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Towards an Understanding of Steroid Hormone Mediated Mechanisms of Myopia Progression: A Scleral Perspective

By

Patrick Michael Carney

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Vision Science

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Christine Wildsoet, Chair
Associate Professor Diane Bautista
Professor John Flannery

Summer 2019

Towards an Understanding of Steroid Hormone Mediated Mechanisms of Myopia Progression:
A Scleral Perspective

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Abstract

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Professor Christine Wildsoet, Chair

The eye is an incredibly complex organ, composed of a wide spectrum of tissues. This ranges from the vascular (e.g. choroid) to the extracellular matrix (ECM) rich (e.g. cornea) to the neural (e.g. retina). Working together in concert, these tissues coordinate a wide variety of disparate activities, with exquisite temporal, spatial and functional precision, in order to facilitate vision. Given this high degree of complexity and variability across its components, the eye in many ways approximates the body in miniature. And just like the body at large, ocular tissues utilize the full complement of steroid hormones to facilitate these activities; both in the normal and under pathological conditions.

The impact of steroid hormones has been particularly well studied in the context of (1) sexually dimorphic processes and structures and (2) tissue growth and remodeling of ECM rich tissues (e.g. solid tumors and skin), as they appear exquisitely sensitive to steroid hormone signaling to drive their growth. Interestingly, myopia, an ocular pathology that is one the leading drivers of blindness worldwide, is characterized by (1) excessive ECM tissue remodeling of the sclera, leading to scleral thinning and ocular elongation (often, with greatest effect, during childhood and/or adolescence), and (2) its sexually dimorphic character; on the average it presents both more commonly and with greater phenotypic severity in females. Despite these characteristics, the affect and regulation of steroid hormones and their receptors on myopia progression, and with particular emphasis on the sclera, has received sparingly little study.

In this dissertation we work to remedy this. Here we explore the changes that occur in steroid hormones and their cognate receptors, both systemically and locally (i.e. at the level of the sclera), as myopia progresses, in a well validated form-deprivation, chicken based model of the pathology.

Leveraging a host of biometric (i.e. high frequency a-scan ultrasonography and retinoscopy), biochemical (i.e. western blotting), analytical chemistry (tandem mass spectrometry) and molecular biology (i.e. quantitative real-time polymerase chain reaction) tools we discover a strong associations between glucocorticoid metabolism in the sclera and myopia progression. More explicitly we observe lower scleral levels of the major glucocorticoid species in chicken,

corticosterone, concomitant with a drop in transcripts of its biosynthetic enzyme (HSD11β1) and myopia progression, irrespective of sex (chapter 3).

We also find several sex-specific disparities including: higher degrees of myopia in female subjects (Chapter 2), differences in the transcriptional expression levels of several sex hormone receptors (Chapter 4) and elevated progesterone levels in female form deprived sclera (Chapter 3). In aggregate our findings argue for a possible role of steroid hormones regulating ocular growth and tissue remodeling during myopia progression, both in manners that are independent from and dependent upon subject's sex.

To my parents, Walt and Diane Carney, who have always supported me in my academic endeavors and who have a been a constant source of support and encouragement in life.

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List of Abbreviations

AC	Anterior Chamber
AL	Axial Length
AR	Androgen Receptor
CC	Contralateral Control
ERα	Estrogen Receptor Alpha
ERβ	Estrogen Receptor Beta
ECM	Extracellular Matrix
FGF8	Fibroblast Growth Factor 8
FSc	Fibrous Sclera
FD	Form Deprivation
gDNA	Genomic Deoxyribonucleic Acid
GPER	G-Protein Coupled Estrogen Receptor
GPCR	G-Protein Coupled Receptor
GR	Glucocorticoid Receptor
HFAU	High Frequency A-Scan Ultrasonography
mRNA	Messenger Ribonucleic Acid
MR	Mineralocorticoid Receptor
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
SEM	Standard Error of the Mean
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
VD	Vitreous Chamber

Acknowledgements

I would like to thank Drs. Breanna Ford and Dan Nomura for their collaboration, helping with the our mass spectrometry efforts. I would also like to thank Drs. Jiexi Zeng, Yang Shen and Hui Zang for their kind help collecting retinoscopy data.

Chapter 1: Steroids Hormones, Their Receptors and Their Impact on the Eye: An Introduction

1.1 Myopia & the Sclera: An Introduction

Myopia (near-sightedness) describes the optical condition in which a mismatch exists between the optical power of an eye and its length, such that images of distant objects form in front of the fovea, to be out-of-focus, absent of optical, e.g., spectacles, contact lenses, or surgical correction. In the majority of cases, this is a consequence of excessive eye elongation during childhood and/or adolescence. Due to this excessive elongation, myopia is associated with a large number of secondary blinding pathologies, including glaucoma, maculopathies and retinal detachments (Saw et al. 2005). It is for this reason and its rapidly rising prevalence that myopia is now one of the world's leading causes of acquired blindness. For example, it has recently been predicted that over 50% of the world's population will be myopic by the year 2050 (Holden et al., 2016), with the prevalence having already reached epidemic levels across several key demographic entities, perhaps the most prominent example being young adults in industrialized East Asian countries. Indeed, one study based in Seoul, South Korea found the rate of myopia to have reached 96.5% among young adult males (Jong SK et al, 2012). Furthermore, despite the comparatively low prevalence rates in the west, they are still concerning; for example, the prevalence of myopia in the US sits at 42%, which represents an approximate 68% increase over 30 years (Vitale et al., 2009). Despite these figures, coupled with the substantial financial, physical and emotional burdens associated with myopia, currently available treatments for controlling myopia have limited efficacy. Thus there is a clear and urgent need to better understand the molecular mechanisms underlying myopia's genesis and progression, in order to inform screening and interventional guidelines and design more effective therapeutic interventions.

Of direct relevance to this dissertation are two key overarching facts, both of which strongly suggest the possibility of underlying hormone-centric drivers that may contribute to the progression of myopia. First, as noted above, myopia is fundamentally a problem of excessive eye elongation, which ultimately is a product of accelerated remodeling and thinning of the sclera, which represents the outer wall and support structure of the eye. These scleral changes underlie the expansion of the globe during myopia progression, but at the same time, the retina and choroid are subject to stretching forces that are believed to contribute to the development of the aforementioned secondary blinding pathologies (McBrien and Gentle, 2003, Rada et al., 2006). It is also well known that connective tissues with similar composition to sclera, i.e., those tissues rich in collagen and other extracellular matrix components, supported by fibroblasts and myofibroblasts (e.g. skin, tendons, solid endocrine tumors, etc.), rely in part, without any known exceptions, on steroid hormone-mediated signaling to regulate growth and remodeling (Shah MG and Mailback, 2001, Thornton MJ, 2013, Folkerd and Dowsett, 2010, Hansen and Kjaer, 2016). Thus it follows that the same may hold true with sclera, particularly during myopiagenesis and progression, when scleral remodeling is accelerated.

Second, myopia is on average, both more prevalent and presents with more severe clinical phenotypes in females than in males (Zhao J et al, 2000; Sewunet SA et al, 2014). Indeed, per the National Eye Institute webpage discussing myopia prevalence statistics, females present with a 17% higher prevalence of myopia, compared to males (nei.nih.gov). Furthermore, data extracted from a US-based study by Vitale and colleagues (Vitale S et al, 2009) indicates females to be 38% more likely to be highly myopic (defined as ≥ -5 diopters), compared to males. Traditionally these differences have been explained in terms of behavior, e.g. females are more mature and focused at a younger age than their male peers and thus likely spend more time on myopia-inducing near-work. However, an alternative, sex steroid hormone-specific explanation would seem plausible, based on their known actions as summarized later in this chapter. Could, for example, estrogen at least contribute to the greater susceptibility to myopia in females, or alternatively, could testosterone have a protective (anti-myopic) effect? There is strong evidence, both ocular and extraocular in origin, supporting these possibilities.

To date, the impact of steroid hormone signaling on scleral remodeling and its relationship to myopia progression has received minimal attention. As background to related research presented in this dissertation, current knowledge concerning the mechanisms underlying hormone and hormone receptor regulation will be reviewed, along with known relevant processes in the eye, with emphasis on processes relevant to myopia development and progression.

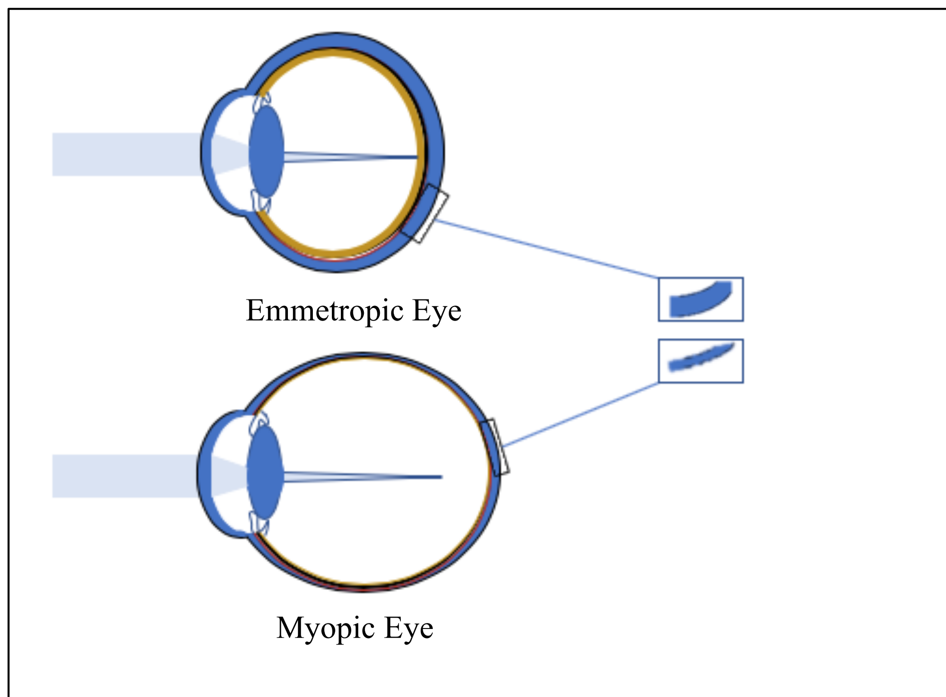


Figure 1.1 Emmetropic and myopic eyes compared. Note the differences in axial length and scleral thickness; myopic eyes are longer, with thinner scleras.

1.2 Steroid hormones: Families, Biosynthesis, Trafficking and Variability.

1.2.1 Hormone Overview

Hormones are traditionally described as chemical messengers, synthesized by cells, that often, although not exclusively, travel via circulation to distal sites of action. They control a wide variety of physiological processes, ranging from growth and metabolism to sexual development and dimorphism, behavior and immuno-regulation (Folkerd et al 2010, Yanase et al. 2015, Purves et al. 2001, Cutolo et al., 2004, Foo et al 2017). Hormones may be divided into three broad classes (Fig. 1.1): amino acid derivatives, peptide hormones and lipid derivatives (Nussey et al. 2001). Each of these classes can then be further subdivided. Thus the amino acid derivatives can be further categorized as either thyroid hormones (e.g. thyroxine), catecholamine derivatives (e.g. norepinephrine) or tryptophan derivatives (e.g. melatonin), the peptide hormones as peptides (e.g. oxytocin), small proteins (e.g. growth hormone), or glycoproteins (e.g. follicle-stimulating hormone), and lastly, the lipid derivatives as either eicosanoids or steroid hormones. The eicosanoid group includes leukotrienes and prostaglandins, which are known to play important roles in homeostasis as well as inflammation and disease. The focus of the research in this dissertation has been the last, steroid hormone group, which represent cholesterol derivatives and analogues. It has many members, including glucocorticoids and mineralocorticoids as well as classical sex hormones (androgens, estrogens and progestogens)(Fig 1.1). As a group, they play important diverse roles, for example in the regulation of sexual differentiation, as well as more generally, tissue growth and remodeling (Berg et al. 2002).

Hormone Class	Subtype	Examples	
Amino Acid Derivatives	Thyroid Hormones	Triiodothyronine (T3) & Thyroxine (T4)	
	Catecholamine Derivatives	Epinephrine & Norepinephrine	
	Tryptophan Derivatives	Melatonin	
Peptide hormones	Peptides	Oxytocin, Anti-Diuretic Hormone & Insulin	
	Small Proteins	Growth Hormone & Prolactin	
	Glycoproteins	Luteinizing Hormone (LH) & Follicle Stimulating Hormone (FSH)	
Lipid Derivatives	Eicosanoids	Leukotrienes	LBT_4
		Prostaglandins	PGE_2
	Steroid Hormones	Androgens	Testosterone, DHT & DHEA
		Estrogens	Estradiol, Estriol & Estrone
		Progestogens	Pregnenolone & Progesterone
		Glucocorticoids	Cortisol & Corticosterone
		Mineralocorticoids	Aldosterone

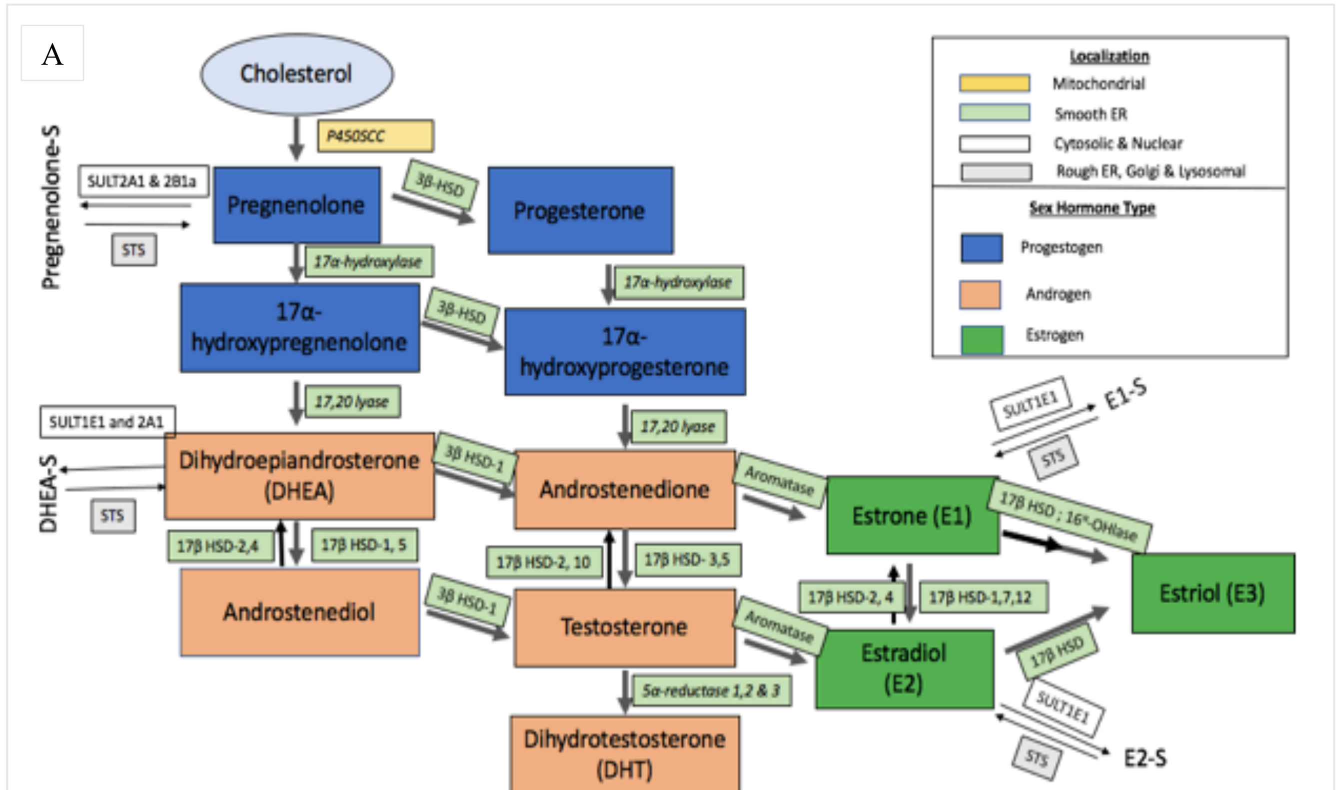
Figure 1.2 Hormone Classification. Summarized are the various classes and sub-classes of hormones, with specific examples; steroid hormones (both sex, in red, and "non-sex", in tan), are the primary focus of this dissertation.

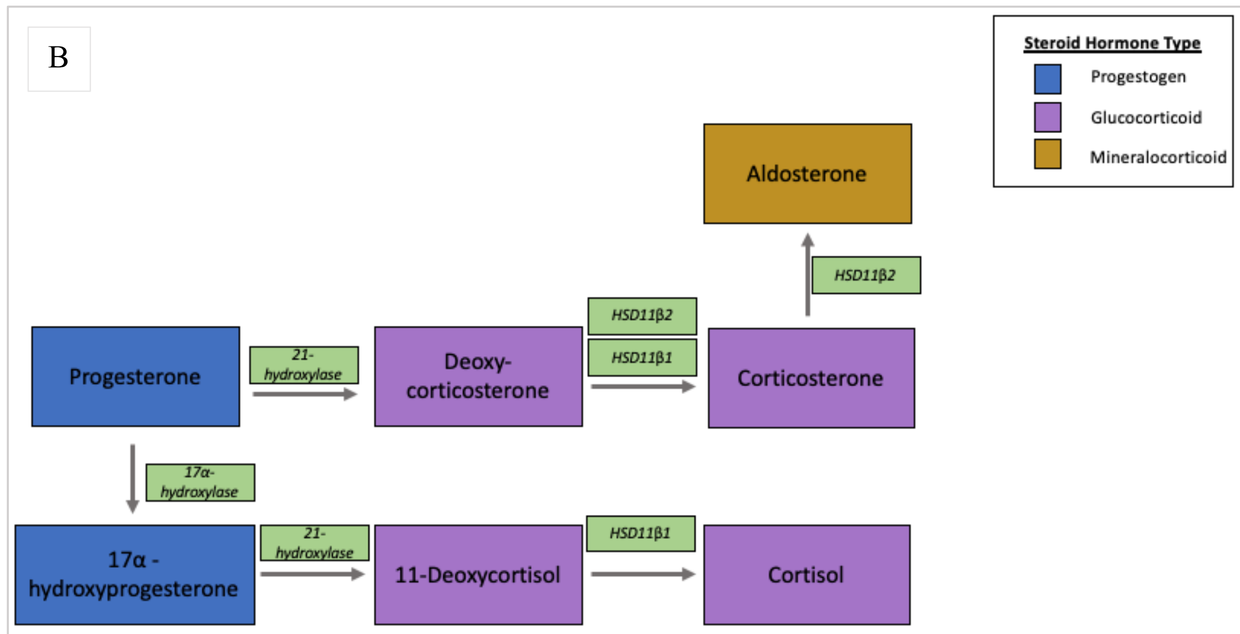
1.2.2 Steroid Hormone Biosynthesis: An Enzymatic View

As noted above steroid hormones are derived from cholesterol. Figure 1.3 summarizes the synthetic process. In brief, cholesterol is first transfer by the steroidogenic acute regulatory (StAR) protein, from the outer to the inner mitochondrial membrane, after which its side chain is cleaved, catalyzed by the cholesterol side chain cleavage enzyme (p450scc, aka CYP11A1), to generate pregnenolone (a progestogen). Following its synthesis, pregnenolone is then exported to the smooth endoplasmic reticulum (ER), where steroidogenesis continues, aided by a host of endogenously expressed catalytic enzymes) (Haggstrom et al 2014).

To date, limited direct quantification of steroidogenic enzymes has been performed in ocular tissues. Schirra et al (2007) detected mRNA transcripts of steroid sulfatase, 3β-hydroxysteroid dehydrogenase (HSD) B1 and 17β-HSD types 1 and 3 in human cornea. Others have detected transcripts of 3β-HSD, aromatase, P450 side chain cleavage (P450scc) enzyme, 5α-reductase and 17,20 lyase in retina (Guarneri et al 2007, Cascio et al 2015, Ishikawa et al 2014). Expression aromatase has also been detected in the nearby retinal pigment epithelium (RPE) and choroid in rat (Ishikawa et al 2014). In spite of these limited gene expression data, other experiments lend strong support for the presence and activity of steroidogenic enzymes in the eye. For example, Guarneri et al (1994) quantified a variety of progestogens and progestogen metabolites in rat

retina, suggesting local metabolism of these hormones. These authors also provided evidence of local hormone synthesis; specifically, treatment of samples with lovastatin, an inhibitor of cholesterol biosynthesis, prevented formation of pregnenolone in their retinal cultures, while treatment of samples with mevalonolactone promoted the formation of pregnenolone. In another study involving adult retinal explants, Cascio et al (2007) characterized the conversion of progesterone to testosterone. These studies follow a much earlier radiolabelling study by Ploc et al (1978), who demonstrated the interconversion of various androgens in bovine corneal epithelium, i.e. testosterone to androstenedione and dihydrotestosterone (DHT), and androstenedione to testosterone and other (unidentified) metabolites.





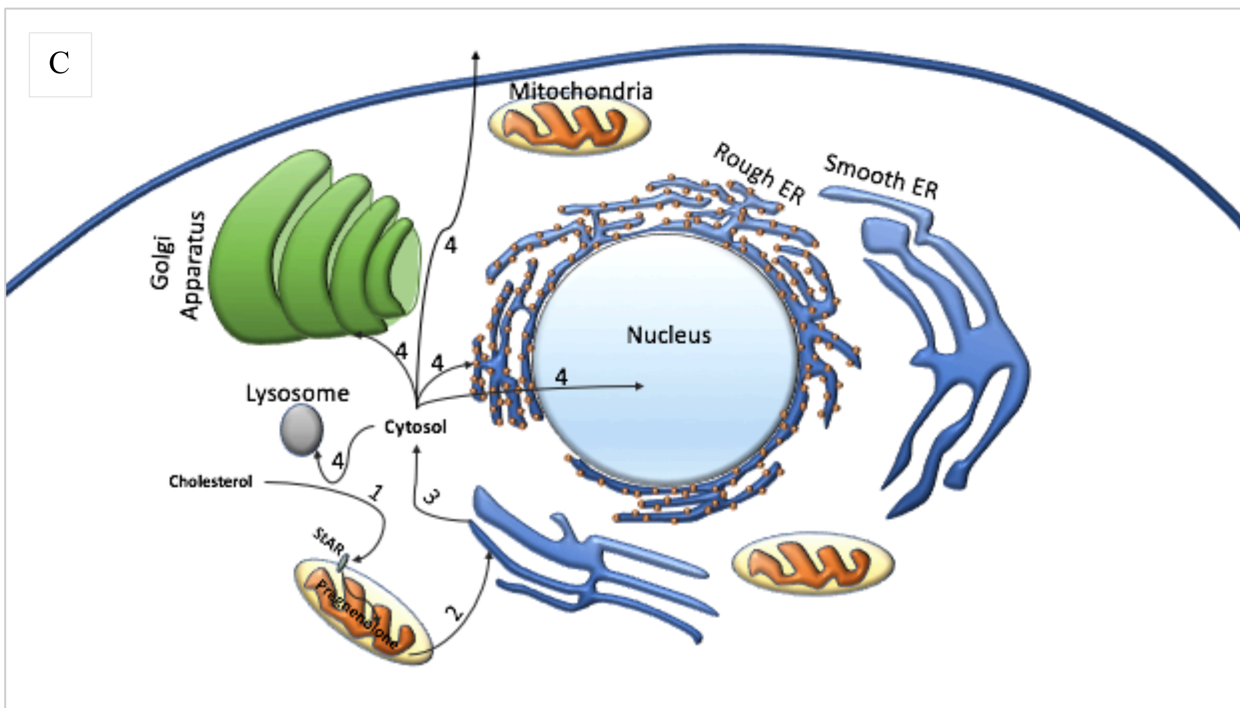


Figure 1.3 Steroid biosynthesis: an enzymatic and cellular organelle view . (A) Sex steroid hormones and (B) "non-sex" steroid hormones are all derived from cholesterol.Various steroidogenic enzymes work in concert to convert one steroid hormone into another. Once sythesized, further control over steroid trafficking and activity is mediated by a variety of sulfotransferases and steroid sulfotases, as well as gluconyryl transferase, which can be found in a variety of cellular organelles and the cytoplasm, at least in higher primates. From a cellular organelle prospective (C) Steroid biosynthesis and processing by subcellular location. Cholesterol is first imported into the mitochondria, via membrane-bound StAR protein, and then converted into pregnenolone (1). Pregnenolone is then exported to the smooth ER (2), for conversion via steroidogenic enzyme catalyzed reactions, to any of a number of steroid hormones, which are then exported to the cytosol (3), and then either out of the cell or into any number of cellular organelles for further processing or participation in receptor-mediated processes (4).

1.2.3 Spatial Steroid Hormone Biosynthesis, Trafficking and Signaling

Steroid hormones are made in a variety of tissues throughout the body. Some are synthesized in gonadal or adrenal tissues, and then distributed via circulation to distal sites of action. Others are synthesized locally and utilized locally. Below, these various modalities of sex hormone biosynthesis, transport and signaling are discussed.

1.2.3.1 Endocrine

Endocrine signaling – Sex Steroid Hormones: This signaling pathway is the best known, with gonadal tissues, namely the testis (Leydig cells) and ovaries (Theca cells), being the main sites of androgen, estrogen and progestogen synthesis. Pulsatile signaling within the hypothalamus represents the first step in the activation of the hypothalamus-pituitary-gonadal or HPG axis, triggering gonadotropin releasing hormone (GnRH) secretion from GnRH neurons in the hypothalamus. Subsequent binding of GnRH to receptors in the anterior pituitary promotes the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into general circulation. Binding of LH to receptors on Leydig and Theca cells leads to an upregulation in expression of the StAR protein, and in turn, increased transfer of cholesterol to inner mitochondrial membranes and initiation of enzyme-mediated steroidogenesis (Rotstein et al., 2012).

Two of the principal hormones synthesized by the above route, testosterone and estrogen, both complex with sex hormone-binding globulin (SHBG), and to a lesser degree, with serum albumin upon entering circulation (Somboonporn et al 2004). Progesterone on the other hand complexes with transcortin, a corticosteroid-binding globulin (Misao et al 1999). Note however, in all cases, a small percentage of these hormones remain unbound and thus in an active state. Via circulation, these bound (and unbound) steroids are able to reach distal site(s) of action, where the hormones dissociate from the parent globulin and either diffuse across the target cell's membrane to reach an intracellular site of action or bind to transmembrane receptors spanning the same. Alternatively, liganded SHBG can complex with SHBG-R to effect signaling.

Endocrine Signaling – "Non-Sex" Steroids Hormones: The hypothalamic-pituitary-adrenal (HPA) axis also determines basal circulating glucocorticoid concentrations, by controlling the synthesis of the neuropeptides, corticotropin-releasing hormone (CHR) and arginine vasopressin, in the hypothalamic paraventricular nucleus. These neuropeptides, in turn, stimulate the release of adrenocorticotropic hormone (ACTH), which is exported into systemic circulation. Its subsequent binding with ACTH receptors in the adrenal glands triggers the release of glucocorticoids into circulation, as either free molecules or complexed with corticosteroid-binding globulins, transcortin or serum albumin (Laue and Cutler, 1997). Mineralocorticoids are likewise released from the adrenal glands in response to a renin-angiotensin cascade (Taves et al., 2011).

1.2.3.2 Intracrine

The adrenal glands also contribute significantly to circulating glucocorticoids and mineralocorticoids via a second, so-called intracrine signaling pathway (Labrie et al 2000). In this case, release of ACTH from the pituitary gland stimulates the synthesis in the adrenal glands of dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S), which subsequently diffuse into circulation to be taken up by distal targets (Fig. 1.5). There, they undergo further transformation by local steroidogenic enzymes, for example, into other androgens, progestogens, estrogens, glucocorticoids and mineralocorticoids.

The important contributions of extra-gonadal sites as sources of sex hormones has only been relatively recently recognized. For example, while postmenopausal women are known to rely entirely on extra-gonadal hormone synthesis as the source of sex hormones (Labrie et al 2000), it is estimated that women synthesize 75% of their estrogen locally prior to menopause (Gibson et al, 2018). Likewise in men, 30-50% of androgen is estimated to synthesized locally (Gibson et al, 2018).

The levels of bioactive hormones and thus their activity are also under local control, via intracellularly-expressed glucuronyl- and sulfo-transferases (Fig. 1.3). These enzymes are responsible for inactivating excess intracellular steroid hormones, limiting the diffusion of active ligands out of cells and thus off-target effects (Labrie et al 2017). In the case of sulfonated sex hormones, this process can be reversed, with the removal of the sulfate functional group by cellularly-expressed steroid sulfatases.

1.2.3.3 Autocrine and Paracrine

Complete local synthesis of steroid hormones, i.e. from cholesterol, represents an alternative mechanism for achieving high tissue and cellular spatial specificity. Cells vary in their capacity to express the necessary steroidogenic enzymes to have this capability. Some cells express the full complement of steroidogenic enzymes, allowing them to synthesize steroid hormones for their own use, the so-called autocrine mechanism. In other cases, cells express only a partial compliment of these enzymes, with neighboring cells being responsible for converting synthesized sex hormone intermediates into the active product/hormone, the so-called paracrine mechanism (Fig. 1.5). There are many examples of these local mechanisms in operation throughout the body, including in the retina of the eye (Cascio et al., 2015).

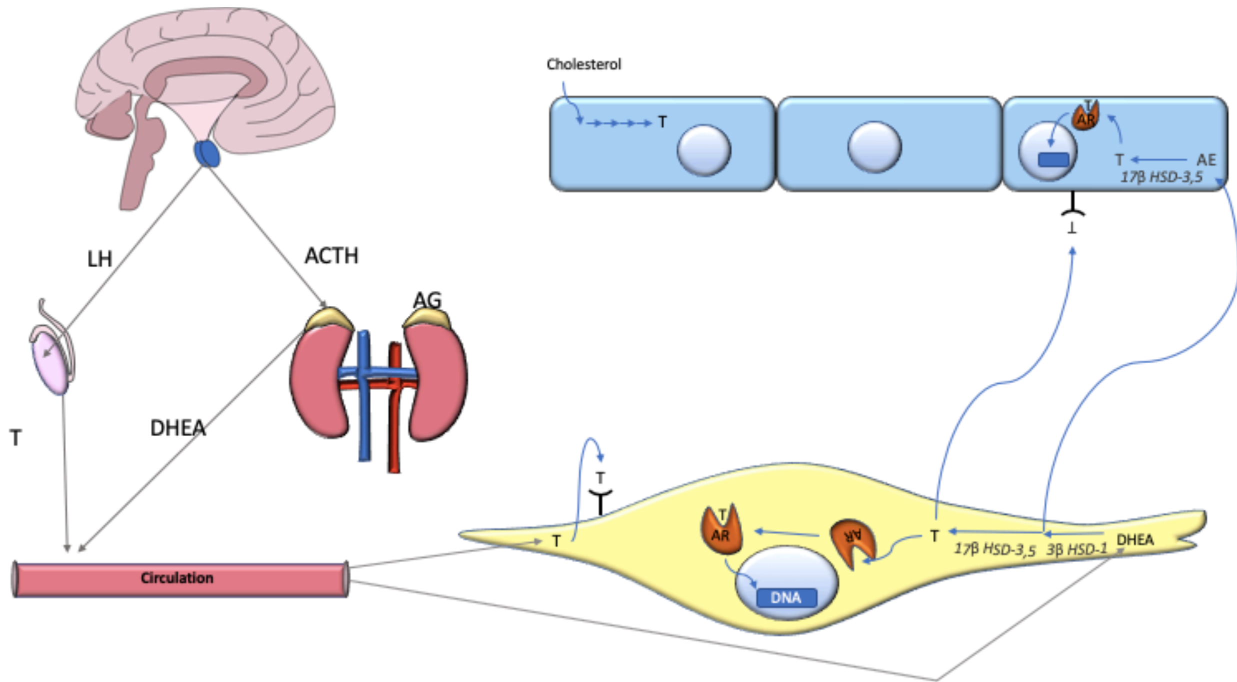


Figure 1.4. Schematic representation of alternative endocrine, intracrine, autocrine and paracrine mechanisms for sex hormone biosynthesis and signaling, using testosterone as an example, synthesized either in the testes, or at distal sites from DHEA released by the adrenal glands into circulation. Abbreviations are as follows: leutinizing hormone (LH), adrenocorticotropic hormone (ACTH), adrenal gland (AG) dehydroepiandrosterone (DHEA), epiandrosterone (EA), Androgen Receptor (AR), Testosterone (T) and hydroxysteroid dehydrogenase (HSD). The yellow and green cells represent a fibroblast and endothelial cells, respectively, though the concept being illustrated is not limited to these two cell types.

1.2.4 Variability: Hormonal Cycling, Age and Sex

Hormone cycling refers to changes in circulating levels, and takes many forms, including purely temporal (e.g. time of day & month/season of year), versus age and sex-dependent (i.e. developmental stage of life, e.g. puberty, menopause, post-menopause). Hormonal cycling has significant implications for *in vivo* research studies, with interpretation of results being potentially influenced by the design of experiments, including the age of subjects and the timing of sampling.

As a general rule, prior to puberty, sex hormone serum levels are (in relative terms) low (Delemarre-van de Waal et al., 2001, Strauss and Barbieri, 2009). As both boys and girls approach puberty serum estrogen levels rise, and eventually peak upon entering puberty. In women estrogen levels then cycle with menstruation, reach an absolute apex during pregnancy, and bottom out post menopause (Strauss and Barbieri, 2009). Likewise, in both males and females, testosterone levels increase around puberty and peak at around 18 – 20 years of age. In males, these levels then start to decline at 30 years of age at a rate of approximately 1% per year (Stanworth et al., 2008, Wang X et al., 2005).

1.2.4.1 Menstrual Cycle

The menstrual cycle is a cycle that women undergo, on a monthly basis, during their reproductively fertile years. Necessary for oocyte production, menarche – on average - starts at about 12.5 years of age in the US (Anderson et al., 2003); a number which represents a historical decrease relative to centuries past (Boaz et al., 1999). During the menstrual cycle, consistent cycling of endogenous circulating sex steroids occurs. Between days 12 and 14 of the menstrual cycle, circulating estrogen levels peak. This is shortly followed by concomitant peak in testosterone levels. Sex steroid levels then dip again until a second period around day 21 & 23 when progesterone and estrogen levels peak temporarily once again (Strauss and Barbieri, 2009).

Inherent to this cycling is a large degree of inter-individual variability. For example, on average, a single cycle is 28 days in duration. Cycles that are between 24 and 38 days, however, are quite normal (Dasharathy et al., 2012). And it is likewise normal for ovulation to occur anywhere between day 12 and 16 of one's cycle (Darsharathy et al, 2012). This is consistent with variability in the timing of the first peak in estrogen levels, during the follicular phase of the cycle.

During the latter part of life, women enter perimenopause, a transitional phase towards menopause and ensuing infertility. During this transition, ovulation becomes less frequent and progesterone levels, during the luteal phase, decline (Prior et al 1998). At menopause ovaries cease estrogen & progesterone production, leading to complete reliance on non-gonadal tissues for their synthesis (Laurie et al., 2000). As one would expect, this results in lower net estrogen and progesterone levels systemically. The age at which both perimenopause and menopause start, varies greatly between individuals. While typically starting in one's forties, it is not irregular for perimenopause to start in one's thirties. Likewise, while the average age of menopause is 51 years of age, for the majority of women it starts when they are between 45 and 55 years old (Gold 2011, Sievert 2006, Varea et al., 2000).

1.2.4.2 Diurnal Sex Hormone Cycling

Just as females display a monthly cycling of estrogen levels, during the menopausal years, there is also a well-established diurnal testosterone rhythm that occurs in adult males on a daily basis. Here, serum testosterone levels peak in the morning and diminish as the day progresses. These levels then rise again in the late night and throughout the early morning (Bremner WJ et al 1983). This pattern is particularly pronounced in younger adult males. As males age and total testosterone levels decline this pattern becomes less pronounced. Interestingly, further data from salivary sample collections show that women display a somewhat similar pattern (Bremner WJ et al, 1983). Likewise, menopausal women have been shown to display repetitive diurnal estrogen cycling (Bao et al 2003).

1.3 Steroid Hormone Receptors: Types and Modes of Regulation

Steroid hormones bind a variety of nuclear hormone receptors (NRs), G protein-coupled receptors (GPCRs) and a novel cell surface receptor, the sex hormone-binding globulin cell surface receptor (SHBG-R). Neuroactive steroids have further been found to also interact with a

variety of ion channels (e.g. $GABA_A$, NMDA and Sigma receptors). As experiments described in this dissertation were limited to NRs and GPCRs, the discussion below is also so limited.

1.3.1 Steroid Hormone Receptor Types

1.3.1.1 Nuclear Receptors

In the classic model of steroid hormone-receptor signaling, NR's are endogenously expressed and localized to the cytosol, where they complex with heat shock proteins (HSPs)(Smith et al 2008), until binding with the cognate steroid hormone causes a confirmation shift in the NR and subsequent dissociation of bound HSPs. Liganded NRs translocate via nuclear pore complexes (NPC) into the nucleus as monomers, homodimers or, less commonly, heterodimers (e.g. ERα-ERβ heterodimer), and once there, the liganded NRs bind to their target DNA sequence and recruits co-regulatory proteins to either initiate transcription (transactivation) or terminate it (transrepression). These processes are summarized pictorially in Figures 1.13 and1.14. Also as evident from inspection of Figure 1.13, the model just described represents a simplified version of what in reality is far more complex and nuanced.

The focus of attention in experiments described in this dissertation were six steroid-liganded nuclear hormone receptors – Androgen Receptor (AR), Estrogen Receptor α (ERα), Estrogen Receptor β (ERβ), Progesterone Receptor (PR), Glucorticoid Receptor (GR) and Mineralocorticoid Receptor (MR), with a G protein-coupled estrogen receptor (GPER) being the one exception.

1.4.1.2 G-Protein Coupled Receptors

More recently, a number of GPCR's have also been found to bind to and mediate sex hormone signaling. These receptors are thought to be especially important in mediating rapid, non-genomic (transcription-independent) responses to steroid hormones. Specifically targeted for study in this dissertation research was the G protein-coupled estrogen receptor-1 (GPER or GPR30), which to-date, is the only well characterized membrane-associated receptor known to selectively bind estrogen with high affinity. It is classified as a rhodopsin-like GPCR, because of the seven transmembrane helices that span the endoplasmic reticulum (ER) membrane, although differing from most GPCRs, which are typically localized to the cell rather than ER membrane (Wang et al., 2014). Several other putative, membrane-bound receptors that appear to bind estrogen with high affinity have also been described, They include the estrogen receptor-X (ER-X) (Toran-Allerand et al 2002), estrogen receptor-x (ERx) (Kampa et al., 2012) and the Gq-coupled membrane estrogen receptor (Gq-mER) (Qiu et al 2006, Micevych et al 2012). Their characterization is ongoing and for this reason, they were not targeted for study in dissertation research. Nonetheless, their existence does highlight an important concept. Approximately one-third of class A GPCRs contain an amino acid consensus sequence that in the past was thought to encode a cholesterol-binding motif (Wang et al., 2013). It is now speculated that this sequence may enable sex hormones to interact more widely with GPCR's than previously thought.

1.4.1.3 Receptor Regulation

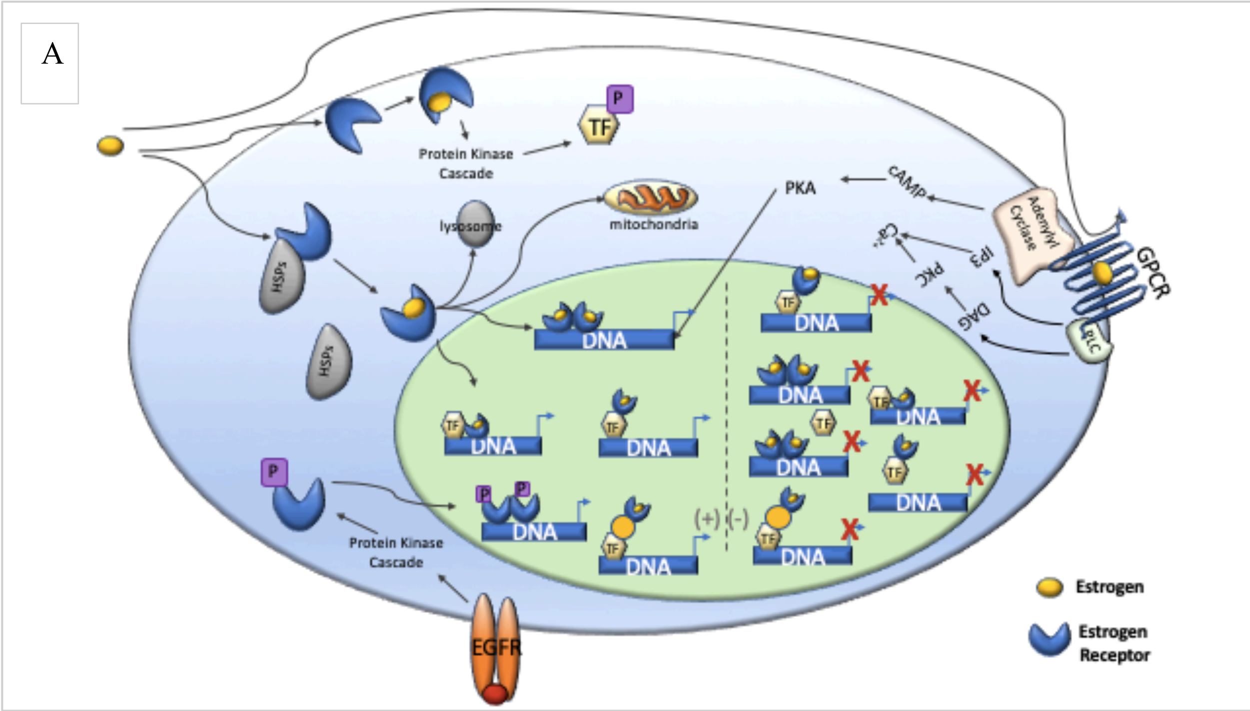
Ultimately beyond the scope of this dissertation, should readers decide to take research outlined herein further it would be extremely prudent to consider alternative splicing and post-translational modification. In the case of the former alternative splicing facilities the synthesis of multiple receptor isoforms during the transcriptional process. This allows for modulation of receptor function, activity and localization, following translation. For example AR isoforms with partial or full LBD deletions are typically constitutively active. And alternative splicing of the receptor's N-terminal poly-glutamine and poly-glycine stretches, result in the modulation of AR functionality via variability in (splice-form dependent) transactivation reactions (S Laurentino et al 2012). In the case of the latter, proteins are modified post-translationally with chemical moieties that allow for control over the sub-cellular localization, stability, protein-protein interactions and activity (Hany A Abdel-Hafiz et al, 2014).

1.4 Steroid Hormone Signaling & Cascades

Steroid hormone receptors (both liganded and unbound) are capable of signaling through a wide variety of mechanisms and pathways. However, these mechanisms can be categorized broadly as either genomic (Fig. 1.14) or non-genomic (Fig. 1.15) in nature.

In the case of the former, responses develop over relatively long periods of time. Sex hormone receptors provide such an example. The ligand-bound receptors must first translocate to the nucleus, where they regulate gene transcription and downstream protein translation in concert with co-regulatory proteins (co-activators and co-repressors). The generation of a physiological output is itself dependent on transcription, translation and a cascade of intra- and inter-cellular events, and possibly many multiples of iterations of the same. Thus it may take hours to days from the time of entry of sex hormone ligands into a cell to realize a relevant physiological output.

In the case of non-genomic signaling, responses are generated much more rapidly, on the order of seconds to minutes. These signaling pathways typically involve intermediary kinase cascades (commonly MAPK, IP3K and various tyrosine kinases), more often than not, involving steroid-liganded ion channels and GPCRs, with outputs as variable and disparate as vasodilation and neuronal survival. Because these mechanisms do not involve sequential iterations of transcription and translation, response times tend to be quite short (Laurentino et al., 2012 & Losel and Wehling, 2003).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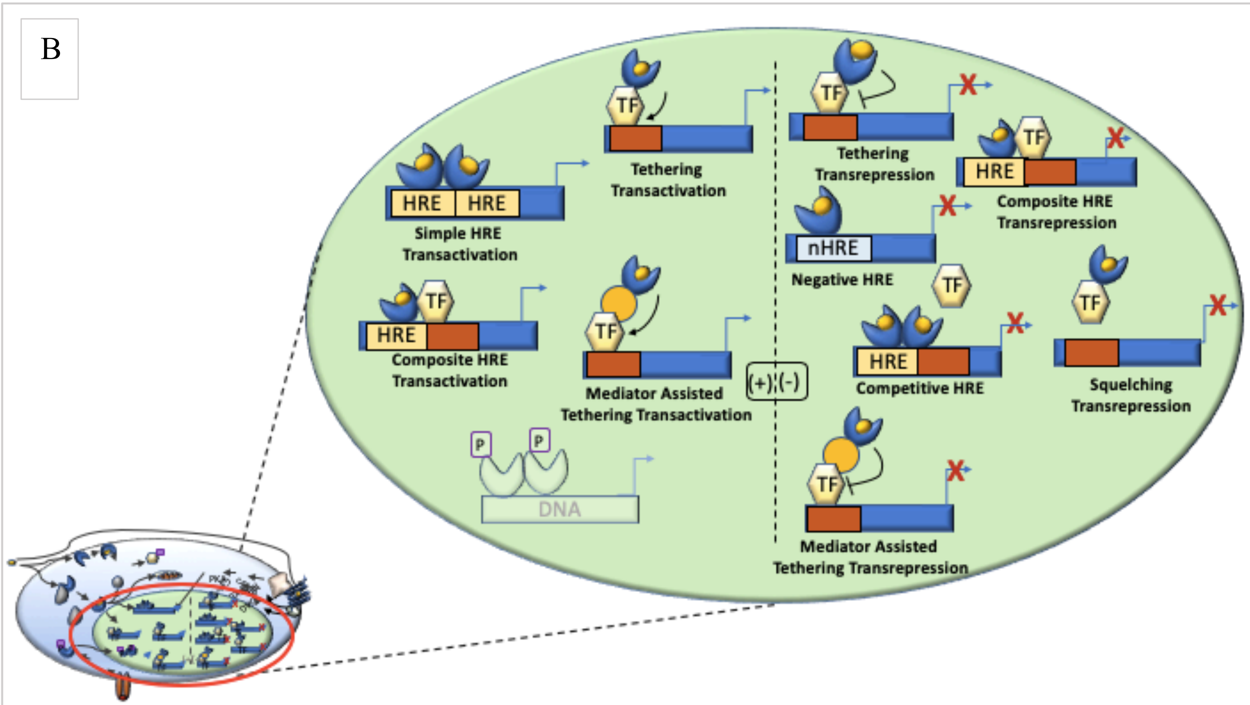
B
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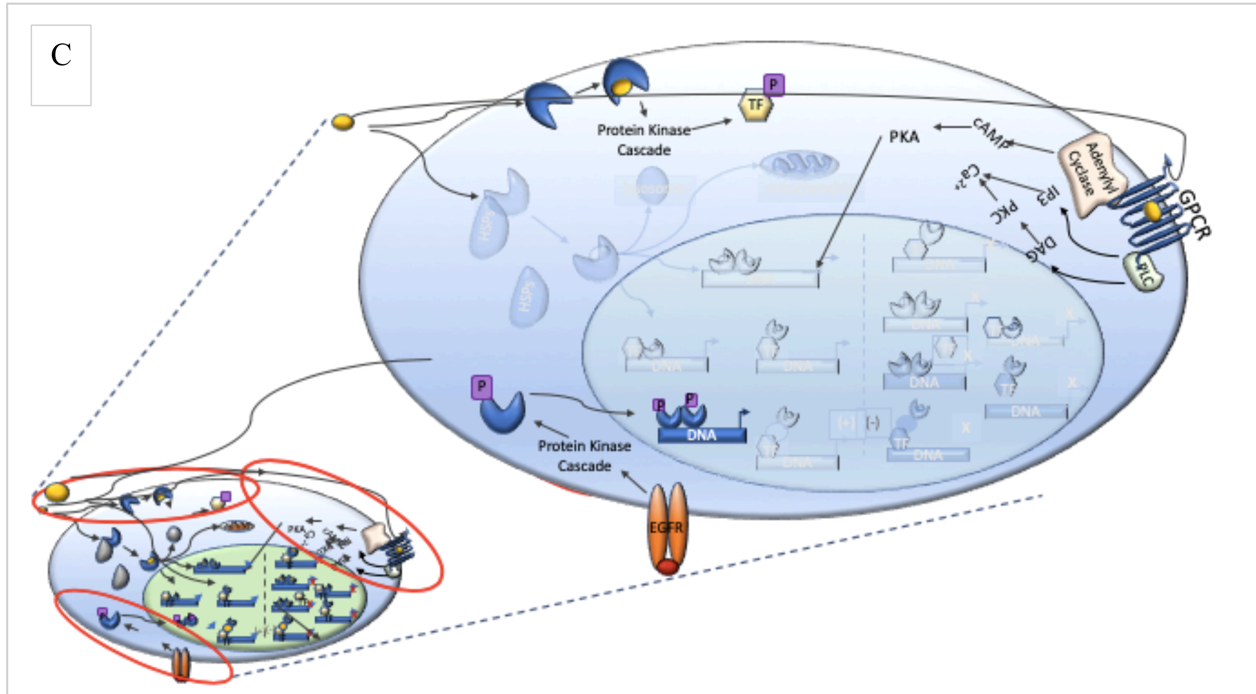


Figure 1.5 Genomic and non-genomic mechanisms of steroid hormone signaling, as exemplified by estrogen and its receptors. (A) Provides a general overview. During (B) genomic steroid hormone signaling, key events are localized to the cell nucleus, as summarized schematically. In contrast (C) non-genomic mechanisms of hormone signaling occure when key events are localized to the cell membrane and/or cytosol, as summarized schematically. Abbreviations: Phosphoryl group (P), non-steroid receptor Transcription Factor (TF), heat shock proteins (HSPs), cyclic adenosine mono-phosphate (cAMP), protein kinase A (PKA), protein kinase C (PKC), Inositol triphosphate (IP3), diacylglycerol (DAG), calcium ion (Ca^{+2}), Epidermal Growth Factor Receptor (EGFR) and G-Protein Coupled Receptor (GPCR), Hormone Response Element (HRE) & non-nuclear receptor Transcription Factor (TF), negative HRE (nHRE), no transcription (X). The orange box indicates a non-HRE DNA binding site, the gold circle, a generic intermediary protein of unspecified identity.

A further way of categorizing relevant receptor-mediated signaling is whether or not they participates in ‘crosstalk.’ Molecular crosstalk occurs when one (or more) components of a signal transduction cascade affect another cascade (Lange CA, 2004, Cottlicher et al., 1998).

Lastly, sex hormones receptors have been found to influence the strength of their own signaling via participating in feedback loops, both positive and negative (Thomas et al 2008; Alimirah et al, 2006).

1.5 An Integrated View: Steroid Hormones, Their Receptors, The Eye & Myopia

The important role that sex hormones and their receptors play in regulating a variety of processes, both normal physiological and pathological, in the eye is just starting to be fully appreciated. Research to-date in this area is still relatively nascent, however, and minimal in the context of myopia.

In the following sections, findings from the limited studies probing the links between steroid hormones, their receptors, and normal ocular health as well as ocular pathologies, are reviewed. The link between various endocrine factors and myopia will also be examined, as the limited literature on this topic allows, with logical inferences being made from other relevant ocular and and as appropriate extraocular studies, as background to the research presented in this dissertation. A tissue-centric approach is followed, with review, in a stepwise fashion, of each ocular tissue layer, starting with the cornea at the very front of the eye and ending with the sclera.

1.5.1 Cornea

As the most anterior, exposed ocular tissue, the cornea is multilayered, ECM-rich, largely avascular and transparent, absent insult (Fig. 1.17). Its primary function is to refract incoming light, as one of two optical components of the imaging forming system of the eye, the retina being the other. In this role, the cornea provides about two-thirds of the eye's refracting power (Meek et al., 2015). It also performs a secondary immunological function, with resident innate immune macromolecules;, at times, it also hosts cells of the adaptive immune system. Anatomically, the cornea can be subdivided into three cellular layers: the epithelium, stroma and the endothelium.

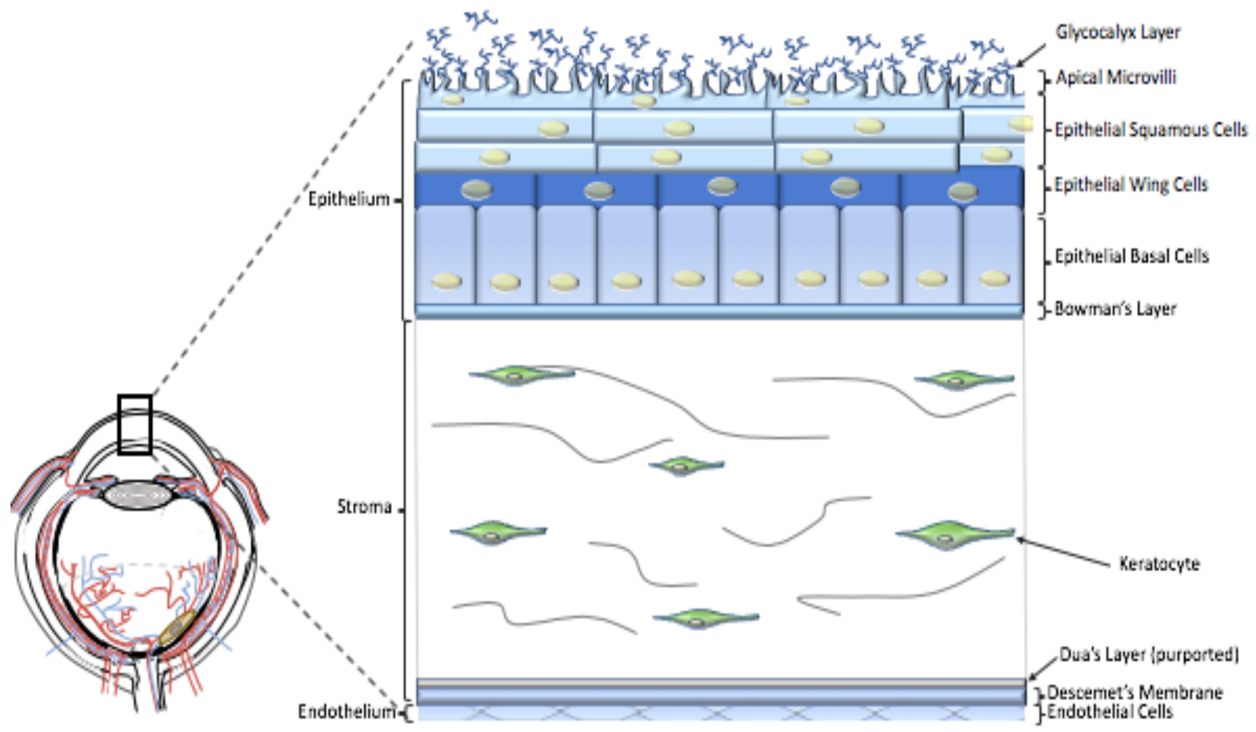


Figure 1.6 Schematic representation of the human cornea, which includes three cellular layers, the middle stromal layer being the most sparsely populated, with specialized fibroblasts (keratocytes), embedded in a highly organized collagen-dense matrix, sandwiched between a multi-layered epithelium anteriorly and a single layer of endothelial cells posteriorly..

1.5.1.1 Corneal Epithelium

The most anterior cellular layer of the cornea, the corneal epithelium, is composed of a single layer of basal cells, overlaid by two to three layers of stratified, supra-basal, wing cells. These are in turn are overlaid with two to three layers of squamous, polygonal, superficial epithelial cells connected through tight junctions. This network of epithelial cells offers a protective barrier to external pathogens, it also plays an important role in the maintenance of the cornea's optical clarity through influences on the ion and water balance of the underlying stroma, as well though interactions with the overlying tear film. Nerve endings in the deeper layers of the epithelium, which represent projections from the trigeminal nerve, represent the sensory arm of a protective blink reflex against mechanical and chemical damage.

Comparatively little endocrine-centric research has been done on the corneal epithelium. However, androgen, progesterone and estrogen (both α and ß) receptors are all reported to be expressed in human corneal epithelial cells (hCEC) isolated from both male and female donor eyes (Suzuki et al., 2001), with differential expression in a pathology-dependent manner (e.g. in keratoconus) (Ayan et al., 2018). Estrogen has also been found to negatively regulate (i.e. delay) epithelial wound healing, following removal of corneal epithelial cells in an *in vivo* mouse model (Wang et al., 2012). Furthermore, Suzuki and Sullivan (2006) found estrogen to decrease IL-6 and -8 gene expression in primary corneal epithelial cell cultures, although they found no effect of estrogen on either IL-1b or matrix metallopeptidase (MMP2 or MMP9) production in the same cell line. While the same authors found similar negative results were obtained with SV40 immortalized hCECs, interestingly Wang et al. (2012) found that treatment of SV40 hCECs, with estrogen decreased not only IL-6 production, but also that of IL-1 and TNF-α.

In still other studies, 5α-reductase (type 1 and 2) gene transcripts (enzymes responsible for catalyzing the conversion of testosterone to dihydrotestosterone, i.e. DHT) were found to be expressed in human corneal epithelium (Rocha et al., 2000), and DHT, working through androgen receptors, was found to drive expression of mucin type 1 (MUC1) and activate Wnt signaling (Qin et al., 2016), Thus, there is strong evidence that sex hormones are expressed and act within the corneal epithelium to regulate a variety of biological processes.

1.5.1.2 Stroma

The corneal stroma is the thickest layer of the cornea, accounting for approximately 90% of its cross-sectional width (Fig. 1.17). The stroma is primarily composed of collagen, organized into uniform, multi-layered structure, which is critical to the need for transparency of this refracting element, as well to the cornea's protective role in preventing pathogens from infecting the eye. Other key elements include proteoglycans and a sparse population of keratocytes, a specialized corneal fibroblast responsible for the maintenance of the stroma's ECM.

The vast majority of work regarding sex hormone regulation in the cornea, to-date, has been performed in the corneal stroma, with most research being focused on the link between sex hormones, phases of the menstrual cycle and corneal thickness. Specifically, Leach et al. (1971) observed that - on average - corneal thickness peaks just prior to ovulation and that this thickening parallels the bimodal spiking of plasma estrogen in the menstrual cycle. In other words, higher estrogen levels positively correlate with thicker corneas. Providing support to

these findings, Kiely et al. (1983) observed that corneas reach peak thickness around the time of ovulation as well as on day 21 of the cycle, corresponding to when serum estrogen is most highly elevated. While findings from some later studies appear to contradict these findings, e.g., by Giuseppe et al. (2007) and Soni (1980), the latter studies also suffer from methodological design short-comings related to the timing of measurements and small sample sizes. Given that hormone levels can spike acutely and for relatively short periods across the menstrual cycle, outcomes can vary significantly, depending on the timing on measurements.

Lending further support to a role for estrogen in maintenance and remodeling of the corneal stoma, Keskin et al. (2009) observed that central corneal thickness (CCT) decreases in postmenopausal women. They further found this decrease in CCT to be positively and linearly correlated with the postmenopausal drop of serum estrogen levels. Additionally, Aydin et al. (2009), found that post-menopausal decreases in serum estrogen correlate with a steepening of the horizontal corneal curvature. In light of these findings it is interesting, but perhaps unsurprising, that hormone replacement (dual estrogen + progestin derivative) therapy (HRT) has been found to increase corneal thickness in post-menopausal women (Affinito et al., 2003). Likewise, treatment of pre-menopausal women of childbearing age with an estrogen + progestin derivative-based contraceptives prevented the cyclical thinning and thickening of cornea normally observed in the menstrual cycle (Soni, 1980). The same author also noted that participants treated with contraceptives containing higher doses of progestin realized an earlier stabilizing effect on corneal thickness, relative to those participants exposed to lower doses of progestin. These findings thus suggest that both hormones (estrogen and progestin) have roles in corneal stromal maintenance.

By what mechanism(s) might this maintenance occur? Tachibana et al. (2000) found that both estrogen receptors-α and -ß are expressed throughout the cornea. More specific immunohistochemistry (IHC) antibody staining revealed their presence in the nuclei of corneal epithelial, stromal and endothelial cells in male and female mouse. Zhou et al. (2011) further found that both 17ß-estradiol and progesterone inhibited interleukin (IL) – 1ß-induced collagen degradation by corneal fibroblasts, in a concentration-dependent manner. The same authors also found treatment with testosterone and DHEA had no effect. That estrogen and progesterone serve to prevent collagen degradation in the corneal stroma is supported by other recent finding by Hongbo et al. (2018) who found that treatment of cultured corneal fibroblasts with estrogen down-regulated the expression of MMP2 (although not MMP9), a critical proteinase involved in collagen degradation. On the other hand, Phillips et al. (1983) found that progesterone, as well as some glucocorticoid analogs, inhibited collagen synthesis following stromal injury in a model of wound healing. Thus, progesterone may not only work to inhibit collagen degradation, but it may also work to inhibit its deposition in response to injury. Thus it can be argued progesterone acts to maintain stromal structural homeostasis in the cornea, through its dual ability to prevent both collagen degradation and synthesis.

Steroid hormones are known to play diverse roles, even within a single tissue. Thus in the cornea, their activity is not solely one of regulating ECM remodeling. For example, both estrogen and DHEA, an androgen, appear to regulate metabolic pathways in corneal fibroblasts (McKay et al., 2017). Specifically, estrogen was found to increase the synthesis of both glycolytic and pentose phosphate pathway intermediates in cultured primary human corneal

fibroblasts. In contrast, treatment with DHEA decreased basal levels of pentose phosphate flux in the same cells, while down-regulating the expression of insulin growth factor 1 (IGF-1) and its endogenous receptor.

In the context of myopia, findings for corneal stroma are of potential most relevance, because of its structural similarity to the sclera, which undergoes substantial remodeling and thinning in the course of myopia development and progression.

1.5.2 Ocular Crystalline Lens

The ocular lens is a bi-convex structure, positioned directly behind the iris. Largely made up tightly packed lens fibers, supported by an anterior layer of epithelial cells, which are also the source of new lens fibers, its function is optical in nature. With the assistance of ciliary muscles, which are under neural control, its shape can be adjusted to images of near objects into focus on the retina, a process known as accommodation.

To date, the vast majority of lens-related research exploring the effects of sex-hormones and their cognate receptors has been in the context of cataracts, for which sex-related difference in prevalence rates have been noted. Specifically, women appear more prone to develop cataracts then their male counterparts, and this sex-linked disparity in prevalence is most prominent in postmenopausal women (Zetterberg and Celojevic, 2015). Mounting experimental evidence suggests a rather complicated relationship between estrogen and this pathology. Specifically, it appears to be the active withdrawal of estrogen that contributes to cataract formation, rather than simply low levels of estrogen as one might expect based on elevated rates of cataracts in post-menopausal females. Thus in experimental mouse-based studies, cataract formation was prevented in female subjects ovariectomized prior to reaching sexual maturity (Davis et al., 2002). The same study further demonstrated that animals treated with diethylsylbestrol (DES), a potent synthetic estrogen, as neonates and irrespective of their sex, later developed cataracts. It is therefore interesting, yet perhaps unsurprising, that hormone replacement therapy (HRT) offers some protection against nuclear and posterior sub-capsular cataracts in postmenopausal women (Freeman et al., 2001).

Towards a molecular and cellular explanation for the above effects, gene transcripts for the estrogen receptors-α and -ß, as well as G-protein estrogen receptor 1 (GPER), have been found in both human and mouse ocular lens (Kirker et al., 2013). It has been hypothesized that estrogen confers protection against cataract formation by preserving the proper functioning of mitochondria. In models of oxidative (i.e., peroxide-induced) stress, estrogens prevent rapid mitochondrial depolarization and maintain mitochondrial membrane potentials (Moor et al., 2005). This protective effect appears to be at least partly non-genomic in character; specifically, estrogen both drives ERK1/2 phosphorylation and positively regulates MAPK (Moor et al., 2005), with both actions serving to stabilize mitochondrial membrane potentials and prevent local depletion of adenosine triphosphase (ATP) (Moor et al., 2004). In still other studies, estrogen was found to increase in cultured hLECs the activity of superoxide dismutases (SOD), which are responsible for processing and neutralizing free radicals (Skilijic et al., 2018). Skilijic et al. (2018) also observed ERα expression levels to decrease, while ERß expression increased

with estrogen treatment. Of note in this context, Cammarata and colleagues (2004) found that while ERα and ß were present in the nuclei of lens epithelial cells (LECs), only the ß form of ER was present in the mitochondria. Also tellingly, as women enter menopause estrogen levels plummet, along with a concomitant rise in ERα expression and a decline in ERß expression (Skilijic D et al., 2018). Overall, these various observations point to a mechanistic explanation for the observed protective effect ("anti-cataract") of high steady state levels of estrogen.

Separately, estrogen has been found to prevent TGFß2-induced cataract formation (Chen et al., 2004) and shown to increase aromatase (but not sulfatase) activity in cataractous LECs (Loitz et al., 2015). Interestingly, but unsurprisingly, TGFß2 participates in crosstalk with the aforementioned ERK/MAPK/SMAD signaling pathways.

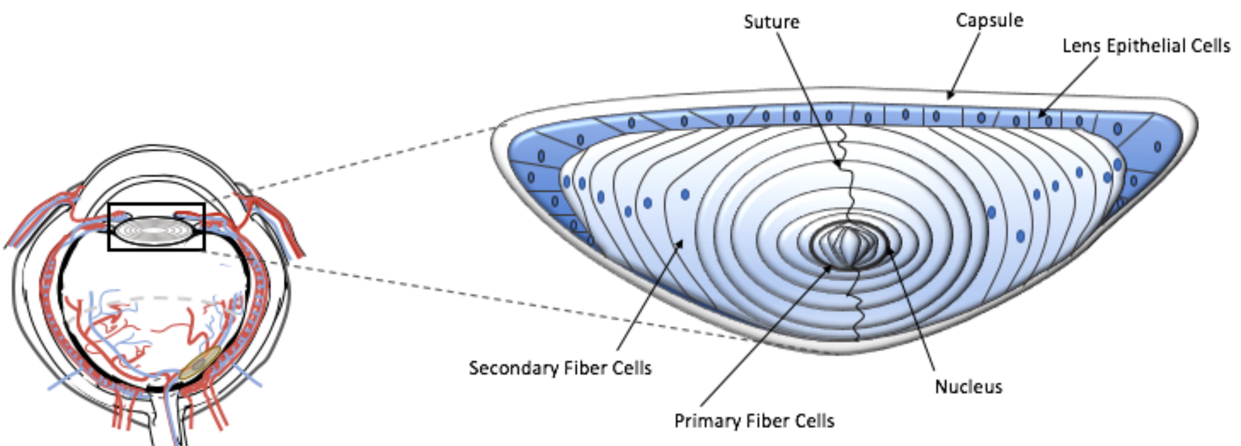


Figure 1.7 Schematic representation of the human crystalline lens, which is includes an outer elastic capsule, an anterior layer of epitthelial cells and layers of lens fibers, which are continuously generated throughout life, the newest fibers making up the outer shell (cortex), the oldest making up the center (nucleus).

While the above evidence suggests estrogen therapy as a plausible prophylactic strategy t protect against cataracts, such interventions would invariably have multiple effects, not only within the lens, due to the complex nature of estrogen signaling, but also elsewhere both within and outside the eye..

1.5.3 Retina

The retina is one of the most complex tissues in the human body. Neural in origin, in many respects it approximates the brain in micro. Its function is to register light that enters the eye, generating electrochemical signals that are subsequently relayed and further processed both within the retina and in vision-related areas of the brain. Anatomically, the retina is composed of a multilayered array of interconnected light sensitive cells and neurons, of which there are many subtypes (i.e. rod and cone photoreceptors, bipolar cells, ganglion cells, amacrine cells and

horizontal cells), as well as supporting glial cells (Müller cells, astrocytes and microglia). The human retinal is also vascularized, with branches from the central retinal artery (CRA) serving the inner retinal layers through a capillary network embedded in the inner retinal (ganglion cell layer and inner nuclear) retinal layers. The avascular retinal periphery and outer retinal layers, which include the photoreceptors, receive metabolic support via the choroid (Cascio et al., 2015).

As in the brain, sex hormones play neuroactive roles in the retina (Bucolo et al 2007; Cascio et al 2015). While the supporting vascular networks just described are likely sources of sex hormones for the retina, pan-retinal expression of cholesterol processing and steroidogenic enzymes are known to promote *de novo* autocrine and paracrine synthesis of steroids in the retina. To this end, the mitochondrial cholesterol side-chain cleavage enzyme, i.e. P450scc, is expressed in retinal ganglion cells (RGCs) and amacrine cells, where it catalyzes the conversion of cholesterol to pregnenolone (Guarneri et al., 1994). This catalytic reaction is stimulated via activation of $GABA_A$ receptors on these cells (Guarneri et al 1994; Guarneri et al 1995). Following its synthesis, pregnenolone is converted to progesterone by 3ß-HSD, which is widely distributed throughout cells of the retina (Mellon et al, 2001; Guarneri et al 1995). Progesterone is subsequently converted to androstenedione by P450c17, which shows a similar expression pattern to 3ß-HSD: under select conditions, pregnenolone is also converted to DHEA (Cascio et al, 2015). Also present in the retina are the enzymes necessary to convert androstenedione to testosterone, i.e., 17ß-HSD type 5, testosterone to dihydrotestostone (DHT), i.e., 5-α reductase type I and II, progesterone to allopregnanolone, i.e., 3α -HSD, and testosterone to estrogen, i.e., aromatase (Cascio et al, 2015). In summary the retina has the necessary enzymatic machinery to synthesize a wide range of sex hormones.

Predictably, given the capacity of the retina to synthesize a broad array of sex hormones, it also expresses most, if not all sex hormone binding receptors, including ERα, ERß, AR, PRa/b, the membrane proteins progesterone receptor membrane component proteins 1 and 2 (PRMCP-1 and -2), progesterone membrane receptors-α, -ß and -γ, and the novel G-protein coupled estrogen receptor (GPER/GPR30). While typically ligand activated, some of these receptors have been shown to be regulated in a ligand-independent manner via ERK1/2 mediated phosphorylation (Lucas TF et al, 2010). With regards to the estrogen receptors, Cascio et al found, in rat retina, ERα and ERß to show cell type-specific differential expression, with high levels of ERα expression in amacrine and ganglion cells and in the outer synaptic layer of the retina, while ERß was found to display more prominent expression in the inner synaptic layer.

Suffice to say, the retinal possess the cellular, molecular and chemical machinery necessary to detect and respond to endocrine input. With respect to the process of emmetropization and myopia progression, there is no direct evidence to suggest that steroid hormones influence pro-myopiagenic signaling. Although the latter possibility cannot be ruled out, simply put, relevant studies have not been done to-date. Nonetheless, there is ample evidence to show that dopamine-responsive neural elements are regulated by steroid sex hormones (Sotomayor-Zarate et al., 2014), and myopia in experimental models has been linked to depleted retinal dopamine levels (Stone et al., 1989). Thus could there be a link between steroid hormones, retinal signaling and myopia progression? There is also significant literature highlighting links between steroid hormones, neuroprotective effects and various ocular pathologies. Thus the possibility that sex

hormones play a role in the retinal pathology seen in high myopia, which is more prevalent in women, also warrants investigation.

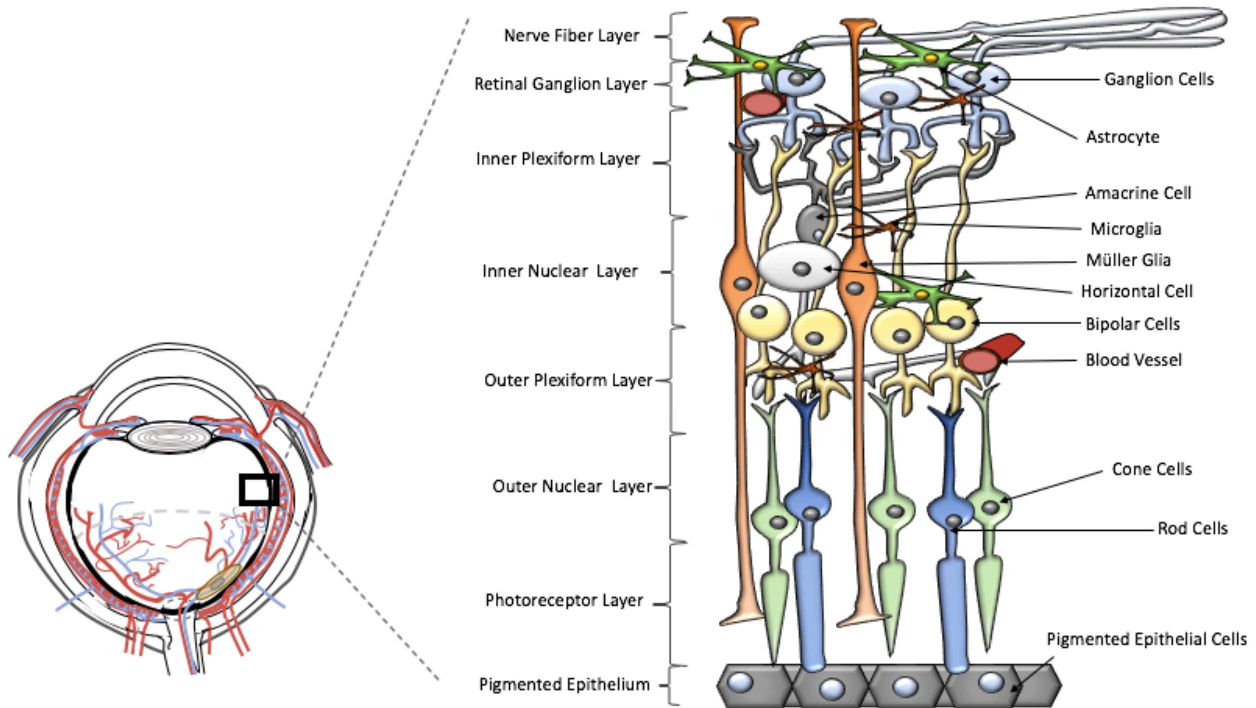


Figure 1.8 Schematic representation of the human retina, showing its cellular components, sandwiched between the outer retinal pigment epithelium layer and inner nerve fiber layer.

1.5.4 Retinal Pigmented Epithelium

A monolayer cuboidal cells, interconnected by tight junctions, the retinal pigment epithelium (RPE) sits between the choroid and photoreceptor cells of the retina. Its functions, numerous and integral to normal retinal function, include the storage and conversion of retinoids, the trafficking of ions, fluids and various nutrients, phagocytosis of outer segment photoreceptor membranes, and the absorption of scattered light.

Relatively little work has been performed on the RPE in the context of sex hormones and cognate receptors. In both human and rat RPE, ERα and ß show co-expression profiles (Cascio et al., 2015). The RPE has been linked as a site, to the putative cytoprotective effects of both estrogen and DHEA-S against oxidative damage, which appear dependent on the sigma-1 receptor (Bucolo et al., 2005). Furthermore, estrogen signaling through the ERß receptor has shown to upregulate the synthesis of endogenous ROS scavenging macromolecules in cultured ARPE-19 cells (Giddabasappa et al., 2010). These observations take on particular importance in the context of AMD for which post-menopausal women show a two-fold increased risk relative to their pre-menopausal counterparts (Elliot et al., 2008).

In addition to its antioxidative role, a potential role for progesterone in retinal neovascularization is also suggested by results from a study by Lange et al. (2007). Acting through a newly discovered membrane bound progesterone receptor, the membrane-associated progesterone receptor component 1 (PGRMC1), which has been localized primarily to RPE and Müller glia, progesterone has been shown to upregulate VEGF expression through a signal pathway that involves phosphorylation of PKC and ERK-1/2.

Finally and tangential to the above discussions, the RPE is reported to show relatively higher MMP-2 activity compared to pro-MMP2, MMP14, TIMP2 in the presence of estrogen, this effect reflecting ERß activity, which subsequently transactivates the MMP-2 promoter through a transcription factor specificity protein-1 (SP-1) site (Elliot et al. 2010). However, in apparent contradiction, both estrogen and progesterone were reported to inhibit TGF-ß2-mediated release of MMP2 and MMP9 from RPE in another study involving an *ex vivo* model of proliferative vitreoretinopathy (PVR) (Kimura et al., 2014). Taken together these two, seemingly opposing, findings provide yet further evidence of the complexity of sex hormone signaling and influences.

1.5.5 Choroid

The choroid, which is sandwiched between the sclera and RPE, is largely comprised of blood vessels, fed by the posterior ciliary arteries (and ultimately the ophthalmic artery). Functionally it serves to delivery oxygen, nutrients and trophic factors to the outer retinal layers, as well as serving as a thermoregulator. In relation to the influences of sex hormones on the choroid, it is of potential interest that aged female mice were found to develop more severe choroidal neovascularization (CNV) than age-matched males (Espinosa-Heidmann et al., 2002). Surprisingly, however, estrogen replacement therapy (ERT) in ovariectomized animals was found to worsen CNV, and in rats, ERT increased choroidal VEGF expression (Dundar et al., 2010). The latter results suggest that ERT may place individuals at increased risk of CNV. Further, the observation in rabbits of choroidal thinning (and RPE thickening) after induction of hypoestrogenemia is also in general agreement with these findings (Obrubov et al., 2013), Interestingly, a study performed in mice reported inhibition of laser-induced CNV with 2-methoxyestradiol (2ME2), treatment (Funakoshi et al., 2006). Said *et al.* also showed that 2ME2 was able to reverse CNV in experimentally-induced retinopathy. 2ME2, which is a naturally occurring metabolite of estrogen, has strong affinity for GPER but negligible affinity for the ERα and ß receptors.

As an aside, choroidal blood flow is reported to be higher in women during their menopausal compared to their post-menopausal years (Schmidl et al., 2015). This finding is consistent with results from other tissues suggesting that estrogen acts as a vasodilator; on the other hand, progesterone appears to act as a vasoconstrictor on ocular vasculature (Schmidl et al., 2015). There appear to be no equivalent age-related changes in men.

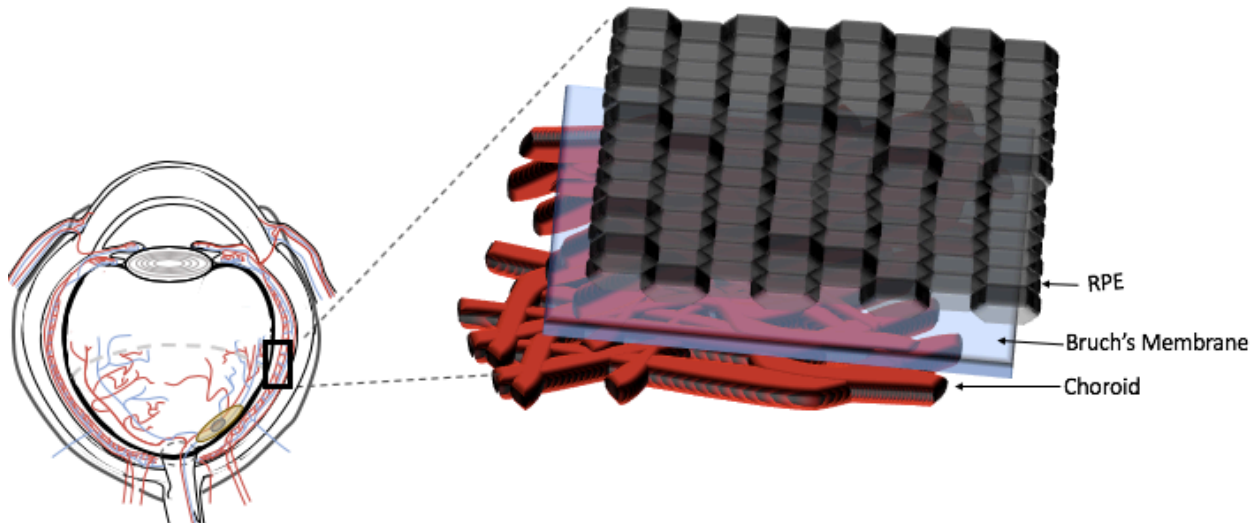


Figure 1.9 Schematic representation of the human choroid, a richly vascular layer, sandwiched between the inner retinal pigment epithelium (RPE) and Bruch's membrane, and the outer sclera. It is supplied by the posterior ciliary arteries.

1.5.6 Sclera

Forming the outermost coat of the eye (except anteriorly), the sclera has largely been excluded from steroid hormone–centric studies to-date. Populated by a sparse population of fibroblasts and myofibroblasts, the sclera is largely comprised of collagen, which accounts for approximately 90% of its weight and mostly type I (>99%)(Rada Summers et al., 2006; Metlapally et al., 2015). In this way, the sclera bears close resemblance to the corneal stroma, yet the resemblance ends there, as scleral collagen lacks the orderly lamellar organization of the cornea stroma; instead, there is an irregular network of overlapping, interweaving and interlinked fibrils. The result is an opaque, mesh-like structure that is biomechanically robust yet flexible. Scleral fibroblasts, the most numerous cell type in the sclera are embedded in this collagenous network, where they responsible for maintaining the scleral ECM.

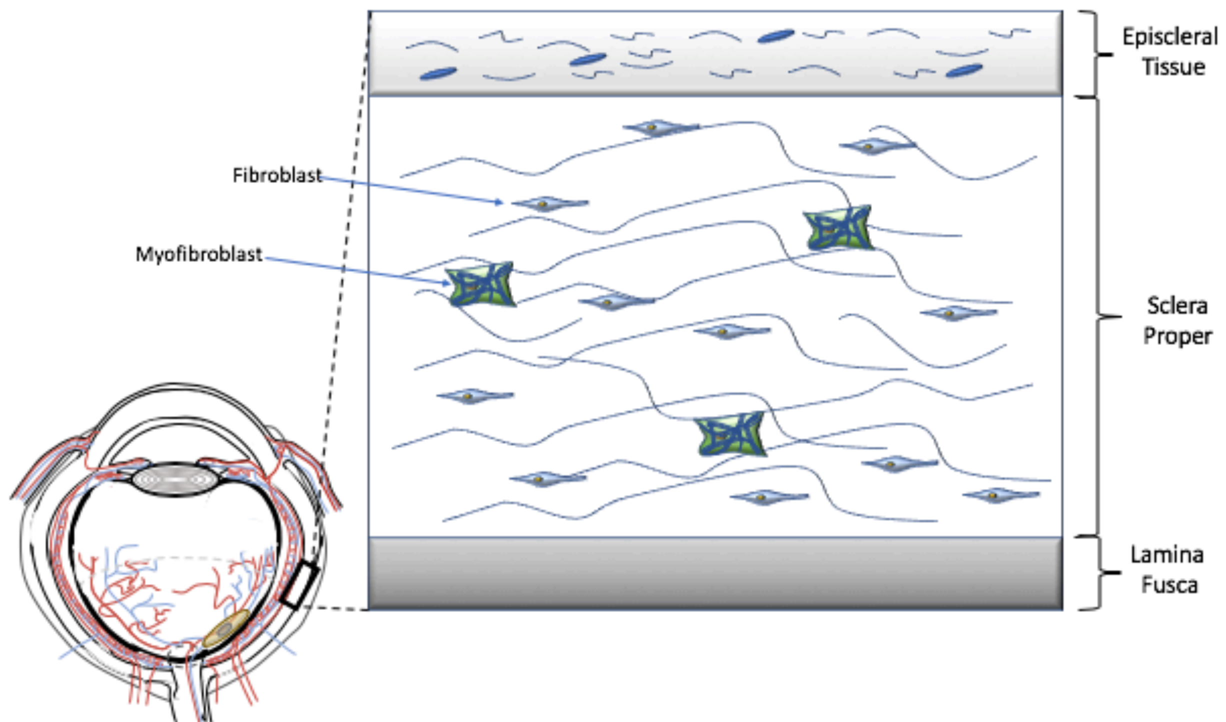


Figure 1.10 Schematic representation of the human sclera, the outer wall of the eye, and an ECM-rich tissue composed of three distinct layers, the outer episclera, the sclera proper and the inner lamina fusca.

The sclera is particularly relevant to myopic progression in humans. As eye elongation accelerates, during myopic progression, the sclera undergoes extensive remodeling, degradation outpacing the synthesis of new collagen fibers, with the net effect being a reduction in the average diameter of individual collagen fibrils and an overall reduction in collagen content (Rada Summers et al., 2006). These changes lead to an overall reduction in the thickness of the posterior sclera, of up to 31%, in eyes with moderate to high myopia (relative to their non-myopic counterparts), and biomechanically weakening as a result (Metlapally et al., 2015).

The process of scleral ECM during myopia progression has been extensively studied in animals of the same. Specifically, animal models have shown myopia progression to be correlated with an increase in MMP2 levels and a decrease in the levels of its endogenous inhibitor, tissue inhibitor of metalloproteinase 2 (TIMP-2) (Qian et al., 2015, Liu et al., 2017). MMP2 is important for local ECM degradation and collagen remodeling (Rastogi et al., 2013). Interestingly, as mentioned in the earlier section on cornea, MMP2 expression in fibroblasts may be regulated by steroid hormones. Exposure to estrogen specifically has been shown to decrease MMP2 synthesis by fibroblasts (in an ER and MAPK specific manner), leading to a relative increase in collagen synthesis (Hongbo et al., 2018, Mahmoodzadeh et al., 2010). On the other research in guinea pigs by Ding et al. (2018), showed that hydrocortisone acting through glucocorticoid receptors could inhibit normal eye growth, with the opposite result being obtained in a lens induced myopia model (i.e. hydrocortisone treatment, in negative lens treated subjects, increased axial length elongation). Perhaps tellingly, however, Ding and colleagues also noted an overall increase in serum estrogen levels in this latter treatment paradigm. And other studies involving extra-ocular tissues have reported glucocorticoid-induced inhibition of collagen

synthesis by fibroblasts as well as enhanced collagen degradation (Tiganescu et al., 2013, Zhou et al., 2011, Mi et al., 2018, Verbruggen 1981). Together, these data seems to support the idea that glucocorticoids to be, broadly speaking, ocular growth halting, with the caveat that the effect can be overcome by elevated levels of estrogen; a potentially pro-myopic molecule.

As discussed earlier, women (i.e., higher estrogen levels) experience myopia at higher rates and with greater phenotypic severity. Also of potential relevance, girls, who, on average, undergo puberty earlier than boys, have been reported to reach peak growth rates earlier, in addition to showing earlier onset of myopia and earlier peak rates of myopic progression (Yip et al., 2012). Consistent with the latter observations, an inverse relationship between the age of puberty onset and severity of myopia has also been reported (Yet et al., 2011). A role for estrogen in these observations is yet to be established, and it is possible that other steroid hormones could also be involved, given that, as stated earlier, it is well known that steroid hormones can significant affect the growth and remodeling of ECM- fibroblast-rich tissues, where ever they occur in the body.

Chapter 2: Impact of Form Deprivation on Ocular Dimensions and Optics – A Story of Two Sexes

2.1 Introduction

As discussed previously, myopia is ultimately a problem of excessive ocular growth, where the vitreous chamber (VC) expands, introducing a mismatch between the eye's optical power and its length, and the sclera thins as pathology progresses. Beyond the visual consequences of this optical mismatch when left uncorrected, myopia is linked to increased rates of a range of blinding, secondary pathologies (as discussed in chapter 1). Interestingly it has been found that myopia presents both more frequently and – on the average – with greater clinical severity in women, compared to men (Zhao et al. 2000, Sewunet et al. 2014, Vitale et al., 2009). This naturally raises the question of what drives this disparity. Are there significant differences between the axial lengths, ocular tissue thicknesses or optics of male and female myopic eyes? And if so, how is this driven differentially by the underlying biochemistry? More specifically, could sex-related differences in endocrine or, more broadly, steroid hormone signaling be responsible for this apparent difference in myopia susceptibility?

In order to begin to address the above question, we leveraged a well-validated animal model of myopia, specifically a form deprivation-induced myopia chicken model. As noted by Wallman and Winawer (2004), the eyes of young chickens can be induced to grow by up to 300 μm in as little as two days. In the form deprivation-induced model of myopia, a diffuser, either completely opaque or frosted, is affixed over one eye of test subjects. In the presence of the diffuser, the retina is deprived of a clear image, as compared with a sharp (optically in-focus) image. In an attempt to rectify this situation, retina-derived signals drive the eye to elongate at a faster rate. In the case of form deprivation-induced myopia, this growth response does not correct the problem, i.e., the aforementioned clear image never forms, and thus the eye continues to grow at an accelerated rate in what is termed 'open loop' growth (in contrast to lens-induced myopia, where the induced growth is compensatory)(Morgan et al 2013). Importantly, over the time span of experiments described in this dissertation paradigm, the form-deprived eyes continue to expand and their scleras remain in a state of increased remodeling and continued thinning, in the case of its fibrous layer. Given that we hypothesize a strong endocrine component to contribute to these processes, this open-loop form deprivation model of myopia progression is a particularly advantageous model, given that for other tissues, endocrine signals have been shown to be most prominent and relevant during active tissue growth and remodeling (Dhingra 2017, Fenner 2011, Shah and Mailbach 2001). Thus we hypothesize that endocrine signals play an important, active role in fibrous scleral growth and remodeling, in response to myopia-inducing stimuli..

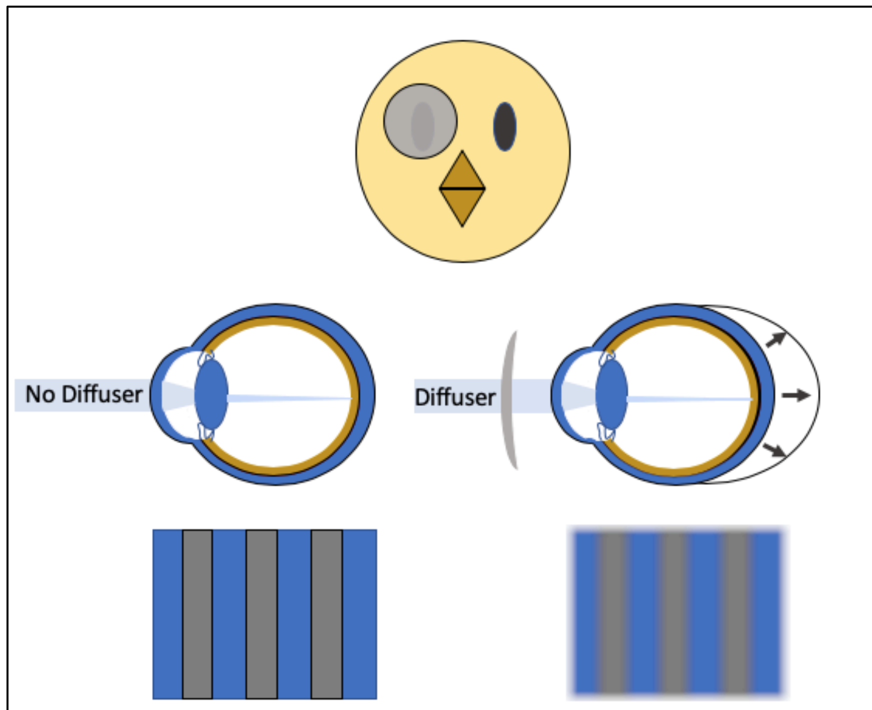


Figure 2.1 Form deprivation model of myopia induction, involving a monocular diffuser. In the presence of diffuser induced "blur" (i.e. attenuation of spatial detail and contrast)(right), form-deprived eye elongate in an (ultimately ineffective) attempt to improve retinal image quality. In normal development, eyes grow to correct any neonatal focusing errors, thereby establishing emmetropia (left).

As the eye elongates axially in form deprivation induced myopia, as with general human myopia, both the ocular axial length and the thicknesses of the tissues making up the wall of the vitreous chamber change. The thicknesses of these tissues, specifically the retina, choroid and sclera, along with the on-axis dimensions of the various compartments of the eye, i.e., anterior and vitreous chamber, and crystalline lens, can be tracked *in vivo*, using high frequency A-scan ultrasonography (HFAU). The custom-built machine used in the described research offers a resolution of approximately 10 µm. Also by summing the dimensions of appropriate ocular elements, axial length data can be obtained.

Finally, while such ocular dimensional data as discussed above, are well documented for myopiagenic conditions in a large number of published studies covering different animal models and spanning several decades, there has been only limited study of sex-related differences (Wallman and Winawer 2004, Edwards 1996, Goss and Crisswell 1981, Schmid and Wildsoet, 1996, Xhu et al. 1995, Prashar et al. 2009, Chen et al. 2010). Furthermore, the association between sex-related differences in susceptibility to experimental myopia and steroid hormone signaling has been almost entirely neglected.

In order to study the link between sex and the ocular metrics discussed above, one needs a reliable method of sexing subjects. The options vary according to the animal model being used, and also the timing needs of the project. Several options exist for the chicken (post-hatch), including manual examination of the cloaca, i.e. 'vent-sexing', which is of insufficient reliability,

even in the hands of professionals, analysis to determine sex by polymerase chain reaction (PCR), and analysis to determine sex by manual examination of gonads, post-mortem. The latter approach has the advantages of being exceptionally fast, reliable and resource efficient. Furthermore, while it has not been used in myopia research involving the chick, it is well documented in the animal science and veterinary literature (Ahiagbe et al., 2018; Apperson et al., 2017, Sohn et al., 2002, Sohn et al., 2012), as well as in the developmental biology literature (Gonzalez-Moran et al. 2011, Guioli et al. 2014, Ayers et al. 2013, Intrapat et al. 2013). Importantly, a recently published study that examined the validity of sexing via visual inspection of testes and ovaries against PCR analysis of a female chicken (i.e. W chromosome) specific 600 base pair long *EcoRI* DNA fragment, reported a 100% match (Ahiagbe et al., 2018).

In brief, sex-specific differences in gonadal structures have been described in most birds, excluding birds of prey. Both gonadal structures (ovaries and testes) are located intra-abdominally, adjacent to the kidneys. In the case of female birds, including chickens, only the left ovary fully develops into a functional gamete-producing organ, with cortex and medulla components, while the right ovary is significantly smaller and lacks a cortex. The latter asymmetry in the is apparent as early as embryonic day 8, is clearly visible at hatching, and becomes more distinct with increasing age thereafter, due to regression of the right and growth of the left ovary (Gonzalez-Moran MG et al., 2011). In contrast, male chickens develop two symmetric testes (Apperson et al., 2017).

2.2 Methods

2.2.1 Animals

White Leghorn chicken eggs were obtained from the University of California (UC) Davis Department of Animal Science's Avian Facility. Upon receipt, eggs were transferred to a humidity and temperature (dry temp: 37.8C, humidity: 72%) controlled incubator (1502 GQF Sportsman Cabinet, Savannah, GA, USA), for 19 days. On the 19th day, eggs were transferred to a separate temperature and humidity (wet temp: 30C, dry temp: 37C) controlled incubator (same model) configured for hatching. On hatching (three days later, day 21), they were transferred to wire mesh cage housing in a temperature (30-35C), humidity (30-70%) and light (12hr/12hr on-off diurnal cycle) controlled room. Both food (Start & Grow, Nestle Purina, St. Louis, Missouri, USA) and water were provided ad libitum.

Form deprivation: On day six post hatch, subjects had a Velcro ring (19.1 mm outside diameter, Velcro USA Inc, Manchester, NH, USA), affixed around one eye with collodion glue (Spectrum Chemical MFG Corp, Gardena, CA, USA). Their nails were also trimmed, with small animal pet nail clippers to help prevent removal of the Velcro rings. A diffuser, with a mating Velcro ring attached, was attached on the following day (day 7 post-hatch), and left in place on for 14 days, excluding a brief period on day 20, to accommodate biometric data collection. Thirty-eight chickens (19 males (M) & 19 females (F)) were used in this study.

All animal care and treatments in this study conform to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision

Research. Experimental protocols were approved by the Animal Care and Use Committee of UC Berkeley.

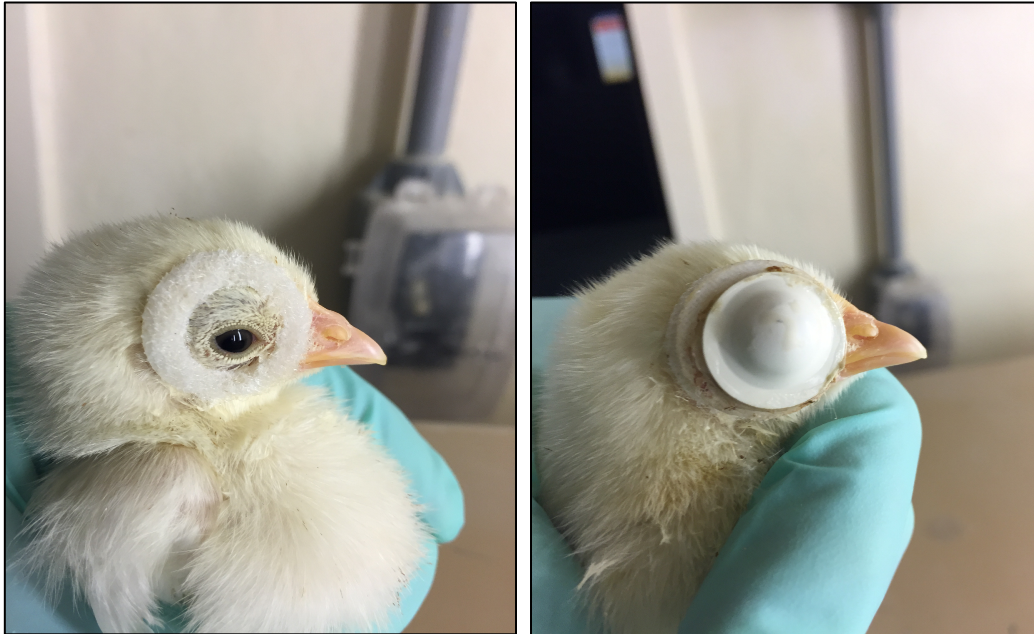


Figure 2.2. Seven day-old chick with Velcro ring (Left) affixed around its right eye (left), and with form depriving, white diffuser attached (right).

2.2.2 Refraction by Streak Retinoscopy

At 20 days of age, chickens were anesthetized with 1.5% isoflurane (Piramal Enterprises Limited, Andhra Pradesh, India) in oxygen. During ocular measurements, eyelids were held open with a custom-built ophthalmic speculum. Streak retinoscopy was used to quantify the refractive errors of both the form-deprived and fellow eyes of each bird (retinoscope Model 11730, Welch Allen, Skaneateles Falls , NY, USA; L2 retinoscopy rack, Luneau Ophtalmologie, Chartres, France). Spherical equivalent refractive error, calculated by averaging readings in the two principal meridians, are reported.

2.2.3 Hi-Frequency A-Scan Ultrasonography

Following streak retinoscopy, high frequency A-scan ultrasonography (HFAU) was performed on the same subjects. Ultrasound gel (Aquasonic 100, Parker Laboratories, Inc, Fairfield, NJ, USA) was first applied to the ultrasound probe cover; the probe, which was affixed to a custom device enabling its movement in X, Y & Z dimensions, was then maneuvered into alignment with the optical axis of the eye and brought into contact with the cornea. Ultrasound traces were captured from both form-deprived and fellow control eyes. At least five traces were saved per eye for follow-up analysis (on a separate computer). The axial dimensions of the anterior chamber (AC), crystalline lens, vitreous chamber (VC), retina, choroid and sclera were then obtained from the traces and both external axial length (i.e. AC depth + lens + VC depth + retina

+ choroid + sclera thicknesses) and internal axial length (i.e. i.e. AC depth + lens thickness + VC depth) calculated.

2.2.4 Post-Mortem Chicken Sexing

Following sacrifice at 21 days of age, the chickens (n = 38, 19M & 19F) were sexed via intrabdominal inspection of their gonads and scleral tissue and serum were collected (see Chapters 3 and 4). In brief, the chickens were placed in the supine position, with body extended horizontally and abdominal cavity uppermost. Surgical scissors were then inserted into the cloaca and used to make an initial small cut that was then extended up towards the ribcage. Additional cuts were made to the left and right of the rib cage, to expose the thoracic cavity and subsequently reveal the gonads and kidneys, which lie deep in the cavity, adjacent to the backbone. Chickens with two symmetric, oval-shaped, orange-yellow colored gonads were recorded as males. Chickens with a large (dominant), singular, amorphous, pink-tannish-greyish gonad on the left side (with a smaller, partly regressed ovary on the right), were recorded as female (Figure 2.3).

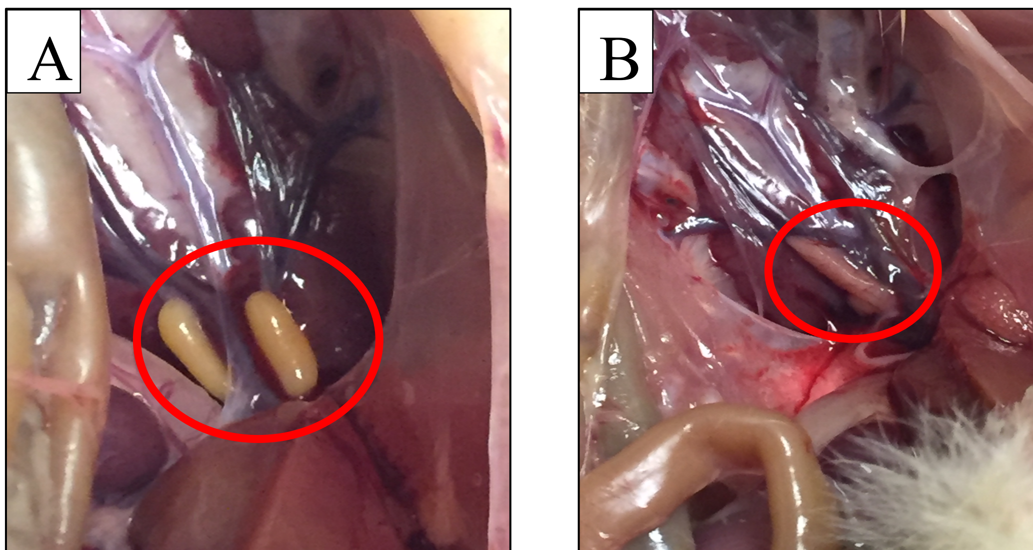


Figure 2.3 Chicken gonads are located intra-abdominally, proximal to the kidneys, adjacent to the backbone. At 21 days of age, male chickens are identifiable by the presence of two symmetrical testes (A, circled in red), while female chickens have a dominant left ovary (B, circled in red)

2.2.5 Body Mass

Immediately prior to sacrifice, the weight (body mass) of each subject was also measured using a digital scale (model SC4010, Ohaus Corp, Florham Park, NJ, USA).

2.2.6 Statistics

Statistical analysis was performed with the aid of Microsoft Excel. Measurement variability was calculated and reported as a standard error of the mean (SEM). Two-tailed paired t-tests were

used to compare measurements from form-deprived and their fellow non-form-deprived eyes, and thus p-values obtained. When comparing measurements from males and female subjects (i.e. making sex specific comparisons) p-values were calculated using two tailed unpaired t-tests Data was considered to be significant when $p \leq 0.05$.

2.3 Results

2.3.1 Effects of diffuser-induced form deprivation on refractive error and ocular growth

The biometric and refractive error data recorded at the end of the 14-day treatment period for form-deprived eyes and their fellows (pooled across sexes) are summarized in Table 2.1. As has previously been shown by a multitude of investigators, we also found that the diffuser treatment significantly increased the axial lengths of the form-deprived eyes relative to those of the fellow eyes ($p < 0.001$). Both AC and VC depths were also significantly increased ($p < 0.001$ for both measurements), thereby contributing to the axial length increases. Significant differences were also found in choroidal thickness, with the choroids of form-deprived eyes being significantly thinner than that measured in their non-form-deprived (i.e. fellow) eyes ($p <0.001$). Further, while sclera also appeared thinner in form-deprived eyes, this relationship did not reach significance. Finally, as expected from the increase in axial length in form-deprived eyes, their refractive errors showed significant myopic shifts relative to the refractive errors of their fellows (i.e. mean: - 25.51 D vs. 1.74 D, $p < 0.001$).

The above biometric and refractive error data, broken down by sex and form deprivation status are summarized in Tables 2.2 & 2.3. In this related analysis, male birds were found to have naturally deeper anterior chambers, as reflected by their non-form-deprived (i.e. fellow) eyes, relative to those of female birds, with this difference being statistically significant ($p < 0.014$). These same (fellow) male eyes also displayed significantly thinner choroids and scleras ($p < 0.05$ & $p < 0.01$, respectively), relative to those of female birds. While these trends did follow through to form-deprived eyes, the differences here (i.e., choroidal and scleral thickness differences between male and female birds), were not statistically significant. The form-deprived eyes of male birds were also longer than the form-deprived eyes of female birds; however, the latter difference did not reach statistical significance. Finally, male birds also appeared to be heavier than female birds, but this difference also did not reach statistical significance (M: 146.529 +/- 3.041g, F: 138.208 +/- 3.311g),

Table 2.1 Ocular component dimensions of both form-deprived and fellow eyes of all subjects, pooled across sexes (n=38). Values represent means +/- SEMs.

Ocular Parameters	Formed Deprived Eyes	Fellow Control Eyes	p-values
Refractive error (D)	-25.513 +/- 1.078	1.737 +/- 0.105	7.66E-41***
Outer Axial Length (mm)	11.229 +/- 0.120	10.136 +/- 0.042	4.54E-13***
Inner (i.e. optical) Axial Length (mm)	10.724 +/- 0.114	9.568 +/- 0.039	5.98E-15***

Anterior Chamber (AC) Depth (mm)	1.668 +/- 0.043	1.534 +/- 0.019	5.68E-03***
Vitreous Chamber (VC)§ Depth (mm)	6.706 +/- 0.074	5.705 +/- 0.027	7.27E-21***
Choroidal Thickness (mm)	0.144 +/-0.004	0.223 +/- 0.008	1.72E-13***
Scleral Thickness (mm)	0.117+/- 0.003	0.122 +/- 0.003	2.84E-01

§VC represents the distance between the back of the lens and front of the retina.

Table 2.2 Ocular component dimensions of the fellow eyes of male and female subjects (n=19, 19), broken down by sex. Values represent means +/- SEMs.

Ocular parameters	Female Fellow Eyes	Male Fellow Eyes	p-value
Refractive Error (D)	1.750 +/- 0.170	1.725 +/- 0.129	9.48E-01
Outer Axial Length (mm)	10.108 +/- 0.18	10.162 +/- 0.057	5.13E-01
Inner Axial Length (mm)	9.546 +/- 0.056	9.589 +/- 0.054	5.82E-01
Anterior Chamber (AC) Depth (mm)	1.487 +/- 0.028	1.580 +/- 0.022	1.43E-02*
Vitreous Chamber (VC) Depth (mm)	5.704 +/- 0.040	5.705 +/- 0.040	9.86E-01
Choroidal Thickness (mm)	0.242 +/- 0.011	0.205 +/- 0.011	1.74E-02*
Scleral Thickness (mm)	0.114 + /- 0.005	0.130 +/- 0.002	4.64E-03**

Table 2.3 Ocular component dimensions of form-deprived eyes of male and female subjects (n=19, 19), broken down by sex. Values represent mean +/- SEMs.

Ocular parameters	Female Form-deprived Eyes	Male Form-deprived Eyes	p-value
Refractive Error (D)	-29.206 +/- 1.110	-22.175 +/- 1.488	6.36E-4***
Outer Axial Length (mm)	11.115 +/- 0.185	11.338 +/- 0.155	3.54E-01
Inner Axial Length (mm)	10.663 +/- 0.179	10.782 +/- 0.146	6.04E-1
Anterior Chamber (AC) Depth (mm)	1.657 +/- 0.072	1.679 +/- 0.052	8.05E-01
Vitreous Chamber (VC) Depth (mm)	6.738 +/- 0.110	6.674 +/- 0.100	6.66E-01
Choroidal Thickness (mm)	0.151 +/- 0.004	0.137 +/- 0.006	5.11E-2
Scleral Thickness (mm)	0.113 +/- 0.003	0.122 +/- 0.006	2.06E-1

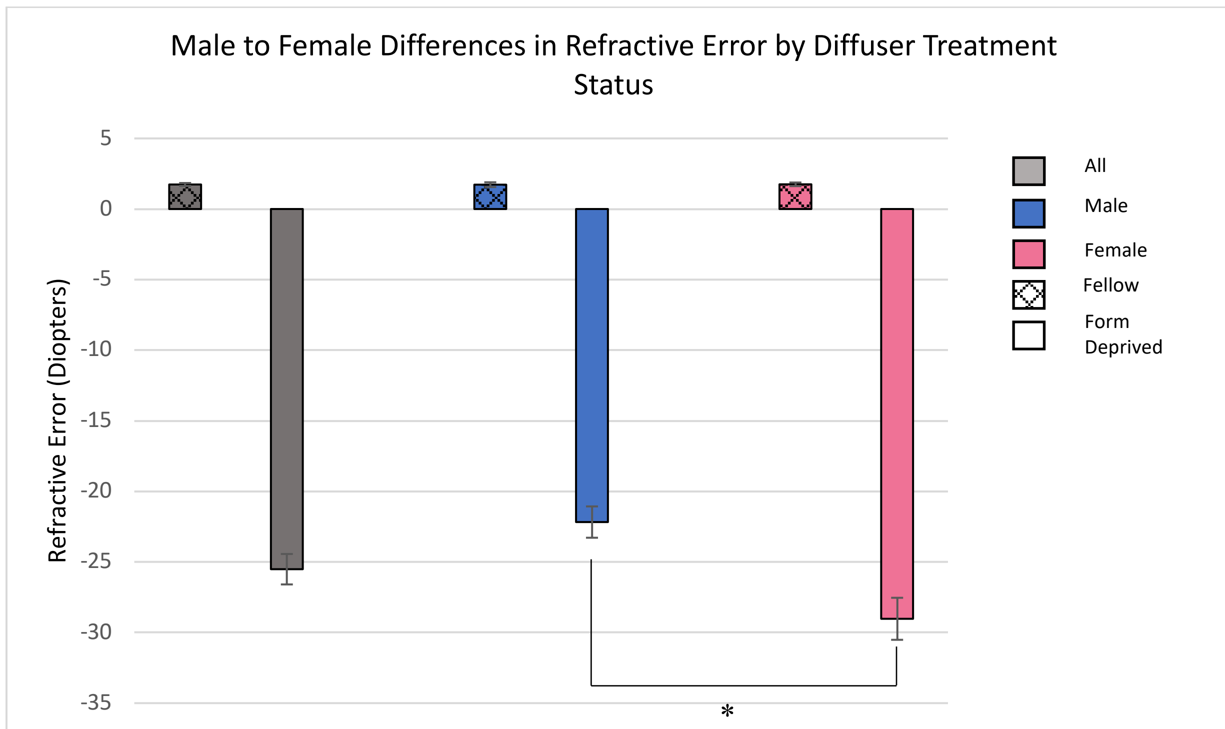


Figure 2.4. Mean refractive errors of fellow (untreated) and form-deprived (diffuser treated) eyes for all subjects, irrespective of sex (All), as well for female and male subjects. Error bars represent SEMs. * $p < 0.001$

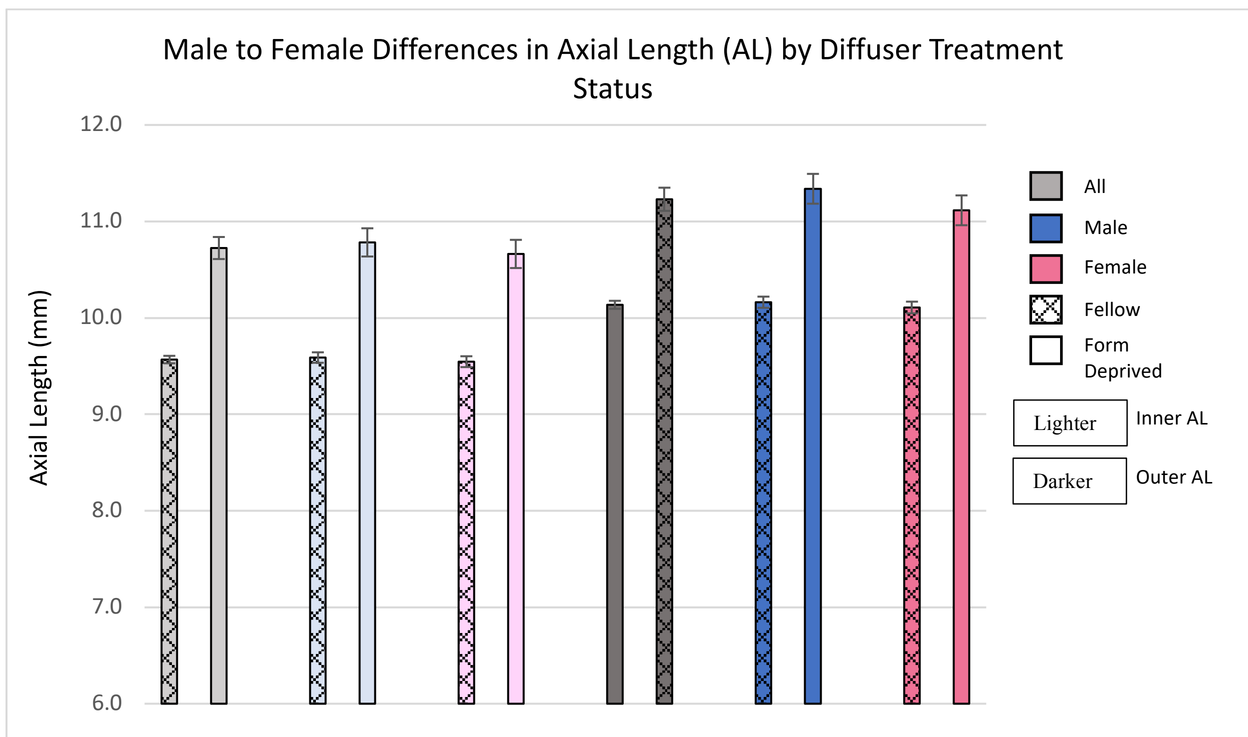


Figure 2.5. Mean inner and outer axial lengths of fellow (untreated) and form-deprived (diffuser treated) eyes for all subjects, irrespective of sex (All), as well for female and male subjects. Error bars represent SEMs

2.4 Discussion

The above results confirm the potency of form deprivation as a myopia-inducing treatment in young chicken. This is not particularly surprising, however, given the results of previous related studies, both within and outside our group (e.g. Schmid and Wildsoet 1996, Nickla and Schroedl 2012, Chen et al. 2009). What is perhaps more surprising are the sex-related differences identified.

In short, we found the form-deprived eyes of female chickens to display greater average amounts of myopia than their male counterparts. While data are in line with what has been observed in humans (Vitale et al. 2009), such findings both agree and disagree with results of related myopia studies involving the chicken. For example, using a lid suture-based approach to form-deprivation, Schmid and Wildsoet (1996) found female subjects to display (statistically non-significant) greater degrees (relative to males) of myopia, in general agreement with our findings. On the other hand, in a diffuser model of form-deprivation, Zhu et al. (1995) reported the opposite trend, i.e. the form-deprived eyes of male chicks had significantly greater myopic errors than female chickens.

In our study, we also found female subjects to display slightly shorter axial lengths (as measured both to the inner retina and outer sclera), then their male counterparts. However, it is important to note that this difference was not statistically significant. Overall, these findings are in agreement with the literature. For example, both Schmid and Wildsoet (1996) and Zhu et al. (1996) reported longer axial lengths for the form-deprived eyes of male compared to female chickens, although this difference reached significance only in the Zhu et al. study. An additional, more recent study conducted by Chen et al. (2010) also reported a similar, statistically significant trend.

What is perhaps most puzzling about the above findings is why the form-deprived eyes of female birds were more myopic, yet tended to be shorter in length compared to their male counterparts, when typically greater degrees of myopia correspond to greater axial lengths. What could explain this sex-related difference?

In our study we did find the choroids of fellow eyes to be significantly thicker in female birds compared to those of their male peers. Could this difference perhaps explain the above mismatch? It has been suggested that a thicker choroid may act to physically push the retina forward (i.e. directionally towards the cornea), decreasing the inner axial length (Wallman et al., 1995). This would be consistent with what we observed in our measurements, given that we observe female subjects to have slightly shorter inner axial length measurements. Nonetheless, while there was also a trend towards thicker choroids in the form deprived eyes of female birds, this difference between male and female birds did not reach statistical significance. Furthermore, one would predict less rather than more myopia in females, given their shorter eyes.

A second possible explanation involves differences in the corneal refracting properties between male and female subjects. While corneal curvature data were not collected in our experiments, in previous research by Wildsoet (1992), male chicks were found to develop greater corneal enlargement and thus greater overall axial lengths, albeit with flatter corneas in response to lid suture-induced form deprivation. Female chicks did not show the same exaggerated corneal response to this stimulus. Thus it is possible that the less myopic, yet longer form-deprived eyes of male subjects in the current study may be a consequence of sex-related differences in corneal curvature.

Apart from observed sex-related differences in refractive error and inner axial length, there are additional interesting findings involving the sclera in our data. First, we found female subjects to have thinner scleras, both in their fellow and form deprived eyes, than their male counterparts. On the other hand, the extent of scleral thinning in response to form deprivation was greater in male subjects than in female subjects. What could explain this disparity?

It has been hypothesized previously by others (Jiang et al. 2019, Troilo et al. 2000) that thicker choroids may act as a diffusion barrier, limiting the levels of growth and remodeling chemical effectors released from the retinal pigment epithelium (RPE) reaching the sclera. Given the thicker choroid observed in female subjects, that they showed lesser scleral thinning in response to form deprivation would be consistent with this hypothesis. A similar explanation for the naturally thinner scleras of female subjects could be offered, if tonic release of growth modulating effectors from the RPE drives normal scleral growth, i.e. in the absence of experimental visual manipulations.

Less directly supported, albeit also plausible explanations for the above observations include possible differences in the myofibroblast content and/or hormonal signaling in the sclera. It has previously been suggested that transdifferentiation of fibroblasts into myofibroblasts (Baum and Duffy 2012), is involved in myopic progression (Yuan et al. 2018). During myopia progression, the biomechanical properties of the sclera change, and myofibroblasts are known to be capable of exerting contractile forces on the surrounding matrix (McBrien NA et al. 2009). Furthermore, analogous cells (i.e. extraocular myofibroblasts) have been linked to both collagen synthesis and degradation (Xu et al. 2014). Might there be differences in myofibroblast and/or fibroblast content and their relative activities in male and female scleras? Furthermore might there be sex-related differences in hormonal signaling in sclera. Hormones and more specifically sex-steroid hormones (e.g. estrogen, progesterone and testosterone) have been found to regulate the and collagen synthesis and degradation activities in ECM-rich extraocular tissues, similar to sclera (Dhingra 2017, Thornton 2013). This latter point is explored in greater depth in subsequent chapters of this dissertation.

In this study and as discussed above, both sex-related differences in scleral thickness in fellow eyes and sex-related differences in scleral thinning in response to form deprivation were described. In understanding the biological significance of these observations, it is important to note that chickens have a bilayered sclera, with inner cartilaginous and outer fibrous layers. The former thickens during myopic progression, while the former thins (Gottleib et al., 1990), and thus net thinning of total sclera, necessitates thinning of the fibrous sclera. While the two layers are not independently resolvable using A-scan ultrasonography, the latter fibrous sclera is of

most interest from a translational perspective, as this layer is most closely analogous to the human sclera in structure; it also is reported to undergo similar biochemical changes in response to myopia-inducing stimuli, as observed in mammals, which also have a primate-like fibrous sclera (Rada et al. 1999). Thus in the experiments described in later chapters of this dissertation, the chick fibrous layer will be the focus.

Chapter 3: Steroid Hormones and Their Association with Myopia Progression in the Sclera

3.1 Introduction

As discussed previously in the introductory chapter, steroid hormones regulate a wide variety of physiological processes throughout the body, being present and active in most tissues and cell types. Interestingly and of potential relevance to myopia, steroid hormones have been shown to directly regulate the growth and remodeling of extracellular matrix (ECM) of fibroblast-rich tissues. Most famously perhaps, androgen binding to the (nuclear hormone receptor) androgen receptor (AR) has been shown to drive the growth of prostate tumors (Dhingra 2017; Fenner 2011). Skin, another ECM- and fibroblast-rich connective tissue, thins and wrinkles in post-menopause, in part as a function of reduced estrogen levels. Estrogen both promotes collagen synthesis and decreases the level of collagenolytic matrix metalloproteinases, specifically of MMP2 and MMP9 (Shah and Mailbach 2001, Thornton 2013). As a final, although certainly not exhaustive, example, glucocorticoids have been found to exert profound effects on ECM composition and remodeling. For example, increased cortisol levels have been linked to reduced collagen synthesis and enhanced collagen degradation (Verbruggen et al. 1981; Kosinski et al. 2015; Terao et al. 2014, Yabing et al. 2018). Glucocorticoids are also known to decrease fibroblast proliferation (Zhou et al. 2011) and the fibroblast-to-myofibroblast transition (Franke and Abraham 2014).

Given the above information, it is not unreasonable to assume that steroid hormones may play a role in myopia-dependent remodeling and more specifically, a role in remodeling of the sclera, an ECM- and fibroblast-rich connective tissue. We hypothesize that steroid hormones may facilitate scleral remodeling in both sex-dependent, as well as sex-independent (i.e., myopia dependent) ways. As myopia progresses, scleral MMP2 levels rise and collagen levels decline, leading to a net thinning of the sclera (Jones et al. 1996, Rada and Brenza 1995, Guggenheim and McBrien 1996). These changes underlie the posterior vitreous chamber axial expansion, as expressed by increasing myopic refractive errors linked to an enhanced risk of secondary, blinding pathologies.

Below we describe a variety of experimental methods used to explore the above hypothesis, including quantification of changes in both systemic and local (scleral) steroid hormone levels, as a function of myopia progression. We also examined how scleral messenger ribonucleic acid (mRNA) levels of steroidogenic enzymes changed as a function of myopia progression.

3.2 Methods

3.2.1 Subject treatments & numbers

Subjects were obtained and raised as described in Chapter 2, section 2.2.1, of this dissertation. In brief, chicks were either monocularly form deprived with a diffuser or left untreated to serve as controls. The monocular diffuser treatments were initiated on day 7 post-hatch and halted at the time of sacrifice and tissue isolation, on day 21 post-hatch. Table 3.1 below summarizes the number of birds and sample types utilized in these experiments. Note that samples referred to as "control serum" and "control sclera" were collected from the same birds. Likewise, "form-deprived serum", "form-deprived sclera" and "fellow sclera" were collected from the same birds. The mismatch between subject numbers and samples analyzed reflects in part, samples used in optimization of experimental protocols. In addition, fibrous sclera samples were not successfully collected in some cases, and not all collected samples were successfully analyzed by Tandem Mass Spectroscopy (TMS).

Table 3.1: Summary of sample numbers, organized by experiment. The numbers in brackets represent the number of samples for which reliable data were obtained.

Experiments	**Total Subject n**	**Male n**	**Female n**
qRT-PCR: Steroidogenic Enzymes	**20 (20)**	**10 (10)**	**10 (10)**
TMS: Control Serum	**32 (26)**	**15 (14)**	**17 (12)**
TMS: Control Sclera	**32 (24)**	**15 (12)**	**17 (12)**
TMS: Form-Deprived Serum	**40 (29)**	**22 (15)**	**18 (14)**
TMS: Fellow Sclera	**40 (12)**	**22 (6)**	**18 (6)**
TMS: Form-Deprived Sclera	**40 (18)**	**22 (8)**	**18 (10)**

3.2.2 Blood collection & serum isolation.

Blood serum was collected from both monocularly form-deprived and untreated (control) subjects. At 21 days of age, subjects were decapitated using a small animal laboratory guillotine. A 50 ml polypropylene conical tube (Thermo Fisher Scientific, Waltham, MA, USA) was used to collect blood from bisected jugular veins. Blood was allowed to sit at room temperature for 30 minutes to allow clotting, after which the liquid component was separated and centrifuged in a 1.5 ml microcentrifuge tube at 3,000 g for 10 minutes. The supernatant (i.e. serum) was then transferred to a fresh 1.5 ml microcentrifuge tube, snap frozen with liquid nitrogen and transferred to a -80 ^{o}C freezer for later steroid hormone extraction.

3.2.3 Fibrous scleral sample isolation & homogenization

Fibrous scleral samples were collected from matched diffuser-treated and fellow eyes of sacrificed birds. Following blood collection, the eye lids were trimmed away and eyes enucleated and placed in a 35 x 10 mm polystyrene petri-dish (Falcon Corning Brand, Corning, NY, USA) containing phosphate-buffered saline (PBS, pH 7.4; Gibco, Life Technologies Limited, Paisley, UK). Attached extraocular muscles and adipose tissue, as well as the optic nerve, were trimmed away from the eyes, which were then bisected along the ocular equator. The anterior portion of

the eye, including the cornea, lens and some sclera, was then gently removed along with the vitreous body. A 6 mm diameter circular piece of the wall of the remaining posterior eye cup was then isolated, using a disposable 6 mm diameter dermal biopsy punch (Integra Militex,York, Pennsylvania USA). Tissue samples were taken from a central location, near the tip of the pecten (see Figure 3.1). The attached retina, retinal pigment epithelium (RPE) and choroid were then gently separated from the sclera with the aid of tweezers. The resulting isolated sclera was then transferred to a petri dish of fresh PBS, after which the cartilaginous and fibrous scleral layers were carefully separated with the aid of a dissecting microscope (Omano, China). Isolated fibrous scleral samples were then transferred to empty 1.5 mL microcentrifuge tubes (VWR, Randor, PA, USA), snap frozen in liquid nitrogen, and transferred to a -80 ^{o}C freezer for storage until subsequent homogenziation.

All analyses required homogenized fibrous scleral samples. An Omni Bead Ruptor 24, with a 7 ml bead kit adaptor was used to this end (Omni International, Kenessaw, GA, USA). Frozen scleral samples were first transferred from microcentrifuge storage tubes to 7 ml prechilled (on ice), ceramic-bead (2.8 mm)-filled tubes (Omni International, Kenessaw, GA, USA), along with 350 ul of appropriate homogenization buffer (either PBS, RLT or RIPA buffer with protease inhibitor, dependent on the experiment in which a given tissue was to be used). Tubes were then capped and sealed with Parafilm M (American National Can, Greenwich, CT, USA), and homogenized using the following Ruptor settings: S = 4.50, C = 02, T = 0:45 & D = 0:20, after first prechilling the Omni Bead Ruptor 24 to 6 ^{o}C. After homogenization was complete, sample tubes were first placed on ice to further cool the resulting solution, and then the contents of each tube were transferred to 1.5 ml microcentrifuge tubes on ice for further, experiment-specific, processing.

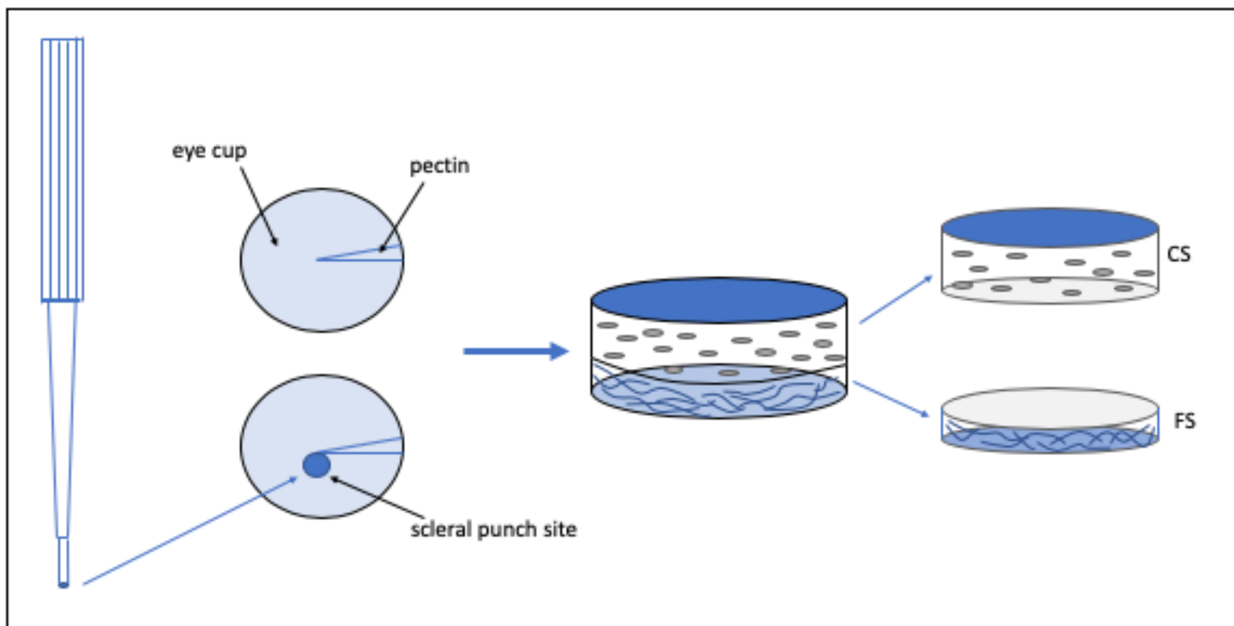


Figure 3.1 Schematic diagram summarizing the procedure for isolating the fibrous sclera sample from chicken eyecup. The collected tissue sample (punch) was separated into to its component parts, with retina, RPE and choroid first removed from the inner wall of the sclera, and then the fibrous scleral layer (FS) separated from the inner cartilaginous scleral (CS) layer...

3.2.4 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Endogenous ribonucleic acids (RNA) from scleral homogenates were extracted using RNeasy Mini Kits (Qiagen, Maryland USA). Genomic DNA (gDNA) was digested using a Turbo DNA-Free Kit (Invitrogen, Carlsbad, California USA). RNA quantification and the A260/A280 optical density ratio were measured using a spectrophotometer (NanoDrop 2000, NanoDrop Technologies Inc, Wilmington, Delaware USA). Total RNA was then reverse transcribed to cDNA using an Omniscript RT Kit (Qiagen, Maryland USA), which was then analyzed by quantitative real-time (qRT) PCR using a StepOnePlus RT-PCR System (Applied Biosystems, Waltham, MA, USA) and iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA).

In total, the transcriptional expression levels in collected scleral samples of eight steroid hormone receptors were quantified (samples from form-deprived and fellow eyes of 19 F & 19 M birds)). Chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference (house-keeping) gene. Primers were designed using the Primer Express 3.0 software program (Applied Biosystems, Foster City, CA). The efficiency of primers was calculated using ten-fold serial dilutions of cDNA. Gene expression levels were calculated as described previously (Schmittgen TD & Livak KJ, 2008). Mean normalized expression values were calculated from technical repeats (i.e. triplicate) of target genes and mRNA expression levels subsequently calculated for all biological repeats. Lastly, differential gene expression levels in "control" sclera (from fellow eyes) versus "experimental" sclera (from form-deprived eyes), were derived. Here expression levels in diffuser-treated, form-deprived eyes are expressed as a percentage of expression in fellow eyes.

Table 3.2. Primers used in qRT-PCR evaluation of steroidogenic enzymes transcript levels.

Protein	**Primer**	**Sequence**
Cytochrome P450 Family 11 Subfamily A Member 1 (CYP11A1)	Forward	CAC AGG CTG ACA AAT GTA TCC AA
	Reverse	GTG CTC CTC GGT GCT CTT G
17,20 Lyase (CYP17A1)	Forward	CGC CAG GGT GGT CAT CA
	Reverse	GTT TAT CCC ACT CCT TCT CAT CGT
21 Hydroxylase (CYP21A2)	Forward	GCG GGA TCC ATC GTT ATC C
	Reverse	GCT CGG GCA GGA ACT CAT C
3-Beta-Hydroxysteroid Dehydrogenase (HSD3B2)	Forward	TCT GGA GGA TCT TCA CCT CTT TG
	Reverse	GCC CTG GAA CTT GCC AAA G

17β-Hydroxysteroid dehydrogenase 1 (HSD17B1)	Forward	GCA GTG TTT GAG GTG AAC GTA TTC
	Reverse	CAC GGT GGC GCT TCA TG
17β-Hydroxysteroid dehydrogenase 3 (HSD17B3)	Forward	GCT TTG ACC TTT TGG CTG GTT
	Reverse	CCT GAA GAC TGC AAG TCA GCA A
5-Alpha Reductase (isoform 1) (5AR1)	Forward	TCG CTC GGT GTT AAT TTA TGG TT
	Reverse	CGG AGG ACC GTG TGT CTC A
5-Alpha Reductase (isoform 2) (5AR2)	Forward	AGC AGG AAT TGA CGG TCT TGA
	Reverse	GAG GGC TGC CAG AAC ATC A
5-Alpha Reductase (isoform 3) (5AR3)	Forward	TCC GCA TTT TTC CAG GAT CT
	Reverse	GCT GCC CGC CTC TCT TG
Hydroxysteroid 11β - Dehydrogenase 1	Forward	GGT GGT GAA AGA GGC TGA GAA C
	Reverse	GGA GGC GAC TTT ACC TGA AAC AG
Hydroxysteroid 11β - Dehydrogenase 2	Forward	GGT GGT GAA AGA GGC TGA GAA CA
	Reverse	GGA GGC GAC TTT ACC TGA AAC AG
Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH)	Forward	AGA TGC AGG TGC TGA GTA TGT TG
	Reverse	AAG GCA AGG ACT GCG TCT TC

3.2.5 Steroid Hormone Extraction & Tandem Mass Spectroscopy Analysis

Nonpolar lipid metabolites were extracted from scleral homogenates and serum; 100 ul aliquots of the former and 25 µl aliquots of the latter were each combined with 3 ml of 2:1 chloroform:methanol and 1 ml of PBS, with inclusion of internal standards C12:0 monoalkylglycerol ether (MAGE) (10 nmol, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and pentadecanoic acid (10 nmol, Sigma-Aldrich, St. Louis, MO, USA). Organic and aqueous layers were separated by centrifugation at 10,000 *g* for 5 min and the organic layer then collected, dried under a stream of N_2 and dissolved in 120 µl chloroform. A 10 µl aliquot was injected into LC-MS/MS instrument (Agilent Model 6430 QQQ, Agilent Technologies, Santa Clara, CA, USA), to separate components by liquid chromatography as previously described (Benjamin et al., 2013). MS analyses were subsequently performed on the same machine, using

an electrospray ionization (ESI) source, with the following settings (capillary voltage: 3.0 kV, fragmentor voltage: 100 V, drying gas temperature: 350ºC, drying gas flow rate: 10 l/min, nebulizer pressure: 35 psi). Metabolites were identified by selective reaction monitoring of the transition from precursor to product ions at associated optimized collision energies and retention times, as previously described (Benjamin et al., 2013; Louie et al., 2016). The level of each metabolite was determined by first integrating the area under the relevant curve, which was then normalized to the internal standard value. Metabolite levels are expressed as relative abundances as compared to exogenous steroid hormone controls (Steraloids, New Port, RI, USA).

3.2.6 Statistics

Statistical analyses were performed with the aid of Microsoft Excel. Variability between samples was calculated and reported as standard errors of the mean (SEMs). Two-tailed paired t-test were used to compare results from form-deprived and their fellow (non-form-deprived) eyes. When comparing results obtained from males and female subjects (i.e. sex-specific comparisons) two-tailed unpaired t-tests were used. Differences were considered to be statistically significant when $p \leq 0.05$.

3.3 Results

3.3.1 Serum Steroid Hormone Levels

Strong signals for seven hormones – estrone, estradiol, testosterone, DHEA, progesterone, corticosterone and cortisol, were detected in serum samples by LC:MS/MS. However, no statistically significant differences in steroid hormone levels between untreated (control) male and female birds (i.e., those not fitted with diffusers were observed),(Fig. 3.2).

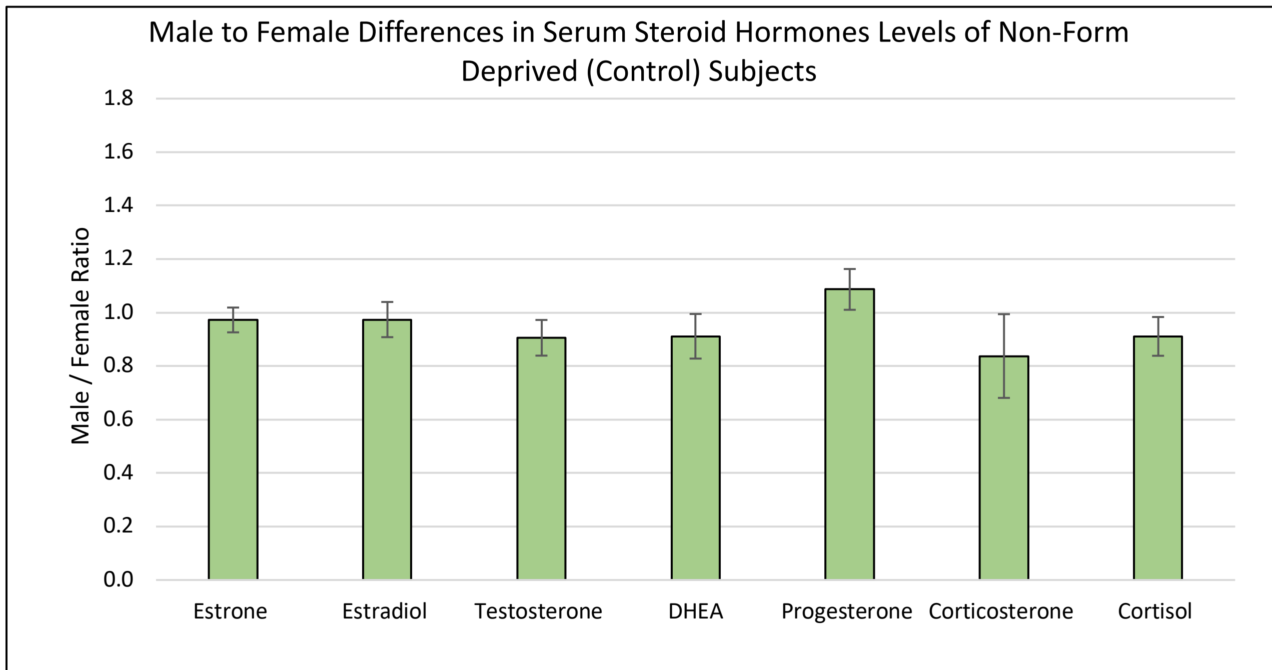


Figure 3.2. Ratio of male to female levels of sex steroid hormones detected in the blood serum of untreated (control) 21 day-old chicks. No significant sex-related differences found. Error bars represent SEMs.

While relative steroid hormones were statically equivalent across sexes for untreated subjects, treated male subjects displayed a statistically significant, increase in serum cortisol levels relative to that of untreated males (by 1.38X, p <0.01) (Fig. 3.3). However, no significant differences between treated and control females were observed in serum levels of any of the same hormones.

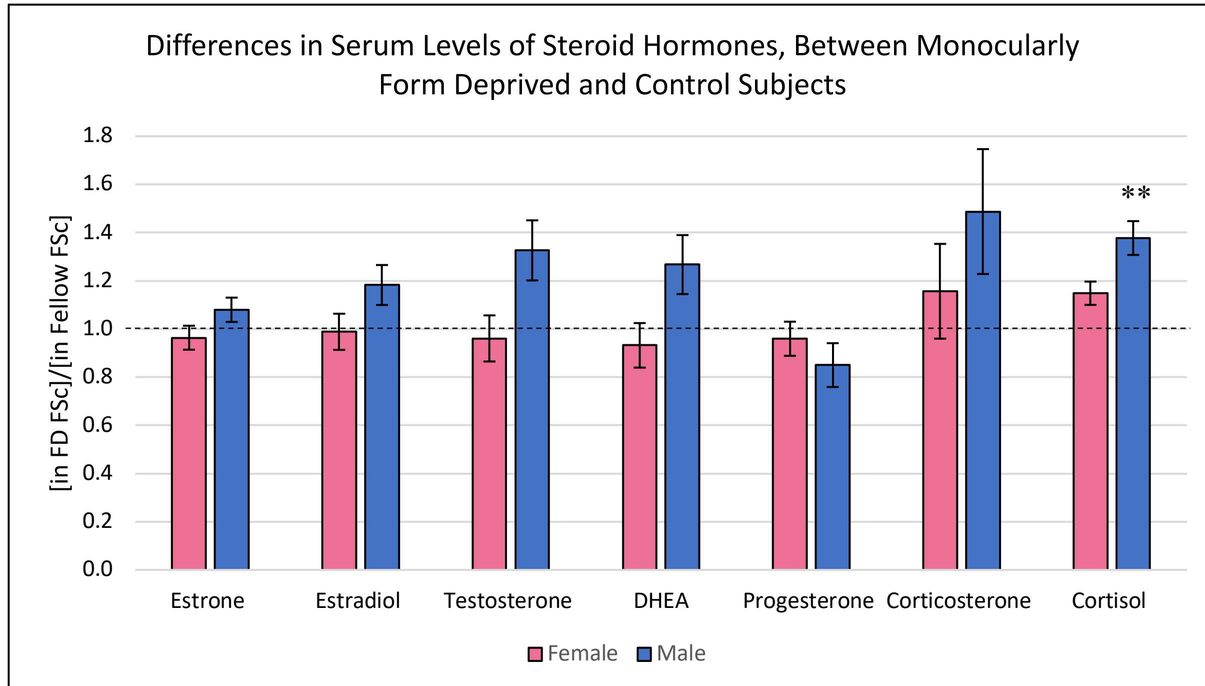


Figure 3.3. Ratio of serum steroid hormone levels in monocularly form-deprived subjects, to levels in untreated (control) 21 day-old) chicks, for each of the seven hormones reliably detected in serum samples. Cortisol levels significantly increased in treated male birds. Error bars represent SEMs. **p<0.01

3.3.2 Scleral Steroid Hormone Levels

Of perhaps more interest in the context of eye growth are the steroid hormone levels in scleral samples. Here strong signals for estradiol, testosterone, DHEA, progesterone and corticosterone were detected by LC:MS/MS. Normal steroid hormone levels, i.e., as indicated by levels in the scleras from untreated fellow eyes of form-deprived subjects, were also largely statistically equivalent for male and female birds, with one exception being corticosterone. Corticosterone levels tended to be higher (1.43X) in the scleras of male compared to female subjects (Fig. 3.4), although this difference did not reach statistical significance.

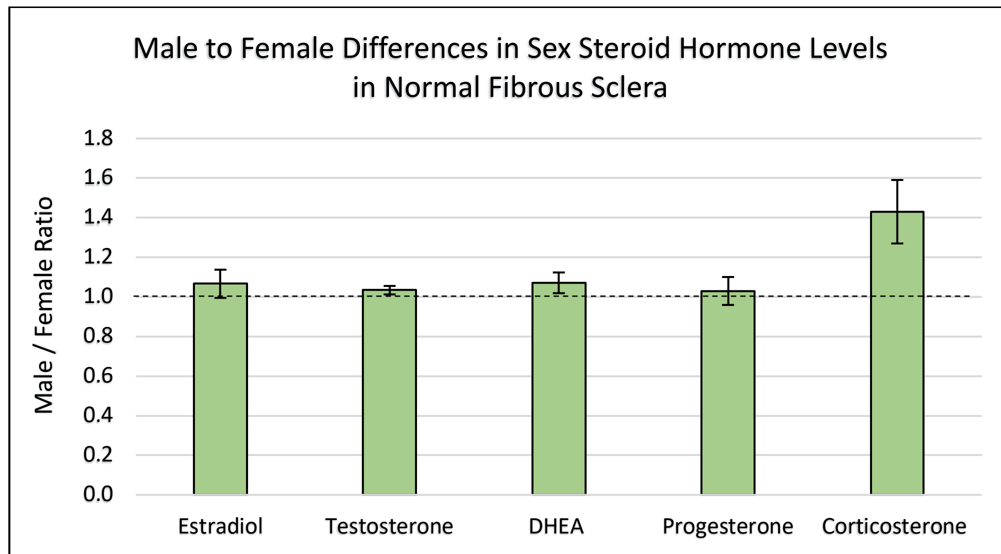


Figure 3.4. Ratio of male to female levels of steroid hormones detected in fibrous sclera from untreated (fellows to form-deprived) eyes of 21 day-old chicks. Corticosterone levels tended to be higher in the fibrous scleras of males, relative to females, but not significantly so. Error bars represent SEMs.

Intriguingly, some very prominent differences emerged when comparing relative hormone levels in the scleras of form-deprived eyes of male and female subjects. Specifically, corticosterone levels were greatly depressed in the scleras of form-deprived eyes for both sexes (0.55X, females; 0.35X, males; $p < 0.05$ & < 0.001 respectively). For female birds, there was also a statistically significant elevation in progesterone levels in the scleras of form-deprived eyes (Fig. 3.5). Likewise, there were statistically significant differences in both corticosterone and progesterone levels between male and female subjects ($p<0.05$ for both steroid hormones), in these eyes.

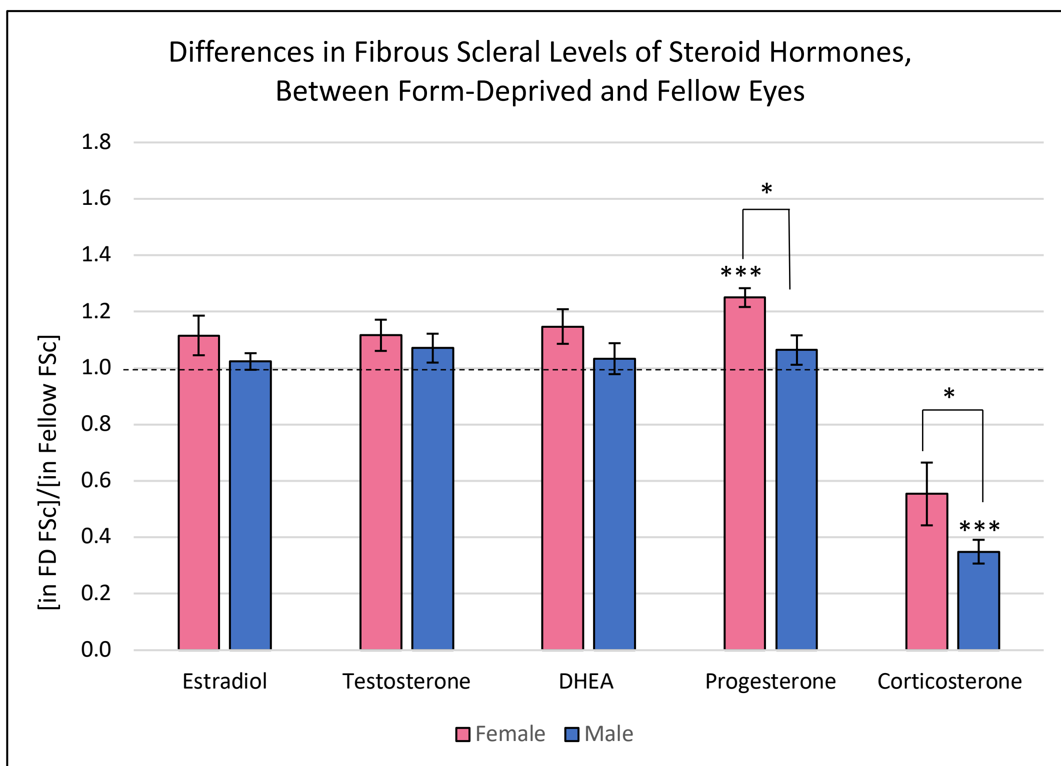


Figure 3.5. Ratio of levels of steroid hormones in fibrous scleras of form-deprived, myopic eyes to levels in fellow eyes of 21 day-old chicks. Progesterone levels significantly elevated in treated eyes of female subjects, corticosterone levels significantly depressed in treated eyes of both sexes and differences between males and females significant for both progesterone and corticosterone levels in scleras from treated eyes. Error bars represent SEMs. *** p < 0.001, *p<0.05

When scleral steroid hormone levels in the eyes of untreated (control) chicks were compared to levels in the fellow eyes of monocularly form-deprived chicks, corticosterone levels appear to be depressed in the latter relative to the former for both sexes, reaching statistical significance for males (Male: 1.66X, $p < 0.01$, Female: 1.35X, $p = 0.075$). The opposite is true for female birds and scleral testosterone levels, which are significantly elevated in fellow to form-deprived eyes compared to eyes of untreated chicks (0.87X, $p<0.05$)

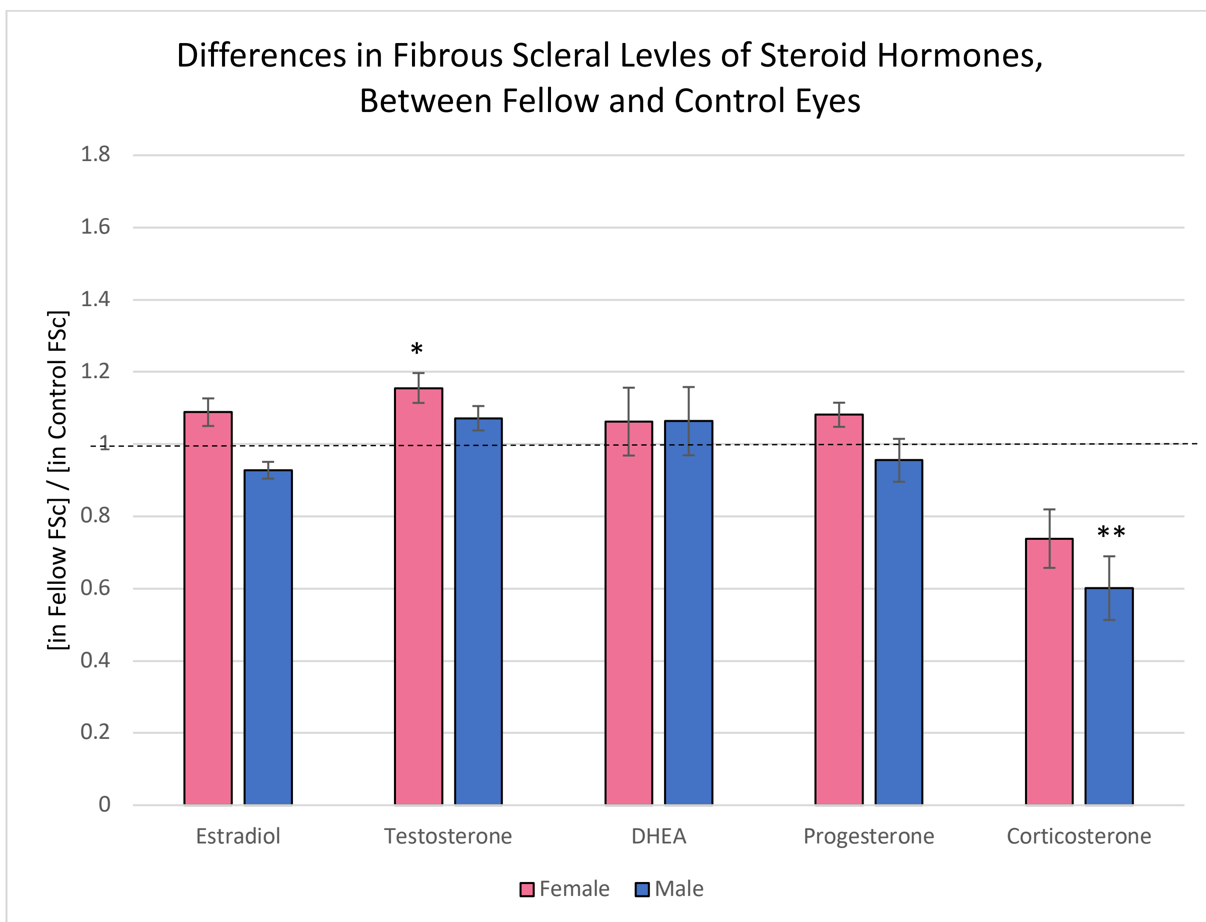


Figure 3.6 Ratio of levels of steroid hormones in fibrous sclera from fellow eyes of monocularly form-deprived chicks to that in control fibrous sclera (of untreated subject). Scleral corticosterone levels are significantly lower in the fellow eye fibrous sclera relative to the analogous tissue in control eyes of males, while scleral testosterone

levels are significantly higher in control relative to fellow eyes for females). Error bars represent SEMs. ** $p < 0.01$, *$p<0.05$.

3.3.3 Scleral Steroidogenic Enzyme Expression

A variety of steroidogenic enzyme transcripts were detected by qRT-PCR in scleral samples, although in samples from fellow (untreated) eyes, no statistically significant differences in expression between male and female subjects were observed (Fig. 3.6).

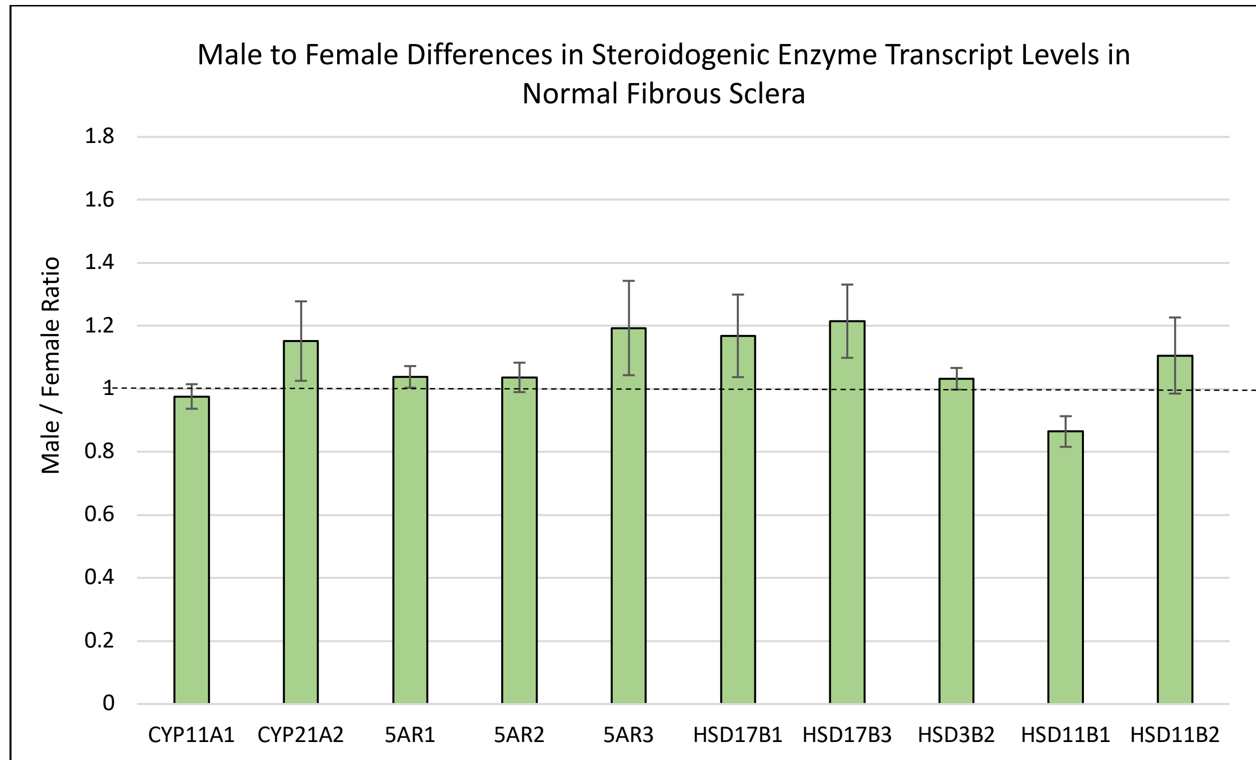


Figure 3.6 Ratio of male to female levels of steroidogenic enzyme transcripts in the fibrous scleras of untreated (fellow) eyes of monocularly form-deprived subjects. Error bars represent SEMs. No statistically significant differences found between males and females.

In relation the effects of form deprivation, one gene, HSD11β1, which is responsible for the synthesis of corticosterone from 11-deoxycorticosterone, was found to be differentially expressed in sclera. Expression levels were significantly lower in samples from form-deprived eyes relative to their fellows, for both males and females ($p< 0.001$ for both) (Fig. 3.7).

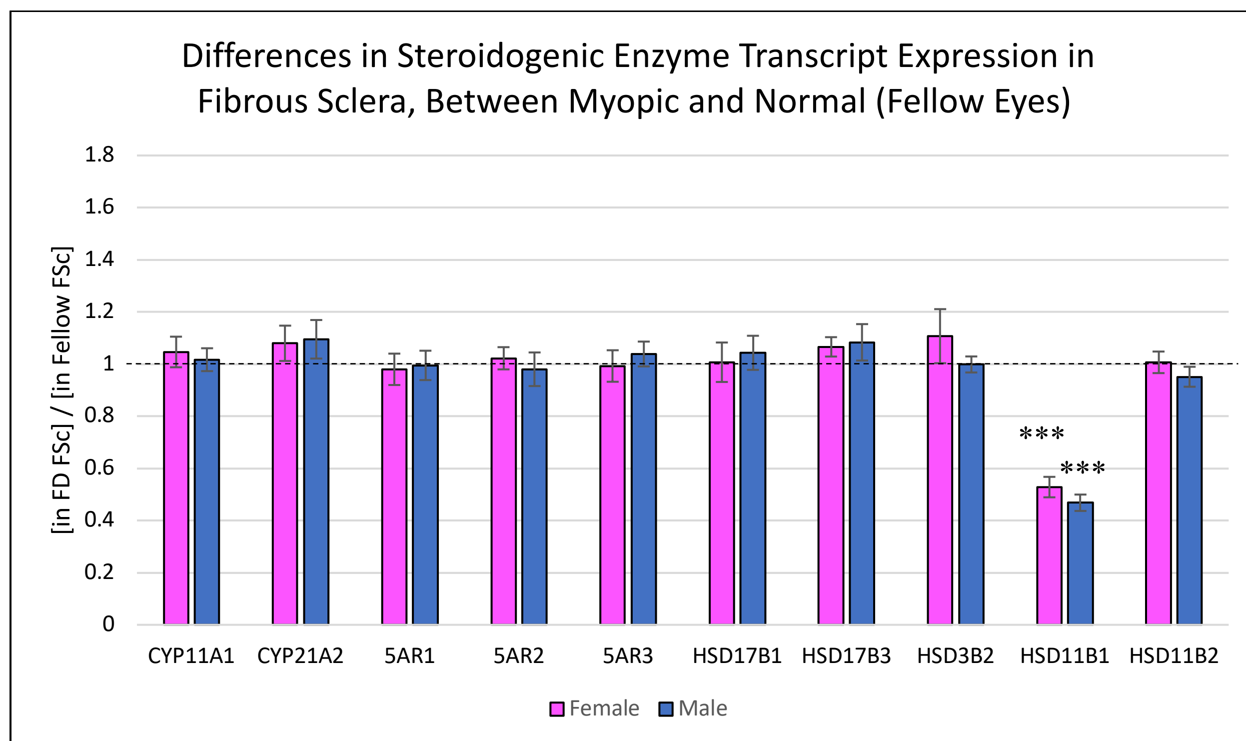


Figure 3.7. Ratio of levels of scleral steroidogenic enzyme transcript expression in form deprived eyes, normalized to that of fellow eyes. Scleral HSD11B1 expression significantly reduced in former, in both males and females. Error bars represent SEMs. *** < 0.001.

3.4 Discussion

The experiments reported on above represent the first investigation into the potential role of steroids as modulators of scleral growth and as novel mediators of myopia-related scleral remodeling and thinning. Key results are discussed below in this context.

First we found that form deprivation was associated with a significant drop in local (i.e., scleral) corticosterone levels, as indicated by results of direct analyte measurement. Expression of its biosynthetic enzyme, HSD11β1, was also reduced at the transcriptional level. These changes were seen in both sexes. Interestingly, corticosterone is the major endogenous glucocorticoid species in chickens, analogous to cortisol in mammals (Scanes 2016). Furthermore, it is known from previous mammalian-based studies that glucocorticoids are potent regulators of ECM-rich tissues. For example, they have been shown to inhibit collagen and sulfated glycosaminoclgycan synthesis (Verbruggen et al., 1981, Kosinski et al., 2015), as well to depress MMP2 levels and activity, in a corticosterone-dependent manner (Shikatani et al., 2012), these processes being also integral to myopia-related scleral changes. By analogy, relative increases in MMP2 levels, as a byproduct of depressed corticosterone levels, as observed in the scleras of our male and female form-deprived chicks, is predicted to result in increased remodeling of the fibrous sclera, as reported in myopic eyes (Rada et al., 1999, Rada and Brenza 1995, Schippert et al., 2006).

It is further worth noting that glucocorticoids have been shown to depress both fibroblast proliferation and the fibroblast-to-myofibroblast transition (Verbruggen et al., 1981, Kosinski et al., 2015, Terao et al., 2014, Yabing et al., 2018, Franke and Abraham 2014). Thus, given the observed depression of corticosterone levels in “myopic” fibrous scleras, it is predicted that fibroblast-to-myofibroblast transdifferentiation would be promoted. This prediction is also consistent with results from a previous study involving pigmented guinea pigs in which the scleras of myopic eyes were found to express greatly elevated levels of α-smooth muscle actin (α-SMA), a marker of myofibroblasts (Yuan et al. 2018).

The above arguments lead to the further prediction that enhanced signaling through the glucocorticoid receptor will inhibit myopia progression. In line with this prediction, other recent research, also involving young guinea pigs (Ding et al., 2018), found that treatment of normal eyes (i.e. no lens or diffuser treatment) with hydrocortisone, a glucocorticoid agonist, inhibited the axial length elongation and concomitant reduction in hyperopia observed during normal development in such animals. However, in a guinea pig model of lens-induced myopia, hydrocortisone treatment had the opposite effect; in this case, treatment with hydrocortisone enhanced axial elongation, leading to a larger myopic shift and greater scleral thinning. While these results are seemingly paradoxical, the authors also noted that with their myopia-inducing lens paradigm, plasma estradiol levels were increased. While it is not clear if these elevated plasma estradiol levels translated into the elevated levels in sclera, estrogen is known to enhance both MMP-2 synthesis and MMP2 activity (Nilsson et al., 2007, Marin-Castano et al., 2003), as well as collagen synthesis and turnover (Shah and Mailbach 2001, Hansen and Kjaer, 2016). Although it is unknown if these processes occur simultaneously. As noted earlier, though, elevated MMP2 activity and collagen turnover have also been associated with myopia-related changes in the sclera. Could the observed increased estrogen levels effectively counteract the inhibitory effect of hydrocortisone on myopia progression in the above lens-induced myopia model? It would seem plausible and warrants further study, in light of the bias towards the greater prevalence and severity of myopia in women who have higher average serum estrogen levels. The data reported here and that of Ding et al. (2018), also point to a role for glucocorticoid signaling in myopia-related scleral changes, which also warrants further investigation.

Furthermore, of some interest is the observation that scleral corticosterone levels were higher in the eyes of untreated (control) birds relative to the fellow eyes of form-deprived birds of both sexes. Although this difference only reached statistical significance in males, that the scleras of fellow eyes followed the trend of form-deprived eyes, suggests inter-ocular yoking, as has been reported previously by members of our group (Zhang et al., 2012) and others (Frost and Norton, 2012, Srinivasalu et al., 2018).

Several of our findings reported here may help explain observed differences in scleral thinning in myopic eyes between male and female birds (Chapter 2). Specifically, scleral corticosterone levels dropped to a greater extent in males than females. Thus greater scleral remodeling and possibly a greater net thinning of the fibrous scleras of form-deprived eyes of male chicks is expected, based on the above discussion. Furthermore, scleral progesterone levels were upregulated in female chicks in response to form-deprivation. Progesterone is known to inhibit both collagen synthesis (Liman et al., 2005, House et al., 2014, Dubey et al., 1998) and collagen

degradation (Halme and Woessner 1975, Zhou et al., 2011, and Zhou et al., 2012) in fibroblasts and ECM-rich tissues. The latter effect is apparently due to a reduction in MMP expression and activity (Zhang et al., 2000, Zhou et al., 2011); while there is some very limited evidence that these two apparently opposing effects (inhibited synthesis and degradation) can occur simultaneously in the same tissue (Ji et al, 2013), underlying mechanisms are less clear.

Interestingly, and in the context of the eye, work performed by Soni (1980) showed that high doses of progesterone were able to dampen the cyclical thinning and thickening of the cornea linked to the menstrual cycle in women. While it is not clear whether this effect of progesterone reflect changes in ECM remodeling, Zhou et al (2011) demonstrated that progesterone inhibits collagen deposition and degradation by corneal fibroblasts, via inhibition of IL-1ß in this case. Progesterone receptors are also known to be expressed in the corneas of a variety of animals as well as human (Rocha et al. 2000, Wickman et al. 2000 and Schirra et al., 2006). Thus it would seem plausible that elevated progesterone levels may affect myopia-related scleral remodeling and thinning. In the studies reported here, progesterone levels were found to be elevated in the fibrous scleras of form-deprived eyes compared to those of fellow eyes for female but not male birds, while female birds showed less relative overall scleral thinning (fibrous+cartilage layers) in their form-deprived eyes compared to male birds. Although it was not possible to measure the thickness of the fibrous scleral layer *in vivo* with our current technology, and so it is not possible to comment on treatment-related thickness changes in this sclera sublayer, it is nonetheless interesting to speculate that the elevated sclera progesterone levels in females offers some protection against myopia-related scleral changes,

Finally, based on observed sex-related differences in the susceptibility to myopia in humans, large, sex-specific and hormone-dependent (e.g. estrogen and testosterone) differences in local scleral hormone levels were predicted but not seen. Sex-specific differences were few in number and relatively small in size (e.g., see Figs. 3.2 & 3.3). However, it is important to note that the subjects in this study were only 21 days old when sacrificed, and so would not have reached puberty (Dunningham and Seigel, 1984, Parker et al., 1942), when sex-steroid hormone levels in males and females, both in serum and locally in tissues, are expected to radically diverge (Scanes et al., 1984, Sharp PJ, 1975). Thus follow-on studies in pubescent subjects are a logical next step in these investigations.

Chapter 4: The Impact of Myopia Progression on Scleral Steroid Hormone Receptors

4.1 Introduction

The focus of Chapter 3 was steroid hormones and changes under both myopic and non-myopic conditions, as well as sex-dependent differences. However, understanding ligand dynamics is only half of the equation, with the receptors on which they act the other half. The focus of this chapter is their cognate receptors. In addition, the expression patterns of fibroblast growth factor 8 (FGF8), an androgen-regulated protein, were considered.

As covered in the introductory first chapter of this dissertation there are six classical steroid hormone receptors – estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), progesterone receptor (PR), androgen receptor (AR), glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Estrogen and some its metabolites are also able to bind the G-Coupled Protein Estrogen Receptor (GPER). The experiments described in this chapter examined how the expression of these receptors changed in the chick fibrous sclera, as a function of myopiagenic stimulation (i.e. form deprivation by monocular diffuser treatment).

Better understanding how these receptor populations change under experimental and control conditions can provide additional insight into the overall system under study. For example, if ligand levels increase, but receptor expression concomitantly and proportionally decreases then the net physiological effect may be negligible, while a different outcome is predicted if ligand levels decrease with no change in receptor numbers. In short, understanding only isolated components of a system provides only an incomplete picture, which by itself, is insufficiently informative. Thus understanding how both scleral ligand levels and those of their cognate receptors change in response to myopia-inducing stimuli, is critical to our overall understanding of the mechanisms underlying related scleral changes.

4.2 Methods

All subjects were obtained and raised as described in Chapter 2 (section 2.2.1), of this dissertation. Chicks were either monocularly form-deprived with a diffuser or left entirely untreated to serve as control subjects. The diffuser treatment was initiated on day 7 post-hatch and halted on day 21 post-hatch, at time of sacrifice and tissue isolation.

Table 4.1 below, summarizes the number of animals and tissue types collected for the two experiments, the first involving scleral gene expression analysis of seven steroid receptors (6 nuclear hormone receptors and one membrane bound hormone receptor) along with FGF8 (an androgen regulated protein), and the second involving Western blot quantification of the one of these receptors. The numbers in brackets in the table represent the number of birds from which

reliable experimental data were obtained, with the discrepancies between these and starting subject numbers reflecting use of samples for experimental protocol optimization and unsuccessful isolation of the fibrous sclera during tissue dissection.

Table 4.1: Sample numbers, by experiment. Numbers in brackets represent numbers from which experimental data were obtained.

Experiment	**Total Subjects n**	**Male n**	**Female N**
qRT-PCR: Steroid Receptors	**42 (38)**	**22 (19)**	**20 (19)**
Western Blot: GR	**10 (10)**	**5 (5)**	**5 (5)**

4.2.1 Quantitative Real-Time Polymerase Chain Reaction

Endogenous ribonucleic acids (RNA) from scleral homogenates were extracted using RNeasy Mini Kits (Qiagen, Maryland USA). Genomic DNA (gDNA) was digested using a Turbo DNA-Free Kit (Invitrogen, Carlsbad, California USA). RNA quantification and the A260/A280 optical density ratio were measured using a spectrophotometer (NanoDrop 2000, NanoDrop Technologies Inc, Wilmington, Delaware USA). Total RNA was then reverse transcribed to cDNA using an Omniscript RT Kit (Qiagen, Maryland USA), which was then analyzed by quantitative real-time PCR using a StepOnePlus RT-PCR System (Applied Biosystems, Waltham, MA, USA) and iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). .

Table 4.2 lists the eight primers used to quantify transcriptional expression levels in scleral samples from form-deprived and fellow eyes. Primers were for six classical nuclear hormone receptors, one membrane bound and steroid hormone liganded G Protein-Coupled Receptor (i.e. GPER) and one androgen regulated protein (i.e. FGF8). Chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene. Primers were designed using the Primer Express 3.0 software program (Applied Biosystems, Foster City, CA). The efficiency of primers was calculated using ten-fold serial dilutions of cDNA. Gene expression levels were calculated as described previously (Schmittgen TD & Livak KJ, 2008). Mean normalized expression values were calculated from technical repeats (i.e. in triplicate) of target genes. mRNA expression levels were subsequently calculated for all biological repeats. Lastly, differential gene expression levels in “control sclera” (from fellow eyes) versus “experimental sclera” (from form-deprived eyes), were derived. Here scleral expression levels in diffuser-treated form-deprived eyes are expressed as a percentage of expression in fellow eyes.

Table 4.2 Abbreviations and primers for seven steroid hormone receptors, one steroid-regulated proteins and one house-keeping protein, used in qRT-PCR analyses.

Protein	**Primer**	**Sequence**
Androgen Receptor (AR)	Forward	AGA CGT GCC GCC AGT TCT T

	Reverse	GTA AAG GTA CCC CAC ACC CAA A
Estrogen Receptor Alpha (ERα)	Forward	ACC AAG GAG ACC CGG TAC TGT
	Reverse	ACC CCA TAG TGG TAG CCT GAA G
Estrogen Receptor Beta (ERß)	Forward	TGA CCT AGG GCA AGT CCT CAT C
	Reverse	TCG CAA GCT TTT CCC AAG A
G Protein-Coupled Estrogen Receptor 1 (GPER)	Forward	TGG AAT TAA GGT CCA AGG ATG TG
	Reverse	GGT CGG AAG ATG GCA GAG TTC
Progesterone Receptor (PR)	Forward	GCG CTC CGT AAC AGC GAT T
	Reverse	CCT GGT CGA GGA GTG CAA TAC
Glucocorticoid Receptor (GR)	Forward	GGA AGG GCA GCA CAA CTA TCT C
	Reverse	TTC CGC CGA ATT TTG TCA AT
Mineralocorticoid Receptor (MR)	Forward	GCG AAG GTA GCG GTT TTC C
	Reverse	CGT CAG GCT CCT GCT TAA TCC
Fibroblast Growth Factor 8 (FGF8)	Forward	CTG CAA GCC CAG GTA ACT GTT
	Reverse	TGC TCC CTC ACA TGC TGT GT
Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH)	Forward	AGA TGC AGG TGC TGA GTA TGT TG
	Reverse	AAG GCA AGG ACT GCG TCT TC

4.2.2 Western Blots

As for qRT-PCR analyses, Western blots made use of homogenized scleral samples. However, different from qRT-PCR analyses, five similar samples, e.g., five scleras for each treatment condition (i.e. one sample from each of 5 form-deprived eyes and their 5 fellows, for both male and female groups), were pooled into their four respective pools. In this way, samples representing scleras from form-deprived eyes of male and female subjects and the fellow eyes from the same male and female subjects were generated. For homogenization (see Chapter 3, Section 3.2.3), tube contents were each supplemented with 350 µl of Radioimmunoprecipitation Assay (RIPA) buffer (Thermo Fisher Scientific, Rockford, IL, USA) and cOmplete protease inhibitor (Roche, Mannheim, Germany), capped and sealed with Parafilm M and then homogenized in the prechilled (to 6 °C) Omni Bead Ruptor 24, as described previously (Chapter

3,). Following homogenization of their contents, tubes were placed on ice for one hour, during which they were briefly and gently shaken every 3-5 minutes. At the end of this period, 250 µl of the homogenate was transferred to a 1.5 mL microcentrifuge tube and subsequently spun down on a model 5430R centrifuge (Eppendorf, Hauppauge, NY, USA) at 4 ^{o}C and 10,000 g for 10 minutes.

Supernatant from the homogenized, centrifuged samples was collected for use in protein analyses. The protein concentration was first measured using a NanoDrop (Thermo Fisher Scientific, Rockford, IL, USA). A suitable volume of the above supernatant was added to a microcentrifuge tube containing Laemmli Sample (LS) Buffer (Bio-Rad, Hercules, CA, USA) and an appropriate volume of lysis buffer to yield a final protein concentration of 1 mg/ml and buffer ratio of 1:4 (LS to lysis buffer). This mixture was then heated to 95 ^{o}C for 5 minutes to accelerate protein denaturation. Following heating, sample tubes were transferred to an ice bath and contents subsequently loaded (along with a protein molecular weight ladder, Rainbow Full Range RPN800N, GE Healthcare Life Sciences, Marlbourough, MA, USA) onto a 4-20% mini-PROTEAN TGX stain-free precast protein gel (Bio-Rad, Hercules, CA, USA). Samples were loaded in quadruplicate, as technical repeats. The gels were then run at 80 V for 10 minutes, followed by 120 V until the dye front ran off the bottom of the gel. Protein bands were then transferred to a nitrocellulose membrane (Thermo Fisher Scientific, Waltham, MA, USA), using an iBlot dry western blotting system (Thermo Fisher Scientific, Waltham, MA, USA), and the two regions of interest of the loaded membrane – containing our target receptor, the glucocorticoid receptor (86 kDA), and the internal control, GAPDH (36 kDa), isolated for further processing. They were first washed 3 times with tris-buffered saline 0.1% tween-20 (TBST) solution and then blocked with reconstituted nonfat dried milk (5% m/v) in TBST (i.e. blocking buffer) for one hour at room temperature, before being washed again 3 times with TBST and incubated overnight at 4 ^{o}C with a primary antibody, either rabbit anti-Glucocorticoid Receptor (PA5-24793, Invitrogen, Carlsbad, CA, USA) or mouse anti-GAPDH loading control (MA5-15738, Invitrogen, Carlsbad, CA USA), presented as a 1:100 or 1:1000 dilution, respectively. The nitrocellulose membrane was washed again the following day, 3 times with TBST, and then incubated for approximately one hour with a secondary antibody, either 1:5,000 goat anti-mouse IgG HRP (G21040, Invitrogen, Carlsbad, CA, USA) or goat anti-rabbit IgG HRP (G21234, Invitrogen, Carlsbad, CA, USA), as appropriate, in a solution of 5% bovine serum albumin (BSA) and TBST. Following incubation, the membrane was again washed 3 times with TBST, followed by a two-minute incubation with ProSignal Pico (Genesee Scientific, San Diego, CA, USA).

Resulting blots were imaged using a ChemiDoc MP System (Bio-Rad, Hercules, CA, USA), utilizing the chemiluminescence setting. Images were analyzed using the Bio-Rad Image Lab (Bio-Rad, Hercules, CA, USA) analysis software suite. In brief, the chemiluminescent signals corresponding to the various bands, including control (GAPDH) and GR bands were quantified. Values corresponding to the GR bands were divided by those for the GAPDH bands, by way of adjusting for / normalizing against differences in the protein loaded in each well. The resulting ratios, representing a given experimental condition (e.g., sclera from form-deprived male subjects) were then averaged and normalized against ratios representing the scleras of matching fellow eyes. In this way, relative protein levels for each condition were derived.

4.2.3 Statistics

Statistical analyses were performed with the aid of Microsoft Excel. Variability between samples was calculated and reported as standard errors of the mean (SEMs). Two-tailed paired t-tests were used to compare results between form-deprived eyes and their fellow, non-deprived eyes, within a given sex. When comparing results obtained from males and female subjects (i.e., performing sex-specific comparisons) two-tailed unpaired t-tests were used. Differences were considered to be statistically significant when $p \leq 0.05$.

4.3 Results

4.3.1 Scleral hormone receptor transcript expression in untreated eyes of monocularly form-deprived birds

Transcript expression levels for androgen, progesterone, glucocorticoid and mineralocorticoid receptors in scleras of non-deprived eyes of 21-day old, chicks were statistically identical in male and female subjects. There were, however, statistically significant differences in the expression levels of ERα & β transcripts, between the two sexes. Specifically, scleras from male subjects showed relatively higher levels of expression of ERβ (1.33x, $p = 0.021$) and lower expression of ERα (0.68x, $p = 0.027$) (Fig. 4.1). However, none of the remaining steroid receptors showed significant sex-related differences in transcriptional expression, and neither did FGF8 and GPER (Fig. 4.2).

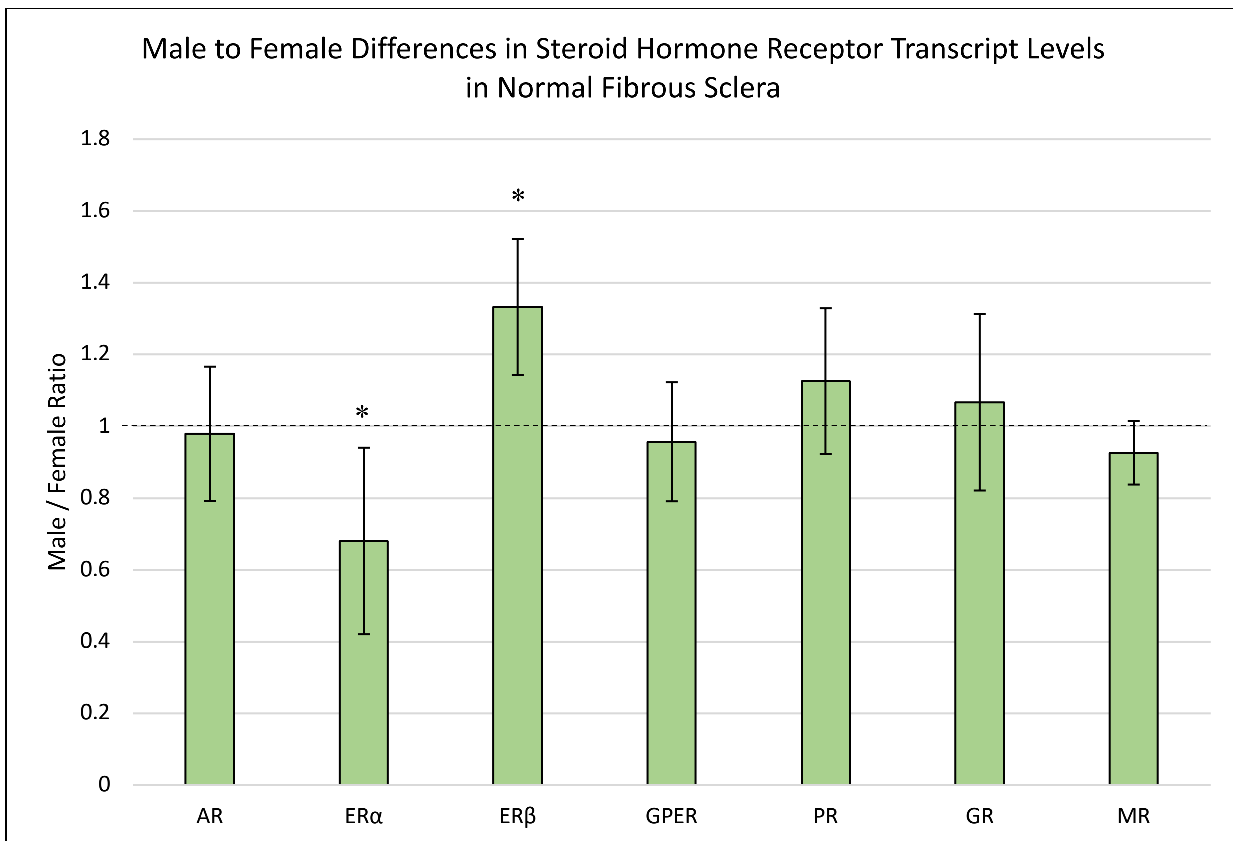


Figure 4.1. Ratio of male to female levels of transcripts in fibrous sclera of normal (fellow) eyes of 21-day-old chicks, for six steroid nuclear hormone receptors, and one estrogen-liganded G protein-coupled Receptor (GPER). ERα and ERβ transcript expression significantly and oppositely regulated in male and female subjects. Error bars represent SEMs. *$p < 0.05$

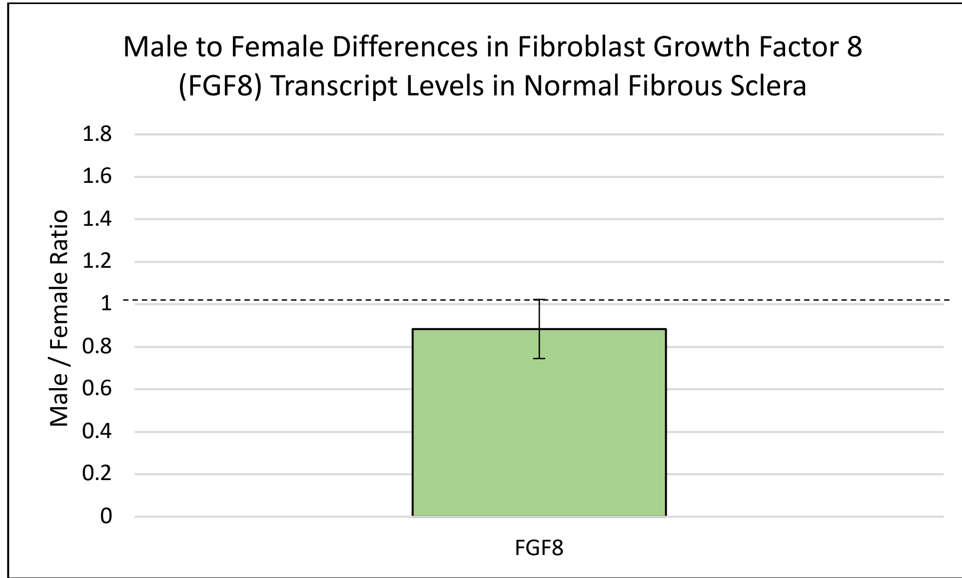


Figure 4.2. Ratio of male to female levels of FGF8 transcript in fibrous sclera of fellow (control) eyes of 21-day-old chicks. Error bars represent SEMs Male-female difference not statistically significant.

4.3.2 Scleral hormone receptor transcript expression in form deprived eyes: similarities and differences from normal eyes and across sexes

For female birds, form-deprived eyes showed an increase in scleral transcriptional expression for both GR (1.85x, $p < 0.001$) and ERβ (1.34x, $p < 0.05$), relative to levels in fellow eyes (Fig. 4.3). In contrast, ERα, AR and FGF8 all showed decreased scleral expression in form-deprived eyes compared to levels in untreated fellow eyes (0.83x, $p < 0.001$; 0.72x, $p < 0.001$; 0.62x, $p < 0.001$ resp.). In the case of male birds, none of the same transcripts showed significant differences in scleral expression levels between form-deprived eyes and their fellows (Fig. 4.3). Differences between male and female in relative scleral expression levels, i.e., in form-deprived eyes relative to their fellows, were also significant for ERβ and GR transcripts.

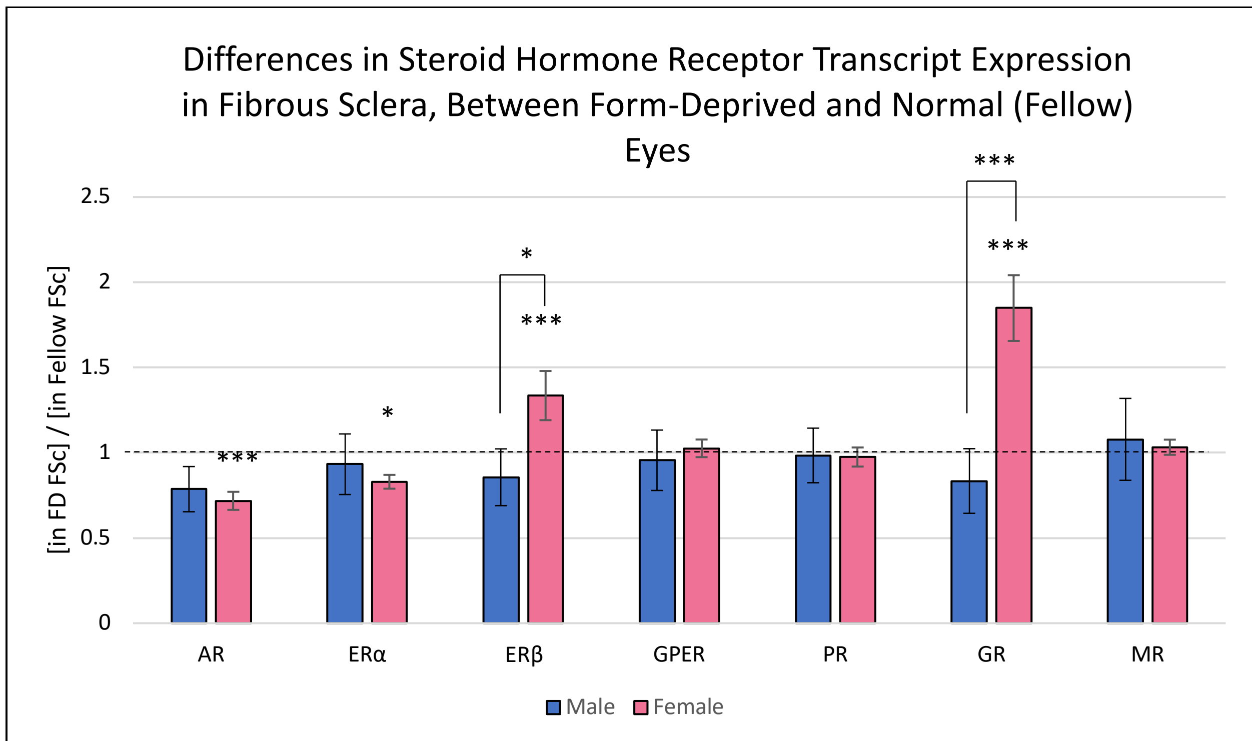


Figure 4.3. Ratio of levels of steroid hormones receptor transcripts in fibrous sclera of form-deprived eyes compared to fellow eyes of 21-day-old chicks. In form-deprived eyes, scleral GR was upregulated, and AR, downregulated in females. Differences in relative scleral expression levels, between male and female, significant for ERβ and GR transcripts. Error bars represent SEMs *$p<0.05$, ***$p < 0.001$

4.3.3 Scleral FGF8 expression in form-deprived eyes: similarities and differences from normal eyes and across sexes

For female subjects, the scleras of form-deprived eyes showed a decrease in FGF8 expression relative to their fellows (0.62x, $p<0.001$). Furthermore, the difference between male and female in relative scleral expression levels, i.e., in form-deprived eyes relative to their fellows, was also significant ($p<0.05$).

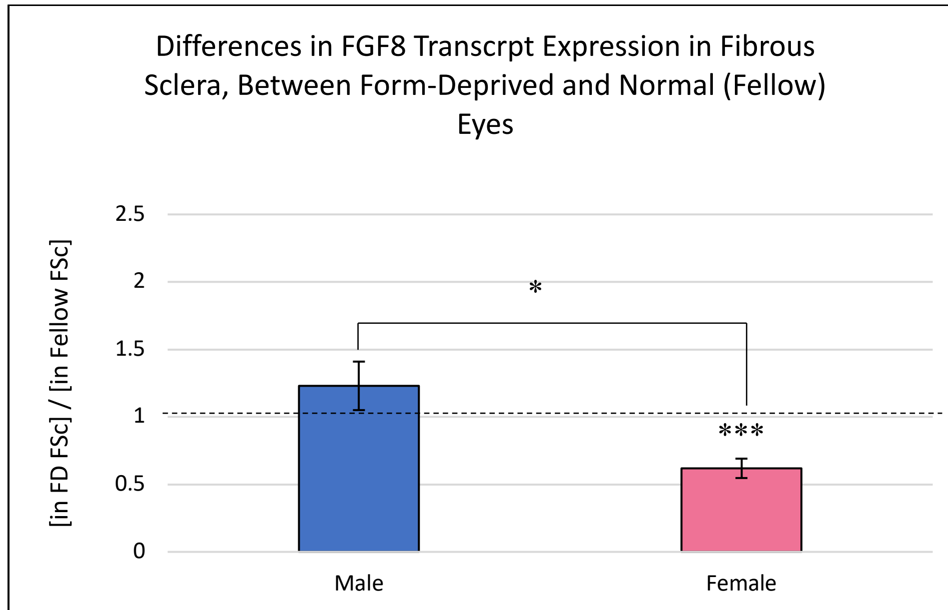
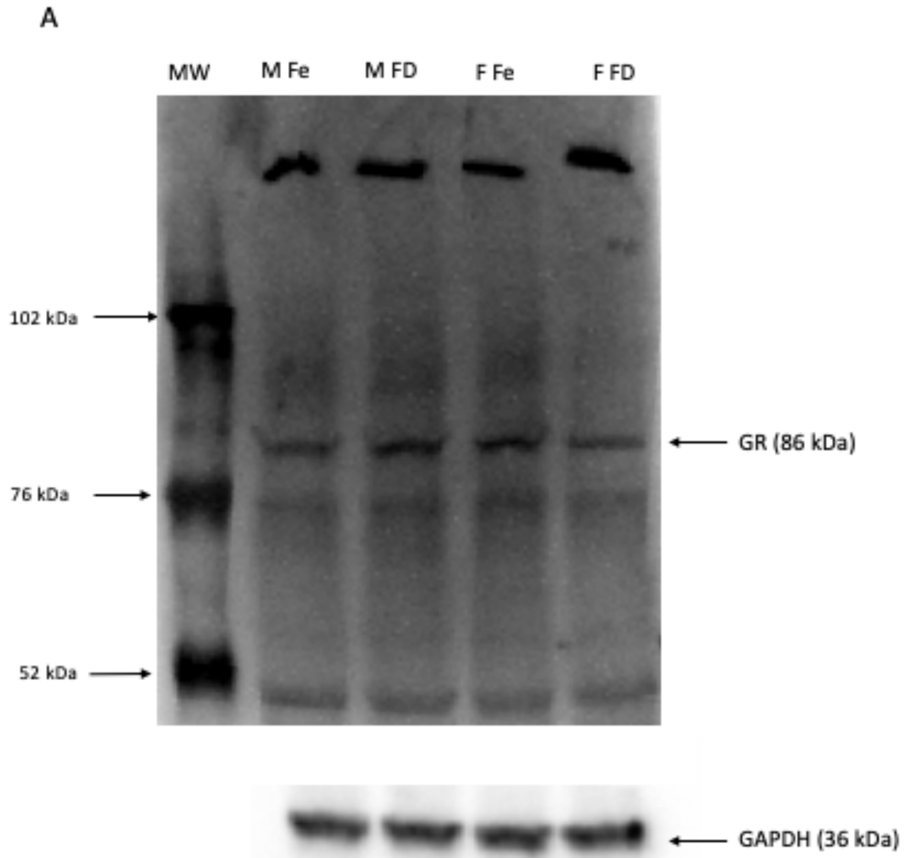


Figure 4.4. Ratio of levels of scleral FGF8 transcript expression in form-deprived eyes compared to fellow eyes. FGF8 transcriptional expression significantly downregulated in female subjects; difference between male and female also statistically significant. Error bars represent SEMs, *$p < 0.05$, ***$p < 0.001$.

4.3.3 Glucocorticoid Receptor Expession in Sclera

Glucocorticoid Receptor expression was detected and quantified via western blotting. We observed relatively equal expression of GR across conditions, i.e. in both male and female form deprive and fellow sclera. That said, we did observe what appears to be a slight elevation of GR in female form deprived sclera. However, this relationship did not rise to the level of significance (Fig. 4.5B). Note also in Figure 4.5A, there are four distinct bands (at approximately 50, 80, 86 and 130 kDA), with the 86kDa banding corresponding to the glucocorticoid receptor. Per the manufacture, the latter three bands are expected. While the band at 50 kDA is not noted on the manufacturer's website, the western blot example on this site was cut off above 50 kDA, offering a plausible explanation for this omission.


A
MW
M Fe
M FD
F Fe
F FD
102 kDa
76 kDa
52 kDa
GR (86 kDa)
GAPDH (36 kDa)

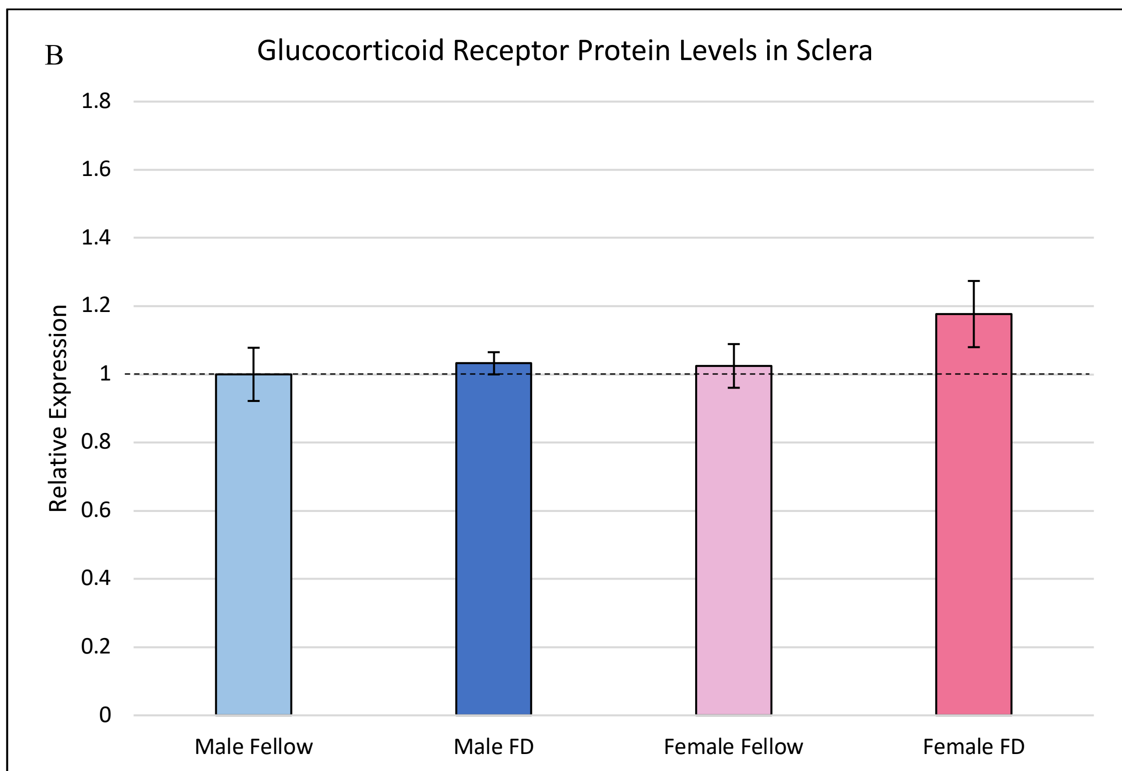

B
Glucocorticoid Receptor Protein Levels in Sclera
Relative Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Male Fellow
Male FD
Female Fellow
Female FD

Figure 4.5. Image of western blot membrane, with both Glucocorticoid Receptor (GR) and GAPDH bands labeled (A). Graphical representation of western blot results for GR levels in form-deprived (FD) eyes and fellows for male and female subjects (B). Slightly higher levels in sclera of FD compared to fellow eyes of females not statistically significant. Error bars represent SEMs.

Discussion 4.4

In the previous chapter, we reported that, by-and-large, in the scleras from untreated fellow eyes of monocularly form-deprived subjects, there was no difference in steroid hormone concentrations between samples from males and females. Unsurprisingly this pattern was repeated in steroid hormone receptor transcript expression in analogous tissues. Specifically in the scleras from control eyes, the transcriptional expression levels of most steroid hormone receptors measured were statistically equivalent in both males and female subjects. As previously noted, our subjects were 21 days of age at time of sacrifice and tissue collection, well prior to animals reaching puberty. Indeed, female White Leghorn chickens reach puberty around the five to six months of age (Dunningham and Siegel, 1984), while males reach this point at between four and five months of age (Parker et al., 1942).

There were two notable exceptions to the above observation, being the scleral expression of ERα and ERβ, which were lower and higher respectively in males compared to females. To our knowledge, this is the first report of its kind in such young chicks, although the significance of this difference is currently unknown. In human, however, signaling through these receptors is known to influence the glycoprotein, collagen and matrix metalloproteinase contents of ECM rich tissues (Markiewicz et al., 2013, Voloshenyuk and Gardner, 2010), although the manner in which they do this is not well understood and likely to be complex. Furthermore, the relative expression of these receptors appears to vary as a function of tissue and cell type, as well as pathology. For example, cancerous tumors have been found to show very different expression patterns compared to that in directly adjacent, benign non-tumorous tissues (Kousidou et al. 2008). Without further study in the ocular scleral system targeted in the current study, our finding of differential sex-specific ERα and ERβ regulation is difficult to interpret.

In the context of myopia, the more interesting observations are the findings in female chickens, of significant differences in scleral steroid hormone receptors expression between form-deprived eyes and their fellows. Here, scleral expression of both androgen and estrogen receptors (AR & ERα in Fig 4.3), were significantly down-regulated in form-deprived eyes relative to fellow eyes, as was FGF8, an androgen-regulated protein. On the other hand, the scleral expression of both GR and ERβ were significantly up-regulated in form-deprived eyes relative to fellow eyes, but apparently unaltered in males, with these sex-related difference here also statistically significant. As background to the following discussion, changes in ligand concentrations, as reported in Chapter 3, as well as the changes in receptor expression, as reported in this chapter, are summarized in Table 4.3.

Table 4.3 Directional changes in steroid hormone receptor protein expression in fibrous sclera from form-deprived relative to fellow eyes, as well as analogous changes in ligand concentrations in these tissues. Non-significant changes are indicated by triple dash (---).

Protein	**Scleral Expression**		**Ligand**	**Scleral Concentration**	
	Male	Female		Male	Female
ERα	---	↓	Estradiol	---	---
ERβ	---	↑	Estradiol	---	---
GPER	---	---	Estradiol	---	---
PR	---	---	Progesterone	↑	---
AR	---	↓	Testosterone	---	---
GR	---	↑	Corticosterone	↓	↓
FGF8[f]	---	↓	Testosterone	---	---

[f]*regulated, rather than liganded, by testosterone.*

For its part, FGF8 has been relatively little studied outside of embryonic development (Hao et al., 2019, da Costa et al., 2018, Huang W et al., 2018). Two of the few exceptions to this appear in the oncology (specifically endocrine tumors) and chondrocyte literature. Of potential relevance to our studies, FGF8 has been found to play a role in extracellular matrix homeostasis, with its overexpression linked to increased ECM degradation (Ellman et al., 2013, Mattila et al, 2007). In work performed by Ellman et al. (2013), inhibition of FGF-8 with a neutralizing antibody inhibited the degradation of ECM. As FGF8 signaling was found to be down-regulated in the fibrous sclera isolated from form-deprived eyes of females, one might also predict reduced thinning of the fibrous layer of the female "myopic sclera". However, this is counter-intuitive, given the comparison is with the scleras of fellow eyes from the same subjects. Although we do not have data covering treatment-related thickness changes in the fibrous sublayer of the sclera, a relative thinning in form-deprived eyes is expected based on observations of others, implying that the picture is much more complex than initially proposed.

As noted above we also observed a relative decrease in the scleral expression of AR, an androgen liganded receptor, in the form-deprived eyes of both males and females, although this effect was only significant in the later. Interestingly, studies performed in extraocular ECM rich tissues have shown that overexpression of AR increases collagen production (Wang et al., 2018 and Lin et al., 2012), whereas its inhibition led to a relative reduction in the collagen content in ECM-rich tissue (Lin et al., 2012 and Markova et al, 2004). In drawing a parallel between receptor inhibition and a reduction in receptor numbers, one would then predict a relative reduction in collagen in the fibrous scleral component of form-deprived eyes, as is consistent with observations in chicks and also tree shrews, which has a fibrous only sclera (Marzani and Wallman, 1997, Metlapally and Wildsoet 2015).

In contrast to the AR population discussed above, scleral expression of the second of the estrogen receptors examined, ERβ, as well as of the glucocorticoid receptor (GR) was up-regulated in the form-deprived relative to fellow eyes of females, with the latter showing the greatest change, almost two fold. The latter change in gene expression was also confirmed at the protein level, although the difference in scleral protein levels between form-deprived eyes and their fellows did not reach statistical significance.

Combined with findings from experiments described in Chapter 3 (i.e. down-regulation in corticosterone expression in the fibrous sclera of the myopic eyes of both sexes), the additional observation related to GR expression reported in this chapter paint an interesting picture, linking scleral glucocorticoid metabolism and signaling through its cognate receptor to myopia progression. Indeed it appears that depressed scleral glucocorticoid levels (in the case of chicken, corticosterone) in form-deprived eyes may serve to remove a brake on scleral remodeling and thinning. In female birds, however, this may in part be counteracted by an increase in GR expression in the fibrous sclera of form-deprived eye. Assuming that thickness changes in the fibrous sublayer of the sclera mirror measured total treatment-induced thickness changes, this scenario is consistent with the lesser degree of scleral thinning (and conceivably remodeling) observed in females compared to males.

In summary, the above findings yield a complex picture, where regulation of scleral remodeling (in response to form-deprivation) appears to be influenced by a variety of simultaneous changes in the scleral levels of endogenous steroid receptors, their cognate ligands and a steroid regulated protein, FGF8.

Chapter 5: Summary & Future Directions

5.1 Dissertation Summary

Myopia is one of the leading causes of secondary blinding pathologies worldwide. It describes the optical condition in which there is a mismatch between the optical power of the eye and its length, the majority of cases being a consequence of excessive eye elongation during childhood and/or adolescence. Increased remodeling and thinning of the outer scleral wall, an extracellular matrix (ECM) rich tissue, is key to this increase in eye length (Metlapally and Wildsoet 2015, Harper and Summers, 2015, Guggenheim and McBrien 1996). This excessive elongation is also coupled to stretching of the inner retinal and choroidal layers, at least in part contributing to the increased risk in myopes of retinal detachment, myopic maculopathies and glaucoma (Saw et al. 2005, Metlapally and Wildsoet 2015). On relevance to the work described in this dissertation, both the incidence of myopia is greater and its clinical phenotype, on average, more severe, in women relative to men (Zhao et al. 2000, Sewunet et al. 2014, Vitale et al., 2009). Underlying the work described is the hypothesis that steroid hormone signaling in sclera plays a significant role in the rate of progression of myopia, with both sex-dependent and independent-mechanisms explored. The chick was used as the model for this research, which made use of a diffuser treatment paradigm to form deprive eyes and so induce myopia. For translational purposes, only the fibrous layer of the chick's bi-layered sclera was analyzed at the molecular level, in keeping with the fibrous-only scleras of mammals and primates. Key findings are summarized below:

(1) Form-deprived female chicks developed higher myopic refractive errors relative to males, although their eyes tended to be shorter in length (although statistically so).

(2) Female chicks had thicker choroids relative to males, with this sex-related difference apparent in both form-deprived and fellow (control) eyes. Female chicks also show thinner scleras overall (fibrous + cartilage layers), under the same conditions, although the degree of form deprivation-induced scleral thinning was greater in male subjects.

(3) Glucocorticoid signaling appears to be significantly associated with myopia-related scleral changes in our experimental paradigm.

- Levels of the major glucocorticoid species in chicken, corticosterone, were depressed in the sclera of form deprived eyes relative to levels in that of fellow eyes, irrespective of sex, although the change was greater in male.

- The reduction of corticosterone levels in the sclera of form-deprived eyes paralleled the decreased scleral expression of the hydroxysteroid 11-beta dehydrogenase 1 (HSD11β1) transcript, HSD11β1 representing a key glucocorticoid biosynthetic enzyme.

- The glucocorticoid receptor (GR), which represents the cognate receptor, of corticosterone, showed sex-specific differential gene regulation in the scleras of form-deprived eyes. More specifically female fibrous sclera from form-deprived eyes, showed increased GR transcript expression, relative to fibrous sclera from fellow eyes. Similar differences were evident at the protein level but far less pronounced. Here, the GR protein appeared to be up-regulated in the fibrous sclera of form deprived eyes, relative to fellow eyes in females, yet this difference failed to reach statistical significance.

- The above findings, in combination with findings from other labs (Ding M et al., 2018), support a role for GR-mediated signaling in scleral remodeling, specifically in protecting against excessive remodeling, as occurs during myopia progression.

(4) Other steroid hormone receptors also displayed significant, sex-specific regulation in the sclera of myopic eyes

- Three gene transcripts, that of the androgen receptor, estrogen receptor (ER) α and fibroblast growth factor 8, all showed significant down-regulation in the scleras of form deprived eyes in females. In contrast, the ERß transcript was up-regulated under the same conditions.

- In sclera of fellow (untreated) eyes, male and female subjects displayed seemingly opposite transcriptional regulation of their estrogen receptors. Specifically, ERα expression was higher, and ERß lower, in females relative to males.

(5) Other steroid hormones also showed sex-specific differences in levels within the sclera.

- Compared to female subjects, males displayed higher systemic levels of cortisol, a minor glucocorticoid species in chickens, although this finding did not translate to the fibrous sclera.

- At the scleral level, female subjects showed higher progesterone levels in their formed-deprived compared to fellow eyes.

In summary, the findings of this research point towards a role for glucocorticoids in modulating scleral ECM remodeling, with implications for myopia progression. Several sex-specific differences in steroid hormone and steroid hormone receptor levels and/or gene expression were also identified. Together these findings suggest a complex regulatory mechanism, with glucocorticoids and their receptors playing key roles and sex hormones offering another level of control that is also sex-specific.

5.2 Future Directions

While the findings as summarized above point to a regulatory role for glucocorticoids in the sclera, with implications for myopic progression, it is also apparent that the picture as it relates to

the roles of other steroid hormones, including sex hormones, is not clear cut. Nonetheless, it is also possible that the latter outcome reflects the young age of the subjects; they were prepubescent. Taking this point into consideration, three future lines of research are proposed.

(1) Explore the role of steroid hormones as general regulators of scleral growth (remodeling).

Glucocorticoids

- Leveraging insights from experiments reported in both this dissertation and by those in Ding M et al. (2018), explore the effect of local treatment with glucocorticoid agonists on myopia progression and progression-related scleral remodeling (and thinning).

- Following up on the finding of Ding et al. (2018), of increased serum estrogen levels and greater myopic progression in response to systemic glucocorticoid treatments, testing of a combination treatment comprising a glucocorticoid agonist and an estrogen antagonist (e.g., tamoxifen) could offer new insights into the interacting influences of these steroid hormones.

Progestogens

- Study impact of progesterone treatment on scleral remodeling and thinning during myopia progression. We found that females showed less overall scleral thinning in response to form-deprivation compared to males, while the fibrous sclera of their form deprived eyes showed increased levels of progesterone. Given also that progesterone is known to inhibit ECM remodeling and thinning in other tissues (Liman et al., 2005, Halme and Woessner 1975, Ji et al, 2013), its effect on myopia progression-related changes in sclera warrants more direct investigation.

(2) Explore the role of GR-mediated signaling in myopia progression-dependent, scleral remodeling.

- In addition to a general decrease in corticosterone levels in fibrous sclera of myopic eyes of both sexes, there was a hint of increased GR expression in female subjects. Exploring the effect of this form-deprivation treatment paradigm on downstream signaling may be informative. For example, are there differences at the level of post-translation modification? Are non-genomic mechanisms (e.g. mitogen-activated protein kinases / extracellular signal regulated pathway, i.e. MAPK/ERK pathway), involved? Such information could inform future anti-myopia treatments.

(3) Explore hormonal differences in subjects that have entered puberty.

- Systemic steroid hormone levels are expected to diverge in male and female subjects around puberty, when myopia rapidly progresses in humans. Although more challenging to study from a practical perspective, experiments on older

chicks would likely yield additional and important insights into sex-specific steroid hormone signaling differences

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