Effects of ML-7 on the Actomyosin Networks of the Avian Crystalline Lens

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Assemblies of contractile proteins such as actin, myosin and myosin light chain kinase (MLCK) are found on the posterior surface of the lens fibre cells. The mechanical properties of chicken lenses, as well as their focal lengths, were found to be affected by the disruption of the network using inhibitors such as 1-(5-Iodonaphthalene-1sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-7). The purpose of this study is to determine if ML-7, a MLCK inhibitor influences actomyosin organization in the chicken lens, which may have lead to the reported changes in stiffness and focal lengths. The Nearest Neighbour Distance (NND) values will be used to test the following hypotheses: ML-7-treated lenses have further NNDs compared to their vehicle counterparts, and that the NNDs of the 100µM-treated lenses would be lower than the lenses treated with lower concentrations (1µM and 10µM) of ML-7.

Eyes of 7-day old white leghorn chickens (gallus gallus domesticus) were obtained. The anterior segment of one eye is treated with 1µM, 10µM, or 100µM of ML-7, and the other, with vehicle. The lenses were stained for actin and myosin. The NNDs for actin-actin, actin-myosin, myosin-actin and myosin-myosin were determined from confocal images of the networks to quantify the network distribution.

Myosin-myosin NNDs of 10µM-treated lenses showed significantly lower values com-

pared to 100µM-treated lenses. However, it is uncertain whether the significant change observed was indeed due to ML-7 activity or due to variation between birds. Additionally, only myosin-actin NNDs showed a significant reduction in treated lenses. These results suggest that actomyosin interactions affected by ML-7 may have been too subtle to detect, or compensation by other kinases occurred. Thus, this study was unable to determine if the stiffness and focal length changes in ML-7-treated lenses observed in previous studies were related to changes that ML-7 imparts on the actomyosin networks.

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I can write a novel for this part alone, but then this thesis would never be completed, so I'll try to keep it short.

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Dedication

For my Oma, Truli. Until we meet again.

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List of Abbreviations

ADP adenosine diphosphate

ATP adenosine triphosphate

BMC basal membrane complex

EtOH ethanol

F-actin filamentous actin

G-actin globular actin

MLCK myosin light chain kinase

NMII non-muscle myosin II

NND nearest neighbour distances

PBS phosphate buffered saline

PFA paraformaldehyde

RM ANOVA repeated measures analysis of variance

SAB sequestered actin bundles

SD standard deviation

TS Tyrode's solution

Chapter 1

Introduction

Human lenses undergo a steepening of curvature to bring near objects into focus through a process called accommodation [1]. The change in the lens' shape is attributed to the contraction of a smooth muscle ring attached to the lens known as the ciliary muscle [1]. However, whether the lens itself plays an active role in changing its shape is currently uncertain. With age, gradual loss of accommodative ability occurs, eventually leading to the inability to focus on nearby objects, a condition known as presbyopia [2]. It has been suggested that increased lens rigidity and lifelong lens growth are contributors to presbyopia [2]. Chickens also experience age-related loss of accommodative amplitude [3]. While there are differences in the accommodative mechanism of human and chickens, they both deform their lenses to accommodate, making them an appropriate model for presbyopia studies

[4].

Contractile proteins such as actin and non-muscle myosin II (NMII) are found in many eukaryotic cells, sometimes contributing to cell mechanical properties [5, 6]. For example, actin-NMII interactions contribute to the shape and deformability of mammalian red blood cells [6]. This is also the case for endothelial and epithelial cells of other animals [7, 8]. Within the lens, actin assembly and disassembly seemingly affects the mouse lens mechanics [9]. In the chicken lens fibre cells, these proteins are found arranged in hexagonal arrays on the posterior face [10]. While the actomyosin network may interact with other proteins on the posterior face of the lens fibre cells to attach to lens capsule and assist with cell migration, they may also play a role in lenticular biomechanics, similar to red blood cells, endothelial and other epithelial cells [6–8, 10–12]. After all, fibre cells arise from the lens' anterior epithelium. Decreases in the focal lengths of chicken lenses were observed when treated with a low concentration of a myosin light chain kinase (MLCK) inhibitor called ML-7 [12]. Moreover, the pharmacological disruption of the network with the same inhibitor decreased overall lens stiffness at low concentrations, which may have possible consequences on accommodative amplitudes [11].

The purpose of this study is to determine if ML-7 affects the distribution of the actomyosin networks. The 2015 study by Won et al. also showed that there is a relationship between the distribution of the actomyosin networks and lens stiffness by examining the net-

work's uniformity [11]. However, some changes in distances between actin and myosin may not alter the uniformity of the network, which is why this study will compare the nearest neighbour distances (NNDs) of actin-actin, actin-myosin, myosin-actin and myosin-myosin in vehicle and ML-7-treated lenses. These distance measurements will be used to test the hypothesis that ML-7-treated lenses would have higher NNDs compared to their vehicle counterparts, as ML-7 should hinder actin-myosin interactions. As contractions generated by the actomyosin systems are associated with tension, and possibly rounding of the lens, the second hypothesis tested in this study is that the NNDs of lenses treated with 100µM of ML-7 would be lower than lenses treated with the lower concentrations (1 µM and 10 µM) of ML-7.

Chapter 2

Literature Review

2.1 The Chicken Eye

Animals with anatomical and physiological similarities to humans have provided insight to numerous research questions in vision science. The chicken is a model often used in ocular research for some similarities to the human eye, in addition to its availability, low cost and ease of handling [4, 13]. Chickens are diurnal, visual organisms like humans [13]. Their use of vision is reflected by the large size of their eyes – a characteristic shared by aves in comparison to other vertebrate eyes [13, 14]. Similar to other vertebrates, the chicken eye is a sphere composed of three layers of tissue (also called tunics) and three chambers [14–16]. The three tunics are as follows:

1. The outer fibrous tunic consisting of the sclera and cornea.

2. The middle vascular tunic (also called the uvea), is composed of the iris, ciliary body and the choroid.

3. The internal sensory tunic is the retina [1, 15].

The anatomy and function of the structures within the chicken eye have similarities to the human eye [1, 13, 15]. First, the sclera encloses all but the anterior portion of the eye, where the transparent cornea allows light into the eye [1, 15]. The amount of light that passes through to the retina is controlled by the muscular iris that adjusts the size of the pupil [1, 15]. The iris separates the two chambers that exist between the cornea and the lens: the anterior and posterior chambers - their names describing their location relative to the iris [1, 15]. The chambers are filled with aqueous humour, which maintains intraocular pressure and provides metabolic support for avascular portions of the eye such as the lens and cornea [1, 15]. The aqueous humor is produced by the ciliary body, which also plays a role in changing the shape of the lens in both chickens and humans [1, 15]. The transparent lens is flexible so that its curvature can be changed to refract light at varying powers to focus onto the retina in a process called accommodation [1]. Posterior to the lens is the vitreous chamber, filled with the gel-like vitreous humour that the lens depends on for metabolic support and waste removal [17]. The posterior wall of the vitreous chamber

is mostly covered by the sensory retina, where photoreceptors convert the light into neural signals for processing by the brain [17]. The main nutrient supply for the retina is provided by the highly vascularized tissue layer, the choroid [1, 18]. There is a difference, however, in the source of nourishment for the retina between the humans and chickens, among other differences which will be discussed next.

As with any animal models, there are differences that distinguish the chicken eye from those of humans. The chicken sclera has a hyaline cartilage layer in addition to the fibrous layer also found in primates [19, 20]. Additionally, scleral ossicles surround the chicken cornea provides structural support during corneal accommodation, which is absent in humans [21]. In chickens, changes in the corneal curvature assist with accommodation and is brought about by the ciliary muscle, which has attachment points on the ossicles [13, 18, 21]. Striated ciliary muscles result in stronger and faster accommodation in chickens compared to smooth ciliary muscle fibres in humans [1, 22]. While the ciliary body is involved with lenticular accommodation in both humans and chickens, it differs in the way it associates with the lens. The ciliary body makes direct contact with the chicken lens at the annular pad, a specialized, thickened region of epithelial cells at the equator of the chicken lens (Figure 2.1) [23]. Humans lack an annular pad; instead, the ciliary body, which is not in contact with the lens, induces shape changes in the human lens by modifying the tension in the zonules suspending the lens [1].

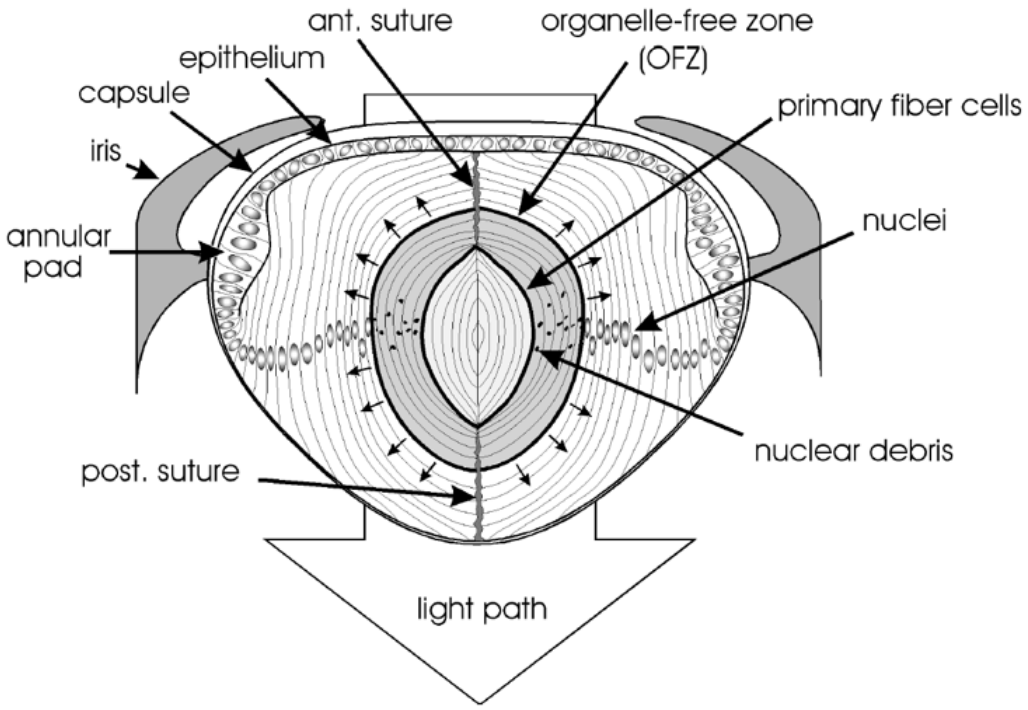

Figure 2.1: Cross-section diagram of the chicken crystalline lens. Republished with permission of Journal of Cell Biology, from Chromatin degradation in differentiating fiber cells of the eye lens, Bassnett, S.; Mataic, D., 137 (1), 1997; permission conveyed through Copyright Clearance Center, Inc.

Like the ciliary muscle, the iris muscles are striated in chickens and smooth in humans, though they both also play the role of controlling the amount of light that goes into the retina [13]. There are quite a few differences between the chicken and human retina, one of them being the absence of a foveal pit in the chicken retina. Instead, there is a thickened part of the retina with higher cone density called the afoveate area centralis, which is considered analogous to the human foveal pit [13, 24]. Six types of photoreceptors are

present in the chicken retina, including double cones for motion perception compared to the four photoreceptors in humans [13]. Vasculature in the retina also differs, as the chicken retina is avascular. Instead of the central retinal artery, the vascular pecten oculi, which arises from the optic nerve head, is thought to provide nutritional support for the retina in addition to the choriocapillaris [13, 18, 25].

2.2 The Vertebrate Crystalline Lens

The vertebrate lens is a biconvex, transparent structure within the eye [15]. It is composed of tightly packed fibre cells with an anterior monolayer of epithelium, contained in a acellular, transparent basement membrane called the lens capsule [16, 26]. The lens serves to focus light onto the retina with minimal scatter, a task that requires transparency and a high refractive index. Programmed organelle loss, highly regular, and tightly packed lens fibre cells are some adaptations that contribute to the proper functioning of the lens [27].

2.2.1 Lens Embryology

The lens is derived from surface ectodermal cells. The formation of the rudimentary lens begins with the thickening of these cells to form the lens placode [28]. The lens placode then invaginates to form a lens pit. The lens pit closes to develop into the lens vesicle, which

pinches off the surface ectoderm. At this point, the lens vesicle is a spherical monolayer of epithelial cells with a hollow lumen surrounded by their basement membrane, which becomes the lens capsule [28, 29]. The posterior cuboidal epithelial cells of the lens vesicle elongate to become the primary lens fibre cells as they begin filling the lumen of the vesicle, forming the embryonic nucleus [28]. The secondary fibre cells arise from cells that migrated from the anterior epithelium to the lens equator, where they elongate, eventually wrap around the embryonic nucleus [28]. The fibre cells also eventually lose their organelles; this process is observed by the 12th day of development (E12) in chicks [30]. The ends of the secondary fibre cells meet anteriorly and posteriorly, resulting in areas of disarray called sutures.

Among vertebrates, different suture types exist due to the variation in the ends of the fibre cells such as how much their end widths taper off, which affects where they meet [31, 32]. The only type of suture that has the cells' ends meeting at a single point on the anterior and posterior pole is the umbilical suture, found in aves and reptiles [31–33]. In other animals, most of the lens fibre cells do not extend all the way to the anterior and posterior poles, and their meeting points result in the line (in rabbits and amphibians) or Y-shaped sutures (in many mammals, including humans) [31]. While suture arrangement influences optical quality and are different in humans compared to chickens, they both experience an age-related decline of their lens' optical function and focusing capability [3,

34, 35]. This decline is partially attributed to the continuous addition of concentric layers of fibre cells by elongation and differentiation of the epithelial cells [2, 36].

2.3 Accommodation

Vertebrates change their lenses' refractive power through a process called accommodation, which is achieved through the displacement or the deformation of the crystalline lens [37]. Human (Figure 2.2) and chicken (Figure 2.3) lenses belong in the latter group, and increase their refractive power by steepening the curvature of their lenses. There are, however, some differences between their accommodative apparatus, which has been outlined in section 2.1.

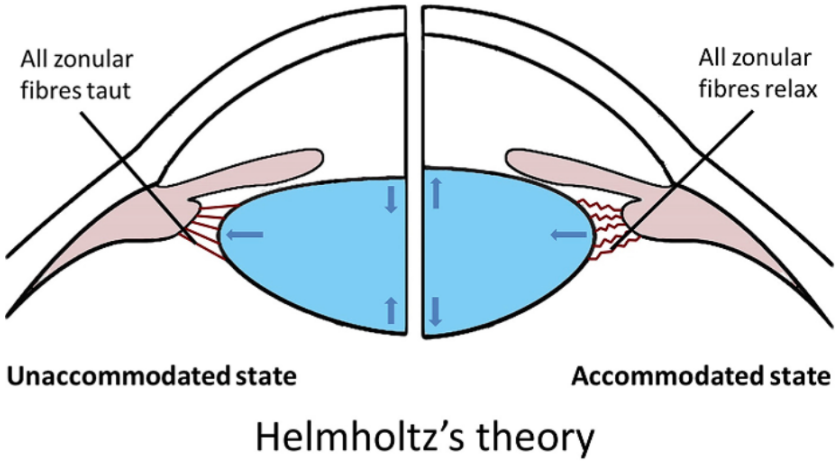

Figure 2.2: Diagram depicting the human eye in its unaccommodated (left) and the accommodated state (right), according to the Helmholtz theory. Republished with permission of Progress in Retinal and Eye Research, from Biomechanics of the human lens and accommodative system: Functional relevance to physiological states, Wang, K.; Pierscionek, B.K., 71, 2019; permission conveyed through Copyright Clearance Center, Inc.

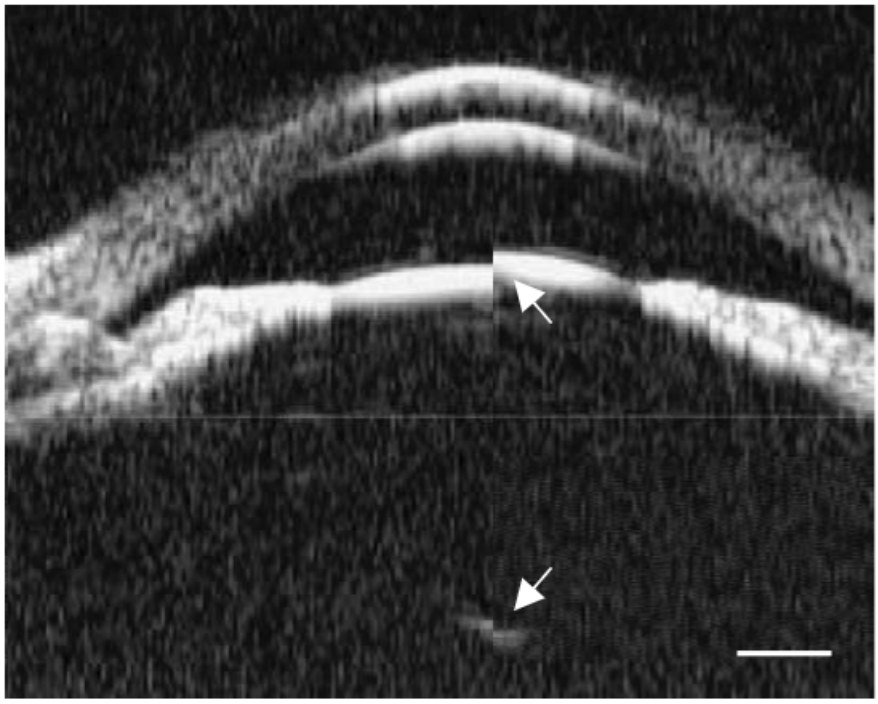

Figure 2.3: Ultrasound biomicrograph of the chicken eye showing the lens in its unaccommodated (left) and the accommodated state (right). Arrows show movement of the lens surfaces. Scale bar = 0.5mm. Republished with permission of Ophthalmic and Physiological Optics, from Ultrasound biomicroscopy of the anterior segment of the enucleated chicken eye during accommodation, Choh et al., 22, 2002; permission conveyed through Copyright Clearance Center, Inc.

2.3.1 Accommodation Theories

The full mechanism remains to be elucidated, however, the crystalline lens' involvement in accommodation has been speculated as early as the 17th century by Descartes, who proposed that focusing on nearby objects is made possible by an increase in lenticular curvature [38]. Using Purkinje images, Cramer (1853) provided the demonstration for this

increase in curvature, at least on the anterior surface of the lens [39]. He theorized that the ciliary muscle acts on the choroid to push the vitreous against the lens [39].

Like Cramer, Helmholtz (1855) also examined Purkinje images to form his theory, which is currently the most accepted theory for the mechanism of accommodation [2, 40, 41]. Helmholtz incorporated the zonules into his theory, which hold the lens taut in its unaccommodated state. During accommodation, the ciliary muscle contracts and zonules relax, which allows the lens to increase in thickness, curvature, and decrease in equatorial diameter (Figure 2.2) [40].

Although the Helmholtz theory is the most widespread, there were others who opposed it. Among the challengers is Tscherning, who argued that ciliary muscle contraction causes zonular fibres to increase, rather than decrease in tension to flatten the softer lens cortex, while the rigid nucleus forms a bulge to result in an "anterior lenticonus" [2, 41, 42]. In agreement with parts of the two opposing theories is Fincham's capsular theory [43]. Fincham noted that the monkey lens takes on an unaccommodated shape when the lens capsule is removed, and that the capsule was thicker at the periphery than the central area [43, 44]. From these observations, he gathered that accommodation involves the zonules relaxing as Helmholtz has proposed, but suggested that the passive lens was moulded by the capsule [43–45]. Fincham also thought that the uneven thickness of the capsule would shape the lens into a conoidal shape as described by Tscherning [43, 44, 46]. However,

it was later shown that both the lens and capsule have their own elastic properties, and that the capsule's role in accommodation is to distribute forces evenly onto the lens, unlike what has been suggested by Fincham [47–49].

There is also Coleman's theory, which highlights the role of the vitreous in modifying the anterior lens curvature. He theorized that ciliary muscle contraction produces a pressure gradient, resulting an increase in vitreous chamber pressure and a decrease in anterior chamber pressure [50]. Since the vitreous pressure supports the back of the lens, the lens is pushed forward during accommodation, resulting in the steepening of the centre of anterior lens curvature, and limited posterior curvature change [50]. However, Fisher showed that there was no significant difference in accommodative amplitude and anterior lens movement between a normal eye and an eye that has undergone vitrectomy [45]. Additionally, Martin and colleagues used mathematical models to simulate the change in power that can be obtained using Helmholtz' mechanism compared to Coleman's [51]. The study found that the simulation using Helmholtz' mechanism resulted in changes in refractive power that match physiological values, unlike the use of Coleman's mechanism [51].

More recently, Schachar also proposed a theory opposing Helmholtz'. Similar to Tscherning, he proposes an increase in zonular tension with ciliary muscle contraction [52]. He specifies that there are groups of zonular fibres which have differing roles; the equatorial fibres would cause the middle of the lens to deform the lens, while the non-equatorial

zonules would provide stability [41, 52, 53]. Schachar also outlined that there would be an increase in lens diameter during accommodation [52, 53]. Evidence against Schachar's theory include studies by Glasser (1994) and Wilson (1997), who showed that the lens diameter decreased during accommodation as proposed in Helmholtz's theory [21, 54]. Additionally, the mathematical assumptions made by Schachar to support his theory has been questioned by Burd (1999), who showed a mathematical model that also supported Helmholtz's theory [41, 55].

2.3.2 Presbyopia

The capacity for accommodation inevitably diminishes in humans as they age, leading to difficulties focusing on nearby objects - a condition known as presbyopia [2, 56]. Presbyopia affected about 1.04 billion people worldwide in 2005 [57]. Although there are differences in the accommodative apparatus of humans and chickens (outlined in section 2.1), the age-related reduction in accommodative amplitude was also observed in chickens by Choh and colleagues [3]. Despite its ubiquity, the pathophysiology of presbyopia is still uncertain [2, 13, 58]. Several theories offer explanations for presbyopia which include age-related changes within the lens and/or the surrounding accommodative apparatus [41, 46].

The Hess-Gullstrand and Duane-Fincham theories are considered to be the two main theories explaining the mechanism of presbyopia [41, 46]. The Hess-Gullstrand theory as-

sumes that the amount of force required from the ciliary muscle to achieve a dioptre change does not change with age [41]. These authors suggest that age-related changes within the lens render the contraction of ciliary muscle to be less effective in deforming the lens [41, 59]. Saladin and Stark's muscle impedance measurements supported this theory, the authors showed that ciliary muscle contraction is proportional to accommodative amplitude changes and that ciliary muscle force increased, even when maximum accommodation has been reached, leading to no changes in accommodative amplitude [59, 60]. Opposing the Hess-Gullstrand theory, the Duane-Fincham theory suggests that the onset of presbyopia arises from the ciliary muscle not producing enough force to focus on nearby objects, however, Duane and Fincham disagree as to what causes this problem [41]. Duane [61] believes that the ciliary muscle weakens with age, but Fincham [43] believes that changes in the lens and capsule properties result in a higher force demand to achieve a change in power. Fincham's proposal was supported by an experiment by Fisher, who showed that the force generated by the ciliary muscle at the onset of presbyopia is greater compared to those generated at younger ages [62].

Koretz and Handelman consider the relationship of age-related changes in the lens with several structures surrounding it in their Geometric Theory [63]. This theory states that as the anterior lens curvature and thickness increases with age, zonule insertion becomes more parallel to capsule surface, resulting in increased zonular tension, reducing the efficacy of

the ciliary muscle contraction [63]. Strenk and colleagues [2] added to and modified the Geometric Theory to consider lens growth's effects on the uvea as a whole. Their Modified Geometric Theory proposes that lifelong growth and thickening of the lens increases forces it transmits on the iris; this force, coupled with scleral rigidity, causes anterior and inward movement of the uveal structures [63]. This movement results in an age-related decrease in circumlental space, which has been proposed to contribute to the decrease in zonular tension [64]. This decrease in zonular tension, similar to original Geometric Theory, results in some of the force generated by the ciliary muscle to be ineffective [2, 64].

While the main theories are focused on the mechanical aspect of presbyopia, the Geometric Theories considers more elements in explaining the onset of presbyopia. This theory moves closer to Weale's suggestion of multiple factors contributing to presbyopia [65]. Weale's suggestion comes from his observation that age-related changes occur in several structures involved in accommodation, such as, but not limited to the lens itself, the capsule, ciliary muscle and zonules [65]. He also pointed out that there are also biochemical and biophysical aspects to consider that may be the cause of certain observed age-related changes [65].

2.4 Proteins in the Crystalline Lens

The crystalline lens has about twice the protein content of most tissues [66]. Based on their solubility in water, two types of protein can be found in the lens, the majority being the water-soluble crystallins [66]. The water-insoluble proteins consist of membrane and cytoskeletal proteins [66]. Insoluble proteins include, but are not limited to adhesion proteins such as N-cadherin, and cytoskeletal proteins such as actin and myosin. The interaction of three proteins may be relevant for the biomechanical changes in the lens.

2.4.1 Crystallins

Transparency, and a high refractive index are key requirements for the lens to serve its purpose. To achieve this characteristic, the lens requires a high concentration of stable, water-soluble proteins such as the crystallins [67, 68]. The crystallins consititute over 90% of the protein content within the lens, and are resistant to aggregation [66, 67]. The high concentrations of crystallins and their "short-range, liquid-like or glass-like order" [68] is central to the lens' transparency and high refractive index [66, 69]. The concentration of crystallins, however, varies throughout the lens; it gradually increases from the lens cortex toward the nucleus, which results in a refractive index gradient, as protein concentration is related to refractive index. In spherical lenses such as those in fish, this gradient offsets

spherical aberration [70, 71]. The gradient arises from a combination of events. Protein synthesis increases in differentiating fibres [72]. Synthesis continues, slows down and eventually stops as the fibre cells mature [72]. Although production stops, there is no protein turnover in the lens. This lack of turnover increases the protein concentration in the older fibre cells in the nucleus [71, 72].

Previously thought to be exclusive to the lens, crystallins originated from already existing heat, stress proteins and enzymes expressed in various tissues. Through evolution, their genes were recruited into the lens to also serve their lenticular function [73]. There are three main superfamilies found in vertebrate lenses α, $\beta\gamma$ and δ-crystallins [74]. All vertebrates possess α and β-crystallins.

Chicken lenses contain crystallins from the α, β and δ superfamilies. The δ-crystallins are structural proteins found in avian and reptilian lenses, but not in mammals, teleosts and amphibians [75]. In embryonic chicken lenses, the δ-crystallin is the first and dominant crystallin to be expressed, but its synthesis stops between 3-5 months after hatching [76]. Initially thought to be completely missing from birds, low levels of γ-crystallins were found in chicken lens tissue, however, their role within the lens is uncertain [77]. Like all vertebrates, chicken lenses also possess α and β-crystallins. Alpha-crystallins are derived from a family of heat shock proteins which function as molecular chaperones [67, 73]; mutations of the α-crystallin genes have been related to cataracts [78, 79]. Alpha-crystallins

are described to be highly polydisperse, existing in different sizes which makes it resistant to aggregation [67]. The chaperone function, along with its high polydispersity are believed to be the reasons for its recruitment into the lens [67, 79]. Alpha-crystallin peptides were also observed to inhibit apoptosis [80]. With no protein or cell turnover, the chaperone function of α-crystallins is thought to be important in preventing the aggregation and misfolding of other proteins [69, 80]. Although the β and γ-crystallins are also widespread, not much is known other than their relation to stress protein superfamilies found in other tissues [67, 81]. They are, however, speculated to play a role in regulating the packing of cytoplasmic components within the lens [67].

2.4.2 Actin

Known as the most abundant protein in eukaryotes, it is no surprise that the cytoskeletal protein actin is found within the crystalline lens of various organisms [82–85]. Actin is abundant due to its various roles, including maintaining the cell's structural integrity. Without actin, cells would not have a shape, let alone carry out essential biological processes such as cell migration, adhesion, division, shape changes and vesicle movement [86, 87]. The dynamics of actin reorganization is important in many of its roles as it has effects on cytoskeletal and therefore cellular mechanics. Actin crosslinking and its interactions with other proteins contribute to cellular shape and mechanical integrity, [88] and the dis-

ruption of actin networks has been shown to affect the stiffness of various cells [89–93] and chick lens [11].

Actin monomers, or globular actin (G-actin) reversibly polymerizes into filamentous actin (f-actin or microfilaments), depending on several factors including the ionic strength of their surroundings and concentration of free g-actin monomers [82, 83]. G-actin monomers have an adenosine triphosphate (ATP)-binding site on one end and a site that associates with another G-actin monomer on the opposite end [82, 83]. Consequently, additional actin monomers are only added to one end during polymerization, which creates a polar microfilament. The growing end is called the plus (+) end, and the other end is designated the minus (-) end [82, 83]. This polarity is important in its interactions with myosins, a motor protein important in many of actin's functions, which will be discussed later in this review.

In the crystalline lens, microfilaments are found in the epithelium and the fibre cell basal membrane complex (BMC) [10, 94]. Rafferty's group examined the organization of microfilaments in the apical ends of the lens epithelium and found three types of arrangements: sequestered actin bundles (SABs), stress fibres and polygonal arrays [5, 85, 94–96]. First observed in mouse lens epithelial cells, then rabbits, a SAB is a single bundle of actin, often curved, stained brightly in each epithelial cell [5, 85]. Stress fibres consist of crosslinked microfilaments in a parallel arrangement, sometimes with myosin, bound

together by various contractile proteins [97]. Among the animals they studied, most of those that accommodate by lens deformation had actin organized into polygonal arrays [85]. Following these findings, Rafferty and Scholz also found myosins adjacent to actin filaments and at the vertices of said polygonal arrays, at least in squirrel and rabbit lenses [94]. They proposed that these actomyosin polygons may contract the epithelium as a whole, but suggested that its role is more likely to provide tensile strength to maintain the integrity of the lens when deformed during accommodation [94]. A similar polygonal arrangement was later discovered on the posterior face of the lens fibre cells by Bassnett and his colleagues, in the BMC [10]. Cheng et al. (2018) showed actin's possible role in the mechanical integrity of the murine lens through tropomyosin knockout studies [9]. Tropomyosin 3.5 protects f-actin disassembly, and its knockout leads to a change in the lens' actin networks, which appeared to soften the lens [9].

2.4.3 Myosin

The protein most commonly associated with actin is myosin [84]. Although there are many classes of myosins, all members of the myosin superfamily can be described as motor proteins that convert chemical energy in the form of ATP into mechanical energy via the enzyme ATPase [83]. This ability gives myosins many functions, the most well-known one being the generation of contractions in muscles. However, they interact with actin

in nonmuscle cells as well, driving movement and force generation in processes such as cell adhesion, migration, stiffness, and shape changes [83, 98, 99]. Myosins have one or two larger subunits, or heavy chains that are stabilized and/or regulated by one or more smaller subunits, also referred to as light chains [83, 98]. The heavy chains consist of three domains: the head, neck and tail, which have variations in the different myosin types [83]. The head region is highly conserved, as it contains the binding sites for actin and ATP and allows for myosin's main function as a motor protein [83]. Interaction of the heavy chains with the light chains happens in the neck domain, which varies between myosin types and contributes to movements along actin filaments [83, 98]. The least conserved domain, the tail also moderates binding in the heavy chain, and has a binding site, which determines its specialized function(s) and location [83, 98].

Found in all but plant eukaryotic cells, the most ubiquitous and well-studied myosin class is myosin II, or conventional myosin [98, 100]. Non-muscle myosin II interacts with actin, and has three isoforms: IIA, IIB and IIC, which vary in tissue and cell distribution [100, 101]. Myosin IIA and IIB are both found within the mouse lens, with IIA widespread in the developing lens epithelial and fibre cells, while IIB is mostly found in the epithelium and posterior portion of the fibre cells [10, 102, 103]. Myosin IIB antibody successfully labels myosin in the chick lens BMC, suggesting that myosin IIB distribution may be similar in vertebrate lenses (see section 3.3).

2.4.4 N-cadherin

A superfamily of cell-cell adhesion proteins, cadherins have key roles in tissue morphogenesis such as cell recognition, sorting and movement [104, 105]. The adhesive function of cadherins are attributed to extracellular homophilic interactions and intracellular interactions with the actin cytoskeleton [105]. Several subtypes of cadherins exist with varying structures and tissue distribution, some tissues expressing more than one subtype [105, 106]. Found in the embryonic chicken lens, subtypes N-cadherin (neural cadherin) and B-cadherin (brain cadherin) are involved in lens development and morphogenesis [107]. Between the two subtypes, N-cadherin is more widespread in the cell, expressed by both undifferentiated epithelial cells and differentiated fibre cells at cell-cell junctions [10, 107, 108]. In the BMC, N-cadherins are found midway between cell vertices, where actin bundles are concentrated [10]. Its position relative to actin and myosin leads to the possibility of its role in stabilization and transmission of contractile forces generated by the actin-myosin system [10].

2.5 Actin-myosin Interactions

Interactions between actin and myosin are ubiquitous. Most nonmuscle actomyosin systems are "small-scale versions" of those found in muscles that also generate contractions when

myosin II slides the actin filaments in opposite directions [82]. A well-known example of nonmuscle actomyosin contraction is the contractile ring that forms to separate the two new cells at the end of cell division [82]. Of interest to this project is their contribution to mechanical properties of cells; for example, they govern the shape and deformability of red blood cells, endothelial and epithelial cells [6–8]. The most extensively studied actin-myosin II systems, however, are those found in muscle cells (also called muscle fibres), where they are responsible for generating contractions in all three types of muscle fibres: smooth, cardiac and skeletal [82]. In these muscle fibres, actin and myosin II interact with other proteins to form thin and thick filaments, respectively. The two types of filaments are aligned parallel to each other, and the polarity of f-actin is shown by the position of myosin heads, which all point to the same direction along the thin filaments. The f-actin polarity and organization of the myosin heads create the appearance of barbed (+) and pointed (-) ends of the thin filaments, and gives muscle contraction its directionality [82, 83, 109]. During contraction, the myosin heads swivel and pull the thin filaments, as it moves towards the (+) end of the thin filament [109].

2.5.1 Skeletal Muscle Contraction

Skeletal muscles are attached to bones by connective tissue [56]. Controlled by the somatic nervous system, these muscles are responsible for voluntary movements, aside from shiv-

ering to increase body temperature [56]. The thick and thin filaments are arranged in a parallel order, sometimes overlapping, creating the striations seen under the microscope. The fundamental contractile unit for the skeletal muscle is called a sarcomere [56]. A sarcomere has two boundaries, where the (-) ends of microfilaments are aligned and anchored. The thin filaments do not overlap with the thick filaments near the ends; the interaction of myosin heads with the thin filaments occur closer to the centre of the sarcomere. This overlap stops in the middle of the sarcomere, where the myosin tails are bundled. The sarcomere is the foundation for the sliding filament theory, which is the most widely accepted model for muscle contraction [56].

The steps of the sliding filament theory are described next, beginning with the rigor state, where myosin is strongly bound to actin and no nucleotides (ATP or ADP). It should be noted, however, that in living muscle tissue, the rigor state is transient, as the levels of ATP in living muscles allow most of the myosin heads to bind ATP almost immediately after the products of ATP hydrolysis are released. When ATP binds to myosin, myosin detaches from actin. Myosin breaks down the ATP into adenosine diphosphate (ADP) and a phosphate group, moving myosin heads move into a cocked position and weakly bind to actin to form a "cross-bridge". Calcium ions (Ca^{2+}) initiate a contraction by unblocking myosin sites, increasing the strength of the actin-myosin bond. The myosin head swings as it releases the phosphate group from ATP hydrolysis. The myosin head

pulls the actin filament with it, creating the "power stroke" and the filaments slide past each other. Myosin then returns to its original position, and ADP is released, returning to the rigor state [56].

2.5.2 Cardiac Muscle Contraction

Involuntary contractions propagated by cardiac muscle tissue allows the heart to pump blood. While they are found only within the heart, the myofilaments in cardiac fibres are also arranged in sarcomeres, thus sharing their striated appearance and contraction mechanism with skeletal muscles [56]. There are, however, differences in contraction regulation and the isoforms of the contractile proteins involved [110, 111]. Additionally, compared to multinucleate skeletal muscles, cardiac muscle fibres are uninucleate, shorter and are linked together via gap junctions like some smooth muscles [56]. The linkage between the muscle fibres allow the pacemaker cells of the heart to regulate the muscle fibres to contract as a unit [56].

2.5.3 Smooth Muscle Contraction

Smooth muscles are found in organs and vasculature. They are the most variable group of muscle fibres, as they require specializations based on their location and function. They

are also under the control of the autonomic nervous system like the cardiac muscles, but there are differences in the number of thick and thin filaments and how they are arranged. Smooth muscle fibres are characterized by a greater actin to myosin ratio compared to cardiac or skeletal muscles, and the absence of sarcomeres [56]. Instead, the myofilaments are organized diagonally, resembling a net that wraps around the cell [56]. This arrangement causes the cell to become rounder when the filaments contract [56]. The mechanism of contraction is quite similar to the skeletal and cardiac muscles, with some differences. Instead of troponin, Ca^{2+} binds calmodulin to uncover the myosin binding site on the actin filament. The Ca^{2+}-bound calmodulin then activates MLCK, which phosphorylates the regulatory light chains of myosin heads to increase ATPase activity, generating a contraction [56]. The power stroke and the release of ADP and phosphate group is the same in smooth muscle systems as the skeletal and cardiac muscle fibres.

2.5.4 Nonmuscle Contraction

Two main types of contractile actomyosin systems exist outside of muscles: stress fibres and adhesion belts, both of which are found adjacent to cell membranes [82]. Stress fibres are commonly found in the basal membrane in many epithelial cells, including some lens epithelia, while adhesion belts are commonly found in the apical membrane of many epithelial cells [82]. Nonmuscle contractile arrangements of actin-myosin resemble sarcom-

eres of skeletal and cardiac muscles, and functions similarly, where myosin heads pull actin filaments to slide past them [82, 83]. The mechanism and regulation of nonmuscle contraction, however, are similar to smooth muscles. The regulation of nonmuscle contraction is dependent on the phosphorylation of the myosin regulatory light chain by MLCK, which is activated by Ca^{2+}-bound calmodulin [82, 112].

2.6 Fibre Cell Basal Membrane Complex (BMC)

Lens fibre cells are hexagon-shaped epithelial cells that have undergone differentiation and elongation. Like other epithelial cells, regions of their plasma membrane have varying biochemical compositions, allowing the membranes to be distinguished into apical, lateral and basal domains [113, 114]. The apical membranes of the fibre cells meet each other at the anterior suture. Actin networks also exist in the apical domain of fibre cells, arranged in a less regular fashion than those at the BMC [94, 114]. Although scarce, some gap junctions exist between the anterior epithelium and the fibre cells, suggesting some molecular transport between the anterior epithelial and fibre cells [28, 114]. Additionally, gap junctions within the lateral domain are responsible for metabolic support and communication between the fibre cells in the avascular lens [28]. Tight junctions separate the apical domain of the fibre cells from the lateral domain, which are characterized by the numerous

interdigitations and desmosomes that join the fibre cells together [28]. This interlocking contributes to the regular packing of the fibre cells to minimize light scatter, and keeps them in place during accommodation-related shape changes [28, 114]. While there are no junctions that distinguish the lateral and basal domains of the fibre cells, the biochemical composition of the basal membrane is distinct. The posterior face of the fibre cells and its associated cytoskeletal components is called the basal membrane complex (BMC) [10]. This domain contains specializations for interactions with the lens capsule at the BMC [10].

As is common in many cells, proteins capable of generating and regulating contractions, such as actin and myosin II, caldesmon and myosin light chain kinase (MLCK) are present in the BMC [10]. In the BMC, actin is colocalized with N-cadherin (neural cadherin), forming hexagons with myosin II at its centre to "[resemble] a two-dimensional muscle" (Figure 2.4) [10].

It is possible that the contractile tone generated by this network could contribute to altering the shape of the posterior lens during accommodation, while N-cadherin stabilizes and transmits said contractile forces [10]. Previous studies have suggested the networks' influence on lenticular biomechanics [11, 12]. First, Luck and Choh showed that treatment with a low concentration of ML-7, an MLCK inhibitor decreases the focal lengths of chick lenses [12]. Furthermore, Won et al. showed that pharmacological manipulation of the

network and the same MLCK inhibitor decreases overall lens stiffness at low concentrations, which could have consequences on accommodative amplitudes [11].

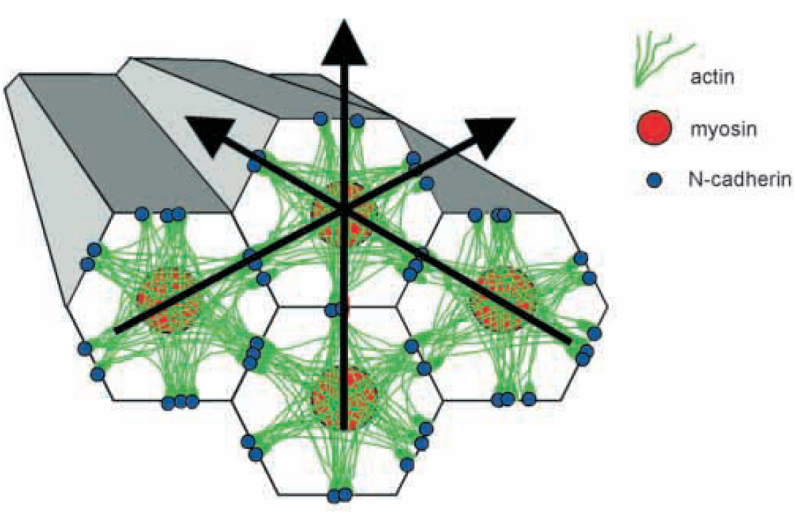

Figure 2.4: Diagram representing the muscle-like arrangement of actin, myosin and N-cadherin on the posterior face of the lens. The black arrows show the possible direction of contractile force transmission. Republished with permission of Journal of Cell Science, from Molecular architecture of the lens fiber cell basal membrane complex, Bassnett et al., 112(13), 1999; permission conveyed through Copyright Clearance Center, Inc.

Chapter 3

Methods

3.1 Animals

This study was conducted in agreement with the Guidelines of the Canadian Council on Animal Care and was approved by the University of Waterloo Animal Care Committee. White leghorn (*Gallus gallus domesticus*) hatchlings were procured from a hatchery (Maple Leaf Foods, New Hamburg, ON) and raised to 6-8 days prior to euthanasia by decapitation. Previous studies which reported the effects of ML-7 on chick lenses also used 6-8-day old chick lenses [11, 12]. Chicks were housed in stainless steel brooders with a heat source, kept on a 14:10-hour light:dark cycle, and provided with food and water *ad libitum*.

3.2 ML-7

Hexahydro-1-[(5-iodo-1-naphthalenyl)sulfonyl]-1H-1,4-diazepine hydrochloride (ML-7), is a competitive, reversible inhibitor that targets the ATP binding site of MLCK [115, 116]. Inhibiting MLCK prevents the phosphorylation of the MLC, thereby preventing actin and myosin from generating contractions.

3.3 Lens Dissections and Treatments

Following decapitation, the eyes of the chicks were enucleated and placed in Tyrode's solution (TS). The posterior segment was removed, and the anterior segment was exposed to the appropriate solution. For each bird, one eye was treated with ML-7, the other treated with vehicle for 15 minutes. The treatment solutions were: 1 µM ML-7 in 0.001% (v/v) ethanol (EtOH) in TS, 10 µM ML-7 in 0.01% (v/v) EtOH in TS or 100 µM ML-7 in 0.1% (v/v) EtOH. The vehicle solutions contained: 0.001% (v/v) EtOH in TS, 0.01% (v/v) EtOH in TS or 0.1% (v/v) EtOH TS. Following treatment, the anterior segments were briefly dipped in TS and fixed with 4% paraformaldehyde (PFA) solution in phosphate buffered saline (PBS) for 10 minutes. After fixation, the anterior segments were rinsed with TS (3 x 5 minutes) and lenses were isolated from the surrounding structures for immunostaining.

3.4 Immunohistochemistry and Confocal Microscopy

Extracted lenses were rinsed with TS (3 x 5 minutes), then permeabilized in 0.1% Triton-X in PBS for 30 minutes. They were rinsed with PBS (3 x 5 minutes) prior to incubation in a mouse anti-myosin antibody (Developmental Studies Hybridoma Bank CMII 23, 1:100 dilution in PBS, 2 hours at 37°C). Three more washes with PBS preceded the addition of goat anti-mouse secondary antibody conjugated with Texas Red (ab6787, Abcam, 1:200 dilution in PBS overnight at room temperature). Three final PBS rinses preceded counter-staining with Phalloidin-iFluor 488 Reagent (ab176753, 1:1000 dilution in PBS, 15 minutes at room temperature). Whole lenses were mounted onto slides using 5% (w/v) agar solution in water. The posterior pole was topped with a coverslip coated with Prolong Gold (P36934, Life Technologies). Images were captured using a Zeiss LSM510 Meta confocal microscope and the Zen 2009 software.

3.5 Centroid Detection Validation

Two available methods of analyzing and quantifying myosin distributions were compared to a custom MATLAB software coded by Dr. Alexander Wong. Lenses from 7-day old white leghorn (*Gallus gallus domesticus*) chickens were labelled for myosin as outlined above. Nearest neighbour distances (NNDs) for myosin centroids at the posterior surface

were determined using the following methods on the same image (Figure 3.1):

1. ImageJ: manual using the Elliptical tool

2. ImageJ: automatic using the Analyze Particle tool

3. MATLAB: automatic using custom-coded software

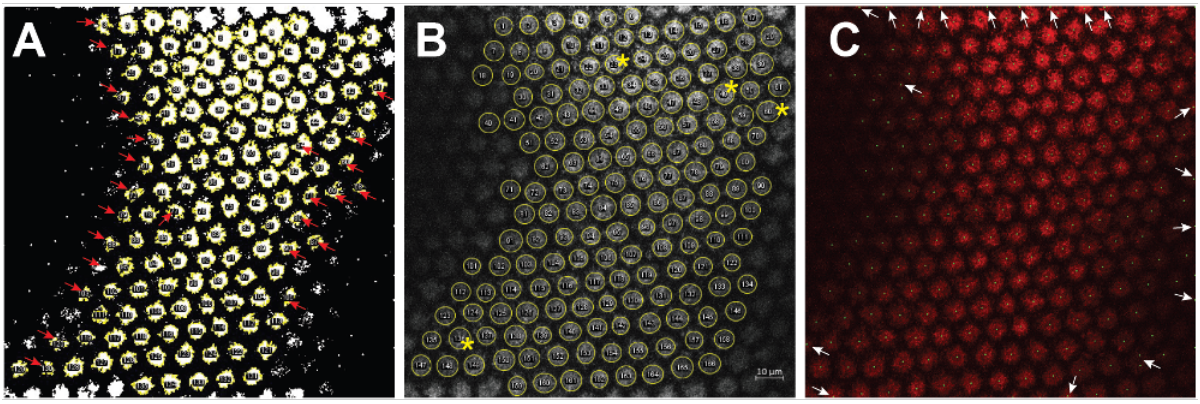

Figure 3.1: Myosin centroid detection using ImageJ Analyze Particles (A), ImageJ Elliptical tool (B), custom-coded software on MATLAB (C) Regions of interest are outlined in yellow in (A) and (B), and centroids are denoted by the green dots in (C).

For both methods on ImageJ, the NND plugin was used, following the centroid detection using the methods outlined above. First, the Elliptical tool (manual) on ImageJ was used to determine XY centres of ellipses superimposed on the image by the user. Three ellipses were superimposed on each centroid to account for user error, and these results were averaged. For the automated ImageJ method, the image was converted to a binary format using the program's "Make Binary" function. The "Fill Holes" and "Eraser" tools in ImageJ were

used to correct centroids altered by the thresholding process before determining the myosin centres using the Analyze Particles tool. For analysis in MATLAB, the image was opened and the custom-coded script was run to obtain the myosin centres and NNDs. Corrections were made using MATLAB's "Brush Tool". This allowed the user to select and remove erroneously detected centroids, such as those on the edges of the image, shown by arrows in Figure 3.1C). The three methods of calculating NNDs from the same 135 centroids were analyzed using repeated measures ANOVA.

The Analyze Particles tool detected the fewest centroids (135) compared to the Elliptical (166) and MATLAB methods (163). Ranges and mean NNDs (±standard deviation (SD)) for the same 135 centroids are listed in Table 3.1. There were no significant differences found between the three methods (P=0.2754).

Parameter	**Analyze particles**	**Elliptical tool**	**MATLAB**
Range	7.98-10.31	8.13-9.87	8.07-10.03
Mean NND ± SD (μm)	9.02±0.47	8.97±0.35	8.96±0.38

Table 3.1: Ranges and means for the NNDs of the same 135 myosin centroids using three methods of centroid detection

Although the elliptical tool allowed for the detection of more centroids, the ellipses do not always fit properly (asterisks, Figure 3.1B), possibly leading to inaccurate NND values. The MATLAB program uses adaptive thresholding to consider variations in illumination, allowing it to detect more centroids with fewer detection errors than the Analyze Particles

tool (arrows, Figure 3.1A, 3.1C).

The custom MATLAB program was chosen for determining the distances between the proteins in the actomyosin network. In the event that centroids were missed by the MATLAB software, the Elliptical tool from ImageJ was used to manually determine the coordinates of these centroids. The coordinates of the missing centroids were then added onto the variables table on MATLAB, and the code was run again to obtain the NNDs.

3.6 Actomyosin Network Analysis and Quantification

Confocal images of the actomyosin networks were taken 0.5mm away from the suture in the direction of the four cardinal points. Due to the curvatures of the lens, images at different depths were taken to fill the whole frame. These stacks were combined into a single image using the "Z Project" tool on imageJ using the "Max Intensity" setting prior to analysis.

The custom MATLAB software coded by Dr. Alexander Wong was chosen from the comparison outlined in section 3.4. The code uses adaptive thresholding, which is able to handle images with uneven illumination [117]. The centroids were then detected. The MATLAB software was then further developed to detect actin and compute four types of NND measurements to determine the effects of ML-7 on network distribution:

1. actin-actin

2. actin-myosin

3. myosin-actin

4. myosin-myosin

The distances are computed in pixels, which was converted to micrometres (µm). By measuring scale bars thrice in 10 different images using ImageJ, it was determined that 1 µm was equal to 3.3875 pixels.

3.7 Statistical Analysis

For each image, the nearest neighbour distances of actin and myosin centroids were computed by the MATLAB code, then these values were averaged. Four images of the actomyosin networks were collected from each eye, and the mean nearest actin-actin, actin-myosin, myosin-myosin and myosin-actin distances from the four images were averaged. To determine if there were significant changes in the network distributions, a two-way repeated measures analysis of variance (RM ANOVA) was used (GraphPad Software Inc., San Diego, CA). Values were matched by subjects to determine how much variation was due to the different birds. Tukey's and Bonferroni's multiple comparisons test was also used to detect any differences arising from the different concentrations of ML-7 (Jamovi).

To determine if there are any differences in effects between concentrations, distances were presented as relative NNDs (percent of the vehicle-treated lenses) to account for variability between birds. The Kruskal-Wallis and Dunn's Multiple Comparisons tests were used to assess if any differences in relative NNDs between concentrations were statistically significant (GraphPad Software Inc., San Diego, CA). For all experiments, differences were considered significant if $P<0.05$.

Chapter 4

Results

4.1 Analysis of NND Measurements

Confocal images of the actomyosin networks show no obvious deviations in the ML-7-treated lenses from the vehicle-treated lenses (Figure 4.1). The minimal effect was also reflected by the similar mean NNDs of the treated and vehicle lenses, except for myosin-actin. Myosin-myosin had the furthest mean NNDs(±SD) (9.30µm±0.39 treated; 9.45µm±0.37 vehicle), followed by actin-myosin (6.17µm±0.26 treated; 6.25µm±0.26 vehicle), myosin-actin (4.86µm±0.21 treated; 4.94µm±0.22 vehicle) and actin-actin(4.67µm±0.16 treated; 4.72µm±0.17 vehicle)

The distributions of the distances for all NND measurements in the 1, 10 and 100 µM groups were different, but the ranges between the eyes for each group were relatively similar (Figure 4.2). When mean NND measurements for the treated lenses were compared to the vehicle lenses, there were no significant differences in the actin-actin, actin-myosin, myosin-myosin NNDs (P>0.1994). Mean myosin-actin distances, however, were lower in the treated (4.86µm±0.21) lenses versus the vehicle lenses (4.94µm±0.022; P=0.0454) across the three concentrations (Figure 4.3).

4.2 Effects of Different ML-7 Concentrations

No difference in mean NNDs was found at the various concentrations (P>0.0973), nor was there any interaction between concentration and treatment (P>0.2010), except for the myosin-myosin NND measurements (Figure 4.2). For this comparison, the NND values for the 10 µM-treated eyes (mean±SD 9.01 µm±0.32) were shorter than those for the 100 µM-treated eyes (9.66 µm±0.33)(P=0.034). No differences were observed between vehicle eyes at the various concentrations. However, when NNDs for treated eyes were expressed as a percent of their vehicle counterparts (Figure 4.4), no significant differences were observed between the concentration groups for the myosin-actin group (P 0.3112), nor the myosin-myosin group (P 0.1066); mean relative NNDs from highest to lowest were: 99.7%±1.9

(100µM), 99.2%±1.9 (1µM) 97.2%±3.1 (10µM). The same relative order was observed for the other NND measurements except for actin-myosin, which showed the highest relative means in the 100µM (100.6%±1.7), followed by 10µM (99.3%±5.4) and 1µM (96.7%±4.3).

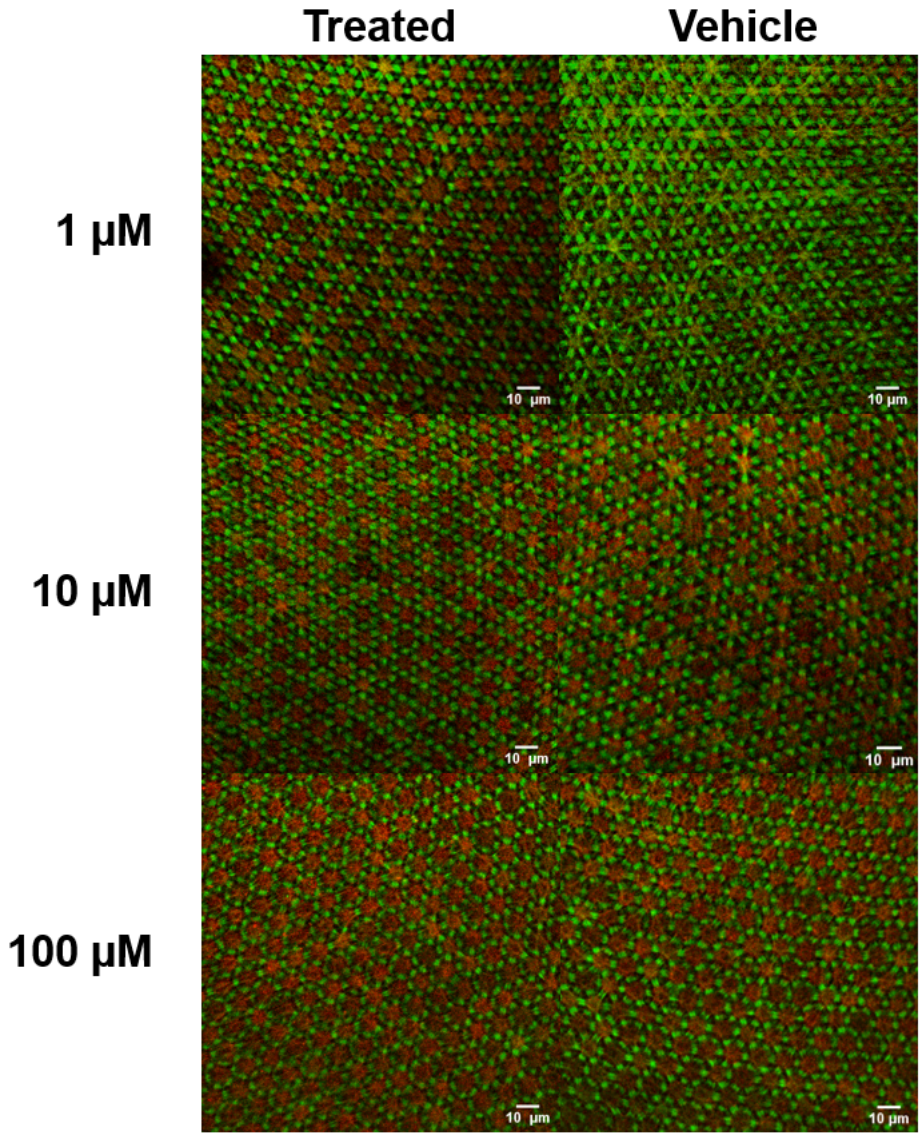

Figure 4.1: Confocal images of actin (green) and myosin (red) networks of 7-day old chicks. Treated and vehicle pairs are eyes from the same bird. Scale bar = 10µm.

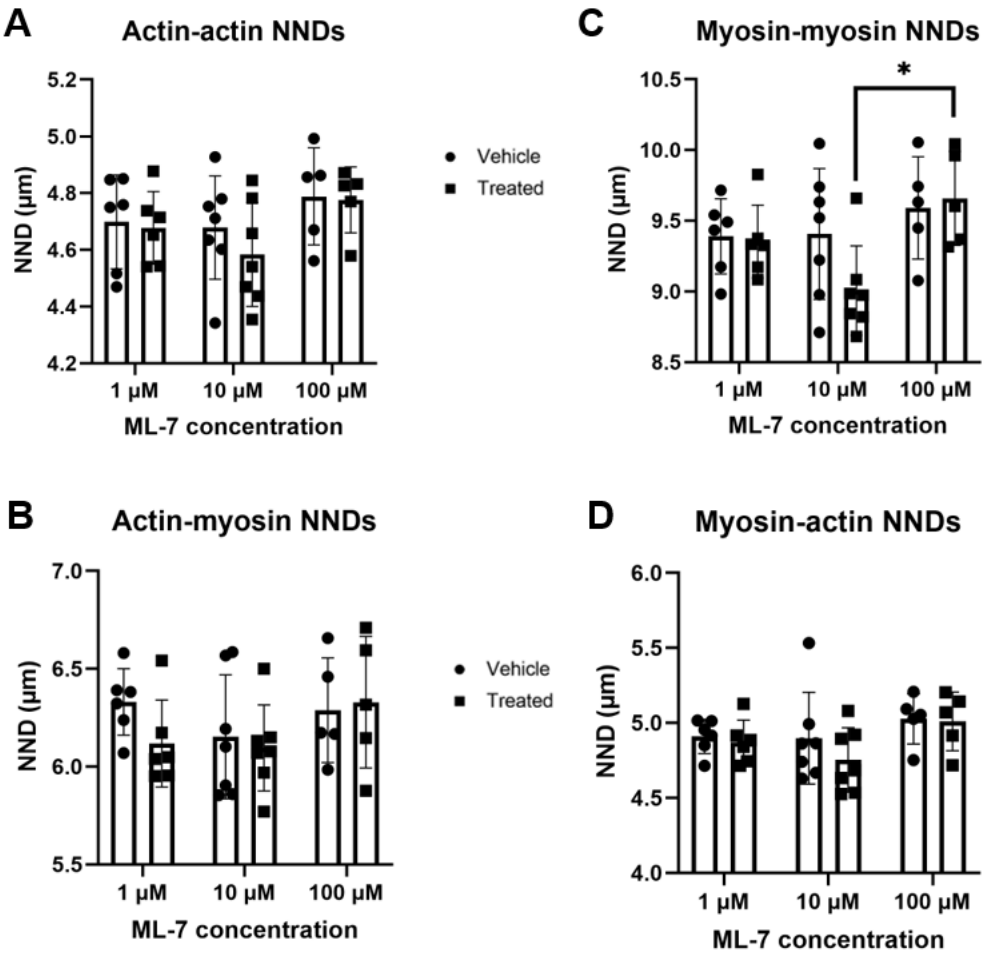

Figure 4.2: Mean NNDs (±SD) in µm for actin-actin (A), actin-myosin (B), myosin-myosin (C), myosin-actin (D) for lenses treated with vehicle, 1µM (n=6), 10µM (n=7) or 100µ(n=5) of ML-7. The asterisk in (C) denotes a significant difference in myosin-myosin NNDs between 10µM and 100µM-treated lenses (P=0.034)

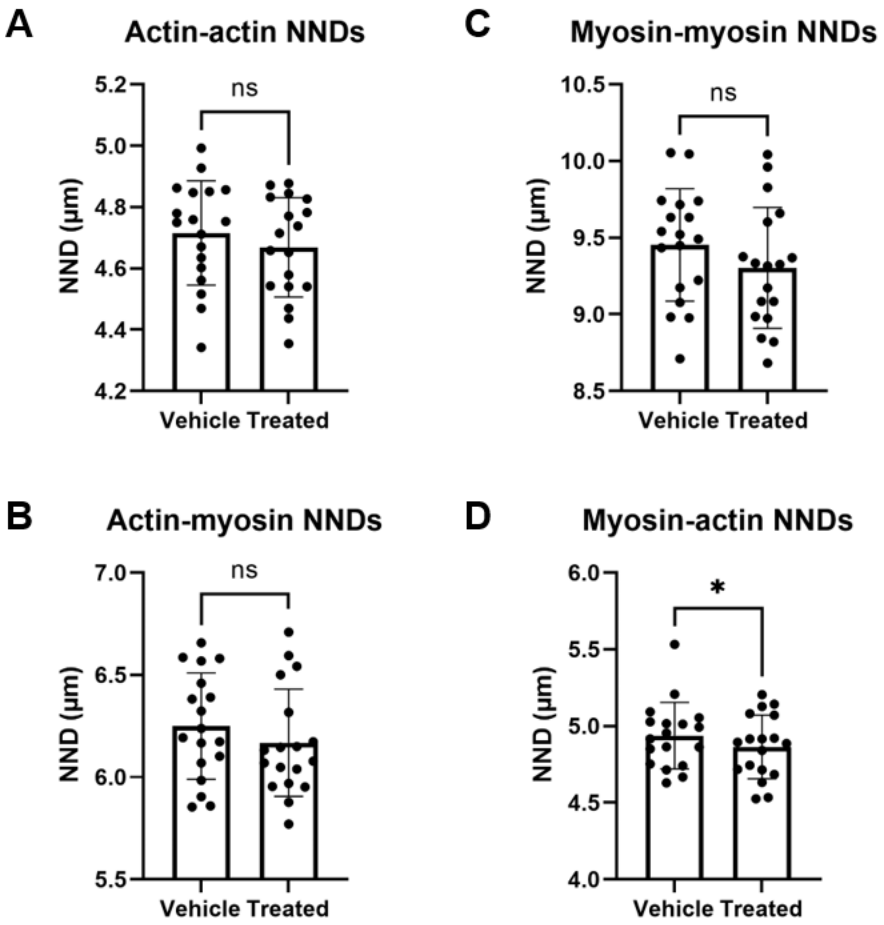

Figure 4.3: Mean NNDs (±SD) in µm for actin-actin (A), actin-myosin (B), myosin-myosin (C), myosin-actin (D) for lenses treated with vehicle, 1µM (n=6), 10µM (n=7) or 100µ(n=5) of ML-7. The asterisk in (D) denotes a significant difference in myosin-actin NNDs between vehicle and treated lenses across all three concentrations (P=0.0454)

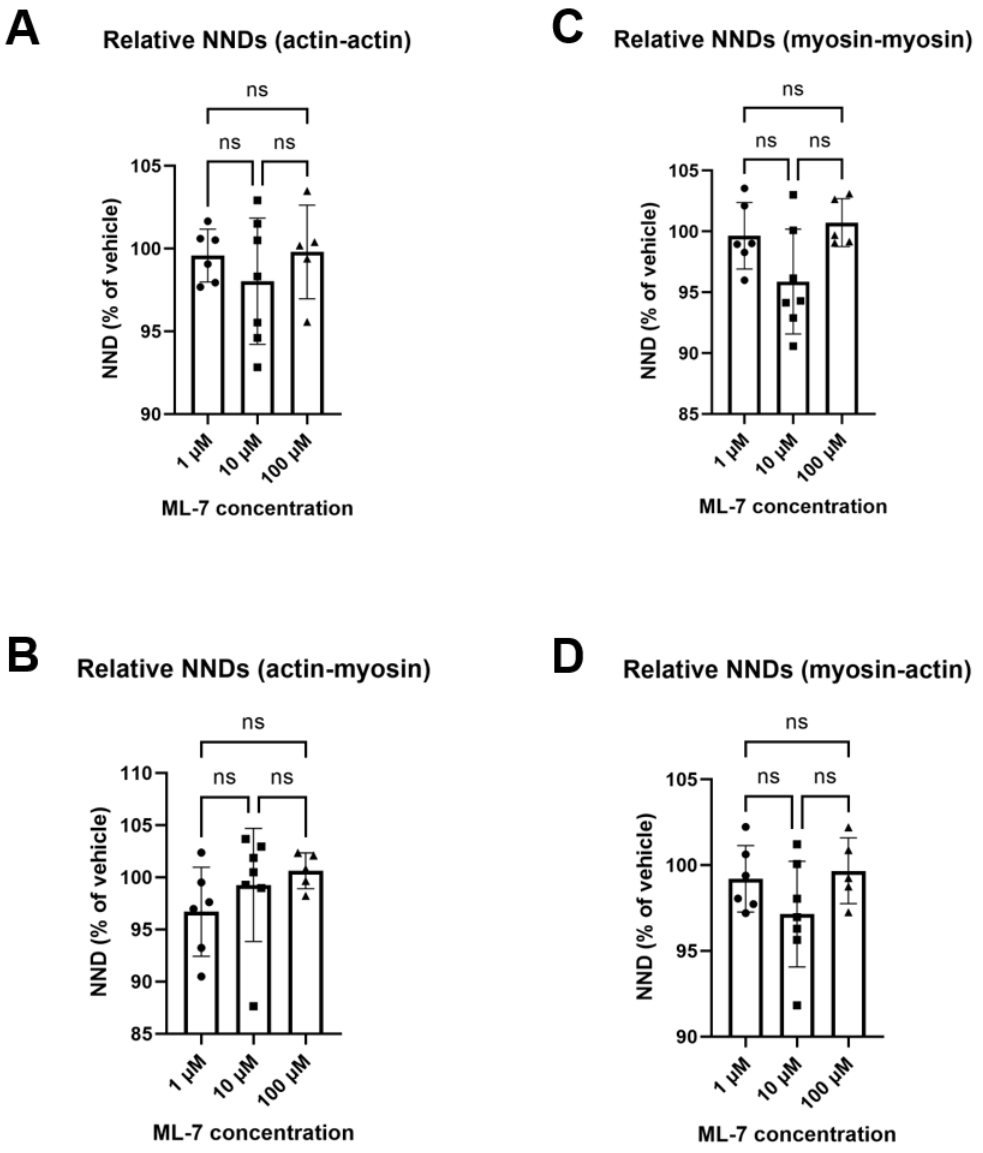

Figure 4.4: Mean Relative NNDs (±SD) as % of vehicle for actin-actin (A), actin-myosin (B), myosin-myosin (C), myosin-actin (D) for lenses treated with 1µM (n=6), 10µM (n=7) or 100µ(n=5) of ML-7.

Chapter 5

Discussion

5.1 ML-7 and the Actomyosin Network Distribution

MLCK is involved in the phosphorylation of the myosin light chain (MLC), which results in myosin's interaction with actin, and contraction of the actomyosin network (Figure 5.1) [56]. The contractile tone generated by this system increases the stiffness of various muscle and nonmuscle cells [82, 98, 99]. Given that ML-7 inhibits MLCK, which has a key role in actomyosin network contractions, treated lenses were expected to have decreased actin-myosin interactions, resulting in larger NNDs. However, a significant reduction was found when comparing mean myosin-actin NNDs of the treated and vehicle lenses (Figure 4.3). This finding does not support the idea that ML-7 treatment would result in further NNDs

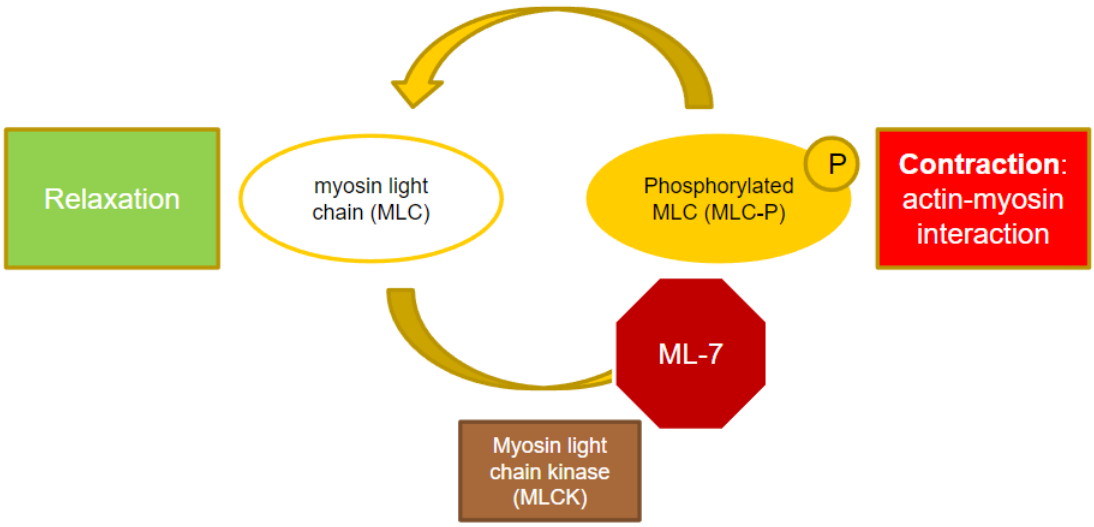

Figure 5.1: Diagram depicting the mechanism of MLC phosphorylation by MLCK, and inhibition of MLCK by ML-7. Redrawn with modifications under Creative Commons Attribution 4.0 (http://creativecommons.org/licenses/by/4.0/), from Polycystin-1 Regulates Actomyosin Contraction and the Cellular Response to Extracellular Stiffness, Nigro et al., Scientific Reports 9, 2019.

between actin and myosin by preventing their interactions. ML-7 treatment also did not have significant effects on the other NND values. The presence of other kinases may explain why there was a decrease in myosin-actin NND values, and why it did not have a significant effect on the other NND values. ML-7 is specific to MLCK, and MLCK is not the only kinase which phosphorylates the MLC [118]. Another major family of kinases within the lens and many other cells is the Rho-associated protein kinase (ROCK) family of kinases or Rho-kinases [102]. These kinases regulate actin-myosin interactions in lens epithelial cell proliferation and migration [102]. Similar to MLCK, Rho-kinases are also involved in the

phosphorylation of the myosin light chain [102, 118]. It is possible that the effect of ML-7 was diminished by the Rho-kinase activity, which may have compensated for the inhibited MLCK. Additionally, experiments performed in fibroblasts involving Rho-kinase may also explain why a reduction or no changes in NND would be found in concentrations that were previously reported to cause softening of the lens. Studies from the Radmacher group found that fibroblasts were softer when treated with ML-7, but not when a Rho-kinase inhibitor Y27632 was used [99, 119]. Taken together, the results of these fibroblast studies seem to suggest that ML-7-associated softening of tissues may not be related to its effects on the actomyosin networks.

Lenses treated with 100 µM ML-7 were shown to be stiffer and have shorter focal lengths, and these characteristics are associated with higher tension in the actomyosin networks [11, 12]. Therefore, shorter NNDs were expected to accompany lenses treated with 100µM ML-7, and greater NNDs to accompany the lower concentrations. If the results were consistent with this hypothesis, it would support the idea that stiffening and softening of the lens by ML-7 is a result of its effects on the actomyosin network. In this study, significant changes between 10µM and 100µM-treated lenses were only found in myosin-myosin NNDs. These changes were accompanied by some spread in the data (Figure 4.2), possibly due to variation between birds, which can be seen qualitatively by comparing the confocal images of the networks of the vehicle-treated lenses in Figure 4.1.

A possible cause for these differences may lie in the experimental procedure. Adjustments to the experimental protocol had to be made due to COVID safety restrictions. These changes included having to transport samples across campus prior to processing tissues. This results in extended time between sacrifice and tissue processing, and possibly, exposure to variations in outdoor temperatures, including freezing temperatures. A consequence of this lengthened time after sacrifice was a limit on the number of chicks that was able to be processed in one study repetition, as dissections take some time before staining. In the interest of maintaining tissue integrity, the number of chicks that was studied in one experiment was limited to 2-3. Thus, data was collected from separate batches of chicks, possibly resulting in the variation observed. The smaller batches also resulted in the experiments being conducted through different seasons, therefore, circannual rhythms may have contributed to variations as well. To account for the between-bird variation, relative NND values were calculated (percentage of the vehicle eye NNDs). The lack of significance when the mean relative myosin-myosin NNDs at different concentrations were compared seems to support the idea that the significant difference in myosin-myosin NND values between the 10 and 100 µM ML-7 treatments may be due to the variation between birds. Consequently, these findings cannot be used to conclude if various ML-7 concentrations affect the actomyosin networks differently.

5.2 Future Work

It is possible that the effects of ML-7 were dampened by the presence of other kinases that phosphorylate MLC such as ROCK. Matsui and Deguchi (2019) found that inhibition of ROCK in vascular smooth muscle cells had a greater effect than inhibition of MLCK via ML-7 [118]. They also suggested the rebound of phosphorylation activity may be due to compensation by other kinases [118]; this may also explain the lens stiffening previously reported in higher ML-7 concentrations [11, 12]. Investigating the actomyosin networks in lenses treated with MLCK only, Y27632 only, and both inhibitors would help rule out ROCK's possible role in compensation. Additionally, comparing the stiffness in lenses treated with ML-7 to Y27632, both inhibitors, and vehicle, may also help determine whether the softening of the lens is due to the effects of ML-7 on the actomyosin networks.

5.3 Conclusion

This study was able to quantify distributions and recognize changes in the actomyosin network, detecting lower myosin-myosin NNDs of 10µM-treated lenses compared to 100µM-treated lenses. However, it is uncertain whether or not the difference between concentrations was due to variation between birds. Therefore, these results cannot be used to conclude whether or not various ML-7 concentrations affect networks differently. A signifi-

cant reduction was also found in the myosin-actin NNDs of ML-7-treated lenses compared to their vehicle counterparts, which is inconsistent with the role of ML-7 preventing actomyosin interactions. Additionally, there were no changes observed between treated and vehicle lenses in the other types of NND measurements, including actin-myosin. The results suggest that ML-7 has little to no effect on the networks. Additionally, while the changes seen in the network could be due to ML-7 activity, it could have also been influenced by the small sample and/or other kinases. Therefore, it is uncertain if the stiffness and focal length changes in ML-7-treated lenses observed in previous studies were related to the influence of ML-7 on the actomyosin networks. Future experiments with other kinase inhibitors would determine if ML-7 changes the actomyosin network distribution.

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Title	Effects of ML-7 on the Actomyosin Networks of the Avian Crystalline Lens	**Institution name**	University of Waterloo
		Expected presentation date	2022-04-25
Instructor name	Adeline Suko		

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Editor of portion(s)	Wang, Kehao; Pierscionek, Barbara K.	**Author of portion(s)**	Wang, Kehao; Pierscionek, Barbara K.
Volume of serial or monograph	71	**Issue, if republishing an article from a serial**	N/A
Page or page range of portion	114-131	**Publication date of portion**	2019-07-01

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Volume of serial or monograph	22		
Page or page range of portion	401-408	**Publication date of portion**	2002-09-04

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