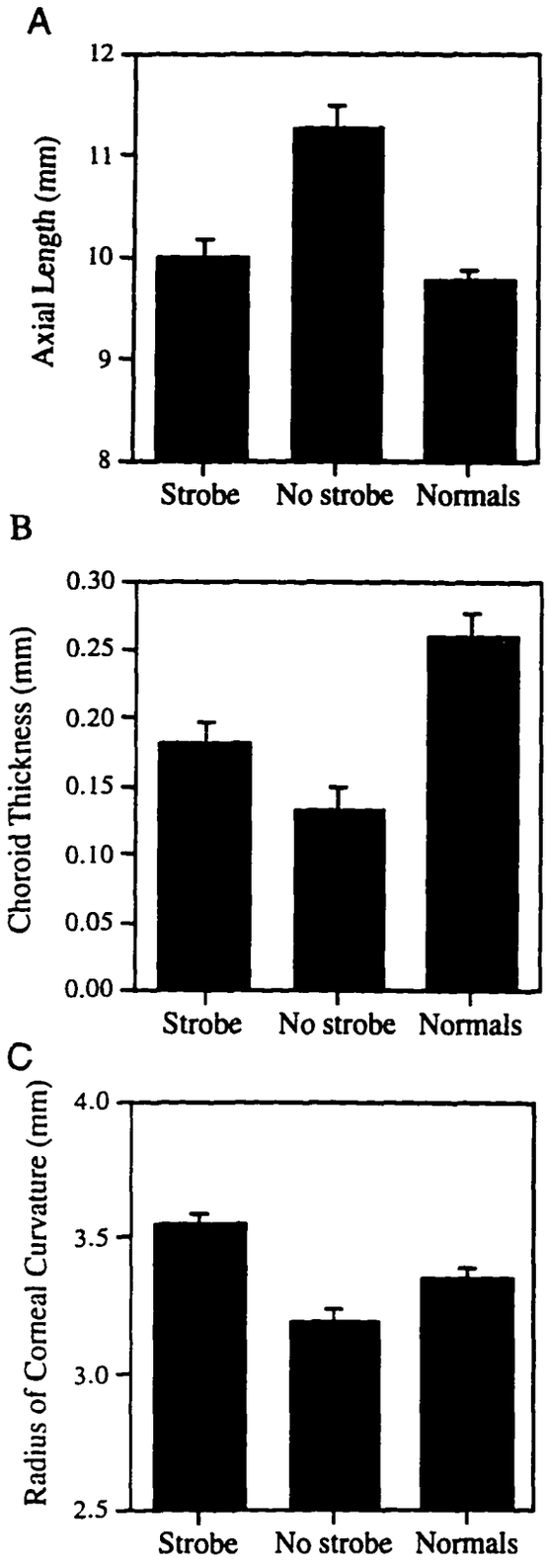


Figure 2.3. The effects of stroboscopic stimulation on ocular dimensions of deprived eyes compared to untreated deprived eyes (no strobe) and their fellow normal eyes (normals). Strobe is given at dawn and dusk ("strobe").

A. Mean axial length. Strobe stimulation decreases axial elongation during form-deprivation.

B. Mean choroidal thickness. Strobe stimulation reduces the deprivation-induced choroidal thinning (compare "strobe" to "no strobe") and thus reduces the myopia. Note however, that choroids in both "strobe" and "no strobe" form deprived eyes are significantly thinner than normal.

C. Mean corneal curvature. Strobe stimulation increases the radius of corneal curvature (flattens the cornea) which decreases the deprivation-induced myopia.

III. Light/dark transitions are only effective during the night

Pulses of light given during the night significantly reduced the degree of myopia (figure 2.4a: "night" vs "no stimuli", -8.5D vs -17.2D, $p<0.01$) while pulses of dark during the day had no effect (figure 2.4a: -18 D vs -17.2D). The reduction in the myopia by light at night was primarily due to a decrease in axial length; these eyes were significantly smaller than the eyes given pulses during the day (figure 2.4b: 9.34 mm vs 9.71 mm, $p<0.05$). Therefore, using brief alternating periods of light and dark to produce visual transients in form-deprived eyes is effective in reducing myopia only if they occur either for the entire cycle (15 minute alternations for 24 hours, Gottlieb et al., 1992) or during the night.

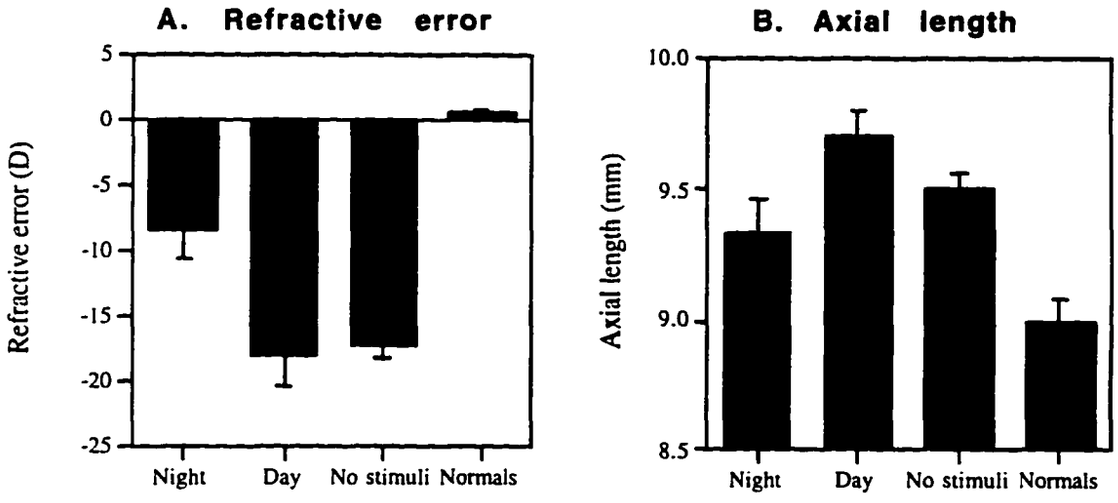


Figure 2.4 The effects of light/dark transitions during the day or night on (a) refractive error and (b) axial length in form-deprived eyes. "No stimuli" are untreated deprived eyes, "normals" are the fellow normal eyes. Pulses of light during the night reduce the myopia by decreasing axial elongation, while transitions during the day are ineffective.

Discussion

Three different visual manipulations that increase visual transients--strobe, daily periods of vision, and light/dark alternations--all significantly reduce the amount of deprivation-induced myopia. We find that these manipulations are more effective at reducing the ocular growth of a form-deprived eye when they are given during the night than when they are given during the day. Furthermore, half-hour trains of strobe stimulation are more effective at dawn and dusk than at random other times of day. By what mechanism could these visual manipulations reduce the effects of form deprivation?

One hypothesis that could account for the ameliorating effects of these visual manipulations on deprivation-induced myopia is that the rate of ocular elongation of the form-deprived eye might be reduced in proportion to the amount of retinal activity experienced each day. This hypothesis has been put forth to account for the effects of stroboscopic stimulation on form-deprived eyes. Evidence in support of this is that the efficacy of strobe is frequency-dependent; the most effective frequencies being between 10-20 Hz, which are lower than the flicker fusion threshold for chickens; high frequencies such as 60 Hz are not effective (Gottlieb and Wallman, 1987). This hypothesis can also account for the ameliorating effects both of brief daily periods of vision as well as alternating 15 minute periods of light and dark.

Alternatively, it is possible that the timing of the retinal stimulation, rather than the amount of retinal activity, is important in the efficacy of the visual manipulations in reducing deprivation-myopia. It had been shown that strobe stimulation over the entire light period of the light/dark cycle reduced the amount of deprivation myopia (Gottlieb and Wallman, 1987;

Brennan et al., 1993). We found that merely 1 hour per day of 15 Hz strobe stimulation was similarly effective. In addition, the efficacy was dependent on phase, being most effective at night. More specifically, we found that half-hour trains of strobe at "transition times" of "dawn" (lights on) and "dusk" (lights off) were more effective than at other times of day. Therefore, the effects of strobe might not be due solely to an overall increase in retinal activity, but also might impact on a phase-dependent retinal process. We speculate that the efficacy of the strobe given at the transition times of dawn and dusk is due to the strobe acting as a "skeleton" *Zeitgeber* for ocular rhythms that were possibly desynchronized as a result of depriving the eye of form vision.

Entrainment of circadian rhythms can be achieved by exposing the animal to daily brief pulses of light at dawn and dusk in otherwise constant darkness ("skeleton" photoperiod). The notion of skeleton photoperiods acting as effective entraining agents is the main evidence for the "nonparametric" model of entrainment of circadian rhythms, which holds that entrainment by light/dark cycles is accounted for by discrete daily phase shifts caused by exposure to light at certain phases of the rhythm (Pittendrigh, 1981). The magnitude and direction of the shifts in response to stimulation are dependent on the phase at which they are given; for example, pulses of light given during the early subjective night usually delay rhythms, while pulses given during late subjective night advance them. It is thus plausible that strobe stimulation at the transition times of dawn and dusk may act as a skeleton photoperiod for the form-deprived eye. If so, it would imply that the light seen through the diffuser is not an adequate *Zeitgeber*, and that vision, and not just light, acts as a *Zeitgeber* for some retinal rhythms.

Although the ameliorating effects of both brief daily periods of vision and alternating periods of light and darkness ("transitions") on deprivation-induced myopia (Nickla et al., 1989; Napper et al., 1995) can be seen as evidence that deprivation-myopia is reduced by increasing retinal activity, they also support the hypothesis that timing is a crucial factor in their efficacy. We found that, similar to strobe stimulation, the efficacy of these treatments was dependent on the phase at which they were given: both were most effective when given during the night. In fact, the "transitions" were ineffective when given during the day. This latter fact, that daytime "transitions" were ineffective, contradicts the hypothesis that the preventative effects were solely due to increasing retinal activity, because the amount of stimulation in both the day and night "transitions" was similar. Similarly, both constant light and constant dark, neither of which cause an increase in retinal activity, also reduce deprivation myopia. Therefore, we conclude that the increase in retinal activity *per se* is not the sole factor accounting for the effects of these three visual manipulations on form-deprived eyes.

An alternative hypothesis that is compatible with the fact that all three manipulations--strobe, vision and light/dark transitions--are more effective at reducing deprivation-myopia when given during the night than when given during the day is that the development of deprivation-myopia requires a certain amount of darkness. It can be postulated that all of these stimuli are effective by virtue of their preventing a "critical" duration of darkness that might be required for the buildup of a "growth" factor. In contradiction to this, however, trains of strobe at "transition" times of dawn and dusk, which were <u>not</u> during the dark period, were significantly more effective than one hour during the day, and tended to be more effective than

one hour of strobe at night (although the difference was not significant). Furthermore, we had previously shown that 2 hour transitions of light/dark (2 hours on, 2 hours off for 24 hours) had no effect on form-deprived eyes; these eyes became as myopic as in a normal light/dark cycle (Gottlieb et al., 1992). Therefore, it is unlikely that the preventative effect of stimulation during the mid-night (which would split the "darkness" into two 5-hour periods) is due to preventing a critical period of darkness. Taken together, these results are inconsistent with the hypothesis that a critical duration of darkness is required for the development of myopia.

In summary, the results of all of the experiments described above are consistent with the hypothesis that visual manipulations are more effective during the night than during the day because they impact on a phase-dependent ocular event that underlies ocular growth. In further support of this phase-related hypothesis is the fact that the growth of the chicken eye is diurnally rhythmic (Weiss and Schaeffel, 1993), and that dopamine, one of the putative signals involved in regulating the growth of chick and monkey eyes, shows a diurnal rhythm in synthesis (Iuvone et al., 1978).

Nighttime visual stimulation is more effective in reducing deprivation-induced abnormal ocular growth than daytime visual stimulation. We know that the rate of ocular elongation is correlated with the rate of scleral proteoglycan synthesis (Rada et al., 1991). We also know that the rate of ocular elongation is lower during the night than during the day (Weiss and Schaeffel, 1993). Consistent with these latter two facts, we found that the rate of scleral proteoglycan synthesis is lower at night than during the morning (Nickla and Wallman, 1995b). Consistent with the first two facts, it was found that 3 minutes of light per hour during the night results in a large (and significant) reduction in proteoglycan synthesis in scleras from

form-deprived eyes compared to eyes receiving no stimulation (Marzani and Kusakari, unpublished results). This biochemical result is in accord with our anatomical results; both showing that brief nighttime visual stimulation results in a rapid and large reduction in scleral proteoglycan synthesis and in ocular growth.

There are two phase-related mechanisms that might account for the greater efficacy of visual stimulation in inhibiting ocular growth during the night. We will assume that the retina synthesizes a molecule that inhibits scleral proteoglycan synthesis, and that retinal stimulation induces release of this molecule. First, it is possible that the retina contains a greater amount of this inhibitor at night (that is, that its synthesis is diurnally rhythmic). Alternatively, the levels of inhibitor might not fluctuate, but the sclera might be more susceptible to its effects during the night by virtue of it being at a certain phase in its metabolic rhythms. It is known, for instance, that cartilage shows diurnal rhythms in cell division, proteoglycan synthesis and DNA synthesis, and that these events occur in specific phase relationships to one another. It is plausible then that the stimulus-evoked response of the sclera is dependent upon the phase at which the stimulus occurs. Specifically, the greater nighttime efficacy of visual stimulation may be a consequence of the inhibitory molecule acting on the sclera during the trough of the rhythm in scleral proteoglycan synthesis. Evidence in support of this conjecture is that there is a significant correlation between the rate of ocular growth and the difference in phase between the rhythms in elongation and intraocular pressure; specifically, the rate of elongation is lowest when these two rhythms are close in phase (see Chapter 5). Hence, ocular elongation could be determined by the phase relationships between various ocular rhythms.

In conclusion, the abnormal elongation resulting from form deprivation can be decreased by certain types of visual stimulation; these manipulations are most effective during the night. (Theoretically, it is possible to ascertain if there is a most effective time for stimulation to affect growth in much the same way as a phase response curve can be generated for the efficacy of a stimulus in phase-shifting a circadian rhythm). We propose that this phase dependence reflects a phase dependent sensitivity of the growth system to stimulation, in much the same way that light is effective in phase shifting a rhythm only during subjective night. It follows that the growth regulatory mechanisms are themselves rhythmic. The implications of this hypothesis are 1) that visual transients can act as *Zeitgebers* for certain ocular rhythms, 2) that "form vision" may have phase-dependent effects on normal growth (for instance, inhibiting growth if "vision" is present at dawn and/or dusk), and 3) that the deprivation of form vision is functionally a type of "constant" condition which may have various effects on ocular rhythms, including allowing them to free-run. In conclusion, it is likely that ocular diurnal rhythms are involved in regulating the growth of the eye, and that deprivation of form vision affects eye growth by altering these rhythms.

CHAPTER 3

Visual influences on ocular diurnal rhythms in axial length and choroidal thickness in chick eyes

Introduction

The eyes of many species start out hyperopic and grow towards emmetropia during early post-natal life, suggesting that visual experience may help regulate the growth of the eye to match the length to the optics. In support of this hypothesis, depriving the eye of form vision by a variety of manipulations that permit the transmittance of light, but not vision, causes the eye to elongate excessively and become myopic (Wiesel and Raviola, 1977; Wallman et al., 1978; Yinon et al., 1979; Hodos and Kuenzel, 1984; Yinon and Koslowe, 1984; Sivak et al., 1989; Norton, 1990). Restoration of normal vision results in a decrease in ocular growth and the eventual return to emmetropia. To date, the strongest evidence for the visual control of eye growth comes from studies showing that the eyes of chicks compensate for artificially induced refractive errors produced by spectacle lenses. Virtually nothing, however, is known of the signals involved in the regulation of eye growth or of the cellular pathways that subserve the transmission of the signal from the retina (where the primary signal is presumably produced) to the effector tissue in the sclera. One hypothesis that has been put forth is that ocular elongation is regulated by separate stimulatory and inhibitory retinal signals, and that the rate of elongation is determined by the balance between these signals, which is in turn dependent on an error signal provided by normal vision. As a

consequence, in an impoverished visual environment the eye adopts a growth pattern in which it elongates without restraint presumably as a result of the absence of the "stop growth" signal initiated by the visual "error signal". This "tonic" regulatory mechanism assumes an integration or summation of at least two opposing signals acting continuously on the sclera (Schmid and Wildsoet, 1996), a view also supported by data from Roher and Stell (1994) who hypothesized that bFGF and TGFb act as "stop" and "go" signals.

In contrast, several recent studies, mostly on chicks, have implicated the involvement of ocular diurnal rhythms in eye growth, and by extension, in the deprivation-induced abnormal elongation. First, it was found that dopamine, a retinal neuromodulator whose levels fluctuate in a diurnal rhythm (being high during the day and low at night), shows reduced daytime levels in form-deprived chicken and monkey eyes (Stone et al., 1989; Iuvone et al, 1989). Furthermore, administration of apomorphine, a dopamine agonist, to lid-sutured chick and monkey eyes reduces the degree of axial myopia (Stone et al., 1989; Iuvone et al., 1990, 1991). Second, Weiss and Schaeffel (1993) found that ocular elongation in normal chick eyes is diurnally rhythmic, showing greater rates during the day than during the night. This elongation rhythm appeared to be absent in form-deprived eyes, which were reported to grow during the night as well as during the day. The loss of growth inhibition at night was proposed as an explanation for the overall increase in ocular growth in these eyes. Third, constant light (the absence of temporal cues) results in excessive ocular elongation in normal eyes; these eyes too, show both a loss of rhythmicity in elongation and reduced levels of retinal dopamine (Bartmann et al., 1994). Finally, eyes exposed to defocus by both positive

and negative spectacle lenses showed a loss of rhythmicity during the first several days of lens wear, prior to full compensation to the defocus (Schaeffel et al., 1995). Together these studies implicate a "phasic" influence on the regulatory mechanism of eye growth, such that signals or cues may be more effective at certain times of day than at others. It is plausible that alterations in the phase of the ocular rhythms may have an influence on the growth response of the eye.

To summarize, it has been reported that the excessive axial elongation caused by depriving the eye of form vision or exposing it to constant light is associated both with reduced retinal dopamine levels and a loss in the diurnal rhythmicity in elongation. The effects of both these conditions could be interpreted as being a consequence of the loss of rhythmicity in underlying ocular processes that regulate the growth of the eye. In the case of constant light, it is easy to imagine that the absence of the diurnal cues of light and dark acting as a *Zeitgeber* could result in a loss of ocular rhythmicity. In the case of form deprivation (or spectacle lenses), however, it is a larger leap in faith because neither diffusers nor lenses prevent the transmittance of light/dark cues. Therefore, any change in ocular rhythms under these conditions must be the result of the absence of visual transients, and not of light and dark *per se*. Alternatively, changes in ocular rhythms could be secondary to the increased rate of elongation. One way to elucidate the involvement of diurnal ocular rhythms in eye growth would be to characterize these rhythms in eyes growing abnormally fast (form-deprived eyes) and eyes whose rate of growth has slowed (eyes recovering from deprivation myopia) and compare these to normal eyes. To this aim, we examined in detail the diurnal rhythms in axial length, choroidal

thickness and anterior chamber depth in normal, myopic and recovering eyes.

Our study augmented the study by Weiss and Schaeffel (1993) in two major ways. First, the "axial length" changes measured by Weiss and Schaeffel were changes in the distance from the cornea to the retina. Because this dimension did not include the tissues behind the vitreal/retinal interface, namely, the retina and choroid, the changes observed could be the result either of changes in length or of changes in the thickness of the retina and/or choroid. To distinguish between an ocular elongation component (i.e. scleral growth) and a possible retinal/choroidal contribution, we refined our ultrasound system to be able to distinguish peaks that we have ascertained represent the interfaces between the respective layers of the ocular "tunic".

Second, the conclusions drawn from the Weiss and Schaeffel study are based on data sampled at only twice the period of the diurnal rhythm (i.e. at the Nyquist frequency); such under-sampling carries the risk of under-estimating the amplitude of the rhythm, and even overlooking an existing rhythm if the times of measurement fall at the mesor (the value midway between the peak and the trough). Therefore, we measured the eyes 3 or 4 times per day. In one set of experiments, the dimensions of the eye were measured at 6 hour intervals: 6 am, noon, 6 pm and midnight over 5 days, but in doing so, we interrupted the "night" at two times (midnight and 6 am). Because it is known that stimulation during the night can shift the phase of circadian rhythms, in other experiments we measured only during the "on" phase of the light/dark cycle: 8 am, 2 pm and 8 pm. Although the sampling rate is lower in this paradigm, it is still above the Nyquist frequency for daily rhythms.

Finally, we examined growth rhythms in eyes that had decreased their growth and were recovering from form deprivation myopia. We know that restoring normal visual input by removing the diffusers results in a rapid thickening of the choroid, and an eventual cessation of axial growth, both of which contribute to a return to emmetropia. We asked whether the diurnal rhythms in these "recovering" eyes differed from those in normal and/or form-deprived eyes.

Methods

Animals

White Leghorn chickens (*Gallus gallus*) were obtained as 1-day-old hatchlings from Truslow Farms (Chestertown, MD) and housed in temperature controlled brooders with a light/dark cycle of 14L/10D. Food and water were provided *ad libitum*. Lights came on at 8 am, and went off at 10 pm for all experiments. Three groups of birds were used in this study: normal birds (no manipulation), myopic birds (one eye was deprived of form vision), and birds that were "recovering" from induced myopia.

To induce myopia, white plastic dome diffusers (light attenuation 0.6 log units) were glued to the feathers around one eye of each bird on day 2. For the myopic group, diffusers remained in place until the time of measurement (day 12); the diffusers were then removed and replaced with diffusers attached to Velcro, and a Velcro ring was glued to the feathers around the eye. This allowed for their easy removal and subsequent replacement upon completion of the measurement, and before the birds recovered from anaesthesia. For the "recovering" group, diffusers were affixed on day 2 and removed on the evening of the first day of

measurement at age 13 days. These remained off for the duration of the experiment.

Ultrasound Measurements

For ultrasound biometry, chickens were lightly anaesthetized with halothane (0.6%, Halocarbon Laboratories) inhalation anaesthesia; the eyes were not under cycloplegia, although the halothane causes some mydriasis and therefore some cycloplegia. To attain the high resolution required, A-scan ultrasonography was done using a 30 MHz polymer transducer (Panametrics Model 176599), linked to a computer interface with a Sonix 8100 A/D board which allowed sampling of the output signal at 100 MHz. Attached to the transducer was a cone of plexiglass which was filled with water and sealed with parafilm; this "water bath" is separated from the cornea of the eye by an intervening layer of ultrasound gel. A purpose-designed head holder for the chicks, which permitted rotations of the head about any axis, facilitated alignment of the transducer with the eye's optic axis. As an optical aid to alignment of the transducer with the optic axis, we used a telescope surrounded by lights which forms an image of a ring when focused on the cornea. Centering of this ring in the pupil indicates alignment of the transducer with the pupillary axis. With this method of alignment, reliable tracings could be obtained within 5 minutes per eye, thereby minimizing the duration of anaesthesia and its possible adverse affects on eye growth. Between eight and ten traces per eye are stored and analyzed to obtain mean data of the parameters of interest. The velocity of sound used to convert data to distances was 1534 meters per second (m/s) for the aqueous and vitreous chambers, retina and choroid, and 1608 m/s for the lens (Wallman and Adams, 1987).

Internal ocular dimensions measured in this manner were anterior chamber depth, lens thickness and vitreous chamber depth (posterior lens to retinal inner limiting membrane), as well as retina, choroid and scleral thickness. The reliability of our measurement system is discussed in the Results section.

Experimental Paradigms

Three different studies were undertaken. The light cycle was 14L/10D: lights went on at 8 am, off at 10 pm for all experiments.

(1) "Interrupted Night". Five normal and 4 form-deprived myopic birds were measured at 6 hour intervals for 5 days and 4 nights. Times of measurement were 6 am, noon, 6 pm and midnight. The measurements at midnight and 6 am exposed the chicks to short periods of visual stimulation during their night; this was unavoidable with the ultrasonography technique used but was minimized by holding the birds in a light-tight box until their turn for measurement, and returning them to darkness immediately upon completion of the measurement (usually within 5 minutes). In a companion study, 10 form-deprived myopic birds were measured at 6 hour intervals over 1 cycle only (started at 6 am on day 12).

(2) "Uninterrupted Night". For purposes of comparison with the Weiss and Schaeffel study and as a control for visual stimulation during the night, a second series of experiments involved measurements done at 8 am, 2 pm and 8 pm over 5 days. These sampling points were selected on the basis that they provided data at two 6 hour intervals during the day and did not interrupt the night. Seven myopic birds were used in this study (yielding 7 "deprived" eyes and 7 "fellow control" eyes). In a companion study, 5 normal birds (10 eyes) were measured at these times over only 2 cycles.

(3) Eyes "recovering" from deprivation. In this group, one eye of each bird was form-deprived from day 2 until day 13. On day 13, the diffusers were removed immediately prior to the evening measurement (8 pm), and measurements were done at 8 am and 8 pm (12 hour intervals) for 3 days and 4 nights. On the final day and night (24 hours) data were collected at 6 hour intervals (8 am, 2 pm, 8 pm, 2 am and 8 am).

Results

Ultrasound Measurements: Performance Limits

Accuracy

Although A-scan ultrasound biometry usually offers an accuracy of 50 μm or so, we have refined it to permit finer resolution. We did this in three ways, (1) by using a high frequency transducer (30 MHz), (2) by using a computer interface operating with a higher frequency sampling rate (100 MHz, nearly 5 times the sampling frequency previously used, allowing for greater precision in measuring distances), and (3) visualizing the peaks at high resolution while aligning the probe with the eye. These modifications allow fine resolution of the retinal, choroidal and scleral boundaries. We here present evidence for the consistency of the various wave forms that enables us to detect changes on the order of 10 microns. We also present the rationale for choosing the various peaks that we assign to the retinal, choroidal and scleral interfaces. We estimated accuracy by measuring one eye 8 times (4 traces per measurement, with realignment in between each of the 8 sets). The means and standard deviations of these 8

averages are then obtained for each component (Table 3.1) to give estimates of accuracy.

Table 3.1. Means and standard deviations for one eye.

Component	Mean (mm)	S. D. (μm)
Anterior chamber	1.537	19.6
Lens	2.26	18.2
Vitreous chamber	5.353	18.2
Retina	0.243	3.5
Choroid	0.256	8.3

Table 3.1. Means and standard deviations for the components of one eye re-aligned and measured 8 times. Four traces were obtained from each measurement. The means and standard deviations of the averages of these 8 measurements are shown. We refer to the standard deviations as "accuracy".

Peak selection and reproducibility

Figure 3.1a shows a representative trace from one eye to illustrate the pattern of peaks that represent the reflections from the major ocular surfaces (see also Wallman et al., 1995). The peaks labeled C1 and C2 represent the front and back of the cornea, respectively, L1 and L2, front and back of the lens, R is the front of the retina and S2 is the outer scleral surface. The two peaks between R and S2, seen in an expanded state in figure 3.1b, are the retinal/choroidal interface (Ch) and the choroidal/scleral interface (S1). For one eye (figure 3.1a), the distances and standard errors from 7 such traces are: C to L1 (anterior chamber depth) = 1.439 mm (+/-.018), L1 to L2 (lens) = 2.139 mm (+/-.009), L2 to R (vitreous chamber depth) = 5.174 mm (+/-.009), R to S2 (retina to back of sclera) = 0.526 mm (+/-.018).

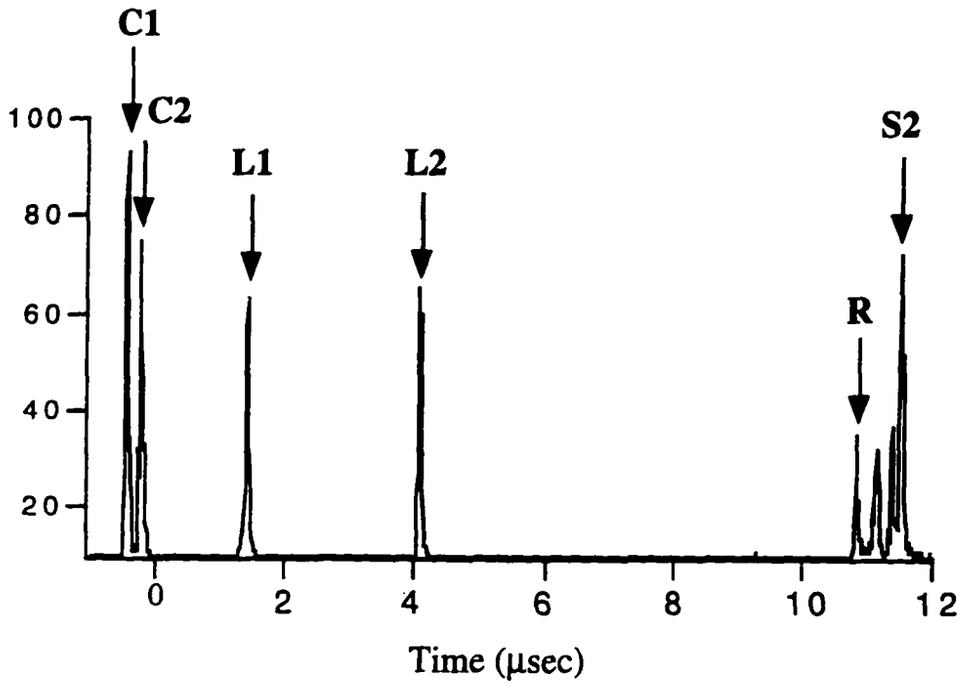


Figure 3.1a. Ultrasound trace from one eye illustrates the general pattern of peaks representing the corneal surfaces (C1 and C2), front and back of the lens (L1 and L2), retinal interface (R) and back of the globe (S2). The y axis is signal amplitude in arbitrary units.

At the back of the eye (figure 3.1b), the first echo is generally composed of 2-3 peaks, two of which are generally relatively large. We choose the first of the two large peaks to represent the retina/vitreous chamber interface. In cases in which only 2 peaks are apparent (for example, in the topmost trace, figure 3.1b), the first peak is chosen. The distance from L2 (lens back) to R therefore delimits the vitreous chamber (figure 3.1a). The accuracy of measurement for the vitreous chamber depth obtained from the re-measurement exercise is 18 μm (Table 3.1).

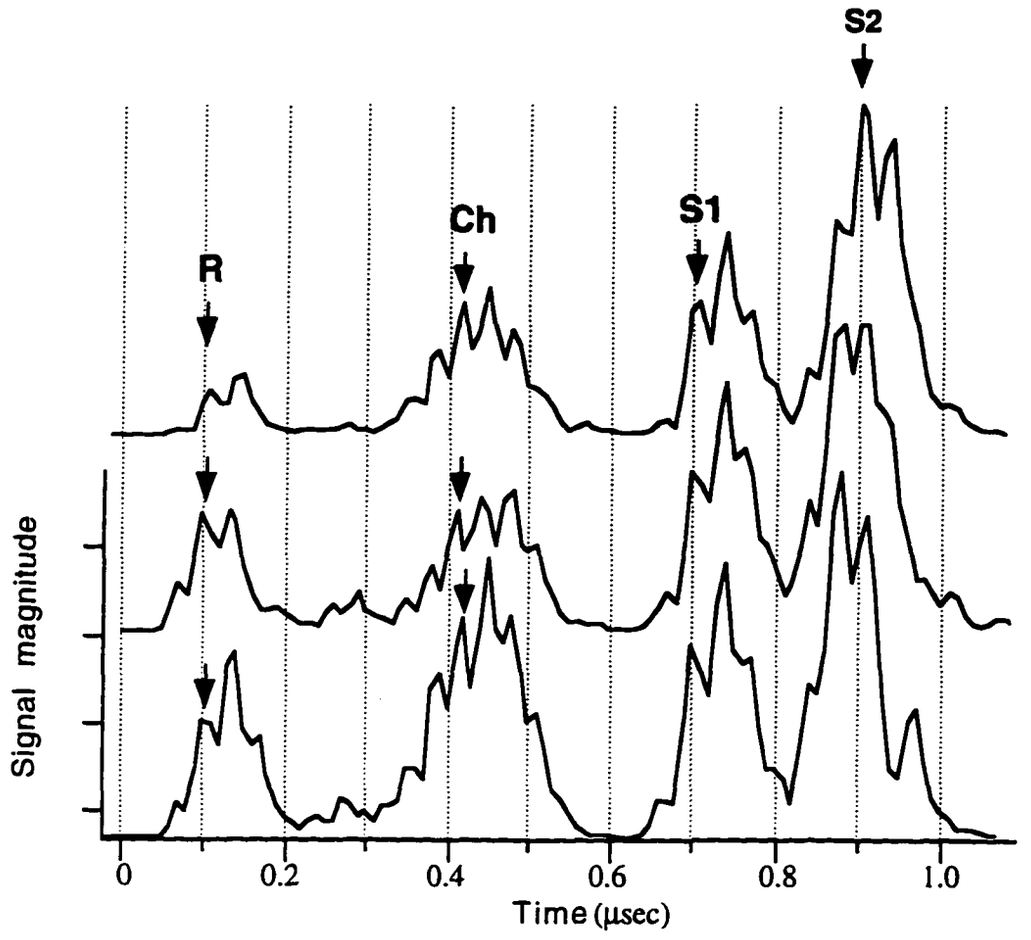


Figure 3.1b. Expanded traces of the back of three different eyes to illustrate the assignment of peaks for each of the interfaces: R, front of retina (vitreal-retinal interface); Ch, front of choroid (retinal-choroidal interface); S1, inner sclera (choroid-scleral interface); S2, outer sclera. See text for details.

The second echo (behind the retina) is generally composed of a series of 5 peaks, the third and fourth of which are usually the largest. We chose the first of these largest peaks (Ch) as representing the retina-choroidal interface. Sometimes there are 3 large central peaks (such as in the middle trace); in these cases the first peak of this "trio" (and third in the series) is chosen. The distance from R to Ch is the retinal thickness. We find that in normal eyes aged 1-4 weeks, the distance between R and Ch is relatively

invariant, and is approximately 245 - 255 μm. The accuracy of this measurement is 4 μm (Table 3.1). These values for retinal thickness are in accord with the values obtained from measuring histological sections, both by us and by Barrington (1990). In addition, they are in agreement with the dimensions obtained using laser interferometry, a completely independent method of measurement (Schmid et al., 1996). The relative invariance in retinal thickness in normal eyes serves as a partial check on the validity of the assignment of the choroidal/retinal interface peak assignment.

The third echo is usually composed of 3 peaks, the first two of which are more distinct. The first of these 2 large peaks is chosen to delineate the front of the sclera (S1). The distance between Ch and S1 represents choroidal thickness. The accuracy of measurement for the choroid is 8 μm. S1 tends to be the most problematic of the peak assignments, because this echo appears most susceptible to changes in eye alignment.

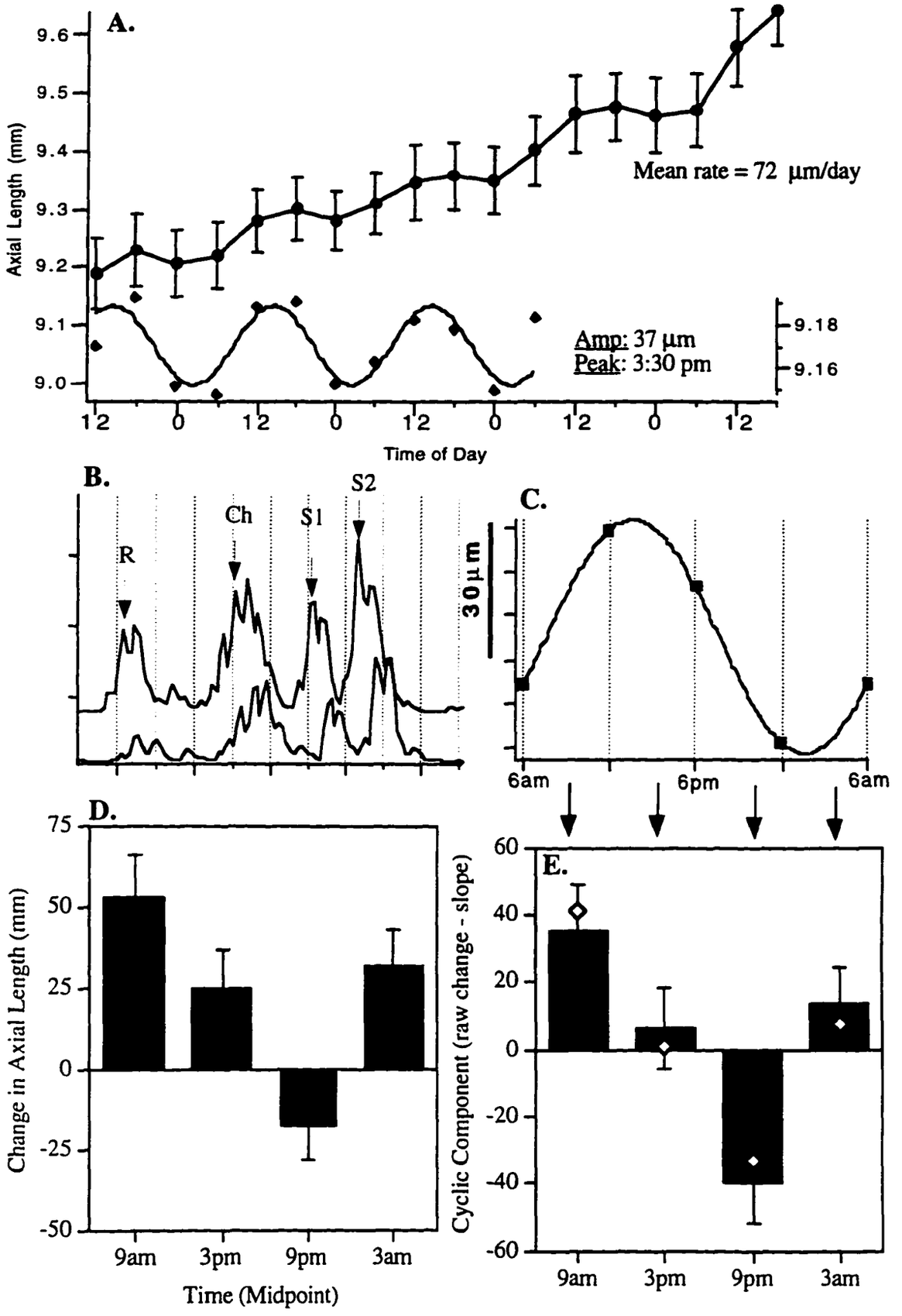
The last echo at the back of the eye is usually composed of 3 peaks, again, the first largest one of these is chosen (S2). The distance between S1 and S2 represents scleral thickness, and the accuracy associated with this measurement is 3 μm. The assignment of S2 is not relevant to this paper, as we always use S1, the front of the sclera, to represent the back of the eye in estimating axial length (defined as the sum of the dimensions from the cornea through S1). Note that axial length **does** include the thickness of retina and choroid and as such is a true measure of the axial length of the globe.

DIURNAL VARIATIONS IN AXIAL LENGTH

Normal eyes

The axial elongation of normal eyes is diurnally rhythmic, with the greatest increase occurring in the early part of the day, and a decrease in length occurring during the early night. This pattern of elongation is shown in figure 3.2a, which shows the mean axial length and standard errors for 10 normal eyes as a function of time over the 4 cycles; at each midnight point (0:00) there is a mean reduction in length. We parsed the growth into a "steady state" component and a daily "cyclic" component, both of which will be shown graphically for normal and myopic eyes in the "interrupted night" experiment only. Because similar procedures for data manipulation and representation will be used throughout this paper, we will discuss these procedures in some detail using the axial length data for normal eyes (figure 3.2) as an example.

Legend: Figure 3.2. Diurnal changes in axial length in normal eyes. A. Mean change in axial length for 10 normal eyes over 5 days and 4 nights; error bars are standard errors of the mean, x axis is time of day, with 12 denoting noon and 0 denoting midnight. Inset, "Residuals" (diamonds) of the data after subtracting the straight line fit regression (only the first three cycles are shown), and the sine wave fit to the data. The peak to peak amplitude is 37 μm, and the peak occurs at 3:30 pm. B. Expanded traces from the back of one eye measured at 6 am (top trace) and 6 pm (bottom trace) to illustrate change in length over 12 hours. R, retina; Ch, choroid; S1, scleral front; S2, back of sclera. C. Mean data for the 3 cycles shown in inset (squares) and sine wave fit to the mean. D. Mean change in the rate of elongation over 6 hour intervals for the 5 days. The x axis shows the midpoints of these 6 hour intervals; for instance, 9 am denotes the change in length between 6 am and noon for all eyes over all 4 mornings. E. Cyclic changes, obtained by subtracting each data point in the data from the inset from the following data point, and averaging these. Bars show the mean oscillation around the mean rate of elongation (slope). Diamonds show the equivalent differences for the sine wave fit to this data. The arrows above compare the cyclic changes as obtained in this manner to those shown in C. For more detail, see Results.



The "growth curve" shown in figure 3.2a can be assumed to be composed of a diurnal rhythm superimposed on a constant rate of growth. To separate these, we first fit a linear regression line to the data in figure 3.2a for each eye; the mean slope of this line defines the mean steady rate of growth; this "mean rate" is noted to the right in figure 3.2a. We then subtracted each individual regression line from the data points for each eye and averaged these residuals to estimate the pure cyclic component; thus the diamonds in the inset to figure 3.2a represent the mean fluctuation around the mean slope. As a tool for quantifying parameters of the rhythmic component, a sine wave having a period of 24 hours was fitted to these mean residuals (wave fit to diamonds). This analysis yields an amplitude (which we express as the peak to peak difference) and a phase (which we express as time of occurrence of the peak), which are noted to the right of the inset. The amplitudes and phase peaks as derived from this analysis are also given in Table 3.2. It should be noted that because one is unlikely to have made the measurements at exactly the peak and trough of the rhythm, this procedure allows the phase to be specified with a precision greater than the 6 hour sampling interval, and the amplitude may be greater than the average daily differences measured. In this analysis we have not attempted to estimate rhythmic components at frequencies higher than 1 per day.

As an illustration of the changes distinguishable in the ultrasound traces, figure 3.2b shows expanded traces of the back of one eye measured at 6 am (top) and 6 pm (bottom). These traces are aligned at the cornea (C1), thus the shift to the right of peaks S1 and S2 in the bottom trace reflects a 22 μm increase in axial length over this interval.

For didactic purposes, figure 3.2c shows the mean of the three cycles shown in the inset: the squares represent the mean of the data points for each time of measurement (x axis), the wave is the sine wave fit to this "collapsed" data. This rhythmic component was used to show changes in the rate of elongation over the 6 hour intervals; these were obtained by subtracting the "residual" length at each time point from the residual length at the next time point for each eye (figure 3.2e, bars). These differences represent the "pure" cyclic component: in these eyes the axial length can be fit by a sine wave around the steady state growth, increasing in the morning and decreasing in the evening (figure 3.2e, white diamonds). In these eyes, the rate of axial elongation during the morning interval is approximately 35 μm above the steady rate of elongation (72 μm/day), and the sine fit to these data over the same interval shows a similar value (about 40 μm, first white diamond, figure 3.2e). The degree of agreement of these two measures (diamonds and bars) gives a graphical idea of the goodness of fit of the sine function to the data.[1]

The "raw" changes in growth rate across the day are shown in figure 3.2d; these represent the mean rates of change in length, obtained by subtracting the length at each time point from the length at the next time

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[1] The fact that there are slight discrepancies between the data points in figure 3.2c and the bars in 3.2e is because the bars in figure 3.2e represent the differences between the data points while the points in figure 3.2c are the data themselves (from the inset). This results in slight differences in the number of points included at certain times of day. For example, the first noon point in the inset is included in the mean represented by the second point in figure 3.2c, however, this same data point is not represented in deriving the change in length between 6 am to noon (9am midpoint, figure 3.2e), because there is no preceding 6 am measurement. This will result in a slight difference between the two graphs where either noon or 6 am data points are involved.

point for each eye (figure 3.2d). In these eyes, there is a mean increase in length between 6 am and noon of about 50 μm, and a decrease in length between 6 pm and midnight of about 20 μm. Note that if there is indeed a sinusoidal oscillation around a mean slope, the sum of the data in the "cyclic" analysis (figure 3.2e) should equal approximately 0, which it does. In contrast, the "raw" changes shown in figure 3.2d include the "steady" state rate of growth, and hence their sum will always be a positive number.

As an aside, it should be noted that using the same procedures as described above, one could divide the day into only two parts and measure the observed growth rate during these 12 hour intervals. One could also compute the growth rate expected if the growth modulation was the sinusoid as derived for the 6 hour intervals. One 12 hour interval, corresponding to the peak and trough, that would yield maximum differences (3:30 am and 3:30 pm, in the example shown). Another choice of 12 hour interval--6 hours distant (9:30 am and 9:30 pm in this case)--would yield zero differences; these correspond to the phase at which the sine wave intercepts the rhythm "mesor" (point midway between the peak and trough). This exemplifies the inherent risks of under-sampling in the characterization of rhythmic phenomena.

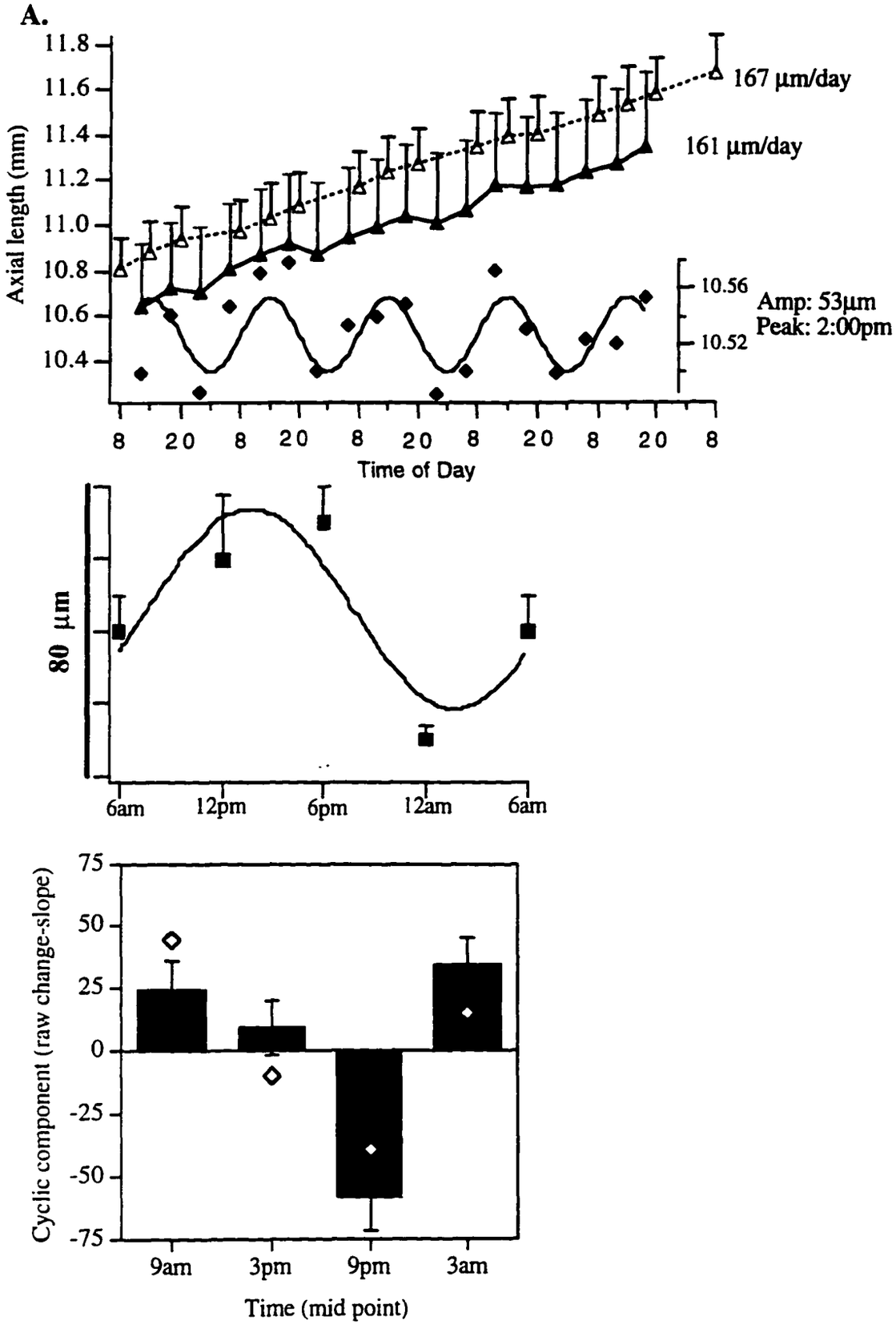
In summary, in normal eyes axial elongation is diurnally rhythmic, the eye increases during the day and decreases during the night (figure 3.2). All four cycles shown in figure 3.2a had a net shrinkage at midnight compared to the prior 6 pm: in the 10 eyes followed for 4 days, the length at midnight was *less* than at 6 pm in 29 out of 40 cases, despite the continuing growth of the eye. The highest rate of elongation (67 μm/6 hours) occurs in the morning, it decreases in the afternoon and *reverses* (eyes shrink by 18 μm/6 hours) in the evening (figure 3.2d & e). Each of

the "net" rates (figure 3.2d) is significantly different from the adjacent one, and all but the evening interval (midpoint at 9 pm) differ from zero (t-test).

Form-deprived eyes and fellow normal eyes

Interrupted night

Form-deprived eyes: The elongation of form-deprived eyes also shows a diurnal rhythm, increasing during the day and decreasing at night (figure 3.3), although the rhythm is less apparent in figure 3.3a than it was for normal eyes because the rapid growth of myopic eyes requires a compressed ordinate scale. For the eyes measured 4 times per day (figure 3.3a, solid lines) the steady rate of growth is 161 μm/day, almost 3 times that of normal eyes. The cyclic component (inset and figure 3.3b) shows an amplitude of 53 μm, which is approximately 1.5 times the amplitude of normal eye growth. The peak of the rhythm occurs in the afternoon (2:00 pm, inset), slightly phase advanced compared to the rhythm in normal eyes (compare to figure 3.2a, inset, and see Table 3.2). These eyes, too, show a decrease in length in the early part of the night (between 6 pm and midnight: figure 3.3a - 3.3c, figure 3.4, black bars). For the 4 eyes followed over 4 days, the length at midnight was less than at the prior 6 pm in 9 out of 16 cases, despite the continuing growth of the eye.



Fellow eyes: The fellow normal eyes of myopic eyes also show a diurnal rhythm in axial length (figure 3.4a and 3.4b, grey bars), growing more during the day than during the night. Figure 3.4a shows the changes in the rate of axial elongation for both eyes in the four birds whose data is shown in figures 3.3a (solid line) through 3.3c, whereas figure 3.4b shows the data from the 10 birds in the "companion study" that measured over one cycle only. The pattern of growth in the fellow eyes (grey bars) resembles that of normal eyes (compare grey bars in figure 3.4a and 3.4b to figure 3.2d), although the mean rate of growth is slightly higher (82 μm/day, Table 3.2). Similar to both normal and form-deprived eyes, the peak of the rhythm occurs in the afternoon (3pm, Table 3.2). The difference in growth rate between fellow normal and form-deprived eyes (82 μm vs 161 μm/day) is most manifest in the late night, in which the myopic eyes grow more than their fellow eyes (figure 3.4a: 3 am midpoint: 72 μm vs 26 μm/6 hrs, $T= 3.2$, $p<0.005$). In addition, the variability for the fellow eyes is greater than for form-deprived eyes (compare standard error bars of grey and black bars; data have equal n's).

Figure 3.3. (Previous page). Diurnal changes in axial length in form-deprived myopic eyes. A. Mean change in axial length for 4 form-deprived eyes over 5 days and 4 nights in "interrupted night" (solid lines) and in uninterrupted night (dashed lines). Error bars are standard errors of the mean, x axis is time of day. Inset, residuals (diamonds) of the data from interrupted night (after subtracting the regression), and the sine wave fit to the data. The amplitude is 53 μm, and the peak occurs at 2:00 pm. B. Mean of all 5 cycles shown in inset (squares) and sine wave fit to the data. C. Changes around the mean rate of elongation, obtained by subtracting each data point in the data from the inset (residuals) from the following data point for all eyes, and averaging these. Bars show the mean oscillation around the mean rate of elongation (slope). Diamonds show the sine wave fit to the data.

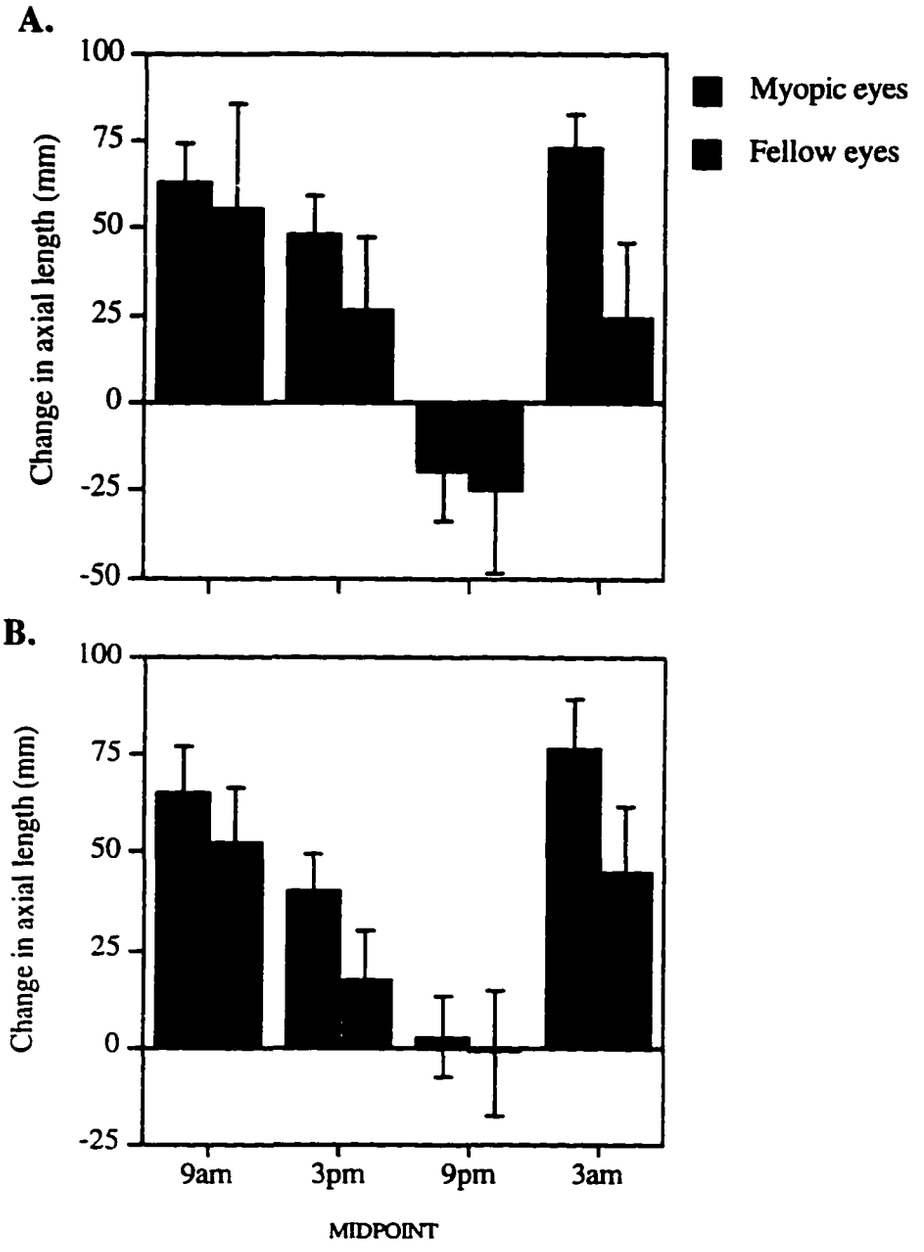


Figure 3.4. Changes in axial length in form-deprived (black bars) and fellow eyes (grey bars) in two experiments; common x axis shown in 3.4b represents midpoints of 6 hr intervals. A. Axial length changes in 4 birds whose data for the form-deprived eye is shown in figure 3.3. B. Axial length changes in 10 birds measured over 24 hours. The pattern of change in length are almost identical in the two experiments for both eyes. Error bars are standard errors of the mean.

The phase of the rhythm in axial length in form-deprived myopic eyes is advanced relative to that for fellow normal eyes (figure 3.5, figure 3.6). Figure 3.5 shows the mean data (residuals, symbols) for the 10 birds from the "one cycle" experiment (data in figure 3.4b), and for the first cycle only for the 4 birds from the "interrupted night" experiment (data in figure 3.4a) combined. The waves show the sine waves fit to the data. For all 14 birds (figure 3.5, shown individually in figure 3.6), the mean difference in phase between the two eyes is 2.3 hours (T=1.8, p=0.09).

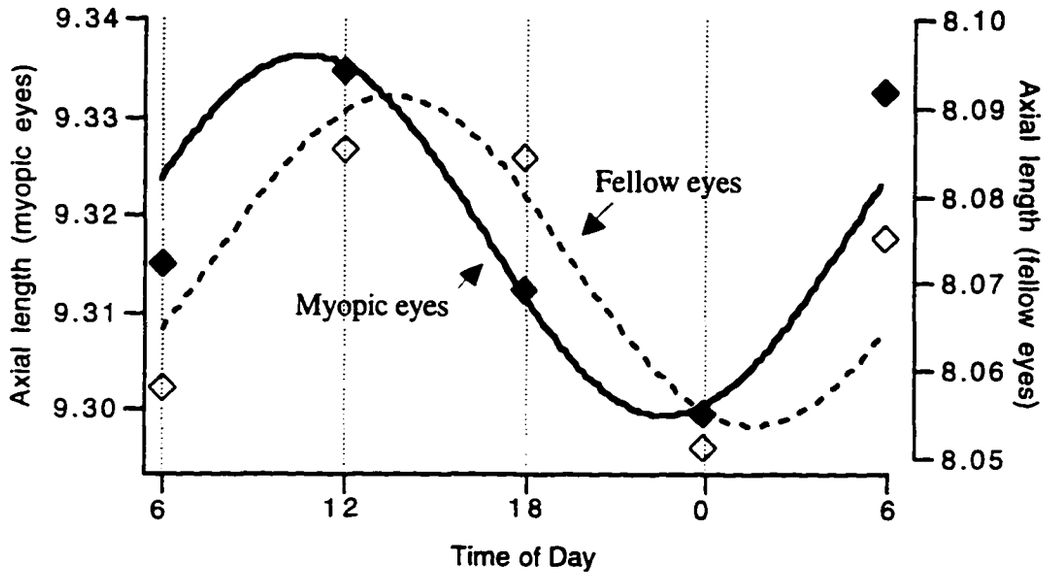
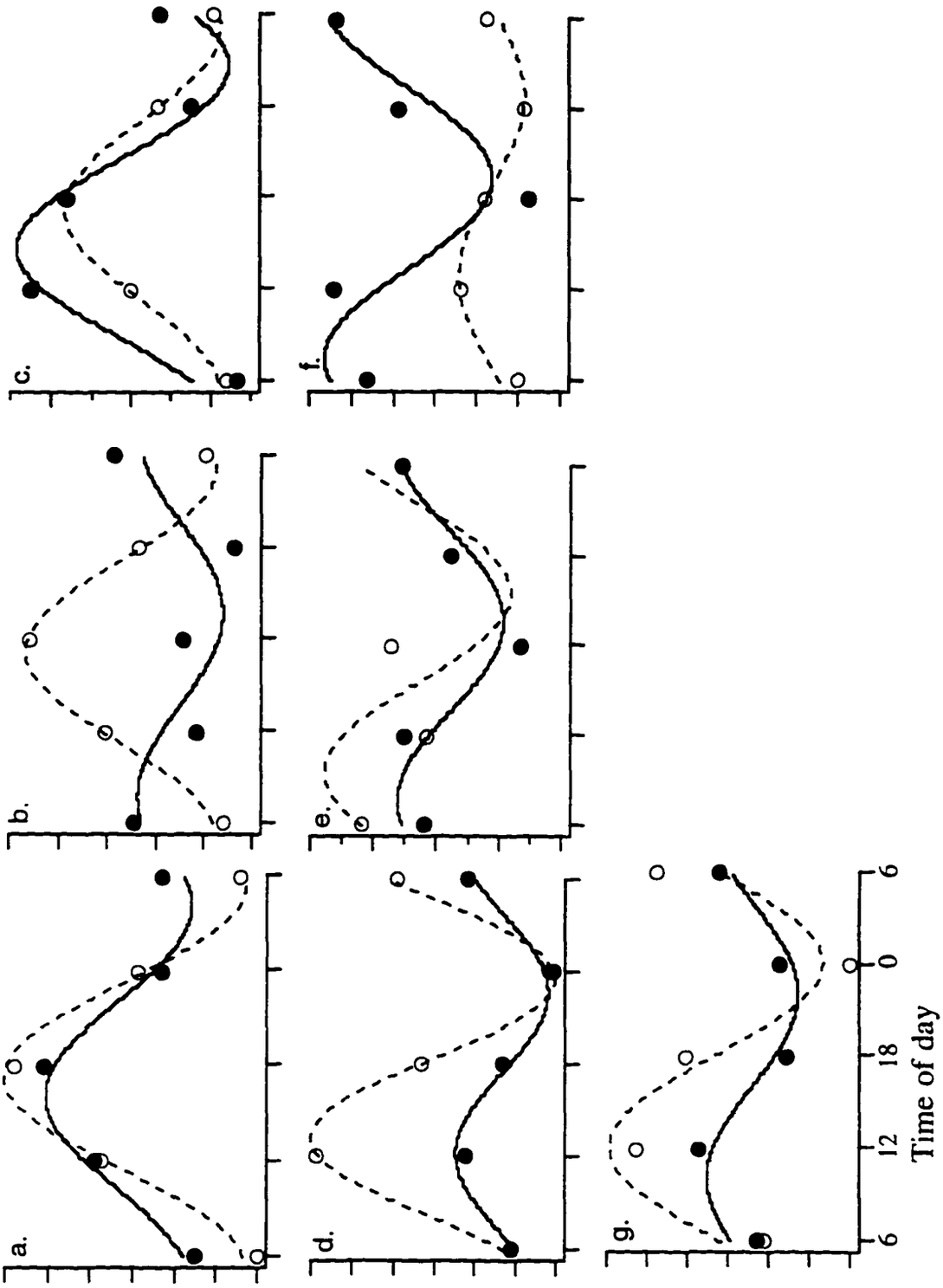
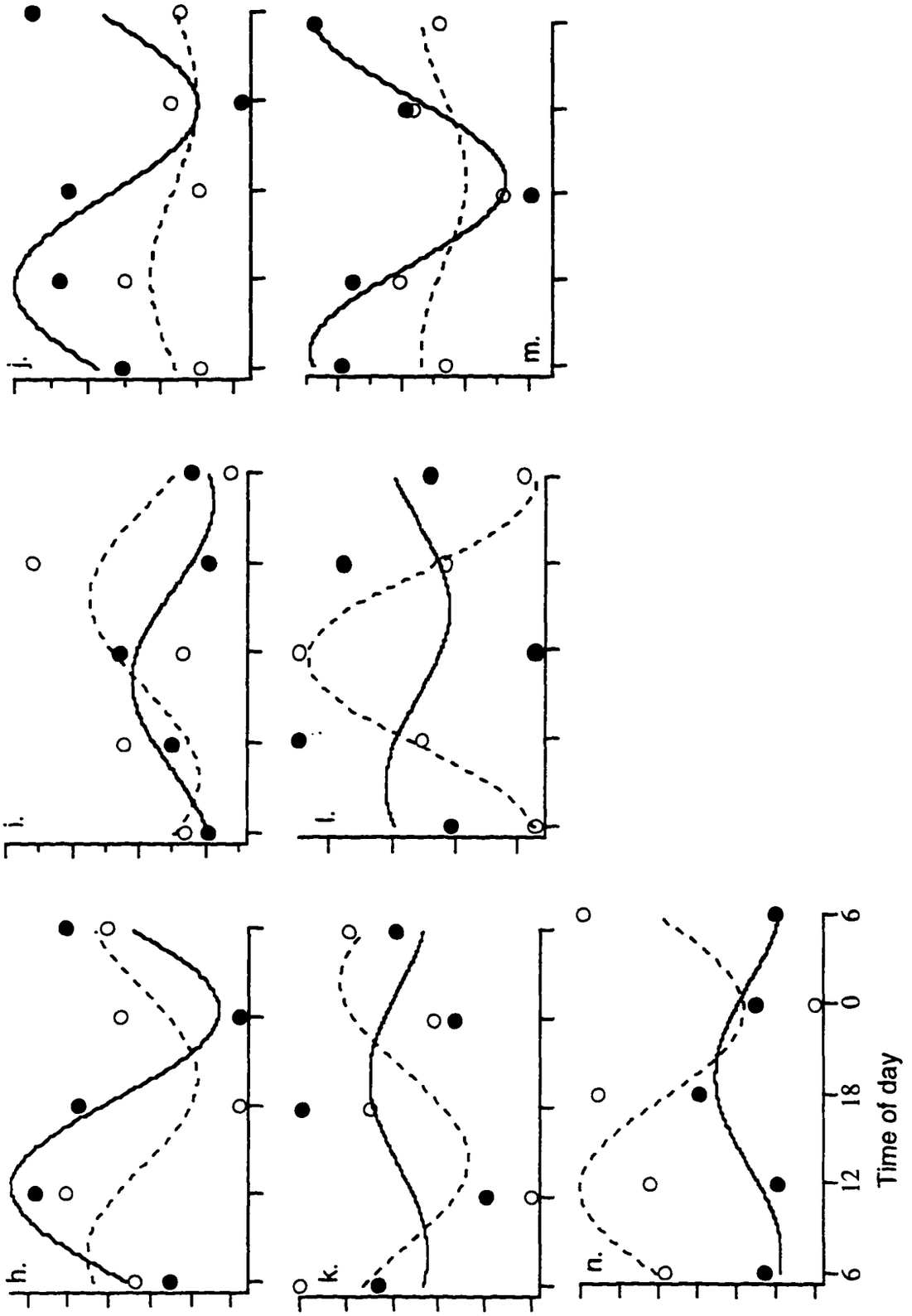


Figure 3.5. Mean residuals (symbols) and sine wave fit to the data for one cycle of the axial length rhythm in both eyes of form-deprived birds whose data is shown in figure 3.4 (n=14). For the 4 birds in figure 3.4a, only the first cycle, beginning at 6am, is used. Form-deprived eyes, solid line and symbols; fellow eyes, dashed line and open symbols.

Figure 3.6 shows the individual sine waves fit to the data for all 14 birds; solid lines and symbols are form-deprived eyes; dashed lines and open symbols are fellow eyes; the scales are the same. As an estimate of goodness of fit, we calculated the standard deviations of the residuals of the sine wave fit to the data, and divided this by the standard deviation of the "raw" data. We chose a criterion of < 65% as being an acceptable fit to the data, and separated the data accordingly. Birds with both eyes having "acceptable" fits are shown in graphs 3.6 a-g; birds with either eye having a "poor" fit are shown in graphs 3.6 h-n. For the 7 birds with acceptable fits, the phase of the rhythm in the form-deprived eyes is advanced relative to the fellow eyes by a mean of 3.4 hours (t-test, $p<.05$).

Figure 3.6. (Next 2 pages). Sine waves fit to the residuals (symbols) of the axial length rhythm in form-deprived (solid lines and symbols) and fellow normal eyes (dashed lines and open symbols) in 14 birds measured at 6 hour intervals over 24 hours (the mean of which is shown in figure 3.5). Graphs a-g show birds with both eyes having acceptable fits to the data; birds in graphs h-n have at least one eye with an unacceptable wave fit (see text). For the birds with acceptable fits (graphs a-g), there is a mean phase advance of 3.4 hours in form-deprived eyes relative to fellow eyes.





Uninterrupted night

In form-deprived myopic eyes measured at 8 am, 2 pm and 8 pm, the rate of elongation appears to be more linear than that for the interrupted night experiment (figure 3.3a, compare dashed to solid lines), although the mean overall rate is similar (167 μm vs 161 μm/day). In contrast to form-deprived eyes measured 4 times per day, these eyes showed no significant difference in the rate of elongation during the day versus during the night (figure 3.7a, black bars: between 8 am and 8 pm: 99 μm versus 73 μm/12 hours, p=0.07). As an illustration of this, the cyclic component is negligible (figure 3.7b, black bars). The fellow eyes show a similar pattern; they too show no diurnal differences in the rate of elongation (figure 3.7a, grey bars: 56 vs 48 μm/12 hours), and the cyclic component is nearly non-existent (figure 3.7b, grey bars). (It should be noted here that because the measurement intervals are unequal, subtracting the regression from the data results in a larger change in the "night" data (8p-8a), which encompasses 12 hours, compared to the 6 hours of the morning and afternoon. A consequence of this is that the difference between the "raw" data (figure 3.7a) and the "cyclic" data (figure 3.7b) is relatively greater for the 12 hour interval (8p-8a) than for the 6 hour intervals (8a-2p and 2p-8p).

What accounts for the finding of diurnal rhythmicity in form-deprived eyes measured at 6 hour intervals but not in eyes measured at 8 am, 2 pm and 8 pm? When form-deprived eyes were measured at 6 hour intervals, we found that the mean estimated peak and trough of the rhythm occurred at 2 pm and 2 am, respectively (Table 3.2). It follows that the mesor of the rhythm falls at 8 am and 8 pm; hence theoretically, measurements done at

these times would show no difference between these 12 hour intervals. This is, in fact, what we found. In conclusion, the fact that neither form-deprived nor fellow eyes show a diurnal rhythm in length when measured at 8 am and 8 pm does not preclude the existence of a rhythm; the rhythm may be imperceptible if the measurement times fall near the rhythm mesor.

Interestingly, for normal eyes measured at 8 am, 2 pm and 8 pm, there are significant diurnal differences in the rate of elongation (figures 3.7a and 3.7b, white bars); these eyes elongate during the morning and afternoon, and decrease over the night. The day versus night difference is highly significant (85 μm vs -10 μm/12 hours, $p<0.0001$). The fact that normal eyes show a diurnal rhythm in elongation but myopic eyes do not is presumably a reflection of a phase difference between normal and myopic eyes (Table 3.2) although the difference is not statistically significant.

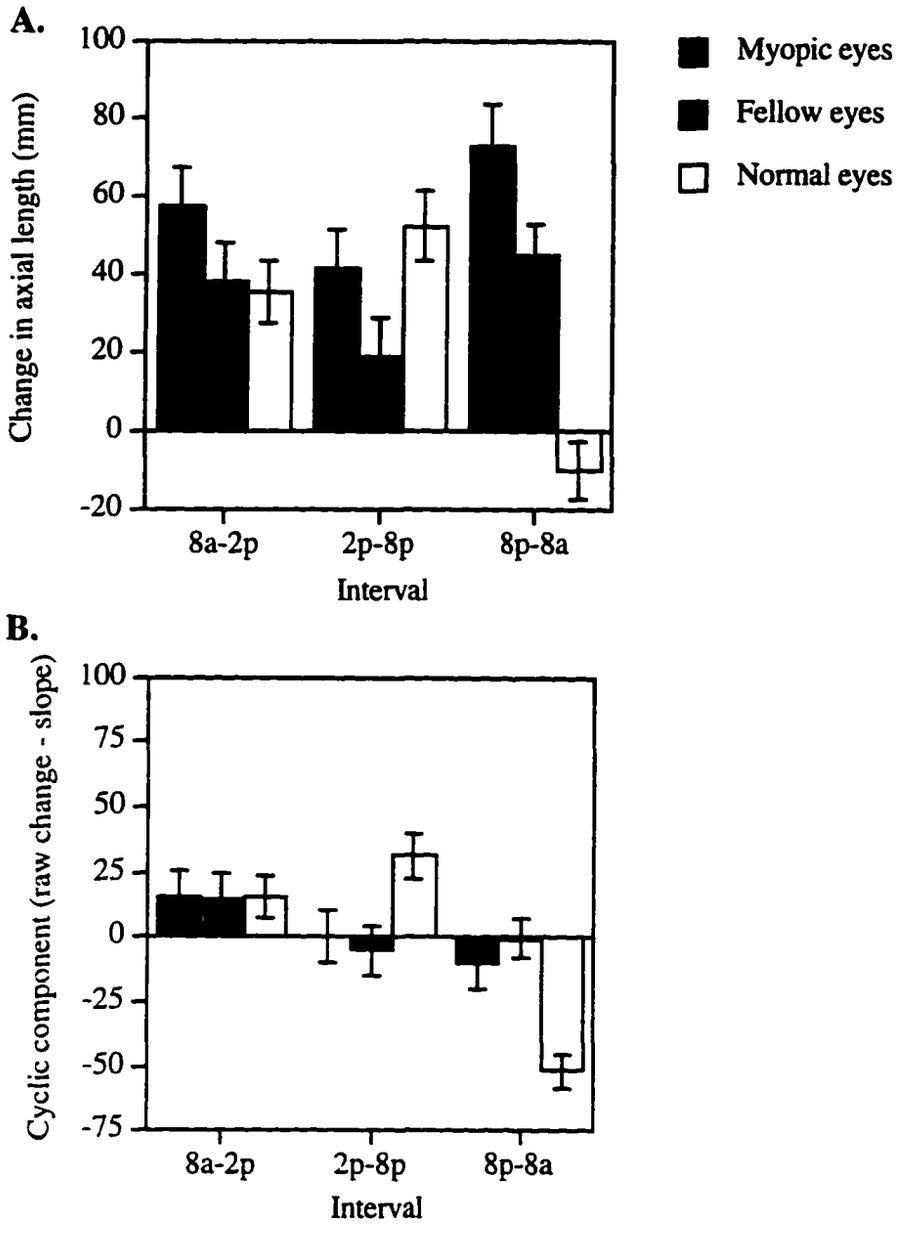


Figure 3.7. Diurnal changes in axial length in form-deprived eyes (black bars), fellow eyes (grey bars) and normal eyes (white bars) measured 3 times per day. The first two intervals are 6 hours, the last is 12 hours (8p-8a). A. Raw changes in the rate of elongation over the day. B. Changes in the cyclic component (after subtracting the regression). There are no significant diurnal differences in elongation in form-deprived or in fellow eyes. Normal eyes, however, elongate significantly more during the day than during the night.

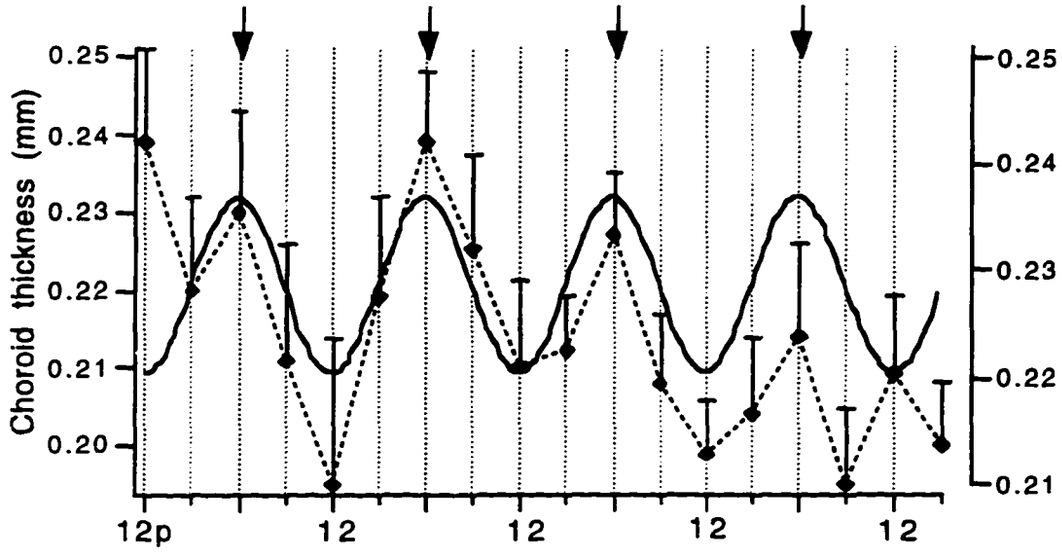
DIURNAL VARIATIONS IN CHOROID AND VITREOUS CHAMBER

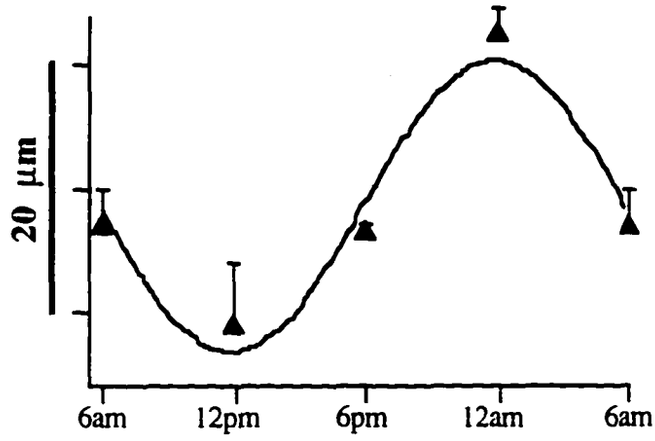
Normal eyes

Choroid

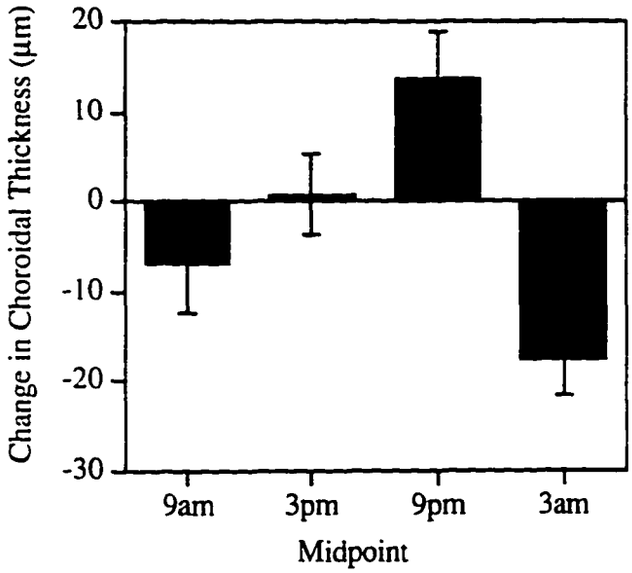
A novel finding of this study is that the choroid, the vascular tissue behind the retina, shows large fluctuations in thickness both in normal and in form-deprived eyes. In normal eyes, the choroidal thickness increases in the evening and decreases during the night and early morning (figure 3.8). Of the 10 eyes followed for 4 days (dotted lines, Figure 3.8a), the choroid thickness was greater at midnight (arrows) than at the prior 6 pm in 29 out of 40 cases. The greatest amount of thickening occurs in the evening and the greatest thinning at night (figure 3.8c: 9 pm vs 3 am, 14 μm vs -18 μm/6 hours, respectively, $T=4.7$, $p<0.0001$). The cyclic component of these changes (figures 3.8a and 3.8b) shows a pattern almost identical to that describing the absolute changes (figure 3.8c), reflecting the very small steady change in choroidal thickness (-6.0 μm/day, Table 3.2). The amplitude of the rhythm is 16 μm and the peak occurs at midnight (figure 3.8a; Table 3.2). The rhythm in choroidal thickness is approximately anti-phase to the rhythm in axial length (Table 3.2, column 4); the choroid is increasing in thickness at the same time that the eye is decreasing in length (compare figure 3.8c to figure 3.2d and 3.2e). This difference in phase is statistically significant (Table 3.2, column 5, $T=3.5$, $p<0.01$).

Figure 3.8. (Next page). Diurnal changes in choroid thickness in normal eyes. A. Dotted line, mean choroid thickness for 10 normal eyes over 5 days and 4 nights; error bars are standard errors of the mean, x axis is time of day, with 12 denoting noon; arrows denote midnight. Solid line is sine wave fit to the data, axis on right. The amplitude is 16 μm and the peak occurs at midnight. B. Mean residuals (triangles) for all 5 cycles shown in 3.8a and sine wave fit to the mean. C. Mean change in thickness over 6 hour intervals for the 5 days obtained by subtracting each data point in 3.8a from the successive data point. The x axis shows the midpoints of the 6 hour intervals.





C.



Vitreous chamber depth

The vitreous chamber (lens to retina) shows a diurnal rhythm in elongation similar to that of the axial length; it increases most in the morning and decreases in the evening (figures 3.9b and 3.9c, compare to figures 3.2d and 3.2e). The cyclic analysis shows that the amplitude is 64 μm, which is larger than that of the axial length amplitude, and the peak occurs at the same time as that for axial length (2:00 pm, inset figure 3.9a; Table 3.2).

Changes in the vitreous chamber depth are the sum of changes of opposite sign in axial length and choroidal thickness. Because the choroidal changes are relatively small compared to the changes in axial length, the rhythm in the vitreous chamber depth is in phase with the rhythm in axial length, and of greater amplitude. The oscillations in choroidal thickness account for approximately one quarter of the change in the vitreous chamber dimensions (the choroidal rhythm amplitude is 16 μm, the vitreous chamber amplitude is 64 μm). The steady growth rate is 44 μm/day, which is somewhat lower than that for axial length, presumably because the increasing anterior chamber depth contributes an additional component to the increasing axial length. (The influence of the anterior chamber rhythm is discussed in more detail in a subsequent section.)

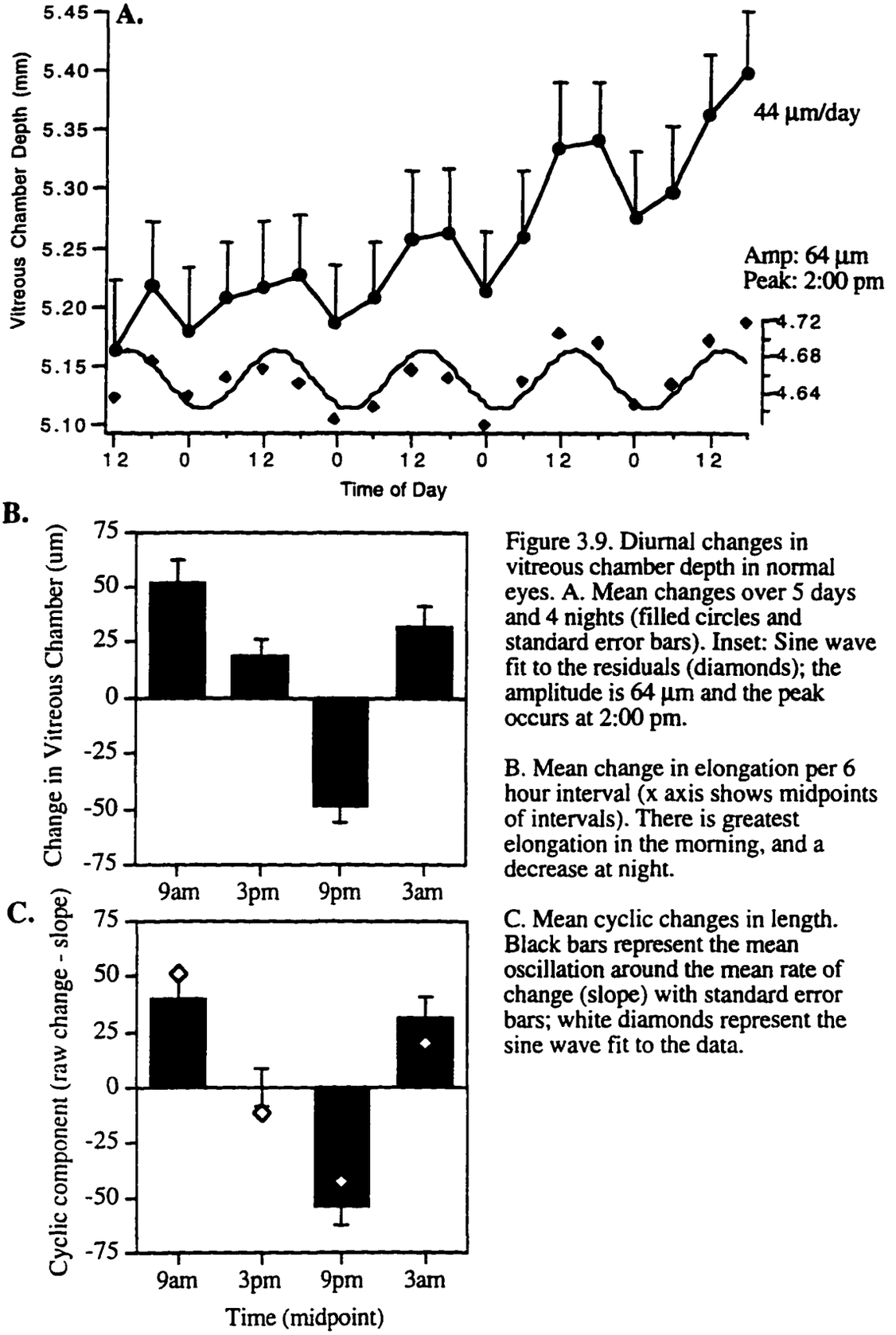


Figure 3.9. Diurnal changes in vitreous chamber depth in normal eyes. A. Mean changes over 5 days and 4 nights (filled circles and standard error bars). Inset: Sine wave fit to the residuals (diamonds); the amplitude is 64 µm and the peak occurs at 2:00 pm.

B. Mean change in elongation per 6 hour interval (x axis shows midpoints of intervals). There is greatest elongation in the morning, and a decrease at night.

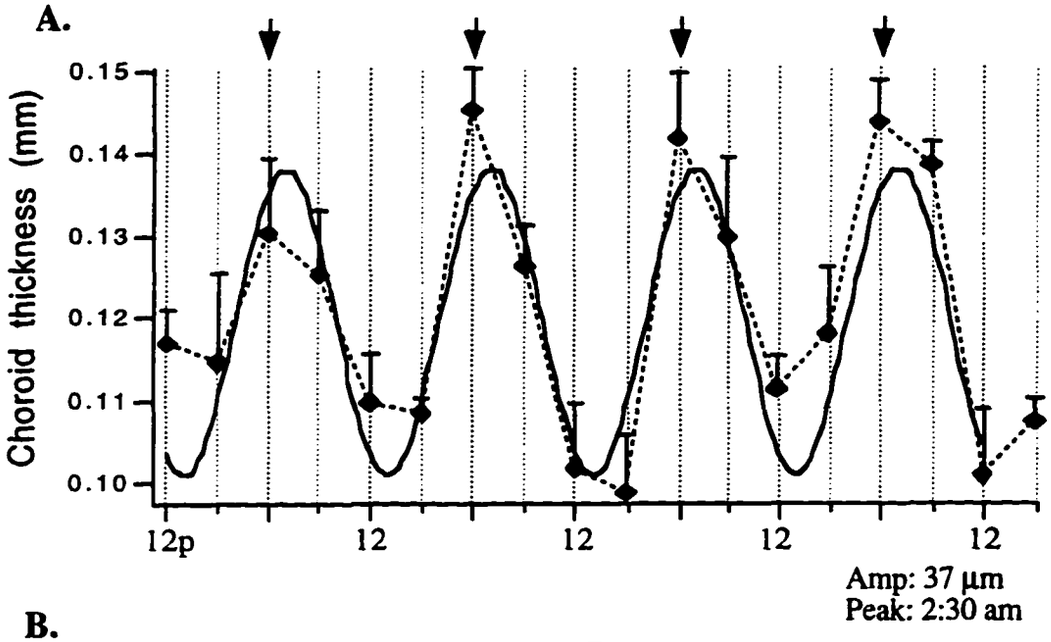
C. Mean cyclic changes in length. Black bars represent the mean oscillation around the mean rate of change (slope) with standard error bars; white diamonds represent the sine wave fit to the data.

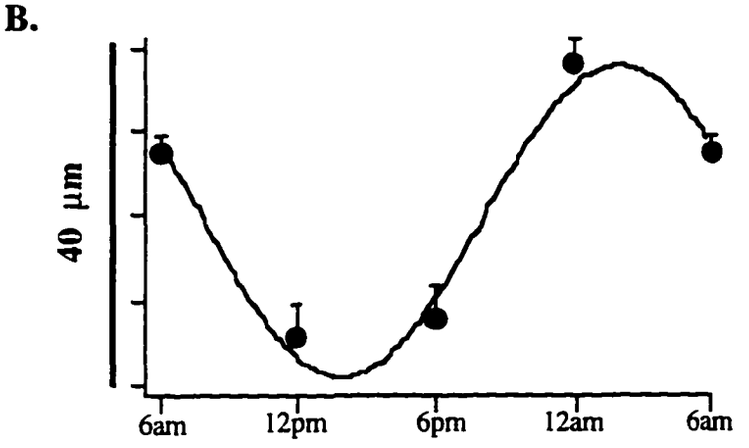
Form-deprived eyes and fellow normal eyes

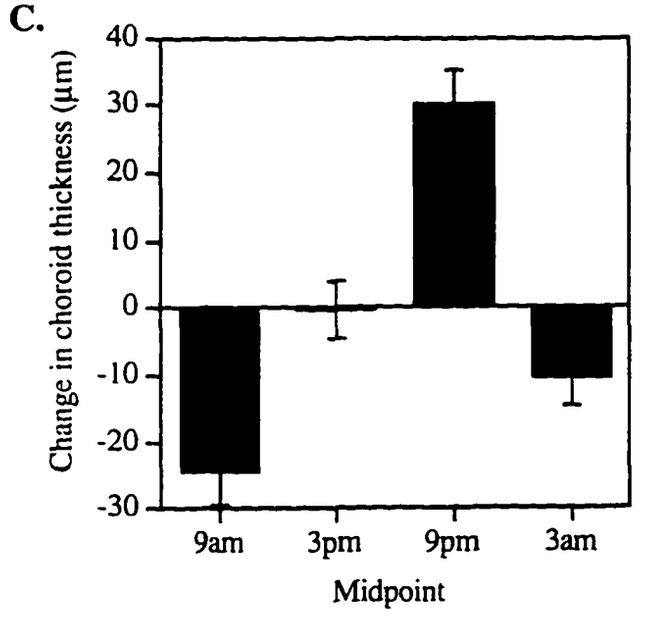
Choroid: interrupted night

The choroids of form-deprived eyes show diurnal fluctuations in thickness that are approximately twice as large than those in normal eyes (figure 3.10), despite the fact that their mean thickness is about half that of choroids in normal eyes (compare y axes in figures 3.10a and 3.8a). Choroids thicken during the evening (30 μm/6 hours) and thin during the morning (-24 μm/6 hours, figure 3.10c). All rates of change differ significantly from their adjacent intervals. For the 4 eyes followed for 4 days, the choroidal thickness was greater at midnight than at the prior 6 pm in 15 out of 16 cases. The cyclic component of this rhythm is almost identical to the "raw" changes (compare dotted and solid lines in figure 3.10a) because there is almost no steady rate of change (0.8 μm/day). The amplitude of this rhythm is 37 μm (solid line, figure 3.10a), approximately twice as large that as in normal eyes (compare to solid line, figure 3.8a), and the peak occurs at 2:30 am (Table 3.2). The choroidal and axial length rhythms in myopic eyes are nearly exactly opposite one another in phase (Table 3.2); this phase difference is statistically significant (Table 3.2, column 5, $T=11.8$, $p<0.001$).

Figure 3.10. (Next page). Diurnal changes in choroids in form-deprived eyes. A. Mean thickness over 5 days and 4 nights (dotted line and symbols), and the sine wave fit to the data (solid lines). The x axis shows time of day, with 12 denoting noon; the arrows denote midnight. Choroids thicken during the evening and thin during the morning. B. Mean residuals over the 5 cycles (circles) and sine wave fit to the data. C. Mean change in thickness over 6 hour intervals with standard error bars.







The choroidal rhythm in fellow eyes is similar to that in both normal and form-deprived eyes; the thickness increases in the evening and decreases at night (figure 3.11a, grey bars; compare to black bars, and to figure 3.8c), although the pattern of change over the day is not as regular as that seen in the myopic eyes, and there is a general increase in variability (compare standard error bars of grey and black bars in figure 3.11a; these two groups have equal n's). The amplitude of the rhythm is 18 um, and the peak occurs at 1:00 am (data not shown graphically, see Table 3.2).

Uninterrupted night

Choroids in form-deprived eyes measured at 8 am, 2 pm and 8 pm show fluctuations in thickness, however, the differences are smaller (figure 3.11b, black bars). Choroids thin during the day and thicken at night (-11 μm vs 7 μm/12 hours, p=0.01). Choroids of fellow eyes measured at these times show a similar pattern (figure 3.11b, grey bars), although the diurnal difference is not significant. In these choroids, there was a gradual decrease in thickness, with a greater thinning during the day than during the night (-9 μm vs 0 μm/12 hour, figure 3.11b, grey bars). (Note that a gradual thinning was also a feature of normal choroids (figure 3.8a).

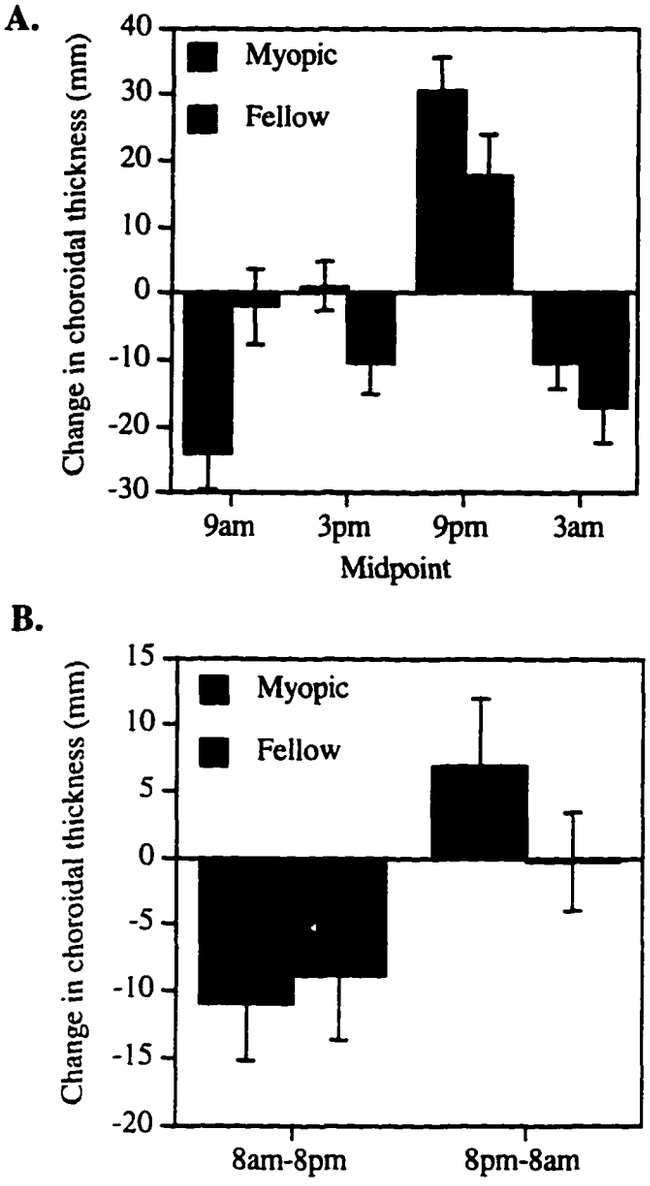


Figure 3.11. Diurnal changes in choroidal thickness in form-deprived (black bars) and fellow (grey bars) eyes. A. Data from same birds shown in figure 3.10; "interrupted night". Choroids in both eyes thicken most in the evening, and thin in the late night and morning. B. Data from "uninterrupted night" showing changes over 12 hour intervals. Choroids in form-deprived eyes thicken at night and thin during the day; diurnal changes in choroids in fellow eyes are not significantly different.

Vitreous chamber depth: interrupted night

The vitreous chambers of myopic eyes show diurnal fluctuations that are similar in phase to the fluctuations in axial length, increasing in the night and morning, and decreasing in the evening (figures 3.12a, solid lines; figures 3.12b and 3.13a, black bars). (Compare to axial length data in figures 3.3a and 3.3b). The cyclic component shows a peak at 2:00 pm and an amplitude of 110 μm (inset, figure 3.12a; Table 3.2). Again, because the changes in vitreous chamber are the sum of changes of opposite sign in the axial length and choroid, and because changes in the choroid are relatively small relative to axial length, this rhythm is in phase with the axial length rhythm and of greater amplitude. The fluctuations in choroidal thickness account for approximately one third of the change in the vitreous chamber dimensions in these eyes (amplitudes of 37 μm and 107 μm, respectively). The steady growth rate for the vitreous chamber is 107 μm/day (Table 3.2), less than that for axial length, and greater than that of normal eyes.

The vitreous chambers of fellow eyes show diurnal fluctuations similar to those of fellow myopic eyes and normal eyes (figure 3.13a, grey bars, compare to black bars; compare to figure 3.9b) and similar to the axial length rhythm (compare grey bars, figure 3.13a, to grey bars, figure 3.4a), increasing during the day and decreasing at night. The peak occurs in the afternoon (4:30 pm) and the rate of elongation (40 μm) is similar to normal (Table 3.2). Similar to the other components in these fellow eyes, there is greater variability (compare standard error bars of grey and black bars, groups have an equal n).

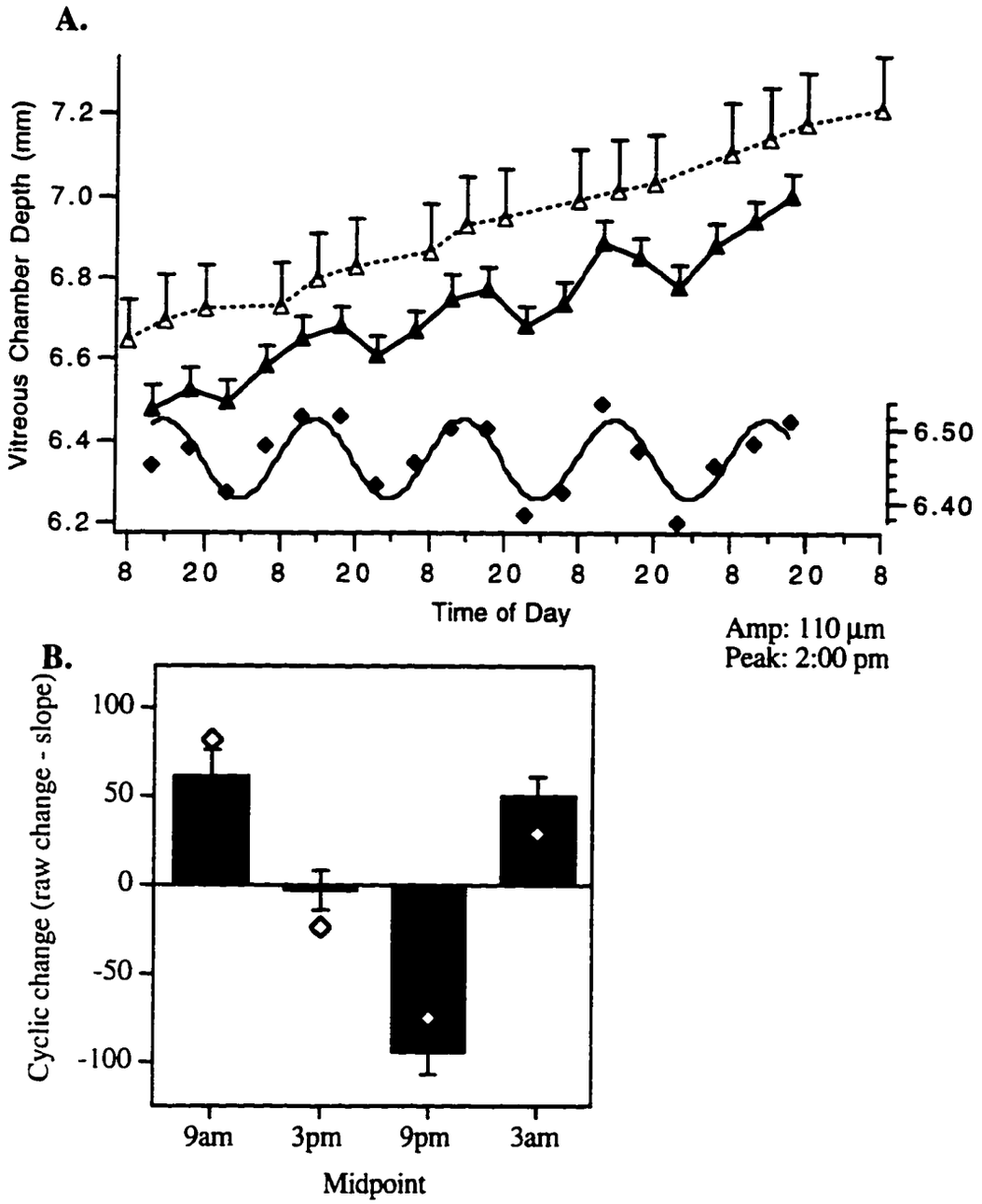


Figure 3.12. Diurnal changes in vitreous chamber depth in form-deprived myopic eyes. A. Changes over 5 days and 4 nights in interrupted night (solid symbols and lines) and uninterrupted night (open triangles and dashed line) experiments. The rhythmicity is most apparent in the interrupted night data (solid line); the peak occurs at 2 pm. B. Mean "cyclic" changes (changes around the mean rate of elongation) over 6 hour intervals in form deprived eyes (with standard errors). The white diamonds represent the sine wave fit to this data.

Uninterrupted night

The vitreous chambers of form-deprived eyes measured at 8 am, 2 pm and 8 pm also show diurnal differences in elongation (figure 3.12a, dashed line, figure 3.13b, black bars), increasing more during the day than during the night (72 μm vs 39 μm/12 hours; $p<0.01$, figure 3.13b). The fact that there is a significant diurnal rhythm in vitreous chamber depth but not in axial length in these form-deprived eyes is probably because the changes in the choroid, being of opposite sign to those of the axial length, add enough of a change to become significant.

The vitreous chamber depth of fellow eyes does not show significant diurnal differences in its rate of elongation (37 μm vs 30 μm/12 hours, figure 3.13b, grey bars).

Summary of myopic and fellow normal eyes

In summary, form-deprived eyes show diurnal rhythms in axial length, vitreous chamber depth and choroidal thickness when measured 4 times per day; the rhythms in axial length and choroidal thickness are out of phase with one another. These rhythms are less apparent when measured at 8 am, 2 pm, and 8 pm, presumably because 8 am and 8 pm are near the rhythm mesor. The fellow eyes of form-deprived eyes are similar to normal, however, with increased variability. This increase in variability presumably reflects an effect exerted by the contralateral myopic eyes.

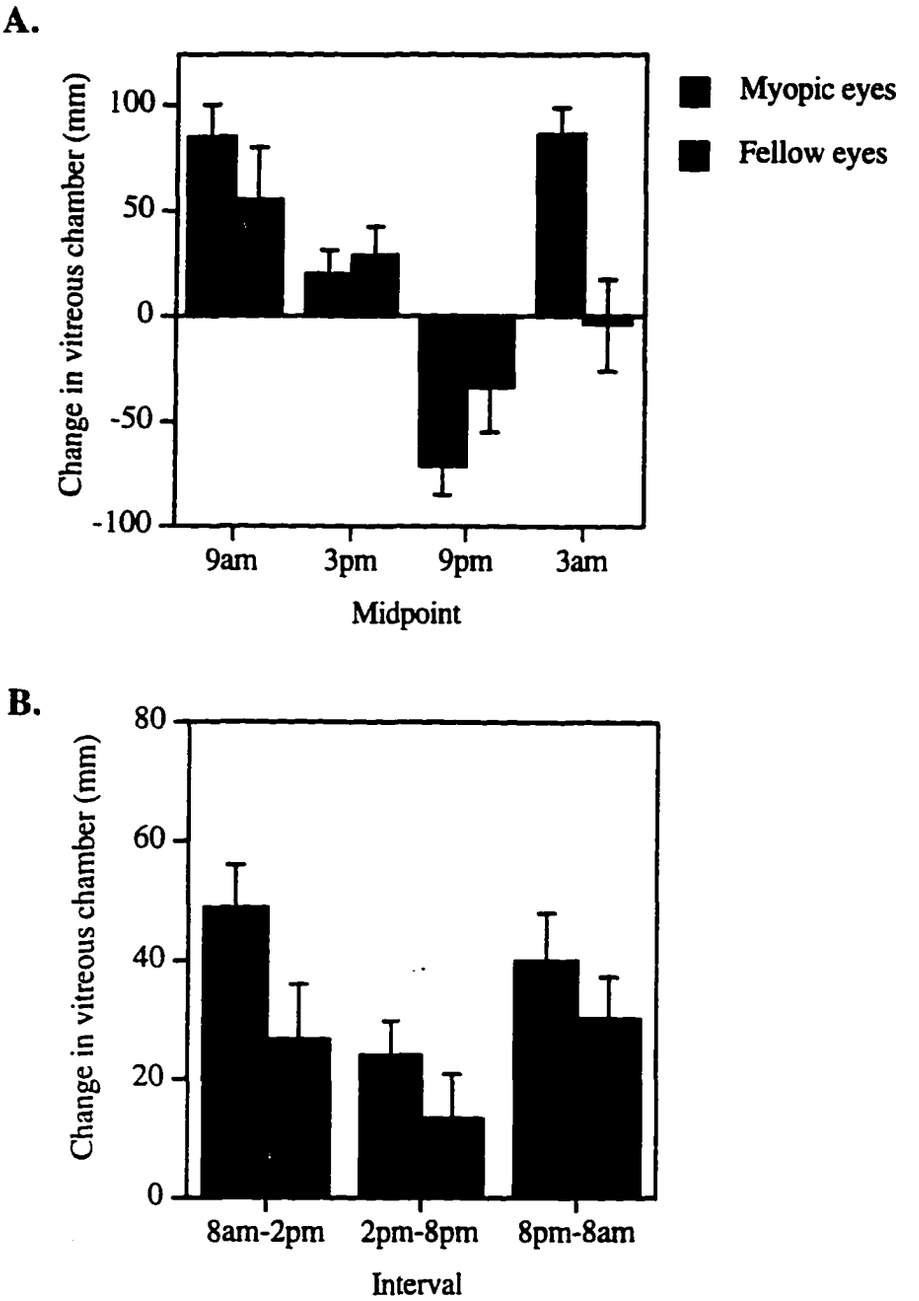


Figure 3.13. Diurnal changes in vitreous chamber depth in form-deprived eyes (black bars) and fellow eyes (grey bars). A. Data from same birds shown in figure 3.12; "interrupted night". B. Data from "uninterrupted night" showing changes over three measured intervals. Vitreous chambers of form-deprived eyes elongate more during the day than during the night in both experiments. There are no significant diurnal differences in fellow eyes.

RECOVERING EYES AND FELLOW NORMAL EYES

Diurnal Variations in Axial Length

Recovering eyes

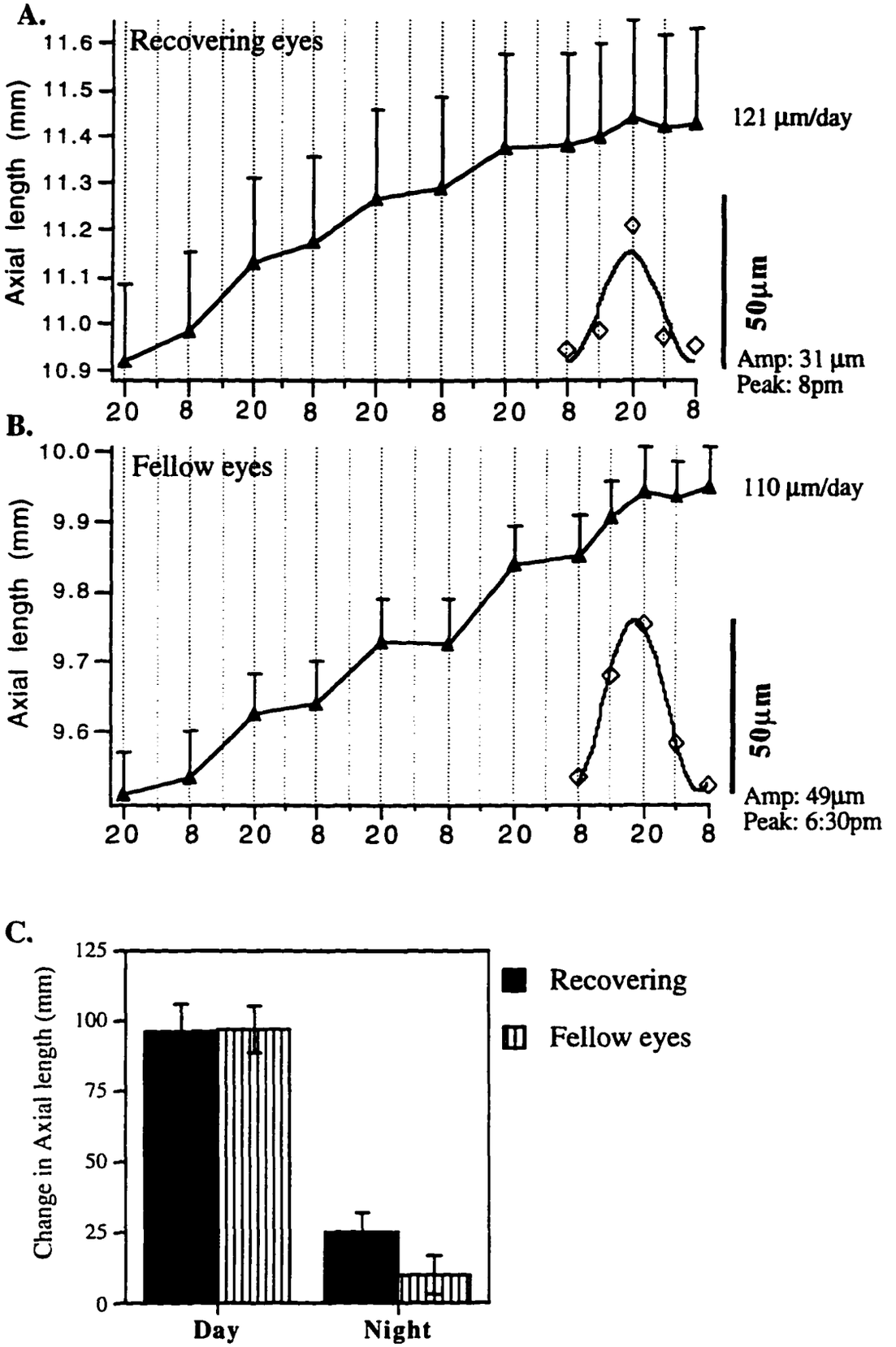
From previous work we know that after we remove the diffusers, eyes return to emmetropia over the following week. We now report that "recovering" eyes also show diurnal fluctuations in axial length, increasing more during the day than during the night (96 μm vs 25 μm/12 hrs, $T=5.9$, $p<0.0001$, figure 3.14a; 3.14c, black bars), similar to normal and myopic eyes. The cyclic component of the rhythm (measured over the last 24 hours) shows a peak at 8 pm (Table 3.2, column 4), which is phase-delayed relative to normal eyes by a mean of 4-5 hours (Table 3.2, column 5, $T=2.3$, $p<0.05$), and to myopic eyes by about the same (Table 3.2, column 5, $T=2.5$, $p<0.05$). The amplitude of the rhythm is 31 μm, similar to that of normal eyes (inset, figure 3.14a; Table 3.2).

In these recovering eyes, the mean rate of elongation per 24 hours is 121 μm (figure 3.14a, Table 3.2), which is less than that of myopic eyes (161 μm), and greater than that of normal eyes (72 μm). The mean daytime rate of elongation is similar to that of myopic eyes measured over the same intervals (96 μm vs 99 μm/12 hours, respectively), however, the mean rate of elongation during the night is significantly lower (25 μm vs 73 μm/12 hours, $T= 3.8$, $p<0.0005$). Therefore, restoring normal vision by removing the diffuser results in a decrease in the rate of nighttime elongation, which accounts for the overall reduction in the rate of elongation in these eyes.

Fellow eyes

The axial elongation of fellow eyes of recovering birds is also diurnally rhythmic, increasing more during the day than during the night (97 μm vs 13 μm/12 hrs, T= 7.9, p< 0.0001, figure 3.14b; 3.14c, striped bars). This rhythm has a peak at 6:30 pm, and an amplitude of 49 μm (inset, figure 3.14b; Table 3.2). Therefore, the axial length rhythms of both eyes of birds recovering from myopia are similar to each other, and have a mean phase delay relative to normal and myopic eyes (Table 3.2). The mean rate of elongation is 110 μm/24 hours, which is greater than in normal eyes, and less than in recovering (and myopic) eyes (figure 3.14b; Table 3.2).

Figure 3.14. (Next page). Diurnal changes in axial length in eyes recovering from myopia and in fellow eyes. A. Mean changes (and standard error bars) over 4 days and 5 nights in recovering eyes. Inset: Residuals (diamonds) of the data over the last 24 hours and the sine wave fit to the data; y axis on the right. The amplitude of the rhythm is 31 μm, the peak occurs at 8pm. Eyes elongate more during the day than during the night. B. Mean changes (and standard error bars) in fellow eyes. Inset: Residuals of the data over the last 24 hours (diamonds) and the sine wave fit to the data. C. Mean changes in length over 12 hour intervals (8:00 to 8:00) in recovering eyes (black bars) and fellow eyes (striped bars).



Diurnal Variations in Choroidal Thickness

Recovering eyes

We know from previous work that the immediate response of the eye to removing the diffuser is for the choroid to increase in thickness. The choroids of recovering eyes in this study increased by a mean of 525 μm over the 4 days, at a rate of 124 μm/day (figure 3.15a). In addition, the thickness of these choroids show diurnal fluctuations, thickening more during the day than during the night (means: 109 μm vs 15 μm/12 hrs, $T=7.6$, $p<0.0001$, figure 3.15c, black bars). The cyclic component of the rhythm (inset, figure 3.15a) shows a peak at 8:00 pm (Table 3.2), similar to the peak in the axial length rhythm. This constitutes a significant phase advance of 3 hours relative to choroids of normal eyes (Table 3.2, column 5, $T=2.1$, $p=0.05$) and an advance of 6 hours relative to myopic eyes (Table 3.2, column 5, $T=12$, $p<0.001$). These shifts in phase in axial length and choroid thickness result in the choroidal and axial rhythms being in phase with one another (mean phase difference = 0 hours), the opposite of the pattern found in normal and myopic eyes (mean phase difference in normal eyes is 8.75 hours; t-test of axial versus choroidal phase differences between recovering and normal eyes, $p<0.005$).

Figure 3.15b shows the mean rate of change in thickness for each consecutive 12 hour night and day beginning the first night after removing the diffusers (at 8 pm). In general, the choroid thickens more during the day than during the night, with the exception of the first night: over the first night the choroids thickened by approximately 75 μm (figure 3.15a & 3.15b). Thereafter, the nighttime rate of change was approximately zero and consistently lower than the daytime rate of change.

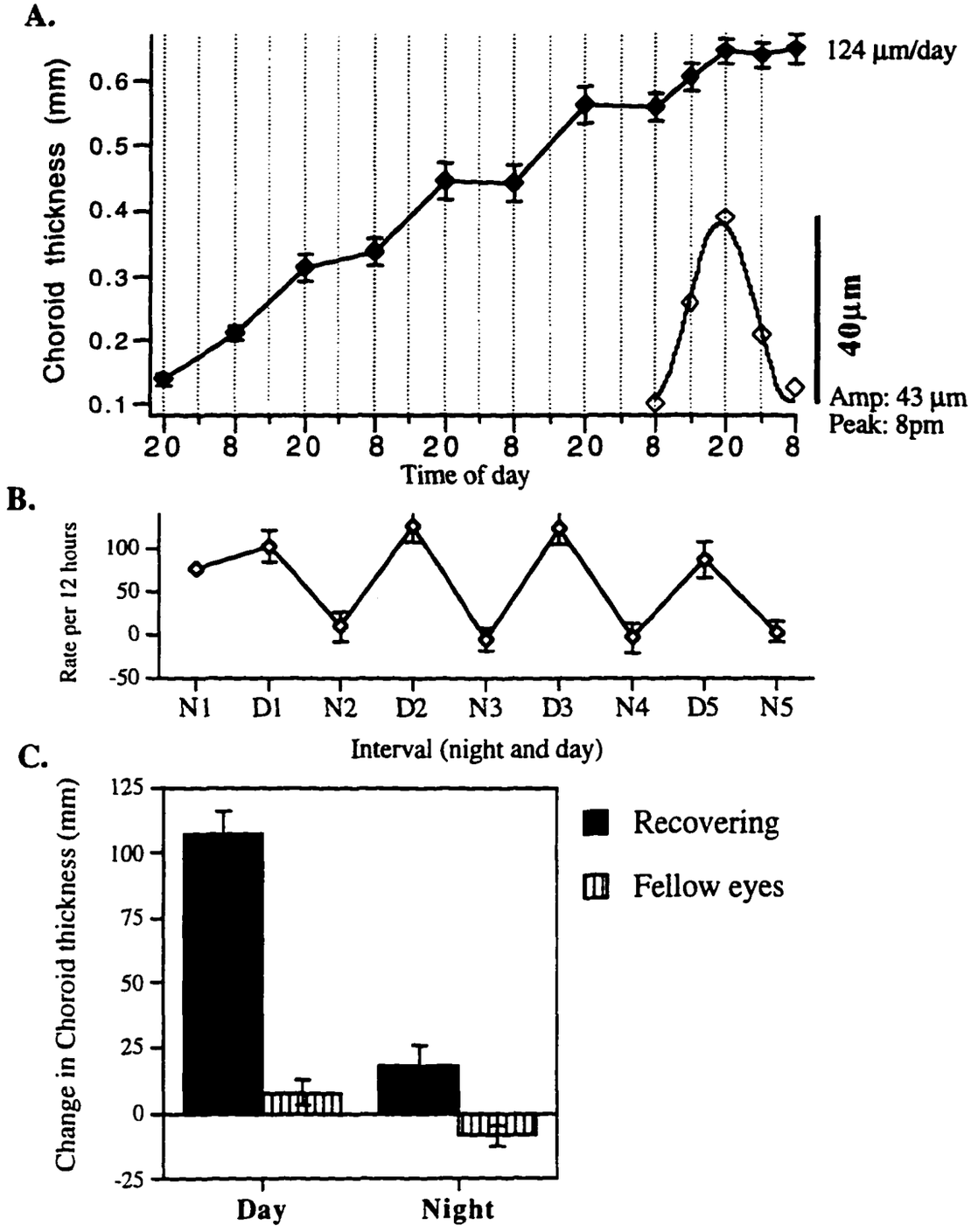


Figure 3.15. Diurnal changes in choroid thickness in recovering and fellow eyes. A. Changes in thickness in recovering eyes over 4 days and 5 nights. Inset: Residuals (diamonds) of the data for the last 24 hours and the sine wave fit to the data. B. Rates of change in recovering eyes for successive 12 hour intervals of night (N) and day (D). Note the very regular pattern of change in the rate of thickening after the first night; the choroids thicken much more during the day than night. C. Mean changes over 12 hour intervals (8 to 8) in recovering eyes (black bars) and fellow eyes (striped bars).

Fellow eyes

The choroids of fellow eyes showed diurnal fluctuations in thickness similar to recovering eyes, thickening during the day and thinning at night (8 μm vs -9 μm/12 hours, T=2.8, p=0.006, figure 3.15c, striped bars; figure 3.16), although the changes are significantly smaller than in recovering eyes (day: 8 μm vs 109 μm/12 hours, T=9.4, p<0.0001; night: -9 μm vs 15 μm/12 hours, T=2.8, p<0.01). The 6 hour interval data for the last 24 hours shows higher frequency oscillations (figure 3.16, last 5 data points) which precluded the fitting to the data of a sine wave having a 24 hour period. It is possible that these higher frequency oscillations are characteristic of these eyes, although we cannot be certain.

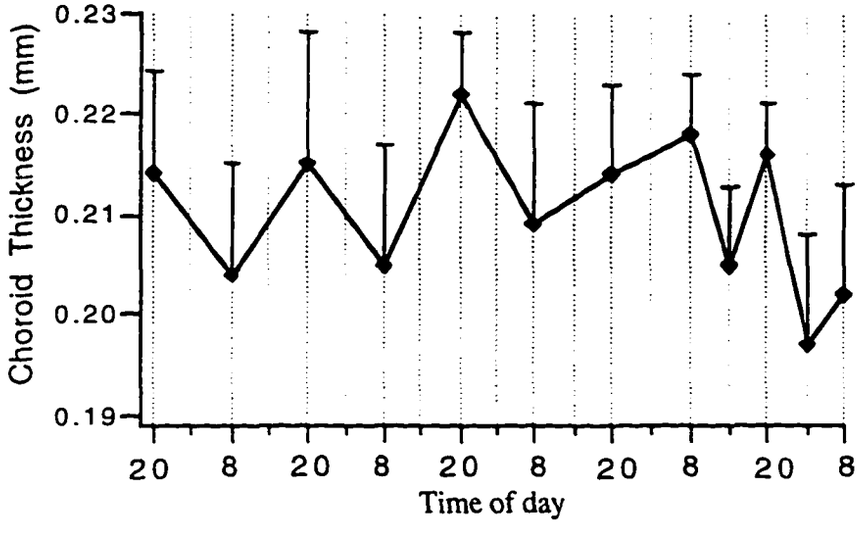


Figure 3.16. Choroidal thickness in fellow eyes of recovering eyes measured over 5 nights and 4 days. Choroids thin during the night and thicken during the day. Note the high frequency oscillations over the last 24 hours.

Diurnal Variations in Vitreous Chamber Depth

Recovering eyes

The vitreous chamber of recovering eyes decreases by approximately 200 μm over the measurement period, with a larger decrement during the day than during the night (figure 3.17a and figure 3.17c, black bars: -55 μm vs -5 μm/12 hours, T=-5.3, $p< 0.0001$). The peak of the rhythm occurs at 9 am and the trough at 9 pm (inset, figure 3.17a). Therefore, the phase of the rhythm in vitreous chamber depth is opposite that of the axial length rhythm (compare to inset in figure 3.14a; Table 3.2), the opposite of what is found in normal eyes, in which the two rhythms are approximately in phase with one another. This difference in recovering eyes is the consequence of the very large fluctuations in choroidal thickness; choroids thicken at a higher mean rate than does the axial length.

Fellow eyes

The rhythm in vitreous chamber depth in fellow eyes is similar to the rhythm in axial length in these eyes, increasing more during the day than during the night (59 μm vs 0 μm, T=6.8, $p<0.0001$, figure 3.17c, striped bars). The cyclic component of the vitreous chamber rhythm shows a peak at 5:00 pm (inset, figure 3.17b; Table 3.2). The amplitude of the rhythm is 33 μm, similar to that for both recovering and normal eyes (34 μm and 37 μm, respectively, Table 3.2).

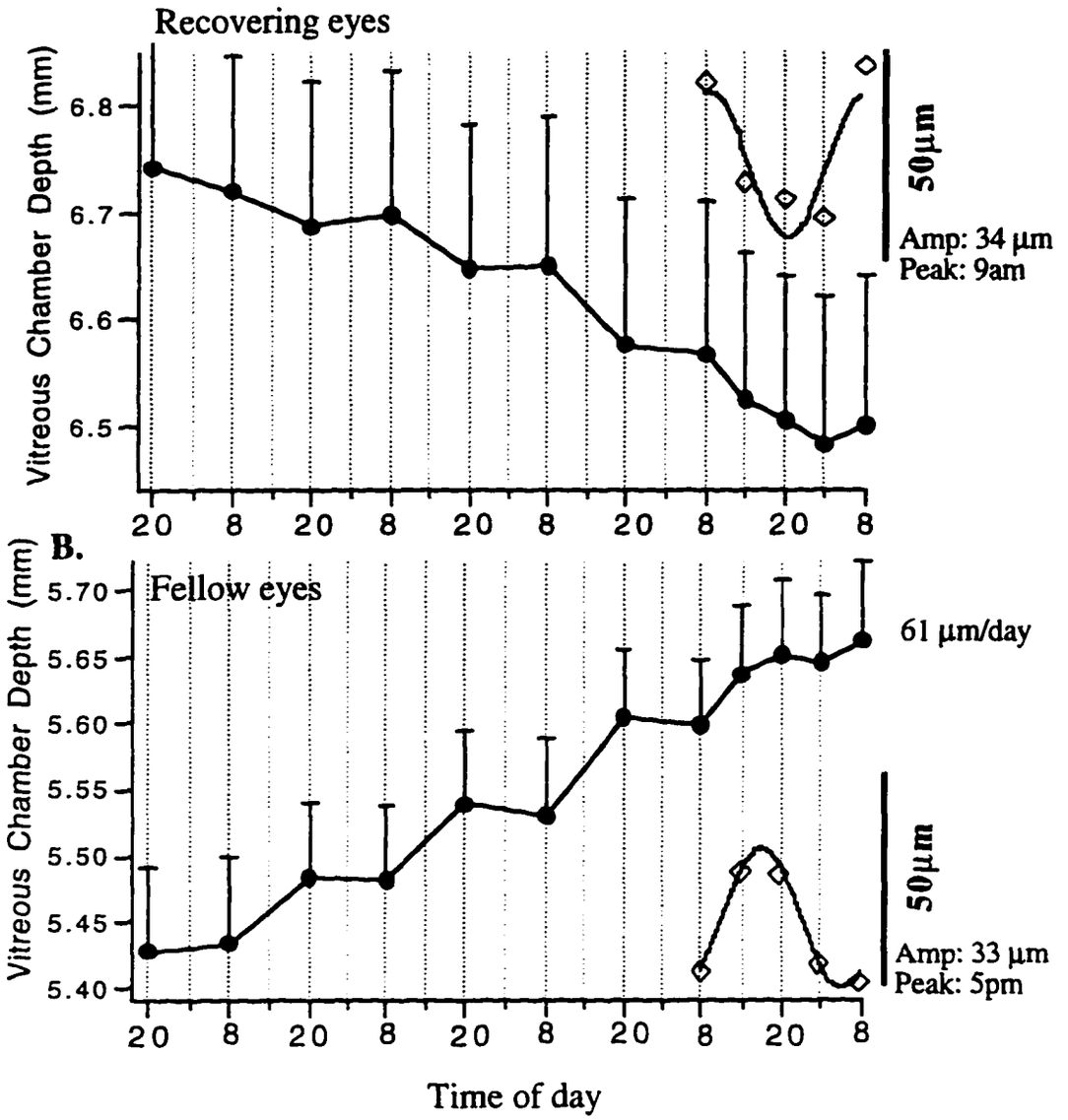
The vitreous chambers of fellow eyes are increasing at the same mean rate as those in recovering eyes are decreasing (compare figures 3.17b to 3.17a). This difference is a consequence of a difference in the daytime changes, which are similar in magnitude but of opposite sign for the two

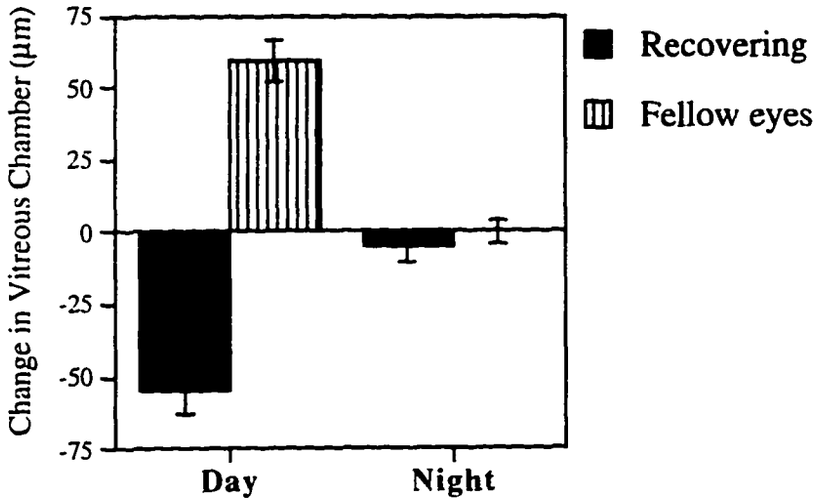
groups (59 μm vs -55 μm/12 hrs, T=-10.7, $p<0.0001$, striped and black bars; figure 3.17c); the nighttime rates of growth for both eyes are close to zero.

Differences between Recovering, Myopic and Normal eyes

In summary, eyes that are recovering from form deprivation myopia exhibit several notable differences from normal and form-deprived eyes. First, there is a large steady increase in choroidal thickening, which reflects an immediate compensatory response to the myopic defocus imposed on the eye upon removing the diffuser. Second, there are shifts in the phases of both axial length and choroidal rhythms that result in the phase relationships between the two becoming reversed compared to normal eyes. To illustrate this, figure 3.18 shows the sine waves fit to the mean data for the axial length and choroidal rhythms for normal (top), form-deprived (middle) and recovering (bottom) eyes. In both normal and form-deprived eyes, the mean rhythm in axial length is approximately 180 degrees out of phase relative to the respective mean choroidal rhythm (phase difference of 8.5 hours for normal eyes and 12 hours for form-deprived eyes). In recovering eyes, however (bottom), these rhythms are exactly *in phase* with one another. This difference in recovering eyes is the result of almost equal shifts in both axial and choroidal rhythms relative to those for normal or form-deprived eyes; the axial length rhythm being *delayed* by about 6 hours and the choroidal rhythm being *advanced* by about 6 hours (Table 3.2).

Figure 3.17. (Next page). Diurnal changes in vitreous chamber depth in recovering eyes (A) and fellow eyes (B). A. Mean changes in recovering eyes over 4 days and 5 nights. Inset: Residuals (diamonds) of the data over the last 24 hours and sine wave fit to the data; y axis on right. B. Fellow eyes; same conventions as in A. C. Mean changes in vitreous chamber depth over 12 hour intervals for recovering eyes (black bars) and fellow eyes (striped bars).





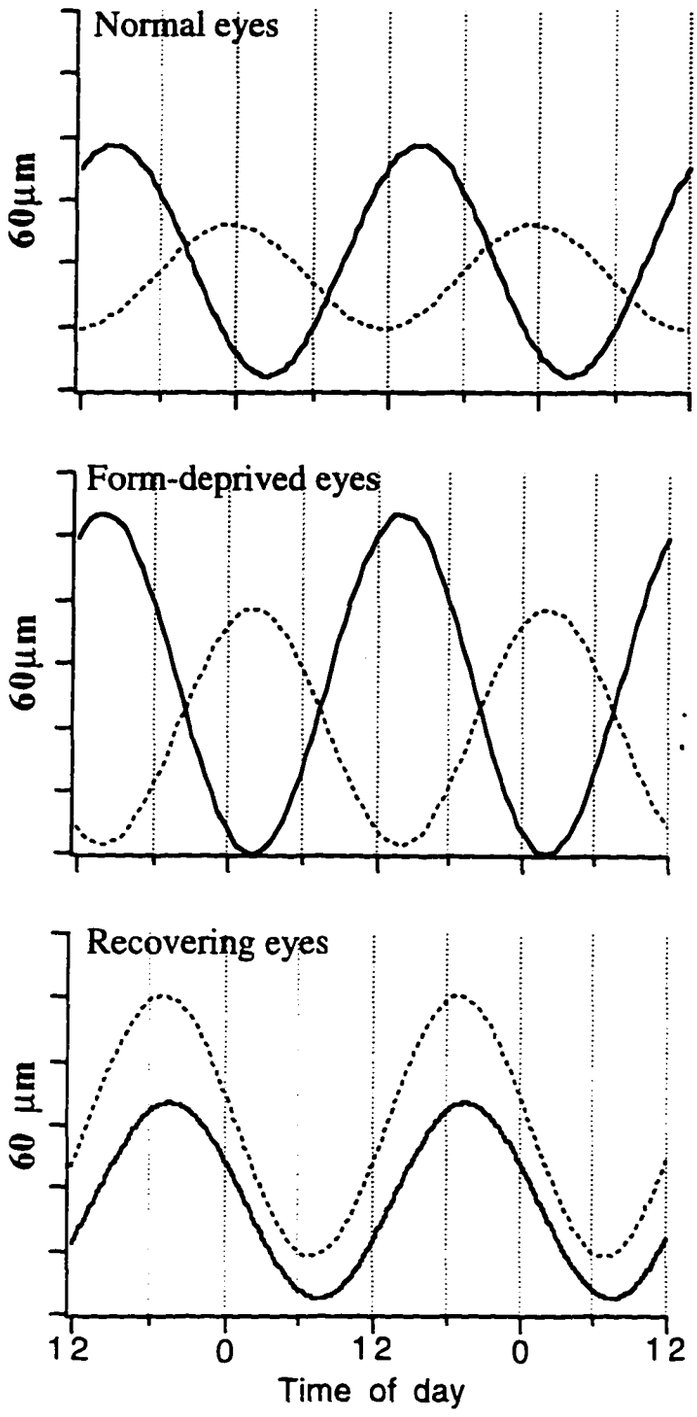


Figure 3.18. Axial length (solid lines) and choroidal (dashed lines) rhythms in normal (top), form-deprived (middle) and recovering (bottom) eyes. In normal and form-deprived eyes the axial length and choroidal rhythms are out of phase (9 hours in normal eyes, 12 hours in deprived eyes). In recovering eyes, the two rhythms are in phase. Both y axes are 60 µm, in 10 µm increments, for all. X axis is time of day: 12 is noon and 0 is midnight; x axis the same for all graphs.

DIURNAL VARIATIONS IN ANTERIOR CHAMBER DEPTH

Axial length is the composite of the anterior chamber, lens, vitreous chamber, choroidal and retinal thicknesses. Because the thickness of the retina and lens are relatively stable, these can be disregarded in terms of contributing to changes in axial length. Does the anterior chamber constitute a separate component of the changes in axial length? A rhythm in anterior chamber depth might be the result of either movement of the lens forward and back, or of independent diurnal oscillations in the growth of the cornea. A separate contribution to changes in axial length would only apply if there is a growth rhythm in the cornea. We find that there are independent fluctuations in the anterior chamber, however, these are more variable than the other ocular components and are not always apparent. We will here report only on eyes showing clear diurnal rhythms over the 12 hour measurement periods corresponding to "day" and "night" (6:00 to 6:00 or 8:00 to 8:00).

In normal eyes, the anterior chamber increases more during the night than during the day, although the difference is not significant (figure 3.19, white bars: measured at 6 am and 6 pm, 20.3 μm vs 2.5 μm/12 hours, respectively, p=0.1). In form-deprived eyes, the anterior chamber also increases more during the night than during the day (figure 3.19, grey bars, 14 μm vs -19 μm; T=3.1, p=0.005), however, these eyes differ from normal in that their anterior chambers decrease during the day (figure 3.19). These rhythms in anterior chamber depth are therefore approximately out of phase with their corresponding rhythms in axial length, which increases more during the day than during the night over the

same intervals. In contrast, the anterior chamber depth of both recovering and fellow eyes increases more during the day than during the night (recovering eyes: figure 3.19, black bars: 37 μm vs 3 μm/12 hrs, T=3.8, p=0.0004; fellow eyes, striped bars: 23 μm vs 0 μm/12 hrs, T=2.1, p<0.05); these rhythms are therefore in phase as opposed to out of phase with their corresponding rhythms in axial length.

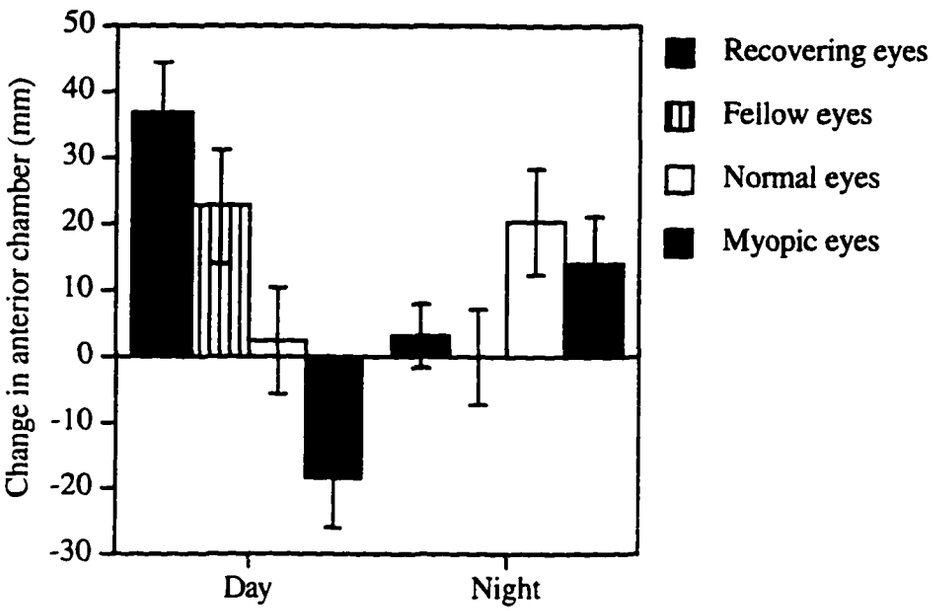


Figure 3.19. Diurnal changes in anterior chamber depth in recovering (black bars), fellow (striped bars), normal (white bars) and myopic (grey bars) eyes over 12 hour intervals (day versus night). Day is 8am-8pm for the recovering birds, 6am-6pm for normal and myopic birds. Error bars are standard errors of the mean. Note that for both eyes of recovering birds, the increase in the anterior chamber is greater during the day than during the night, however, in both normal and form deprived myopic eyes the opposite is true (anterior chambers increase more during the night than during the day).

Changes in the vitreous chamber depth are the sum of changes in choroidal thickness and axial length, but axial length also encompasses changes in anterior chamber depth; it follows that the difference between the changes in the axial length and vitreous chamber depth should be equivalent to the sum of the changes in choroid and anterior chamber dimensions. For recovering eyes, the difference between changes in axial length and vitreous chamber depth per 12 hour daytime interval is 151 μm: (96 - (-55)=151 μm) and should be equivalent to the sum of the daytime changes in the anterior chamber (37 μm) and the choroid thickness (109 μm), as it is: (37+109=146). In other words, if there were no change in the anterior chamber, the choroidal and axial changes would cancel each other out (each is approximately 100 μm) resulting in no net change in vitreous chamber depth. Because the vitreous chamber in fact, *decreases* by 50 μm per day implies that approximately half of the observed axial elongation (50 μm of the 100 μm) must be due to an increase in anterior chamber depth. The actual increase of 37 μm is close to this value. For myopic eyes, a similar analysis reveals a difference between axial and vitreous chamber changes of 37 μm (nighttime change) which can be accounted for by changes in choroid (20 μm increase) and anterior chamber depth (14 μm increase). It should be noted however, that because it is impossible with our techniques to distinguish between changes due to corneal growth versus possible changes in lens position, it is difficult to determine the true relevance of these data to ocular growth patterns.

Discussion

We here report 6 main findings: (1) Both normal and form-deprived eyes show prominent diurnal rhythms in axial length: the greatest elongation occurs in the morning, and the eye shrinks in the evening. The phase of the rhythm in form-deprived is phase-advanced relative to that for normal eyes. The diurnal rhythm is not discernible in form-deprived eyes measured at 8 am, 2 pm and 8 pm because 8 am and 8 pm are near the mesor of the rhythm. (2) Eyes that are recovering from myopia also show large diurnal rhythms in axial length; these rhythms show a significant phase delay relative to normal eyes. The overall reduction in the rate of growth in these eyes is the result of a decrease in the nighttime elongation rate. (3) There is a diurnal rhythm in choroid thickness in all eyes. In normal and form-deprived eyes the peak occurs around midnight; this rhythm is approximately anti-phase to the corresponding rhythm in axial length (figure 3.18a and b). (4) In recovering eyes the choroidal rhythm shows a significant phase advance relative to normal eyes, with a peak occurring in the evening. The outcome of these shifts in phase in recovering eyes is that the axial length and choroidal thickness rhythms are now in phase with one another (figure 3.18c). (5) In normal and form-deprived eyes, the rhythm in vitreous chamber depth is in phase with the rhythm in axial length. In recovering eyes, the rhythm in vitreous chamber depth is out of phase with the rhythm in axial length, because the rate of choroidal thickening during the day exceeds the rate of elongation of the eye. (6) There are diurnal rhythms in anterior chamber depth. In normal and form-deprived eyes these rhythms are approximately out of phase with

the rhythms in axial length. In recovering eyes, the rhythm in anterior chamber depth is approximately in phase with the rhythm in axial length.

Axial length rhythms in normal, form-deprived and recovering eyes

By measuring the dimensions of the chick eye at 6 hour intervals over 5 cycles, we show that the growing eye undergoes diurnal oscillations in length and in choroid thickness. Weiss and Schaeffel had previously shown diurnal fluctuations in ocular length as defined as the distance from cornea to retina; our findings indicate that this rhythm is composed of at least two oscillating components: that of the globe itself (our axial length), and that of the choroidal thickness. The rhythms of these two components are normally anti-phase to one another, the result of which is an even larger amplitude rhythm in the vitreous chamber depth. (That the latter is a close correlate of the dimension measured by Weiss and Schaeffel might explain why they were able to find diurnal differences when measuring close to the mesor of the rhythm). Our results indicate that these rhythms are susceptible to visual influences: when the eye is deprived of form vision with a diffuser, the rhythm in axial length becomes slightly advanced in phase relative to normal. Furthermore, myopic defocus (from removing the diffuser) causes the rhythm in axial length to become delayed relative to normal, and the rhythm in choroid thickness to become advanced; the axial length and choroidal rhythms become in phase with one another.

Although the phases of some components are susceptible to visual influences, the diurnal rhythmicity itself is uncompromised: it is retained in both rapidly elongating visually-deprived eyes and in eyes that have slowed

their elongation in response to myopic defocus (recovering eyes). Form-deprived eyes measured at 6 am, noon, 6 pm and midnight show a large-amplitude rhythm in axial elongation. Our finding of rhythmicity in form-deprived eyes contradicts that of Weiss and Schaeffel, who reported that the "axial length" rhythm was abolished in myopic eyes. This difference in results cannot be accounted for by a difference in deprivation procedure, as in both cases the rate of elongation in the deprived eyes was at least twice that of normal eyes (the rate of elongation in Weiss and Schaeffel's myopic eyes was twice that of normal eyes, in ours it was nearly three times that of normal). When we duplicated their experimental procedure (measuring at 8 am and 8 pm), we too, found no significant diurnal differences in the axial elongation of form-deprived eyes (compare "our axial length" (cornea to sclera, figure 3.20a) to "their axial length" (cornea to retina, figure 3.20b, striped bars). Similarly, when we used the same dimension they used: cornea to retina, we too found no significant diurnal differences (figure 3.20b, our data, black bars; their data, striped bars). The day and night changes in length are not significantly different for either set of data.

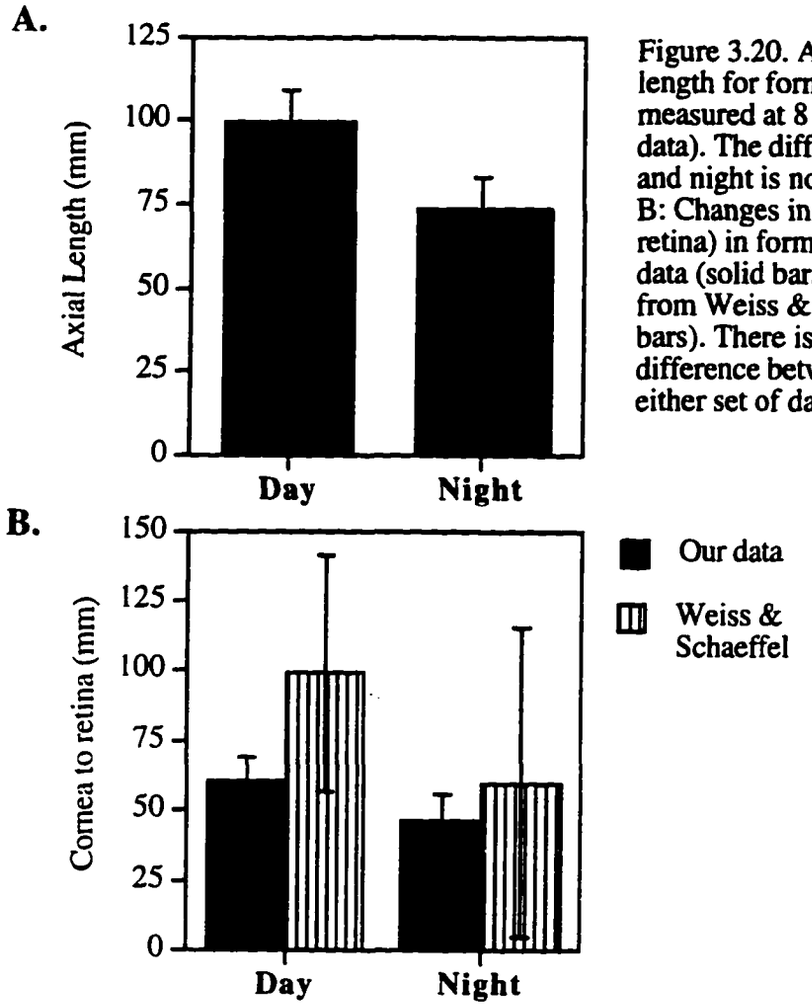


Figure 3.20. A: Changes in axial length for form deprived eyes measured at 8 am and 8 pm (our data). The difference between day and night is not significant.
B: Changes in "length" (cornea to retina) in form deprived eyes; our data (solid bars) compared to data from Weiss & Schaeffel (striped bars). There is no significant difference between day and night for either set of data.

For a sinusoidal rhythm with a 24 hour period, the greatest differences in the rates of change between successive 12 hour intervals should be seen at measurement times closest to the peak and trough, with the smallest differences being expected at measurement times 6 hours equidistant from the peak (closest to the mesor, the rhythm mean, at which measurement times would show zero change). Therefore, the lack of rhythmicity in myopic eyes measured at 8 am and 8 pm is probably a consequence of these times being close to the mesor of the rhythm. In support of this conjecture, the sine wave analysis for myopic eyes in the

"interrupted night" experiment shows a peak and trough at 2 pm and 2 am respectively, making 8 am and 8 pm (6 hours from these times) fall exactly on the mesor. Measurements at other times, for example at 6 am and 6 pm, should result in intermediate differences, as is seen (figure 3.21). The most parsimonious explanation for the lack diurnal differences in the form-deprived eyes in Weiss and Schaeffel's study is that the measurement times 8 am and 8 pm coincided with the mesor of the cycle.

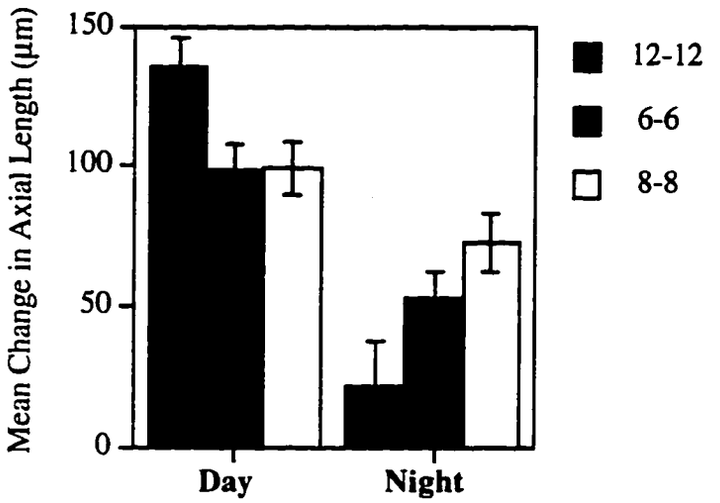


Figure 3.21. Changes in axial length for all 12 hour intervals measured (for the 12 to 12 intervals, midnight to noon is arbitrarily denoted as "day".) The smallest day versus night difference is between 8:00 and 8:00 (white bars), next, between 6:00 and 6:00 (grey bars), and the largest difference is between 12:00 and 12:00 (black bars). The determining factor in the magnitude of changes seen is the time at which the peak occurs (e.g., the largest changes in dimension will be seen when the measurements are taken closest to the peak and trough in any sinusoidal rhythm).

We, like Weiss and Schaeffel, show that growth in normal eyes measured at 8 am and 8 pm is significantly higher during the day than during the night, while myopic eyes do not show a significant diurnal difference. If the absence of rhythmicity in myopic eyes measured at 8 and 8 is indeed because these times fall on the mesor, then the fact that normal

eyes show significant diurnal differences indicates a difference in phase between normal and myopic eyes. In fact, the mean peak for myopic eyes measured at 6 hour intervals occurs at 2:00 pm, which is slightly phase advanced relative to normal eyes (peak=3:30 pm). Furthermore, a comparison between the form-deprived and fellow normal eyes of the 14 birds measured at 6 hour intervals (data in figures 3.5 and 3.6) shows a mean phase advance of 2.5-3.5 hours for the form-deprived eyes. Taken together, these data suggest that there is a slight difference in phase between these two types of eyes, and so one might expect to find diurnal differences in normal but not in myopic eyes when measuring at 8 am and 8 pm.

We argue above that the apparent lack of rhythmicity in myopic eyes measured at 8 am and 8 pm is due to an unfortunate choice of measurement times, however, two alternative explanations warrant consideration: (1) Form-deprived eyes are indeed arhythmic, as reported by Weiss and Schaeffel, and our experimental procedure (in "interrupted night") imposed a rhythmicity. One might suggest, for example, that the visual stimulation during the night related to the measurement procedure induced a rhythm. (2) The arhythmicity is a consequence of the desynchronization of the rhythms of individual eyes (secondary arhythmicity), and our experimental procedure synchronized these eyes. In support of the first hypothesis is that the elongation of individual form-deprived eyes in the "uninterrupted night" appears to be arhythmic, while individual eyes in the interrupted night show clear fluctuations. However, 4 lines of evidence support the conclusion of rhythmicity in myopic eyes and refute the notion of a procedure-induced rhythm. (1) The rates of growth for myopic eyes in both experiments were almost identical, so it is unlikely that the

nighttime stimulation resulted in a "normalizing" of the deprived eyes. (2) As already discussed, the predicted mesor for myopic eyes measured in the interrupted night is exactly at 8 am and 8 pm. (3) Myopic eyes are phase advanced relative to normal eyes in the interrupted night experiment. This could explain the finding of rhythmicity in normal but not myopic eyes measured at 8 am, 2 pm, and 8 pm. Finally, (4) although it is known that a *Zeitgeber* given at transition times can synchronize rhythms that had drifted out of phase, there is no precedent for light at night inducing a rhythm that does not exist under natural cyclic light conditions. The second hypothesis (synchronization of de-synchronized rhythms) may be supported by point #4, however the other 3 points are evidence against a de-synchronization. Taken together, the evidence indicates that the lack of rhythmicity in myopic eyes measured at 8 and 8 is solely due to an unfortunate choice of measurement times, although we cannot exclude the possibility that nighttime stimulation induced rhythms in otherwise arhythmic eyes. In order to rule out this possibility, measurements would have to be done under conditions of total darkness, and the results compared to those of the interrupted night experiment. This is not an easy endeavor, as it would entail using infrared lights for alignment and positioning, and a means for completely shielding the bird from the equipment lights.

Removing the diffuser from a form-deprived eye results in the eventual return to emmetropia from myopia. This "recovery" from myopia has two components: a fast choroidal component, whereby the choroid thickens to move the retina forward, and a slower scleral component, whereby there is a decrease in axial elongation. The initiation of the scleral response (i.e. cessation in axial elongation) has been shown to take several

days (Wallman et al., 1994). Similarly, the reduction in scleral proteoglycan synthesis is also delayed by several days (Nickla et al., 1992; Rada et al., 1992) Therefore, it is not surprising that for the first 4 days of recovery the mean rate of elongation in recovering eyes remains *higher* than that of normal eyes but lower than that of myopic eyes (121 vs 72 and 161 μm/day, respectively, Table 3.2). The decrease in the rate of elongation in recovering eyes is solely due to a decrease in the nighttime elongation; the daytime rates of growth are similar for the two eyes. An additional effect of the restoration of vision on the axial length was a delay in phase of about 6 hours relative to myopic eyes.

Choroidal rhythms in normal and form-deprived eyes: what is the functional significance?

It has long been thought that the primary function of the choroid is to nourish and support the retina by means of the capillaries in the choriocapillaris (Bill, 1985). We have recently shown, however, that the choroid plays a dynamic role in visual development by providing a means of adjusting the position of the retina with respect to the image plane (Wallman et al., 1995). Specifically, the choroid thickens in response to myopic defocus and thins in response to hyperopic defocus. We here report that there is a diurnal rhythm in the thickness of the choroid: it thickens most in the evening and thins in the morning. In normal and myopic eyes this rhythm is approximately anti-phase to the rhythm in axial length. In recovering eyes, the rhythms in elongation and choroidal thickness are approximately in phase with one another (figure 3.18).

We hypothesize that the choroidal rhythm in thickness may influence the rhythm in ocular elongation. We find that the time of greatest choroidal thickness is concomitant with the smallest axial length, and vice versa. By the same token. thinner choroids are associated with faster growing eyes, and thicker choroids with slower growing eyes. Specifically, eyes exposed to myopic defocus via either positive lenses or removing the diffuser have thicker choroids and decreased axial elongation; conversely, eyes exposed to hyperopic defocus by negative lenses have thinner choroids and increased axial elongation. This anatomical correlation has a biochemical counterpart: when pieces of scleral tissue from normal eyes are co-cultured with thick choroids from recovering eyes, the proteoglycan synthesis in the sclera is lower than in scleras co-cultured with thin choroids from normal or myopic eyes (Gottlieb et al., 1993).

We speculate that there are two mechanisms at work in the choroidal regulation of axial elongation. First, we propose that the thickness of the choroid is inversely proportional to scleral growth (with an indeterminate time lag), with the degree of choroidal thickening over the day determining the amount of scleral inhibition at night. Second, we propose that the amount of choroidal thickening in the evening is determined by the amount of refractive error sensed during the day. In this way, the retina/choroid determines the appropriate rate of growth for the sclera by sensing and responding to the amount of refractive error. The oscillations in choroidal thickness would thus serve a similar function to the "trial and error" we must use in focusing a microscope; specifically, it provides a mechanism whereby the direction of the required response of the eye growth system (depending on whether the eye is too long or too short) can be determined and acted upon.

In normal eyes, because the oscillations in choroid thickness are small, (the change in refractive error would be less than one diopter), the interaction between choroid and sclera would essentially serve to "fine tune" the growth rate. As an example, at noon, when the choroid is thinnest and the axial length longest, the eye might be slightly myopic; the magnitude of the refractive error would determine the amount of choroidal thickening. The amount of choroidal thickening would in turn determine the amount of inhibitory signal released by the choroid which would decrease scleral growth by an appropriate amount. In consequence, at midnight (when the choroid has thickened), the scleral elongation has decreased. What then accounts for the subsequent increase in elongation during the early morning hours? It is plausible that after the myopic error signal was reduced by a certain amount (perhaps in the late afternoon), an inhibitory signal for the choroid is released, which would decrease the rate of choroidal thickening and in turn increase the rate of axial elongation, all with a certain lag time. The increase in elongation and concurrent thinning choroid over the night would result in a larger eye, thinner choroid and myopic refractive error in the morning, which would perpetuate the cycle. It is apparent that in this kind of a feedback system the phase relations between the choroidal thickening and elongation are of crucial importance. (As an aside, this model cannot explain the shrinkage in eye length at midnight, which is probably at least partially attributable to a decrease in intraocular pressure (IOP). The involvement of other ocular rhythms in ocular elongation is discussed in more detail in Chapters 4 and 5).

In form-deprived eyes, the rate of ocular elongation is more than twice that in normal eyes and the amplitude of the rhythm is larger. In addition, the choroids are thinner than normal, and the amplitude of their

rhythm is larger. Are the thinner choroids and larger amplitudes a cause or a consequence of the change in the rate of elongation? It could be imagined that the increase in the rate of axial elongation could cause the choroid to stretch and become thinner. However, the almost 3-fold increase in amplitude makes a purely mechanical effect unlikely. We propose that the two deprivation-induced effects on the choroid (increased amplitude and thinning) are the result of two separate mechanisms. First, we know that the choroid responds to lens-induced blur by changing its thickness in the direction appropriate for compensation. We propose that the default response of the choroid in the absence of an error signal is to thin. Second, with regard to the amplitude of the choroidal oscillations, large amounts of defocus would preclude the eye being emmetropic at any point in the cycle. We propose that under these circumstances, the choroidal rhythm amplitude increases in an attempt to locate the image plane. We know that myopic defocus imposed by either removing a diffuser or via positive spectacle lenses results in choroidal rhythms of higher than normal amplitude (this paper and unpublished observations). This larger amplitude is superimposed on a choroid that is steadily thickening in compensation for the refractive error. In form-deprived eyes, it is plausible that increasing the amplitude of the oscillations are a response to the apparent large amount of "defocus". If, as we previously postulate, choroidal thickness is inversely related to the rate of scleral growth, and if the amplitude of the oscillation influences the amplitude of the elongation rhythm, then these form-deprived choroids would result in greater rates of elongation as well as a larger amplitude rhythm in elongation, which is in fact, what we find.

Visual influences on phase: Myopic defocus causes phase shifts in axial and choroidal rhythms.

Removing the diffusers from form-deprived eyes results in an approximate 6 hour advance in the choroidal rhythm, and 6 hour delay in the axial length rhythm, bringing both components into phase with one another (figure 13). We find a similar phase relationship between axial length and choroid thickness rhythms in eyes wearing plus 15 D spectacle lenses (Nickla et al., 1996), therefore, the shifts appear to be a specific response to myopic defocus. Furthermore, the choroid in both conditions of myopic defocus thickens by about 120 μm per 24 hours, with the greatest increase occurring during the day. Furthermore, both recovering and plus lens eyes show a thickening of 75 μm over the first night. Therefore, myopic defocus initiates two notable changes in the choroid: (1) it results in a large steady increase in thickness, and (2) it shifts the phase of the rhythm bringing it into phase with the elongation rhythm.

In order to explain these results, we propose that there are two independent influences on the choroid: a "rhythmic" one that is driven by an endogenous clock, and a "visual" one that is driven by myopic defocus. If we make the assumption that form deprivation is analogous to constant conditions (both lacking a *Zeitgeber*, so that the rhythms free-run) then the "endogenous" choroidal rhythm will be reflected by the rhythm in form-deprived eyes. Because the choroidal rhythm in form-deprived eyes is phase-delayed relative to that in normal eyes, it follows that the period of the endogenous rhythm is longer than 24 hours. (If an endogenous rhythm has a period that is longer than 24 hours, it requires daily advances for

entrainment to a 24 hour cycle, because under free-run conditions, the phase would occur progressively later). If deprivation does reflect a kind of "constant condition", then visual transients (i.e., patterned vision) must function as the *Zeitgeber*, hence in normal eyes vision at dawn would advance the phase of the rhythm. Finally, we make three conjectures: first, that the visual influence is initiated by myopic defocus, in which case the choroid thickens until eventually the retina reaches the image plane. Second, continued choroidal thickening requires periodic exposure to the "defocus" (i.e., it does not continue indefinitely in darkness). Third, the "visual" choroidal thickening is phase-dependent: thickening can only be initiated by visual defocus during the day.

The above set of conjectures can explain several observations on recovering eyes and eyes wearing plus lenses: In both conditions, removing the diffuser results in myopic defocus so that the visual mechanism can be activated; the choroid increases by 75 μm over the following 12-24 hours. Both eyes receive a strong defocus signal at the time of lights on (8 am) the following day, which will cause a phase advance in the choroidal rhythm. The dual effects of the visual and endogenous mechanisms result in the choroid thickening over the day, concomitant with the increase in axial length.

We report that myopic defocus, which causes choroidal thickening and decreased axial elongation, also delays the rhythm in axial length and advances the rhythm in choroidal thickness, so that the rhythms in axial length and choroidal thickness become in phase. In normal eyes, the mean phase difference between the two rhythms is about 9 hours. In form-deprived eyes, the phase difference is greater than normal (about 12 hours). If we accept that the choroidal thickness rhythm in normal eyes

directly drives the rhythm in elongation (so that a thicker choroid causes greater inhibition of elongation), it is difficult to explain how the choroid is influencing the axial length rhythm in recovering eyes. It is plausible that the shift in these rhythms, which also occurs in eyes wearing positive spectacle lenses, is part of a mechanism to slow axial elongation. We postulate that the efficacy of the inhibitory influence of the choroid on axial elongation is dependent on the phase relationships between the two rhythms, and that perhaps the shift in the phases of these rhythms in response to myopic defocus is a mechanism for slowing the elongation rate of the eye.

In conclusion, the persistence of the choroidal and axial oscillations in all types of eyes suggests that both rhythms are somewhat independent of one another, and can be shifted in different directions by certain types of visual stimuli. It is possible that the phase relationships between the two rhythms are crucial in determining the rate of elongation. In reality, the rhythm in axial length is probably the result of an interaction between several factors, including, but not limited to, rhythms in choroid thickness, intraocular pressure, and scleral proteoglycan synthesis. The evidence for the involvement of the latter two factors will be summarized briefly in the following section, and be presented in detail in the subsequent two chapters of this thesis.

Ocular rhythms and ocular growth: is there a connection?

The fact that the elongation of the eye is rhythmic makes it likely that the mechanisms underlying ocular growth (scleral synthesis rates, for instance) are also rhythmic. It follows that alterations in the rates of

growth concomitant with various visual manipulations may be the result of alterations in these underlying rhythmic processes. The existence of circadian rhythms in cell division and growth in vertebrates has been known since the 1950s. In cartilage, for example, DNA synthesis, cell division and proteoglycan synthesis all show diurnal rhythms that have specific phase relationships to one another, indicating that their temporal coordination must be important in regulating growth (Simmons, 1968). Because the chick sclera contains a cartilaginous layer, and because the rate of elongation of the globe is correlated with the rate of synthesis of scleral proteoglycans (Christensen and Wallman, 1991; Rada et al., 1991), it is parsimonious that the rhythmic oscillations in the size of the eye reflect the diurnal oscillations in proteoglycan synthesis. We, find, in fact, that the synthesis of matrix proteoglycans in the chick sclera is indeed rhythmic, with higher synthesis during the day than during the night. This underlying rhythmicity at the molecular level is evidence that the elongation of the eye is the end result of several ocular rhythms. The evidence for the rhythmic production of scleral proteoglycans in normal and form-deprived eyes will be discussed in the following chapter.

Another ocular process that shows diurnal fluctuations is the intraocular pressure (IOP). In normal chicken eyes, intraocular pressure is low during the night and high during the day. The fact that the trough in the IOP rhythm is coincident with the time that the eye is smallest (around midnight), and that the increase in IOP occurs at the time of greatest axial elongation (morning), suggests that the rhythm in IOP might play a role in the rhythm of elongation. In fact, we found that artificially imposed changes in intraocular pressure of the same magnitude as the diurnal changes resulted in changes in axial length similar to those seen during the

diurnal cycle. It could be imagined that the fluctuations in axial length are due to the rhythmic changes in IOP inflating and deflating the eye, superimposed on a steady rate of growth. We will present evidence that this is not the case; that in fact, the elongation rhythm is the result of a combination of rhythmic factors, which includes rhythms in proteoglycan synthesis and in intraocular pressure.

In conclusion, the rhythm in axial elongation is the outcome of several underlying diurnal ocular rhythms, including, but not limited to, choroidal thickness. Different visual manipulations result in various shifts in the phases of the rhythms in axial elongation and choroid thickness. We speculate that the rate of eye growth is influenced by the specific phase-related interactions between these underlying rhythms, and that shifts in the phases of the rhythms cause changes in the rate of growth.

Table 3.2. Mean amplitude, phase and rate of change for components of eyes.

Condition	Component	Ampl.(μm)	*Time of mean peak	Mean time of peak (s.d.)(n) **Good fits	All data	Mean rate (μm/24 hrs)
Normal	Axial length	37 (5.0)	15:30	15:00 (3.5)(8)	14:00 (4.1)(10)	72
	Choroid	16 (3.4)	0:00	22:30 (3.7)(6)	22:30 (3.0)(10)	-6.0
	Vitreous	64	14:00			44
Myopic	Axial length	53 (4.7)	14:00	14:00 (1.3)(3)	14:00 (1.1)(4)	161
	Choroid	37 (4.8)	2:30	2:00 (1.0)(4)	same	0.8
	Vitreous	110	14:00			107
Recovery	Axial length	31 (4.9)	20:00	19:00 (3.0)(8)	same	121
	Choroid	43 (5.2)	20:00	19:30 (.8)(8)	same	124
	Vitreous	34	9:00			-60
Fellow Myo	Axial length	64	15:00			82
	Choroid	18	1:00			-8.0
	Vitreous	66	16:30			40
Fellow Reco	Axial length	49	18:30			110
	Vitreous	33	17:00			61

Table 3.2. Mean amplitude, phase and "steady" rate of change for axial length, choroid thickness and vitreous chamber depth. The mean amplitude is the peak to peak difference of the sine wave fit to the mean of all the data. The standard deviations given for axial length and choroid for normal, myopic and recovering eyes were obtained from the sine waves fit to the individual data. Phase is given as the time of the peak of the rhythm and is obtained in 2 different ways: "Time of mean peak" is the peak of the sine wave fit to the mean "cyclic" data for all eyes in a group. "Mean time of peak" is derived from the sine waves fit to the data for each individual eye; the means and standard deviations of these are shown. Peaks are given to the nearest half hour; standard deviations to the nearest tenth of an hour. "Good fits" represent only those eyes in which the sine wave fit to the data was subjectively determined to be acceptable (assessed by 2 observers)."All data" represents all of the eyes in a group regardless of the goodness of fit; the standard deviations are in first set of parentheses, n's are in the second set of parentheses. "Mean rate" is the slope of the linear regression fit to the mean of the data for all eyes in a group. *Phase data referred to in text of Results section excluding statistics on phase differences. **Phase data used in statistical comparison of phases.

CHAPTER 4

A circadian rhythm in scleral proteoglycan synthesis may mediate the rhythm in ocular elongation in chicks

Introduction

There is now strong evidence that the post-natal growth of the chicken eye is regulated by visual input, so that the physical length is matched to the focal length. The modulation of ocular length is believed to occur via changes in the rate of synthesis of extracellular matrix molecules in the sclera, the outermost tunic of the eye. It has been shown that visual manipulations that increase the rate of growth of the eye, for instance, depriving the eye of patterned visual input using plastic diffusers, or the wearing of negative spectacle lenses, are associated with increases in the synthesis of scleral proteoglycans. Form deprivation has also been found also to increase the production of protein and DNA in the sclera (Christensen and Wallman, 1991). Conversely, manipulations that decrease the rate of growth of the eye, for instance, removing the diffuser, or the wearing of positive spectacle lenses, are associated with decreases in the production of scleral proteoglycans (Rada et al., 1991; Nickla et al., 1992). To date, all of the evidence in support of the hypothesis that the modulation of eye growth occurs via changes in the rates of scleral matrix synthesis has been largely based on the chicken model in which the scleral responses to visual manipulations occur rapidly and are profound. Furthermore, these scleral responses have been restricted to those involving relatively severe alterations of the visual image, either by form deprivation or large

defocusing errors. We asked the question, do changes in scleral synthesis rates play a role in the normal growth of the eye as well?

During post-natal development, the elongation of the eyes of chicks shows prominent diurnal rhythms, elongating significantly more during the day than during the night (Weiss and Schaeffel, 1993; Nickla and Wallman, 1995). The purpose of the present study was to ascertain whether we could detect rhythmic changes in the synthesis of scleral proteoglycans that might underlie the rhythm in ocular elongation in normal eyes. If this were so, it would strengthen the evidence indicating that the final pathway of the signal cascade from the retina involves modulation of the rates of synthesis of scleral matrix molecules. In addition, if we found a rhythm in proteoglycan synthesis that persisted in culture and was coincident with changes in axial length, it would imply that one of the essential aspects of the growth of the eye was circadian, hence that the upstream factors regulating this growth might also be circadian.

In order to study putative time-dependent changes in proteoglycan synthesis, two different *in vitro* paradigms were used. In the first, pieces of sclera were dissected at different time of day and cultured in medium labeled with inorganic sulfur-35. In this paradigm, the growth activities of the cultured tissue presumably reflect their previous conditions in the intact organism. To examine the temporal pattern of the rate of synthesis in more detail and to ascertain if the rhythm was endogenous (that is, if it would persist in the absence of exogenous temporal cues), the second paradigm used an automated flow-through perifusion system in which we could continuously assay the production of proteoglycans by measuring the amounts put out into the medium flowing over the tissue. If we were able to detect rhythmic changes in the synthesis of proteoglycans over several

diurnal cycles, it would imply that the rhythm was an endogenous circadian one. The success of this paradigm as a means to study the dynamics of proteoglycan synthesis was dependent on the requirements that the relative quantities of the proteoglycans in the aliquots of culture medium were representative of the rate of synthesis at those times.

We found that the rate of synthesis of scleral proteoglycans is diurnally rhythmic, being higher during the day than during the night, and highest in the morning. The changes in proteoglycan synthesis are approximately temporally coincident with the changes in the rate of axial elongation. Furthermore, this diurnal rhythm persisted in culture for at least 3 cycles with a period of approximately 24 hours. However, the conditions in the perifusion paradigm caused strong phase shifts and "reset" the rhythm, and so was inadequate as a means of assessing the phase of the rhythm with relation to time of day. This rhythm is apparent as well in scleras of form-deprived myopic eyes. We conclude that the rhythm in scleral proteoglycan synthesis contributes to the rhythm in axial elongation.

Methods

Studies: *In Vitro* Synthesis of Glycosaminoglycans

Experiment 1: Analysis of scleral tissue at different times of day

To make eyes myopic, translucent diffusers were glued onto the feathers around one eye at 2 days of age and remained on for 2 weeks. The light/dark cycle was 14L/10D, with lights on at 8 am and off at 10 pm for all experiments.

In the first paradigm, 8 mono-occluded birds were killed with an overdose of sodium pentobarbital at 9 am (n=4) or 10 pm (n=4) on day 14. Eyes were immediately dissected out, and the globe was cleared of the extraocular muscles. Eyes were then bisected, the scleras were scraped clean of the retina/RPE, and a six-mm punch was taken from the posterior pole of each sclera. Punches were put into 0.5 ml medium (N2, described below) with radiolabeled Na_2SO_4 (approximately 20uCi/ml) and incubated in separate wells at 37 C in 5% CO_2 for 10 hours. Proteoglycans were assayed as described below.

The second paradigm differs from the first in that the duration of incubation was 2 hours, rather than 10 hours. This includes 4 experiments, in which 3 times were chosen to correspond to "morning", "afternoon", and "night". Each time point consists of 4 pieces of sclera. Times of culture were: experiment 1: 8 am, 4 pm and midnight, (n=4 normal eyes at each time); experiment 2: 10 am, 2 pm and 9 pm (n=4 normal eyes at each time); experiment 3: 11 am, 4 pm and midnight (n=4 normal and 4 myopic eyes at each time); experiment 4: 10 am, 1 pm and 9 pm (n=4 normal and 4 myopic eyes at each time). Proteoglycans were assayed as described below.

Experiment 2: Comparison of "tissue" and "medium" proteoglycans

In order to determine the relative amounts of proteoglycans that are released into the medium versus those that are retained in the tissue matrix, we dissected and cultured two 4-mm punches of sclera per eye; one of these punches was assayed after 2 hours for labeled proteoglycans, the other was used to measure the labeled proteoglycans released into the medium after 2 hours.

Dissections were done at 10 am and 4 pm; 4 normal eyes were used for each time, yielding two punches from each sclera at 10 am and 4 pm. One punch was put into labeled medium for 2 hours and the tissue was then assayed for glycosaminoglycans ("tissue" proteoglycans: n=4 per time point). The second punch was put into labeled medium and the "conditioned" medium was collected after 2 hours and assayed for labeled proteoglycans using the same procedure as for the tissue. This medium was then replaced by unlabeled medium until 4 pm (for the 10 am piece) or the following 10 am (for the 4 pm piece). At these times, labeled medium replaced the unlabeled medium, was collected 2 hours later, and assayed for labeled proteoglycans. This paradigm yielded data on the amount of labeled proteoglycans in both the tissue (10 am, n=4; 4 pm, n=4) and the medium (10 am: n=8; 4pm: n=8) from the same eyes.

Experiment 3: Long-term culture

Rationale: Experiment 2 established that there is a measurable amount of newly synthesized glycosaminoglycans released into the culture medium from the scleral tissue in a 2 hour incubation period. We hypothesized that the relative quantities of these newly synthesized "leakage" glycosaminoglycans reflect the relative rates of synthesis in the tissue over

time. If so, then diurnal fluctuations in synthesis could be monitored by sampling the labeled culture medium perfusing the tissue.

Tissue Preparation: Six-mm punches of tissue from the posterior sclera were dissected from 1-2 week old chickens. Time of dissection was either in the morning (8 am to 10 am, n=14), afternoon (2 pm to 4 pm, n=12) or night (8pm to 10 pm, n=13) for scleras from normal eyes, with no more than 1 hour elapsing between dissection and the beginning of the tissue culture. Scleras from form-deprived eyes were all dissected in the afternoon (n=8). The duration of the experiments was 48-80 hours.

Tissue Viability: Previous experiments (and investigations) have shown scleral tissue to be viable in organ culture for at least 4 days, as determined both by continued incorporation of label into proteoglycans, and by the vital dye methylene blue. As a control, approximately one third of the pieces were exposed to methylene blue after termination of the experiments. All but one piece showed uptake of the dye into the chondrocytes; we assumed that unless the tissue was visibly compromised (i.e. mushy), it was still viable at the end of the experiment. Pieces that looked unhealthy were not assayed.

Perifusion Apparatus: A schematic diagram of the apparatus is shown in figure 4.1 (from Kierszenbaum and Tres, 1987). An automated perifusion system (APW10, manufactured by Endotronics, Minneapolis, Minnesota) was modified by the authors for use with permeable substrates for dissociated cells or tissues. The control module and peristaltic pump allows continuous delivery of fresh medium to scleral tissues in individual growth

chambers. The peristaltic pump is supplemented by multi-channel pumps that pump medium through a heat-gas exchanger which allows gas and heat diffusion across the permeable silicon tubing containing the culture medium. The gas used was 5% CO_2 and 95% air, and the temperature was maintained at 37 C via a water bath. The plexiglass growth chambers contain wire grids at the bottom to support the tissue. All components of the system that come into contact with the medium are autoclavable. Prior to each experiment, 1N NaOH, 1N HCl, and 95% ethanol (in that sequence) were pumped through the system to insure sterility. Following this, unlabeled medium was pumped through. A 300 ml glass bottle containing the sulfur-35-labeled medium was then exchanged for the bottle containing cold medium. The scleral tissues were placed in separate growth chambers, and the flow rate was set at 2 ml per hour. Medium was collected at 2 hour intervals for up to 80 hours (approximately 4 ml per sample). Collected samples were placed in a refrigerator; no sample was left at room temperature for over 8 hours. At any sign of contamination in the system the experiment was terminated, and any contaminated samples were discarded.

Biochemistry

The medium used for all experiments was N2, the chemically defined medium of Bottenstein (Bottenstein, 1983). This medium is a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 with added sodium bicarbonate (15 mM), sodium selenite (30 nM), progesterone (20 mM), putrescine (100 uM), pyruvate (1 mM), glutamine (1 mM) and insulin (5 mg/ml). Antibiotics added were streptomycin (0.1 mg/ml), garamycin (0.01 mg/ml) and fungizone (0.1 mg/ml).

To assay newly synthesized glycosaminoglycans (GAGs), tissue was digested in 0.05% proteinase-K (protease type XXVIII, Sigma, in 10 mM EDTA, 0.1M sodium phosphate, pH 6.5) overnight at 57 C. This treatment results in complete digestion of the cartilaginous and fibrous sclera. $^{35}SO_4$-labeled glycosaminoglycans were precipitated by the addition of 0.5% cetylpyridinium chloride (CPC) in 0.002 M Na_2SO_4 in the presence of unlabeled chondroitin sulfate (1mg/ml in dH_2O). Samples were incubated for 1 hour at 37 C and the precipitated GAGs were collected on Whatman filters (GF/A) using a Millipore 12-port manifold. Filters were rinsed 5 times with 0.1% CPC containing 0.05M NaCl and dH_2O. Radioactivity in the filters was measured by liquid scintillation counting in 10 ml scintillation fluid (CytoScint, Fischer Biotech).

This assay measures the incorporation of labeled inorganic sulfur into the GAG side chains of proteoglycan molecules. Hence, an increase in the amount of label could reflect either increased synthesis of GAGs or an increase in the length or sulfation of pre-existing GAGs. Unless the newly synthesized GAGs are added to newly synthesized core proteins, the increase in label would not reflect newly synthesized proteoglycans, therefore we are unable to say with certainty that that is what we are measuring. However, for the sake of simplicity, we will hereafter refer to the incorporation of labeled sulfur into GAGs as "proteoglycan synthesis".

Characterization of proteoglycan

To characterize the labeled molecule in the medium, molecular weight fractionations were performed on undigested samples of medium, and on medium digested with either chondroitinase or keratanase, two enzymes that specifically cleave chrondroitin-6-sulfate and keratan sulfate. For the

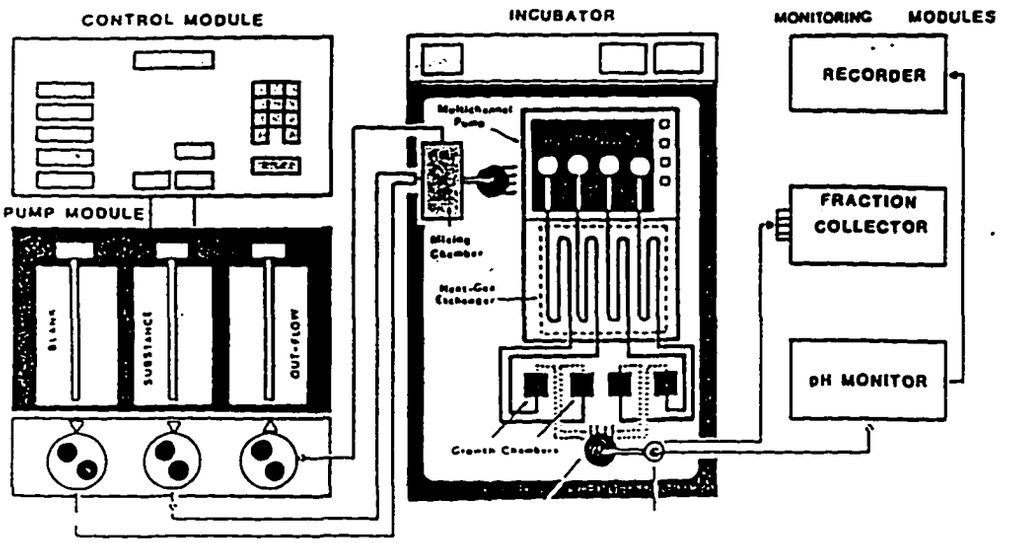
enzyme digestions, labeled samples of medium were concentrated using 10,000 MW microconcentrators (Centriprep-10; Amicon, Beverly, MA). Concentrated samples were then digested with chondroitinase ABC (Seikagaku Inc.. 5 units/200 ul) or keratanase (Seikagaku Inc., 10 units/200 ul) in 0.1M Tris, pH 7.4, containing 500 mM phenylmethylsulfonyl fluoride, 100 mM N-ethylmaleimide, 100 mM EDTA, and 36 mM pepstatin A. 50 ul of buffer and 2 ul of the enzyme were added to 50 ul of the medium sample and incubated at 37 C overnight. Subsequently, 50 ul of the enzyme-digested medium sample was run on a Sephadex-50 column. As a control, 50 ul of the undigested medium were also run on the column. 500 ul fractions were collected and counted for radioactivity.

We compared the profile of our labeled molecule to that for the cartilage proteoglycan aggrecan. To do this, un-incorporated isotope was removed from the sample by repeated centrifugation and washing in 0.1M phosphate buffer. Approximately 10 ml media was spun at 12,000 x g using a microconcentrator (10,000 MW cutoff filter). The resulting supernatant was applied to a sepharose CL-4B molecular sieve column (100 x 1.6 cm) in 4M guanidine HCl containing 0.02M Tris and 0.01% CHAPS, pH 6.8. A 200 ul aliquot from each fraction was counted for radioactivity.

Data Analysis and Statistics

For experiment 3 (long term culture), data for individual pieces (disintegrations per minute, DPMs) were first normalized by dividing by their mean values; this corrected for inter-experimental differences in specific activity among experiments. The resultant data were smoothed using a 3 point running average and the mean for each group was obtained. The data for normal and myopic pieces were subjected to an ANOVA with

repeated measures. The parameters of the rhythms were determined by fitting a sine function to the mean normalized data; this analysis yielded the amplitude, period and phase of the rhythm.



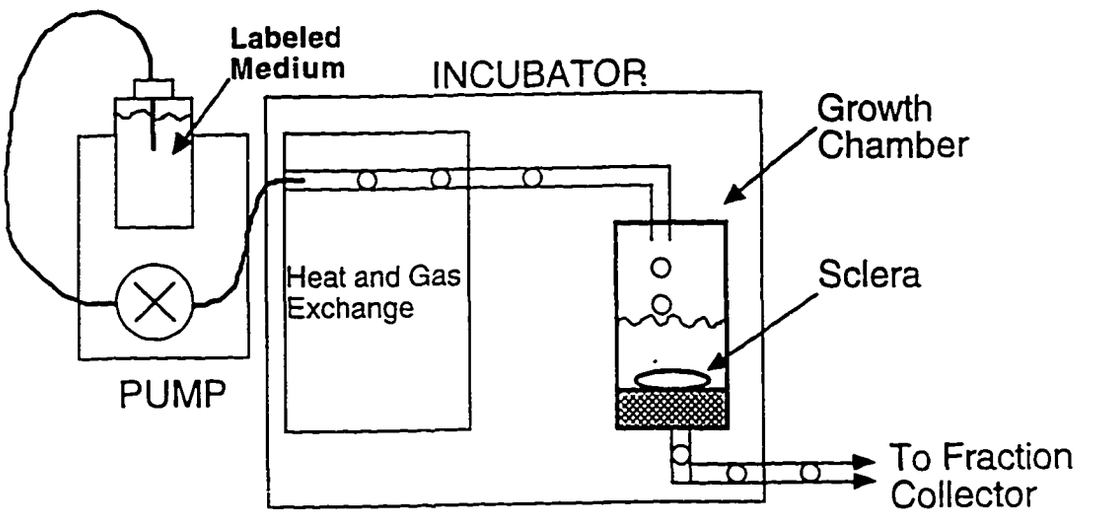


Figure 4.1. Schematic diagrams of perifusion apparatus. Top, from Kierszenbaum & Tres, 1987, shows the modular components of the perifusion system. The arrangement consists of a control module and pump system, "growth chambers" for the tissue that are set into a water-bath temperature-controlled unit through which medium is continuously perfused via gas permeable plastic tubing, and a fraction collector. B. Schematic diagram shows the path of the labeled medium from the reservoir through the heat-gas exchange manifold, to the culture chambers in which tissue incubates, and finally to the fraction collector.

Results

Tissue Assays: 10 hour incubations:

Proteoglycan synthesis is greater during the day than during the night.

For all pieces of sclera incubated for 10 hours, proteoglycan synthesis during the day (9 am to 7 pm) is 72% higher than during the night (10 pm to 8 am) (figure 4.2a, t-test, $p<0.005$). This day/night difference is relatively greater in normal scleras than in myopic scleras (figure 4.2b, normals: 91% higher during the day, $p=0.01$; myopic: 54% higher during the day, $p=0.005$), however, the difference is not significant (ANOVA, $F=0.07$, $p=.8$). In addition, in accordance with previous work (Rada et al., 1991; Nickla et al., 1992), we find that proteoglycan synthesis in scleras from form-deprived eyes is significantly greater than in scleras from normal eyes (figure 4.2b, ANOVA, $F=42.7$, $p<0.0001$).

2 hour incubations:

Proteoglycan synthesis is highest in the morning

In scleras from normal eyes, proteoglycan synthesis is highest in the morning, lowest in the afternoon and intermediate during the night (figure 4.3, black bars). This finding was consistent in each of the 4 experiments; with morning corresponding to between 8 and 11 am, afternoon between 1 and 4 pm, and night between 9 pm and midnight. In all 4 experiments the synthesis during the morning was significantly higher than during the afternoon. In scleras from form-deprived eyes, although there was a similar trend as in normal eyes in one experiment (striped bars, bottom right, figure 4.3), in another experiment (striped bars, bottom left, figure 4.3c), there were no significant differences as a function of time of day.

A. Day versus Night

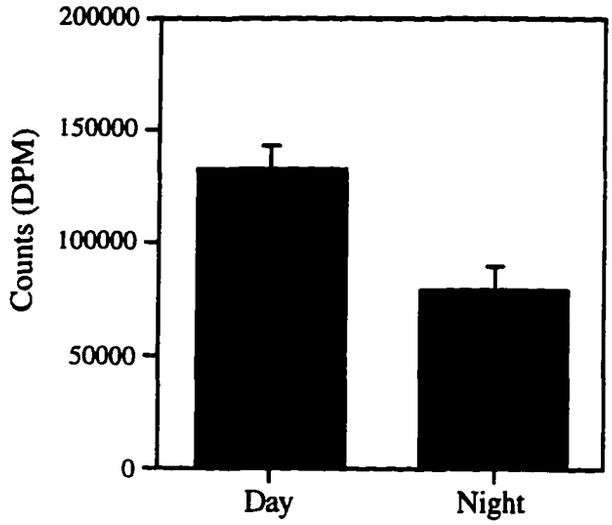


Figure 4.2a. Synthesis of proteoglycans by scleral punches (combining normal and myopic eyes) over 10 hours of the day versus 10 hours of the night. The amount of proteoglycans synthesized during the day is significantly greater than the amount synthesized during the night.

B. Normal versus Myopic eyes

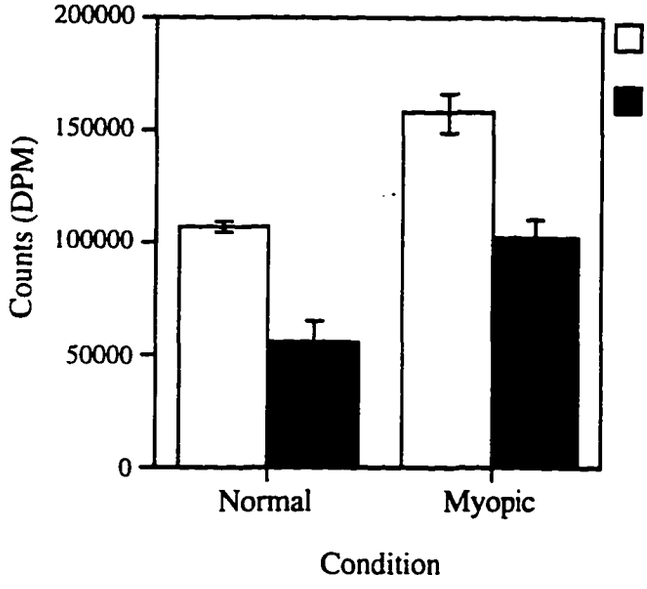


Figure 4.2b. Synthesis of proteoglycans during the day (white bars) and during the night (black bars) for scleras from normal and myopic eyes. Synthesis is higher in both eyes during the day. In addition, the synthesis in scleras from myopic eyes is significantly higher than in scleras from normal eyes for both day and night.

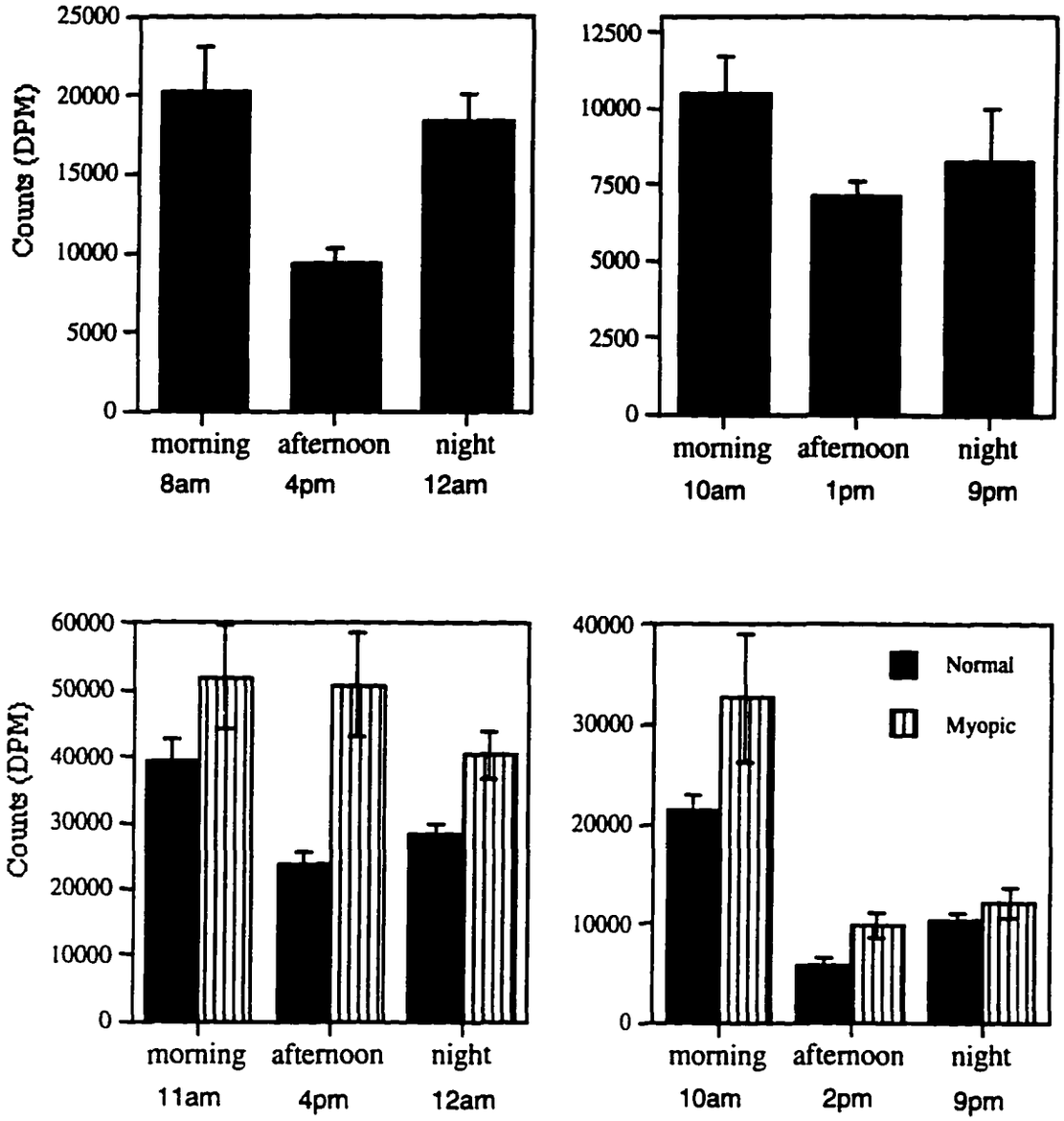


Figure 4.3. Proteoglycan synthesis over 2 hours in pieces of sclera at different times of day. The four graphs represent different experiments; starting times of incubation are indicated below the x axis. Solid bars, scleras from normal eyes; striped bars, scleras from form-deprived myopic eyes; error bars are standard errors of the mean. In scleras from normal eyes (n=4 for each time point, solid bars), the highest rate of synthesis occurs during the morning, with less synthesis during the afternoon and night. In scleras from myopic eyes (n=4 for each time point) there is no consistent trend.

Comparison of tissue and medium proteoglycans

A comparison between the newly synthesized proteoglycans in the tissue with those released into the medium shows that synthesis is higher in the morning (10 am) than in the afternoon (4 pm) in both cases (figure 4.4, dotted and black bars, $p<0.05$; $p=0.007$, respectively). The amount of labeled proteoglycans in the medium was approximately 15% of that in the tissue. Because the phase-dependent difference in proteoglycan synthesis in the medium is similar to that found in the tissue, we conclude that the medium-derived proteoglycans are probably an accurate representation of the relative rates of synthesis in the pieces as a function of time.

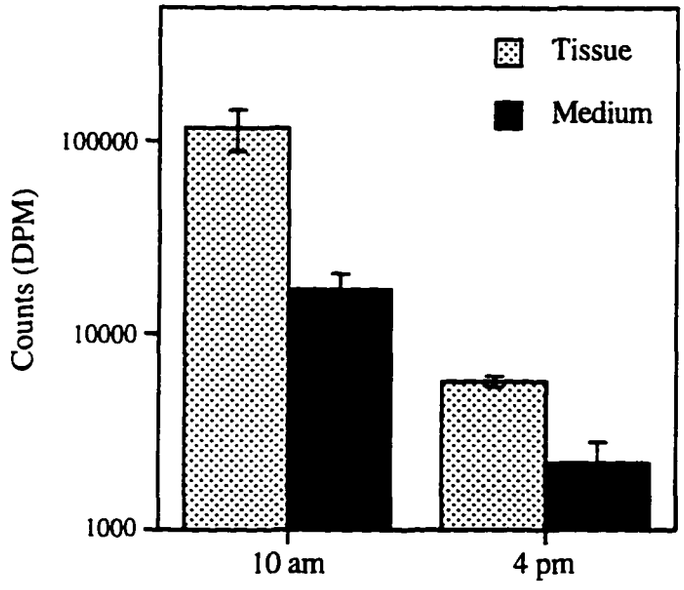


Figure 4.4. Uptake of sulfur-^{35} into proteoglycans in the tissue (stippled bars) compared to those released into the medium (black bars) over 2 hour intervals at two times of day. For both tissue and medium, proteoglycan synthesis is higher in the morning than in the afternoon. In addition, the amount of proteoglycans in the medium is lower than in the tissue (note that the y axis is logarithmic).

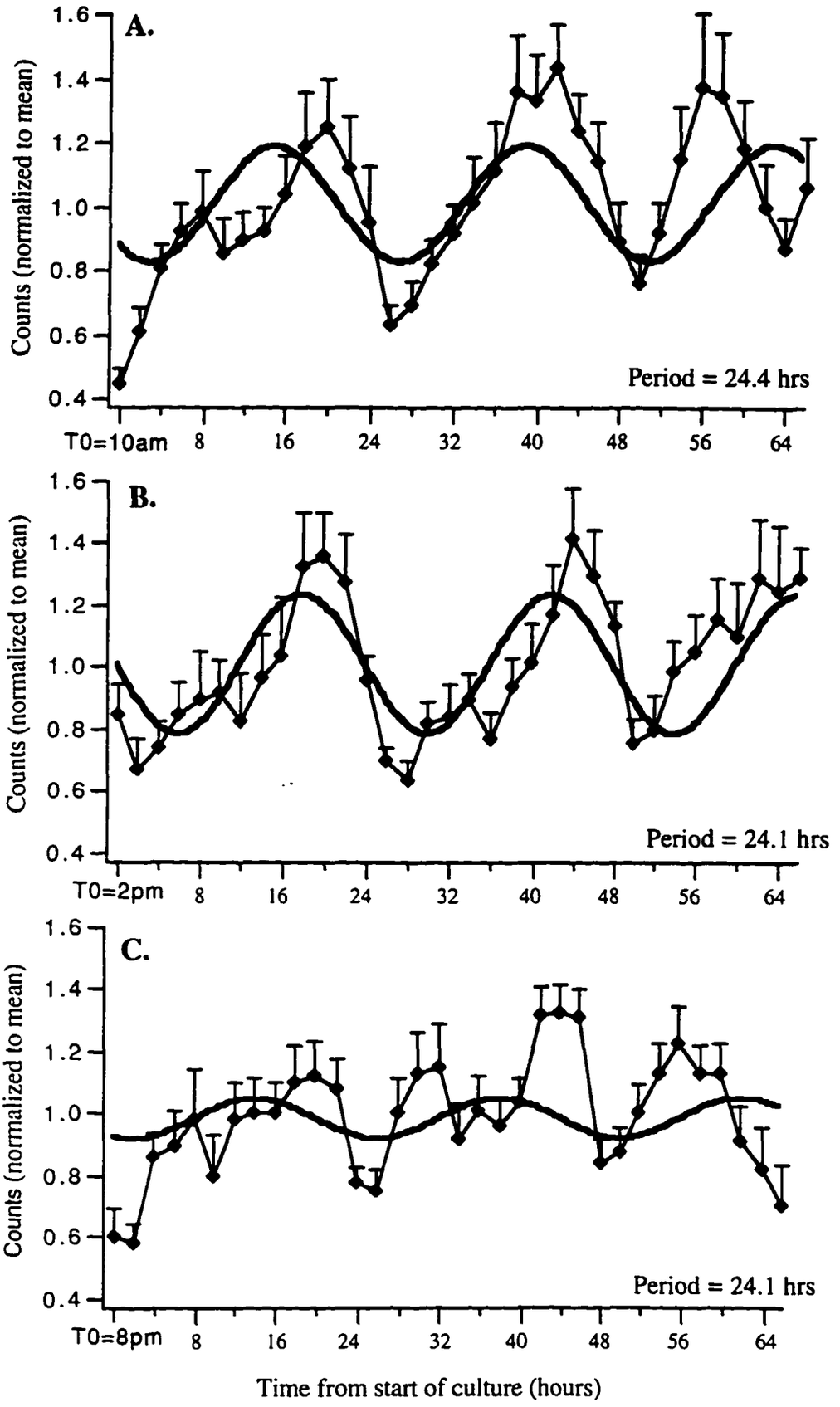
Long-term perifusion culture

Proteoglycan synthesis shows a diurnal rhythm that is retained in culture for several cycles.

Proteoglycan synthesis shows a diurnal rhythmic in culture. This rhythm has a period of approximately 24 hours that persists *in vitro* for at least 3 cycles (Figure 4.5). Figure 4.5 shows the mean of the normalized data with the sine wave fit to this data for the three experiments for normal scleras; time 0 (time of dissection) is indicated below the x axis, the period (peak to peak) obtained from the sine fit is indicated on the right of each graph.

Pieces of sclera dissected in the morning (figure 4.5a) show a normalized mean amplitude of 0.4, meaning that the fluctuation around the mean is 40% of the mean rates of synthesis (synthesis increases and decreases by 20% of the mean rate). The mean peak of the rhythm occurs at 1:00 am (analysis by sine fit). Fourier analysis shows the major frequency component to be the diurnal one (one cycle per day, the period is approximately 24 hours), with additional harmonics at the ultradian frequencies of 1.875 cycles/day (period approximately 12 hours) and 1.5cycles/day (period=16 hours) (figure 4.6a). Pieces of sclera dissected in the afternoon (Figure 4.5b) show a mean amplitude of 0.45, and a mean peak occurring at 8 am, a phase delay of 7 hours in relation to the morning experiment. Fourier analysis shows the major frequency component again to be the diurnal component, with additional harmonics at 1.875 cycles/day (period=12 hours) (figure 4.6b).

Figure 4.5. (next page). Mean counts of all experiments (counts S-35 normalized to the mean for each experiment) for scleras from normal eyes. A. Culture started in the morning; B, culture started in the afternoon, C, culture started at night. Time 0, start of culture indicated at left on x axis. Period of the sine wave fit to the data on bottom right for each graph. Note that the acrophase for each is approximately 16 hours from time 0.



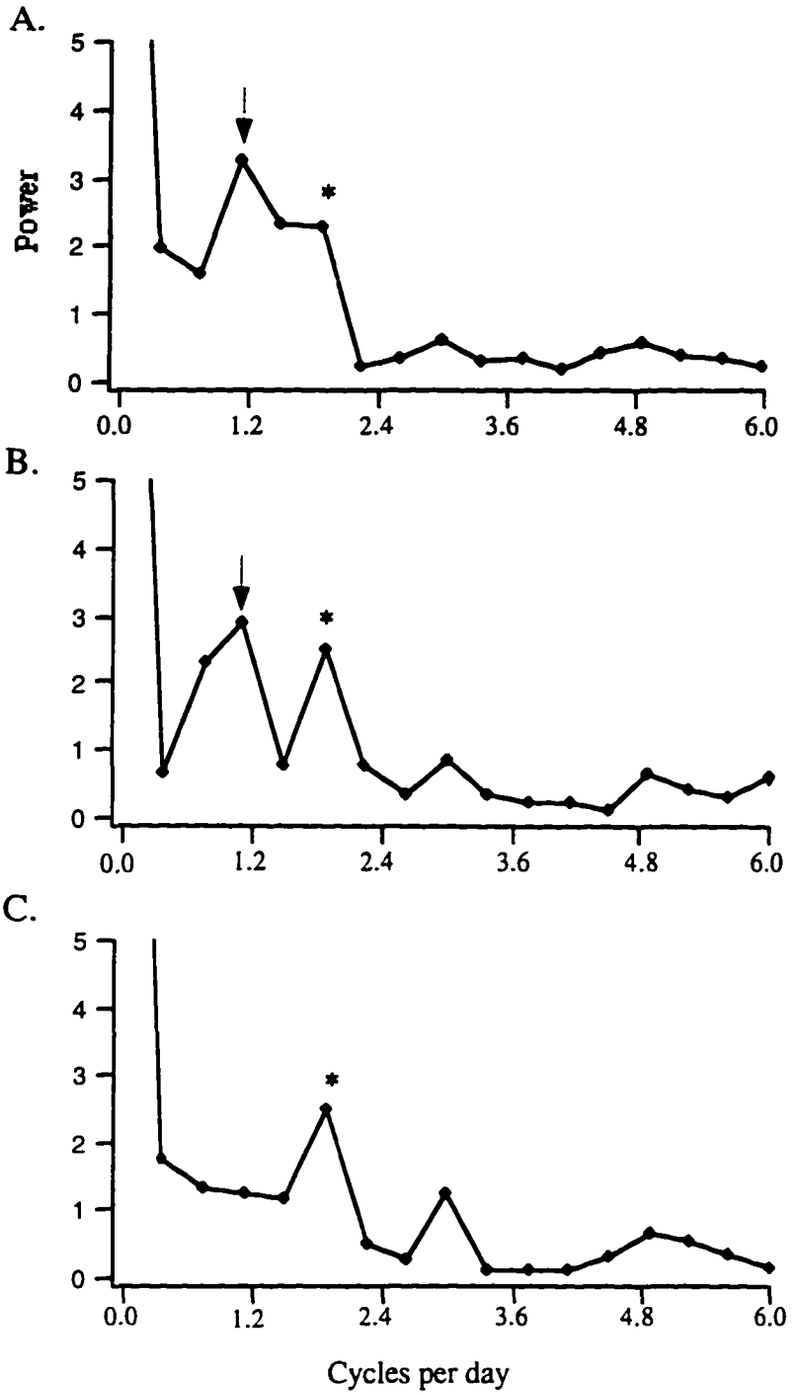
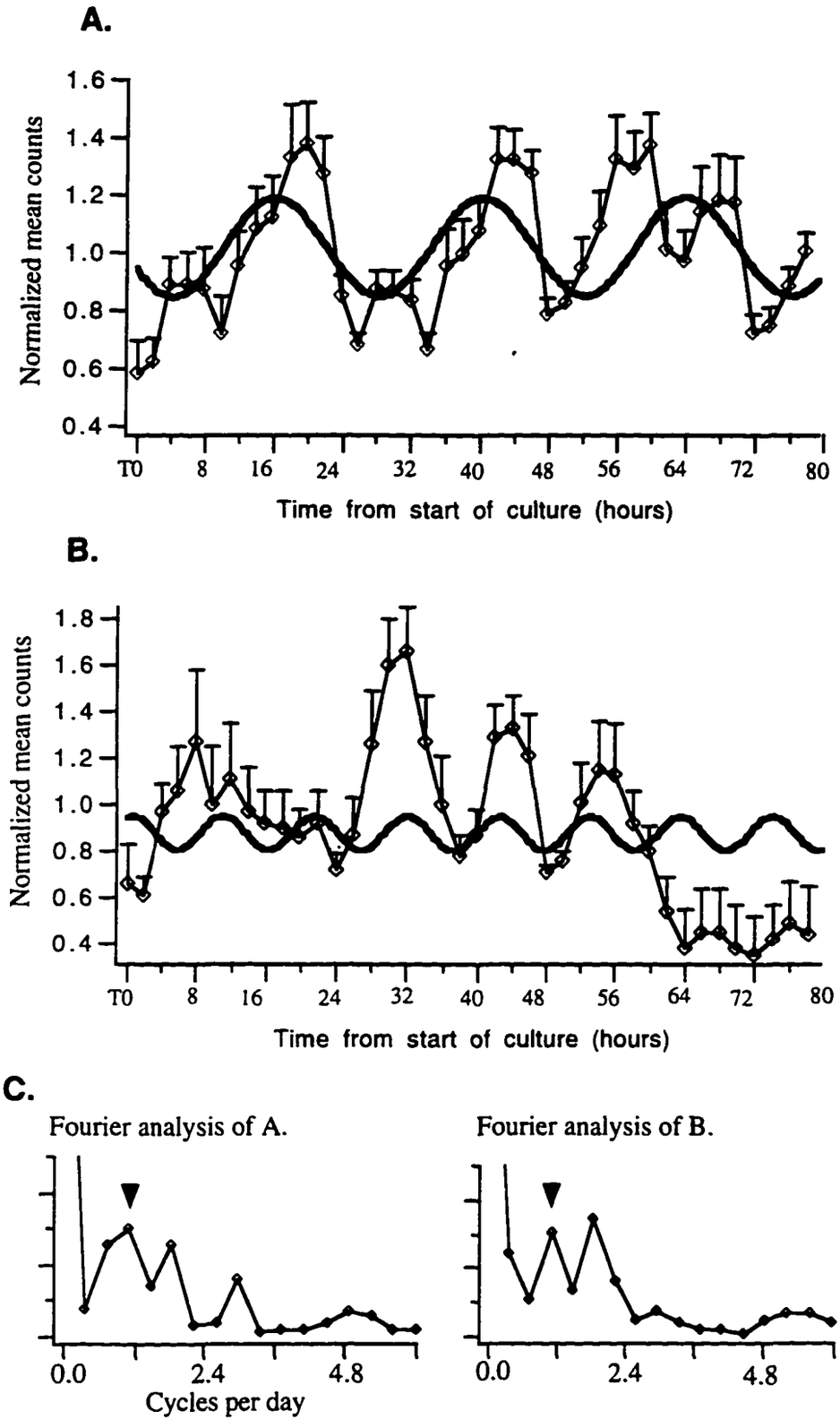


Figure 4.6. Spectral frequency analysis of mean data from graphs in figure 4.5 (A, morning; B, afternoon; C, night). The diurnal frequency (1 cycle per day) is indicated by arrows. The ultradian frequency of 2 cycles per day is indicated by asterisks. The major frequency component for the morning and afternoon data is the diurnal one. For night, the major frequency component is 2 cycles per day.

Pieces of sclera dissected at night (Figure 4.5c) show a much smaller mean amplitude (0.13), and a mean peak at 9:30 pm, a phase advance of about 3 hours in relation to the morning experiment. The major frequency component is ultradian, at 1.875 cycles/day, with a secondary one at 2.25 cycles/day (period=8 hours, figure 4.6c). An examination of the individual pieces in this group reveals two distinct patterns; this variability accounts for the poorer fit of a sine wave and the apparently less cyclic nature (compare figure 4.5c to 4.5a and 4.5b). If the individual experiments for this group are separated into two groups based on a subjective assessment of "shape", the data for the two groups appear as shown in figures 4.7a (7 pieces) and 4.7b (6 pieces). The mean of the data for the first night group (figure 4.7a) appears very similar to that for the morning and afternoon groups (compare to figures 4.5a and 4.5b). There is a clear diurnal rhythm, with a mean peak at midnight. The major frequency component in this group is the diurnal one, with a secondary one at 1.875 cycles/day (figure 4.7c, left). The second group (figure 4.7b) however, appears more variable in phase than the first, and does not exhibit a clear diurnal rhythm. The major frequency component is 1.875 cycles/day (figure 4.7c, right).

Figure 4.7. (Next page). Data from the "night" experiment (from figure 4.5c) divided into 2 groups depending on pattern of peak frequency. A and B: Data is normalized to the mean for each experiment (symbols). The sine wave fit to the data is shown by the waves. Note that the data in a appears similar to that of the other experimental groups in figure 4.5a and 4.5b while the data in b shows higher frequency components. C. Fourier analysis of data from a and b. The arrow indicates the diurnal frequency; both sets of data show both diurnal and ultradian frequency components.



The rhythm is re-set by being put into culture.

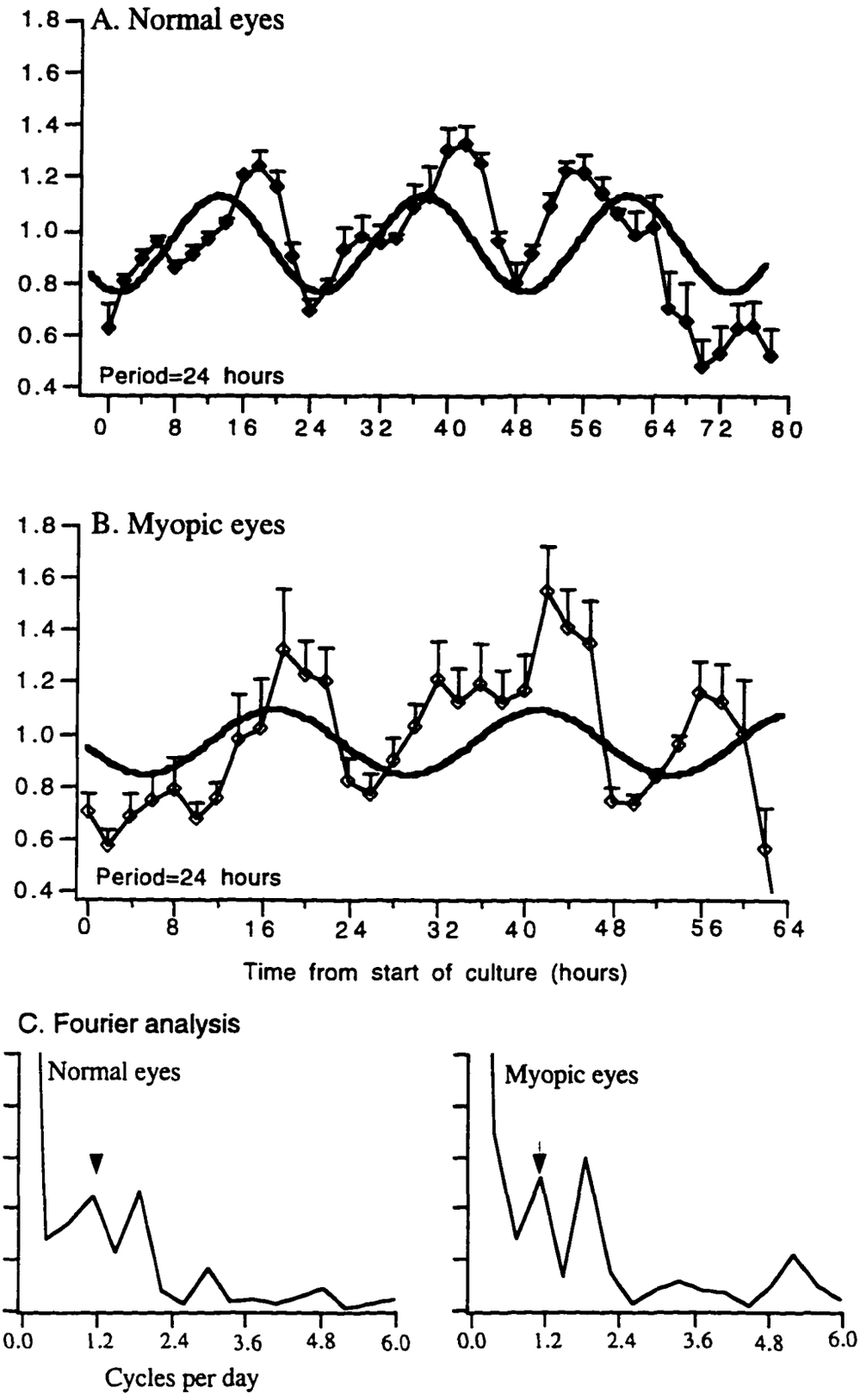
Surprisingly, a comparison of the mean scleral synthesis rhythms from experiments that differed only in the time of dissection (figures 4.5a, b and c) showed that the rhythm peaks occurred at different times of day, with the "afternoon" group being phase delayed and the "night" group phase advanced relative to the "morning" group. There was a consistency, however, in the times of peak synthesis relative to the start of the experiment: all peaks occurred between approximately 14 and 18 hours after the beginning of the culture, regardless of local time of day (figure 4.5). The only plausible interpretation of this result is that the rhythm in proteoglycan synthesis was strongly phase-shifted by the conditions of the experiment, so that they were "reset" to time 0. Despite this phase shifting, all of the experiments showed a rhythm with an approximate 24 hour period, as assessed both by the sine wave fit (thick lines in figure 4.5) and by Fourier analysis (for "night" group, only if divided into two groups). In addition, all three groups show a second ultradian frequency component at approximately 2 cycles per day.

The mean for all of the pieces from normal eyes is shown in Figure 4.8a. The period of the sine wave fit is 24 hours (solid line). The major frequency component is the diurnal one, with a secondary one at 1.875 cycles/day (figure 4.8c, left). In summary, pieces of sclera from normal eyes shows a endogenous circadian rhythm that persists for at least 3 cycles in culture. Furthermore, some aspect of the culturing procedure, either removing the eye, or a component of the medium, shifts the phases of these rhythms, and effectively resets the "clock".

There is a rhythm in scleral GAG synthesis in myopic eyes

Scleras from form-deprived myopic eyes also showed a diurnal rhythm in proteoglycan synthesis (figure 4.8b). This rhythm has a smaller amplitude than the rhythm in the normal pieces (0.24 vs 0.36, compare figure 4.8b to figure 4.8a), although, similar to normal eyes, the period of the sine wave fit to the mean data is 24 hours (figure 4.8b). Similar to sclera from normal eyes dissected in the afternoon, the peak occurred at about 8 am. Fourier analysis (figure 4.8c, right) shows the major frequency component to be ultradian, at 1.875 cycles per day, with a second one at the diurnal frequency (arrow, 1 cycle/day). An ANOVA (repeated measures) shows that there is no significant difference in the rate of synthesis as a function of time for pieces from normal versus myopic eyes.

Figure 4.8. (Next page). Mean normalized counts (symbols and standard error bars) for all scleras from normal eyes (A, n=39) and all scleras from form-deprived myopic eyes (B, n=8) . The period of the sine wave fit to the data is 24 hours, and is shown at the bottom left. C. Fourier analysis of the data in a and b, left and right, respectively. Both normal (left) and myopic (right) scleras show a frequency component of 1 cycle per day.



Characterization of the labeled molecule

Size fractionation of the glycosaminoglycans after enzymatic digestion by chondroitinase and keratanase indicates that the most prevalent GAG is chondroitin-6-sulfate, the predominant GAG in the cartilage proteoglycan aggrecan (triangles and solid line, figure 4.9a), with a small amount of keratan sulfate (circles and dashed line, figure 4.9a). Size fractionation of the proteoglycans in undigested medium on a Sepharose CL-4B column (figure 4.9b) shows that much of the radioactivity eluted in the void volume at a position similar to that seen for aggrecan[1] (asterisk, figure 4.9b, compare to graph on right, from Rada et al. (1992)). We can conclude with some certainty that the labeled molecules we are measuring in the medium in which we incubate scleral tissue are the chondroitin-6-sulfate glycosaminoglycan chains that comprise a large part of the cartilage proteoglycan, aggrecan.

[1] The larger peak at a smaller molecular weight (tube #19) probably represents breakdown of the aggrecan molecule (personal communcication, Jody Rada).

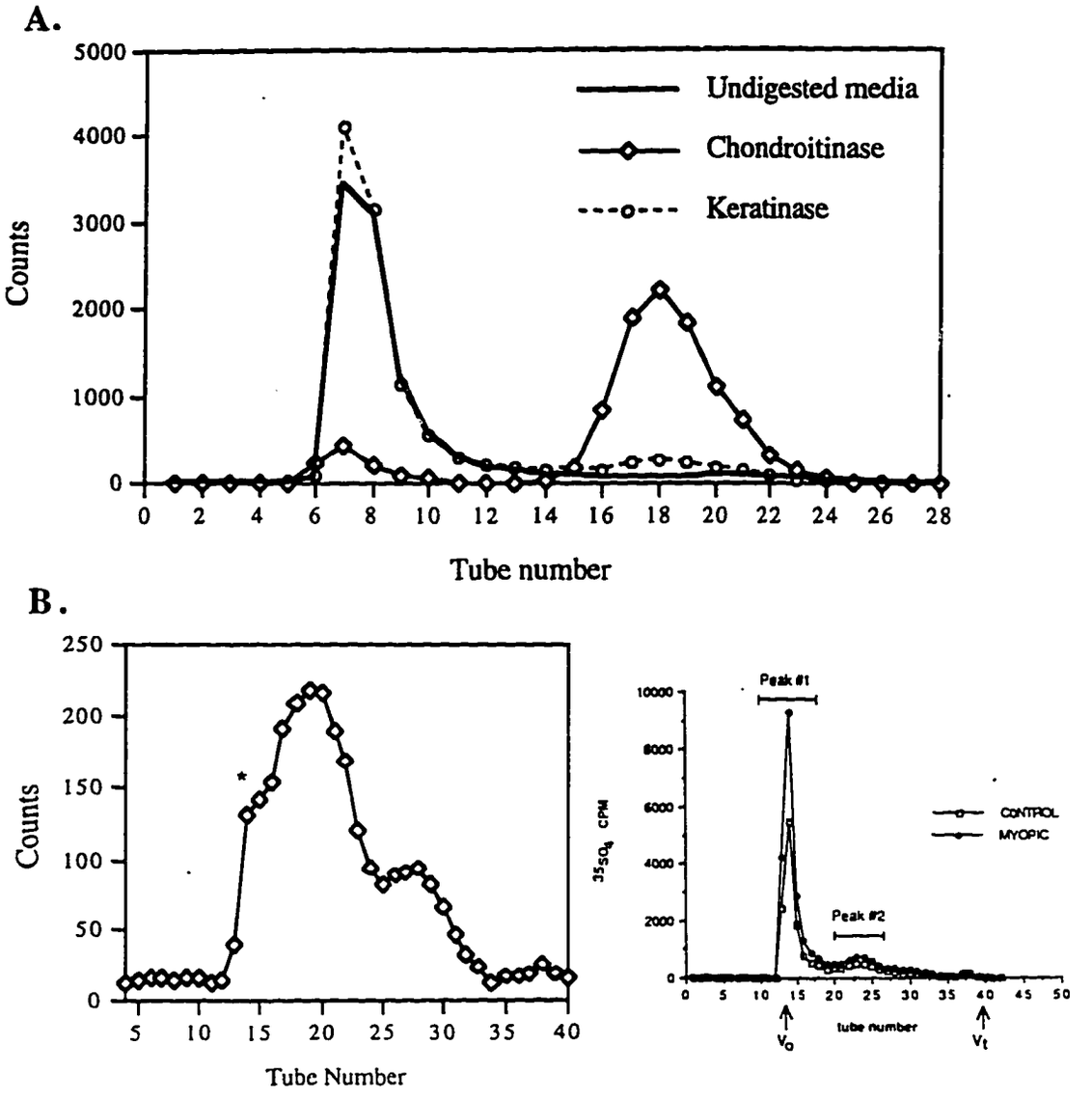


Figure 4.9. Molecular weight fractionation of labeled proteoglycans in medium from perifusion experiment. A. Medium digested with chondroitinase (diamonds) and keratanase (circles), compared to undigested medium (bold line) on a Sephadex G-50 column. B. Fractionation of molecules in medium on Sepharose CL-4B column (left), compared to the profile for the cartilage proteoglycan Aggrecan (right, from Rada et al., 1991).

Discussion

The rate of ocular growth in birds has been shown to be correlated with the rates of synthesis of scleral proteoglycans (Rada et al., 1991; Nickla et al., 1992; Rada et al., 1992), therefore the regulation of growth is probably via a modulation of synthesis rates. It has recently been found that ocular elongation shows a diurnal rhythm. We find that the sclera of chick eyes shows rhythmic fluctuations in proteoglycan synthesis *in vitro*, being high during the morning and low at night. This rhythm has a period of approximately 24 hours, and persists in culture for at least 3 cycles. We propose that these rhythmic oscillations in proteoglycan synthesis contribute to the rhythm in axial elongation found in normal chick eyes. Furthermore, we propose that the cells responsible for the rhythmic production of matrix molecules are the chondrocytes of the cartilaginous layer of the sclera.

The chick sclera consists of two layers, an inner cartilaginous one and an outer fibrous one (Walls, 1942). The cartilaginous layer is composed of chondrocytes embedded in a matrix of collagen fibers and the proteoglycan aggrecan. The fibrous layer is composed of fibroblasts, collagen and the proteoglycan decorin. Proteoglycan molecules are highly negatively charged macromolecules that consist of a core protein to which glycosaminoglycan chains (GAGs, repeating disaccharide units that may be sulfated) are attached. Aggrecan contains many chains of the sulfated glycosaminoglycans chondroitin-6-sulfate and keratan-sulfate, and is much larger than decorin. Decorin consists of only one glycosaminoglycan chain of dermatan sulfate, and consequently is much less sulfated than aggrecan.

The proteoglycan synthesized by our scleral tissue consists of sulfated glycosaminoglycans that are cleaved by the enzymes chondroitinase and keratanase. In addition, the intact proteoglycan is the same size as the cartilage proteoglycan aggrecan. Therefore, we conclude that the proteoglycan that is being rhythmically synthesized is aggrecan, and hence that the cells responsible for the production of these molecules are the chondrocytes in the cartilaginous layer of the sclera.

Synthesis of proteoglycans *in vitro*

Pieces of scleral tissue release proteoglycans into the medium; these released molecules show a diurnal rhythm in synthesis. We are assuming that the quantity of synthesized molecules in the medium reflect the rate of synthesis of proteoglycans in the tissue, and that they are the same molecule. Proteoglycans are synthesized in the Golgi apparatus where the GAG chains are connected to the core protein, and are secreted into the matrix against a concentration gradient (Kimura et al., 1981). Because they are molecules that are actively and rapidly secreted from the cell (Kimura et al., 1981) it is plausible that the medium from cultures of scleral explants would contain some proportion of the newly synthesized molecules that would reflect the rate of synthesis by the chondrocytes. Alternatively, or in addition, the molecules in the medium might constitute a degraded form of the proteoglycan which would not be discernible from the intact molecule, or a different proteoglycan.

What is known regarding the synthesis and secretion of proteoglycans in *in vitro* situations? Studies examining the differences between proteoglycans secreted into the medium versus those in the tissue matrix have been done on articular cartilage and retinal pigment epithelial (RPE)

cells *in vitro*. In one such study, normal and osteoarthritic cartilage was pulse-labeled with inorganic sulfur-35 for 4 hours and the proteoglycans "lost" to the medium were followed for 8 days (Carney et al., 1984). It was found that the quantity of labeled proteoglycans in the medium was positively correlated with the quantity of labeled proteoglycans in the tissue, however, some of the molecules in the medium were smaller, presumably the result of degradation in the arthritic cartilage. In addition, the secretion of these proteoglycans into the medium was an active cell mediated process, as evidenced by the decrease in output in tissue that was killed by freeze-thawing. Therefore, the proteoglycans in our medium could reflect either their rate of synthesis and/or their rate of turnover. Although we cannot definitively distinguish between these two possibilities, the fact that the molecule in the medium is approximately the same size as aggrecan (figure 4.9) indicates that degradation is probably not a major factor. Furthermore, the fact that the rate of turnover is often correlated with the rate of synthesis (Carney et al., 1984) makes the issue of minor importance in the interpretation of our data.

It is also plausible that the secreted proteoglycan differs qualitatively from that put into the matrix. In RPE cells *in vitro* it was shown that the glycosaminoglycans secreted by the cells differed both from those maintained inside the cell (intracellular) and from those put into the extracellular matrix (Stramm, 1987; Stramm et al., 1989). Furthermore, it has been reported that the ability of some proteoglycans to aggregate by binding to hyaluronic acid is dependent on the application of compressive forces on the tissue. Specifically, cartilage that was subjected to intermittent compressive force *in vitro* synthesized proteoglycans that had a greater ability to bind matrix components than cartilage that was not subjected to

intermittent force (van Kampen et al., 1985). It is possible that in the absence of the normal physiological milieu, the proteoglycans produced are qualitatively different from those produced *in vivo*. Nonetheless, the molecule we measured in the medium is a chondroitin-sulfate containing proteoglycan of similar molecular weight as aggrecan.

Because one of the main functions of cartilage is the ability to withstand the compressive forces acting on joints, much of the work in this field of research has examined the effect of forces on the metabolic properties of the chondrocytes in cartilaginous tissue. *In vivo*, cartilage is normally exposed to intermittent compressive forces. It has been found that normal growth in cartilage requires the application of force; unloading (removing the force) results in the loss of proteoglycans and tissue degradation (reviewed in: Tammi et al., 1987). Furthermore, the effects of force are dependent on whether the force is static or intermittent. In general, both static compressive forces and unloading (no force) result in a decrease (or no change) in proteoglycan synthesis. In contrast, intermittent compressive forces result in an increase in proteoglycan synthesis (reviewed in: van Kampen and van de Stadt, 1987). (These effects are also dependent on the frequency and magnitude of the applied force). These responses are believed to be a reflection of the ability of the chondrocytes *in vivo* to sense and respond to the magnitude of the strain with "repair" activities that increase the production of matrix. For one example, chondrocytes exposed to hydrostatic compressive forces of 0.3 Hz showed an increase in proteoglycan synthesis and matrix volume (Burger and Veldhuizen, 1993). Similarly, chondrocytes grown on a membrane that are subjected to a cyclic (0.2-1 Hz) strain for 8 hours show a 2-3 fold increase

in proteoglycan synthesis (Lee et al., 1982; DeWitt et al., 1984) and a decrease in protein and collagen synthesis (Lee et al., 1982).

The relevance of these studies to the work presented here resides in the fact that the eye is under the influence of the intraocular pressure (IOP), which is maintained by the continuous production and drainage of the aqueous humour in the anterior chamber. Intraocular pressure undergoes a diurnal rhythm in the eyes of many species, including chickens (Nickla and Wallman, 1995a). Furthermore, higher-frequency transients of IOP are presumably induced by the normal changes in accommodation and blinking of the eye. Therefore, two of the inherent characteristics of intraocular pressure, that it exerts a force on the ocular tunic, and that this force is by its nature, cyclic, are qualities that have been shown to influence growth in cartilage in other systems. In fact, it has been shown that brief pulses of hydrostatic pressure of a physiological magnitude for intraocular pressure (approximately 15 mm Hg) applied once every 27 hours (approximately the diurnal frequency) to cartilage *in vitro* results in a 50% increase in proteoglycan synthesis (Takano-Yamamoto et al., 1991). We propose that the rhythm in intraocular pressure is an important regulatory factor in the growth of the eye by virtue of its effect on scleral proteoglycan synthesis. It is also possible that the absence of this force in culture may alter synthesis rates.

Rhythmic growth parameters in cartilage

It has long been known that cartilage and bone *in vivo* exhibit circadian rhythms in the synthesis of DNA and proteoglycans that are highly temporally coordinated with the cell division cycle and to one another (reviewed in: Simmons, 1992). Specifically, in cartilage, DNA

synthesis occurs in the morning, followed by proteoglycan synthesis at mid-day, and finally, mitosis (Simmons, 1962, 1964, 1968). The amplitude (peak to peak excursion) of the daily fluctuations in cell division and proteoglycan synthesis is between 100-300% (reviewed in: Simmons, 1992). It appears, therefore, that the cells cannot simultaneously synthesize macromolecules and undergo mitosis, and so the coordination of these different growth events with the cycle of light and dark is advantageous. It should be noted that these studies were done *in vivo*, and hence do not demonstrate the existence of a self-oscillating system. We here show that the cartilaginous layer of the chick sclera shows a circadian rhythm in proteoglycan synthesis that is retained in culture for at least 3 cycles and has a peak to peak amplitude of approximately 50% of the mean. By measuring the amount of newly synthesized GAGs in scleral tissue we find that the peak in synthesis occurs in the morning. The fact that the rhythm persists in the absence of exogenous cues indicates that it is an endogenous circadian rhythm.

One component of the diurnal periodicity in the growth of cartilage is the 24 hour generation time in individual chondrocytes. Isolated prechondroblasts, the progenitors of chondrocytes, have a generation time of approximately 24 hours. Other bone progenitors such as skeletoblasts show an increase in generation time as a function of time in culture, presumably a result of the formation of intercellular junctions that facilitate the transmittal of a cell division "restraining" signal. The lack of comparable intercellular junctions between prechondroblasts prohibits this intercellular signal, and so the generation time remains approximately one day. This has also been demonstrated for chondrocytes *in vivo* (reviewed in: Petrovic et al., 1984).

The existence of an endogenous circadian rhythm in cartilage *in vitro* was similarly demonstrated by Stutzmann and Petrovic (1978). These authors measured the incorporation of tritiated thymidine (DNA synthesis) into chondrocytes from rat synchondrosis by labeling the culture medium for the last hour in culture. They found that the diurnal periodicity in cartilage growth was retained in organ culture in the absence of physiological (i.e. hormonal) and environmental (i.e. light and dark) signals, showing a peak at 9:00 am and troughs at 11:00 pm and 1:00 am (Stutzmann and Petrovic, 1978). The fact that this was demonstrated in tissue that was in culture from 48-72 hours indicates that the diurnal rhythm in DNA synthesis was endogenous. They also showed that tissue from animals in an inverted light/dark cycle showed an inverted rhythm, controlling for the possibility that undetectable exogenous factors were responsible. Furthermore, the addition to the medium of serum containing somatotrophin and somatomedin (insulin-like growth factor) caused a phase shift of several hours. Our culture medium also contains insulin, the relevance of which will be discussed later. This study showed that (1) the rhythm in cell division persists in the absence of extrinsic factors, and (2) the susceptibility of cartilage to certain hormones is also circadian. Our findings extend this previous work on cartilaginous growth rhythms by demonstrating that a growth rhythm exists in chick scleral cartilage; in addition, because the rhythm in scleral proteoglycan synthesis is in phase with the diurnal rhythm in ocular elongation, we propose that the rhythm in proteoglycan synthesis is a major component of the diurnal rhythm in ocular elongation.

Eye growth and proteoglycan synthesis

In chickens, the rate of synthesis of proteoglycans in the sclera parallels the rate of ocular elongation (Rada et al., 1991). In rapidly elongating form-deprived eyes, the rate of synthesis of scleral proteoglycans was increased by approximately 50% above normal (Rada et al., 1991). Similarly, eyes elongating in response to negative spectacle lenses show an increase in their rate of proteoglycan synthesis (Nickla et al., 1992). Conversely, eyes that have slowed their growth, for example, eyes recovering from deprivation myopia (Nickla et al., 1992; Rada et al., 1992) and eyes responding to positive spectacle lenses (Nickla et al., 1992), show a decrease in the rate of proteoglycan synthesis.

Axial elongation in normal chick eyes shows a diurnal rhythm, elongating more during the day than during the night (Weiss and Schaeffel, 1993; Nickla and Wallman, 1995a). Form-deprived myopic eyes also exhibit a rhythm in axial length that is similar to normal (Nickla and Wallman, 1995a). Changes in scleral proteoglycan synthesis are associated with changes in the rate of growth of the eye (Nickla et al., 1992; Rada et al., 1992). We here show that there is greater synthesis of proteoglycans during the day than during the night, and that the highest rate of synthesis occurs in the morning. These diurnal changes in proteoglycan synthesis parallel the diurnal changes in axial elongation. We conclude therefore that the circadian rhythm in proteoglycan synthesis contributes to the diurnal rhythm in axial elongation.

By sampling the culture medium at 2 hour intervals, we were able to characterize the period and amplitude of the rhythm. Scleras from both normal and form-deprived eyes synthesize proteoglycans in a cyclic manner and both show a major frequency component at the diurnal

frequency of 1 cycle per 24 hours (also, an ANOVA shows no difference between them). However, scleras from myopic eyes synthesize significantly more proteoglycans than those from normal eyes, in keeping with the almost 2-fold higher rate of elongation in these eyes. Therefore, the higher rate of elongation in form-deprived eyes is accountable for not by a difference in the phase or the amplitude of the rhythm in proteoglycan synthesis, but by a higher rate of synthesis.

Rhythms in proteoglycan synthesis and intraocular pressure underlie the rhythms in axial elongation in chick eyes

In normal chick eyes, the highest rate of axial elongation occurs during the morning, between 6 am and noon, and the lowest occurs at night, between 6 pm and midnight. The time of greatest elongation is concomitant with an increase in IOP, and the time of least growth with a decrease in IOP. We here report that the synthesis of scleral proteoglycans is highest in the morning and lowest in the afternoon. The temporal coincidence between these three ocular components of growth: elongation, IOP and scleral synthesis, points to an interrelationship between the changing rates of axial elongation and the diurnal rhythms in scleral proteoglycan synthesis and intraocular pressure. We propose that the rhythm in IOP influences the rhythm in proteoglycan synthesis via changes in force exerted on the sclera, and that the result of these changes in intraocular pressure and proteoglycan synthesis is the rhythm in axial length.

The evidence that intermittent force produces changes in proteoglycan synthesis has already been discussed. Much of the evidence indicates that it is not force *per se*, but the change in force that is crucial to changes in

synthesis rates in cartilage. In our scleral tissue, we find that the diurnal fluctuations in proteoglycan synthesis occur in the absence of any type of change in pressure. Hence, we know that both IOP and proteoglycan synthesis constitute independent endogenous rhythms. It is impossible to predict how the *in vitro* changes might differ from those occurring in the intact organism. We propose that the rate of scleral proteoglycan synthesis shows a phase-dependent sensitivity to changes in intraocular pressure. Specifically, we have evidence that the degree of synchrony (phase difference) between the endogenous rhythms in IOP and proteoglycan synthesis determines the overall rate of eye growth; we find that when the rise in IOP during the early morning is concomitant with the increase in scleral proteoglycan synthesis the overall rate of growth is lower than if the two are not coincident (see Discussion in Chapter 5).

In summary, we hypothesize that the diurnal changes in IOP influence the rate of proteoglycan synthesis in the sclera, but that the oscillations in synthesis rates are not dependent on the changes in pressure. It is possible that the rate of synthesis (and hence eye growth) has a phase-dependent sensitivity to changes in pressure, and that the change in rate is determined by when changes in pressure occur with respect to various events in the cell cycle. In this way, the scleral synthesis rhythm is influenced by the intraocular pressure rhythm, the outcome of which are diurnal fluctuations in axial length.

Re-setting the rhythm in perifusion culture

The peak of the rhythm in proteoglycan synthesis occurs approximately 16 hours after the tissue is put into culture, regardless of what time of day the culture is started. The only plausible interpretation of

this result is that some aspect of the culture conditions strongly phase-shifts the rhythm. If all culture conditions, including those for measuring incorporation into the tissue, caused a similar resetting, it should not be possible to distinguish a difference in synthesis for "day" versus "night" (sidereal time). The fact that we do find a phase-dependent difference in proteoglycan synthesis when we assay the tissue itself, rather than the medium, indicates that the rhythms were not "reset" under these conditions. We propose two explanations for this difference in results: First, it is possible that there are separate clock-driven rhythms in proteoglycan synthesis and the aggregation of the proteoglycans with hyaluronic acid in the tissue matrix. If the aggregation rate is higher during the day than during the night, this would account for our findings of a day/night difference in the tissue and possibly not the medium. It is also plausible that the rhythm in proteoglycan synthesis that is reflected in the levels in the medium are more susceptible some factor in the medium that causes phase shifts. Evidence supporting this hypothesis is work showing that many factors influence the qualities of proteoglycans; for instance, cyclic compressive force enhances the aggregation properties of proteoglycans in chondrocyte culture, improving their ability to associate with other matrix components (van Kampen et al., 1985). If some factor in the medium caused a similar effect, it might explain why this rhythm would be less susceptible than the synthesis rhythm to factors that might shift the phase of the rhythm. Nonetheless, the fact that both "tissue" and "released" proteoglycans show a consistent diurnal rhythmicity indicates that the rhythmicity is an inherent characteristic of the chondrocytes.

Alternatively, the difference between the results of the "tissue" and "medium" experiments might be that the medium contains a factor that

causes a concentration-dependent shift in phase. The large phase shifts seen in the perifusion experiments could reflect the continuous replenishment of this factor, whereas in static culture the factor might be rapidly exhausted. Many types of substances have been shown to shift circadian rhythms, both *in vitro* and *in vivo*. Our medium, although "defined", contains many supplementary components such as progesterone and insulin, both hormones having documented effects on cartilage metabolism (reviewed in: Buchanan and Preece, 1992). Insulin, for example, has phase-dependent effects on DNA synthesis in many tissues (reviewed in: Scheving et al., 1982). It was shown that DNA synthesis in bone marrow was increased only when insulin was injected at the transition from light to dark. In liver, insulin caused either increases or decreases in DNA synthesis depending on the time of the injection. Furthermore, insulin-like growth factor I (somatomedin) was shown to shift the DNA synthesis rhythm in cartilage *in vitro* (Stutzmann and Petrovic, 1978). Other examples of phase shifting compounds are cytosine arabinoside, which causes a 4 hour phase shift in the circadian rhythm in mitosis in rat corneal epithelium (Scheving and Pauly, 1973) and dopamine agonists, which cause phase shifts in the rhythm of retinal melatonin synthesis (Cahill and Besharse, 1991).

Diurnal rhythms in non-neuronal tissues

Although most physiological processes, from cell division and protein synthesis to sleep/wake cycles and body temperature, have been shown to be diurnally rhythmic, only a few tissues have been identified as containing independent self-oscillating clocks. We find a circadian rhythm in proteoglycan synthesis in cartilaginous tissue that persists for several cycles in the absence of extrinsic time cues, including physiological (i.e.

hormonal) ones. What are the implications of this finding in terms of what is known regarding the mechanisms for controlling circadian rhythms? The requirements for demonstrating that a tissue contains a clock include the persistence of an oscillation *in vitro* for several cycles under constant conditions. Stronger evidence would be the demonstration that the tissue responded to a *Zeitgeber in vitro*, that is, that the rhythm could be phase-shifted by light, or by a pharmacological agent. These criteria have been satisfied for the suprachiasmatic nucleus in mammals, the pineal gland in lower vertebrates (including chickens), and the retinal pigment epithelium (RPE) and retina in several species (birds, fishes, amphibians, now mammals) (reviewed in: Cahill and Besharse, 1995). The element common to all of these tissues is that they are either neural, or, in the case of the pineal gland and RPE, of neural derivation. That clocks do exist outside of the nervous system has recently been shown in invertebrates; for example, the prothoracic gland in insects shows a circadian rhythm in ecdysteroid synthesis that is maintained in culture for several cycles and is directly photosensitive (Vafopoulou and Steel, 1991). In addition, isolated cultured reproductive systems of the gypsy moth contains a light-sensitive pacemaker that controls the release of sperm from the testes; these isolated organs also respond to pulses of light by phase shifts (Giebultowicz et al., 1989; Giebultowicz and Foster, 1995). Furthermore, as mentioned earlier, Stutzmann and Petrovic (Stutzmann and Petrovic, 1978) demonstrated that cartilage continued to show rhythmic fluctuations in DNA synthesis *in vitro*. Independent oscillators exist at the level of the single cell in both vertebrate (bird pineal) and invertebrate (Gonyaulax, Neurospora, Euglena) systems. All of the above hint at the possibility that non-neural

tissues may contain the machinery for functioning as an oscillator; the avian sclera may be one such tissue.

It is also possible that the clock resides in the cell division cycle, which is a clock in the sense that it measures time, albeit, imprecisely (not all cells in a tissue have the same generation time). It is known that the generation time of cartilage cells *in vitro* is approximately 24 hours (Stutzmann and Petrovic, 1982; Guiguet et al., 1984), and that, *in vivo*, the synthesis of DNA and mitosis is diurnally rhythmic. Hence, if the synthesis of proteoglycans is coordinated with the cell cycle then synthesis would show a diurnal rhythm. This coordination might have a structural significance: In cartilage, cell division produces groups of chondrocytes that are initially clustered together in "nests". We speculate that the coupling of cell division to proteoglycan synthesis could separate the newly divided cells and so function to organize the tissue. Because we know that chondrocytes continue to divide in culture, we might expect that the synthesis of proteoglycans would remain rhythmic for several cycles, even dissociated from the retinal "clock". The fact that some component of the medium resets the phase of the rhythm is plausibly the consequence of it acting to synchronize the rhythm in mitosis. For example, IGF I has been shown to phase-shift the rhythm in DNA synthesis (Stutzmann and Petrovic, 1978). Because proteoglycan synthesis shows a consistent phase relationship to DNA synthesis, it is plausible that by virtue of phase-shifting the rhythm in DNA synthesis and cell division, insulin also shifts the phase of the rhythm in proteoglycan synthesis.

In conclusion, there is a rhythm in proteoglycan synthesis in the chick sclera that is correlated in phase with the rhythm in ocular elongation and intraocular pressure. This rhythm is circadian, as it persists in culture for

at least 3 cycles and has a period of 24 hours. We speculate that there is an interaction between the rhythms in intraocular pressure and scleral proteoglycan synthesis, with scleral synthesis (and perhaps cell division) being influenced by the rhythm in intraocular pressure. Both factors would determine the parameters of the rhythm in ocular elongation, and so be crucial in the regulation of the growth of the eye.

CHAPTER 5

The circadian rhythm in intraocular pressure and its relation to diurnal ocular growth changes in chicks

Introduction

Myopia, or nearsightedness, is the result of the eye being too long for its optics, so that the image of a distant object falls in front of the retina. Although numerous theories have been advanced regarding the causative factors involved in the etiology of myopia, none have been definitively linked to the etiology of myopia. Two of the more compelling hypotheses that continue to receive attention come from separate avenues of research in clinical and animal studies: first, evidence from decades of clinical research support the notion that intraocular pressure (IOP) has an influence on the size of the eye (review: Prüett, 1988). Second, evidence mainly from animal models support the hypothesis that vision strongly influences the growth of the eye such that different "growth" signals are sent from the retina to the sclera depending on the visual circumstances (review: Wallman, 1993). Evidence supporting this notion is that depriving an eye of form vision by using diffusers or suturing the lids results in axial elongation and myopia. More compelling evidence is that the rate of growth of the eye compensates for artificially-induced refractive errors: chicks, tree shrews and monkey eyes all become myopic in response to negative lenses and hyperopic in response to positive lenses (chicks: Schaeffel et al., 1988; Irving et al., 1992; Wildsoet and Wallman, 1992; Wallman et al., 1995); monkeys: Hung et al., 1995) tree shrews: Siegwart and Norton, 1993).

Two alternative models can explain how IOP could be involved in the development of myopia: first, excessive eye enlargement could be the result of higher than normal IOP in an otherwise normal eye, or second, it could be the result of normal IOP in a more compliant (stretchable) eye. With regard to the first model, there is evidence in the clinical literature that myopia is associated with higher than normal intraocular pressure and there is also purported to be an association between glaucoma and myopia (review: Curtain, 1985). However, there is no consensus that the etiology of myopia is connected to high IOP. This issue has not been extensively investigated in animal models of myopia, and in the chick, lower rather than higher than normal IOP has been reported to be associated with form deprivation myopia (Wilkinson and Hodos, 1991). Furthermore, while chickens exposed to continuous light develop enlarged eyes (light-induced bupthalmous) and higher than normal IOP, the increase in eye size precedes the increase in IOP (Lauber, 1987).

With regard to the second model involving an increase in scleral compliance, several studies have reported abnormal biochemical and structural features in scleras from myopic eyes, resulting in more elastic scleras that are more prone to creep in response to force exerted by intraocular pressure. For instance, studies on humans have shown an association between axial myopia and scleral thinning (Curtin and Teng, 1957; Bell, 1978; Curtin et al., 1979; Avetisov et al., 1984). These thinner scleras are composed of smaller than normal collagen fiber bundles and collagen fibrils. Similarly, in tree shrews, the scleras of form-deprived eyes have smaller than normal collagen fibrils (Cornell and McBrien, 1994). Finally, the biomechanical qualities (creep rate) of scleras from myopic humans (Avetisov et al., 1984), tree shrews (Siegwart and Norton, 1994;

Phillips and McBrien, 1995), and rabbits (Ku and Greene, 1981) differ from normal scleras. However, the use of lathyritic agents that interfere with cross-linking of collagen (and weaken the sclera) were ineffective in inducing changes in the size of the eye in form-deprived chicks (McBrien and Norton, 1988).

In this paper, we provide evidence that the mechanism involved in regulating eye growth is essentially a synthesis of both of the above hypotheses: that growth is determined by the synchrony between the rhythms in IOP and other ocular diurnal rhythms, and hence involves both retinal signals and IOP. We report that the IOP of normal chick eyes undergoes diurnal fluctuations that are coincident with diurnal changes in axial length and in light of these data propose that IOP is a relevant factor in the normal growth of the eye, not by virtue of either its magnitude, or of abnormal scleral compliance, but by virtue of the daily changes that it undergoes. IOP increases in the morning when the eye is elongating most and decreases at night when the eye is elongating least. These diurnal changes in IOP could account for much of the diurnal changes in axial length of the eye. In form-deprived myopic eyes the rhythm is no longer as tightly synchronized to the cycle of light and dark. We postulate that the growth of the chicken eye is determined, at least in part, by the synchrony between the diurnal fluctuations in IOP and scleral growth.

Methods

Subjects

Subjects were White Leghorn chickens (*Gallus gallus*) obtained as 1 day old hatchlings from Truslow Farms (Chestertown, MD). Birds were housed in temperature controlled brooders with a light/dark cycle of 14L/10D (lights on at 8 am, off at 10 pm), and fed food and water *ad libitum*.

To induce myopia, translucent white plastic dome diffusers were glued to the feathers around one eye at day 2 (attenuation of light is approximately 0.6 log units). Experiments generally involved repeated measurements and the fitted diffusers remained in place until the first of these measurements after which they were replaced with detachable ones. For these, a Velcro ring was glued to the feathers around the eye and the diffusers were attached to the mating ring of Velcro. Diffusers were replaced immediately after measurement.

Studies

A. Light / Dark Study.

Two experiments, differing only in the times of measurement and the duration of the experiment, comprise the data for assessing the IOP rhythm under normal light/dark conditions (14L/10D). Because of these differences, the data for the two experiments will be shown and treated separately, unless otherwise indicated.

Experiment 1: Six 2-week-old birds that had been deprived of form vision in one eye from day 2 comprised the experimental group (6 myopic eyes, 6 untreated fellow eyes). Six normal birds were also included (12 normal

eyes). Measurements of IOP were done at approximate 6 hour intervals over 24 hours: 1:00pm, 7:00pm, 1am, 7:30am, and 1pm.

Experiment 2: Ten 2-week old birds that had been deprived of form vision in one eye from 2 days of age were used. The untreated fellow eyes served as the controls. Measurements began at 6 am and were done at varying intervals over the following 36 hours. Times of measurement were: 6am, noon, 3pm, 6pm, 1am, 6am, 2pm and 6pm.

B. Constant Dark Study.

Twelve 2-3 week old birds that had been deprived of form vision in one eye from hatching were used (12 myopic eyes, 12 fellow eyes) as well as 14 normal birds (28 normal eyes). The night prior to the first measurement, birds were put into darkness and remained in darkness through the subsequent days of measurement. Measurements were done over a period of 48 hours at 8am, 12pm, 6pm, 12am, 3am, 12pm, 12am, 3am, 7am and 3pm. Measurements were made under a photographic safe light and were completed within several minutes after which birds were immediately returned to darkness. The diffusers were not replaced. Food and water were available, and birds ate and drank in the dark.

C. Intraocular Pressure and Axial Length Rhythms Compared

By measuring both the IOP and axial length of eyes at the same time points, we were able to investigate the phase relationship between these two parameters. We measured both IOP and ocular dimensions at 6 hour intervals over 24 hours (6am, 12pm, 6pm, 12am, and 6am) in ten 2-week old birds that had been deprived of form vision. At each time point, immediately after measuring the IOP, eyes were measured using high-

frequency A-scan ultrasonography. Birds were kept in a normal light/dark cycle.

D. Ocular Compliance

Ocular compliance (change in length/change in IOP) data was obtained for one eye of 4 normal and 4 myopic birds (4 normal and 4 myopic eyes), aged 2-3 wks. To measure ocular compliance (change in length/change in IOP), we manipulated IOP using an attached water-filled manometer and simultaneously measured the eye using high frequency A-scan ultrasonography. The manometer was attached via a 3-way valve to the eye using a 25 gauge needle cannula in the anterior chamber, and to a pressure transducer interfaced with a polygraph. Prior to the cannulation procedure, birds were anaesthetized (IM injection of 40% urethane, 100 mg/kg) and "baseline" IOP and axial dimensional data measured by tonometry and A-scan ultrasonography respectively (see below). The birds were placed in a restraining holder for ultrasonography and all subsequent measurements. With the cannula in place, the pressure in the eye was reset to the pre-recorded baseline value by raising or lowering the manometer column as required. Ultrasound measurements were taken at this pressure, and then at 5 mm Hg increments from 10 to 35 mm Hg. Two further sets of data were collected for (i) 5 mm Hg decrements of pressure between 35 mm and 10 mm Hg, and (ii) 5 mm Hg increments back up to 35 mm Hg. In this way, the compliance of the globe was measured for 3 cycles of change in IOP for each eye.

Measurement Procedures

IOP: A clinical applanation tonometer (Tonopen XL, Mentor O&O, Norwell, MA) was used to measure intraocular pressure. This instrument provides confidence interval information based on successive readings; only data with the confidence intervals of 5 % or greater were accepted. Ophthaine (E.R. Squibb & Sons, Inc., N.J.) was topically applied to achieve corneal prior to measurement.

Axial ocular dimensions: To measure ocular dimensions, high-frequency A-scan ultrasonography was used (for details, see Wildsoet and Wallman, 1995). Birds were anaesthetized with halothane (0.8% in oxygen) for this procedure.

Data Analysis and Statistics

To characterize the differences in IOP rhythms between normal and myopic eyes in both the light-dark and constant dark studies, we computed the daily change in IOP in two ways. The first, "day - night difference", represents the difference in IOP between the mid-day time point (1:00 pm) and mid-night time point (1 am or 3 am) for all experiments. The second, "amplitude", is the difference between the *highest* and *lowest* pressure measurements recorded in any one 24 hr day, regardless of time of day. In this way we are able to distinguish between phase-related differences in IOP rhythm and differences in amplitude. A two sample t-test was used to compare data from normal and myopic eyes.

For the same studies, a two-way analysis of variance (ANOVA) with repeated measures was performed on each experimental data set to determine differences between groups (time and condition interaction). For all initial analyses, the fellow untreated eyes of deprived birds were kept

separate from the normal eyes of normal birds. However, because there were no significant differences between these two groups, the data were in some cases subsequently combined in graphs for ease of comparison.

In order to assess the phase relationship between the rhythms in IOP and axial length, both sets of data for normal and myopic eyes was fit to sine waves. Because the eyes were growing, the data for the axial length had to be first separated into a "steady state" component and a cyclic component. This was done by fitting a linear regression line to the individual data and subtracting that from the data for each eye. These residuals were then averaged, and a sine wave was fit to the mean data. A mean phase difference between the rhythms in axial length and IOP was subsequently calculated.

Results

In brief, normal eyes of chicks show a diurnal rhythm in intraocular pressure that is high during the day and low at night. This rhythm persists in constant darkness, and so has a circadian component. This rhythm differs in eyes that are myopic as a result of form deprivation in that the daily minimum is less likely to occur during the night than is the case in normal eyes. We find that the rate of ocular elongation is predicted by the phase difference between the daily rhythms in IOP and in axial elongation.

Normal Eyes

Light/Dark Study

Normal eyes show a diurnal rhythm in intraocular pressure with IOP being highest during the day around noon and lowest at about midnight (figure 5.1a, top, filled squares). At 7 am the IOP is slightly, but not significantly, higher than at midnight, suggesting that the pressure probably begins to rise just prior to dawn. The variable "time of day" accounts for a significant amount of the variance in intraocular pressure for normal eyes (Figure 5.1a, ANOVA $F_{4,55}$ =23, $p<0.0001$).

The fellow untreated eyes of form-deprived birds (figures 5.1a and 5.1b, filled circles) show a rhythm in IOP that is similar to normal eyes, with the exception that at 7 am in experiment 1 the IOP in normal eyes remains lower than that in both eyes of deprived birds (compare squares to circles, figure 5.1a). A one-way ANOVA shows that the variable "time of day" accounts for a significant amount of the variance in IOP for these eyes, similar to normal eyes (exp 1: $F_{4,25}$=4.55, $p<0.01$; exp 2: ANOVA $F_{8,81}$ =4.76, $p<0.001$).

Constant Dark Study

The diurnal rhythm in intraocular pressure in normal eyes persists in constant darkness with a smaller mean amplitude (figure 5.2, filled squares; also compare solid lines in figures 5.1 and 5.2). The fact that it persists in constant darkness fulfills a criterion for circadian rhythms, hence, the rhythm in IOP in the chick has a circadian component. As in the eyes in light/dark, IOP is low during the night (1am and 3am) and high during the

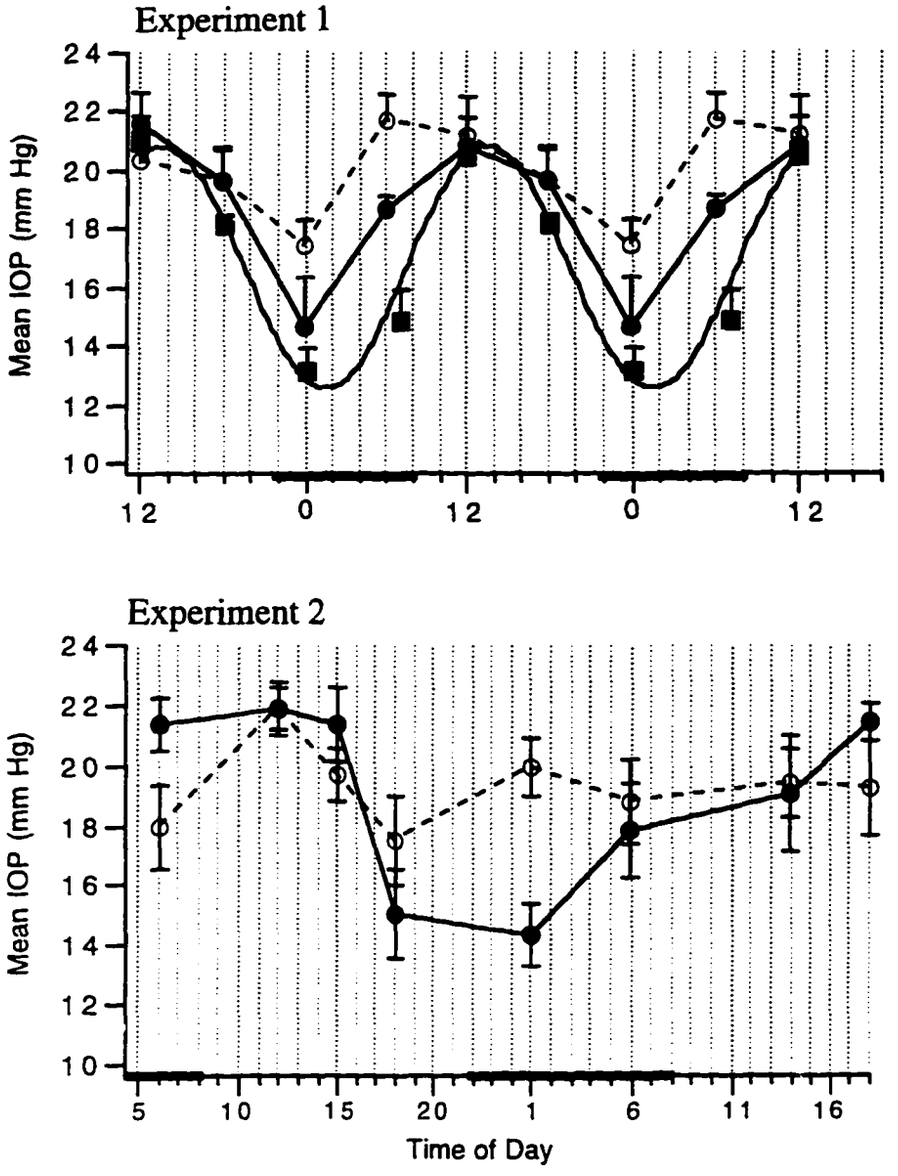


Figure 5.1. Mean intraocular pressure as a function of time of day in normal (solid squares), myopic (open circles and dashed lines), and fellow untreated (solid circles) eyes in a 14L/10D cycle. Black bar on x axis denotes night (lights off). A. Duration of experiment is 24 hours; data is double-plotted to reveal rhythmicity in normal eyes. The wave (solid wave with solid triangles) is the sine wave fit to the data for normal eyes (squares). In normal eyes, the trough occurs at night. The fellow eyes do not differ from normal (compare solid lines). B. Duration of experiment is 36 hours. The mean time-dependent change in IOP is smaller in myopic eyes than in fellow eyes.

day. An ANOVA shows that the variable "time of day" accounts for a significant amount of the variance in normal eyes ($F_{4,275}$=30.8; p=<0.001).

In constant darkness, the IOP rhythm in fellow untreated eyes is indistinguishable from that in normal eyes (figure 5.2, compare filled circles and squares). An ANOVA shows that the "time" variable accounts for a significant amount of the variance in IOP in these fellow eyes ($F_{5,66}$=8.7, p<0.001).

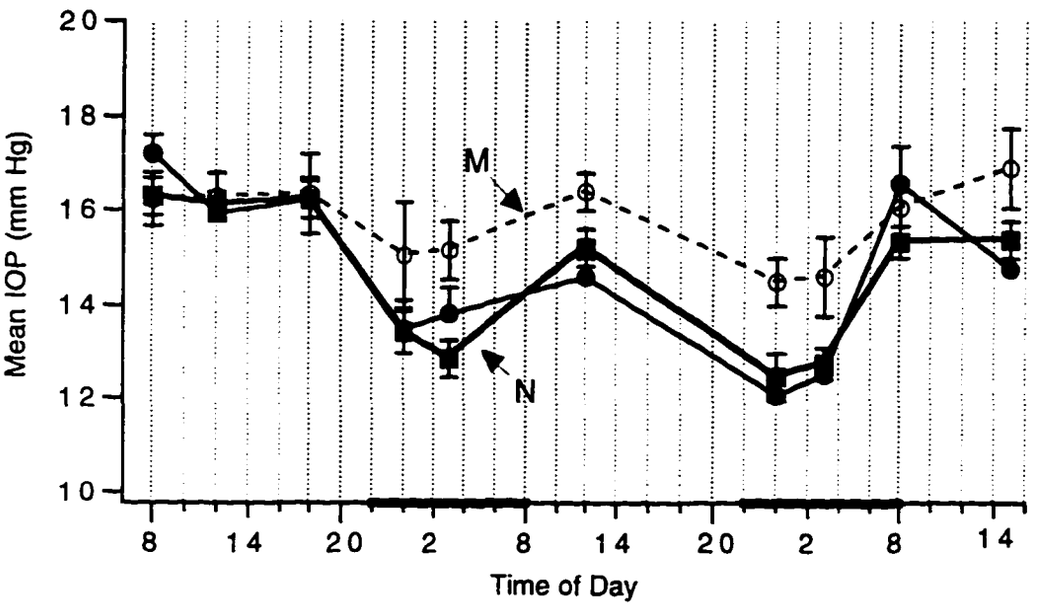


Figure 5.2. Mean intraocular pressure as a function of time of day in normal (solid squares), myopic (open circles) and fellow normal (solid circles) eyes in constant darkness over 2 cycles (48 hours). Black bars on x axis denotes subjective night (time of lights off in L/D). The mean time-dependent difference in IOP is approximately half that in a light/dark cycle (compare y axis to that in figure 5.1). The mean time-dependent difference for myopic eyes is smaller than for normal eyes. There are no differences between normal and fellow eyes. The mean daytime IOP for all eyes is lower than in a light/dark cycle (compare with figure 5.1).

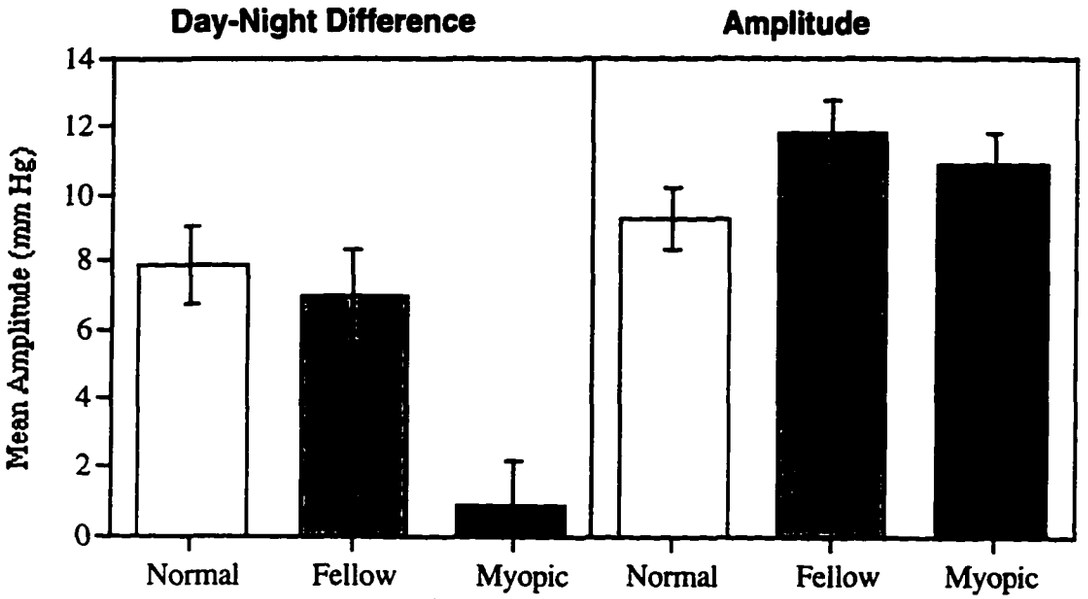
Form-deprived Myopic Eyes vs Normal Eyes

Light/Dark Study

While myopic eyes also show diurnal fluctuations in IOP (figures 5.1a and 5.1b, dashed lines with open circles), the mean time-dependent decrease in IOP is smaller than that seen in normal eyes and here, ANOVA (one way) shows that the variable "time of day" does *not* account for a significant amount of the variance (exp 1: $F_{4,25}$= 1.65, p=0.193; exp 2: $F_{7,72}$=1.05, p=0.4). In addition, a two-way ANOVA shows that the effect of "time of day" on myopic eyes differs from that on normal eyes (exp. 1: two way ANOVA interaction $F_{4,54}$=4.8; p=0.002; exp.2: $F_{8,8}$=2.4, p=0.02). Thus these two results are consistent and indicate a difference between myopic and normal eyes with respect to the time dependent changes in intraocular pressure.

We calculated the daily changes in IOP in two ways: the first is phase-dependent and depicts the mean difference between the mid-day and mid-night values for each eye (figure 5.3a, left), the second is independent of phase and depicts the difference between the maximum and minimum IOP for each eye (amplitude, figure 5.3a, right). In myopic eyes, the day-night difference is significantly smaller than in either normal eyes or fellow eyes (left, 0.87 mm Hg vs 7.8 and 6.9 mm Hg, respectively, p<0.0005), reflecting the difference in *mean* change seen in figure 5.1. However, there is no difference in amplitude (right) between myopic and normal eyes. Therefore, the apparent difference in the pattern of time-dependent changes in IOP between normal and myopic eyes (figure 5.1) must be due not to a difference in amplitude, but to a difference in phase.

A. In Light/Dark



B. In Constant Dark

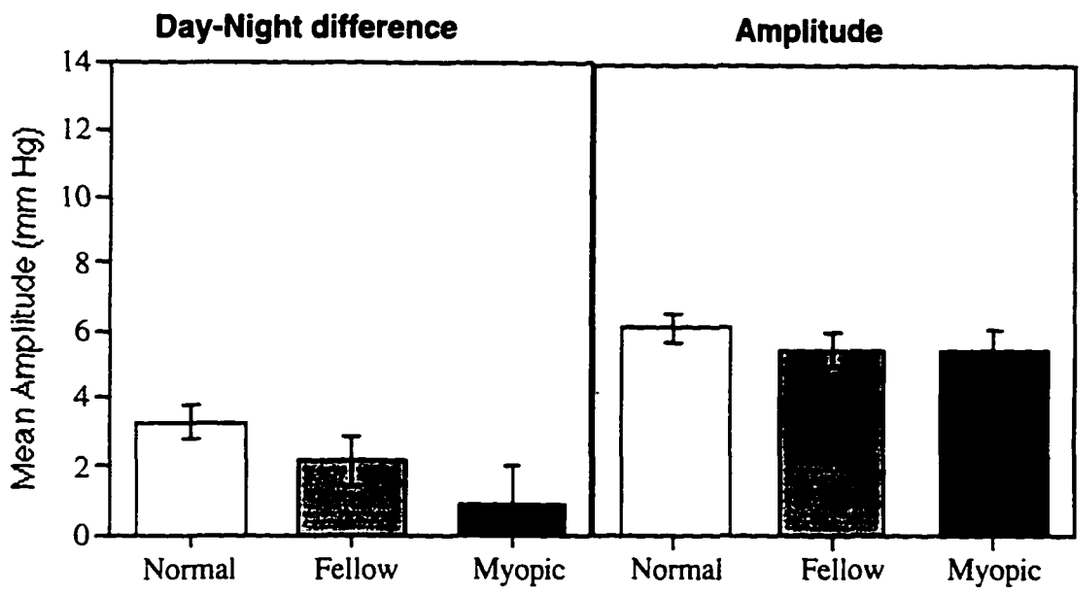


Figure 5.3. Two methods of assessing daily changes in intraocular pressure in normal (white bars) fellow untreated (grey bars) and myopic (black bars) eyes: left graphs show the difference between the mid-day and mid-night IOP; right graphs show the amplitude (maximum - minimum). A. Mean daily change in a 14/10 light/dark cycle. B. Mean daily change in constant darkness. The amplitude (right) does not differ between the 3 types of eyes in either light/dark or constant dark. Note that the mean daily changes in darkness are significantly smaller than in light/dark for all types of eyes.

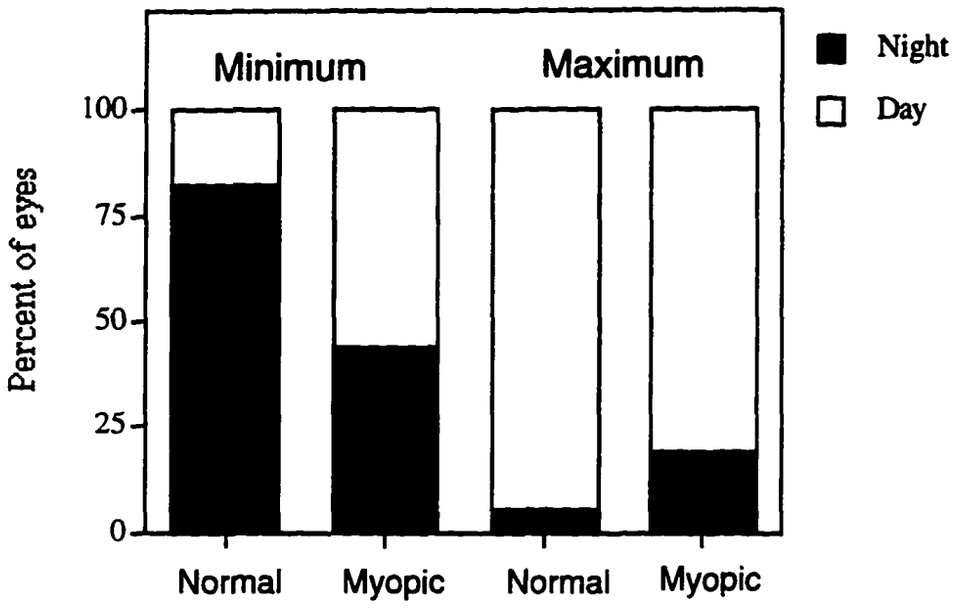
To show the difference in phase between normal and myopic eyes we plot the percentage of eyes having their minimum and maximum IOP during the day or night (figure 5.4a, black bars, night; white bars, day). The minimum IOP occurs during the night in 82% of normal eyes, but in myopic eyes the minimum IOP is almost equally distributed between day and night. In addition, 95% of normal eyes have their peak (maximum) IOP during the day, while relatively fewer myopic eyes do (right pair of bars). The distribution of the minima in IOP for normal versus myopic eyes is significantly different (Chi-square, $p<0.05$).

In summary, the apparent difference in the mean change in IOP as a function of time of day between normal and myopic eyes is due not to a smaller amplitude in myopic eyes, but to the variability in phase across this group. Specifically, in myopic eyes the trough of the pressure rhythm does not consistently occur during the night.

Constant Dark Study

In darkness too, eyes that have been deprived of form vision show a smaller mean daily change in IOP than do normal eyes (figure 5.2, compare dashed lines to solid lines). When both cycles are taken into account, the effect of "time" on pressure does reach significance (one way ANOVA, $F_{4,115}=2.75$, $p=0.03$). (However, a one way ANOVA for the first cycle only shows that "time of day" does not have a significant effect on IOP ($F_{4,55}= 0.62$, $p=0.65$).

A. In Light/Dark



B. In Constant Dark

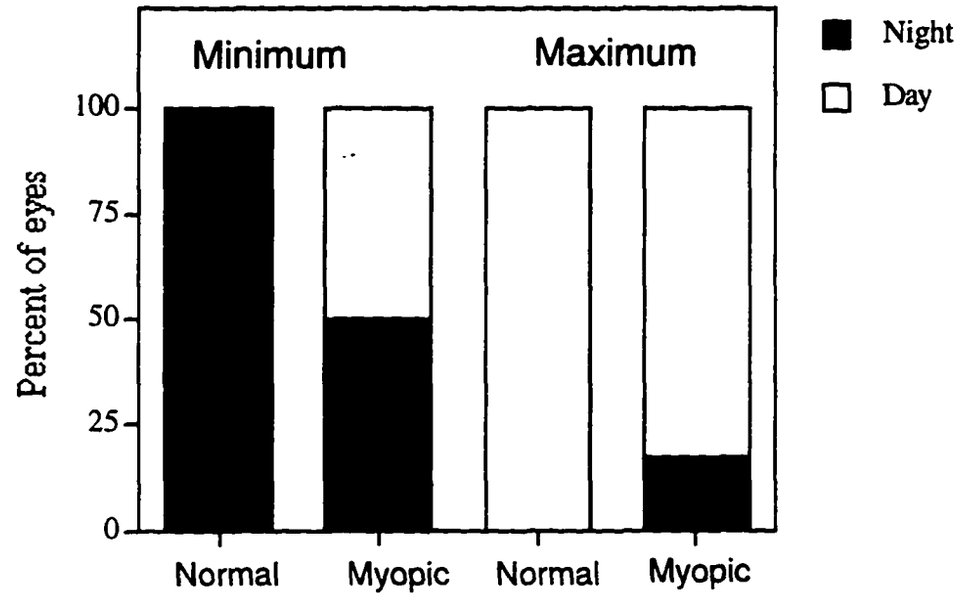


Figure 5.4. Percentage of eyes showing mininum and maximum IOP during the day (white area) and night (black area) for normal (fellow untreated eyes and normal eyes combined) and myopic eyes. A. In a light/dark cycle: 82% of normal eyes show a trough during the night, while only 46% of myopic eyes show a trough at night. B. In constant darkness: All normal eyes show a trough during subjective night while half of the myopic eyes show a trough during the day.

In constant darkness, the IOP changes approximately half as much as in a light/dark cycle; this is so for both measures of daily change (Figure 5.3b, compare to figure 5.3a: normal eyes: day-night= 3.3 vs 7.8, p=0.002; amplitude = 6.1 vs 9.3, p<0.0001; fellow eyes: day-night= 2.2 vs 6.9, p=0.007; amplitude = 5.5 vs 11.8, p<0.0001; myopic eyes: amplitude = 5.5 vs 10.9 mm Hg, p=0.0001). Note that in form-deprived myopic eyes, although the amplitude differs between light/dark and dark, the day-night difference does not.

Finally, as in the light/dark condition, while the mean day-night difference is smaller in myopic eyes than normal eyes (although not significantly so, figure 5.3b, left panel), the amplitude does not differ between these two groups (figure 5.3b, right panel). Therefore, again, it can be concluded that the mean difference in the daily changes between myopic and normal eyes (solid vs dashed lines, figure 5.2) is due to a difference in phase.

To show the difference in phase between normal and myopic eyes in constant dark, we plot the percentage of eyes having their minimum and maximum IOP during the subjective day (time when lights would be on, white bars, figure 5.4b) or subjective night (black bars, figure 5.4b). 100% of the normal eyes have their minimum IOP occurring during the night, while in myopic eyes the minima are evenly distributed between night and day. Similarly, 100% of the normal eyes, but fewer myopic eyes, have their maximum IOP occurring during the day. The distribution of the IOP minima in normal versus myopic eyes is significantly different (Chi-square, p<0.01). Therefore, as in the light/dark condition, the normal and myopic eyes differ with respect to the phase at which the minimum intraocular pressure occurs.

Light results in an increase in IOP in both types of eyes.

We calculated mean day-time IOP and mean night-time IOP by averaging data over all times of day and times of night respectively. These mean values were obtained for both normal and myopic eyes and both the light/dark and constant dark conditions (Table 5.1). When the lights were on ("day" in light/dark), the mean IOP in normal and myopic eyes did not differ. However, in darkness, both during the night in L/D and during subjective day and night in D/D, the mean IOP in myopic eyes was significantly higher than in normal eyes. It follows that light *per se* increases intraocular pressure, and therefore, lights on in the morning results in a further increase in IOP over the endogenous morning rise.

EYE	LIGHT/DARK		CONSTANT DARK	
	Day	Night	Day	Night
Normal	19.8	13.1**	15.8*	12.9**
Myopic	19.8	18.7	16.4	14.8

Table 5.1. Mean intraocular pressure in normal and myopic eyes in light/dark and constant darkness. Normal vs myopic eyes: *=$p<0.05$; **=$p<0.001$.

In summary, the IOP rhythm in myopic eyes differs from normal in not being as well synchronized to the light/dark cycle. Specifically, the trough of the IOP rhythm in myopic eyes occurs at variable times over the 24 hour cycle, while that of normal eyes occurs predominantly during the night. This difference is manifested as a smaller mean time-dependent rhythm in myopic eyes (figures 5.1 and 5.2) while there is no difference in

amplitude of these rhythms between myopic and normal eyes either in a light/dark cycle, or in constant darkness. Finally, light results in an increase in IOP over the endogenous day-time rise which is reflected in the amplitude of the IOP rhythm for normal eyes being smaller in dark than in light/dark.

Changes In Axial Length Attributable To Changes In IOP

Compliance measurements

The eyes of chickens show a diurnal rhythm in axial elongation, elongating more during the day than at night. This is true for both normal eyes (Weiss and Schaeffel, 1993; Nickla and Wallman, 1995) and myopic eyes (Nickla and Wallman, 1995). Because the peaks in the rhythms in IOP and axial length both occur during the day, and the troughs of both rhythms at night, we speculate that diurnal changes in IOP may influence the size of the eye and contribute to the observed rhythm in axial length. To investigate this possibility, we artificially imposed changes in IOP by cannulating the eye, while simultaneously measuring the dimensions of the same eyes using high frequency ultrasonography.

In normal eyes, increases in IOP cause a linear increase in both axial length and vitreous chamber depth (figure 5.5, top, lines next to arrows). Similar relations between these parameters are observed for all 3 cycles although there is hysteresis. In addition, when the imposed IOP is decreased from 35 mm Hg to 10 mm Hg (dashed lines), eye dimensions also decrease but neither axial length nor vitreous chamber return fully to the initial length (mean difference of about 0.2 mm for axial length). The mean compliance in axial length (cornea to sclera, bold line and standard error

bars) is 8.0 um/mm Hg; the value derived for vitreous chamber depth is slightly larger and perhaps due to choroidal thinning at higher pressure.

Myopic eyes showed a similar response in relation to axial length; increases in IOP resulted in increases in axial length, with the converse being true for reductions in IOP and hysteresis again being apparent. A similar value for compliance was also obtained (figure 5.5, bottom left, mean = 6.1 um/mm Hg).

The vitreous chamber of myopic eyes shows a different and apparently paradoxical effect of IOP manipulation (figure 5.5, bottom right). In response to both increasing cycles of IOP, the vitreous chamber *decreased* (solid lines, mean decrease=4 um/mm Hg); and with the decrease from maximum IOP (dashed line) there was an *increase* in length (5.5 um/mm Hg). These changes are in the opposite direction from those described above and also opposite to what might be expected. We hypothesize that this strange result arises from the increase in pressure shifting the position of the lens backward in these eyes (there is little change in the choroid/retina) perhaps because the lens zonules are more flexible. In any event, it is odd that increased pressure in the globe causes the lens to move, as this suggests the presence of a barrier between the anterior and posterior chambers.

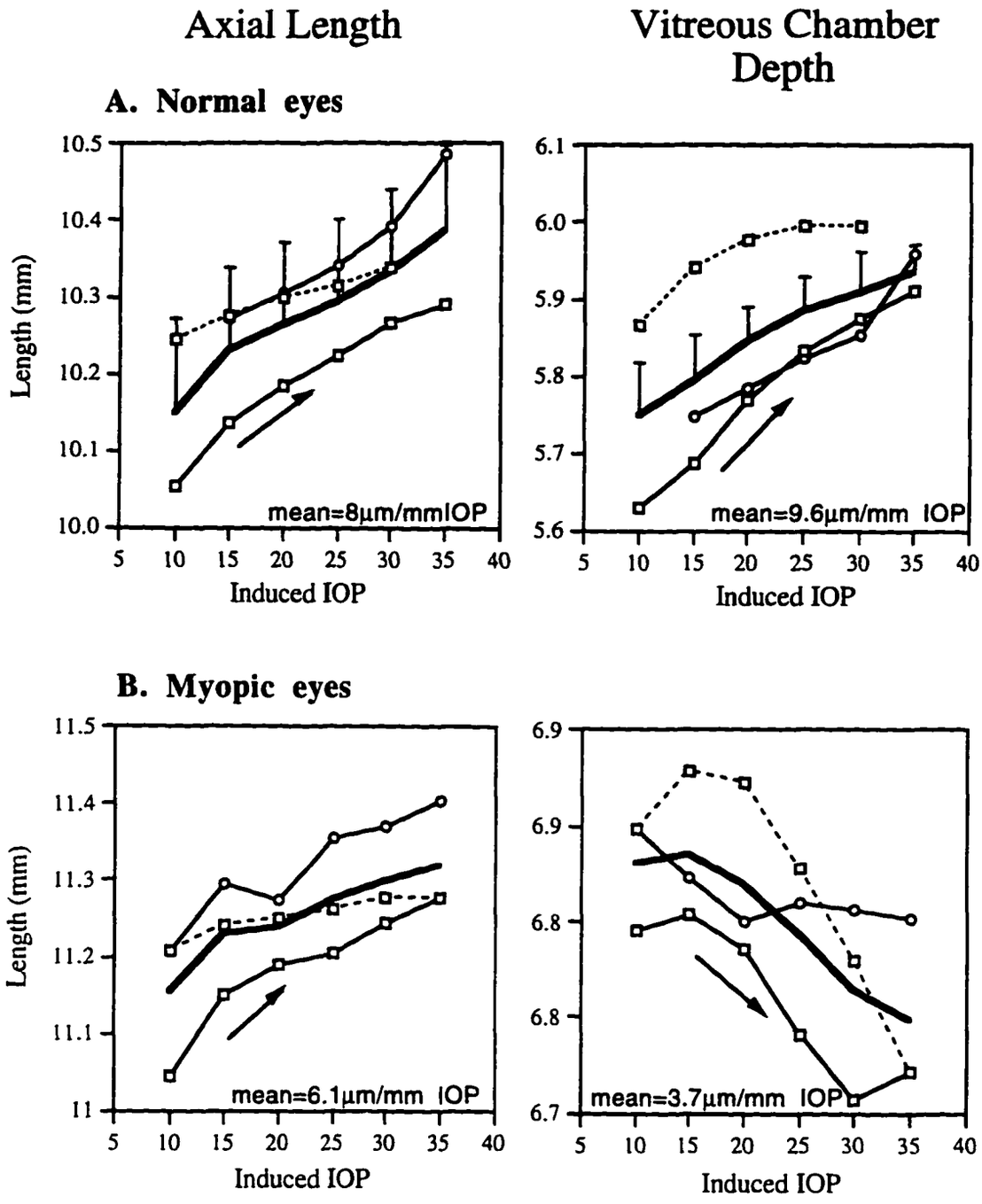


Figure 5.5. Mean compliance in axial length (cornea to sclera, left) and vitreous chamber depth (lens back to retina, right) in normal (top) and myopic (bottom) eyes. All graphs show two ascending (solid lines, first one is near arrow) and one descending run of IOP starting from 10 mm Hg through 35 mm Hg; thick line is the mean (with standard error bars for normal eyes). In normal eyes (n=4, top), the mean compliance for axial length is 8um/mm Hg; for the vitreous chamber it is slightly higher. In myopic eyes (n=4, bottom) the mean compliance for axial length is only 6 um/mm Hg. The vitreous chamber depth of myopic eyes (bottom, right) shows opposite responses (see Results).

Phase relationships between IOP and axial length

To assess phase relationships between the rhythms in IOP and elongation, we measured both variables simultaneously in both eyes of 10 birds (figures 5.6a and 5.6b). For the normal eyes, the mean rhythm in IOP (solid line and symbols, figure 5.6a) is 4 hours in advance of the rhythm in axial length (dashed line and open symbols; t-test of difference from 0, p=0.01). In form-deprived eyes (figure 5.6b) there is no consistent phase relationship between the rhythms because the trough in the IOP rhythm does not always occur at night (the variability is reflected by the non-rhythmicity of the mean data, solid line; see also text in results for figures 5.1 and 5.2), however, the trough for the axial elongation rhythm occurs at about midnight in the majority of these eyes (circles and dashed line). Even when the IOP and axial length measurements are not done on the same eyes, the two rhythms show the same approximate mean phase relationship as the eyes in which the two were simultaneously measured (3 hour difference, figure 5.6c). This graph represents the data from figure 5.1 double plotted in conjunction with the axial length data from 10 normal eyes from another study (figure 3.2a, Chapter 3). The similarity to the data in figure 5.6a indicates that the relationship is quite robust, and that the two rhythms are not exactly coincident.

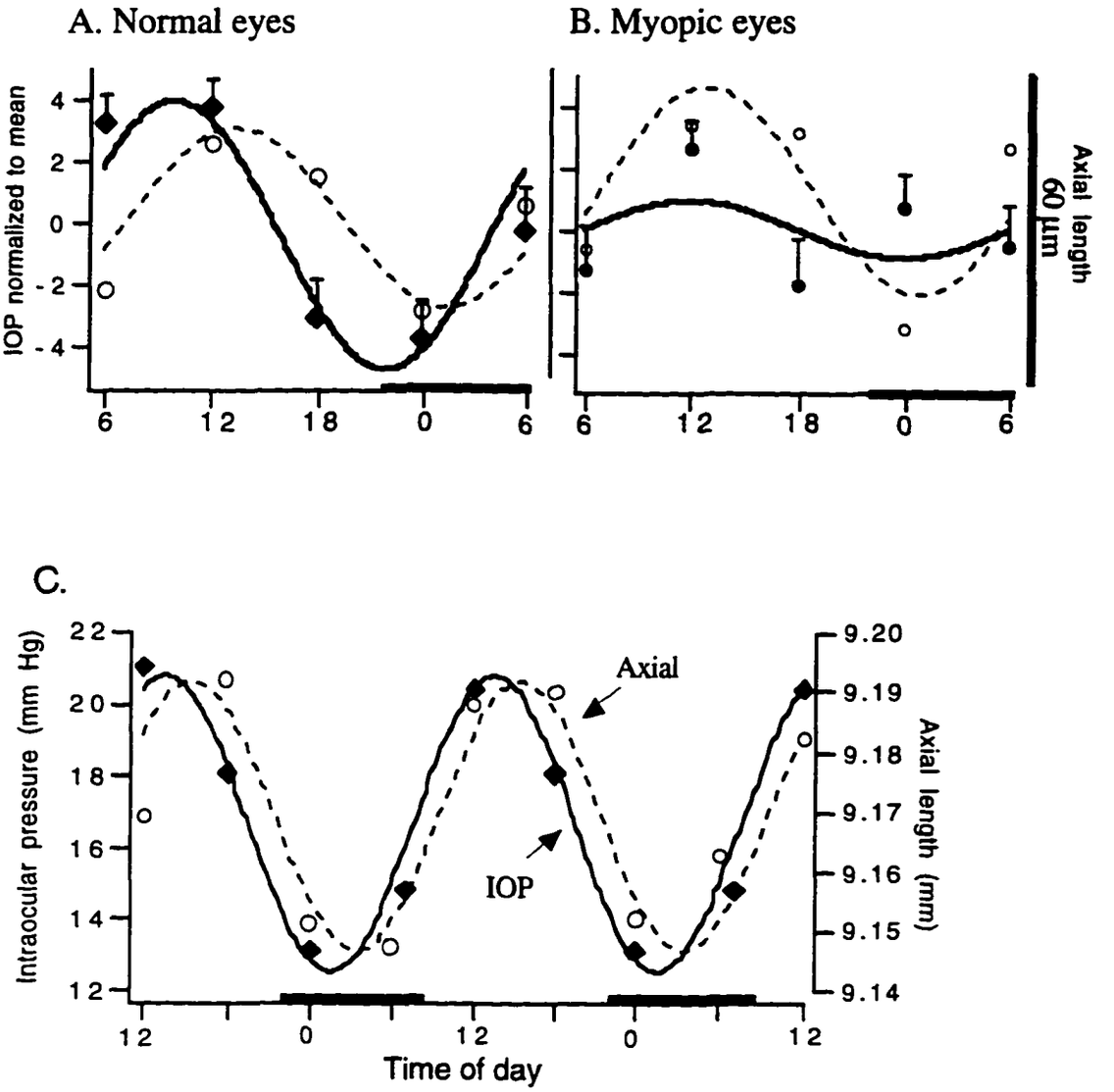


Figure 5.6. Phase relationships between the rhythms in IOP and axial length in normal (A) and form deprived eyes (B) as shown by sine wave fit to mean data. X axis is time of day, black bars denote night. A. Mean IOP (solid symbols) and axial length (open symbols) measured in the same normal eyes (n=10); error bars are standard errors of the mean. The IOP is normalized to the mean for each eye. Axial length is normalized to the linear regression for each eye (see Methods). Waves are sine waves fit to the data (solid lines, IOP; dashed lines, axial length). B. Form deprived eyes (n=10). Conventions same as in A. C. IOP data from figure 1 for normal eyes (solid line) and the sine wave fit to the data for axial length in 10 eyes from another study (dashed lines). In normal eyes, the rhythm in IOP is in advance of the rhythm in axial length by a mean of 3 hours. In myopic eyes three is no consistent phase relationship between the two rhythms.

As an alternative way of analyzing these data, the relationship across individual eyes between the difference in phase between the rhythms in IOP and axial elongation and the rate of elongation was examined. The rate of elongation (increase in length per 24 hours) was obtained by calculating the slope of the linear regression line fit to the data for each individual eye. The phase was obtained by fitting a sine wave to the individual data for both IOP and the "cyclic component" of the data for axial length (see Methods); the temporal spacing of the peaks of these two rhythms provides an estimate of the phase difference between them. Figure 5.7 shows a scatter plot of the elongation rate as a function of the difference in phase, based on the data of 9 normal and 8 myopic eyes (3 eyes having a 0 elongation rate were excluded). We find a significant positive correlation between the difference in phase between the rhythms in IOP and axial elongation and the rate of elongation of the eye ($r=0.57$, $p<0.05$).

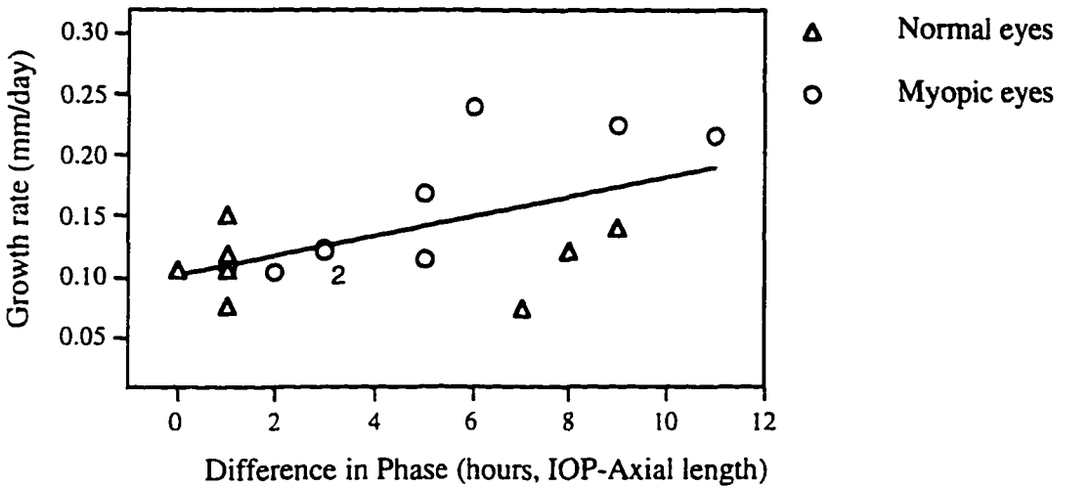


Figure 5.7. Rate of axial elongation (slope of the linear regression fit to data for axial length) as a function of the difference in phase between the rhythms in IOP and axial length in 17 eyes (3 eyes having 0 growth rate are excluded). The greater the phase difference between the two rhythms, the higher the rate of elongation (correlation: $r=0.57$, $p<0.05$). The mean rate of elongation for normal eyes is 0.11 mm/day; for myopic eyes it is 0.16 mm/day.

Discussion

We here report six main findings: (1) the eyes of chickens exhibit a diurnal rhythm in intraocular pressure, with IOP high during the day and low during the night, (2) this rhythm persists in constant darkness, albeit with a reduced amplitude, and therefore has a circadian component, (3) eyes that are deprived of form vision also show diurnal fluctuations, however, the phase is more variable than in normal eyes (i.e they are not as tightly synchronized to the light/dark cycle), (4) the amplitude of the daily rhythm in IOP could produce a large portion of the rhythmic changes in axial length seen *in vivo*, (5) in normal eyes the mean phase of the rhythm in IOP is approximately temporally coincident with that of the rhythm in axial length while in form-deprived eyes there is no consistent relationship, and finally (6) the overall difference between the phases of the rhythms in IOP and axial length predict the rate of growth.

Myopia and Intraocular Pressure

Intraocular pressure exhibits a diurnal rhythm in several species, including humans. This pressure is maintained via a continuous secretion of aqueous humour from the ciliary epithelium and drainage through the Canal of Schlemm and the uveoscleral pathway; changes in the rate of secretion and the resistance to drainage govern the diurnal changes observed. A major function of this system is the maintenance of the shape of the globe. In fact, it has long been thought that intraocular pressure is crucial in the normal development of the size of the eye. Along the same lines, high intraocular pressure has been associated with enlarged myopic eyes.

The idea that intraocular pressure plays a role in the growth of the eye has been around since 1956, when Coulombre showed that lowering IOP by cannulating the eye of chicken embryos resulted in a smaller size eye (Coulombre, 1956). More recently, a similar study on chick embryos by Neath et al. (1991) concluded that from day E4 to E10 the growth of the cornea and the eye is dependent on the maintenance of intraocular pressure. More compelling evidence for an association between IOP and eye size are the claims that in humans, abnormally high levels of intraocular pressure lead to myopia, a notion that has been propounded in the clinical literature for years (reviews: Curtin, 1985; Pruett, 1988). However, definitive evidence is lacking. In animal models of myopia, for instance, there is no evidence linking high intraocular pressure with myopic eyes; in light-induced bupthalmous in chickens, for example, the increase in IOP developed much later than the development of axial enlargement (Lauber et al., 1961; Lauber et al., 1970). Also in chickens, Wilkinson and Hodos (1991) found that the IOP in form-deprived myopic eyes was not significantly higher than in normal eyes (in fact, they found that is was slightly lower). Similarly, we find that the mean daytime intraocular pressure of normal and myopic eyes is the same, and that the mean higher IOP in myopic eyes at night is a consequence of the shifts in phase of the trough of the rhythm.

Associated with the notion of a role for IOP in myopiagenesis is the hypothesis that the sclera of eyes predisposed to myopia is weaker, or more "stretchy", than normal. Our data do not support this hypothesis; we find that there is little difference in compliance between the two types of eyes (in fact, the compliance may be lower in myopic scleras). In summary, there is as of yet no conclusive evidence indicating a causal relationship between

either high intraocular pressure or a more compliant sclera and myopia either in humans or animal models. None of these data, however, preclude the possibility that changes in intraocular pressure effects changes in the length of the eye. Many species show diurnal rhythms in intraocular pressure; we extend the list to include the chicken, which shows a robust circadian rhythm in IOP with the peak occurring during the day, coincident with the time of greatest ocular elongation. We speculate that the rhythm in IOP influences the growth of the eye, and that its desynchronization in form-deprived eyes may be causally related to the abnormal growth in these eyes.

Intraocular Pressure and Ocular Growth

Recently, a rhythm in elongation has been described in the developing chick eye. By measuring the length of the eye at 12 hour intervals, Weiss and Schaeffel (1993) showed that ocular elongation in chickens is diurnally rhythmic, increasing only during the day. The oscillations in axial length also occur in adult birds (Schmid et al., 1995). By measuring the dimensions of the various ocular components at 6 hour intervals, we found that the greatest amount of axial elongation occurs in the morning, between 6 am and noon, and that the eye shrinks in the evening, between 6 pm and midnight. The length oscillates by approximately 35 μm over the 24 hour daily period, in addition to the continuous non-rhythmic growth of about 70 μm (Nickla and Wallman, 1995). In contrast to Weiss and Schaeffel, we found that form-deprived eyes also showed a rhythm in elongation similar to that in normal eyes.

We propose that the diurnal fluctuations in IOP contribute to the diurnal fluctuations in length by means of influencing the underlying scleral

growth. We find that the amplitude of the diurnal rhythm in intraocular pressure is approximately 8 mm Hg and the compliance of the normal eye is approximately 8 um/mm Hg. Hence, the daily changes in IOP would cause a change in length of approximately 64 μm, suggesting that the rhythm in IOP is a major component of the rhythm in axial length. Furthermore, the changes in IOP approximately coincide with the changes we see in axial length, specifically, the elongation of the eye is maximal in the early morning hours when the IOP is increasing, and declines in the evening, when IOP is decreasing (figure 5.6). The oscillations in axial length are not, however, solely due to changes in IOP superimposed on a linear, non-rhythmic growth. Our evidence suggests that there are separate rhythms of IOP and eye growth that interact with one another.

The evidence that the intraocular pressure rhythm is not the sole determinant of the diurnal fluctuations in length is several fold. Eye growth in chickens has been shown to be correlated with the synthesis of matrix proteoglycans in the sclera. Scleras of eyes elongating faster than normal (form-deprived eyes) increase their rate of proteoglycan synthesis (Rada et al., 1991); conversely, scleras of eyes growing slower than normal (recovering eyes) decrease their rate of proteoglycan synthesis (Nickla et al., 1992; Rada et al., 1992). We find that scleral proteoglycan synthesis exhibits a diurnal rhythm *in vitro*, with greater synthesis during the day than during the night. This rhythm persists *in vitro* for at least 3 cycles, implying that it is an endogenous, circadian rhythm (Nickla and Wallman, 1995; dissertation), the expression of which is not dependent on fluctuations in IOP. Furthermore, we know that the rhythms in IOP and axial length are not in phase, and show a lot of variability (figure 5.7). If it were so that the IOP rhythm were the sole determinant of the fluctuations in length, then the

two would by necessity be in phase. We conclude that the rhythm in axial length is not due to changes in IOP superimposed on linear growth. We propose instead, that the rhythm in proteoglycan synthesis underlies the rhythmic changes in axial length, and that the rate of synthesis is determined by the phase relationships between the rhythms in IOP and scleral synthesis.

Support for the hypothesis that IOP influences synthesis of matrix molecules in the sclera comes from studies showing that growth of cartilage and bone is influenced by mechanical force, both *in vivo* and *in vitro*. For example, both tensile and compressive forces applied to cartilage in culture result in increases in DNA and proteoglycan synthesis, with the direction of the change (increase or decrease) determined by whether the force is static or cyclic. In general, cyclic forces increase matrix production while non-cyclic forces reduce it or produce no change[1] (van Kampen et al., 1985; van Kampen and van de Stadt, 1987; Burger and Veldhuizen, 1993). Amazingly, it has been shown that brief pulses of hydrostatic pressure of physiological magnitude for intraocular pressure (approximately 15 mm Hg) applied once every 27 hours cause an approximate 50% increase in proteoglycan synthesis by chondrocytes *in vitro* (Takano-Yamamoto et al., 1991). Therefore, two of the inherent characteristics of intraocular pressure, that it exerts a force on the ocular tunic, and that this force is by its nature, cyclic, are qualities that have been shown to influence growth in cartilage in other systems.

We hypothesize that the rhythm in IOP exerts a mechanical effect (stretch or force) that influences the rate of scleral proteoglycan synthesis, which, in turn, influences the rhythm in elongation. It is known that the

[1] It should be noted that the frequency of the cyclic forces used in most of these *in vitro* studies is much higher (on the order of one cycle every 3 seconds) than the diurnal frequency (1 cycle per day) of the intraocular pressure rhythm.

synthesis of matrix proteoglycans by chondrocytes shows a circadian rhythm. This has been found both *in vivo* as well as *in vitro* (reviewed in Simmons, 1992). Furthermore, the synthesis of DNA and cell division shows a diurnal rhythm. These three cell cycle events show specific phase relationships to one other, such that DNA synthesis occurs earliest, followed by proteoglycan synthesis and finally, cell division (Simmons, 1968). Because the events involved in the cell cycle and growth of the tissue are cyclic, it is likely that the factors regulating growth are themselves also cyclic. It has in fact been demonstrated that cyclic changes in force results in increases in proteoglycan synthesis, and presumably growth. We thus propose that the cyclic fluctuations in IOP regulate the synthesis of matrix molecules by exerting cyclic force on the sclera and furthermore, that the phase of the cell cycle rhythms during which changes in IOP occur is crucial in determining the rate of matrix synthesis.

In normal eyes, the morning increase in IOP is coincident with the peak in the endogenous rhythm in scleral PG synthesis; in form-deprived myopic eyes, however, the two rhythms do not usually coincide. This suggests that normal growth regulation requires the synchronization between the rhythms in IOP and in scleral proteoglycan synthesis. We hypothesize that normal growth requires high (or increasing) IOP during the phase at which PG synthesis is also increasing, and that abnormal growth occurs if the peak in IOP occurs at other times in the cell cycle. We provide evidence, in fact, that the rate of elongation (growth) of the eye is dependent on the degree of synchrony between the rhythms in IOP and elongation (figure 5.7). There is a significant positive correlation between the rate of elongation (growth) and the difference in phase between the IOP and elongation rhythms such that the greater the difference in phase, the

higher the rate of growth. If we make the assumption that the elongation rhythm largely reflects the rhythm in proteoglycan synthesis, this correlation constitutes direct support for the hypothesis that the degree of phase synchronization determines the rate of ocular elongation. More support for this hypothesis comes from studies showing that visual manipulations that decrease ocular elongation (removal of the diffuser and positive spectacle lenses) are associated with large phase shifts in some ocular rhythms. Specifically, we find that the rhythms in elongation and choroidal thickening reverse in relation to one another compared to normal and myopic eyes so that rather than being approximately 180 degrees out of phase with one another, these rhythms shift to become in phase (Nickla et al., 1996).

It is possible that the requirement for the coincidence between the rhythms in IOP and scleral PG synthesis is really the result of changes in the efficacy of IOP on the sclera as a result of increased scleral compliance, for instance. In this case, what would be crucial in determining the force exerted on the chondrocytes would be not the increase in pressure *per se*, but the compliance of the eye at the time of the increase in IOP: a more compliant eye (i.e. a "stretchier" one) would have less force exerted on it, and hence result in less growth. In normal eyes, this increased compliance might occur at around dawn concomitant with the increase in IOP, resulting in a lower rate of PG synthesis (and growth) than if the two were not coincident as in myopic eyes.

We conclude that the growth of the chick eye is influenced by diurnal changes in intraocular pressure and that the regulation of growth depends on the rhythm in IOP being synchronized to the light/dark cycle (and hence, to another diurnal ocular rhythm). We propose that the abnormal

elongation concomitant with form deprivation is not the result either of abnormally high levels of IOP, nor of increased scleral compliance but instead results from the de-synchronization of the IOP rhythm with respect to the light/dark cycle. It is likely that the phase relationships between the rhythms in IOP and various ocular rhythms largely determine the rate of growth of the eye.

CHAPTER 6

General Discussion

Form Deprivation and Altered Ocular Rhythms

Several lines of evidence arising from studies of the effects of visual manipulations on ocular growth have suggested that ocular diurnal rhythms are involved in the regulation of the growth of the eye. First, the eyes of chicks show a diurnal rhythm in the rate of elongation, increasing more during the day than during the night (Weiss and Schaeffel, 1993). Second, it has been shown that the absence of a photoperiod, i.e. constant light and constant dark, results in excessive axial elongation in chickens (Gottlieb et al., 1987; Gottlieb et al., 1991; Lauber and Kinnear, 1979; Lauber et al., 1961), and that constant light results in the loss of the rhythm in elongation (Bartmann et al., 1994). Third, we found that various visual manipulations that reduce the amount of form deprivation myopia by decreasing eye growth (such as strobe stimulation and brief periods of vision) are more effective when given during the night as opposed to during the day (Chapter 2). Finally, in lid-sutured chick and monkey eyes the daytime rise in retinal dopamine was reduced compared to normal (Iuvone et al., 1989; Stone et al., 1989), suggesting that the abnormal axial elongation is a consequence of a dampened rhythm in retinal dopamine.

What role do ocular rhythms play in the regulation of the growth of the eye? The aim of this dissertation was to examine several ocular rhythms that might be related to growth in normal eyes, as well as in eyes growing excessively fast (form-deprived eyes) and eyes whose growth had slowed

(eyes recovering from myopia). We found that both form deprivation and myopic defocus (removing the diffuser from the deprived eye) results not in arhythmicity, but in shifts in phase in the rhythms in elongation and choroidal thickness. Furthermore, form deprivation caused a desynchronization in the rhythm in intraocular pressure (IOP). We argue that the phase relationships between the rhythms in intraocular pressure, choroidal thickness and scleral proteoglycan synthesis are of crucial importance in determining the rate of growth of the eye, and that altering these phase relationships (by various visual manipulations) alters the rate of growth.

Four growth-related ocular rhythms

In this dissertation I demonstrated four independent diurnal ocular rhythms that are associated with ocular growth: axial elongation, choroidal thickness, scleral proteoglycan synthesis and intraocular pressure. The rhythms in axial length and choroid thickness show consistent phase shifts depending on whether the growth is abnormally fast, or abnormally slow. In normal eyes, the choroidal and axial length rhythms are about 8 hours out of phase. In form-deprived eyes, the choroidal and axial length rhythms are 12 hours out of phase as a result of a phase advance for the axial length rhythm and a delay for the choroidal rhythm. In recovering eyes, these two rhythms are exactly in phase with one another as a result of a phase delay in the axial length rhythm and an advance in the choroidal rhythm relative to normal eyes. Hence, the phases of the rhythms in axial length and choroid thickness move in opposite directions from normal in response to visual manipulations that produce opposite effects on the rate of eye growth. Because these rhythms are retained in form-deprived and

recovering eyes, but are phase-shifted with respect to one another, they appear to constitute independent diurnal rhythms.

There is also a rhythm in proteoglycan synthesis in the scleras of normal and form-deprived eyes: synthesis is higher during the day than during the night. This rhythm is presumably one component of the rhythm in axial elongation. The rhythm is retained in culture over several cycles, which indicates that it is a circadian rhythm, and that it too, is independent of exogenous factors.

Finally, there is a rhythm in intraocular pressure: In normal eyes, intraocular pressure is synchronized to the diurnal cycle of light and dark, and is high during the day and low at night. In form-deprived eyes, this rhythm is no longer synchronized to the light/dark cycle.

Rhythm interactions

Which of the interactions among these four rhythms are important in controlling ocular growth? We propose that the phase relationships between all of these rhythms determine the rate of ocular growth. Specifically, we argue first, that the synthesis of scleral extracellular matrix, as reflected by proteoglycan synthesis, is an important correlate of ocular elongation. We find that the daily changes in proteoglycan synthesis are correlated with the daily changes in growth rate: proteoglycan synthesis is highest in the morning when the rate of elongation is highest, and lower at night when the rate of elongation is lowest. Second, we propose that the rate of proteoglycan synthesis (and consequently, the rate of ocular elongation) is stimulated by increases in intraocular pressure, especially at certain times of day. Finally, we propose that the rate of proteoglycan

synthesis is inhibited by daily increases in choroidal thickness, again in a phase-dependent manner.

Rhythms in intraocular pressure in relation to ocular elongation and proteoglycan synthesis

There is a rhythm in intraocular pressure in the normal chick eye that is approximately coincident with the rhythm in elongation (and proteoglycan synthesis): IOP is highest during the day when elongation (and PG synthesis) is greatest, and lowest at night when elongation (and synthesis) is lowest (Nickla and Wallman, 1995a). This rhythm continues in constant dark, with a reduced amplitude. In form-deprived eyes, however, the rhythm is no longer synchronized to the light/dark cycle (nor to the rhythm in proteoglycan synthesis) (see figure 6.1).

The evidence supporting a phase-dependent influence of intraocular pressure on the rate of ocular growth is that there is a significant correlation between the phase angles of the rhythms in IOP and axial elongation and the rate of ocular growth: the closer in phase the two rhythms are, the lower the rate of ocular growth and vice versa. Hence, normal growth requires an approximate temporal coincidence between the rhythms in IOP and in proteoglycan synthesis; this condition restrains growth. When the rhythms are not aligned (as in form-deprived eyes) there is excessive growth.

Form vision as *Zeitgeber* for the circadian rhythm in IOP

One of the more interesting implications of the result that form deprivation causes a desynchronization of the rhythm in IOP is the underlying assumption that form vision can act as a *Zeitgeber* for ocular

rhythms. In the circadian literature, the effects of form vision have not been dissociated from the effects of light in rhythm entrainment. Constant conditions of light or dark affect diurnal rhythms by virtue of the absence of a time cue, or *Zeitgeber*. Because plastic diffusers reduce the transmittance of light by only about 0.6 log units, the change in light intensity at the retina is unlikely to be significant in terms of cueing the clock. If the absence of form vision does cause a desynchronization in ocular rhythms, it follows that visual attributes such as spatial or temporal transients, in addition to light *per se*, play a role in the entrainment of rhythms; that is, are *Zeitgebers*. Form deprivation could then be thought of as a condition in which the *Zeitgeber* might not be strong enough to synchronize the rhythms. Because a primary function of the retina is to discern patterns, and because the ocular clock is believed to reside in the retina, it is not implausible that form vision is as important as light in the circadian response.

If form vision is a *Zeitgeber*, it almost certainly acts in conjunction with light. The finding that the amplitude of the rhythm in IOP is reduced in constant darkness due to a reduction in the IOP during subjective day, suggests that light performs two functions: one, as a *Zeitgeber*, and two, to increase the IOP. In fact, this dual role for light/dark, both as *Zeitgebers* and to increase a rhythm amplitude, is seen in the retinal rhythm in melatonin synthesis (Besharse and Iuvone, 1983) and in retinomotor movements. We speculate that light is responsible for the daily increase in IOP, and that vision is responsible for the entrainment to the light/dark cycle. In the absence of light, the amplitude decreases. In the absence of vision, but not of light, the amplitude is unaffected but the rhythms become desynchronized.

Possible mechanism for the effect of IOP on proteoglycan synthesis

We propose that the daily increases in IOP increase proteoglycan synthesis, the extent of which is dependent on the time of day (more specifically, the time in the cell cycle of the chondrocytes in the sclera). We suggest that increases in IOP stimulate scleral proteoglycan synthesis by exerting a force on the cartilaginous sclera. Support for this notion comes from the large body of literature showing that cyclic oscillations of compressive force increase the synthesis of proteins, proteoglycans and DNA in cartilage (reviewed in: van Kampen et al., 1985; van Kampen et al., 1987). Even brief oscillations in force given only once a day caused significant increases in proteoglycan synthesis. The cyclic nature of intraocular pressure, and the fact that it exerts a compressive force on the sclera, support the hypothesis that diurnal changes in intraocular pressure influence the rate of scleral proteoglycan synthesis. In addition, it is known that in cartilage, macromolecular synthesis and cell division show diurnal rhythms, the peaks of which occur in a specific temporal sequence (Simmons, 1968). In view of this, it is very likely that the efficacy of physiological factors (either force or humoral) in influencing growth parameters would be dependent on the time in the cycle at which they occur.

Rhythms in choroidal thickness in relation to ocular elongation and proteoglycan synthesis

The phase of the rhythm in choroidal thickness in normal eyes is approximately opposite that of the rhythm in ocular elongation, increasing in thickness when elongation is decreasing and vice versa. This is also true

in form-deprived eyes. In recovering eyes, however, the two rhythms are in phase.

The evidence in support of a phase-dependent influence of the choroidal thickness rhythm on the rate of ocular growth is the correspondence between the phase angles of the rhythms in choroid thickness and axial elongation and the rate of ocular growth (see figure 6.1). Specifically, we find that "intermediate" rates of growth occur when the choroidal and axial length rhythms are approximately 8 hours apart (as in normal eyes). Abnormally fast growth occurs when these rhythms are exactly anti-phase to one another (12 hours, as in form-deprived eyes). Slower growth occurs when the two rhythms are in phase (0 hours, as in recovering eyes). We argue that "normal" growth occurs when the choroidal thickness peaks at a specific time in the cellular cycle of macromolecular synthesis (proteoglycans, DNA), and that shifts in the phases of these rhythms in relation to one another result in either higher or lower rates of growth.

Possible mechanism for the effect of the choroid on proteoglycan synthesis

We propose that the choroid may be the intermediary between the retinal defocus signal and the inhibition of ocular elongation. First, the amount of choroidal thickening is determined by the refractive error, so that myopic defocus results in choroidal thickening, and hyperopic defocus in choroidal thinning (Wallman et al., 1995). Furthermore, lens-induced myopic defocus (and thicker choroids) leads to a decrease in ocular elongation, and hyperopic defocus (and thinner choroids) leads to an increase in ocular elongation; the reverse occurs upon removing the lenses (Wildsoet and Wallman, 1995). In this way the degree of choroidal

thickening can respond to the amount of defocus and determine the amount of inhibition required to slow scleral growth in order for the eye to achieve emmetropia. We propose that the choroidal effects on ocular growth occur over a matter of hours, rather than days (as previously supposed), and reflect a mechanism for the daily fine-tuning of ocular growth during normal emmetropization. Further evidence that the thickness of the choroid influences scleral (and hence, ocular) growth comes from *in vitro* studies in which choroids from either recovering or normal eyes were co-cultured with scleras; we found that thicker choroids from recovering eyes resulted in an inhibition in scleral proteoglycan synthesis compared to thinner choroids from normal or myopic eyes.

Conclusions

In conclusion, we propose that the rhythmic fluctuations in axial length are the end result of interactions between other diurnal ocular rhythms related to growth: these include (but are not limited to) rhythms in choroidal thickness, in scleral proteoglycan synthesis, and in intraocular pressure. We find that the deprivation-induced alterations in these rhythms do not entail a loss of rhythmicity, as has been postulated by others: Form-deprived eyes do not show primary arhythmicity in any one of the four major rhythms examined in this dissertation, but do show a desynchronization of the rhythm in IOP with the light/dark cycle, and shifts in phase of the rhythms in choroid thickness and axial length. We propose that these shifts in phase result in changes in the rate of proteoglycan synthesis and ocular growth, and underlie the difference between normal and form-deprived eyes.

In recovering eyes, the rhythms in choroid thickness and axial length shift in the opposite direction of the phase shifts in myopic eyes relative to normal. We propose that the fine-tuning of the growth of the eye requires visual input: the lack of form vision results in a desynchronization of the IOP rhythm with respect to other ocular rhythms and leads to excessive elongation. When vision is restored to form-deprived eyes, the large shifts in phase of the choroidal rhythm with respect to the axial length (and presumably, proteoglycan synthesis) rhythm results in the required decrease in growth. It is plausible that the more subtle regulatory mechanisms that underlie the compensation to lens-induced defocus might also be dependent on phase-shifts in ocular rhythms.

We conclude that the endogenous rhythm in proteoglycan synthesis is the "final common pathway" in the regulation of the rate of ocular elongation, and that the rate of proteoglycan synthesis (and hence ocular growth) is determined by the various phase relationships between itself and the other ocular rhythms. The elucidation of the mechanisms by which ocular rhythms influence ocular growth opens up new areas for research. It is imperative that future studies on the control of ocular growth and emmetropization take into account the influences of these, and possibly other, diurnal ocular rhythms.

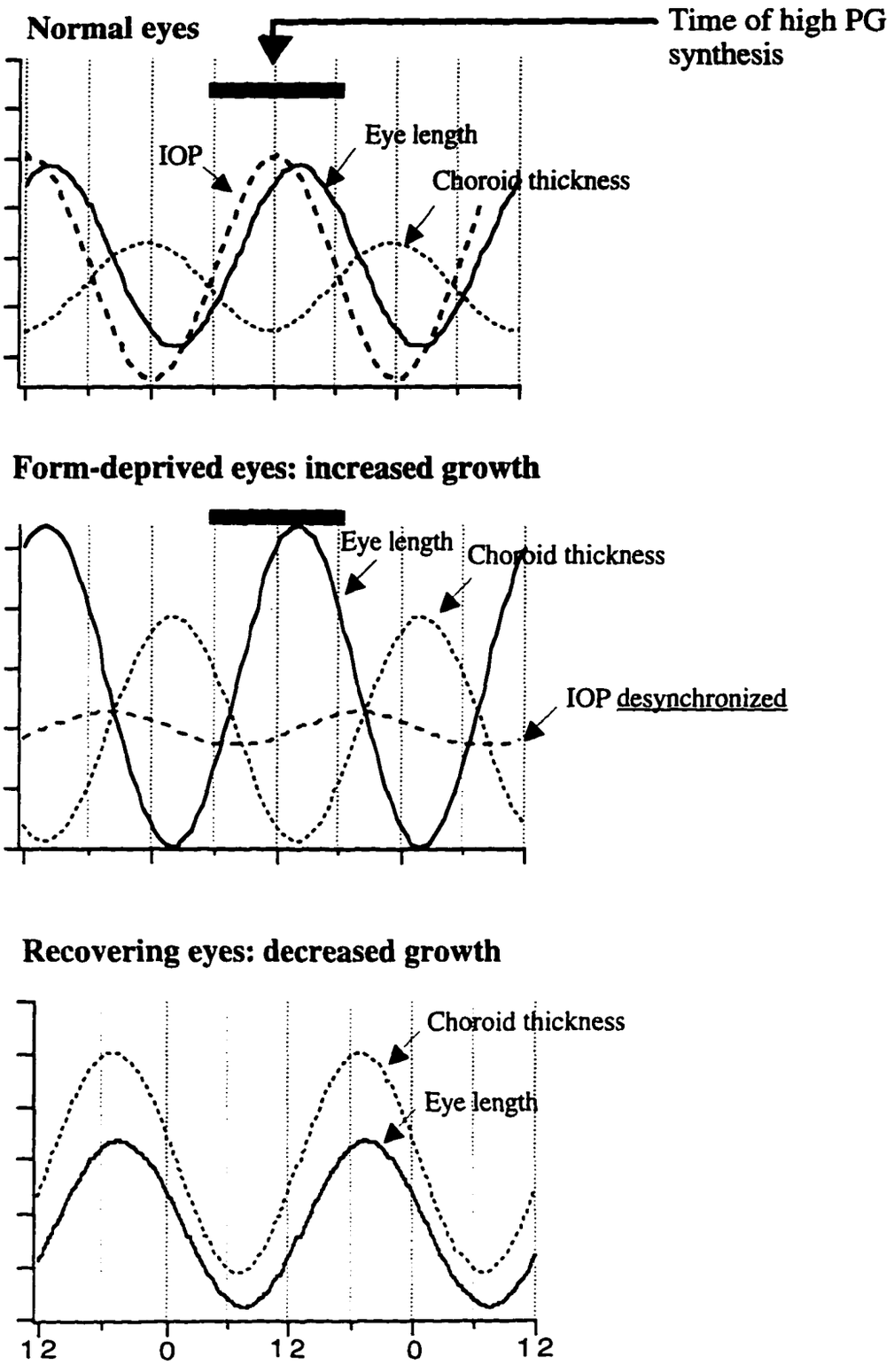


Figure 6.1. Phase relationships between rhythms in 3 types of eyes.

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